The Role of Plasma and Vascular Tetrahydrobiopterin in Vascular Disease States

Colin Cunnington

MBChB MRCP

Department of Cardiovascular Medicine

&

St Catherine's College,

University of Oxford



A thesis submitted for the degree of Doctor of Philosophy

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Abstract

The endothelial nitric oxide synthase (eNOS) co-factor tetrahydrobiopterin (BH4) has been shown to play a pivotal role in maintaining endothelial function in experimental vascular disease models. In BH4-deficient states, eNOS becomes enzymatically 'uncoupled', generating reactive oxygen species instead of nitric oxide, thus promoting endothelial dysfunction. In humans with coronary artery disease (CAD), higher vascular BH4 levels have been shown to be associated with improved endothelial function, and genetic variation in endogenous BH4 synthesis has implicated a causal role. Accordingly, BH4 has been proposed as a potential therapeutic target in vascular disease states. The work in this thesis aims to further elucidate the roles of exogenous and endogenous BH4 in humans.

In a randomised, placebo-controlled clinical trial of oral BH4 therapy in patients with CAD, exogenous BH4 had no effect on endothelial function or vascular oxidative stress. Subsequent pharmacokinetic and pharmacodynamic analysis revealed that oral BH4 significantly augmented BH4 levels in plasma and in venous tissue (but not in arterial tissue), but also increased levels of the oxidation product dihydrobiopterin (BH2), which lacks eNOS cofactor activity. Thus, there was a null effect on overall biopterin redox status.

To further understand the mechanics of exogenous BH4 oxidation, *ex vivo* studies of human blood and vascular tissue demonstrated that exogenous BH4 is very rapidly oxidised to BH2; co-administration with an antioxidant had only a modest effect on preventing BH4 oxidation in blood, with no beneficial effect on biopterin redox state in the vasculature.

Finally, using a "Mendelian randomisation" approach, I studied the effects of a haplotype of *GCH1* (the gene encoding the rate limiting enzyme in BH4 synthesis) on endogenous BH4 bioavailability and vascular function in healthy individuals. In patients with CAD, this haplotype has been associated with decreased BH4 bioavailability and eNOS uncoupling, however in healthy individuals the haplotype exerted no significant effect, likely due to reduced inflammatory stimulation of *GCH1*.

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List of Abbreviations

5-MTHF	5-methyltetrahydrofolic acid
7N	7-nitroinidazole
ACh	Acetylcholine
AdGCH	Adenoviral gene transfer of *GCH1*
AIx	Augmentation index
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
AUC	Area under the curve
B	Biopterin
BAEC	Bovine aortic endothelial cells
BH2	7,8-dihydrobiopterin
BH4	5,6,7,8-tetrahydrobiopterin
BK	Bradykinin
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CCR2	Chemokine receptor 2
cGMP	Cyclic guanosine monophosphate
cIMT	Carotid intima-media thickness
CRP	C-reactive protein
DAHP	2,4-diamino-6-hydroxypyrimidine
DHFR	Dihydrofolate reductase
DOCA	Deoxycorticosterone acetate
DTE	Dithioerythritol
EC	Electrochemical (HPLC detection)
ECG	Electrocardiogram
EDR	Endothelium-dependent relaxation
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FBF	Forearm blood flow
FL	Fluorescence (HPLC detection)

FMD	Flow-mediated dilatation
FMN	Flavin mononucleotide
FOV	Field of view
GCH1	The gene encoding GTPCH
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
GTPCH	Guanosine triphosphate cyclohydrolase-1
H2O2	Hydrogen peroxide
HASTE	Half Fourier acquisition single shot turbo spin echo
hph-1	Hyperphenylalaninaemic mouse mutant
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IL-1β	Interleukin 1β
IMA	Internal mammary artery
iNOS	Inducible nitric oxide synthase
IPC	Ischaemia preconditioning
IRI	Ischaemia reperfusion injury
KO	Knockout
LDL	Low density lipoprotein
L-DOPA	L-3,4-dihydroxyphenylalanine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N-monomethyl-L-arginine
LPS	Lipopolysaccharide
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NH4	Tetrahydroneopterin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
OCDEM	Oxford Centre for Diabetes, Endocrinology & Metabolism
OCMR	Oxford Centre for Clinical Magnetic Resonance Research
PAH	Phenylalanine hydroxylase
PBMC	Peripheral blood mononuclear cell
PE	Phenylephrine
PTPS	6-pyruvoyltetrahydropterin synthase

PWV	Pulse wave velocity
qBH2	Quinonoid dihydrobiopterin
RCT	Randomised controlled trial
ROS	Reactive oxygen species
SHR	Spontaneously hypertensive rat
SIN-1	3-morpholinosydnonimine
SMTC	S-methyl-L-thiocitrulline
SNP	Single nucleotide polymorphism
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SR	Sepiapterin reductase
STZ	Streptozotocin
SV	Saphenous vein
TAC	Transverse aortic constriction
tBio	Total biopterins
TCA	Trichloroacetic acid
Tg	Transgenic
TE	Echo time
TH	Tyrosine hydroxylase
TNFα	Tumour necrosis factor α
TPH	Tryptophan hydroxylase
TR	Repetition time
TrueFISP	True fast imaging with steady state procession
WT	Wild type

CHAPTER 1: INTRODUCTION

1.1 NO and the endothelium

The influence of endothelial cells on vascular homeostasis was first described by Furchgott & Zawadzki in 1980 [1], who proposed that endothelial cells release an endothelial-derived relaxing factor (EDRF) in response to acetylcholine. EDRF has since been shown to be nitric oxide (NO) [2]. In addition to its vasodilatory capacity, NO has a number of anti-atherogenic properties, including down-regulation of leucocyte adhesion molecule expression [3], inhibition of platelet aggregation [4] and suppression of smooth muscle proliferation [5]. It is now widely accepted that endothelial dysfunction, as defined as decreased NO bioavailability, is a critical early step in the development of atherosclerosis, being associated with risk factors for atherosclerotic vascular disease [6-7], and predictive of adverse cardiovascular events in prospective studies [8-9]. Numerous pharmacological and genetic experimental models indicate that reduced endothelial NO production accelerates atherosclerosis [10]. Furthermore, under certain pathophysiological conditions, endothelial nitric oxide synthase (eNOS), the enzymatic source of NO, can instead become a producer of superoxide anion [11-12]. This process, termed 'eNOS uncoupling', can give rise to increased vascular oxidative stress and further loss of NO bioavailability. In this chapter I aim to review the evidence underlying eNOS uncoupling and its importance in the pathogenesis of atherosclerosis.

1.2 Production of NO in the endothelium

In addition to its role in endothelial biology, NO has been shown to function as an important signalling molecule in other physiological systems, mediating the

cytotoxic effect of macrophages [13] and acting as a messenger molecule in platelets [4] and the brain [14]. Characterising the enzymatic source of NO production in these tissues was the focus of much research in the late 1980s and early 1990s.

1.2.1 NOS structure and function

It was established that the amino acid L-arginine is the substrate for NO production, and more specifically that the terminal guanidino-nitrogen atom of L-arginine is used to synthesize NO [15-16], via an N^{ω}-hydroxy-L-arginine intermediate [17], with L-citrulline as a further product [18]. The oxygen atom in NO was shown to be derived from molecular oxygen [19].

Three isoforms of NOS have been defined: neuronal (nNOS, NOS I), inducible (iNOS, NOS II) and endothelial (eNOS, NOS III). eNOS and nNOS are expressed constitutively, whereas iNOS expression is induced by cytokines and other inflammatory stimuli [20]. In addition to requiring L-arginine as a substrate for NO production, all isoforms require the co-factors 6R-5,6,7,8-tetrahydrobiopterin (BH4, discussed below), nicotinamide adenine dinucleotide phosphate (NADPH) [17, 21-22], and the flavins, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) [20, 22-23] . All three isoforms are activated by the presence of intracellular Ca^{2+} ions [21-22, 24-25], however only eNOS and nNOS are regulated by agonist stimulation through calcium-calmodulin activation [25-26], whereas iNOS remains tonically active because calmodulin remains bound at intracellular calcium concentrations, independent of agonist stimulation [22]. In contrast to nNOS and iNOS, which are cytosolic enzymes, eNOS is mainly membrane-associated, localised in plasma membrane

caveolae and other intracellular membranes through post-translational lipid modifications and protein-protein interactions with caveolin and other caveolae-associated proteins [24, 26-27].

All three NOS isoforms function as catalytically active homodimers, comprising an oxido-reductase bi-domain structure, with calmodulin facilitating the transfer of electrons from the C-terminal reductase domain to the haem-containing oxygenase domain of the opposite monomer within the dimer pair [28]. Upon binding of Ca^{2+} to constitutive NOS, oxidation of the electron donor NADPH results in electron transfer via FAD and FMN of the reductase domain to the haem centre of the oxygenase domain where molecular oxygen and L-arginine are bound; NO catalysis can then proceed via the stepwise oxidation of L-arginine (Figure 1-1A) [28].

1.2.2 Requirement of BH4 as a co-factor for NOS

Prior to the discovery of NO, the pteridine co-factor tetrahydrobiopterin (BH4) was known to catalyse the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase [29], as well as acting as a co-factor for other aromatic amino acid hydroxylases. Deficiency of BH4 has been shown to lead to hyperphenylalaninaemia [30]. Other pterin species, particularly neopterin, had been shown to be released by cytokine-activated macrophages [31]. Thus, it was speculated that BH4 may have co-factor activity in other enzyme systems. The requirement for BH4 as a NOS cofactor was first demonstrated for iNOS in macrophages [32-33]; it was shown that both BH4 and NADPH were required for enzyme activity, as defined by the consumption of L-arginine and the production of citrulline and NO_2^-/NO_3^-, the breakdown products of NO. Evidence for BH4 co-

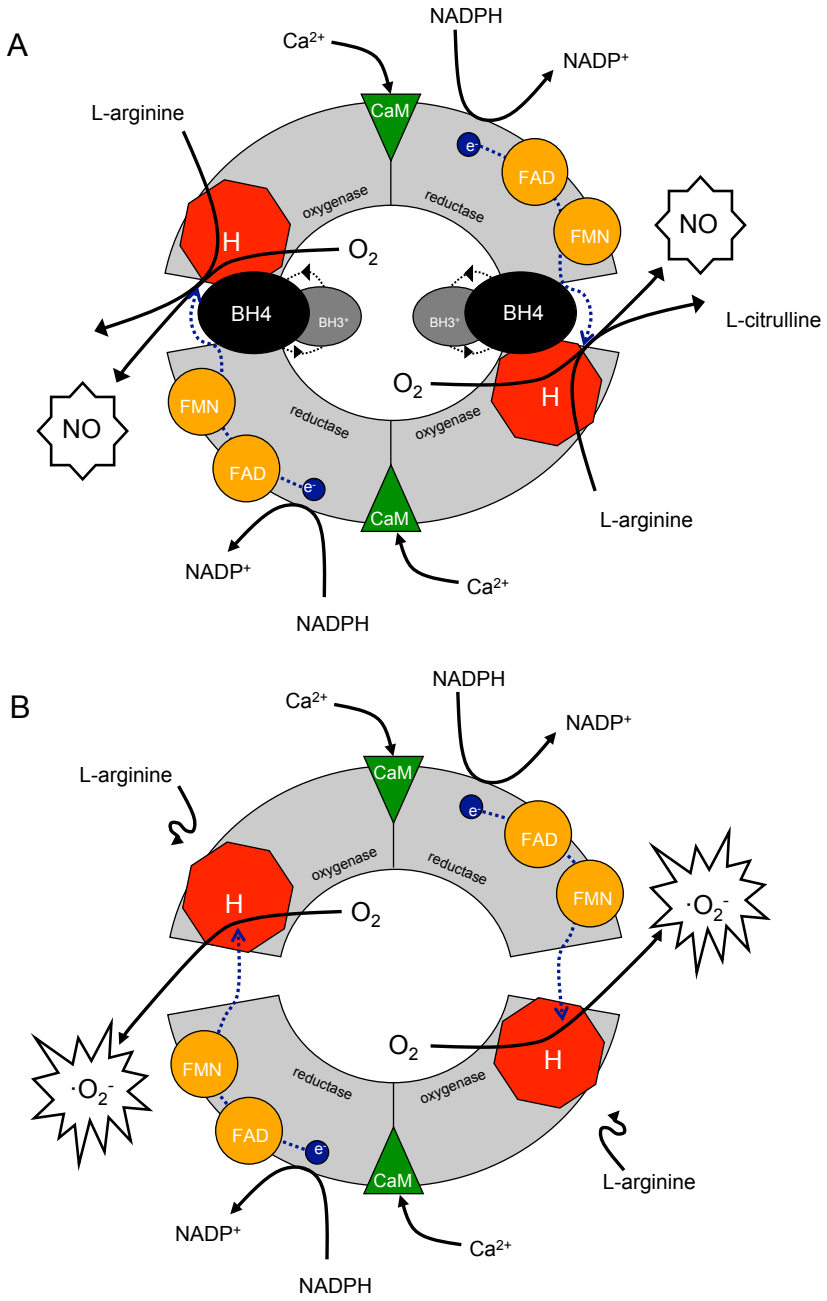


Figure 1-1: Mechanism of endothelial nitric oxide synthase regulation by tetrahydrobiopterin

Under optimal conditions (Panel A), the eNOS enzyme is a homodimer with each monomer comprising a flavin-containing reductase domain and a haem-containing oxygenase domain. Upon binding of calcium (Ca^{2+})/calmodulin (CaM), electron flow (e^-) generated from NADPH is transferred via the flavins FAD and FMN to the oxygenase domain of the other monomer. Tetrahydrobiopterin (BH4) is bound close to the haem group (H) in the active site and also interacts with residues in both monomers, contributing to eNOS dimer formation. Nitric oxide (NO) is generated by the oxidation of the guanidino-nitrogen of L-arginine using molecular oxygen, together with the production of L-citrulline. BH4 undergoes temporary oxidation to the $BH3^+$ radical but is rapidly recycled back to BH4 in a continuous process. In conditions of BH4 deficiency (Panel B), electron flow from flavins to L-arginine becomes uncoupled from L-arginine oxidation, resulting in formation of superoxide ($\cdot O_2^-$) from molecular oxygen.

factor activity was later demonstrated for NOS in brain [25], neutrophils [22], fibroblasts [34] and endothelial cells [35]. Studies of purified NOS suggest that BH4 functions both via allosteric regulation of L-arginine binding [36] and by promoting dimer formation and stability [28, 37].

1.2.3 BH4 synthesis

Intracellular BH4 levels are regulated principally by the activity of the *de novo* synthetic pathway (Figure 1-2). Guanosine triphosphate cylohydrolase I (GTPCH) catalyses the conversion of GTP to dihydroneopterin triphosphate. Two further enzymatic steps, catalysed by 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR), are required to synthesise BH4, although under normal circumstances GTPCH is the rate-limiting enzyme [38]. GTPCH, but not PTPS or SR, is upregulated in some cell types by inflammatory cytokines such as interferon-γ and tumour necrosis factor-α (TNFα) [38-39]. Additionally, BH4 may be synthesised via a 'salvage pathway' involving conversion of exogenous sepiapterin to 7,8-dihydrobiopterin (BH2), and then via NADPH-dependent dihydrofolate reductase (DHFR) to BH4 [40]. DHFR may also play a role in 'recycling' BH4 from BH2 produced within cells by oxidation of BH4.

1.3 NOS uncoupling

It was observed that the amino acid sequence of NOS bore close resemblance to the cytochrome P450 reductases, enzymes known to generate superoxide. It was therefore hypothesised that NOS may also be able to produce reactive oxygen species (ROS) as well as NO. Indeed, in 1992 two groups showed that under certain conditions brain NOS could catalyse the formation of reactive oxygen species instead of NO [41-42]. In the absence of BH4 and L-arginine, purified

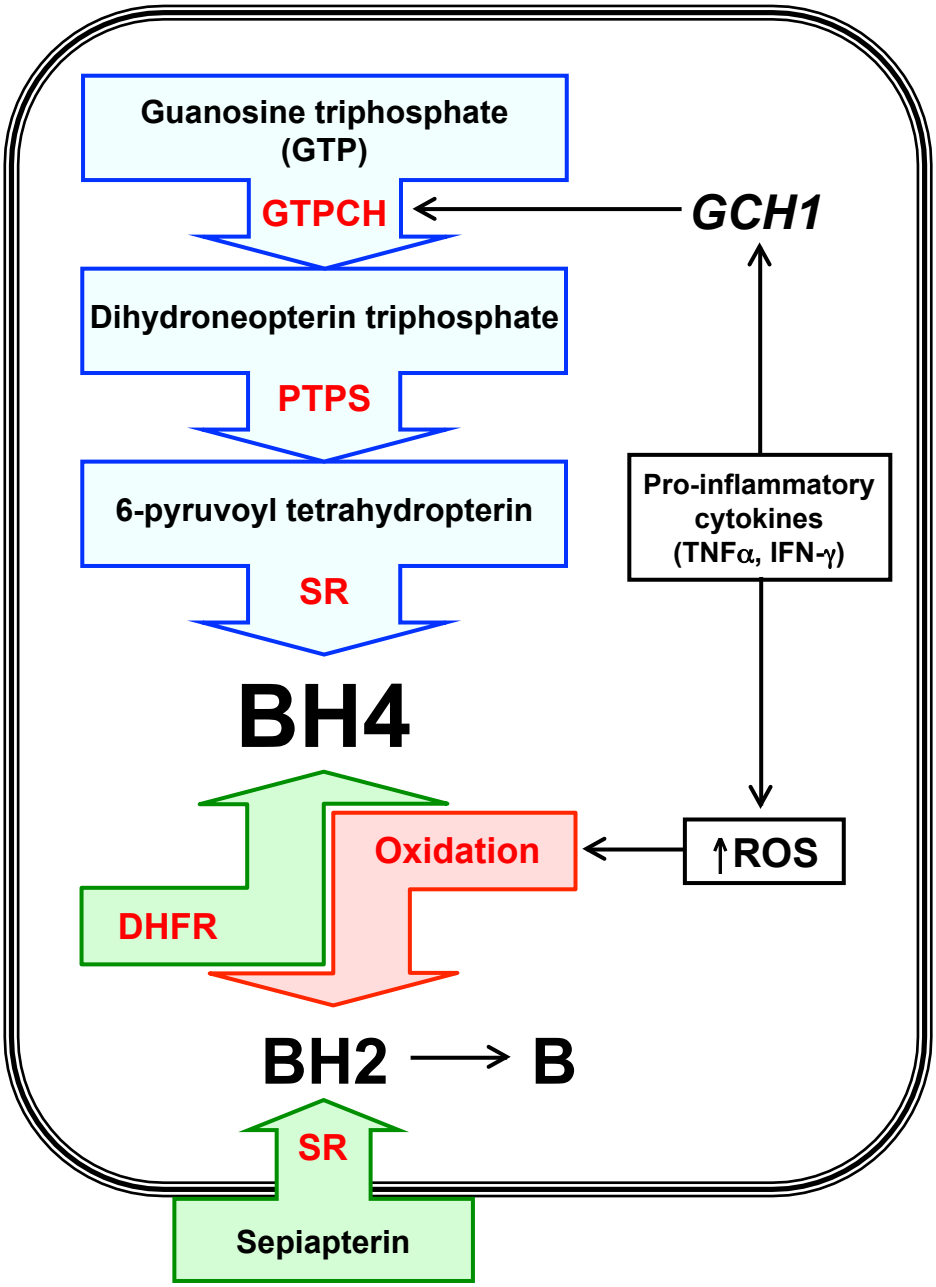


Figure 1-2: Tetrahydrobiopterin biosynthesis and metabolism
De novo BH4 synthesis occurs from the substrate GTP via a three-step enzymatic pathway (blue arrows). GTPCH, encoded by the *GCH1* gene, is the rate-limiting enzyme under normal conditions. BH4 is susceptible to oxidation by reactive oxygen species, forming BH2 and ultimately B. Stimulation by inflammatory cytokines can upregulate *GCH1* and increase BH4 synthesis, however inflammation can also lead to oxidative loss of BH4 via increased production of reactive oxygen species. Synthesis of BH4 is also possible through the 'salvage pathway' from the exogenous synthetic pterin, sepiapterin, which is metabolised first to BH2 by SR and then to BH4 by DHFR (green arrows). GTPCH, GTP cyclohydrolase-1; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase; DHFR, dihydrofolate reductase; BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; B, biopterin; ROS, reactive oxygen species.

porcine cerebellar NOS consumed molecular oxygen at the expense of NADPH, with generation of hydrogen peroxide (H_2O_2, the dismutation product of superoxide by superoxide dismutase (SOD)) [42], a process only partially inhibited by supplementary L-arginine. The addition of BH4 inhibited H_2O_2 formation and restored arginine-to-citrulline conversion [42]. Using electron paramagnetic resonance (EPR) spin trapping techniques it was then demonstrated that NOS produces superoxide rather than H_2O_2 in the absence of L-arginine [41]. The addition of the arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS, inhibited both the production of superoxide and the consumption of NADPH.

These findings from purified NOS were later confirmed in intact cells transfected with nNOS [43], with superoxide being produced only in the absence of L-arginine. Although these investigators did not address the role of BH4 in this study, they demonstrated for the first time that superoxide produced by NOS can combine with NO to form peroxynitrite, a potent oxidant which is significantly more reactive than superoxide itself. Furthermore, peroxynitrite is able to rapidly oxidise BH4 to inactive pterin species, including BH2, 7,8-dihydropterin and dihydroxanthopterin [44] which have no NOS cofactor activity.

1.3.1 eNOS uncoupling

A number of studies over the last 10 years have shown that the endothelium is a source of superoxide under pro-atherosclerotic conditions, including hypercholesterolaemia [45]. It was hypothesised that the enzymatic 'uncoupling' of L-arginine oxidation observed in purified brain NOS, leading to the unopposed reduction of molecular oxygen to superoxide, may implicate the endothelial

isoform of NOS as a source of superoxide in the vascular wall. A fundamental observation was made by Pritchard *et al.*, who noted that incubation of cultured human umbilical vein endothelial cells (HUVEC) with native low-density lipoprotein (LDL) resulted in increased superoxide production, which could be inhibited by L-NAME, implying that eNOS was the source of superoxide [46]. Conversely, L-NAME increased the production of superoxide in control cells due to decreased scavenging of superoxide by NO. LDL also increased levels of nitrotyrosine, a stable oxidation product of peroxynitrite [46].

1.3.1.1 Studies of purified eNOS enzyme

In cell-free systems, three separate groups using recombinant human [11-12] or bovine [47] eNOS demonstrated that BH4, and not L-arginine, was critical in preventing superoxide generation by eNOS; addition of L-arginine in the absence of BH4 was insufficient to inhibit superoxide formation [11], whereas BH4 could abolish the formation of superoxide in the absence of L-arginine [48]. Superoxide production, inhibitable by L-NAME, was reported to occur at the haem centre, as superoxide was shown to be inhibited by the haem iron ligands cyanide and imidazole [11-12]. Additionally, BH4 was able to scavenge superoxide produced from xanthine/xanthine oxidase *in vitro*, indicating a direct antioxidant effect in addition to regulating eNOS coupling [47]. Finally, BH4 increased the eNOS dimer:monomer ratio in purified enzyme heated to 55°C, suggesting a role in maintaining dimerisation [47].

The term 'eNOS uncoupling', therefore, describes the process whereby, in the absence of BH4, electron flow from the reductase domain of eNOS is diverted towards molecular oxygen rather than L-arginine, resulting in superoxide

production rather than NO synthesis (Figure 1-1B). Whilst changes in eNOS dimer structure may be associated with eNOS uncoupling, it is important to note that uncoupling refers specifically to enzymatic function rather than protein structure.

1.3.1.2 Studies of pharmacological BH4 supplementation and depletion

To support these findings in purified eNOS and further investigate the role of BH4 in regulating eNOS function, a number of experimental strategies have been used to examine the effect of altered BH4 levels in cellular or animal models.

Exogenous BH4 administration caused L-NAME-inhibitable relaxations in pre-constricted rat aorta, associated with elevated cyclic guanosine monophosphate (cGMP) levels [49]. A series of studies from the Katusic laboratory investigated the regulation of eNOS by BH4 in canine arteries *ex vivo* [50-53], first demonstrating increased H_2O_2 production in canine coronary arteries depleted of BH4 by incubation with 2,4-diamino-6-hydroxypyrimidine (DAHP, an inhibitor of GTPCH) [50]. BH4 depletion by DAHP, however, resulted in increased endothelium-dependent relaxations (EDR) to calcium ionophore A23187, an effect inhibited by catalase and L-NAME, implying that H_2O_2 generated by eNOS mediates vasorelaxation in these arteries. Second, EDR in canine middle cerebral arteries could be augmented by exogenous sepiapterin (a BH4 analogue converted to BH4 via the salvage pathway), but only in the presence of adequate levels of SOD to prevent scavenging of NO by superoxide [51]. This finding was explained by the fact that BH4 is rapidly oxidised *in vitro* in Krebs buffer, leading to the spontaneous generation of superoxide [51]. This hypothesis was explored further in a third study using canine basilar arteries, where exogenous BH4

produced strong endothelium-dependent contractions inhibitable by SOD [52]. In contrast, endothelium-denuded vessels showed only minor contraction in response to exogenous BH4, with vasorelaxation at higher doses. Lastly, in contrast to coronary arteries [50], canine basilar arteries incubated with DAHP exhibited decreased EDR which were restored by sepiapterin or SOD [53]. The differences observed between coronary and cerebral arteries were thought be due to differential sensitivity of these arteries to superoxide and H_2O_2.

1.3.1.3 Alteration of BH4 levels via genetic manipulation of GTPCH

Further evidence for a causative role of BH4 in eNOS uncoupling was provided by the hyperphenylalaninaemic mouse mutant (hph-1), which displays a 90% deficiency in GTPCH activity [54], the rate-limiting enzyme in BH4 synthesis. In comparison to wild type (WT) mice, the hph-1 mouse had significantly lower aortic BH4 levels, associated with decreased basal arginine-to-citrulline conversion (NOS activity) which could be augmented by exogenous BH4 supplementation [55]. Basal superoxide levels were slightly higher compared with WT; importantly however, the addition of L-NAME increased superoxide in WT mice (due to decreased NO scavenging), whereas superoxide levels in hph-1 were decreased, implying uncoupled eNOS as a principal source of superoxide. Similar to the findings in canine coronary arteries [50], these BH4-deficient mice exhibited increased EDR compared with WT. EDR to acetylcholine was inhibited by catalase and L-NAME, and augmented by SOD, implying that endothelium-dependent vasomotion in these mice is mediated by eNOS-derived superoxide and H_2O_2. Impaired EDR in the presence of catalase could be restored by exogenous BH4. In contrast, EDR in WT mice were augmented by catalase, whereas SOD had no effect on EDR [55].

Cai *et al.* used adenoviral gene transfer of GTPCH (AdGCH) to augment intracellular BH4 levels in human endothelial cells [56]. AdGCH significantly increased both BH4 and total biopterin levels (the sum of BH4, BH2 and biopterin) compared to control cells. AdGCH cells exhibited a parallel increase in eNOS activity, which was inhibited by DAHP or the NOS inhibitor N-monomethyl-L-arginine (L-NMMA), despite these agents having no effect on eNOS protein levels. Despite a similar increase in BH4, sepiapterin incubation resulted in a lesser increase in eNOS activity. Furthermore, AdGCH transfection resulted in both increased eNOS dimer:monomer ratio, providing further evidence of the role of dimerisation regulating eNOS function [56].

1.3.1.4 Paradoxical findings in chronic eNOS overexpression

Studies using animals overexpressing eNOS have also proved useful in clarifying the regulatory function of BH4 *in vivo*. Mice with a deficiency in eNOS have been shown to develop accelerated atherosclerosis [57-58], whereas short-term gene transfer of NOS to augment vascular NO production has been demonstrated to ameliorate endothelial dysfunction and improve markers of atherosclerosis [59-60]. It was therefore expected that mice with transgenic overexpression of eNOS in the setting of atherosclerosis would exhibit decreased lesion formation. However, ApoE-KO/eNOS-Tg mice demonstrated a paradoxical increase in atherosclerosis compared to ApoE-KO controls, despite slightly lower blood pressure [61]. These animals were shown to have both increased vascular NO production and increased superoxide levels (with a larger decrease in superoxide following endothelial denudation) than matched ApoE-KO mice. Despite significantly higher levels of eNOS protein in ApoE-KO/eNOS-Tg mice, both strains had similar aortic BH4 levels (although tending to be reduced in ApoE-

KO/eNOS-Tg animals), implying that decreased BH4:eNOS ratio was contributory to the increased superoxide observed in eNOS transgenic animals. Furthermore, oral BH4 supplementation resulted in a significantly greater reduction of atherosclerotic plaque and superoxide levels in ApoE-KO/eNOS-Tg mice. Thus, the inhibitory effect of BH4 feeding on atherosclerosis is greatest when eNOS is overexpressed, implying that the underlying mechanism is via increased eNOS coupling, rather than a direct antioxidant effect [61].

Bendall *et al.* combined mouse models of eNOS and GTPCH overexpression to further define the quantitative relationship between eNOS and BH4, in the absence of atherosclerosis [62]. First, eNOS-Tg mice had decreased eNOS dimer:monomer ratio compared to WT mice which was normalised in eNOS-Tg/GCH-Tg mice. Second, the aortic BH4 content of eNOS-Tg mice was reduced by 50% compared to WT, whereas both GCH-Tg and eNOS-Tg/GCH-Tg animals exhibited a two-fold increase in aortic BH4. Third, despite an 8-fold increase in eNOS protein, eNOS-Tg mice had similar levels of NO production compared to GCH-Tg mice; eNOS-Tg/GCH-Tg animals had a further significant increase in NO production above eNOS-Tg mice despite similar levels of eNOS protein. Finally, L-NAME-inhibitable superoxide was increased in eNOS-Tg mice and ameliorated by the GCH transgene [62]. Taken together, these data provide compelling evidence to support the notion that eNOS activity is dependent on BH4 availability, even in healthy animals.

1.3.2 Oxidation of tetrahydrobiopterin: the role of peroxynitrite

As BH4 is in itself highly redox sensitive, being easily oxidised in buffer alone to other inactive pterin species with no NOS cofactor activity, it was hypothesised

that BH4 levels may become limiting in the setting of oxidative stress via oxidative scavenging of BH4. Superoxide is known to react rapidly with NO to produce the potent biological oxidant peroxynitrite. It was demonstrated *in vitro* that peroxynitrite can rapidly oxidise BH4 [44, 63], and that peroxynitrite is a far more potent scavenger of BH4 than superoxide itself, with H_2O_2 having no reaction with BH4 [64]. In this study, Laursen *et al.* first demonstrated increased production of superoxide from the aortas of ApoE-KO mice compared to WT, inhibitable by sepiapterin. Second, peroxynitrite production was elevated in ApoE-KO in comparison to WT, and uric acid, a peroxynitrite scavenger, restored the impaired EDR in ApoE-KO aorta. Finally, incubation of WT aortas with peroxynitrite increased superoxide production in vessels with intact endothelium, but not vessels incubated with L-NAME or vessels from eNOS-knockout mice [64].

In a later study, peroxynitrite was shown to oxidise BH4 via the $BH3^+$ radical, and that ascorbic acid was able to reduce $BH3^+$ back to BH4 [63]. Ascorbic acid is known to ameliorate impaired endothelium-dependent vasomotion in subjects with endothelial dysfunction [65-67], thought to be due to its ability to scavenge superoxide [68]. Ascorbate however was only a weak scavenger of peroxynitrite [63]. Incubation with peroxynitrite gave rise to L-NAME-inhibitable superoxide generation in bovine aortic endothelial cells (BAEC), indicating eNOS uncoupling, however superoxide was unable to uncouple eNOS directly. Furthermore, the peroxynitrite donor 3-morpholinosydnonimine (SIN-1) reduced NO production in BAEC *pre-incubated* with BH4 and vitamin C, however *co-incubation* with BH4 (during SIN-1 exposure) partially restored NO production, which was further increased by the addition of ascorbic acid [63].

Taken together, these data suggest that peroxynitrite, resulting from the interaction of NO and superoxide, can induce eNOS uncoupling via oxidation of BH4, and that some of the observed beneficial effects of ascorbic acid on endothelial function may be due to increased recycling of $BH3^+$ back to BH4, thus promoting eNOS coupling. Furthermore, 5-methyltetrahydrofolic acid (5-MTHF), the circulating form of folic acid, has also been shown to be a potent scavenger of peroxynitrite and can promote eNOS coupling and increased BH4:BH2 ratio in human blood vessels [69].

Finally, it has also been observed that peroxynitrite may disrupt eNOS dimerisation via direct oxidation of the zinc-thiolate complex, independently of BH4, thus providing an alternative mechanism underlying peroxynitrite-mediated endothelial dysfunction [70].

1.3.3 The ratio of reduced to oxidised biopterins in eNOS regulation

Considerable debate exists as to whether it is solely the absolute levels of BH4 that are critical in determining eNOS catalytic activity, or alternatively whether the levels of oxidised biopterin species, e.g. BH2, play an important inhibitory role. Total biopterins (the sum of BH4, BH2 and biopterin) represent a measure of overall GTPCH synthetic activity, whereas the ratio of reduced biopterins to oxidised biopterins (expressed either as the BH4:BH2 ratio or BH4:total biopterins ratio) functions as a measure of biopterin redox status. Studies of purified eNOS in a cell-free system have provided robust evidence that BH2 can both bind to eNOS with a similar affinity to that of BH4, and can also promote superoxide generation [48], and thus the ratio of BH4:BH2 is important in

regulating eNOS in this model. It is less clear, however, whether such findings can be applied *in vivo*. Under conditions of oxidative stress, BH4 is oxidised to BH2, thereby resulting in both a diminished BH4 level coupled with a decreased BH4:BH2 ratio; consequently, it has been challenging to determine which process underlies eNOS uncoupling, making data interpretation difficult [71]. Recently published work by Crabtree and colleagues provides the best evidence to date on the role of BH2 *in vivo*. In cells with a fixed BH4 concentration, supplementation of cells with BH2 (in the presence of the DHFR inhibitor methotrexate to prevent reduction to BH4) to decrease the BH4:BH2 ratio, was sufficient to promote eNOS-dependent superoxide formation [72]. Furthermore, in a model of glucose-induced oxidative stress, changes in BH4:BH2 ratio were shown to be more closely correlated to uncoupled eNOS activity than changes in BH4 alone [73]. In the study by Cai *et al.* [56] the rise in BH4 achieved by GTPCH gene transfer was similar to that achieved by incubation with sepiapterin, although sepiapterin incubation resulted in much higher levels of BH2, and thus a lower BH4:total biopterins ratio (<10% vs 30%); this was associated with decreased eNOS activity. In contrast to these findings, the low but detectable concentrations of BH4 in diabetic GCH-Tg mice were sufficient to maintain normal eNOS function in comparison to the eNOS uncoupling observed in diabetic WT mice, despite a comparable reduction in BH4:BH2 ratio following the onset of experimental diabetes in both groups, suggesting that the absolute availability of BH4 may be more important in this model [74]. It is likely to be a combination of both the eNOS:BH4 ratio and BH4:BH2 ratio that is important for regulating eNOS function *in vivo* [72].

1.4 eNOS uncoupling in experimental disease models

In addition to the studies described in detail above, a number of others have provided evidence for the role of eNOS uncoupling, and specifically strategies to augment BH4 levels, in experimental vascular disease states. Table 1-1 provides an overview of these studies.

1.4.1 Diabetes and insulin resistance

Despite the fact that NO-mediated vasodilatation had been shown to be impaired in patients with diabetes [65], stimulation of human endothelial cells with high concentrations of glucose was shown to produce a paradoxical increase in eNOS gene expression and a 40% increase in NO production; superoxide production, however was increased 3-fold [75], resulting in a net alteration in redox balance towards pro-oxidation in the setting of hyperglycaemia. Cai *et al.* extended this work by demonstrating decreased eNOS dimer:monomer ratio and decreased intracellular BH4 and total biopterin content, implying decreased biopterin synthesis by GTPCH [76]. Furthermore adenoviral gene transfer of GTPCH restored eNOS dimer:monomer ratio, increased NO production, and decreased eNOS-derived superoxide.

The Meininger group isolated coronary endothelial cells from the spontaneously diabetic BioBreeding rat, a model of type 1 diabetes mellitus, and found significant BH4 deficiency compared to control cells [77]. Impaired NO production was restored by incubation with sepiapterin. Similarly, impaired EDR in a rat model of streptozotocin-induced (STZ) diabetes was ameliorated by the BH4 analogue 6-methyl-5,6,7,8-tetrahydropterin [78]. eNOS uncoupling was also demonstrated in the fructose-fed rat, a model of insulin resistance, as evidenced

1st author	Species	Tissue	Disease model	Intervention		Effect	Ref
Cai	Human	Endothelial cells	High glucose	GTPCH gene transfer	*In vitro*	↑eNOS dimer:monomer, ↑eNOS coupling	[56]
Meininger	Rat	Endothelial cells	Spontaneous diabetes	Sepiapterin incubation	*In vitro*	↑NO, ↑intracellular BH4	[77]
Pieper	Rat	Aorta	STZ diabetes	Methyltetrahydropterin incubation	*Ex vivo*	↑EDR	[78]
Shinozaki	Rat	Aorta	Insulin resistance	BH4 incubation	*Ex vivo*	↑eNOS coupling	[79]
Shinozaki	Rat	Aorta	Insulin resistance	Oral BH4	*In vivo*	↑eNOS coupling, ↑EDR, ↓lipid peroxidation	[80]
Alp	Mouse	Aorta	STZ diabetes	Transgenic GTPCH overexpression	*In vivo*	↑eNOS coupling, ↑EDR	[74]
Cosentino	Rat	Aorta	Spontaneous hypertension	BH4 incubation	*Ex vivo*	↑eNOS coupling, ↑EDR	[81]
Hong	Rat	Aorta	Spontaneous hypertension	Intraperitoneal BH4	*In vivo*	$\downarrow\cdot O_2^-$, ↑EDR, ↓BP, ↓iNOS expression	[82]
Kase	Rat	Aorta	Angiotensin-II hypertension	Oral BH4	*In vivo*	↑EDR, ↓NO production by iNOS, ↓NADPH oxidase subunit expression	[83]
Landmesser	Mouse	Aorta	DOCA-salt hypertension	Oral BH4	*In vivo*	↑eNOS coupling, ↑EDR	[84]
Zheng	Rat	Carotid artery	DOCA-salt hypertension	GTPCH gene transfer	*Ex vivo*	↑EDR, ↑NO, $\downarrow\cdot O_2^-$	[85]
Mitchell	Rat	Aorta	Glucocorticoid hypertension	Sepiapterin incubation	*Ex vivo*	↑EDR	[86]
Laursen	Mouse	Aorta	ApoE-KO hypercholesterolaemia	Sepiapterin incubation	*Ex vivo*	↑EDR, $\downarrow\cdot O_2^-$	[64]
Ozaki	Mouse	Aorta	ApoE-KO hypercholesterolaemia + eNOS overexpression	Oral BH4	*In vivo*	↓atherosclerosis, ↑eNOS coupling	[61]
Alp	Mouse	Aorta	ApoE-KO hypercholesterolaemia	Transgenic GTPCH overexpression	*In vivo*	↓atherosclerosis, ↑eNOS coupling, ↑EDR	[87]
Ali	Mouse	Vein graft	ApoE-KO hypercholesterolaemia	Transgenic GTPCH overexpression	*In vivo*	↓atherosclerosis, ↑eNOS coupling, ↓macrophage content, ↓MCP-1	[88]
Hattori	Mouse	Aorta	ApoE-KO hypercholesterolaemia	Oral BH4	*In vivo*	↓atherosclerosis, ↑eNOS coupling, ↑EDR, ↓eNOS & iNOS protein, ↓NADPH subunit expression, ↓adhesion molecules ↓MCP-1	[89]

Table 1-1: Summary of studies utilising increased BH4 to modulate vascular function in experimental models of disease

EDR, endothelium-dependent relaxations; STZ, streptozotocin; DOCA, deoxycorticosterone acetate; MCP-1, monocyte chemotactic protein-1.

by L-NAME-inhibitable superoxide production which was inhibited by *ex vivo* incubation with BH4 [79]. In comparison with control rats, fructose-fed rats had decreased GTPCH activity and aortic BH4, and increased levels of oxidised biopterins, associated with impaired EDR. Treatment with oral BH4 in these animals resulted in augmented aortic BH4, restored EDR and NO production, and decreased eNOS-derived superoxide [80]. Furthermore, oral BH4 decreased both lipid peroxidation and the binding of redox-sensitive transcription factors including nuclear factor-κB [80].

Alp *et al.* used a model of endothelial-specific overexpression of human GTPCH to produce a constitutive three-fold increase in aortic BH4 levels in mice [74]. Importantly, systemic levels of BH4, i.e. in plasma and liver, were not affected, thus excluding the potential confounding antioxidant effects of high-dose exogenous BH4 treatment used in other models. STZ-induced diabetes had no effect on GTPCH mRNA or total biopterins in WT mice, however all biopterins were present in an oxidised form, implying that the loss of BH4 in this model of diabetes is due to oxidation alone, rather than decreased synthesis. The resulting impaired EDR and increased eNOS-derived superoxide were restored to control levels by the GTPCH transgene [74].

1.4.2 Hypertension

Studies in the spontaneously hypertensive rat (SHR) [81-82] revealed increased aortic superoxide (inhibitable by L-NMMA) and H_2O_2, and impaired EDR to acetylcholine and A23187 compared to WT animals. One study reported similar endogenous levels of vascular biopterins in SHR and WT [81], whilst the other demonstrated reduced biopterin levels in the plasma of SHR [82], although later

studies in humans demonstrate that biopterin levels in plasma may not act as a reliable surrogate marker of endothelial biopterin availability [90]. Both these studies assayed biopterins via a high performance liquid chromatography (HPLC) technique that oxidises BH4 and BH2 to fully oxidised biopterin, therefore it is not possible to ascertain the relative levels of reduced (BH4) and partially oxidised (BH2) biopterins in these models. However, *ex vivo* incubation with BH4 restored eNOS coupling [81] and chronic intraperitoneal supplementation of BH4 suppressed the development of hypertension in these animals [82]. Interestingly, BH4 supplementation was also seen to suppress the increased basal NO production observed in SHR via decreased expression of iNOS [82], an observation replicated using oral BH4 in a rat model of angiotensin-II-induced oxidative stress [83].

Animals with deoxycorticosterone acetate-salt (DOCA-salt) hypertension have yielded further evidence of eNOS uncoupling as a potential mechanism underlying the impaired vascular function observed in hypertension. By using mice deficient in eNOS or nNOS, Landmesser *et al.* demonstrated that the endothelial isoform was the principal source of NOS-derived superoxide, and importantly that inactivation of NADPH oxidase was able to restore the decreased BH4:total biopterin ratio observed in these animals [84]. These data provide strong evidence to support superoxide-mediated BH4 oxidation as an initial step in eNOS uncoupling in hypertension. Furthermore, oral BH4 supplementation resulted in reduced L-NAME-inhibitable superoxide release, whereas there was no effect of tetrahydroneopterin, which is reported to have similar direct antioxidant properties to BH4, but no effect on eNOS coupling [91]. Additionally, adenoviral gene transfer of GTPCH has been shown to restore decreased GTPCH

activity in DOCA-salt rats, resulting in restored eNOS coupling and EDR [85], and sepiapterin has been shown to improve endothelial function in glucocorticoid hypertension [86].

1.4.3 Hypercholesterolaemia

In addition to the work by Ozaki *et al.* [61] described previously, further studies have investigated the effects of BH4 on atherosclerosis progression using the ApoE-KO mouse model of hypercholesterolaemia [87-89]. Endothelium-specific GTPCH transgenic overexpression was shown to significantly increase the diminished BH4:total biopterins ratio observed in ApoE-KO mice (71% vs 38%), resulting in improved eNOS coupling as evidenced by decreased endothelial and L-NAME-inhibitable superoxide, and augmented EDR. Aortic root plaques in GTPCH transgenic mice were less advanced at 16 weeks compared with ApoE-KO controls, demonstrating that atherosclerosis in the ApoE-KO mouse is modulated by NO-mediated endothelial function through endothelial BH4 availability [87]. Recently, the GTPCH transgenic mouse has been extended to a venous bypass graft model of accelerated atherosclerosis [88]. Experimental venous bypass grafting increases ROS formation that is implicated in inflammation and smooth muscle cell proliferation that leads to vein graft neointimal hyperplasia and accelerated atherosclerosis [92]. In addition to reducing both total and eNOS-derived superoxide, GTPCH overexpression significantly reduced vein graft neointimal hyperplasia and atherosclerosis at both 28- and 56-day timepoints. Furthermore, the inflammatory cell content of these lesions was decreased, specifically via attenuated CCR2-mediated cell signalling, in turn likely mediated through decreased oxidative stress [88].

Reductions in atherosclerosis have also been observed using oral BH4 supplementation [89]. Consistent with observations in hypertensive mice [82-83], BH4 was also shown to decrease both eNOS and iNOS protein levels, in addition to reduced expression of NADPH oxidase subunits, adhesion molecules and CC chemokines [89].

1.5 Human studies

With a plethora of evidence from experimental models implicating eNOS uncoupling as an important mediator of endothelial dysfunction and oxidative stress in conditions predisposing to atherosclerosis, it is not surprising that there has been significant attention given to the possibility that targeting BH4 in the endothelium might represent a realistic treatment strategy to prevent or treat vascular disease. Accordingly, many investigators have sought to elucidate the role of BH4 in human blood vessels. However, human studies have been limited by difficulties in obtaining vascular tissue, and by an inability to directly measure important biological processes *in vivo*, e.g. ROS production. Nonetheless, important evidence exists regarding the role of BH4 in regulating eNOS function in humans, which is reviewed in the following sections.

1.5.1 Studies of isolated blood vessels

Vasomotor studies of coronary arterioles dissected from atrial myocardial samples obtained during cardiac surgery demonstrated impaired EDR in subjects with atherosclerosis compared to those without; furthermore, *ex vivo* incubation with sepiapterin augmented EDR in these vessels [93]. The first demonstration of eNOS uncoupling in human endothelium, however, was in saphenous vein and internal mammary artery conduit samples obtained from patients undergoing

coronary artery bypass graft (CABG) surgery [94]. In this study, Guzik *et al.* reported not only increased vascular superoxide in patients with diabetes compared to those without, but more specifically an increased eNOS-derived component inhibited by L-NAME or endothelial denudation. Similar to L-NAME, sepiapterin incubation inhibited endothelial superoxide production, but there was no effect on superoxide production in the media or adventitia, implying that the action of sepiapterin was mediated via restored eNOS coupling [94].

Two studies performed in our laboratory more recently by Antoniades *et al.* [90, 95] provide further evidence to support the role of BH4 in human endothelium. The first study examined vascular tissue and plasma from 219 patients with atherosclerosis undergoing CABG surgery [90]. 80% of vascular BH4 was present within the endothelium. Patients with the highest levels of vascular BH4 demonstrated the greatest EDR to acetylcholine and the lowest eNOS-derived superoxide production. Furthermore, there remained significant differences in superoxide between patients in the highest and middle tertiles of vascular BH4 content, despite a similar BH4:total biopterins ratio in these groups, suggesting that absolute BH4 levels may be more important than BH4:total biopterin ratio *in vivo*. Surprisingly, there was no association between plasma and vascular BH4 levels, and furthermore there was an inverse relationship between plasma BH4 and EDR, with patients with high plasma BH4 having impaired NO-mediated endothelial function [90]. These data suggest that plasma and vascular compartments of biopterin availability may be regulated separately. Indeed, plasma levels of total biopterins correlated with levels of C-reactive protein (CRP), suggesting that the chronic inflammation associated with atherosclerosis may augment systemic biopterin synthesis through cytokine-mediated activation

of GTPCH [39], whereas inflammation within the vascular wall leads to loss of BH4, perhaps through oxidation.

The second study utilised the concept of "Mendelian randomisation" [96] to determine the effect of genetic variation in GTPCH activity on BH4 levels and endothelial function [95]. A haplotype of the *GCH1* gene (defined by 15 single nucleotide polymorphisms (SNP) and denoted as 'X'), which encodes GTPCH, was previously demonstrated to confer reduced induction of *GCH1* expression in leucocytes following inflammatory stimulation [97]. Amongst patients undergoing CABG surgery, homozygote X haplotype carriers exhibited significantly lower *GCH1* expression and vascular BH4 content, associated with increased eNOS-derived superoxide and impaired EDR [95]. These data provide further evidence to implicate GTPCH and BH4 levels as important regulators of eNOS function in the human vasculature.

1.5.2 Clinical Studies

In addition to the small number of *ex vivo* human studies described in the previous section, there are a larger number of *in vivo* clinical studies using pharmacological supplementation of BH4 to modulate endothelial function. These studies are summarised in Table 1-2. The majority of these studies involved either acute intra-arterial administration of BH4 into either the brachial or coronary arteries, or single dose oral treatment. These studies have frequently reported an improvement of NO-mediated endothelial function in a number of disease states linked with the development of atherosclerosis, although the chief limitation of the methodology is the high doses of BH4 used. Forearm plethysmography protocols using typical infusion rates of 400-500μg/min or

1st author	Clinical setting	Number of subjects	Route of administration	Duration	Effect	Ref
Stroes	Hypercholesterolaemia	26	Brachial artery	Acute infusion	Restores FBF responses to L-NMMA and 5-HT	[98]
Fukuda	Hypercholesterolaemia	18	Coronary artery	Acute infusion	Restores CBF response to ACh and L-NMMA	[99]
Cosentino	Hypercholesterolaemia	22	Oral	4 weeks	Restores FBF response to ACh; ↓plasma F_2-isoprostanes	[100]
Higashi	Hypertension	16	Brachial artery	Acute infusion	Restores FBF response to ACh	[101]
Heitzer	Smokers	58	Brachial artery	Acute infusion	Restores FBF response to ACh	[91]
Ueda	Smokers	17	Oral	Single dose	Improves brachial FMD	[102]
Ihlemann	Glucose challenge (healthy subjects)	39	Brachial artery	Acute infusion	Prevents impairment of FBF response to 5-HT	[103]
Heitzer	Type 2 Diabetes	35	Brachial artery	Acute infusion	Improves FBF response to ACh	[104]
Nystrom	Type 2 Diabetes + CAD	32	Brachial artery	Acute infusion	Increases insulin sensitivity; no effect on brachial FMD	[105]
Maier	CAD	19	Coronary artery	Acute infusion	Prevents vasoconstrictor response to ACh	[106]
Worthley	CAD	47	Coronary artery	Acute infusion	No change in CBF	[107]
Fukuda	Vasospastic angina	28	Coronary artery	Acute infusion	Prevents vasoconstrictor response to low-dose ACh; does not prevent coronary spasm in response to high-dose ACh	[108]
Setoguchi	Coronary endothelial dysfunction	15	Coronary artery	Acute infusion	Restores CBF response to ACh	[109]
Setoguchi	Heart failure	21	Brachial artery	Acute infusion	Improves FBF response to ACh	[110]
Mayahi	Ischaemia-reperfusion injury	48	Brachial artery	Acute infusion	Restores FBF response to ACh following ischaemia-reperfusion	[111]
Higashi	Aging	37	Brachial artery	Acute infusion	Augments FBF response to ACh	[112]
Eskurza	Exercise/aging	31	Oral	Single dose	Restores brachial FMD in older, sedentary subjects	[113]

Table 1-2: Summary of BH4 clinical studies

CAD, coronary artery disease; FBF, forearm blood flow; CBF, coronary blood flow; FMD, flow-mediated dilatation; ACh, acetylcholine; 5-HT, serotonin; L-NMMA, N-monomethyl-L-arginine.

more have demonstrated elevations of plasma BH4 concentrations to between 50-100μM [110-111]; in contrast, plasma BH4 levels in healthy subjects [114] and patients with atherosclerosis [90] have been reported to lie within a range of approximately 10-50nM. Thus the use of supraphysiological BH4 doses may be confounded by non-specific effects, including a direct antioxidant effect on the vessel wall. Some investigators have attempted to account for these effects by comparing the effects of BH4 to other known antioxidants. For example, BH4 restored endothelial function in chronic smokers, whereas tetrahydroneopterin (NH4, which has equipotent antioxidant capability *in vitro*) had no effect, suggesting restored eNOS coupling as the underlying mechanism [91]. Similarly, the 6S-stereoisomer of BH4 showed no effect on endothelial function following a glucose challenge [103]. Whilst these elegantly controlled pharmacological studies have suggested a specific role for BH4, others do not. For example the amelioration of forearm ischaemia-reperfusion injury by 6R-BH4 was also observed with 6S-BH4 and NH4, implying a more non-specific antioxidant effect [111].

To date, there is only one published randomised, placebo-controlled clinical trial of chronic oral BH4 supplementation in vascular function [100]. This study reported an improvement in NO-mediated endothelial function after four weeks of treatment, although it is not possible to be sure whether this effect is due to alteration in eNOS coupling or an alternative mechanism; a reduction in plasma F_2-isoprostanes, a marker of oxidative stress, implies a favourable effect on systemic antioxidant status. One further study showed an improvement in blood pressure with oral BH4 in hypertensive patients, however this study was neither randomised nor placebo-controlled [115].

1.6 Other cardiovascular roles of BH4

In addition to its role in regulating eNOS, BH4 also acts as a co-factor in other enzyme systems, including the two other NOS isoforms (nNOS and iNOS) and the aromatic amino acid hydroxylases (phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH)) [116]. Deficiency in BH4, therefore, may result in hyperphenylalaninaemia and decreased synthesis of the neurotransmitters dopamine, noradrenaline, adrenaline and serotonin (Figure 1-3). Indeed, mutations in genes encoding BH4 synthetic enzymes result in a heterogeneous group of rare disorders characterised by varying degrees of hyperphenylalaninaemia and dystonia which may respond to BH4 therapy [116]. The role of these enzyme systems in cardiovascular pathophysiology is becoming more evident; accordingly, BH4 may be an important regulator of these biological pathways in relation to several cardiovascular disease states.

1.6.1 Role of nNOS in vascular function

The source of NO that mediates vascular tone has, until recently, been presumed to be eNOS, however a specific inhibitor of nNOS suitable for human use (S-methyl-L-thiocitrulline, SMTC) has allowed distinct roles of eNOS and nNOS to become apparent. In healthy subjects, SMTC infusion resulted in a similar reduction in basal forearm blood flow (FBF) to the non-specific NOS inhibitor L-NMMA [117]. In contrast, L-NMMA attenuated the acetylcholine-mediated increase in FBF, whereas SMTC did not. Therefore, it appears that nNOS-derived NO has a greater role in determining basal NO release, and hence resting arterial tone, whereas eNOS-derived NO mediates the response to agonist stimulation. This may explain why basal NO release does not correlate well with acetylcholine-mediated endothelial function, and why conditions associated with endothelial

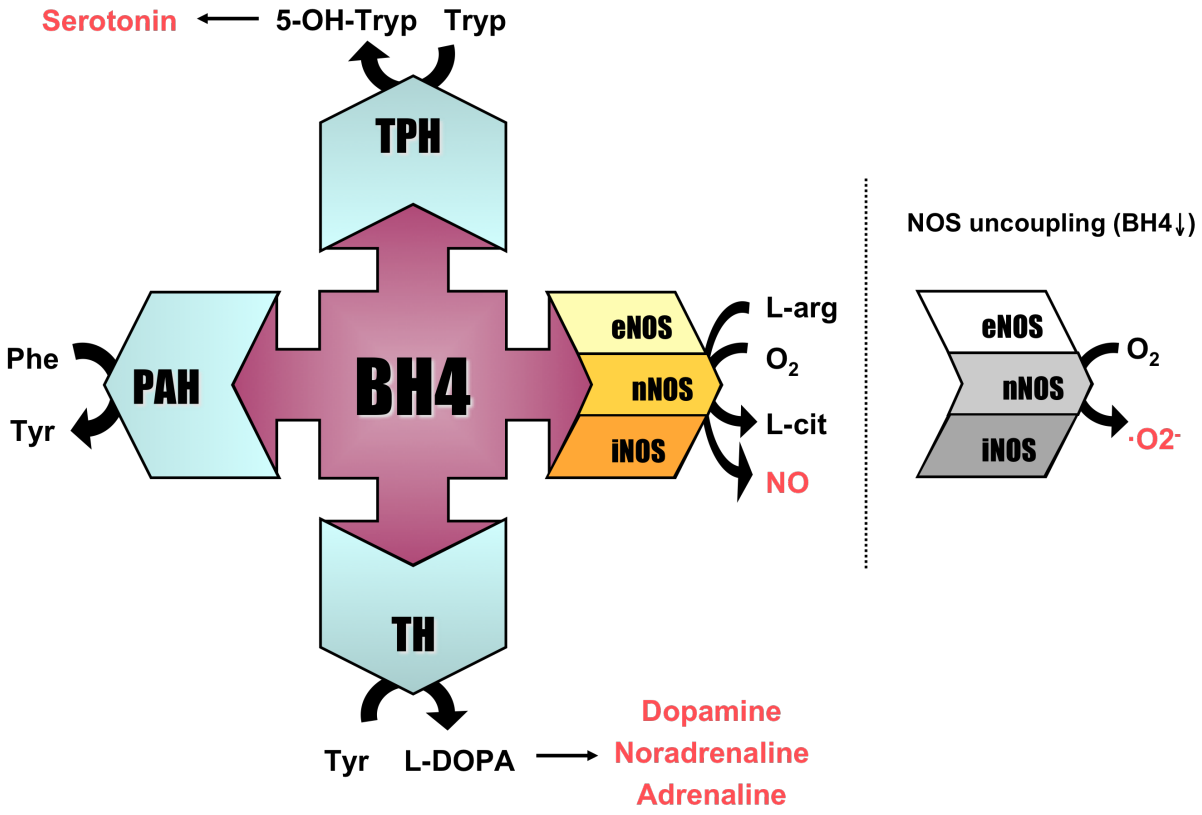


Figure 1-3: Enzyme co-factor activity of tetrahydrobiopterin
BH4 is required for NO production by all three nitric oxide synthase isoforms (gold arrowhead). Under normal conditions these enzymes couple oxidation of the amino acid substrate L-arginine with reduction of molecular oxygen to form NO and L-citrulline. When BH4 is limiting, either through decreased synthesis or increased oxidation, enzymatic uncoupling occurs (grey arrowhead), resulting in the production of superoxide from the unopposed reduction of molecular oxygen, rather than NO. BH4 also has co-factor activity for the aromatic amino acid hydroxylases (blue arrowheads), resulting in neurotransmitter synthesis and preventing the accumulation of phenylalanine. BH4, tetrahydrobiopterin; L-arg, L-arginine; L-cit, L-citrulline; NO, nitric oxide; ·O2-, superoxide; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; Phe, phenylalanine; Tyr, tyrosine; Tryp, tryptophan; 5-OH-Tryp, 5-hydroxytryptophan.

dysfunction, such as hypercholesterolaemia, do not always co-exist with hypertension [117]. As BH4 has been proposed as a novel anti-hypertensive agent [115], further studies to define the relationship between BH4 and specific NOS isoforms in the vascular wall would be informative.

1.6.2 Ischaemia-reperfusion injury and ischaemic preconditioning

Ischaemia-reperfusion injury (IRI) is a common clinical scenario following pharmacological or mechanical reperfusion. IRI is characterised by myocardial necrosis and endothelial dysfunction and may be in part mediated by excessive production of reactive oxygen species (ROS). In rats, myocardial ischaemia leads to depletion of cardiac BH4, decreased NO synthesis and increased superoxide production by NOS which can be partially reversed by exogenous BH4 [118]. Similarly, post-ischaemic coronary endothelial dysfunction can be ameliorated by BH4 analogues *ex vivo* in pigs [119], and incubation with BH4 lessens myocardial IRI in the isolated rat heart [120]. In humans, intra-brachial infusion of 6R-BH4 prevented endothelial IRI in the forearm, although both the 6S-BH4 stereoisomer and NH4 (which both lack NOS cofactor activity but possess similar antioxidant capacity to 6R-BH4) achieved similar results, implying that this effect may be due to a non-specific antioxidant effect rather than improved eNOS coupling [111].

Perhaps more interesting is the role of BH4 in the phenomenon of late phase ischaemic pre-conditioning (IPC), which attenuates IRI. NO derived from both constitutive NOS (early phase) and iNOS (late phase) has been implicated as a mediator of the infarct-reducing effect of IPC [121]. In rabbits, Tang *et al.* demonstrated that left coronary artery IPC resulted in increased myocardial BH4 content in the ischaemic territory following IRI, but not in the non-ischaemic

territory [122]. Furthermore, pharmacological inhibition of BH4 synthesis (using N-acetylserotonin to inhibit sepiapterin reductase) abolished both the rise in BH4 and the infarct-limiting effect of late IPC. Additionally, the presence of hypercholesterolaemia abolished both the BH4 upregulation and infarct-limitation effects of IPC; the mechanism underlying this is unclear, although it is postulated that excess ROS production associated with hypercholesterolaemia may interfere with BH4 bioavailability [122]. More recently, similar findings have been observed in rats [123]. This study reproduced the finding that inhibition of BH4 synthesis (on this occasion using DAHP to inhibit GTPCH) could abolish the protective effect of IPC on the myocardium, and also demonstrated that IPC increases cardiac BH4 via upregulation of *GCH1*. Furthermore, IPC increased myocardial levels of TNFα, providing a possible explanation for this observation [123]. As yet there are no studies in humans addressing the effect of BH4 on myocardial IRI, or the effect of IPC on BH4 bioavailability.

1.6.3 Myocardial function and cardiac hypertrophy

Oxidative stress plays a key role in cardiac biology, particularly in modulating the adaptive responses of hypertrophy and dilatation to pressure overload (reviewed in [124]). eNOS and nNOS are expressed constitutively in myocardial tissue. In a mouse model of transverse aortic constriction (TAC), which results in marked cardiac hypertrophy, dilatation, fibrosis and impaired systolic function, the finding of decreased calcium-dependent NO production and increased NOS-derived superoxide in wild-type animals, but not in mice lacking eNOS, provides evidence of eNOS uncoupling in this pathological state [125]. Furthermore, eNOS-knockout animals exhibited no dilatation or impairment of systolic function, and milder hypertrophy. Co-treatment with oral BH4, but not NH4,

abolished the increase in NOS-derived superoxide and prevented the development of hypertrophy induced by TAC [125], and, in a separate study, reversed established hypertrophy following TAC [126].

Most recently, Silberman *et al.* studied a mouse model of unilateral nephrectomy, subcutaneous implantation of DOCA and saline feeding [127]. This combination of treatments results in mild hypertension with diastolic dysfunction in the absence of hypertrophy and systolic dysfunction, thus allowing characterisation of a more subtle phenotype than in the TAC experiments. These mice had increased cardiac oxidative stress compared with controls; treatment with the selective nNOS inhibitor 7-nitroinidazole (7N) resulted in greater inhibition of superoxide production than with the non-selective NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), implicating nNOS as the principal source. Total biopterin levels were similar between DOCA and control groups, but the ratio of reduced to oxidised biopterins was reduced in DOCA animals, implying that BH4:BH2 ratio may be important in regulating nNOS as well as eNOS. Feeding with BH4 improved total cardiac BH4 levels and redox status, and BH4 (but not NH4) both prevented and reversed diastolic dysfunction. Furthermore, BH4 treatment augmented the phosphorylation status of phospholamban, a key regulator of sarcoplasmic reticulum calcium pump activity and cytosolic calcium levels, in DOCA animals, suggesting a possible underlying mechanism [127].

1.6.4 Pulmonary hypertension

Pulmonary hypertension, a fatal disease, is characterised by vasoconstriction and structural remodelling of distal pulmonary arterioles that leads to right ventricular hypertrophy and, ultimately, right ventricular failure. Findings from

mice deficient in eNOS have implicated reduced endothelial NO bioavailability as a key pathological process in this disease. These animals exhibit increased susceptibility to hypoxia-induced pulmonary hypertension [128] which is in part ameliorated by eNOS gene transfer [129].

Two independent and contemporaneous studies have demonstrated a pivotal role of BH4 in modulating eNOS activity in the pulmonary vasculature [130-131]. The hyperphenylalaninaemic (hph-1) mouse mutant has a systemic 90% reduction in GTPCH expression and activity, with a corresponding decrease in BH4 bioavailability. Compared with WT control animals, hph-1 mice exhibited a pulmonary hypertension phenotype, characterised by increased muscularisation of distal pulmonary arterioles, increased right ventricular mass (in the absence of left ventricular hypertrophy), and increased right ventricular systolic pressure, even in the presence of normoxia [130-131]. Furthermore, the histological and physiological features of pulmonary hypertension were inversely correlated with BH4 levels in lung tissue, and were exaggerated in the presence of chronic hypoxia [130].

Endothelium-specific transgenic overexpression of *GCH1* normalised both lung BH4 content and susceptibility to pulmonary hypertension in hph-1 mice. Despite no differences in levels of eNOS protein, hph-1 animals exhibited increased eNOS-derived superoxide production (indicating eNOS uncoupling) which was normalised by the *GCH1* transgene [130]. This is important as ROS have been implicated in proliferation of pulmonary smooth muscle cells [132]. Furthermore, in the presence of acute hypoxia, hph-1 mice demonstrated decreased pulmonary vasoconstriction in response to pharmacological NOS inhibition by L-NAME, further implicating eNOS-derived superoxide as a key mediator of pulmonary

hypertension [130]. This finding was similarly reversed by overexpression of *GCH1*; more recently, exogenous BH4 has been shown to ameliorate hypoxia-induced pulmonary vasoconstriction [133]. Thus, there is convincing evidence of the role of BH4 in experimental pulmonary hypertension, and targeting BH4 may represent a rational therapeutic approach.

1.6.5 Cardiovascular autonomic function

Alterations in autonomic control of the heart are present in numerous cardiovascular disease states, including heart failure and following myocardial infarction. Furthermore, abnormal clinical measures of autonomic function, such as reduced baroreflex sensitivity and heart rate variability, are predictive of adverse events in these conditions [134], possibly due to an increased propensity to ventricular arrhythmias as a consequence of increased sympathetic drive. Endogenous NO appears to modulate both sympathetic and parasympathetic cardiac control (reviewed in [135]); overall, data from animal studies suggest that, under normal conditions, NO has an inhibitory effect on sympathetic activity whilst increasing parasympathetic (vagal) tone. Furthermore, diseases such as diabetes mellitus and heart failure that are characterised by decreased endothelial NO bioavailability, are also associated with autonomic dysfunction.

BH4 may be an important mediator of cardiovascular autonomic function as it has a critical role in both TH and NOS catalysis, thus regulating both L-DOPA-derived and NO neurotransmitter activity. A study of genetic variation in *GCH1* by Zhang *et al.* identified a further *GCH1* single nucleotide polymorphism (SNP), C+243T, that is associated with decreased baroreflex sensitivity, decreased heart rate variability and higher minimum heart rate in humans [136]. This SNP was

also associated with decreased urinary catecholamine excretion and nitrite/nitrate excretion, implying decreased NO synthesis, although this may be confounded by dietary intake. Furthermore, this SNP had a significant association with higher blood pressure in women. Although this study did not measure BH4 levels, this SNP is in tight linkage disequilibrium [137] with the 'X' *GCH1* haplotype described by Tegeder *et al.* [97], and thus it can be implied that the observed autonomic phenotype is mediated by BH4. However, these findings are from relatively small numbers of subjects and have not yet been replicated in larger studies. Furthermore very large genome-wide association studies have not identified *GCH1* as a candidate locus for hypertension or cardiovascular outcomes such as myocardial infarction [138].

1.6.6 Septic shock and inflammation

Septic shock carries a high mortality and is characterised by profound hypotension secondary to refractory vasodilatation coupled with impaired myocardial contractility. Excessive production of NO by iNOS, which is induced principally by lipopolysaccharide (LPS) and the pro-inflammatory cytokines TNFα and interleukin 1β (IL-1β), is likely to mediate both of these phenomena, although systemic NOS inhibition does not improve survival in these patients [139]. iNOS is dependent on BH4 co-factor activity for NO synthesis [140], and BH4 synthesis is induced by cytokines, in parallel with iNOS expression, via upregulation of GTPCH [39]. Furthermore, glucocorticoids have been shown to almost completely inhibit NO production via suppression of the GTPCH-BH4 axis in cells stimulated with cytokines, despite only modest reductions in iNOS protein [141], implicating BH4 as a key mediator in septic shock. Circulating BH4

levels are higher in patients with septic shock compared to critically ill non-septic control subjects [142].

In an experimental animal model of septic shock, pharmacological inhibition of BH4 synthesis by DAHP reduced NO production and ameliorated hypotension despite no change in induction of iNOS [143]. A further animal study demonstrated that an increase in circulating biopterins preceded the increase in nitrate production [144]. Thus, inhibition of BH4 synthesis may be a rational target to limit iNOS-mediated septic shock.

1.7 Aims of this thesis

The literature summarised in the sections above has demonstrated a pivotal role of BH4 in NOS-mediated endothelial function in experimental cellular and animal models of vascular disease. Recent work in humans has indicated an important role of *endogenous* BH4 in the endothelium of humans with established atherosclerosis, both via observational studies [90] and, using a Mendelian randomisation approach, via genetic variation in BH4 synthesis [95]. However, the effect of *exogenous* BH4 in humans is less clear, and more specifically, very little is known about the potential of chronic oral BH4 therapy as a therapeutic strategy in vascular disease. Furthermore, the role of *endogenous* BH4 in humans without overt atherosclerotic disease is unknown.

Accordingly, this thesis aims to address the overarching hypothesis that increases in BH4 bioavailability – either by exogenous supplementation or by increased endogenous synthesis – leads to improved NO-mediated endothelial function.

In order to address this hypothesis, the experiments performed sought to investigate two specific aspects of BH4 homeostasis:

1. To determine the effect of *exogenous* BH4 in humans via a randomised, placebo-controlled clinical trial of oral BH4. Specifically, I aimed to:

    a. Determine the effect of oral BH4 therapy on vascular function using both *in vivo* and *ex vivo* techniques (Chapter 5).

    b. Investigate the mechanism of action of oral BH4 therapy by determining the pharmacokinetics and pharmacodynamics of this drug in both plasma and vascular tissue (Chapters 4 & 6).

2. To determine the effect of *GCH1* genetic variation on vascular function in healthy volunteers (Chapter 7). Specifically, in subjects without clinically overt vascular disease, I aimed to:

    a. Determine the effect of the 'X' *GCH1* haplotype on BH4 bioavailability.

    b. Determine the effect of the 'X' *GCH1* haplotype on vascular structure and function.

CHAPTER 2: GENERAL METHODS

2.1 Introduction

In this chapter I describe in detail the methods that I used in the experiments that form the results chapters (Chapters 4-7) discussed later in this thesis. These are broadly divided into imaging techniques used to assess vascular structure and function *in vivo*, and laboratory techniques for the *ex vivo* assays and experiments.

2.2 Clinical assessment of vascular function: an overview

The most robust data on drug efficacy come from trials that measure cardiovascular morbidity and mortality. However, such studies typically require very large numbers of participants to have sufficient statistical power, and necessitate enormous resources and expenditure. As such, studies using surrogate outcomes of arterial structure and function have been favoured to test novel therapies prior to undertaking larger scale clinical outcome trials. These surrogate measures are thought to represent an 'intermediate phenotype' in the development of cardiovascular disease. Work in this thesis utilises a number of these measurements which are described in the sections below.

2.2.1 Brachial flow-mediated dilatation

Since first described by Celermajer *et al.* in 1992 [145], flow-mediated dilatation (FMD) of the brachial artery has become the most widely used method to assess endothelial function (or more specifically, endothelial NO bioavailability) *in vivo*. In brief, the technique involves induction of an increase in blood flow by inflation and subsequent release of a sphygmomanometer cuff on the forearm, and

measurement of the subsequent impact of this physiological stimulus on brachial artery diameter above the elbow. The response in the brachial artery is almost completely abolished by L-NMMA, a non-specific NOS inhibitor [146], demonstrating its dependence on local NO bioavailability.

Endothelial dysfunction, as defined by an impaired FMD response, has been shown to be associated with traditional cardiovascular risk factors, such as diabetes [147], hypertension [148], hypercholesterolaemia [149] and smoking [150]. It has also been shown to predict clinical outcomes, mainly in patients with established coronary or peripheral arterial disease [151-152]. It is important, however, to appreciate that changes in FMD reflect changes in endothelial function, which is only one of many factors influencing cardiovascular risk. Whilst most interventions that improve endothelial function have been shown to reduce morbidity and mortality, particularly notable exceptions include antioxidant vitamins and folic acid supplementation. Both have been shown to improve FMD [153-154], however large-scale clinical outcome trials have failed to show a significant benefit [155-156]. Thus, improvements in FMD do not necessarily translated into improved clinical outcomes, but FMD remains an attractive tool for assessment of NO bioavailability, which is particularly pertinent to studying the role of BH4 in the human vasculature.

2.2.1.1 FMD imaging modalities

The original FMD technique employed high-resolution ultrasound to image the brachial artery in the longitudinal plane [145]. This is the most established technique and expert consensus on methodology has been published [157]. More recently, magnetic resonance imaging (MRI) has been used and validated against

ultrasound, with good agreement between techniques and similar reproducibility in healthy subjects [158]. The main advantages of MRI over ultrasound are (i) that full 3-dimensional visualisation of the vessel is possible, enabling the imaging plane to be placed perpendicular to the vessel in a reproducible location and (ii) the ability to measure other parameters of vascular structure and function (e.g. aortic and carotid distensibility) as part of the same examination. The advantages of ultrasound are (i) a higher temporal resolution than MRI, allowing more precise determination of the 'peak of the curve' of dilatation, (ii) lower cost and (iii) better subject tolerability (e.g. no claustrophobia).

It is important to note, however, that the raw data yielded by the two techniques is not the same; ultrasound measures changes in vessel diameter in the longitudinal plane, whereas MRI measures changes in vessel cross-sectional area. Accordingly, an FMD value (i.e. percentage change from baseline) measured by the ultrasound technique will translate to a higher FMD value when measured by MRI, as vessel area is proportional to the square of the vessel radius.

In this thesis, MRI was used for the clinical trial of oral BH4 (Chapter 5), and ultrasound was used for the study of *GCH1* genotype on vascular function (Chapter 7).

2.2.2 Arterial stiffness

Endothelial dysfunction and arterial stiffness represent different aspects of the pathogenesis of arterial disease, although the two entities are not entirely distinct; in humans, measures of arterial stiffness correlate with measures of endothelial function [159], and local NO bioavailability has been shown regulate arterial elasticity in humans *in vivo* [160]. Similar to measures of endothelial

function, large artery stiffness, measured non-invasively by applanation tonometry (carotid-femoral pulse wave velocity (PWV) or augmentation index (AIx)) independently predicts cardiovascular risk in a variety of populations [161-163], and both tonometry measurements have been used as surrogate outcomes in interventional studies [164].

More recently, MRI has been used to study large artery stiffness [165]. Unlike carotid-femoral PWV, which represents a global estimate of overall arterial stiffness, MRI enables more subtle changes in regional stiffness, in that full 3-dimensional visualization of the vessel is possible allowing precise positioning of the image plane, as described with FMD above. Furthermore, blood flow data can be acquired simultaneously, and the path length (the distance between the two aortic locations) can be measured precisely.

As with FMD, MRI was used to measure arterial stiffness (aortic/carotid distensibility and aortic PWV) in the clinical trial of oral BH4 (Chapter 5), whereas carotid-femoral PWV and AIx were measured in the *GCH1* genotype study (Chapter 7) by applanation tonometry.

2.2.3 Carotid atherosclerosis

Measurement of carotid intima-media thickness (cIMT) was used in the *GCH1* genotype study (Chapter 7) to determine the effect of genetic variants on subclinical atherosclerosis in subjects without overt vascular disease. cIMT has been shown in numerous studies to be an independent predictor of cardiovascular risk [166]. As with FMD, consensus guidelines on methodology have been published [167]. Changes in cIMT have also been used as a surrogate

outcome in interventional studies [168], but this aspect was not utilised in this thesis.

2.3 MRI vascular imaging protocols

Building on earlier work done in our research group, I aimed to use vascular MRI to obtain a global assessment of vascular function in the participants of the clinical trial of oral BH4 (Chapter 5). All MRI scans were performed on a 1.5-Tesla clinical magnetic resonance scanner (Siemens Sonata, Erlangen, Germany) with subjects in the supine position.

2.3.1 Overview

In the clinical trial, vascular function was quantified by high-resolution MRI at baseline and at the end of the treatment period. Images of the aorta and carotid arteries were used to determine arterial distensibility and aortic PWV as indices of vascular stiffness. Arterial distensibility was determined in the ascending aorta, proximal descending aorta, distal descending aorta, and both common carotid arteries using a high resolution gradient-echo pulse sequence. A velocity-encoding gradient for phase-contrast MRI was applied to determine PWV in the aorta.

Brachial FMD was used as a measure of endothelial function. Using cross-sectional images of the brachial artery piloted in three dimensions, FMD was defined as the maximal percentage change in luminal area from baseline following five minutes forearm ischemia; endothelium-independent dilation was similarly quantified following administration of 200 mcg sublingual glyceryl trinitrate (GTN).

2.3.2 Aortic image acquisition

For aortic imaging, a combination of a two-element-array surface coil placed on the chest and a spine-coil-array within the patient bed was used (Figure 2-1). The heart was positioned at the isocentre of the magnet in order to maximise image quality. After obtaining localiser images in expiratory breath hold, images were obtained in the following sequence:

2.3.2.1 Transverse HASTE:

Imaging parameters: 8mm slice with 2mm gap

In plane resolution 2.4 x 1.4 mm

TR/TE = 800/25, FOV = 360/100

Free breathing, trigger

Using sagittal localiser position

The second slice was positioned to sit on top of the aortic arch. Sufficient slices were obtained (approx 24) to image down into the abdomen.

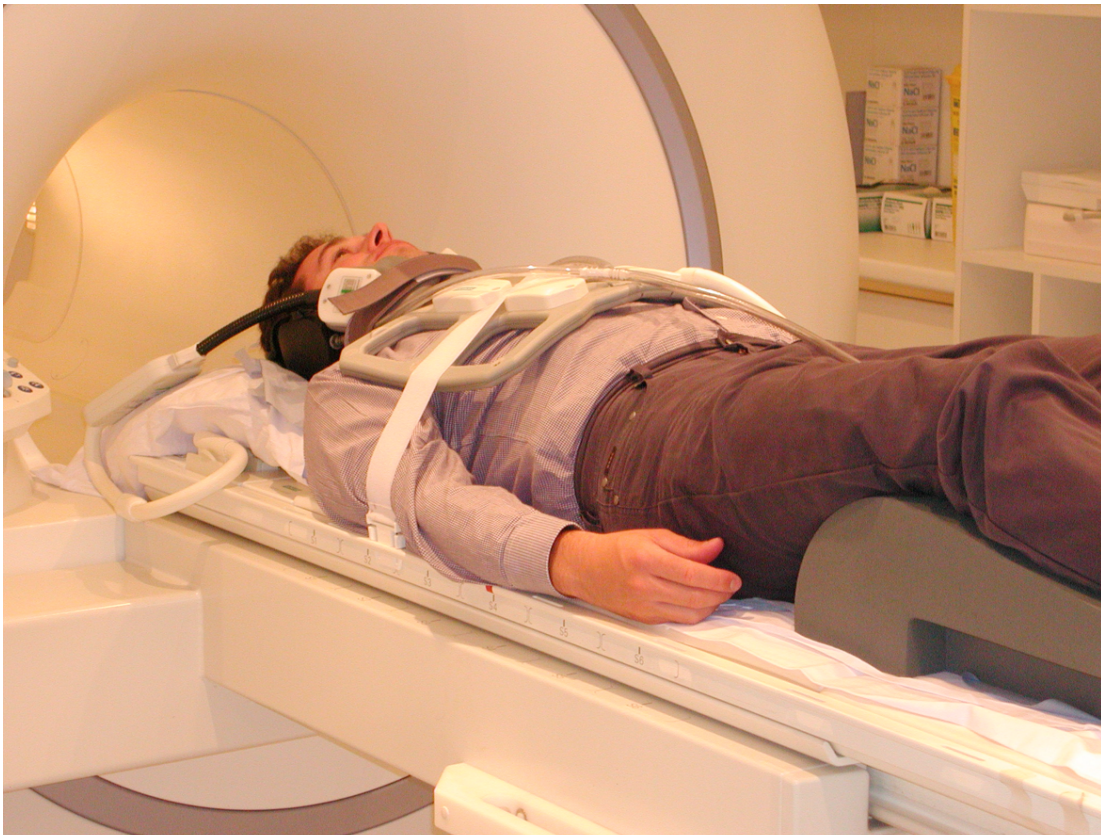


Figure 2-1: Positioning of surface coils for aortic and carotid image acquisition by MRI

Subjects lay in the supine position on the scanner bed which contained the spine coil array. A two-element-array surface coil was placed on the anterior chest wall. Machnet phased array coils were placed on either side of the neck with the head held in a foam support. Body and carotid coils were held in place by velcro straps to minimise movement.

2.3.2.2 Oblique Sagittal HASTE

Imaging parameters: 6mm slice with no gap

In plane resolution 2.5 x 1.5 mm

TR/TE = 800/25, FOV = 360/100

The sequence was planned from the transverse HASTE images. A stack of 7 slices through ascending aorta, arch and descending aorta were then acquired.

2.3.2.3 Flow Sequence

Aortic flow and compliance data are acquired at two levels (Figure 2-2):

i) At the level of the pulmonary artery to assess flow in the ascending aorta and proximal descending aorta. The pulmonary artery is used as an anatomical landmark in order to pilot image sequences acquired in the same patient at different timepoints (Figure 2-2).

ii) In the upper abdominal aorta a set number of slices below the pulmonary artery to assess flow in the abdominal aorta. A set number of slices were used in order to allow images to be acquired in the same anatomical position at the time of the second vascular MR scan.

Imaging parameters: Prospective gating, non-breath-hold at two levels defined above

Through plane velocity measurement (V_{enc} = 200cm/s)

5mm slice thickness; in plane resolution 1.3 x 1.3 mm

FOV = 320/75, TR/TE = 11/2.8

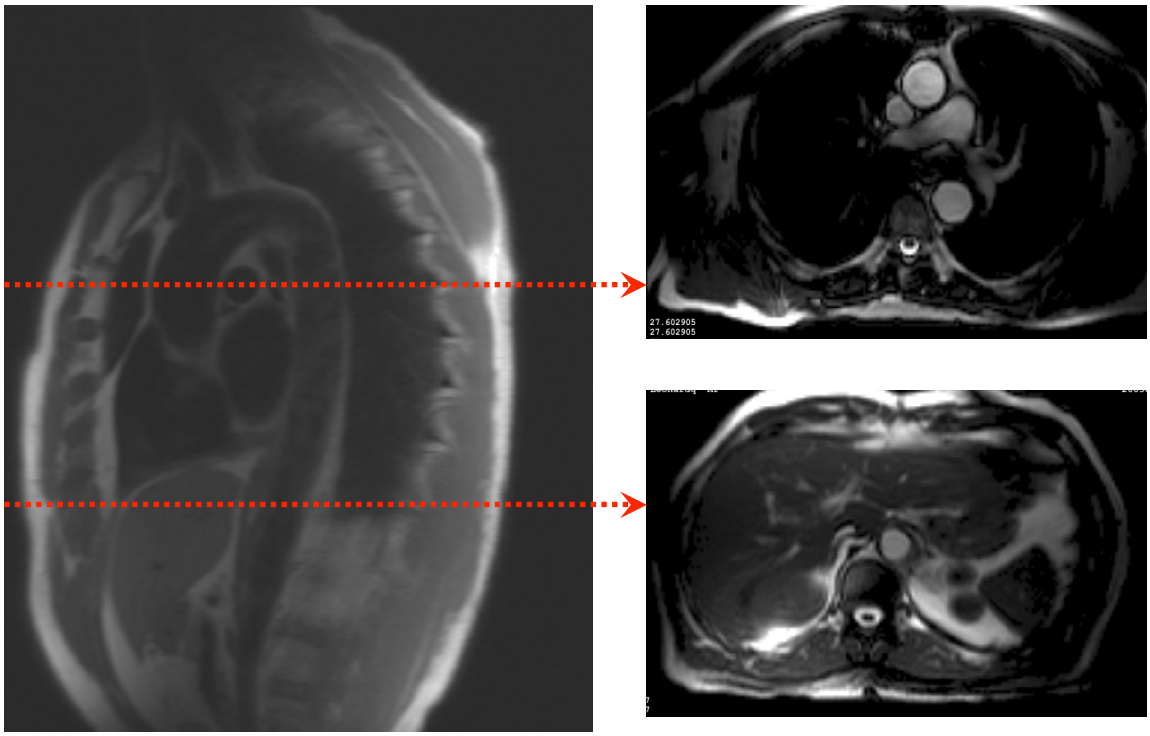


Figure 2-2: Imaging planes for measurement of aortic distensibility by MRI
An oblique sagittal HASTE image of the aorta was obtained (left panel). TrueFISP cine images were obtained at the level of the right pulmonary artery (upper red arrow, for ascending aorta and proximal descending aorta) and a set number of slices below in the abdominal aorta (lower red arrow, for the distal descending aorta).

2.3.2.4 TrueFISP cine

Vascular distensibility of the aorta was assessed in the ascending aorta, proximal descending aorta and distal descending aorta using a TrueFISP (fast imaging with steady state free precession) cine sequence. The anatomical position of the images was identical to those for the flow sequence mentioned above (Figure 2-2).

A transverse section is taken at the level of the pulmonary artery to enable aortic distensibility to be calculated in the ascending and proximal descending aorta. A transverse slice is obtained in the abdominal aorta for distensibility assessment. This slice is obtained a fixed number of slices below the pulmonary artery to enable comparisons to be made between different scans obtained at different timepoints.

Imaging parameters: Retrospective gated, breath-hold in expiration

7mm slice thickness, approx. 25 phases per cine

FOV = 380/87.5 (in plane resolution 2.8 x 2.0 mm)

2.3.3 Aortic stiffness calculation

2.3.3.1 Aortic pulse wave velocity

Flow measurements were analysed with Argus Software (Siemens, Erlangen, Germany). Pulse wave velocities (m/s) were calculated by dividing the distance between measurement levels (Δx, in this case from the ascending aorta to the distal descending aorta) by the time difference between the arrivals of the pulse waves at these levels (Δt) [165]. From the velocity encoded MR data, the velocity

of the aortic blood flow was determined. The differential of this velocity was best-fitted using a Gaussian distribution in order to determine the point of maximum change in velocity with greatest resolution (Figure 2-3).

2.3.3.2 Aortic distensibility

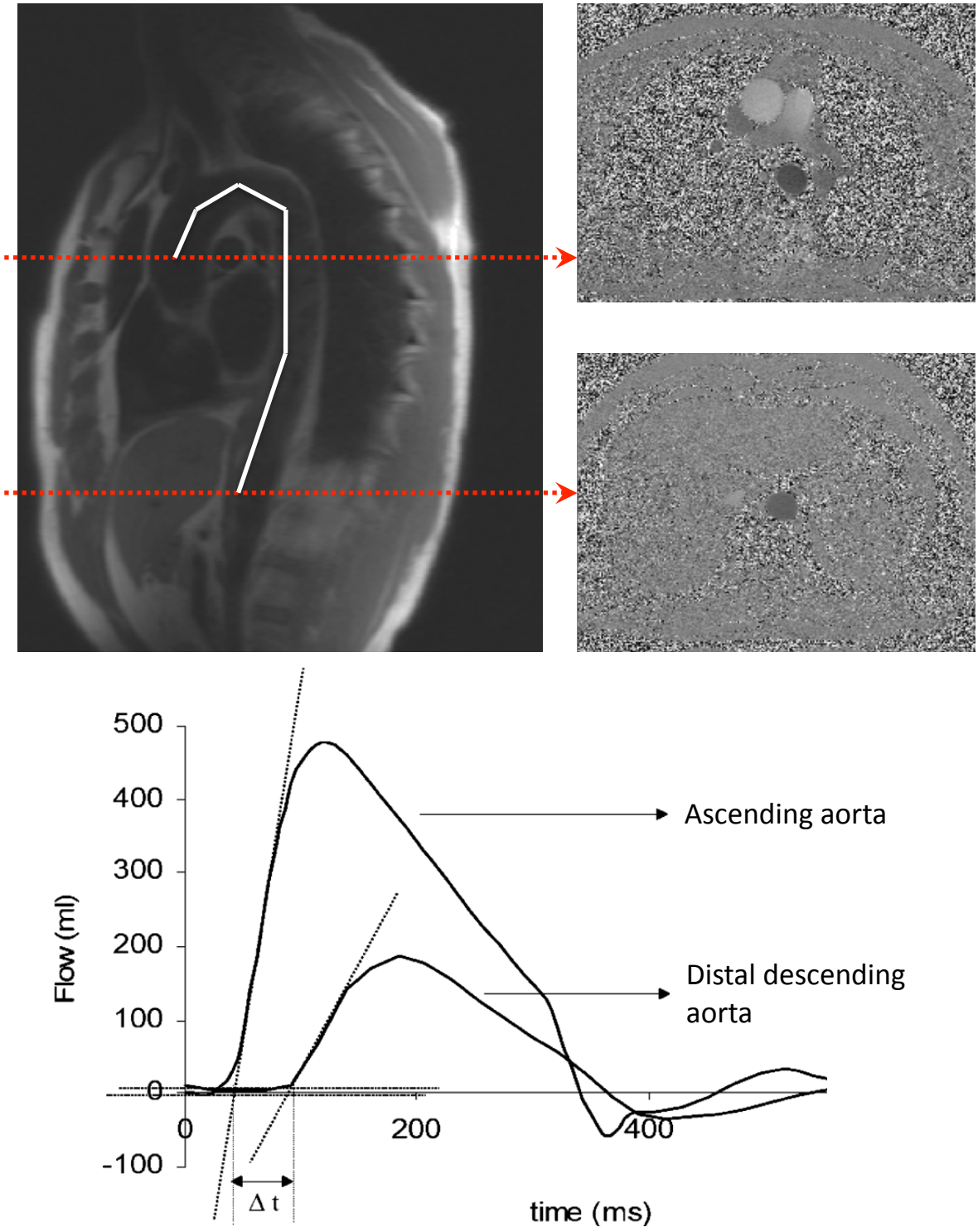
Non-invasive blood pressure was measured at the left brachial artery during TrueFISP cine acquisition. An automated edge-detection method, previously validated in our department, was implemented using Matlab software (The Mathworks, Inc, Natick, MA). Briefly, the data analysis method was based upon vessel wall unwrapping by interpolating onto 72 radial lines from the centre of the vessel at sub-pixel resolution, followed by a gradient detection algorithm which detects the gradient in the radial direction. The peak of this gradient image is traced around the circumference of the vessel automatically. Each frame of the cine sequence was analysed five times; a curve was plotted to demonstrate change in vessel area over the course of the cardiac cycle (Figure 2-4). Distensibility is calculated as $((A_{max} - A_{min})/A_{min})$/pulse pressure.

2.3.4 Carotid imaging

For carotid imaging, Machnet phased array carotid coils were placed on either side of the neck, roughly overlying the position of the common carotid arteries (Figure 2-1).

2.3.4.1 Time of Flight Sequence

A 3-dimensional time of flight sequence was used to locate the bifurcation of the common carotid artery into internal and external carotid arteries. Using the



adapted from van der Meer et al, *JCMR* 2007

Figure 2-3: Calculation of aortic pulse wave velocity by MRI
Axial velocity-encoded flow sequences (upper right panels) were obtained at the level of the right pulmonary artery (to assess flow in the ascending and proximal descending aorta) and a set number of slices below (to assess flow in the distal descending aorta). Flow vs time graphs (lower panel) were plotted using in-built Siemens software. Distance between points was measured using the oblique sagittal HASTE image (upper left panel, white line = Δx). Transit time (Δt) between the arrival of the pulse at each point was measured. PWV was calculated as $\Delta x/\Delta t$.

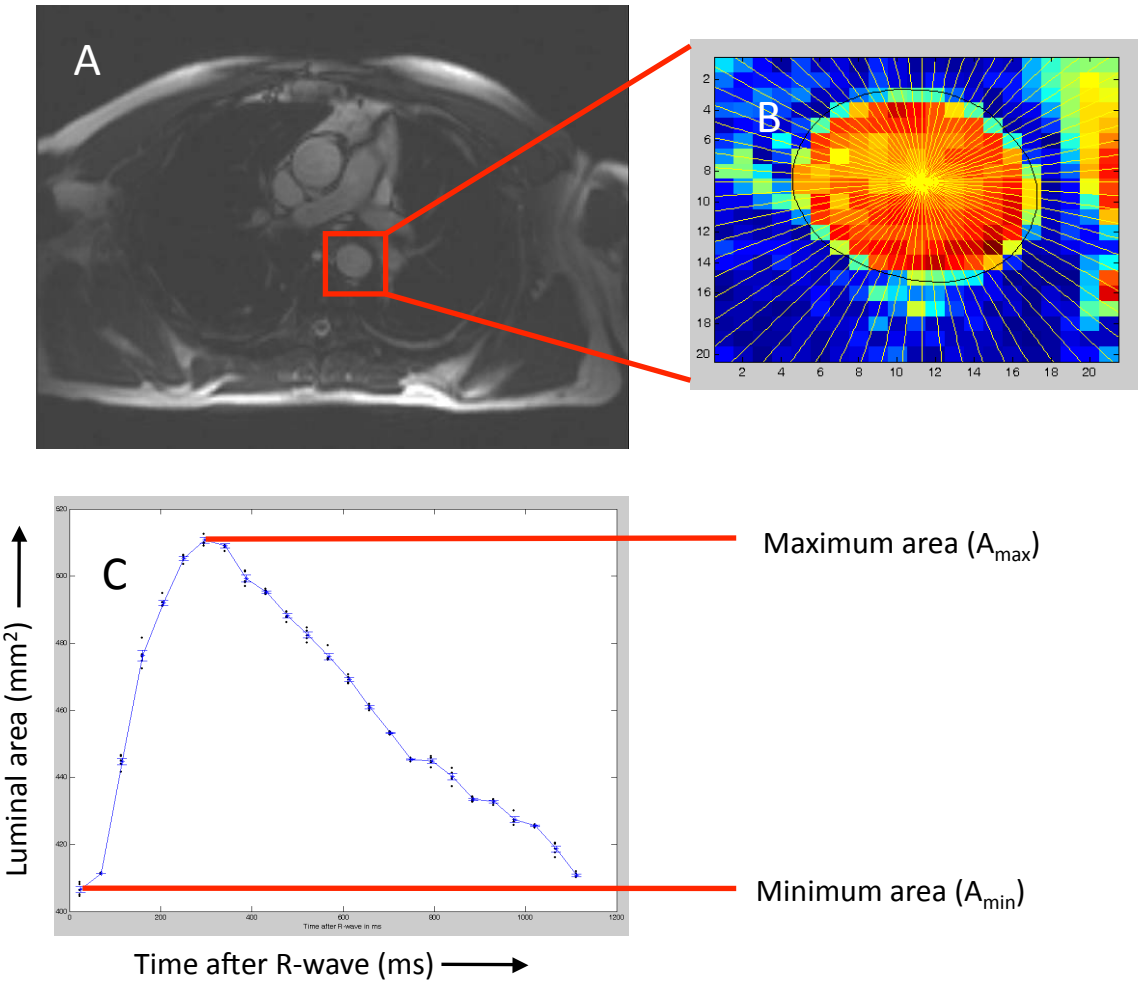


Figure 2-4: Method for calculation of arterial distensibility
A TrueFISP cine image of the target vessel was obtained (Panel A). Semi-automated edge detection software was used to define a region of interest around the target vessel (Panel B); five measurements of vessel luminal area were made in order to generate an area vs. time curve (Panel C), from which maximum and minimum luminal area can be derived. Time was measured relative to the R-wave on the surface ECG. Distensibility was calculated as $((A_{max} - A_{min})/A_{min})$/pulse pressure.

lowest bifurcation as an anatomical landmark, a transverse slice was taken 1cm below the lowest bifurcation.

Imaging parameters: FOV: 200/75; TR/TE = 25/7

60 overlapping slices, 1.5mm thick

In plane resolution 1.0 x 0.8 mm

2.3.4.2 TrueFISP cine images

To assess carotid distensibility, an image is acquired 1cm below the lowest carotid bifurcation.

Imaging parameters: Prospective gating, FOV = 200/65.5, TR/TE = 45.3/2.4

Slice thickness 3mm; in plane resolution 0.5 x 0.5

2.3.4.3 Carotid distensibility

Carotid distensibility was calculated from TrueFISP cine images described above. The method was the same as for aortic distensibility, using semi-automated edge-detection software (Figure 2-4).

2.3.5 Brachial imaging and measurement of FMD

Endothelium dependent and independent relaxation was assessed in the brachial artery. A Machnet phased array carotid coil was placed over the right brachial artery, with a standard blood pressure cuff being placed below the elbow according to standard FMD protocols (Figure 2-5) [145]. Whereas ultrasound-

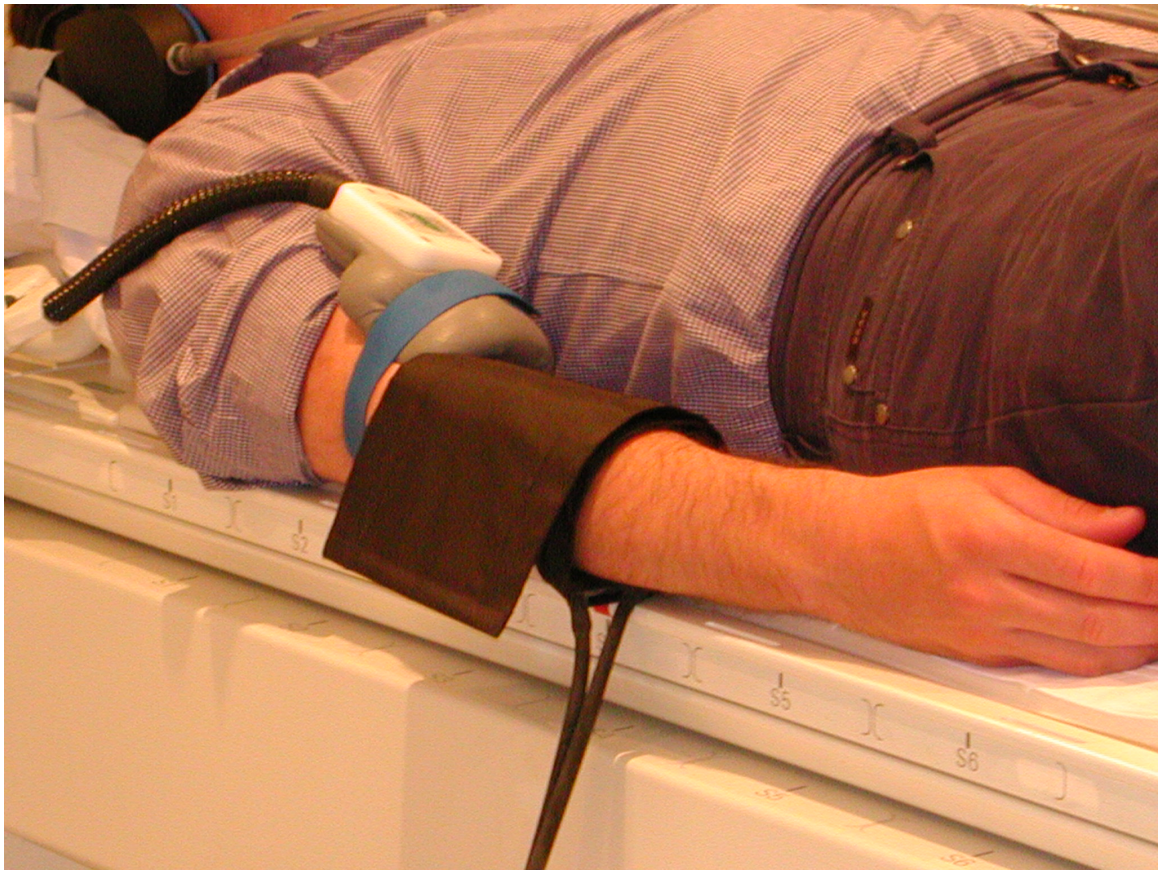


Figure 2-5: Setup for measurement of brachial flow-mediated dilatation by MRI
A Machnet phased array coil was held in place over the brachial artery above the elbow. A sphygmomanometer cuff was placed over the muscle bulk of the proximal forearm; this could be inflated from outside the magnet with the subject's arm at the isocentre of the magnet.

based FMD techniques measure brachial artery diameter, MR-based techniques determine vessel wall cross-sectional area, as described previously.

2.3.5.1 TrueFISP cine images

A 3-dimensional time of flight sequence was used to locate a straight portion of the brachial artery. A transverse section was then taken, the image position being saved for all subsequent images.

Imaging parameters: Prospective gating

FOV = 117/65, TR/TE = 56.3/3

Slice thickness 3mm; in plane resolution 0.3 x 0.3 mm

2.3.5.2 Calculation of FMD

The sequence of image acquisition is depicted in Figure 2-6. FMD was defined as the maximal percentage change in luminal area from baseline following five minutes forearm ischaemia; endothelium-independent dilation was similarly quantified following administration of 200 mcg sublingual GTN.

2.4 Ultrasound determination of vascular structure and function

2.4.1 Brachial FMD

Images of the brachial artery were acquired using a 7-10 MHz linear array probe and standard vascular imaging presets. The forearm was stabilised in a pre-formed channel and the probe fixed in position with a stereotactic clamp. ECG-gated longitudinal images of the brachial artery 5 to 10 cm above the antecubital

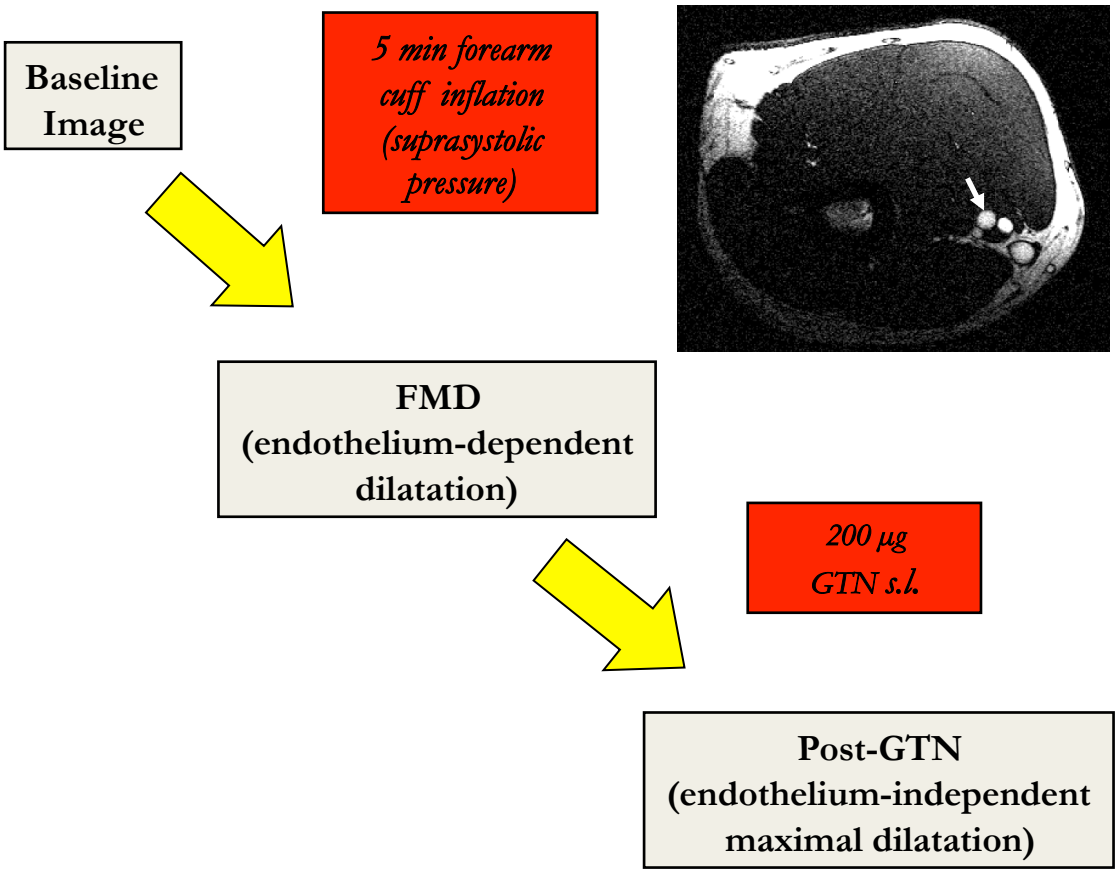


Figure 2-6: Image acquisition sequence for measurement of brachial flow-mediated dilatation by MRI

A baseline TrueFISP cine image of the brachial artery was obtained. The forearm cuff was then inflated to suprasystolic pressure for five minutes. Following cuff release, a stack of cine images of the brachial artery was recorded. Flow-mediated dilatation (FMD) was calculated as the percentage increase between baseline luminal area and maximum luminal area of the brachial artery following cuff release. After the brachial artery had returned to baseline following cuff release, subjects received 200 μg glyceryl trinitrate (GTN) sublingually. Endothelium-independent dilatation was calculated as the percentage increase between baseline luminal area and maximum luminal area following administration of GTN. A representative cine image is shown in the upper right panel with the brachial artery indicated by the white arrow.

fossa were stored for offline analysis using image analysis software (Vascular Analyser, MIA Inc, Iowa). A pneumatic cuff was placed around the forearm immediately distal to the elbow and inflated to 200 mmHg for 5 minutes. FMD (Figure 2-7) was calculated as the proportional change in brachial artery diameter in response to hyperaemic shear stress corrected for resting vessel diameter using the following formula: $(B_{60} - B_0)/B_0 \times 100$ (B0 = baseline brachial diameter; B60 = brachial diameter in response to hyperaemia at 60 seconds following cuff deflation). Endothelial-independent brachial artery dilatation using 200 mcg sublingual GTN was also measured using the brachial diameter 3 minutes following GTN administration.

2.4.2 Carotid intima-media thickness

The right and left common carotid arteries were imaged using high resolution ultrasound (Philips Sonos 5500 equipped with a 10 MHz linear array transducer) 1cm proximal to the carotid bifurcation to obtain the clearest demarcation of the intima-media layer in the posterior vessel wall. Images were stored and analysed offline using image analysis software (Vascular Analyser). Common carotid intima-media thickness (cIMT) was measured as the distance between the lumen-intima and media-adventitia interface and the mean of the right and left cIMT was used for analysis (Figure 2-8).

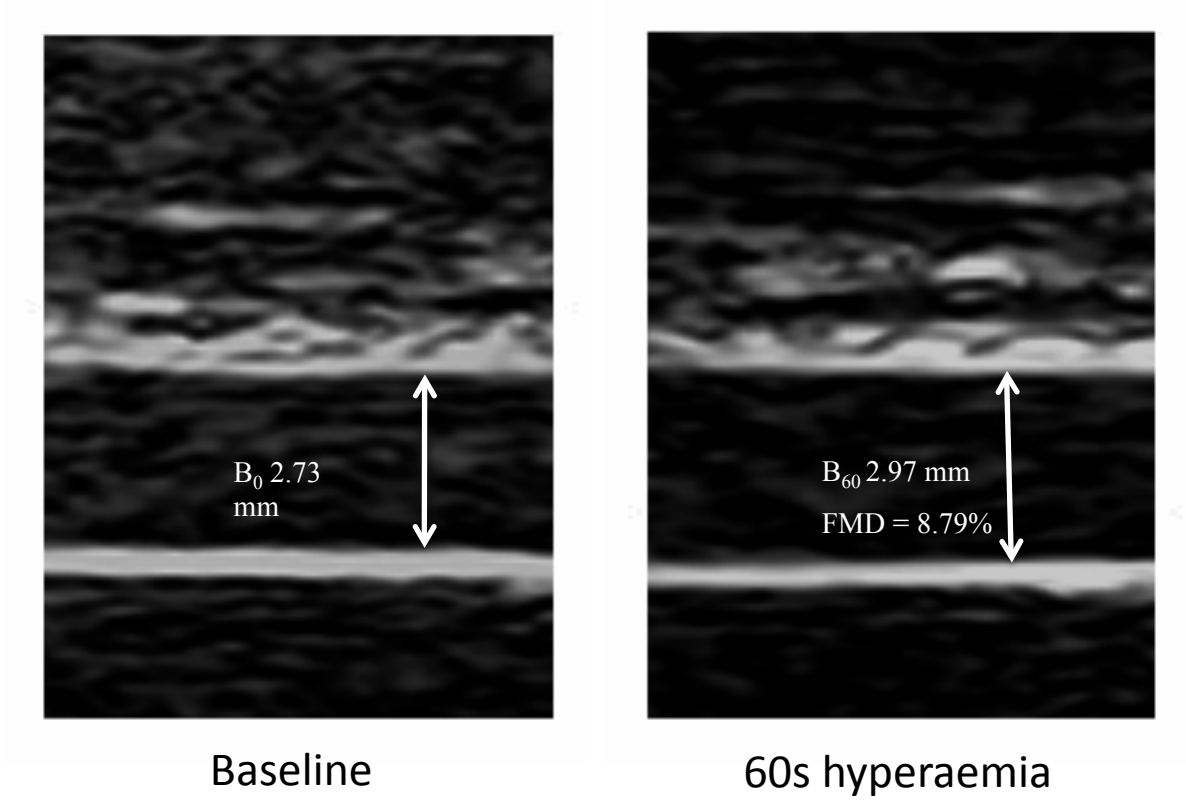


Figure 2-7: Measurement of brachial FMD by ultrasound
A longitudinal image of the brachial artery was obtained under resting conditions (left panel). A blood pressure cuff placed over the forearm immediately distal to the elbow was inflated to 200 mmHg for five minutes. A further image was obtained 60 seconds following cuff release during hyperaemic blood flow (right panel). Luminal diameter was measured at baseline (B_0) and at 60 seconds of hyperaemia (B_{60}). FMD (%) was calculated using the formula $(B_{60}-B_0)/B_0 \times 100$.

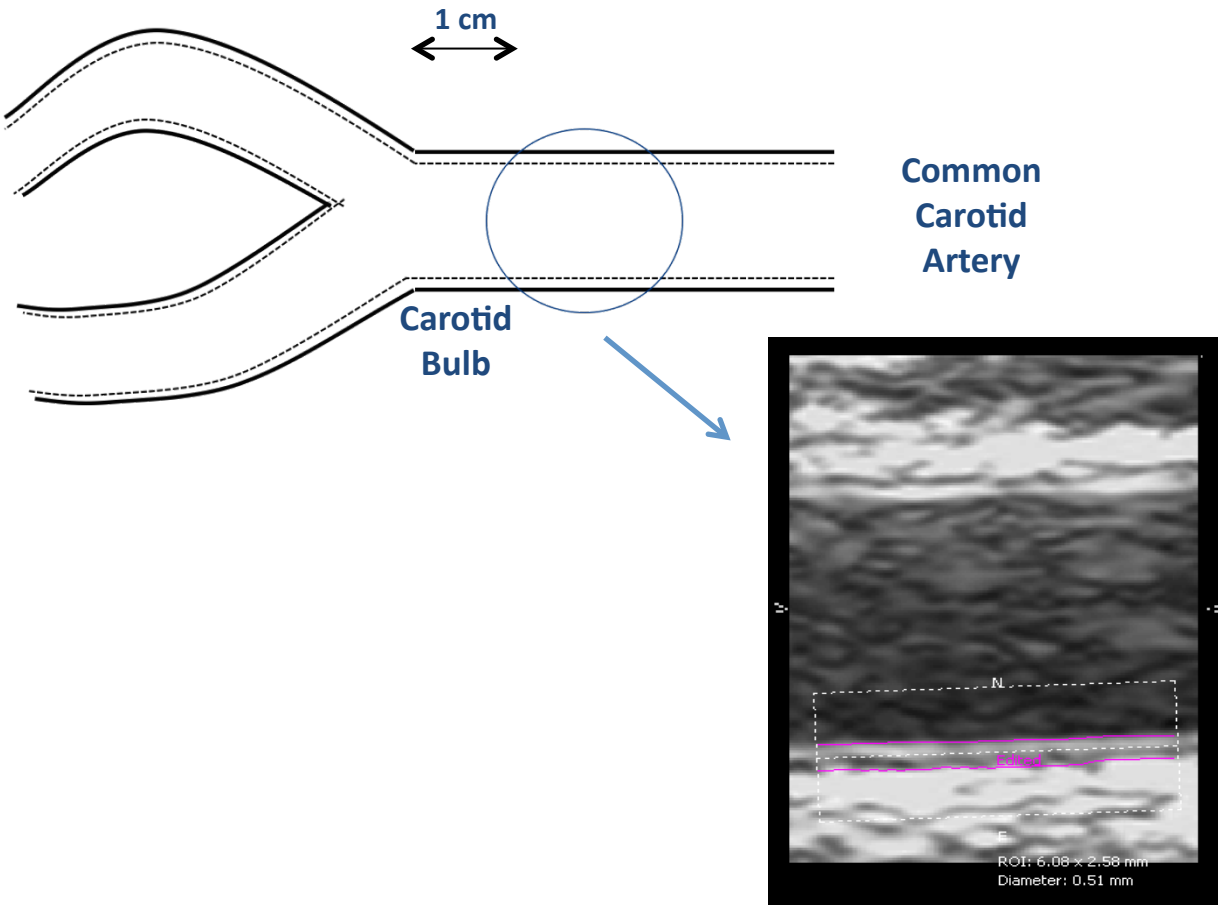


Figure 2-8: Measurement of carotid intima-media thickness using ultrasound
A longitudinal image of the common carotid artery was obtained 1cm below the carotid bulb. Posterior wall intima-media thickness (IMT) of this segment was measured using image analysis software (purple box); the mean posterior wall IMT was used as the readout for carotid IMT.

2.4.3 Arterial stiffness measurements using applanation tonometry

2.4.3.1 Carotid-femoral pulse wave velocity

PWV is a validated measure of arterial stiffness [161, 169]. Carotid-femoral PWV was calculated using measurements of pulse transit time obtained by non-invasive applanation tonometry (SphygmoCor, AtCor Medical, Australia). Following five minutes of supine rest, PWV (Figure 2-9) was derived by recording the time delay between the pressure waveforms at the femoral and carotid pulses relative to the anatomical distance between pulse sites, based on surface measurements via the suprasternal notch.

2.4.3.2 Aortic augmentation index

Augmentation index (AIx) is a systemic measure of arterial stiffness. The pressure waveform recorded at any site of the arterial tree is the sum of the anterograde waveform (generated by cardiac contraction) and the retrograde waveform of the incident wave (reflection from the peripheral vasculature). The merging point of the anterograde and reflected waves can be identified on the pressure waveform as an inflection point. The pressure at the inflection point (Pi) of the central aortic waveform corresponds to the peak blood velocity. (Figure 2-10) Augmented pressure (AP), the pressure added to the incident wave by the returning reflected wave, represents the additional pressure with which the left ventricle must cope that is caused by wave reflection. AIx is defined as the AP as a percentage of the pulse pressure. Larger values of AIx equate to increased wave



Figure 2-9: Measurement of carotid-femoral pulse wave velocity by applanation tonometry

Graphical output from the SphygmoCor applanation tonometry device is displayed. Arterial pressure waveforms were recorded at the femoral artery (upper waveform) and carotid artery (lower waveform). The surface electrocardiogram (ECG) was recorded simultaneously to allow calculation of pulse transit time. Green dots in the pressure waveforms indicate the 'foot of the curve', i.e. the arrival time of the impulse. Recordings were deemed to be of acceptable quality based on visual assessment of the waveform and ECG quality, SD of the mean pulse transit time (<6% of mean, yellow box), and SD of PWV (<10% of PWV, red box).

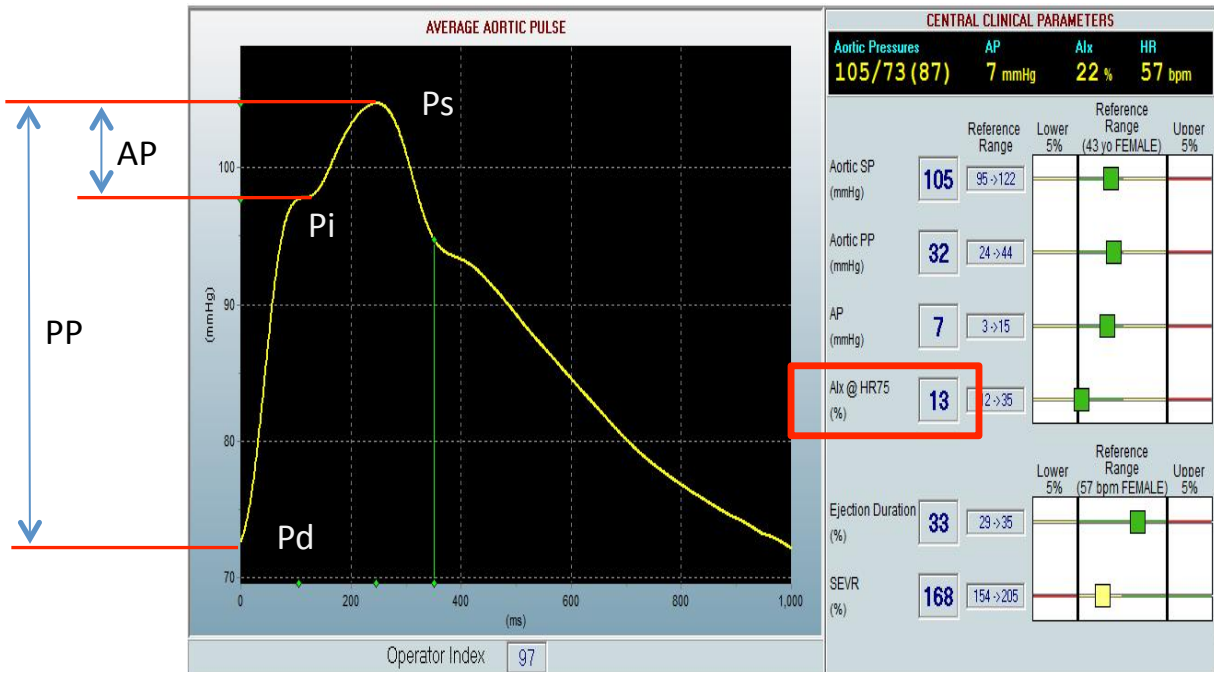


Figure 2-10: Measurement of aortic augmentation index by applanation tonometry

Graphical output from the SphygmoCor device is displayed. Non-invasive blood pressure was measured at the brachial artery. The pressure waveform at the radial artery was recorded using applanation tonometry. Central aortic pressure waveform (displayed) was calculated using a validated generalised transfer function. Pulse pressure (PP) was defined as systolic pressure (Ps) – diastolic pressure (Pd). Augmentation pressure was defined as Ps – pressure at the inflection point (Pi). Augmentation index was calculated as (AP/PP) x 100, and was normalised for resting heart rate (red box).

reflection from the periphery and/or earlier return of the reflected wave (as a result of increased PWV) owing to increased arterial stiffness.

Aortic augmentation index (AIx) was also measured using the SphygmoCor system. The central aortic pressure waveform was derived from the radial artery pressure waveform via the use of a generalised transfer function shown to give an accurate estimate of the central arterial pressure waveform and its characteristics [170] (Figure 2-10). Waveforms of radial pressure were calibrated according to non-invasive blood pressure measured at the brachial artery.

2.5 Vasomotor Studies

2.5.1 Tissue collection and preparation

Samples of saphenous vein (SV) were harvested during CABG surgery using a non-touch technique, before surgical distension, and before topical administration of drugs such as papaverine. Each segment was immediately transferred to ice-cold Krebs-Henseleit buffer and flushed to remove luminal blood. Excess connective tissue was carefully dissected away. Vessels were transferred to the laboratory within 30 minutes. Vessel samples were mounted in 25mL organ baths containing Krebs-Henseleit buffer (mmol/L: NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4, $NaHCO_3$, and glucose 5.5 mmol/L, pH 7.4) maintained at 37°C and oxygenated with 95% O_2/5% CO_2. Vessels were equilibrated for 20 minutes before starting each experiment.

2.5.2 Vascular reactivity recording system

Isometric tension studies were performed using a Grass model 7D polygraph. Vascular reactivity was recorded using Hugo-Sachs system, equipped with electronic tension transducers (Biopac). Data were acquired directly to computer using Biopac MP 100 laboratory data acquiring system and Acqknowledge software, which presented them as tension curves. Tension was calibrated using a 2g weight at the beginning of each experiment. Vascular reactivity was measured simultaneously from four vessel rings in separate organ bath chambers. Tension was applied in increments until a stable resting tension of 3g was achieved. The response to vasoactive drugs was then determined.

2.5.3 Phenylephrine contraction

After establishing resting tension, a viability test was performed by adding KCl 1mM and the maximum contractility of each vessel ring was recorded. After a 20 minute washout period, a concentration response curve to phenylephrine (PE) was determined. Increasing cumulative concentrations of PE (10^{-9}M; $3x10^{-9}$M; 10^{-8}M; $3x10^{-8}$M; 10^{-7}M; $3x10^{-7}$M; 10^{-6}M; $3x10^{-6}$M; 10^{-5}M) were added to organ baths and contractions were recorded. Contractions were expressed as grams of contraction and as % of maximal contracted tension induced by KCl.

2.5.4 Vascular relaxations to endothelium-dependent and endothelium- independent agonists

Following contraction to PE, vessels were washed and equilibrated for 20 minutes. Vessels were submaximally pre-contracted with PE to approximately 60-80% of tension obtained with PE 10^{-5} M (final concentration $3x10^{-6}$M), then

concentration response curves to various agonists were recorded by adding increasing concentrations of individual agonists, as described below:

Acetylcholine (ACh); endothelium-dependent relaxation (10^{-10}M; 3x10^{-10}M; 10^{-9}M; 3x10^{-9}M; 10^{-8}M; 3x10^{-8}M; 10^{-7}M; 3x10^{-7}M; 10^{-6}M).

Bradykinin (BK); endothelium-dependent relaxation (10^{-9}M; 3x10^{-9}M; 10^{-8}M; 3x10^{-8}M; 10^{-7}M; 3x10^{-7}M; 10^{-6}M; 3x10^{-6}M).

Sodium nitroprusside (SNP); endothelium-independent relaxation (10^{-10}M; 3x10^{-10}M; 10^{-9}M; 3x10^{-9}M; 10^{-8}M; 3x10^{-8}M; 10^{-7}M; 3x10^{-7}M; 10^{-6}M) in the presence of NG-nitro-L-arginine methyl ester (L-NAME, 1 mmol/L), a specific competitive inhibitor of NOS.

Relaxation values were calculated as a percentage of phenylephrine induced pre-contraction. Graphical output of a representative experiment is depicted in Figure 2-11.

2.6 Quantification of vascular superoxide production

Superoxide production was measured from intact vascular rings by lucigenin-enhanced chemiluminescence. Lucigenin (bis-N-methylacridium, Sigma, UK) is a chemiluminescent reagent used in the detection of superoxide. Lucigenin has been employed extensively to study superoxide generation from vascular tissues [171]. Paired samples of SV and internal mammary artery (IMA) from the same patient were opened longitudinally to expose the endothelial surface and then equilibrated for 20 minutes in oxygenated (95%O2/5%CO2) Krebs-HEPES buffer (2mL, pH=7.4) at 37^{0}C. Baseline superoxide production was measured with low-concentration lucigenin (5 μM) until a stable signal was achieved (Figure 2-12).

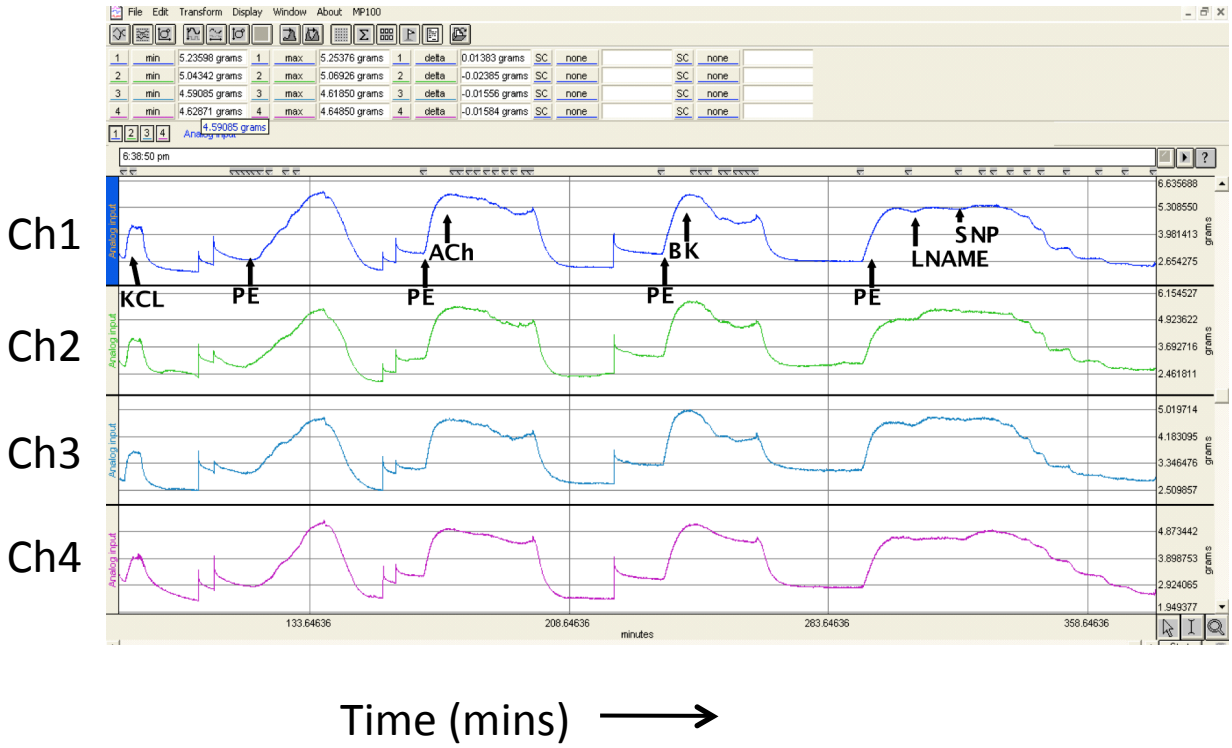


Figure 2-11: Graphical output of isometric tension studies

Rings of saphenous vein obtained at the time of CABG surgery were mounted in each of four organ bath chambers (Ch1-4). Graphical output of a single study is displayed. Y axis in each chamber readout represents vessel tension (g). Tension is applied to vessel rings until a steady resting tension of 3g is achieved. Addition of vasoactive reagents is indicated by the black arrows. Smooth muscle integrity was assessed by the contractile response to potassium chloride (KCl). Following pre-contraction with phenylephrine (PE), endothelium dependent relaxations to acetylcholine (ACh) and bradykinin (BK) are assessed, with twenty minute washout periods between each contraction-relaxation curve. Finally, endothelium-independent relaxation to sodium nitroprusside (SNP) is assessed in the presence of the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (LNAME).

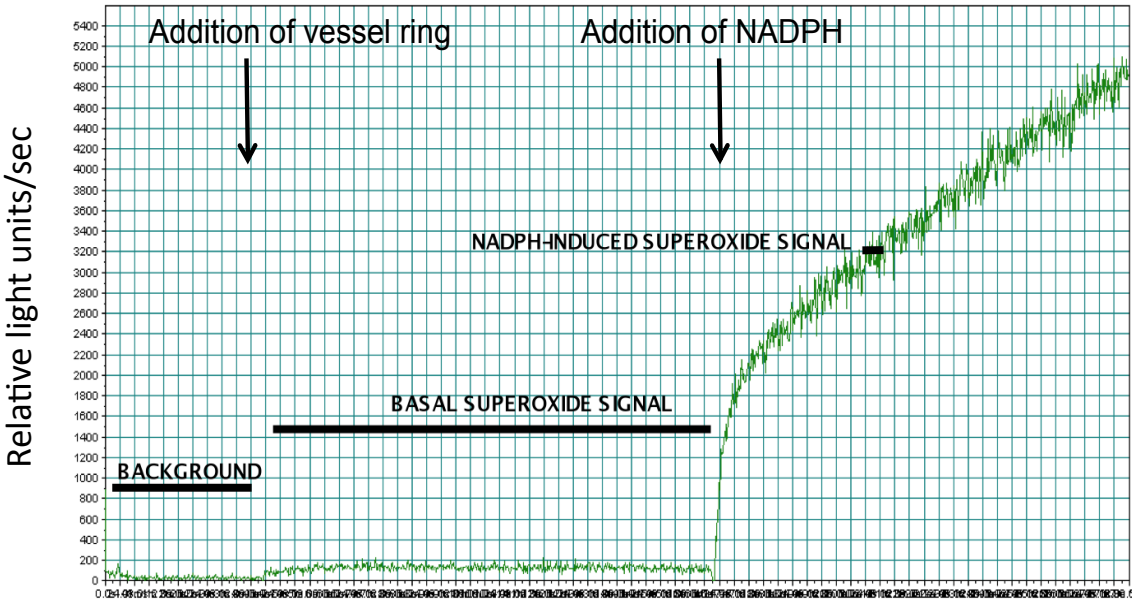


Figure 2-12: Measurement of superoxide production by lucigenin-enhanced chemiluminescence

Graphical output from luminometry software is displayed. Following measurement of background luminescence to ensure stable levels, the vessel ring is added to the chamber. Luminescence is allowed to reach plateau. Total superoxide is calculated as basal superoxide signal minus background luminescence. Nicotinamide adenine dinucleotide phosphate (NADPH) 100 μM is added to assess NADPH oxidase activity. NADPH-induced superoxide is calculated as the superoxide signal at 10 minutes following addition of NADPH.

The NADPH-stimulated superoxide production was estimated 10 minutes after 100 μM NADPH was added to assess NADPH oxidase activity (Figure 2-12). NOS-derived superoxide was measured by adding L-NAME (100μM) in the equilibrating buffer, and by calculating the difference in superoxide signal compared with basal conditions.

2.7 Quantification of plasma and vascular biopterins

BH4, BH2, and B levels in plasma and vascular tissue were each determined separately by high-performance liquid chromatography (HPLC) followed by electrochemical (for BH4) and fluorescence (for BH2 and B) detection. Levels of biopterins were expressed as pmol/g of tissue for vessels and nmol/L for plasma.

2.7.1 Preparation of tissue samples

Samples of SV and IMA snap-frozen in liquid nitrogen at the time of CABG underwent three cycles of freeze/thaw in 300 μl of ice-cold resuspension buffer (50 mM phosphate buffered saline, 1 mM dithioerythritol (DTE), 1mM EDTA, pH 7.4). 180 μl of supernatant was then transferred to new microcentrifuge tubes containing ice-cold acid precipitation solution (1 M phosphoric acid, 2 M trichloroacetic acid (TCA), 1mM DTE) and centrifuged for 5 minutes at 13000 rpm at 4°C. 150 μl of the supernatant was subsequently transferred into a 96-well HPLC plate and placed in the cooled HPLC autosampler for analysis. All processing steps were performed on ice. The sample transfers after the centrifugation were performed in the cold room at 4°C.

2.7.2 Preparation of plasma samples

Plasma samples were first thawed in ice water. Plasma samples were diluted 1:10 by adding 30 μl of sample to 270 μl of resuspension buffer (made as per tissue preparation above). Samples were centrifuged for 5 minutes at 13000 rpm at 4°C to separate any debris. 180 μl of supernatant was then transferred to new microcentrifuge tubes containing ice-cold acid precipitation solution (made as per tissue preparation above) and underwent a further centrifugation. Due to the high protein content of human plasma, 175 μl of subsequent supernatant was transferred to new microcentrifuge tubes for a third centrifugation to minimise protein loading on the HPLC column. Finally, 150 μl of supernatant was transferred to a 96-well plate for analysis. All steps were performed with minimal delay on ice or in the cold room to minimise BH4 auto-oxidation.

2.7.3 HPLC protocol

Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA Inc., UK) and fluorescence (Jasco, UK) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom, UK) and mobile phase comprising 50 mM sodium acetate, 5 mM citric acid, 48 μM EDTA, and 160 μM DTE (pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min.

BH4, BH2 and B were separated using reverse phase HPLC separation. BH4 was measured using electrochemical detection in which BH4 is oxidised by electrode 1 to quinonoid dihydrobiopterin (qBH2) and then reduced back to BH4 at electrode 2. The detector then uses the current generated by this reduction reaction to determine the concentration of BH4. Background currents of +500 to

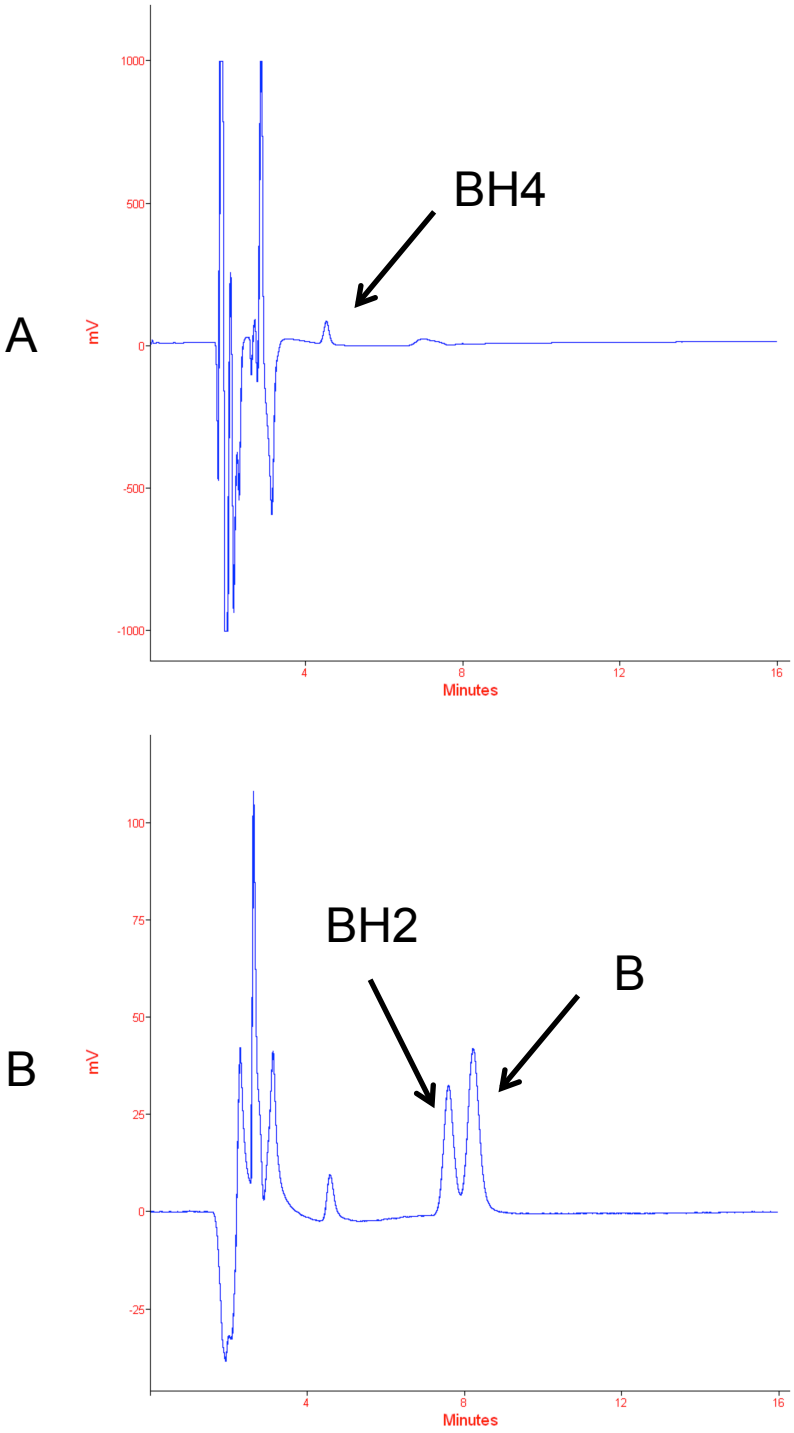


Figure 2-13: Detection of biopterins by high performance liquid chromatography

Panels A and B illustrate typical chromatographic appearances of BH4, BH2 and biopterin (B) detected by HPLC. Retention time (mins) is displayed on the x axes; electrical potential (mV) is displayed on the y axes. Panel A demonstrates electrochemical detection of BH4. Panel B demonstrates fluorescence detection of BH2 and B. Chromatograms represent 10^{-8} M standards in the absence of any biological sample.

+600 μA and -50 to -60 μA were used for the detection of BH4 on electrochemical cells E1 and E2 respectively.

BH2 and B were determined by fluorescence detection. Biopterin species were quantified by comparison with external standards after normalising for sample wet weight for tissue samples or dilution factor (1:10) for plasma. The standards (10^{-10} M to 10^{-7} M)) were prepared from a stock solution containing BH4, BH2 and B (Schirks Laboratories, Jona, Switzerland) in ice-cold resuspension buffer. Sample chromatograms for BH4, BH4 and B are shown in Figure 2-13.

All samples were loaded in the HPLC autosampler in batches (maximum 12 samples to prevent auto-oxidation) in a coded fashion to allow for blinding to sample identity during chromatogram analysis. Each batch was run with an individual standard curve for BH4, BH2 and B.

2.8 General statistical methods

All statistical analyses were carried out using SPSS or GraphPad Prism software. Categorical variables were compared (where appropriate) using the chi-squared test. Continuous variables were tested for normality by visual analysis of histograms and by the Kolmogorov-Smirnov test. Non-normal variables were log-transformed for statistical analyses.

A student's t-test was used to compare two groups affected by one single variable. One-way analysis of variance (ANOVA) was used to compare more than two data groups; two-way ANOVA was used to compare multiple data groups affected by two independent variables. For *ex vivo* experiments, where serial vessel rings from the same vessel or whole blood from the same subject were incubated with different treatments, I used one-way ANOVA for repeated measures. Bonferroni

post hoc correction was applied to compare individual groups if a significant difference was detected by ANOVA.

CHAPTER 3: ANALYSIS OF BH4 IN HUMAN PLASMA

3.1 Background

Quantification of BH4 and its associated oxidation products is key to many of the experiments described in this thesis. Thus, accurate quantification of biopterin species in biological samples is of paramount importance. The general protocol for HPLC analysis of biopterin species is described in Chapter 2. Electrochemical (EC) detection of BH4 [172] is the preferred method in our group as it allows direct quantification of BH4, in contrast to the more established iodine oxidation method [173]; this method necessitates a derived calculation of BH4 following oxidation to biopterin, with subsequent measurement by fluorescence (FL).

The current HPLC technique has posed no difficulties in specimens obtained from animal models in experiments performed in our group. For example plasma from the ApoE-KO mouse yields a clean BH4 chromatographic peaks that is easy to quantify (Figure 3-1A). Likewise, chromatograms from human vascular tissue are similarly easy to analyse (Figure 3-1B). In human plasma, however, electrochemical detection typically produces a 'double peak' appearance (Figure 3-1C), and can occasionally produce even more complex peaks (Figure 3-1D). This represents a significant analytical challenge, as it is unlikely that these multiple peaks all represent BH4.

The options for analysing such peaks include either quantification of the whole 'double peak', potentially including a compound that is not BH4 and falsely elevating the estimate of the true BH4 level, or splitting and quantifying individual peaks, which is liable to error when peaks are difficult to separate by eye. Accordingly, I conducted a series of experiments to optimise the current

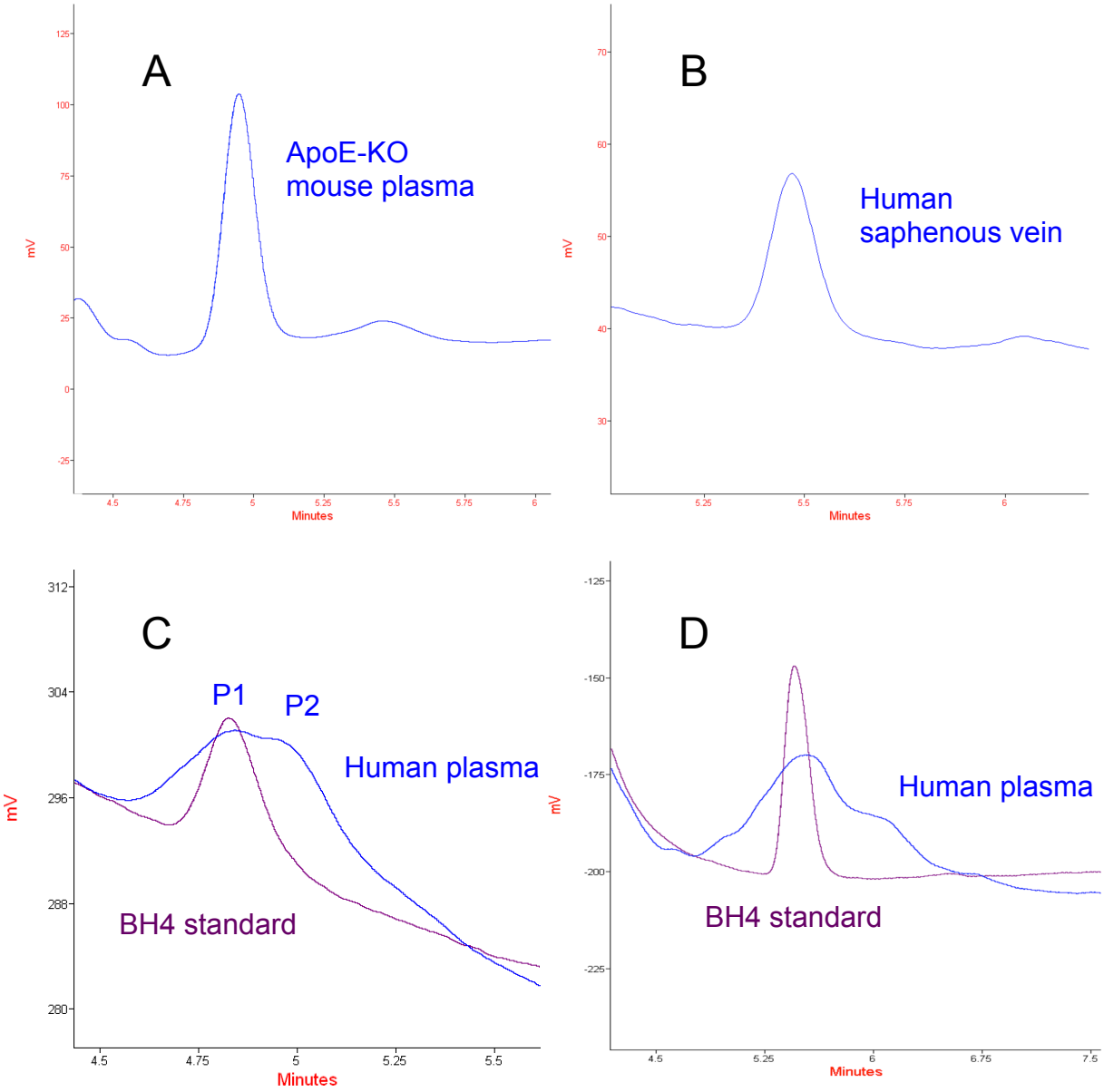


Figure 3-1: Sample BH4 chromatograms from plasma and vessels
Panels A-D illustrate the challenges faced when analysing BH4 in human plasma samples. BH4 chromatograms from electrochemical HPLC detection in ApoE-KO mouse plasma (Panel A) and human saphenous vein obtained at the time of CABG surgery (Panel B) result in single BH4 chromatographic peaks that are easy to quantify. Panel C demonstrates the typical "double peak" in human plasma, with the BH4 standard sample shown alongside for comparison. The peak of the BH4 standard coincides with the 1st peak in human plasma (P1), which is often difficult to separate from the 2nd peak (P2). In some cases, more than two peaks are evident in human plasma (Panel D).

HPLC protocol for analysis of human plasma, and to further characterise these chromatographic appearances in human plasma, in order to ensure consistent and reasoned analysis for the main experiments of this thesis.

3.2 Alternative deproteinisation agents

It was hypothesised that confounding peaks may result from the high protein content of human plasma. I attempted to deproteinise plasma samples with alternative agents, with the aim of reducing the protein content of the HPLC sample below the value of 0.6 mg/ml that is achieved by the TCA/phosphoric acid solution of the existing HPLC protocol. Ammonium acetate and acetonitrile have been used as deproteinisation agents in other techniques [174-175]. Both agents, when combined with the TCA/phosphoric acid solution were able to reduce the protein content of the sample to <0.2 mg/ml. However, when these samples were analysed on the HPLC, the chromatograms with ammonium acetate were degraded by a double peak artefact (Figure 3-2A), and with acetonitrile there was no measurable BH4 peak (Figure 3-2B). Accordingly these agents were not used for further BH4 analysis.

3.3 Characterisation of multiple peaks

3.3.1 Baseline characteristics and effect of exogenous BH4

The typical appearance of the EC BH4 peak in human plasma is a double peak. The retention time of the 1st peak is very close to that of the BH4 standard (Figure 3-1). This is a very consistent finding, even under conditions that result in HPLC artefact, such as a sloping baseline. 'Spiking' of human plasma samples augments only the 1st peak; this is illustrated in Figure 3-3, where supplementation with

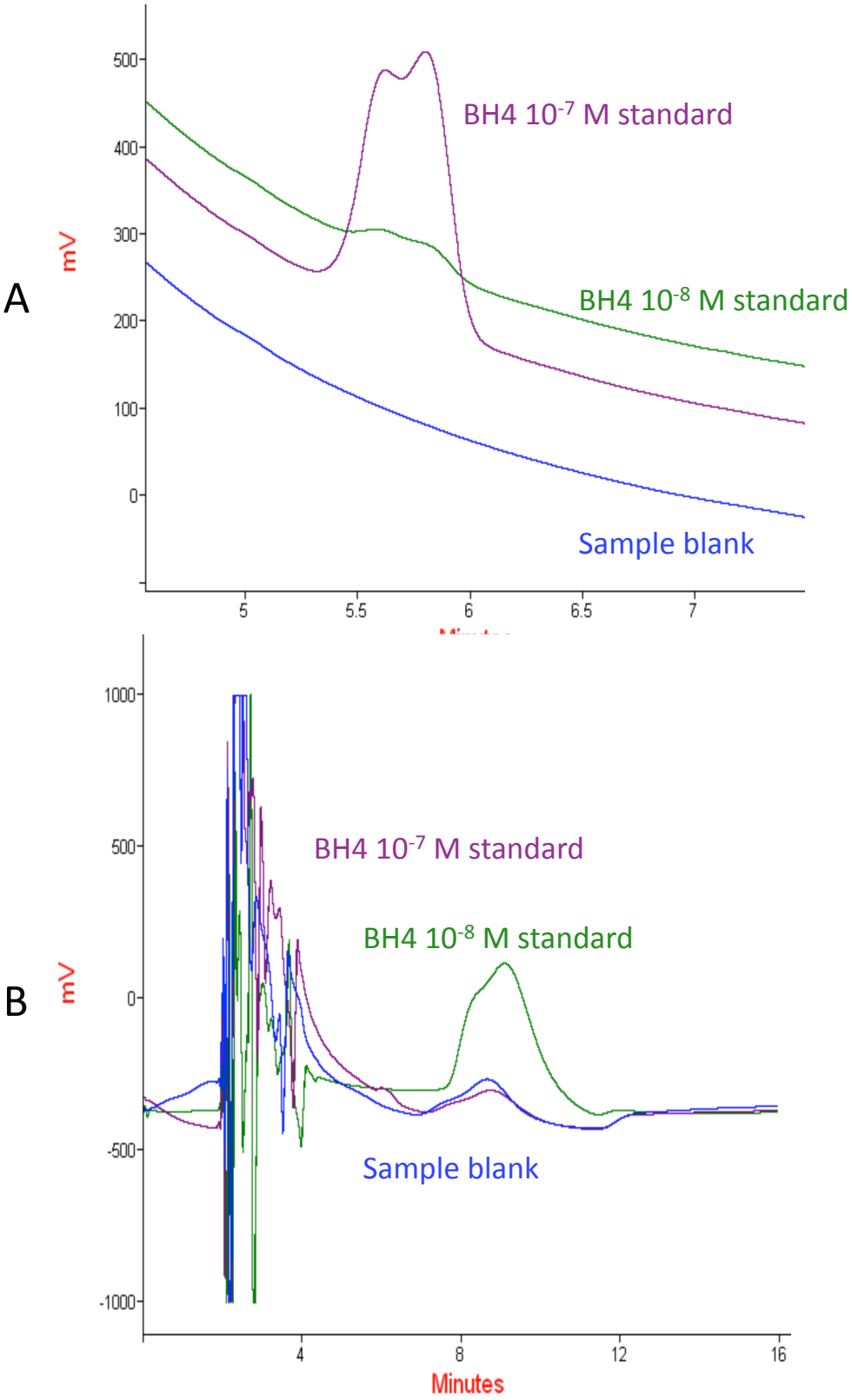


Figure 3-2: Alternative deproteinisation agents for preparation of human plasma for HPLC analysis

Known concentrations of BH4 were analysed by HPLC using different deproteinising agents to asses the qualitative effect on chromatograms. Panel A: ammonium acetate resulted in a double peak in the BH4 standard. Panel B: acetonitrile resulted in no clearly measurable BH4 peak.

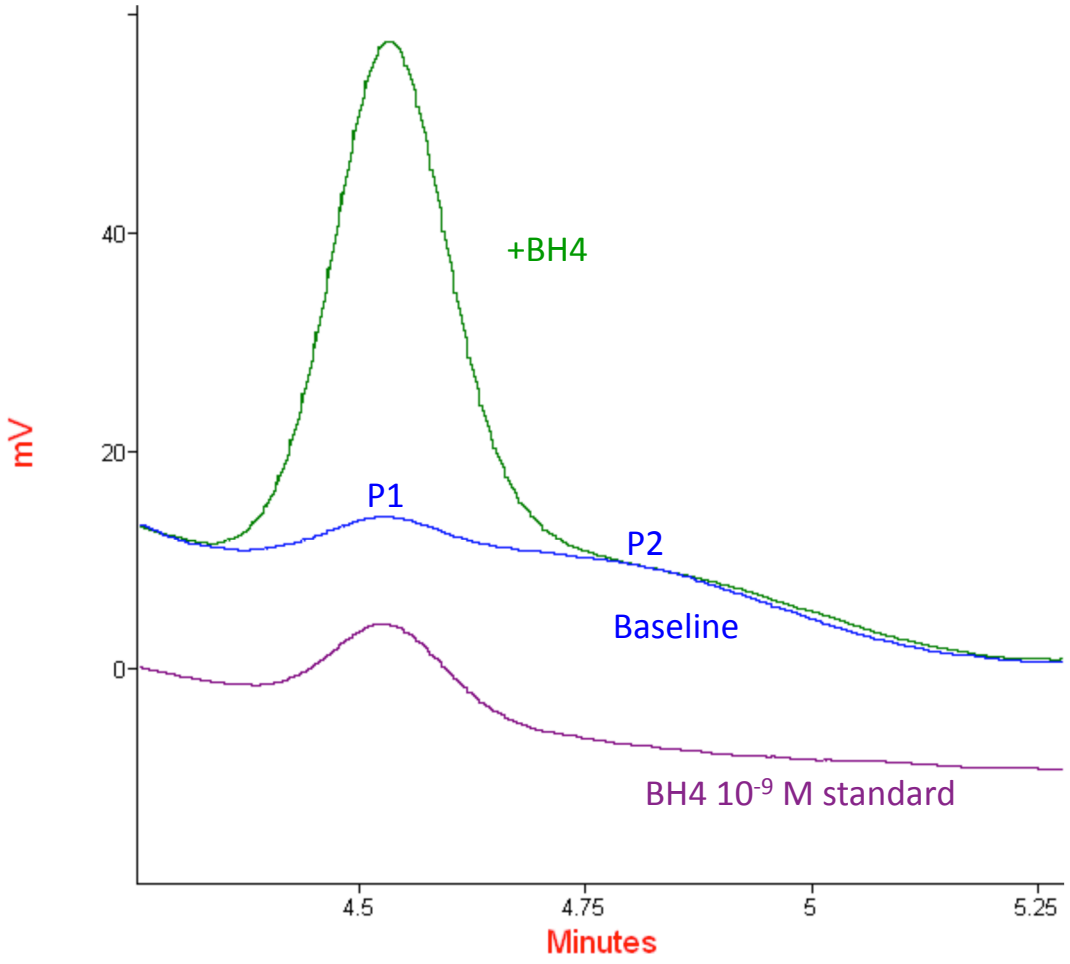


Figure 3-3: Effect of exogenous BH4 on the appearance of the human plasma chromatogram

BH4 was measured by HPLC in human plasma from a healthy volunteer under baseline conditions (blue chromatogram) or following the addition of exogenous BH4 (50nM, green chromatogram). The baseline chromatogram demonstrates the typical BH4 double peak, with the 1st peak (P1) coinciding with the BH4 peak observed in the standard (purple chromatogram). Addition of exogenous BH4 selectively augments the 1st peak in plasma (P1) without augmenting the 2nd peak (P2).

even high concentrations of BH4 ($5x10^{-8}$ M) selectively augments only the 1st peak. Both of these observations suggest that only the 1st peak of the EC chromatogram in human plasma represents BH4.

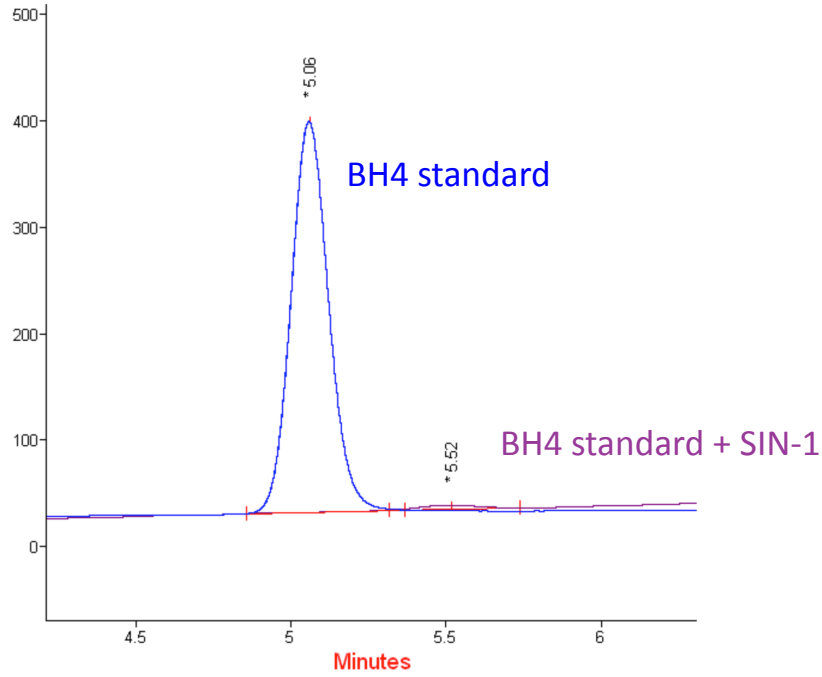
3.3.2 Effect of BH4 oxidation by SIN-1

I next tested the effect of oxidation of BH4 on the appearance of the chromatograms. Addition of the peroxynitrite generator 3-morpholinosyndnomine (SIN-1, final added concentration $2x10^{-5}$ M) resulted in total depletion of the $5x10^{-8}$ M BH4 standard (Figure 3-4A). Closer inspection of this chromatogram reveals a small new peak occurring after the BH4 peak (Figure 3-4B). SIN-1 in the absence of BH4 did not generate any peaks in the EC chromatogram.

3.3.3 Loss of total biopterins due to oxidation

In a separate experiment, I determined the effect of auto-oxidation on endogenous and exogenous biopterins. BH4, BH2 and B were measured in human plasma from two healthy volunteers at baseline and after 4 hours incubation at room temperature. Two concentrations of exogenous BH4 (10^{-9} M or $5x10^{-10}$ M) were added at baseline and compared to endogenous levels (Figure 3-5). BH4 levels decreased over time with corresponding increases in BH2 and B due to oxidation (Figure 3-5, Panels A-C). Total biopterins (the sum of BH4+BH2+B), however, decreased significantly over time Figure 3-5D). Thus, the observed increase in BH2 and B does not fully account for the depletion in BH4, implying that one or more of these biopterin species is converted to an alternative species that is not detected by the current HPLC assay.

A



B

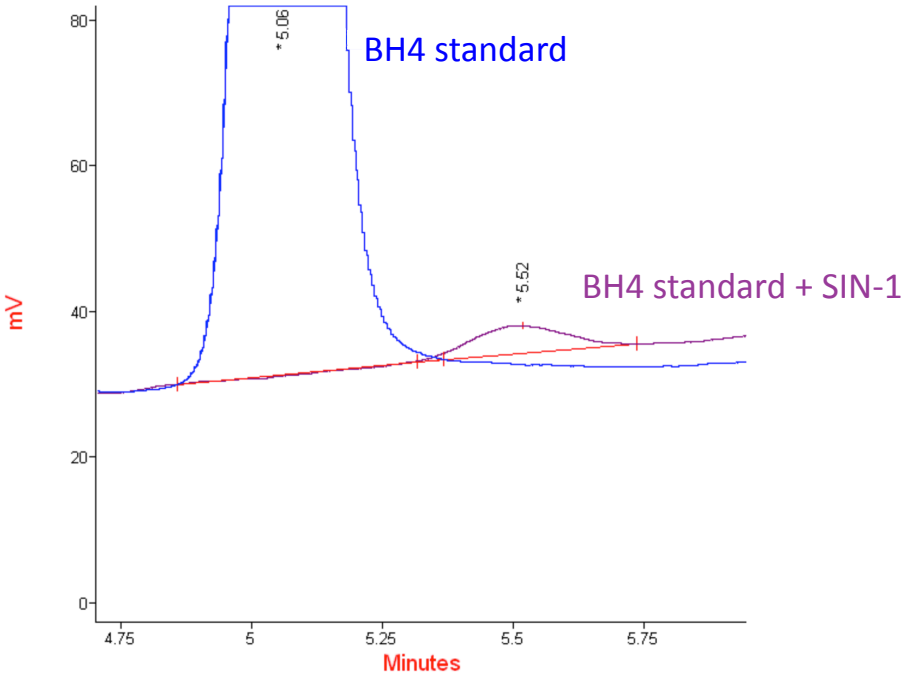


Figure 3-4: Effect of SIN-1 on BH4

Panel A: A $5x10^{-8}$ M BH4 standard was analysed by electrochemical HPLC detection with and without the addition of SIN-1 ($2x10^{-5}$ M). Addition of SIN-1 caused total depletion of BH4 due to oxidation. Panel B: Zoom of panel A demonstrates the appearance of a small peak in the chromatogram at a longer retention time than BH4. SIN-1 alone (in the absence of BH4) did not produce this peak (data not shown).

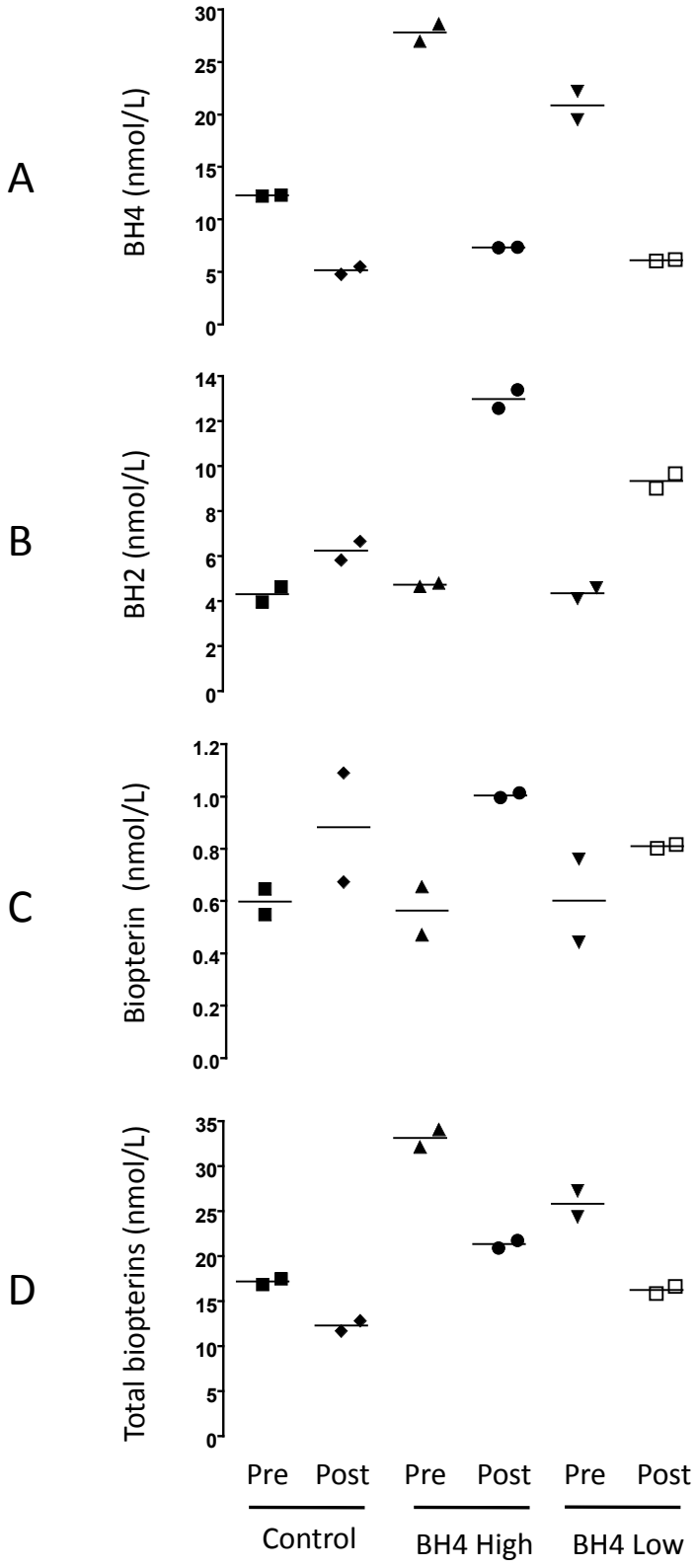


Figure 3-5: Effect of incubation on endogenous and exogenous biopterins in human plasma

BH4, BH2 and biopterin were measured by HPLC in human plasma from two healthy volunteers at baseline and after four hours incubation. Two concentrations of exogenous BH4 (10^{-9} M (BH4 high) or $5x10^{-10}$ M (BH4 low)) were added at baseline and compared to endogenous levels (control). BH4 levels decreased over time (Panel A) with corresponding increases in BH2 (Panel B) and B (Panel C) due to oxidation. Total biopterins (the sum of BH4+BH2+B), however, decreased significantly over time, indicating conversion to other species not detected by the current HPLC assay.

3.3.4 Correlation between loss of biopterins and unidentified peak

I hypothesised that the extra peak created in the EC chromatogram following SIN-1 oxidation (Figure 3-4) may represent an alternative species that may account either fully or partially for the loss of total biopterins following auto-oxidation. In a further experiment, BH4, BH2 and B were quantified by HPLC from five starting concentrations of BH4 ($1x10^{-8}$, $2.5x10^{-8}$, $5\ x10^{-8}$, $7.5x10^{-8}$ and $1x10^{-7}$ M), with and without SIN-1 ($2x10^{-5}$ M). The area under the curve (AUC) for the extra peak in the electrochemical chromatogram was also quantified. The change in total biopterins before and after oxidation was calculated. There was a very strong linear relationship between loss of total biopterins and the extra peak (Pearson's r=0.997) (Figure 3-6). These data would therefore suggest that the extra peak observed following SIN-1 oxidation of BH4 is related to the loss of total biopterins in a quantitatively proportionate fashion. Unfortunately as there is no standard curve with which to quantify the molarity of this unknown species it is not possible to conclude from this experiment whether this unidentified species accounts for all or only part of the observed loss of total biopterins.

3.3.5 Relationship of the unidentified peak to BH4 in plasma

A further experiment was conducted to assess the relevance of the unidentified EC peak – identified following oxidation of exogenous BH4 in the absence of plasma – to the chromatogram observed in human plasma. BH4 was quantified by EC detection in samples of human plasma from healthy volunteers following oxidation by SIN-1 ($2x10^{-5}$ M), both with and without the addition of exogenous BH4 ($5x10^{-8}$ M) at baseline. Sample chromatograms are illustrated in Figure 3-7. These chromatograms demonstrate first that both endogenous and exogenous

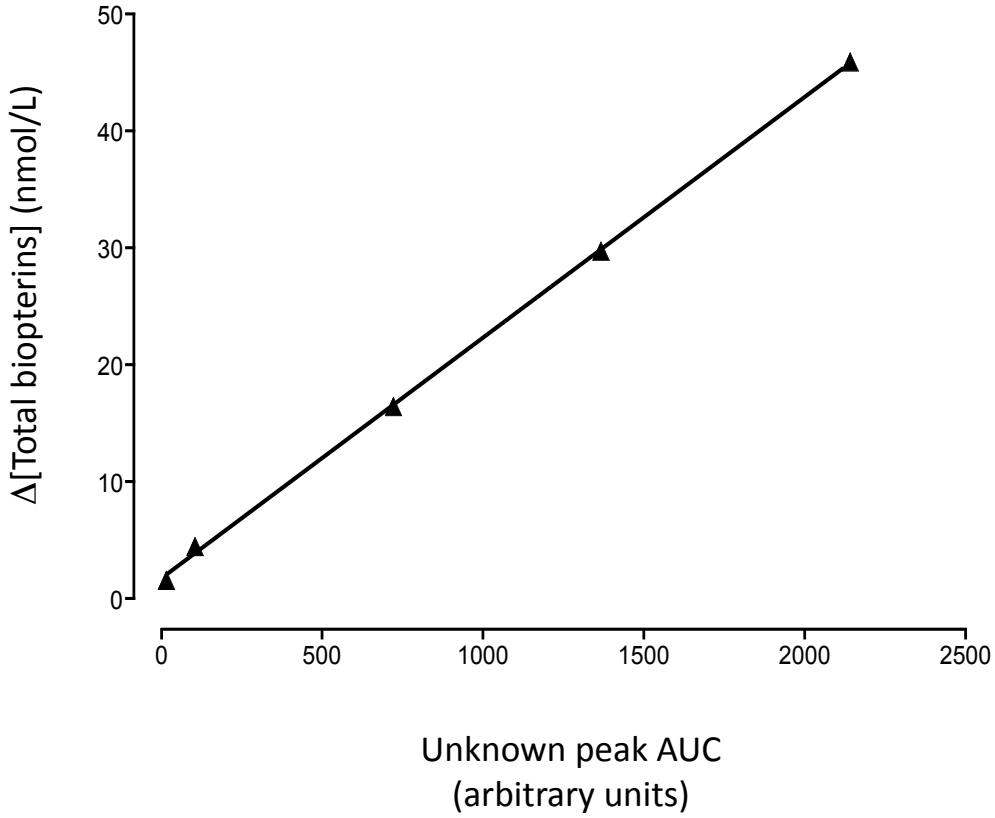


Figure 3-6: Correlation between change in total biopterins over time and the unidentified HPLC species

BH4, BH2 and biopterin (B) were quantified by HPLC from five starting concentrations of BH4 ($1x10^{-8}$, $2.5x10^{-8}$, $5\ x10^{-8}$, $7.5x10^{-8}$ and $1x10^{-7}$ M), at baseline and following oxidation by SIN-1. The area under the curve (AUC) for the extra peak in the electrochemical tracing was also quantified. Total biopterins was calculated as BH4+BH2+B. The change in total biopterins before and after oxidation was calculated. There was a strong linear relationship between loss of total biopterins and the extra peak (Pearson's $r=0.99$, $p<0.0001$).

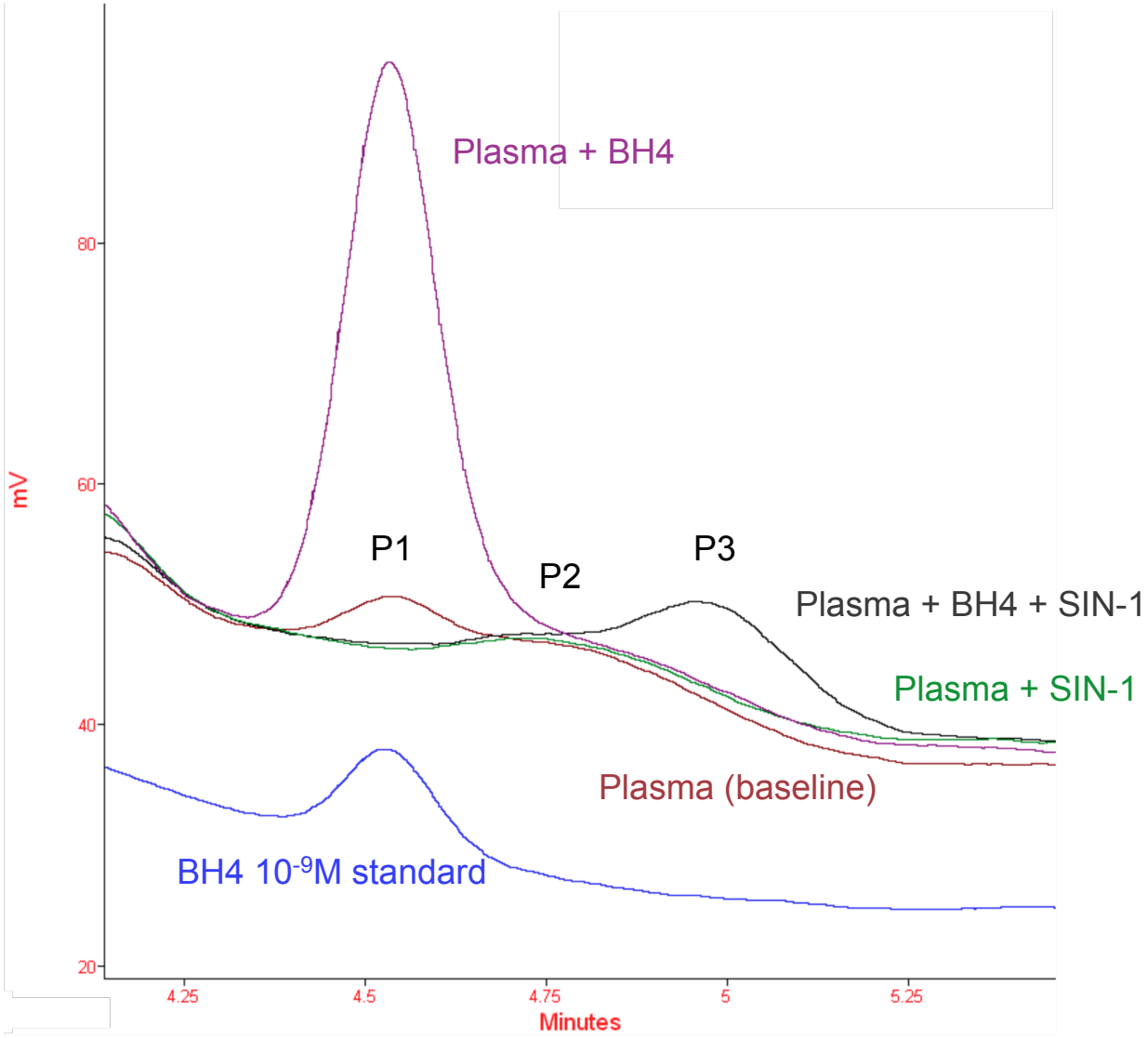


Figure 3-7: Effect of SIN-1 on endogenous and exogenous BH4 in human plasma
BH4 was quantified by electrochemical HPLC detection in samples of human plasma at baseline and following oxidation by SIN-1 in the presence or absence of exogenous BH4 (50 nM). Blue chromatogram represents BH4 alone. Red chromatogram represents plasma alone. Purple chromatogram represents plasma with exogenous BH4. Green chromatogram represents plasma following SIN-1 oxidation. Black chromatogram represents plasma with exogenous BH4 following SIN-1 oxidation. Exogenous BH4 selectively augmented the 1st peak (P1) of the baseline plasma chromatogram, as observed previously. Oxidation by SIN-1 resulted in total depletion of P1 in plasma with no effect on the 2nd peak (P2). A 3rd peak (P3) was visible following SIN-1 oxidation only in the presence of exogenous BH4.

BH4 are totally depleted by SIN-1, as the 1st peak is no longer visible. Second, there is no effect of SIN-1 on the 2nd peak. Third, the extra peak, previously identified by oxidation of BH4 alone, is now visible as a 3rd peak in the chromatogram, but only in the presence of a high concentration of exogenous BH4 (Figure 3-7), and not with endogenous levels of BH4 observed in healthy volunteers.

Interestingly, in subjects with CAD, a 3rd peak is often seen in the EC chromatogram (Figure 3-8). This peak has the same retention time as the unidentified peak observed following oxidation of BH4 alone by SIN-1, and may represent the same species. Thus, one might speculate that this species may be visible only in subjects with high oxidative stress and high plasma BH4 levels (through inflammatory upregulation of the GTPCH-BH4 axis), such as those with CAD, but not in healthy volunteers.

3.3.6 Alternative pterin species

I hypothesised that the extra peak in the EC chromatogram following SIN-1 oxidation may represent an alternative pterin species. Pterin (due to side-chain cleavage from biopterin) and xanthopterin, which has been implicated in BH4 metabolism in rat hearts [118], were postulated as potential candidates for this unidentified species. 10^{-7} M standards of pterin and xanthopterin were analysed using the existing HPLC biopterin assay. The BH4 analogue sepiapterin (10^{-7} M) was also analysed. None of these three pterin species resulted in any measurable peak in the EC chromatogram (Figure 3-9A). In the FL chromatogram, only pterin was detectable, with a peak with a very similar retention time to BH2 (Figure 3-9B). Thus, the extra peak following SIN-1 oxidation is not represented

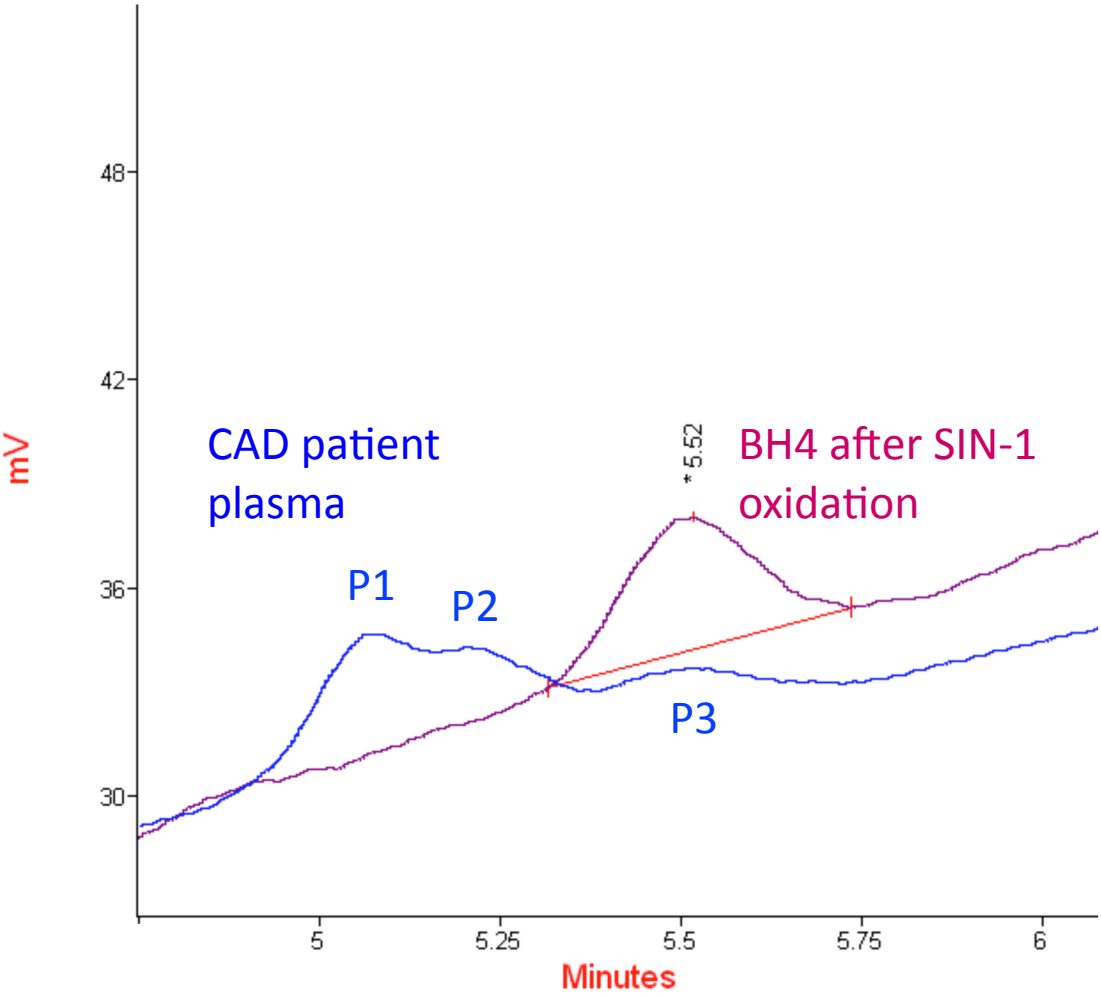


Figure 3-8: The 3rd peak in plasma of humans with coronary artery disease
In subjects with coronary artery disease, a 3rd peak (P3) is often seen in the electrochemical chromatogram (blue chromatogram). This peak has a similar retention time to the unidentified peak seen following oxidation of BH4 by SIN-1 in the absence of plasma (purple chromatogram). This may represent an oxidation product of BH4 in human plasma.

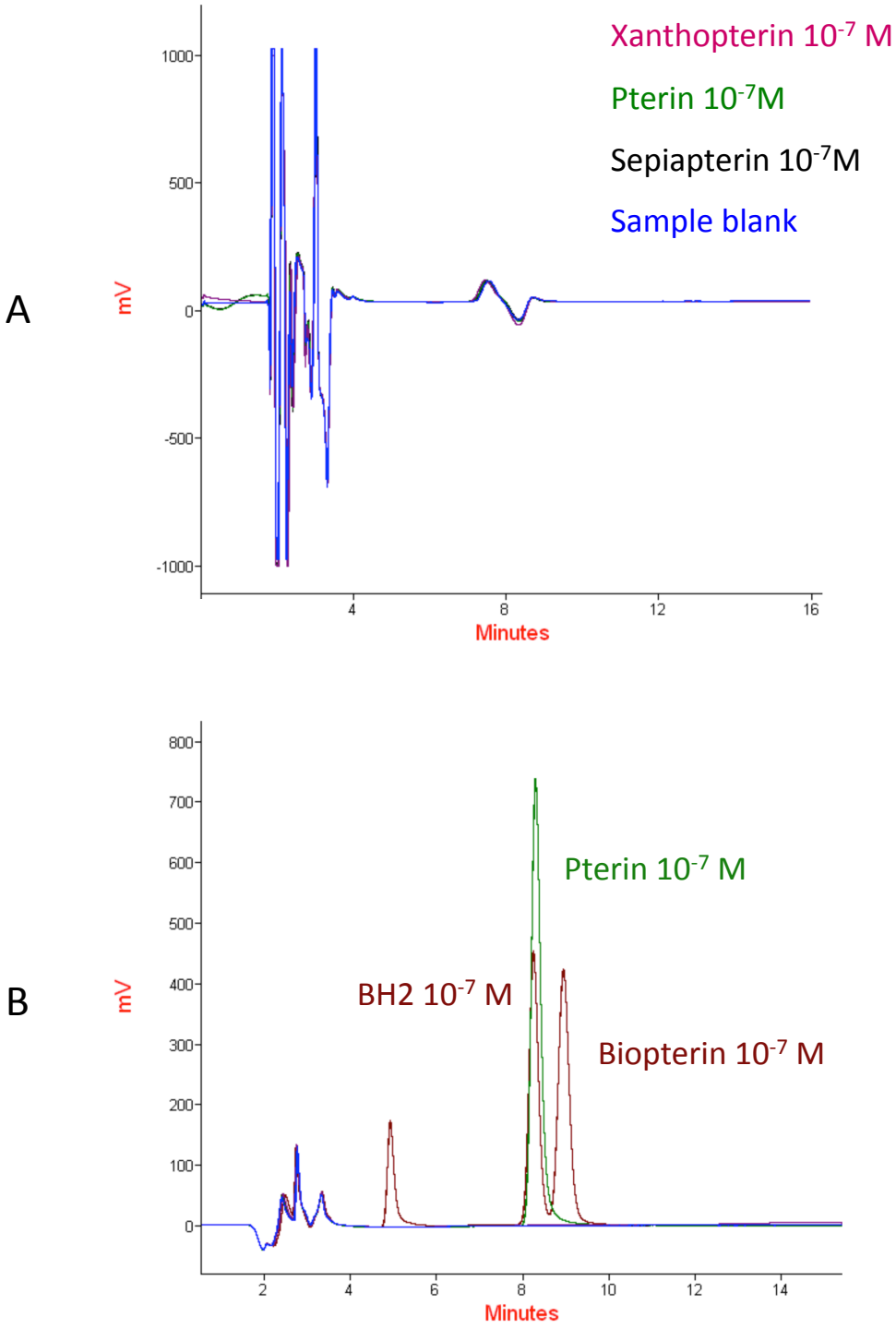


Figure 3-9: Detection of alternative pterin species using existing HPLC protocol
10^{-7} M standards of xanthopterin, pterin and sepiapterin were prepared using the usual biopterin assay. None of these species were detected in the electrochemical trace (Panel A). Only pterin was detected readily in the fluorescence trace (Panel B), with a retention time similar to that of BH2.

by any of these candidate pterin species, and the identity of this species remains unknown.

3.4 Electrochemical versus fluorescence detection of BH4

I next tested whether the fluorescence channel could produce a BH4 chromatogram that was easier to quantify. Samples of human plasma from a healthy volunteer were analysed with simultaneous EC and FL detection. FL was noted to produce a single, readily quantifiable peak, although this peak has a slightly shorter retention time than the BH4 standard (Figure 3-10B), casting doubt as to whether this peak is due to BH4.

To further characterise this peak, exogenous BH4 was added to plasma samples and quantified at baseline and following four hours incubation at room temperature, to allow auto-oxidation of BH4. In the EC tracing, supplemental exogenous BH4 (final added concentration $5x10^{-9}$ M) augmented the 1st BH4 peak AUC approximately 5-fold (Figure 3-10A). In contrast, the same concentration of exogenous BH4 resulted in only minimal augmentation of the FL peak (Figure 3-10B).

Furthermore, incubation of samples for four hours resulted in near total depletion of both endogenous and exogenous BH4 in the EC chromatograms due to oxidation (Figure 3-10A), whereas the AUC for the BH4 peak in control samples remained constant following incubation, with the small increase in baseline AUC with exogenous BH4 returning to baseline (Figure 3-10B).

Taken together, these results suggest that the baseline peak observed in the FL channel does not represent BH4. Accordingly, the FL channel cannot be used for

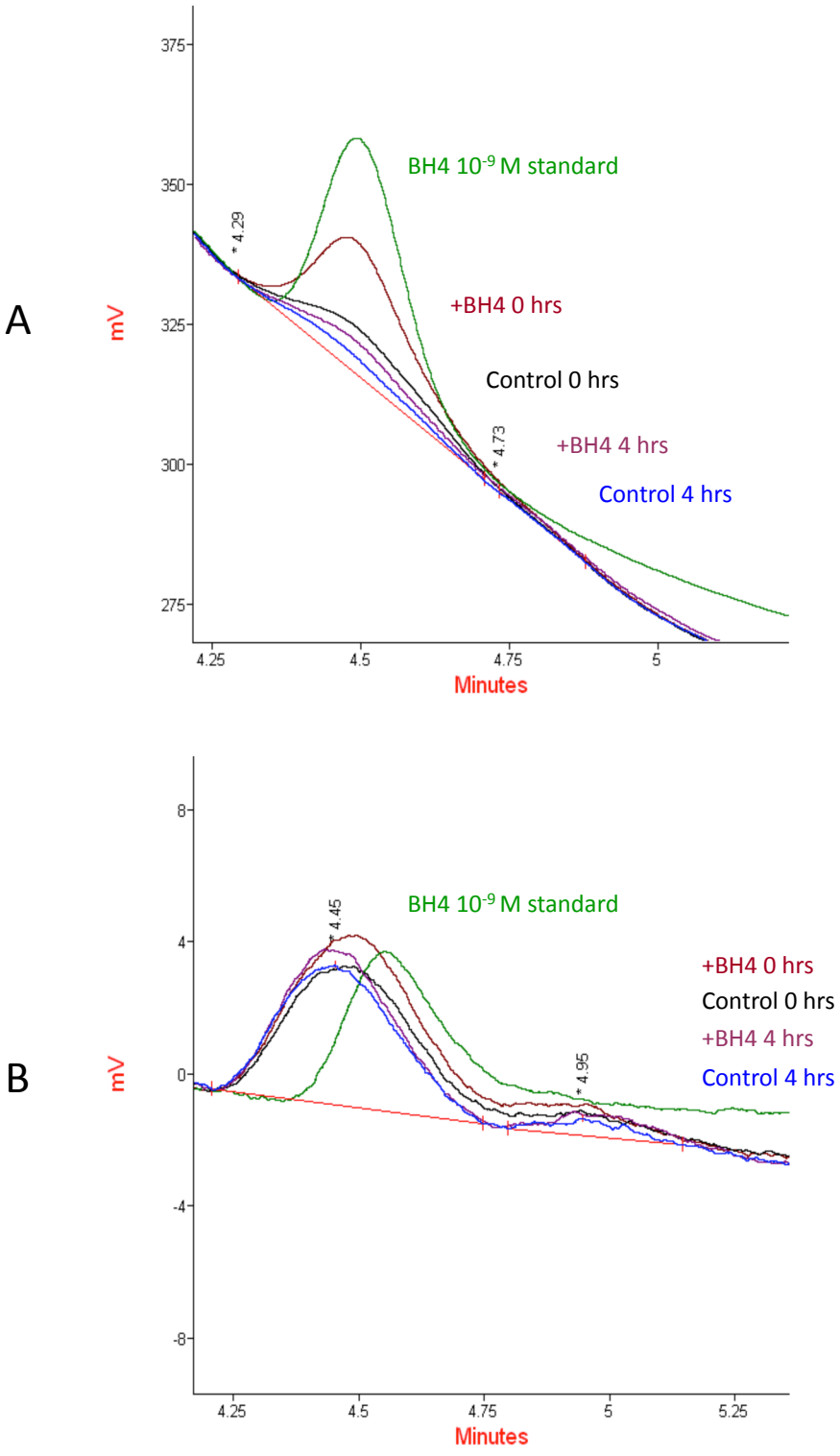


Figure 3-10: BH4 quantification by HPLC using electrochemical and fluorescence detection

Samples of human plasma either with (+BH4) or without (control) the addition of exogenous BH4 (final added concentration $5x10^{-9}$ M) were analysed by HPLC. Using electrochemical detection (EC, Panel A) the plasma 1st BH4 peak coincides with that of the standard, as described above. In contrast, using fluorescence detection (FL, Panel B) the plasma peak has a shorter retention time than the standard. When samples were allowed to auto-oxidise at room temperature (0 hrs vs 4 hrs), there was an expected reduction in BH4 peak in the EC trace, but not in the FL trace.

quantification of BH4, at least in human plasma, and the FL channel was not used for BH4 analysis for any other experiments in this thesis.

3.5 Summary

In summary, BH4 analysis by EC HPLC detection in human plasma is complicated by the presence of multiple chromatographic peaks. The data from this series of experiments suggest that the 1st peak represents the true BH4 level for the following reasons:

- This peak has an identical retention time to the BH4 standard
- Exogenous BH4 selectively augments the 1st peak
- SIN-1 selectively depletes the 1st peak via oxidation

Furthermore, the peak observed in the FL chromatogram cannot be used for BH4 analysis as it appears to be masked by a further species that is not prone to oxidation. Thus, for the purposes of quantification of BH4 in human plasma in this thesis, I have used selective quantification of the 1st BH4 peak in the EC chromatogram.

These experiments have also yielded some interesting insights into BH4 oxidation. Total biopterins become depleted over time, representing likely loss of BH4 to an undetected species, which may have important physiological implications. An unidentified peak is apparent following oxidation of BH4 which correlates strongly with the loss of total biopterins, and this peak appears to be present in the plasma of patients with CAD. Further experiments are warranted to identify this species.

CHAPTER 4: PHARMACOKINETICS AND PHARMACODYNAMICS OF ORAL BH4 THERAPY

4.1 Introduction

Given the importance of endogenous BH4 levels on vascular endothelial function, administration of exogenous BH4 is a potential therapeutic strategy in vascular disease states. Indeed, a number of studies in human subjects with either overt CAD [106] or vascular risk factors [91, 98, 104] have demonstrated that administration of exogenous BH4 by intra-arterial infusion leads to rapid improvements in endothelial function. However, these studies used very high BH4 doses and only tested short-term effects. In forearm plethysmography studies plasma BH4 levels are typically elevated to between 50-100 μM [111], more than 1000-fold higher than physiological plasma BH4 levels of 10-50 nM [90]. These rapid and short-term supraphysiological BH4 doses may be confounded by non-specific effects, including direct antioxidant effects on the vessel wall. Furthermore, intra-arterial infusion is not a relevant therapeutic approach to improve endothelial function in patients with CAD.

The only randomised controlled trial (RCT) of chronic oral BH4 treatment published to date demonstrated an improvement in endothelial function in subjects with hypercholesterolaemia [100]. However, the mechanisms relating oral BH4 treatment to changes in vascular BH4 and eNOS coupling in vascular disease states are unknown, and the applicability to patients with established CAD is uncertain. Accordingly, I conducted a randomised, placebo-controlled trial of oral BH4 treatment in patients with CAD in order to determine the

mechanisms relating exogenous BH4 treatment to human vascular function. This trial is presented in Chapters 4 and 5. The trial was known as the 'OXBIO' study, due to the collaboration between the University of Oxford (the study sponsor) and BioMarin Pharmaceutical Inc., who provided the study drug and funded the research.

4.1.1 Hypothesis & aims

In this first section of the study I aimed to examine the effects of oral BH4 on plasma and vascular BH4 bioavailability. Specifically, I hypothesised that:

- Oral BH4 would elevate plasma BH4 levels compared to placebo.
- Oral BH4 would elevate BH4 levels in vascular tissue compared to placebo.

4.2 Methods

4.2.1 Study design and subjects

Forty-nine patients with multi-vessel CAD undergoing elective CABG surgery at the John Radcliffe Hospital were randomised in a double-blind, parallel design to receive sapropterin dihydrochloride (6R-BH4) at either 700 mg/d (high-dose), or 400 mg/d (low-dose), or matching placebo for 2–6 weeks prior to surgery (clinicaltrials.gov identifier: NCT00423280). The study drug and randomisation plan were provided by BioMarin Pharmaceutical Inc., Novato, USA. Figure 4-1 outlines the study protocol. Study medication was taken at 10p.m., at least one hour following the evening meal. Principal exclusion criteria were recent acute coronary syndrome; heart failure with severe left ventricular dysfunction; renal or liver disease; and contraindications to MRI scanning. Demographic

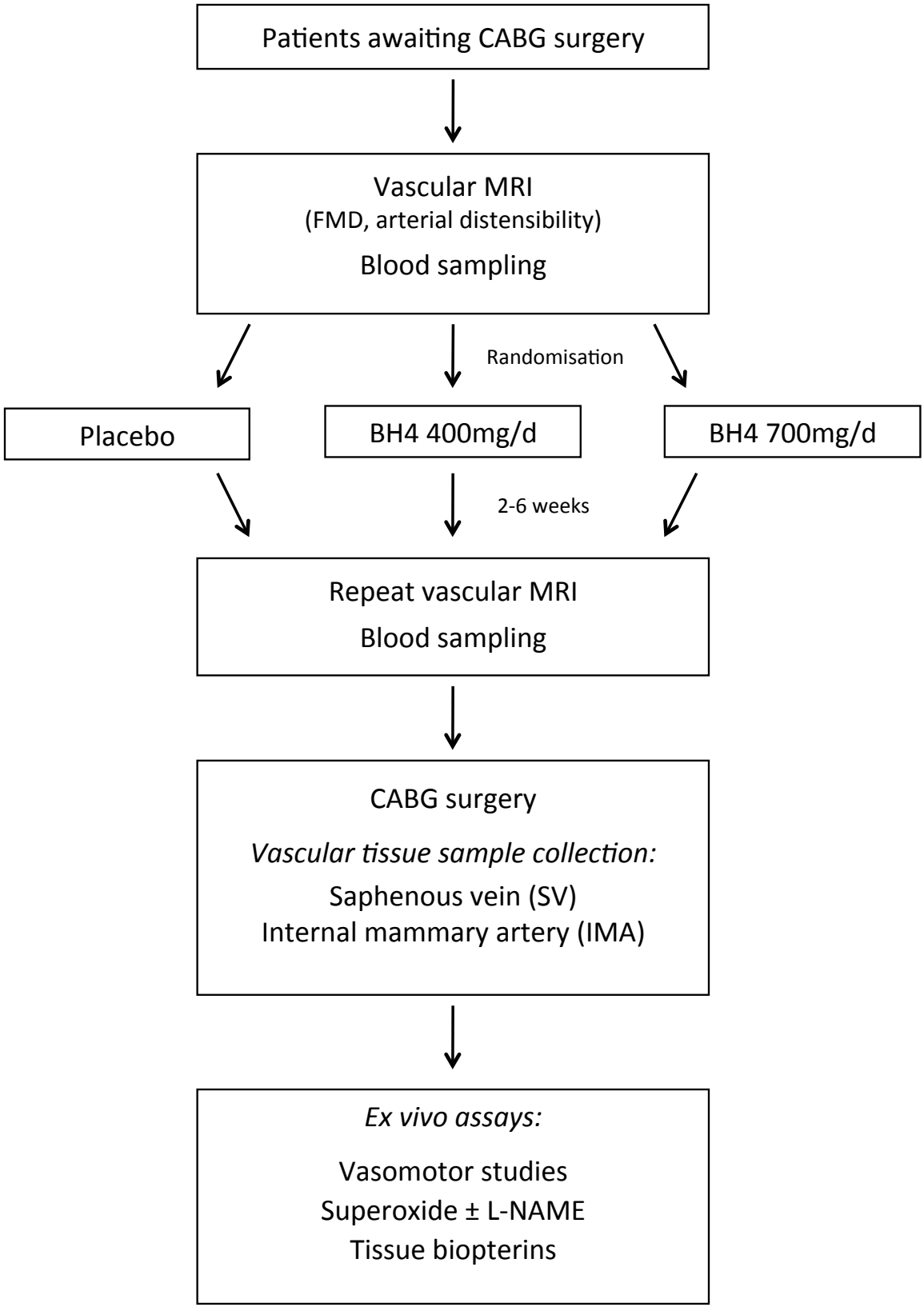


Figure 4-1: OXBIO study scheme

characteristics of the patients are presented in Table 4-1. The study protocol was approved by the Oxfordshire Research Ethics Committee, and each participant gave written informed consent.

4.2.2 Tissue and plasma samples

Paired samples of SV and IMA were obtained from each patient at the time of CABG surgery and snap frozen in the operating theatre in liquid nitrogen to prevent auto-oxidation of BH4. Blood samples were obtained at baseline and immediately before surgery. Blood was centrifuged at 4000 rpm for 5 minutes; plasma was collected and stored at -80°C. Plasma samples for BH4 quantification were collected and stored with the addition of the antioxidant dithioerythritol (DTE, 1 mM), to prevent auto-oxidation of BH4.

4.2.3 Quantification of plasma and vascular biopterins

BH4, BH2, and B levels in plasma and vascular tissue were each determined separately by high-performance liquid chromatography (HPLC) followed by electrochemical (for BH4) and fluorescent (for BH2 and B) detection, as described in Chapter 2. Levels of biopterins were expressed as pmol/g of tissue for vessels and nmol/L for plasma.

4.2.4 Statistical analysis

The clinical trial was powered to detect changes in endothelial function *in vivo* (measured by brachial FMD; results presented and discussed in Chapter 5). Power calculations were based on previous data from the Channon group. It was calculated that with 19 patients per group, the study would be able to detect a 4.7% difference in the change of brachial FMD between any of the two active

	Placebo	6R-BH4 400mg/d	6R-BH4 700mg/d
N (male:female)	19 (16:3)	14 (13:1)	16 (14:2)
Age, years (SEM)	68 (2)	69 (2)	68 (2)
Treatment duration, days (SEM)	30 (3)	30 (4)	29 (3)
BMI, kg/m^2 (SEM)	27.1 (1.0)	29.5 (1.1)	27.2 (0.8)
Systolic BP, mmHg (SEM)	133 (4)	141 (4)	132 (5)
Diastolic BP, mmHg (SEM)	69 (2)	79 (3)	70 (3)
Creatinine, μmol/L (SEM)	109 (4)	102 (5)	112 (5)
Cholesterol, mmol/L			
Median	3.5	4.4	3.8
25^{th}-75^{th} percentiles	3.3–4.5	3.7–5.3	3.3–4.2
HDL, mmol/L			
Median	1.2	1.3	1.1
25^{th}-75^{th} percentiles	1.0–1.4	0.9-1.6	1.0–1.5
Triglycerides, mmol/L			
Median	1.2	1.2	1.3
25^{th}-75^{th} percentiles	1.0–1.4	1.0–2.4	0.9–2.2
Hb_{A1c}, %			
Median	5.7	6.2	5.6
25^{th}-75^{th} percentiles	5.6–6.7	5.6–6.9	5.3-6.4
Diabetes mellitus, n (%)	5 (26)	5 (36)	4 (25)
Hypertension, n (%)	13 (68)	11 (79)	11 (69)
Family history, n (%)	6 (32)	7 (50)	2 (13)
MI, n (%)	9 (47)	6 (43)	3 (19)
Smoking status, n (%)			
Current or recent smoker	2 (11)	2 (14)	4 (25)
Ex-smoker >1 year	13 (68)	10 (71)	10 (63)
Never smoked	4 (21)	2 (14)	2 (13)
Extent of CAD, n (%)			
2-vessel	3 (16)	1 (7)	7 (44)
3-vessel	16 (84)	13 (93)	9 (56)
Medication, n (%)			
Aspirin	19 (100)	11 (79)	14 (88)
Clopidogrel	3 (16)	4 (29)	5 (31)
Statin	18 (95)	13 (93)	16 (100)
ACE inhibitor or ARB	16 (84)	11 (79)	9 (56)
β-blocker	16 (84)	10 (71)	9 (56)
Calcium channel blocker	7 (37)	6 (43)	6 (38)
Diuretic	4 (21)	2 (14)	2 (13)
Nitrate	6 (32)	6 (43)	3 (19)
Nicorandil	2 (11)	0 (0)	2 (13)
Insulin	2 (11)	1 (7)	0 (0)
Oral hypoglycaemic	3 (16)	4 (29)	2 (13)

Table 4-1: Demographic characteristics of OXBIO participants

treatment groups vs placebo, with an assumed normally distributed SD of the change = 4%, α=0.025 and power 90%.

For this specific section of the study, for plasma variables I used two-way ANOVA for repeated measures with “time” x “treatment group” interaction to compare the effect of treatment on these variables between groups. When a significant difference was observed between groups, then individual group comparisons were performed, followed by Bonferroni correction.

For tissue variables, I used one-way ANOVA for multiple comparisons followed by Bonferroni correction to compare variables between groups.

4.3 Results

4.3.1 Effect of oral BH4 treatment on plasma biopterin levels

Both low- and high-dose oral BH4 treatment elevated plasma BH4 levels approximately 2.5-fold compared to placebo ($p<0.001$), with no significant difference between the 400 mg and 700 mg groups (Figure 4-2A). There was a similar significant elevation in plasma BH2 (Figure 4-2B) and B. Accordingly, total biopterin levels (the sum of BH4, BH2 and B) were elevated by treatment (Figure 4-2C), but the ratio of reduced to oxidised biopterins (BH4/(BH2+B)) was not significantly altered by oral BH4 treatment (Figure 4-2D).

4.3.2 Effect of oral BH4 treatment on vascular biopterin levels

In SV, BH4 levels were increased twofold by both low- and high-dose oral BH4 treatment compared to placebo ($p<0.001$; Figure 4-3A), with a similar significant increase in BH2 and B. In IMA, however, there was no significant difference in

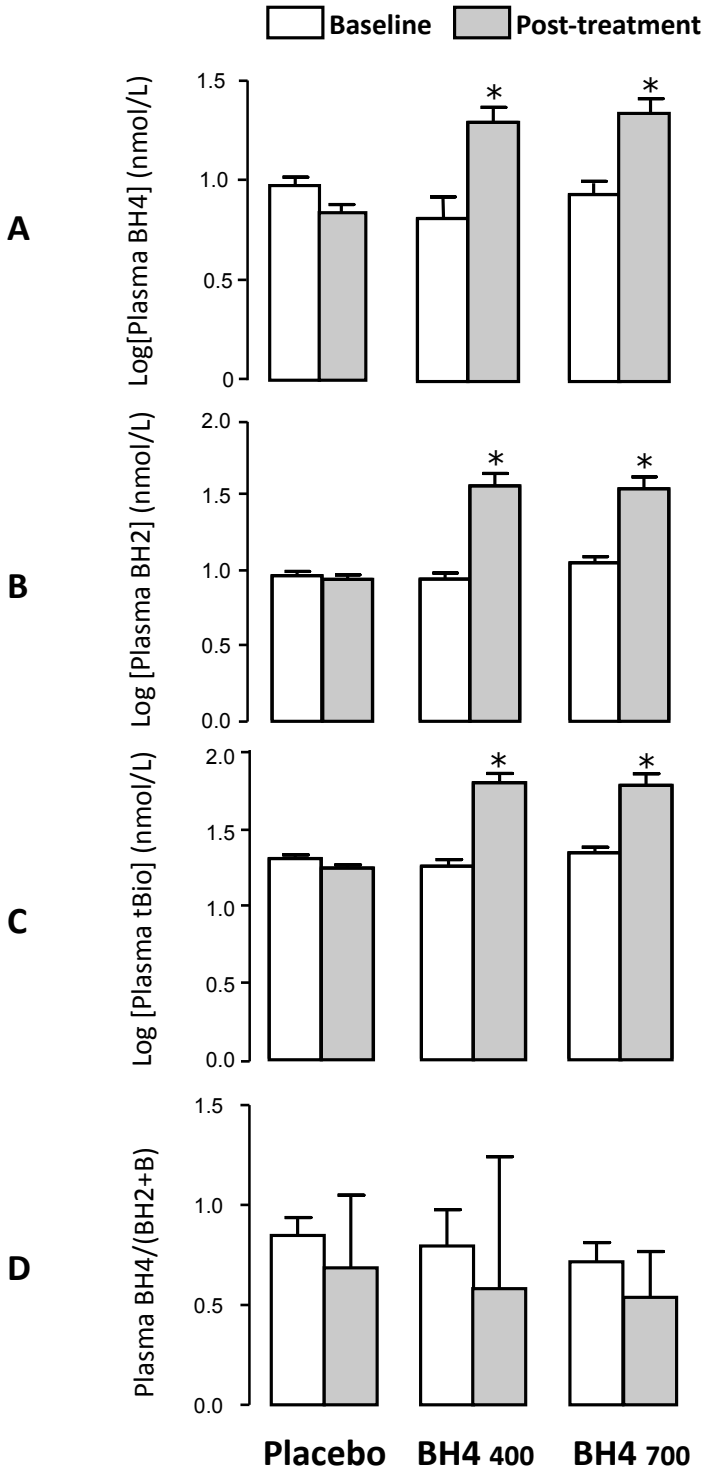


Figure 4-2: Effect of oral BH4 treatment on plasma biopterins

Levels of plasma biopterin species were quantified at baseline and following treatment with oral BH4 (400mg/d, n=13; 700mg/d, n=16) or placebo (n=19). Treatment with oral BH4 400mg/d or 700mg/d resulted in a significant increase in plasma BH4 levels compared to placebo (Panel A), but also a significant increase in plasma BH2 (Panel B) and biopterin (B) (data not shown). Accordingly, total biopterins (tBio, the sum of BH4, BH2 and biopterin) were significantly elevated by BH4 treatment (Panel C), but there was no change in the ratio of reduced to oxidised biopterins (BH4/(BH2+B) (Panel D). There were no significant differences between the two BH4 treatment groups. Values are expressed as mean±SEM of log-transformed values. *p<0.001 vs placebo group for change from baseline.

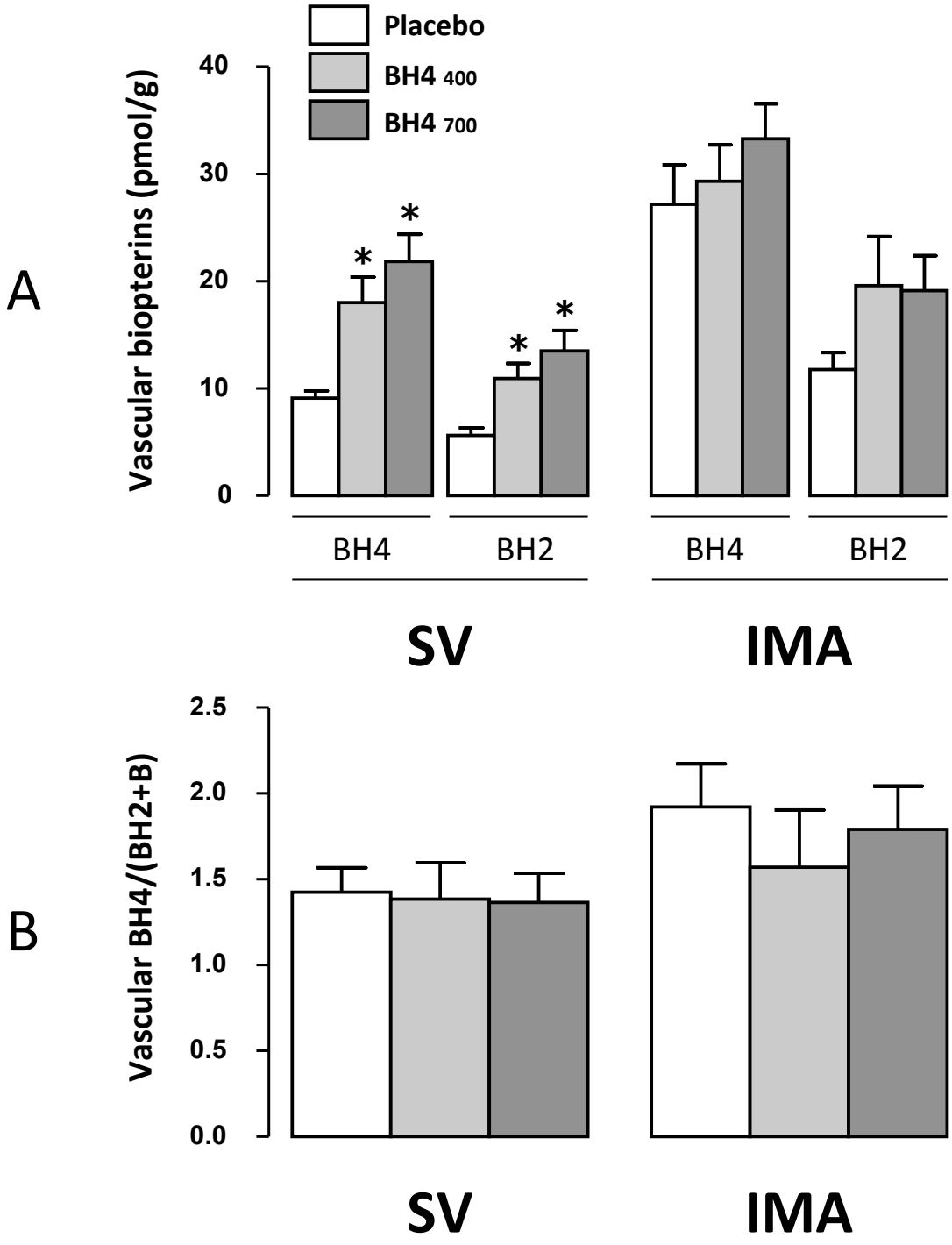


Figure 4-3: Effect of oral BH4 treatment on vascular biopterins
Samples of saphenous vein (SV; placebo, n=18; 400mg/d, n=12; 700mg/d, n=15) and internal mammary artery (IMA; placebo, n=18; 400mg/d, n=11; 700mg/d, n=16) were collected at the time of CABG surgery for quantification of biopterin species. In SV, treatment with oral BH4 resulted in a significant increase in tissue levels of BH4 and BH2 (Panel A) and biopterin (data not shown) compared to placebo. In IMA there were no significant differences between treatment groups (Panel A). In both vessel types, treatment with oral BH4 did not alter the tissue ratio of reduced to oxidised biopterins (BH4/(BH2+B) (Panel B). Values are expressed as mean±SEM. *p<0.001 vs placebo.

BH4 (p=0.334), BH2 (p=0.134) or B (p=0.082) in BH4-treated patients compared to placebo (figure 4-3A). In both SV and IMA there was no difference in the BH4/(BH2+B) ratio between placebo and BH4-treated patients (Figure 4-3B).

4.4 Discussion

The design of the study allows, for the first time in humans, detailed mechanistic evaluation of the pharmacokinetics and pharmacodynamics of oral BH4 treatment in both blood and vascular tissue. Thus, one can directly relate changes in biopterin bioavailability to changes in endothelial function and vascular oxidative stress.

These pharmacokinetic data show that treatment with oral BH4 in subjects with CAD is indeed sufficient to significantly elevate plasma BH4 levels. This is despite the fact that blood samples taken at the time of CABG surgery were effectively trough levels, i.e. 12–16 hours following the last dose. However, this elevation in plasma BH4 is tempered by similar rises in plasma BH2 and B, such that the ratio of reduced to oxidised biopterins (BH4/(BH2+B)) in plasma remains unchanged following treatment.

In the vasculature there are different findings in venous and arterial tissue. Changes in SV mirror those seen in plasma, but in IMA the smaller absolute changes and greater variability in biopterin levels did not reveal a significant effect of oral BH4 treatment. If data from the low- and high-dose BH4 groups are combined to perform an analysis of 'BH4 therapy' versus placebo, the increase in BH2 (but not BH4) in IMA in the combined treatment group reaches statistical significance (19.3 pmol/g vs 11.8 pmol/g, p=0.03). Thus, oral BH4 therapy in

subjects with CAD appears more effective at elevating arterial BH2 levels than BH4 levels. However, despite the changes in BH4 seen after oral BH4 treatment, the BH4/(BH2+B) ratio remains unchanged in both SV and IMA.

The explanation for the discrepancy between venous and arterial tissue is not clear. Biopterin transport is not fully understood, especially in humans and in intact tissues. Previous work has indicated that biopterin transport is cell-type dependent, and that both direct uptake (as BH4) and conversion to BH2 followed by recycling via DHFR are possible mechanisms [176]. The first explanation, therefore, is that there is a genuine biological difference between human veins and arteries in biopterin uptake. If BH4 enters the vessel via passive diffusion from the lumen, then slow venous blood flow in lower limb veins, in comparison to high-pressure arterial flow, may promote uptake. If BH4 enters the vessel via the salvage pathway then it is possible that DHFR is regulated differently between SV and IMA.

The alternative possibility is that the difference observed between SV and IMA is not due to a different biological mechanism of transport between these tissue types. The data do show a small but statistically non-significant increase in a BH4 in IMA (and a slightly larger accumulation of BH2); however, the variability in BH4 was greater in IMA than in SV, and therefore the sample size is too small to exclude a very small effect of BH4 therapy on arterial BH4 levels.

CHAPTER 5: FUNCTIONAL EFFECTS OF ORAL BH4 THERAPY ON VASCULAR FUNCTION

5.1 Introduction

In the previous chapter I demonstrated that chronic treatment with oral BH4, at the doses used in the clinical trial, was able to elevate plasma BH4 levels compared to placebo, with a similar effect on plasma BH2. I also demonstrated similar significant changes in saphenous vein BH4 and BH2 levels, although the changes in internal mammary arteries did not reach statistical significance. The next question was to address how these changes in biopterin availability influenced vascular endothelial function and oxidative stress.

5.1.1 Hypothesis & aims

In this part of the study I sought to determine how oral BH4 treatment affected eNOS-mediated endothelial function in patients with CAD, both *in vivo* and *ex vivo*. Specifically, I hypothesised that:

- Oral BH4 would improve clinical measures of endothelial function and arterial stiffness *in vivo* compared to placebo.
- Oral BH4 would improve *ex vivo* measures of NO-mediated endothelial function and reduce total and NOS-derived vascular superoxide production compared to placebo.

5.2 Methods

5.2.1 Study design and subjects

Study design and subjects were as described in section 4.2.1.

5.2.2 MRI quantification of vascular function

Vascular function was quantified by high-resolution MRI at baseline and at the end of the treatment period. Images of the aorta and carotid arteries were used to determine arterial distensibility and aortic PWV as indices of vascular stiffness. Arterial distensibility was determined in the ascending aorta, proximal descending aorta, distal descending aorta, and both common carotid arteries using a high resolution gradient-echo pulse sequence on a 1.5 Tesla MR scanner (Siemens Sonata, Erlangen, Germany), as described in Chapter 2. A velocity-encoding gradient for phase-contrast MRI was applied to determine PWV in the aorta.

Brachial artery FMD was used as a measure of endothelial function. Using cross-sectional images of the brachial artery piloted in three dimensions, FMD was defined as the maximal percentage change in luminal area from baseline following five minutes forearm ischemia; endothelium-independent dilation was similarly quantified following administration of 200 mcg sublingual GTN.

5.2.3 Vasomotor studies

Paired samples of saphenous vein (SV) and internal mammary artery (IMA) were obtained from each patient at the time of CABG surgery and transferred to the laboratory within 30 minutes in ice-cold Krebs-Henseleit buffer. Endothelium-

dependent and -independent relaxations were assessed in SV rings using isometric tension studies. Four rings from each vessel were pre-contracted with phenylephrine ($3x10^{-6}$ M), following which endothelium-dependent relaxations to acetylcholine (ACh, 10^{-10} to 10^{-6} M) and bradykinin (BK, 10^{-9} to $3x10^{-6}$ M) were quantified. Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, 10^{-10} to 10^{-6} M) were evaluated in the presence of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100 μM).

5.2.4 Quantification of vascular superoxide production

Vascular superoxide production was measured from paired segments of intact SV and IMA using lucigenin-enhanced chemiluminescence, as validated previously [171]. Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 30 minutes in oxygenated (95% O2/5% CO2) Krebs-HEPES buffer (pH 7.4) at 37°C. Lucigenin-enhanced chemiluminescence was measured with low-concentration lucigenin (5 μM). NOS-derived superoxide production was determined by the difference in superoxide production after 30 minutes pre-incubation with L-NAME (100 μM). NADPH-oxidase activity was estimated by measuring superoxide production 10 minutes following the addition of NADPH (100 μM).

5.2.5 Statistical analysis

As discussed in Chapter 4, according to the original power calculation (19 patients per group), the study would be able to detect a 4.7% difference in the change of FMD between any of the two active treatment groups vs placebo, with an assumed normally distributed SD of the change=4%, α=0.025 and power 90%.

However, the study recruited 19 patients in the placebo group, 14 in the 400mg/d group, and 16 in the 700mg/d group. Therefore, *post hoc* analysis showed that the true statistical power to detect the same differences was 83% for the 400mg/d group vs placebo, and 86% for the 700mg/d group vs placebo.

For MRI variables I used two-way ANOVA for repeated measures with "time" x "treatment group" interaction to compare the effect of treatment on these variables between groups. When a significant difference was observed between groups, then individual group comparisons were performed, followed by Bonferroni correction.

For superoxide, I used one-way ANOVA for multiple comparisons followed by Bonferroni correction to compare variables between groups. For the organ bath experiments, the effect of treatment on vasorelaxation in response to ACh, BK or SNP was evaluated using two-way ANOVA for repeated measures (examining the effect of "ACh, BK or SNP concentration" x "treatment group" interaction on "vasorelaxations"), in a full factorial model.

5.3 Results

5.3.1 Effect of oral BH4 treatment on endothelial function and arterial stiffness

Quantification of vascular function by MRI at baseline and after treatment with either placebo or BH4 revealed no effect of oral BH4 treatment on brachial FMD (p=0.325, Figure 5-1A) or GTN-mediated dilatation (p=0.11, Figure 5-1B), or on measures of arterial stiffness measured by (Table 5-1). Correspondingly, samples of SV harvested at the time of CABG surgery for *ex vivo* organ bath studies

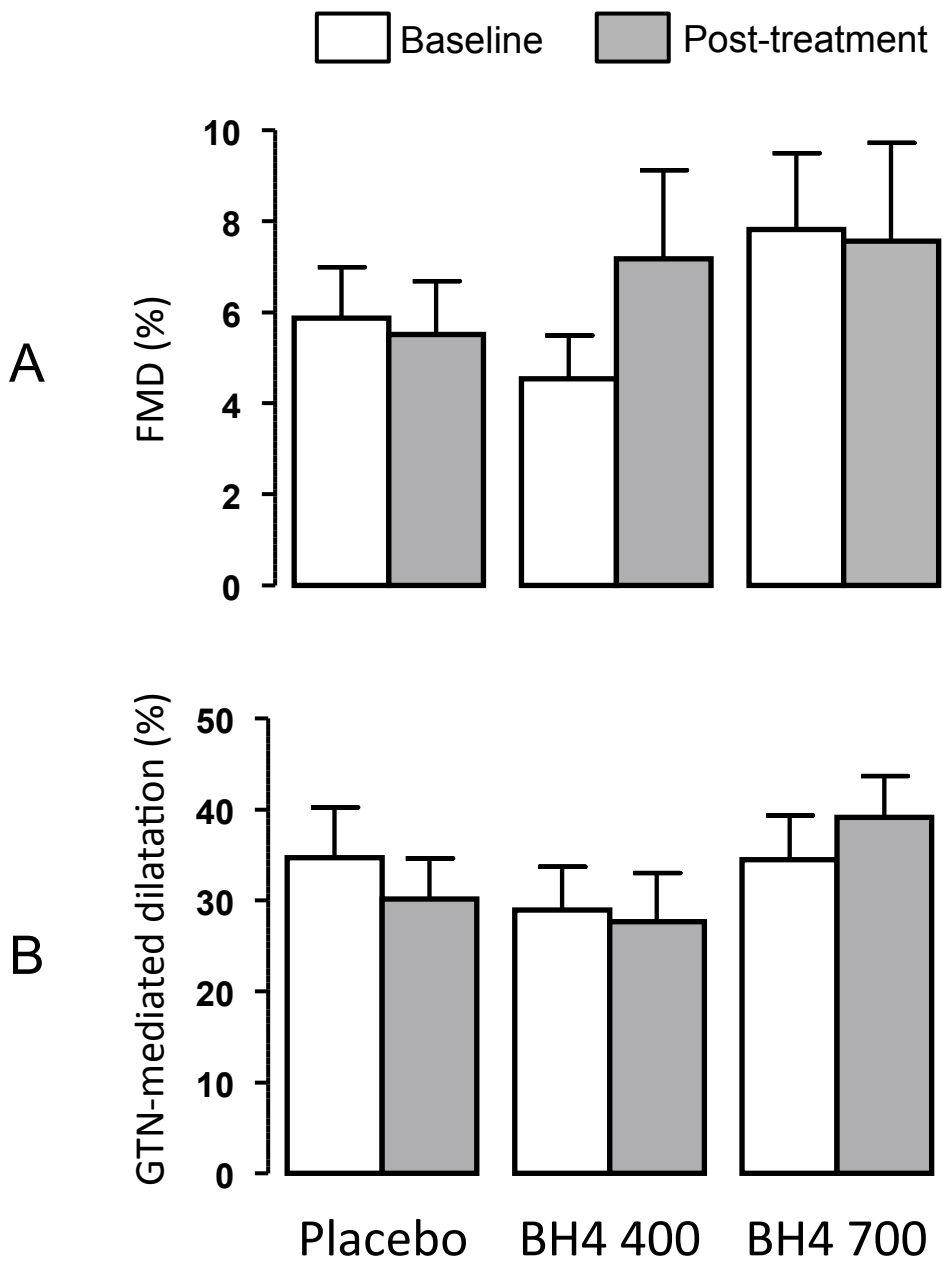


Figure 5-1: Effect of oral BH4 treatment on endothelial function *in vivo*
Endothelium-dependent dilatation was determined *in vivo* (placebo, n=16; 400mg/d, n=11; 700mg/d, n=13) by using MRI to quantify brachial artery flow-mediated dilatation (FMD, Panel A). Endothelium-independent dilatation was assessed following 200μg sublingual glyceryl trinitrate (GTN, Panel B). There was no significant effect of BH4 treatment on either measurement. Data shown in both panels represents maximum percentage change in cross-sectional luminal area from baseline. Values are expressed as mean±SEM.

	Placebo			BH4 400mg/d			BH4 700mg/d			
	Baseline	Post-treatment	Δ	Baseline	Post-treatment	Δ	Baseline	Post-treatment	Δ	p
Distensibility, 10^{-3} $mmHg^{-1}$										
Ascending aorta	1.66 (0.31)	1.54 (0.25)	-0.23 (0.23)	1.79 (0.22)	2.09 (0.45)	0.28 (0.26)	2.04 (0.37)	2.33 (0.38)	0.05 (0.30)	0.40
Proximal descending aorta	2.09 (0.33)	2.17 (0.36)	0.09 (0.12)	2.47 (0.20)	2.62 (0.34)	0.11 (0.23)	2.73 (0.39)	3.01 (0.37)	0.28 (0.29)	0.77
Abdominal aorta	2.69 (0.42)	2.87 (0.42)	0.18 (0.22)	3.00 (0.41)	3.28 (0.43)	0.32 (0.33)	3.32 (0.47)	3.18 (0.46)	-0.14 (0.36)	0.57
Right carotid artery	3.01 (0.25)	2.71 (0.24)	-0.39 (0.24)	3.20 (0.36)	4.48 (0.69)	1.12 (0.59)	4.05 (0.57)	3.83 (0.37)	-0.54 (0.71)	0.11
Left carotid artery	3.59 (0.31)	3.26 (0.40)	-0.53 (0.33)	3.62 (0.33)	4.39 (0.86)	0.68 (0.83)	3.53 (0.42)	3.84 (0.54)	-0.30 (0.47)	0.27
Aortic PWV, m/s	6.57 (0.37)	6.41 (0.37)	-0.01 (0.46)	6.33 (0.40)	7.19 (1.17)	0.73 (1.21)	7.23 (0.72)	6.83 (0.47)	-0.40 (0.59)	0.58

Table 5-1: Effect of oral BH4 treatment on MRI indices of arterial stiffness

Aortic and carotid distensibility and aortic pulse wave velocity (PWV) were measured by MRI at baseline and following treatment with oral BH4 or placebo. Values are expressed as mean±SEM. p-values represent two-way ANOVA for repeated measures with time x group interaction.

showed no significant difference in endothelium-dependent relaxation to ACh or BK, or endothelium-independent relaxation to SNP, between treatment groups (Figure 5-2).

5.3.2 Effect of oral BH4 treatment on vascular superoxide production

There was no significant difference in either total vascular superoxide production between placebo- and BH4-treated subjects in either SV (p=0.70) or IMA (p=0.12) (Figure 5-3A). Furthermore, oral BH4 had no effect on NOS-derived superoxide, as measured by L-NAME-inhibitable superoxide production (Figure 5-3B), or on NADPH-induced superoxide production (data not shown).

5.4 Discussion

In this section of the study, I have demonstrated that oral BH4, at a dose of 400 or 700 mg once daily for 2-6 weeks, has no effect on endothelial function, arterial stiffness, or vascular superoxide in subjects with advanced CAD. It is therefore important to relate these findings to the observed changes in BH4 and BH2 bioavailability.

Despite the changes in absolute BH4 levels in plasma and SV seen after oral BH4 treatment, the BH4/(BH2+B) ratio remains unchanged in plasma and both vessel types (SV and IMA). This finding reveals important insights into the significance of BH2 levels in human vessels. Recent data from cultured endothelial cells [72-73] suggest that the intracellular level of BH2, and more specifically the ratio between reduced and oxidised biopterins, is important in regulating eNOS coupling. These data are consistent with the studies using purified eNOS enzyme

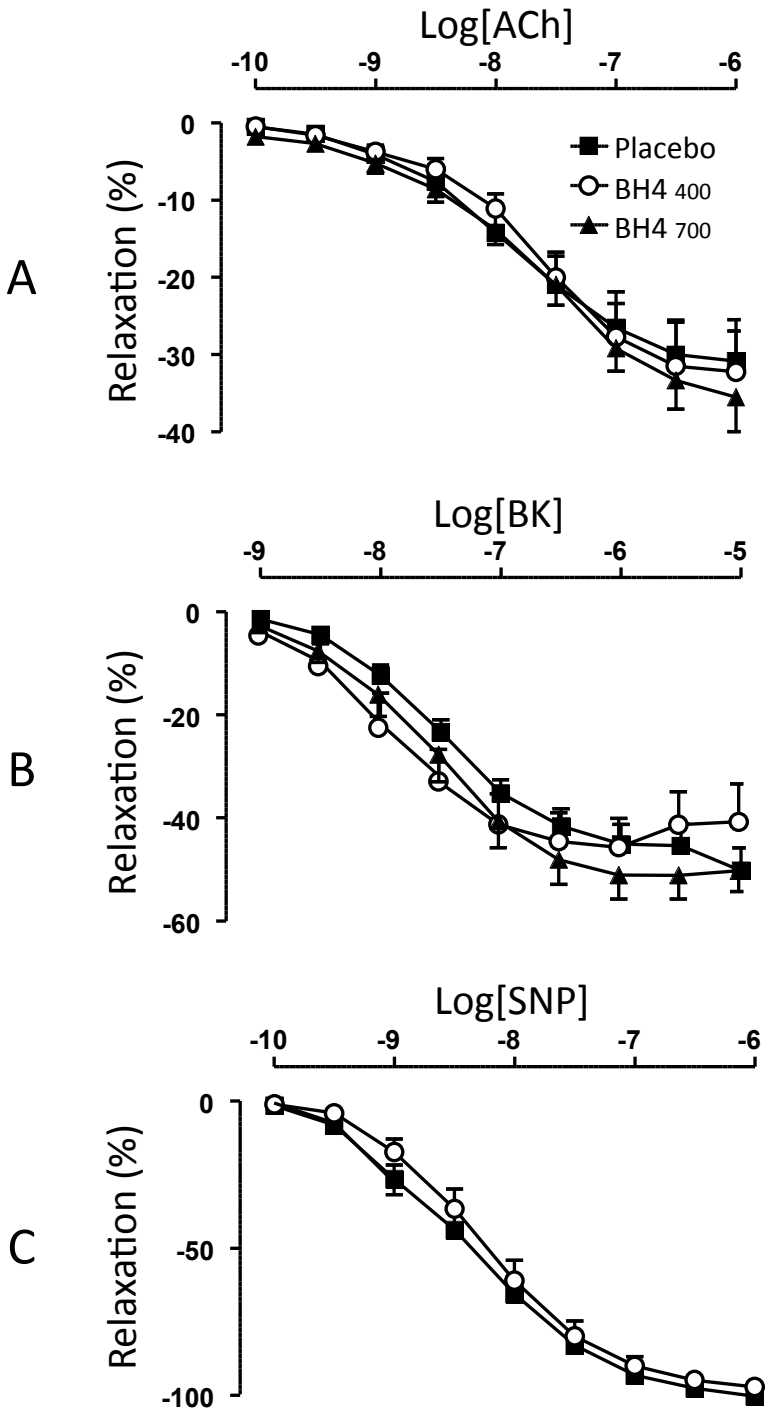


Figure 5-2: Effect of oral BH4 treatment on endothelial function in saphenous vein rings *ex vivo*

Isometric tension studies were performed on saphenous vein rings (placebo, n=14; 400mg/d, n=10; 700mg/d, n=14) to quantify vasorelaxation to acetylcholine (ACh), bradykinin (BK) and sodium nitroprusside (SNP) *ex vivo*. Oral BH4 treatment had no significant effect on endothelium-dependent relaxation to ACh (Panel A) or BK (Panel B), or endothelium-independent relaxation to SNP (Panel C). Values are expressed as mean±SEM.

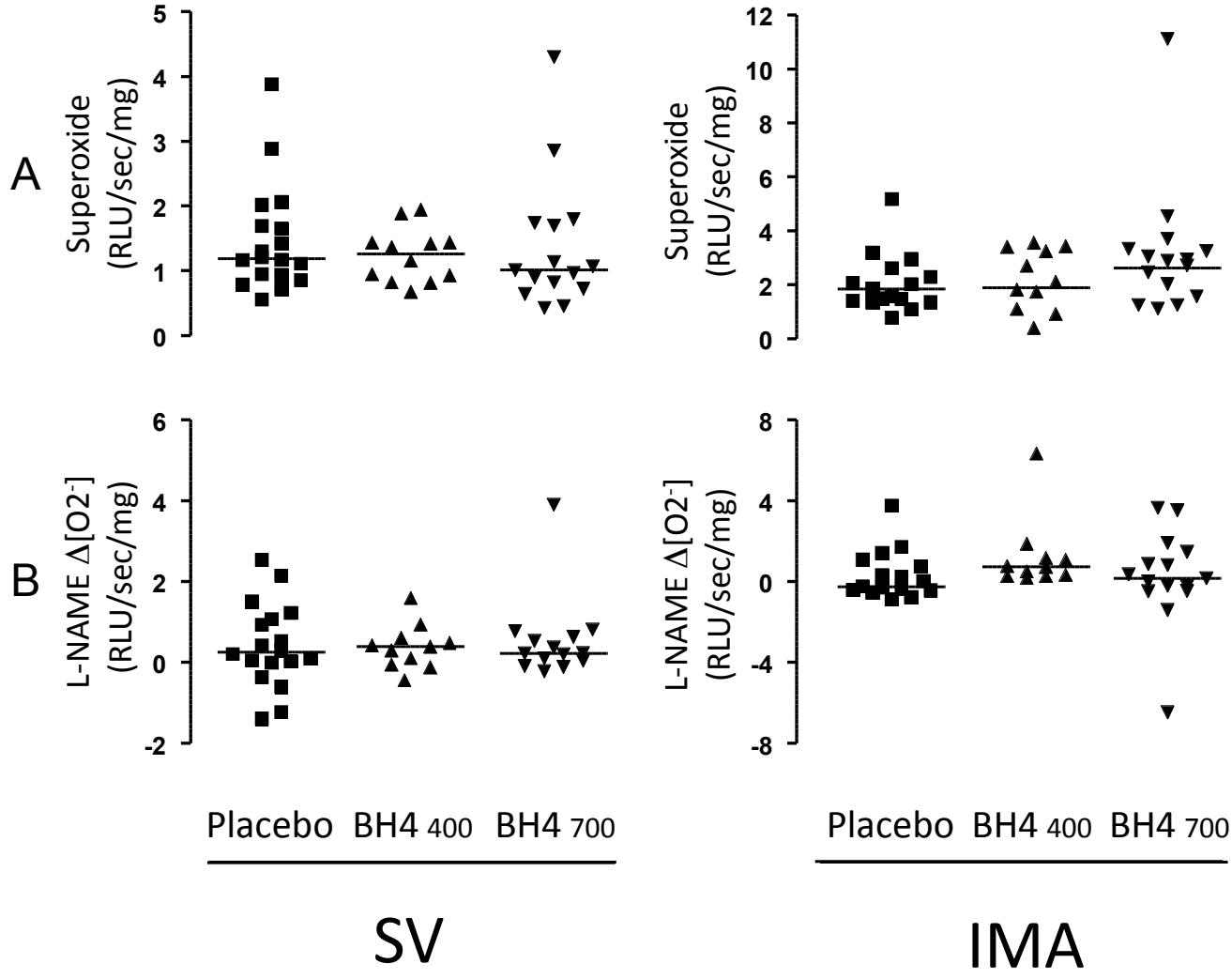


Figure 4-3: Effect of oral BH4 treatment on vascular superoxide production and eNOS coupling

Samples of saphenous vein (SV; placebo, n=18; 400mg/d, n=12; 700mg/d, n=15) and internal mammary artery (IMA; placebo, n=16; 400mg/d, n=11; 700mg/d, n=15) were collected at the time of CABG surgery for quantification of superoxide production by lucigenin-enhanced chemiluminescence in the presence and absence of the NOS inhibitor L-NAME. There was no effect of BH4 treatment on total vascular superoxide production in SV or IMA (Panel A), and no effect on L-NAME-inhibitable superoxide (Panel B). Lines represent median values.

which demonstrated that BH2 can act as a competitive inhibitor of BH4 and promote eNOS uncoupling [48].

In the present study, there is no effect of oral BH4 therapy on total or NOS-derived superoxide in either SV or IMA, despite a twofold increase in absolute BH4 levels in SV. Furthermore, there is no effect of BH4 therapy on endothelium-dependent relaxation in SV rings. Taken together, these findings suggest that BH4/(BH2+B) ratio is a more important determinant of eNOS function than absolute BH4 levels in human vessels. Accordingly, pharmacological strategies that alter the balance between reduced and oxidised biopterins in favour of BH4 may be more effective at improving endothelial function than strategies that have either no effect, or indeed an opposite effect, on this ratio. Both folates [69] – via scavenging of peroxynitrite – and vitamin C [63] – via increased recycling of BH3+ radical to BH4 – have potential to be co-administered with BH4 to achieve this shift in the intracellular oxidative balance. Indeed, chronic oral folic acid treatment has been shown to augment vascular BH4 levels in patients with CAD [177].

The findings of the present study contrast with those of the only previous RCT of chronic oral BH4 treatment [100], which demonstrated an improvement in endothelial function (forearm blood flow response to ACh) in middle-aged subjects with hypercholesterolaemia. That study differed from the current study in a number of respects.

First, the dose of 400 mg BH4 was administered twice daily, potentially reducing the variation in plasma peak-trough concentrations compared to the once-daily

regimen of the current study. Accordingly, vascular measurements will have been performed closer to peak plasma concentrations.

Second, subjects were younger, had no evidence of cardiovascular disease, and had only one risk factor (hypercholesterolaemia), and thus were likely to have lower systemic oxidative stress than subjects in the current study. This may be important as BH4 is known to be highly redox-sensitive, and exogenous BH4 may be oxidised to enzymatically-inactive BH2 (or other biopterin species) in the setting of high oxidative stress.

Third, these younger subjects without established CAD were not taking medications known to improve endothelial function, e.g. HMG-CoA reductase inhibitors (statins) and angiotensin-converting enzyme (ACE) inhibitors, and thus there may be greater 'eNOS reserve' in these subjects compared to CABG patients who already take multiple secondary prevention therapies. Indeed, statin therapy alone appears to have significant beneficial direct effects on BH4 and endothelial function in CAD patients, independent of cholesterol lowering [178].

Thus, the findings of the current study can only be applied to the specific population studied, which represents the most diseased end of the atherosclerosis spectrum; indeed, oral BH4 may have therapeutic potential in earlier stages of vascular disease.

CHAPTER 6: ENDOGENOUS AND EXOGENOUS BH4 HOMEOSTASIS

6.1 Introduction

In Chapter 4 I observed that oral BH4 treatment resulted in significant increases in circulating BH4 and BH2, but had no net effect on biopterin redox status in plasma or vascular tissue. Previous work by Antoniades *et al.* demonstrated an inverse relationship between *endogenous* BH4 levels in plasma and vascular tissue [90]. However, it was unclear from the data in Chapter 4 how variations in *exogenous* plasma BH4, and in particular circulating biopterin redox status (i.e. plasma BH4/(BH2+B) ratio) affects vascular levels.

6.1.1 Hypothesis & aims

In this section I aimed, via further *ex vivo* experiments, to investigate the fate of exogenous BH4 in blood, and the uptake of exogenous BH4 and BH2 in human vascular tissue. Additionally, I aimed to explore whether manipulation of biopterin redox status via antioxidant co-administration could influence vascular levels. Specifically I hypothesised that:

- Antioxidant co-administration would increase exogenous BH4 bioavailability in human blood *ex vivo*.
- Exogenous BH4 co-administered with an antioxidant would increase vascular tissue BH4/(BH2+B) ratio compared to BH4 alone.

6.2 Methods

6.2.1 Incubation of human vessels

To further assess the vascular uptake of exogenous biopterins, SV rings from a further six CABG patients were incubated for 30 minutes in oxygenated Krebs-HEPES buffer at 37°C in either buffer alone, or in the presence of BH4 (100 nM, Schircks Laboratories, Jona, Switzerland), BH4 plus DTE (1 mM), or BH2 (100 nM, Schircks Laboratories). I chose a relatively low concentration of exogenous BH4 (100 nM) to reflect the maximum plasma levels of BH4 achieved after oral BH4 therapy[1], rather than the very high concentrations used in previous intra-arterial studies. Samples of incubation media and vessel rings were stored at -80°C prior to HPLC analysis for quantification of BH4, BH2 and B.

6.2.2 Incubation of whole blood and plasma samples

To further evaluate the oxidation of endogenous and exogenous BH4, whole blood samples from three healthy volunteers were incubated either alone, or with supplementary BH4 (50 nM), BH4 plus DTE (1 mM), or BH2 (50 nM). Blood tubes were placed on rollers during incubation to prevent separation of cellular content from plasma. Plasma was separated from whole blood by centrifugation at baseline and following four hours incubation. Plasma samples were stored at -80°C prior to HPLC analysis.

In a parallel experiment, plasma was separated from whole blood immediately following venepuncture to exclude the effects of the cellular content of blood.

[1] Pharmacokinetic data from BioMarin Investigator's Brochure

Plasma samples were then incubated with exogenous biopterins as per the whole blood experiment above.

6.2.3 Incubation of blood with vitamin C and 5-MTHF

The final stage of these experiments aimed to explore the effect of clinical antioxidant therapies on the redox state of exogenous BH4. Whole blood from three healthy volunteers was incubated with exogenous BH4 (50 nM) for 60 minutes at 37°C either alone, or with additional vitamin C (80 μM), 5-methyltetrahydrofolic acid (5-MTHF, the circulating form of folic acid) (150 nM), or both vitamin C and 5-MTHF. The concentration of vitamin C was selected as this is the plasma concentration of vitamin C at steady state following high-dose oral administration (>1g daily) [179]. The concentration of 5-MTHF was selected as this is the plasma concentration of 5-MTHF achieved by high-dose oral folic acid treatment (5 mg/d) [177]. Plasma was separated from whole blood by centrifugation at baseline and following 5, 15 and 60 minutes incubation for quantification of biopterins by HPLC.

6.2.4 Statistical analysis

Where serial vessel rings from the same vessel or whole blood from the same subject were incubated with different treatments, I used one-way ANOVA for repeated measures. For biopterin measurements in incubation media I used two-way ANOVA for repeated measures with time x group interaction to compare the effect of treatment between groups. Bonferroni *post hoc* correction was applied to compare individual groups if a significant difference was detected by ANOVA.

6.3 Results

6.3.1 Uptake and oxidation of biopterins in human vessels

Incubation of vessels with exogenous BH4 led to rapid and complete depletion of BH4 from the incubation medium, associated with a marked accumulation of BH2. However, the addition of DTE significantly increased residual BH4 concentration ($p<0.001$; Figure 6-1A). In vascular tissue, incubation with exogenous BH4 significantly increased BH2 levels compared to control ($p<0.05$; Figure 6-1C), but only significantly increased BH4 levels in the presence of DTE ($p<0.05$, Figure 6-1B). Incubation with BH2 resulted in a larger increase in tissue BH2 but no increase in tissue BH4. Incubation in exogenous BH4 resulted in a significant reduction in tissue BH4/(BH2+B) ratio compared to control ($p<0.01$), even in the presence of DTE ($p<0.05$, Figure 6-1D).

6.3.2 Oxidation of biopterins in whole blood and plasma

I next investigated the fate of endogenous and exogenous biopterins in whole blood supplemented with BH4 (with or without the antioxidant DTE) or BH2. Endogenous BH4 levels in blood remained unchanged after four hours incubation (Figure 6-2A). In contrast, the addition of exogenous BH4 significantly increased BH2 in human blood, even at baseline, indicating rapid BH4 oxidation (Figure 6-2A). Exogenous BH4 was totally depleted after four hours. The addition of DTE attenuated the rapid oxidation of exogenous BH4 ($p<0.05$), but had no effect on residual BH4 levels after four hours incubation (Figure 6-2A).

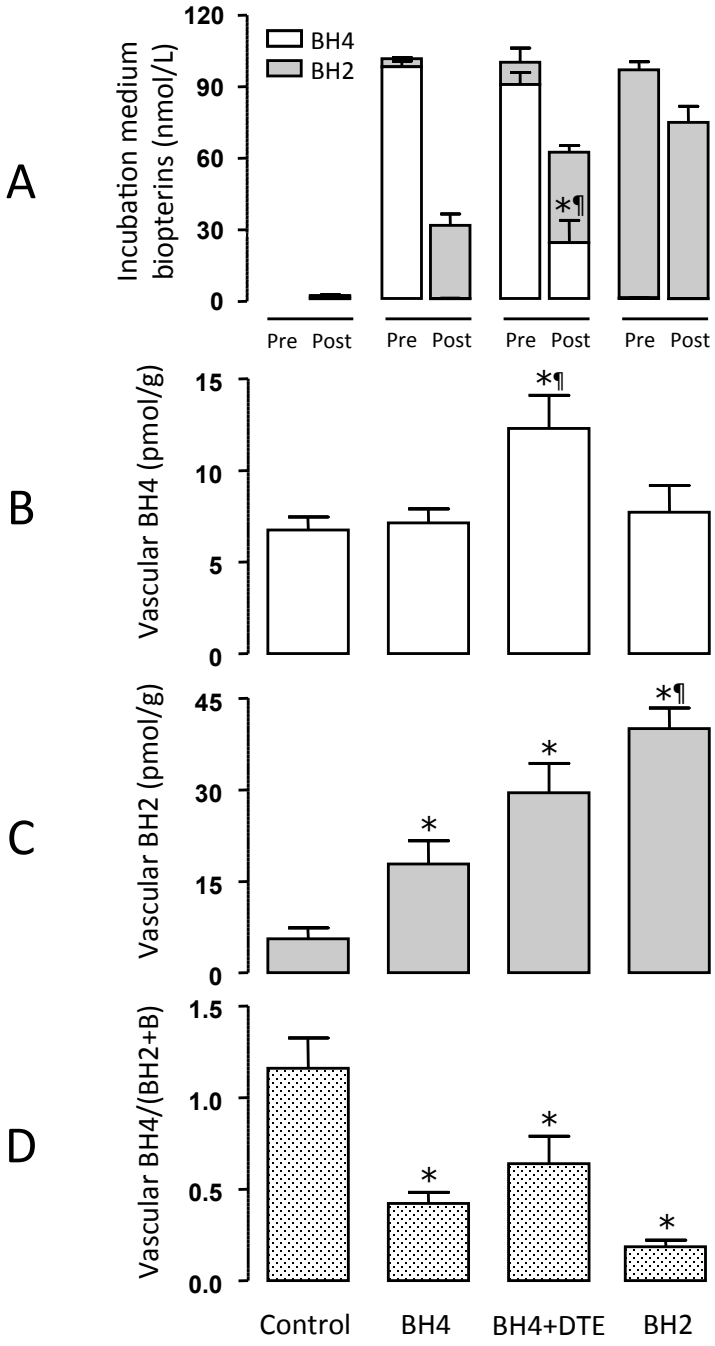


Figure 6-1: *Ex vivo* incubation of vessel rings with exogenous biopterins
Saphenous vein rings (n=6 subjects) were incubated for 30 minutes under the following experimental conditions: **Control**, buffer alone; **BH4**, BH4 100 mM; **BH4+DTE**, BH4 100 mM plus dithoerythritol (DTE, antioxidant) 1 mM; **BH2**, BH2 100 mmol. Samples of incubation media were stored at the beginning and end of the experiment. Panel A: Exogenous BH4 in the incubation medium was totally depleted by the end of the experiment; the addition of DTE provided partial protection from oxidation. Panels B and C: Incubation with BH4 significantly increased tissue BH2 levels compared to control, but only increased tissue BH4 levels in the presence of DTE; incubation with BH2 resulted in a larger increase in tissue BH2 but no increase in tissue BH4. Panel D: Incubation in exogenous BH4 resulted in a significant reduction in tissue BH4/(BH2+B) ratio compared to control, even in the presence of DTE. Values are expressed as mean±SEM. *p<0.05 vs control; ¶p<0.05 vs BH4 alone.

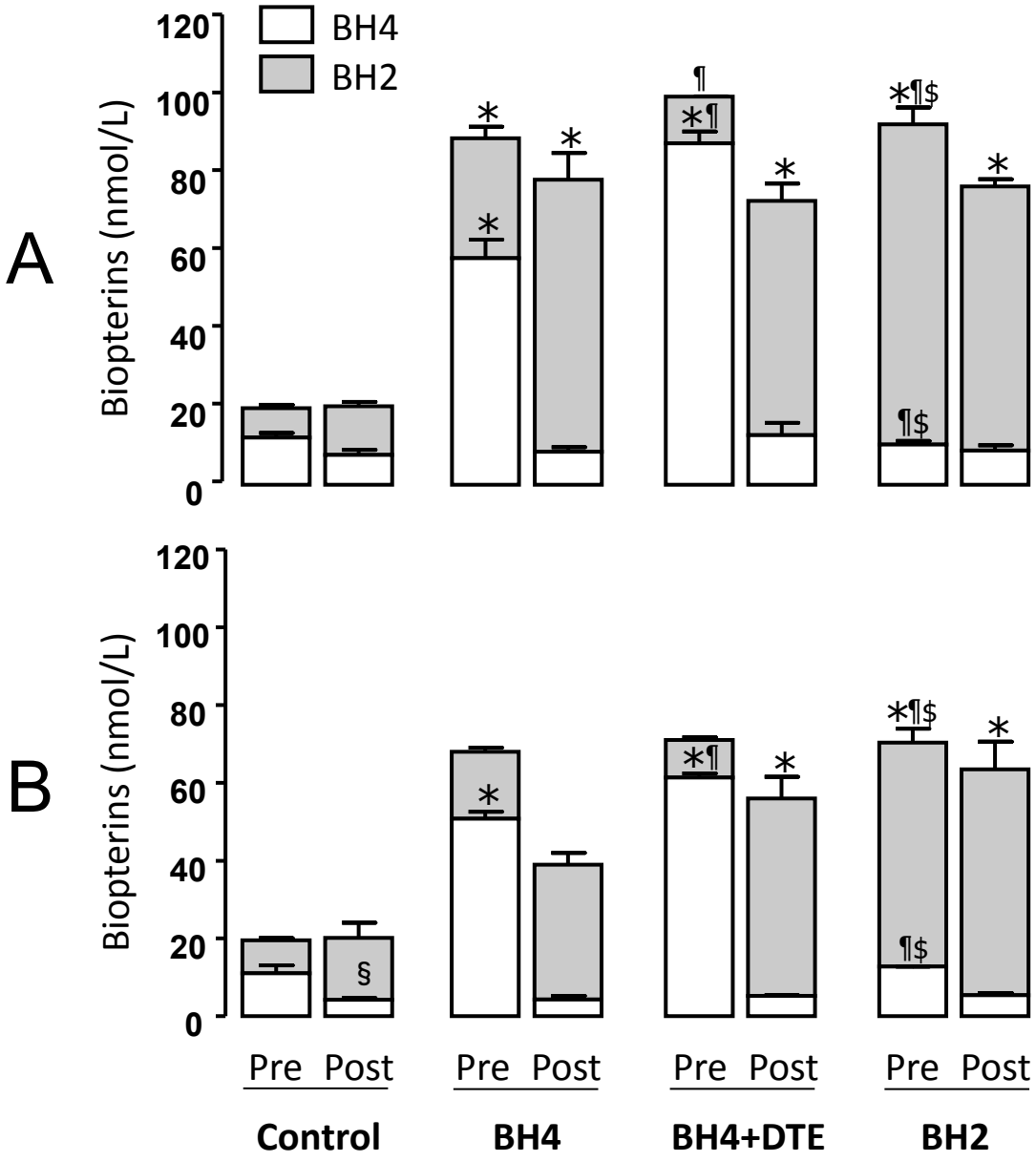


Figure 6-2: Fate of endogenous vs. exogenous BH4 in whole blood and plasma
Samples of whole blood (Panel A) and plasma (Panel B) from healthy volunteers (n=3) were incubated for 4 hours under the following experimental conditions: **Control**, blood or plasma alone; **BH4**, supplementary BH4 50 nM; **BH4+DTE**, supplementary BH4 50 nM plus dithioerythritol (DTE, antioxidant) 1 mM; **BH2**, supplementary BH2 50 nM. In the whole blood samples, plasma was separated at baseline and after 4 hours. Plasma biopterins were then quantified in all samples. In whole blood there was no significant difference in endogenous BH4 or BH2 after 4 hours of incubation, whereas in plasma alone endogenous BH4 was oxidised to BH2. Exogenous BH4 was completely oxidized in both whole blood and plasma after 4 hours; DTE afforded protection from oxidation at baseline, but not after 4 hours. *p<0.05 vs control; ¶p<0.05 vs BH4 alone; §p<0.05 vs baseline.

In contrast to whole blood, when plasma was incubated for four hours there was a significant reduction in endogenous BH4 ($p<0.05$), with evidence of oxidation to BH2 (Figure 6-2B). The effect of addition of exogenous BH4 mirrored that seen in whole blood, i.e. rapid oxidation to BH2 with attenuation by DTE at baseline but not after four hours.

Taken together, these observations demonstrate that exogenous BH4 is rapidly oxidised to BH2 in human blood or blood vessels *ex vivo*, resulting in little or no increase in BH4 levels, but an increase in BH2 and a decrease in the ratio of reduced to oxidised biopterins.

6.3.3 Effect of vitamin C and 5-MTHF on exogenous BH4 bioavailability

In the final experiment I tested the effect of antioxidants used in clinical practice on the bioavailability of exogenous BH4. The depletion of BH4 was most rapid in the first 15 minutes in the control sample (Figure 6-3). Incubation with vitamin C had a small but statistically significant effect on residual BH4 levels after 15 minutes ($p<0.05$), but after 60 minutes BH4 concentrations had fallen to those observed in the control sample. Incubation with 5-MTHF alone had no significant effect, and the addition of 5-MTHF to vitamin C had no greater antioxidant effect compared with vitamin C alone (Figure 6-3).

6.4 Discussion

Understanding the observed effects of oral BH4 treatment on endothelial function in patients with CAD is limited by uncertainty over how exogenous biopterins may enter the vascular endothelium. Studies in mice [180-181] have

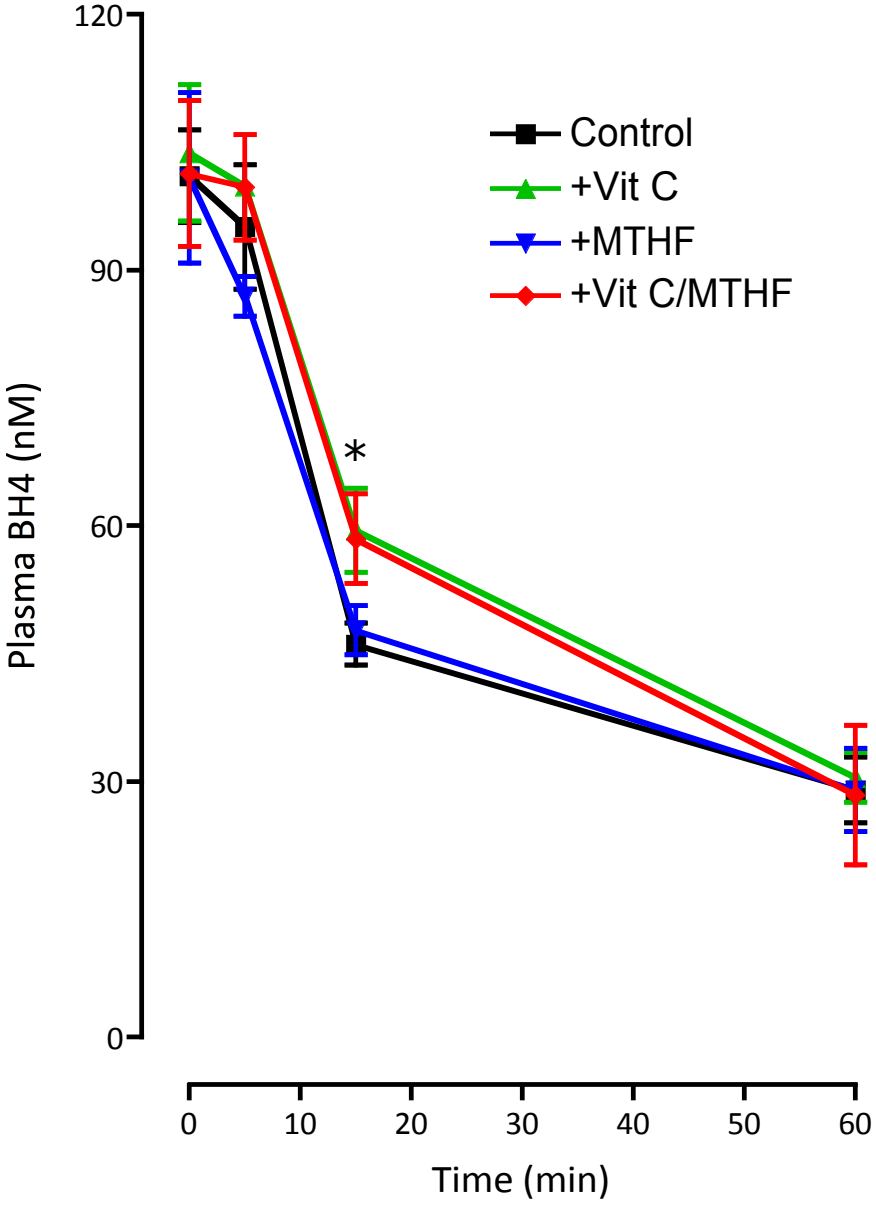


Figure 6-3: Effect of clinical antioxidants on bioavailability of exogenous BH4
Whole blood from healthy volunteers (n=3) was incubated with exogenous BH4 (50 nM) either alone (control) or with the addition of vitamin C (80 μM), 5-MTHF (150 nM), or both vitamin C and 5-MTHF. Plasma for BH4 quantification was obtained by centrifugation at baseline and after 5, 15 and 60 minutes incubation. There was a rapid oxidation of BH4 between 5 and 15 minutes which was attenuated by vitamin C but not by 5-MTHF. This effect was lost after 60 minutes incubation. Data represent mean±SEM. *$p<0.05$ vs control.

suggested that oxidation of BH4 to BH2 and subsequent recycling back to BH4 by dihydrofolate reductase (DHFR) is necessary to augment intracellular BH4; accordingly, this elevation in BH4 can be attenuated by inhibition of DHFR with methotrexate. In Chapter 5 I observed significant oxidation of exogenous BH4 to BH2 in plasma. Thus, one might hypothesise that co-administration of BH4 with an antioxidant (to prevent this oxidation in plasma) may result in a paradoxical reduction in drug uptake into the endothelium. The *ex vivo* experiments using whole blood and vascular tissue shed further light on these important aspects of BH4 homeostasis. Using supplementary doses of exogenous BH4 to raise BH4 levels equivalent to those that are achievable through oral administration (at the doses used in the clinical trial), I have demonstrated a rapid oxidation of BH4 to BH2 when exogenous BH4 is added to human blood, even in healthy volunteers. Co-administration of an antioxidant (either DTE or vitamin C, a widely used clinical antioxidant drug) attenuates the observed oxidation in the initial stages following addition to blood, but has no effect following a prolonged incubation (four hours), during which all exogenous BH4 is totally depleted. In contrast, endogenous levels of BH4 in whole blood are unchanged following incubation, suggesting that endogenous and exogenous BH4 may be oxidised at different rates, possibly due to an interaction with the cellular content of blood. Indeed, the incubations of plasma alone support this theory, with evidence of more rapid oxidation of endogenous BH4 when blood cells are removed immediately following venepuncture. This may be due to either an antioxidant effect of blood cells, or due to release of intracellular biopterins into the plasma.

When vessel rings are incubated in exogenous BH4 for 30 minutes, there is no increase in tissue levels of BH4, but a significant increase in BH2, resulting in a

lower tissue BH4/(BH2+B) ratio. This finding is explained by the rapid oxidation of BH4 to BH2 in the incubation media under the experimental conditions at 37°C in organ chambers, resulting in a predominance of BH2 rather than BH4 for the majority of the duration of the control experiment. Co-administration with DTE significantly attenuates the oxidation of BH4 in the incubation media. The combination of BH4 and DTE is able to increase tissue BH4 levels, but importantly also results in a greater increase in tissue BH2 compared to incubation with BH4 alone, again resulting in a lower BH4/(BH2+B) ratio compared to control. Finally, incubation with BH2 alone results in a large increase in tissue BH2, but no change in tissue BH4.

Taken together, these results suggest that both exogenous BH4 and BH2 can be taken up into human vascular tissue, but accumulation of tissue BH2 does not seem to result in an increase in tissue BH4. The strategy of BH4/antioxidant co-administration, to maintain a favourable BH4/BH2 ratio in circulating blood, might be hypothesised to increase intravascular BH4/BH2 ratio, however these data do not support this. Although DTE affords initial protection of exogenous BH4 from oxidation, this effect appears to be short-lived. More importantly, co-administration of BH4 and DTE still results in an unfavourable ratio of reduced to oxidised biopterins in tissue compared to control, despite increasing absolute BH4 levels, suggesting that in vessels from patients with established CAD there may be continued oxidation of BH4 to BH2 following uptake into the vessel wall, or incomplete regeneration of BH4 from BH2 via the salvage pathway catalysed by dihydrofolate reductase (DHFR). Indeed, recent studies suggest an important role for DHFR in maintaining intracellular BH4 levels, especially in conditions

where BH4 levels are limiting [182-183], or in response to increased oxidative stress where DHFR activity is downregulated [184-185].

CHAPTER 7: ENDOGENOUS BH4 SYNTHESIS AND VASCULAR FUNCTION

7.1 Background

7.1.1 Endogenous BH4 biosynthesis

GTP cyclohydrolase I (GTPCH) is the rate-limiting enzyme in the biosynthesis of BH4 (Figure 1-2), and is a major determinant of BH4 levels, through transcriptional regulation of GTPCH expression. In genetic mouse models, it has been shown that constitutive reduction in GTPCH expression causes BH4 deficiency, resulting in abnormal eNOS regulation [55, 87, 130]. Conversely, transgenic overexpression of GTPCH in the vascular endothelium is sufficient to augment vascular BH4 levels [74], improve endothelial function in models of vascular disease [87], and rescue the vascular effects of genetic BH4 deficiency [130]. Thus, in experimental models, genetic alteration of endogenous BH4 biosynthesis has provided useful insights into the role of BH4 in vascular disease pathogenesis. In contrast, it is unknown whether alterations in endogenous BH4 synthesis in humans are sufficient to alter systemic or vascular BH4 availability, or to have any impact on vascular function or other indices of cardiovascular risk.

Recently published work from our laboratory sought to determine the relationship between vascular function and both plasma BH4 and vascular tissue BH4 levels in patients undergoing CABG surgery [90]. These data showed that low vascular BH4 levels are indeed associated with reduced NO-mediated endothelial function and increased vascular superoxide production. However, these alterations in BH4 in CAD are also influenced by oxidation of BH4 by free

radicals such as peroxynitrite (generating BH2 and B), which causes loss of BH4, independent of any change in biopterin synthesis [63, 69, 84]. This complexity in BH4 biology in CAD is in part related to inflammation (as evidenced by elevated CRP levels) that appears to upregulate GTPCH in liver and lymphoid tissue, leading to increased total biopterin levels, but without a net increase in BH4, since inflammation is also associated with increased BH4 oxidation [87, 186], and with impaired endothelial function [187-189].

These data have revealed interesting relationships between plasma and vascular BH4 in patients with established CAD, and associations between BH4, inflammation, and altered endothelial function. However, the potential for reverse causality prevents any conclusions on the role of BH4 in vascular disease pathogenesis. In particular, the effects of inflammation on BH4 synthesis, BH4 oxidation and endothelial function are major potential confounders. Accordingly, I aimed to address more directly the associations and possible causality of BH4 in vascular disease pathogenesis, using recently identified common genetic variants that are associated with significant differences in endogenous BH4 synthesis.

7.1.2 Genetic variation in BH4 synthesis

Novel variants of the *GCH1* gene that are associated with biologically important differences in *GCH1* expression and BH4 synthesis have been recently described [97, 190-191]. The variant *GCH1* haplotype (denoted the 'X' haplotype) was originally defined by 15 single nucleotide polymorphisms (SNPs) spaced evenly across the *GCH1* gene [97]. *GCH1* had been shown to be upregulated in neurological tissue from rats following experimental nerve injury; stepwise regression analysis demonstrated that one 15 SNP haplotype was associated with

decreased neuropathic pain in humans with chronic lower back pain following surgery. In turn, this haplotype was associated with reduced *GCH1* expression and BH4 accumulation in peripheral blood mononuclear cells (PBMCs) which had been exposed to an inflammatory stimulus *ex vivo* [97].

It was later demonstrated that the 15 SNP haplotype could be reliably detected by screening for only three of the original 15 SNPs: rs8007267G>A in the putative promoter region, rs3783641A>T in intron 1 and rs10483639C>G in the 3' untranslated region (UTR) [190]. Another SNP in the *GCH1* 3' untranslated region (UTR), rs841C>T, identified by a different group, is also associated with decreased urinary NO excretion, and dysregulated cardiovascular autonomic function [136]. Data from our group have since shown that this SNP is in linkage disequilibrium with SNP rs10483639C>G (one of the three defining SNPs of the original X haplotype)[192].

More recently the effects of the *GCH1* X haplotype in subjects with CAD undergoing CABG have been described [193]. The X haplotype was associated with significantly lower *GCH1* mRNA expression in vascular tissue. Furthermore, in patients with the X haplotype there were substantial reductions in both plasma and vascular BH4 levels. These differences in vascular tissue BH4 were also associated with differences in L-NAME-inhibitable superoxide, a measure of uncoupled eNOS, and in NO-mediated vasorelaxations in isolated vessel ring studies [95]. Thus, genetic variation in *GCH1* provides a powerful biological approach to test the effect of high or low BH4 levels on endothelial function and vascular disease intermediate phenotypes, through "Mendelian randomisation". This approach uses the random allocation of alleles between individuals to address long-term causality of known quantitative traits in vascular disease

pathogenesis, and has provided important insights in to the importance of biomarkers such as CRP [194] and homocysteine [195] in cardiovascular disease.

7.2 Hypothesis and aims

The data from CABG patients showed striking differences in BH4 bioavailability in both plasma and vascular tissue, caused by the X haplotype. The differences were greatest between homozygote X carriers, denoted XX, and subjects with no copies of the X haplotype, denoted OO. XO heterozygotes displayed an intermediate phenotype. Importantly however, the greatest differences between genotypes was observed in patients with the highest levels of inflammation, as assessed by plasma CRP concentrations [Antoniades *et al.*, unpublished data]. To further investigate the role of inflammation and endogenous BH4 synthesis on vascular function I studied the effect of the X haplotype in healthy volunteers (without clinically apparent vascular disease).

Specifically, I hypothesised that:

- Healthy subjects with the XX *GCH1* genotype will have altered levels of BH4 compared with those with OO haplotype.

- Differences in plasma BH4 levels, associated with *GCH1* haplotypes, will be associated with alterations in vascular function, as assessed by measurement of endothelial function and vascular stiffness, compared to those with OO haplotype.

7.3 Methods

7.3.1 Subjects and genotyping

All subjects had previously donated DNA samples during participation in the Oxford Biobank study [196], a tissue bank of approximately 1200 healthy volunteers aged 30-50 from the Oxfordshire region. Subjects were recruited based on *GCH1* genotype[2]. The DNA samples were typed for the 3 SNP *GCH1* X haplotype (SNP details described above). Subjects were additionally screened for the fourth SNP (rs841C>T in the 3' UTR) described by Zhang *et al.* [136]. This SNP was shown to be in strong linkage disequilibrium (LD) with rs10483639. The control "O" haplotype was defined as the absence of all four minor allele SNPs detailed above. Detailed haplotype and diplotype frequencies are listed in Tables 7-1 and 7-2. All 25 XX subjects plus approximately 75 age- and sex-matched OO control subjects were subsequently invited to participate. This number of OO control subjects was chosen following consideration of the effect of sample size on the statistical power of the study (Figure 7-1). Twenty XX subjects and 52 OO subjects agreed to participate. Demographic characteristics of participants are detailed in Table 7-3.

7.3.2 Vascular measurements

Subjects attended in the morning following an overnight fast. All measurements were performed in a quiet, temperature-controlled environment. Subjects underwent comprehensive non-invasive vascular assessment using the following techniques (methods described in detail in Chapter 2):

[2] Genotyping was performed by Dr Matthew Neville at the Oxford Centre for Diabetes, Endocrinology & Metabolism (OCDEM).

Haplotype Code	Haplotype*	Haplotype allele count	Haplotype allele frequency (%)
1	0000	1785	76.09
9	1111	382	16.28
7	1100	59	2.51
8	1110	56	2.39
4	0011	30	1.28
3	0010	16	0.68
2	0001	14	0.60
5	0111	2	0.09
6	1011	2	0.09

Table 7-1: Genotyping of 1173 subjects in the Oxford Biobank cohort

GCH1 haplotypes were typed in 1173 subjects (= 2346 chromosomes) by genotyping 4 *GCH1* SNPs:

rs10483639C>G, rs841C>T (both 3' UTR), rs3783641A>T (intron 1) and rs8007267G>A (putative promoter region).

*Haplotypes are coded as 0 or 1 at each SNP, with 1 denoting the presence of the minor allele.

The *GCH1* 'X' haplotype, defined by the minor allele at each of the four SNPs, is highlighted in yellow. There was strong linkage disequilibrium (LD) between SNP rs10483639C>G in the X haplotype originally reported by Tegeder *et al.* [97] and SNP rs841C>T reported by Zhang *et al.* [136]. In only 4 cases (0.18%) was there breakdown in this LD, highlighted in red.

Diplotype	Diplotype count	Diplotype frequency (%)
1_1	674	57.46
1_9	300	25.58
1_7	47	4.01
1_8	45	3.84
9_9	25	2.13
1_4	21	1.79
1_3	13	1.11
8_9	10	0.85
1_2	9	0.77
4_9	9	0.77
7_9	9	0.77
1_5	2	0.17
2_7	2	0.17
2_9	2	0.17
3_9	2	0.17
2_6	1	0.09
3_8	1	0.09
6_7	1	0.09
Total	*1173*	*100.00*

Table 7-2: Diplotype frequencies of *GCH1* haplotypes in 1173 subjects from the Oxford Biobank cohort

The haplotype codes, incorporating 4 SNPs, are shown as defined in Table 7-1.

25 subjects were identified (highlighted in yellow) who were homozygous for the 3 SNP X haplotype originally defined by Tegeder et al [97]. All 25 homozygous subjects were also homozygous for the rs841 minor allele identified by Zhang et al [136]. Thus, the 4 SNP X haplotype incorporates both the Tegeder et al. and the Zhang et al. variants.

We also identified 674 control subjects (OO genotype) who possess no copies of the 4 minor SNP alleles, defined above (highlighted in blue).

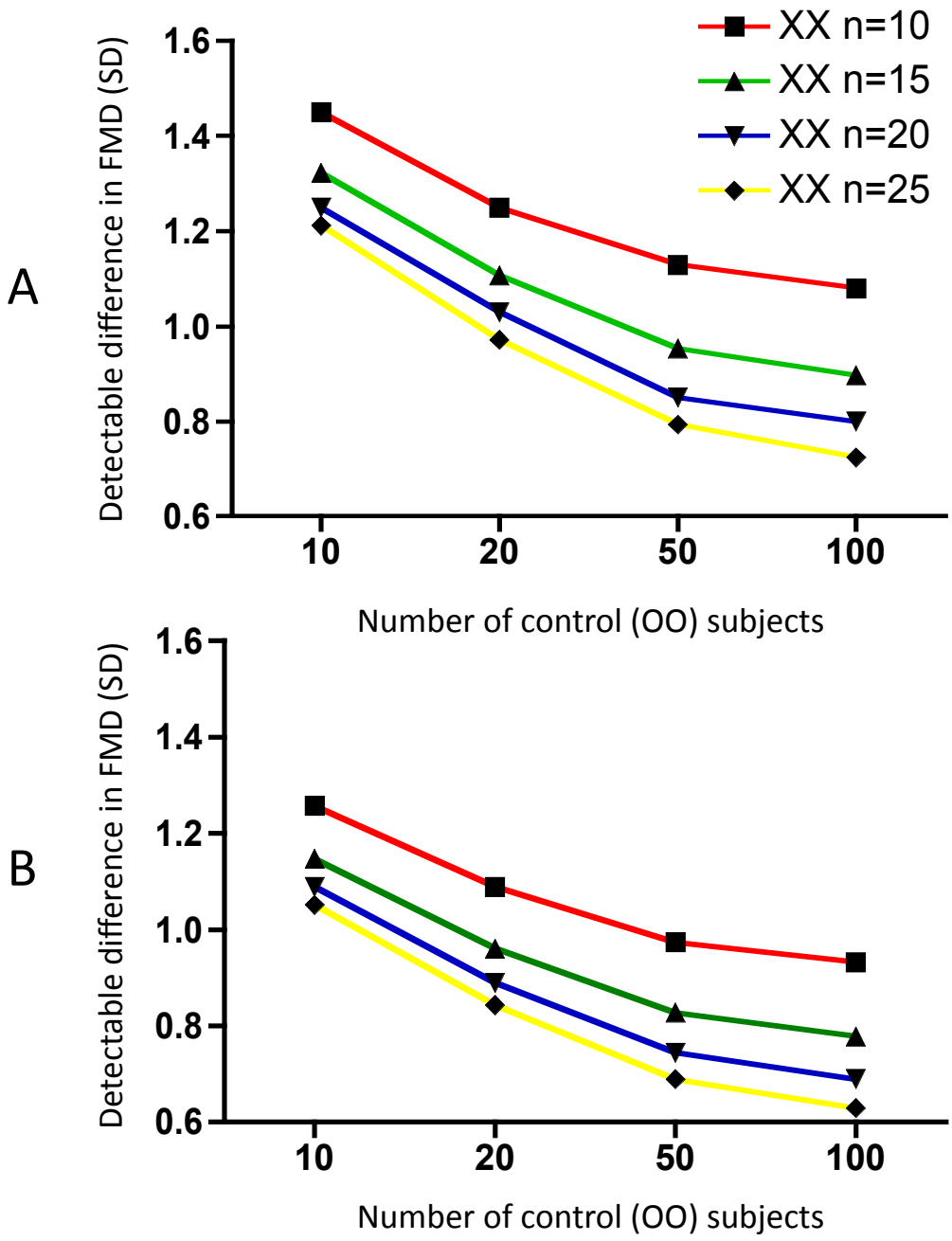


Figure 7-1: Effect of increases in sample size on statistical power
Recruitment for the study was limited by the number of available XX subjects (max = 25) in the Oxford Biobank cohort. The figure demonstrates how the statistical power of the study is dependent principally on the size of the smaller group, but also that the statistical power of the comparison can be enhanced by increasing the size of the larger group. Panel A demonstrates the statistical power according to variation in size of the control group (OO subjects) for given numbers of XX subjects, with 90% power. Panel B shows the same data with 80% power. In the case of 20 XX subjects, the difference detectable with 90% power in brachial FMD with an equal number of OO subjects = 1.02 SD; this can be improved to 0.86 SD by increasing the number of control subjects to 50 (Panel A, blue curve). However, when the number of control subjects is further increased to 100, there is only a small resultant increase in power.

	XX	OO	p
N (male:female)	20 (13:7)	52 (32:20)	0.79
Age, years (SD)	46 (6)	48 (6)	0.13
Smoking status, n (%)			
Current or recent smoker	3 (16)	2 (4)	
Ex-smoker	2 (11)	14 (27)	0.10
Never smoked	14 (74)	36 (69)	
Systolic BP, mmHg (SD)	124 (17)	128 (13)	0.28
Diastolic BP, mmHg (SD)	78 (11)	78 (7)	0.86
BMI, kgm^{-2} (SD)	26.6 (5.1)	25.9 (3.8)	0.50
Waist:hip ratio (SD)	0.87 (0.09)	0.86 (0.09)	0.67
Diabetes mellitus, n (%)	0 (0)	1 (1.9)	0.54
Statin use, n (%)	0 (0)	3 (5.8)	0.27
ACE inhibitor use, n (%)	0 (0)	7 (13.5)	0.08
Aspirin use, n (%)	1 (5)	0 (0)	0.11
Cholesterol, mmol/L			
Median	5.1	5.0	0.64
25^{th}-75^{th} percentiles	4.5–6.1	4.2-5.8	
HDL, mmol/L			
Median	1.2	1.3	0.34
25^{th}-75^{th} percentiles	1.0–1.4	1.1–1.6	
Triglycerides, mmol/L			
Median	1.1	1.0	0.56
25^{th}-75^{th} percentiles	0.8–1.8	0.7–1.5	
Glucose, mmol/L			
Median	5.1	5.1	0.79
25^{th}-75^{th} percentiles	4.9–5.4	4.9–5.5	

Table 7-3: Demographic characteristics of study participants

- Carotid-femoral pulse wave velocity (PWV) by applanation tonometry
- Aortic augmentation index (AIx) by applanation tonometry
- Carotid intima-media thickness (cIMT) by ultrasound
- Ultrasound brachial flow-mediated dilatation and GTN-mediated dilatation

7.3.3 Plasma assays

Plasma biopterins were measured using HPLC as described in Chapter 2. Serum levels of CRP[3] were measured by immunonephelometry using a high-sensitivity method (Dade Behring Marburg GmbH, Marburg, Germany).

7.3.4 Statistical analysis

Categorical demographic variables were compared using the chi-squared test. Continuous variables were compared between groups using a Student's t-test; non-normal data were log-transformed prior to analysis.

7.4 Results

Data analysis was performed after exclusion of seven OO subjects who were treated with ACE inhibitors; these seven subjects included the three subjects also taking a statin. These subjects were excluded from the analysis as both ACE inhibitors and statins are know to have a beneficial effect on endothelial function and would thus be potential confounders.

[3] Performed by Dr Charalambos Antoniades at the University of Athens Medical School.

7.4.1 Arterial stiffness and structure

There was no significant difference in carotid-femoral PWV (p=0.60) between XX and OO subjects (Figure 7-2A), and no significant difference in central aortic AIx (p=0.99) (Figure 7-2B). Furthermore, there was no difference in cIMT of the left (p=0.31) or right (p=0.82) common carotid arteries between genotypes (Figure 7-3).

7.4.2 Endothelial function

There was no significant difference in brachial FMD (p=0.67) between groups (Figure 7-4A). Furthermore, there was no difference in endothelium-independent dilatation following 200 μg sublingual GTN spray (Figure 7-4B).

7.4.3 Plasma biopterins and C-reactive protein

There was no difference in plasma levels of BH4 (p=1.00), BH2 (p=0.57) or B (p=0.78) between XX and OO subjects. Accordingly, there was no difference in total biopterins (p=0.99) between groups, and no difference in BH4/(BH2+B) ratio (Figure 7-5). CRP levels were low (range 0.15 – 7.85 mg/L), as expected in healthy subjects. There was no significant difference between genotypes (p=0.21) (Figure 7-6).

7.4.4 Discussion

This study uses genetic variation in *GCH1* as a biological approach to test the effect of varying BH4 levels on endothelial function and vascular disease intermediate phenotypes through Mendelian randomisation. The study by Tegeder *et al.* [97] showed a markedly attenuated upregulation of *GCH1* mRNA

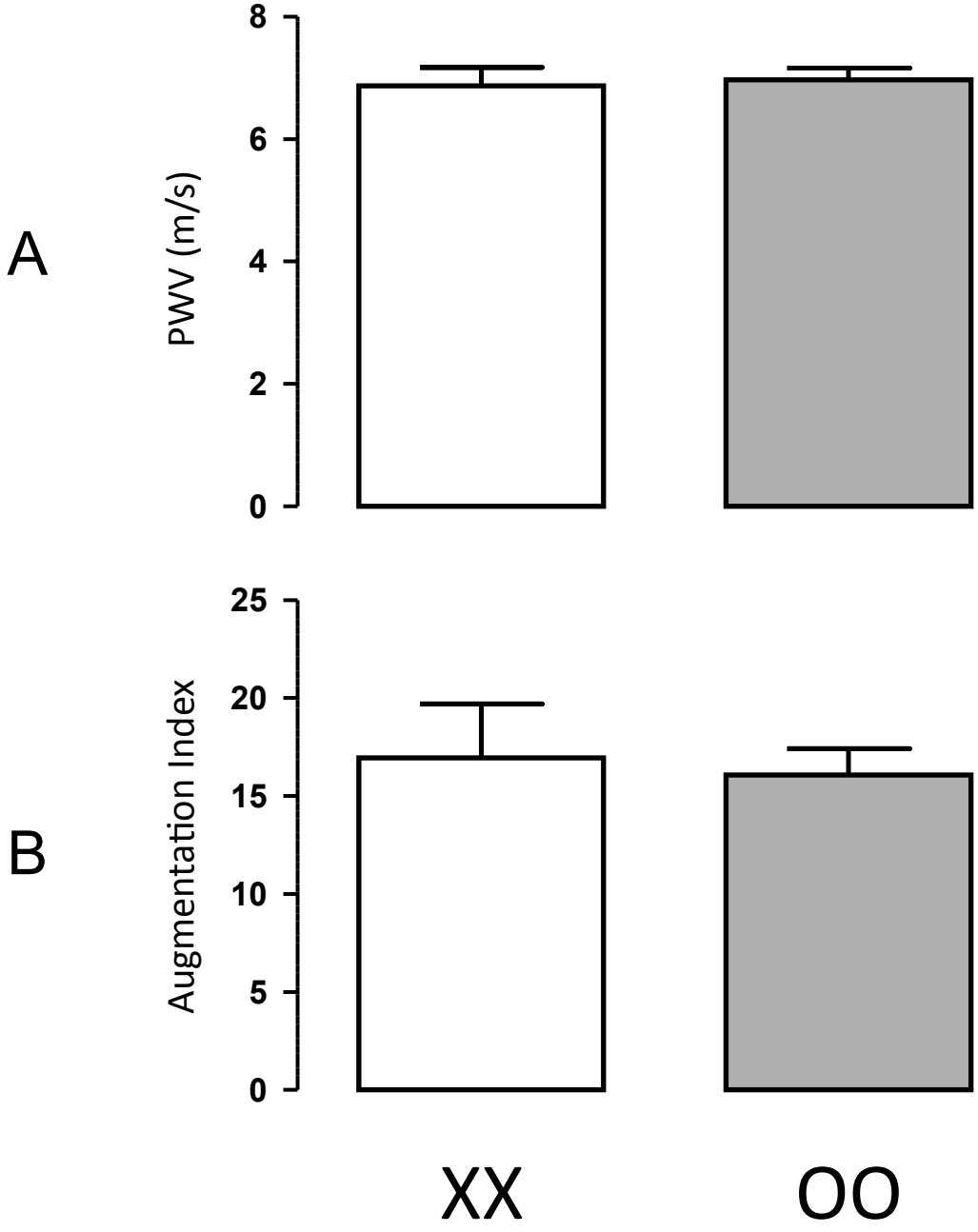


Figure 7-2: Association between *GCH1* genotype and arterial stiffness
Carotid-femoral pulse wave velocity (PWV, Panel A) and aortic augmentation index (AIx, Panel B) were measured non-invasively in healthy subjects with XX (n=20 for both PWV and AIx) or OO (n=44 for PWV, n=45 for AIx) genotype using applanation tonometry. There was no significant difference between genotypes in either measurement. Values are expressed as mean±SEM.

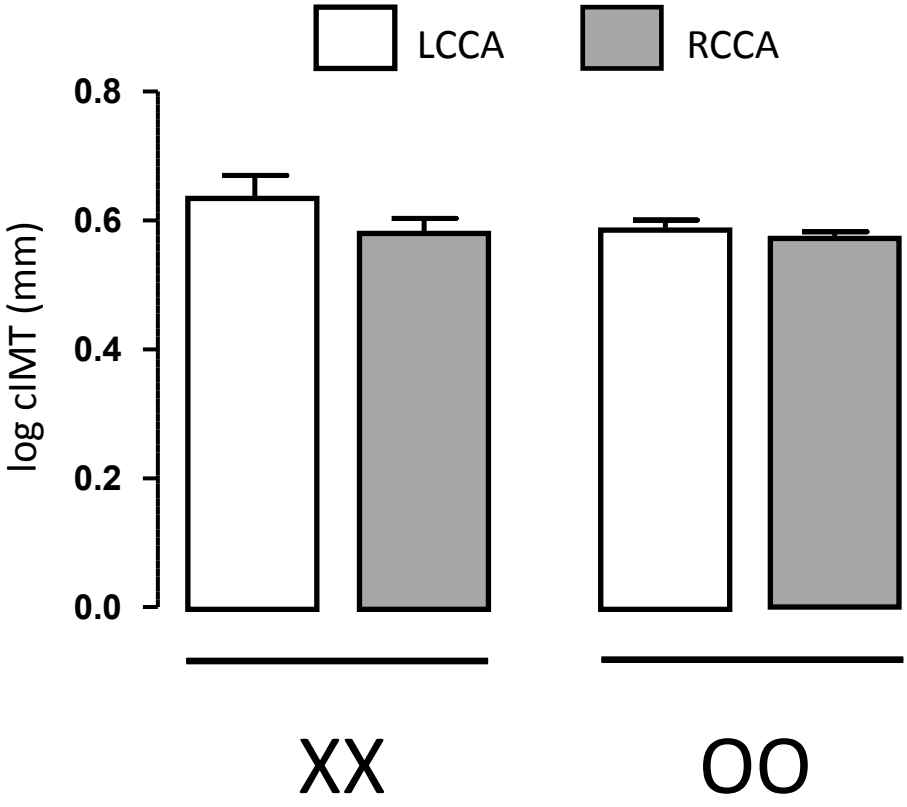


Figure 7-3: Association between *GCH1* genotype and carotid atherosclerosis
Carotid intima-media thickness (cIMT) was measured in the right (RCCA) and left (LCCA) common carotid arteries in subjects with XX (n=19) or OO (n=43) genotype. There was no significant difference in cIMT between genotypes. Values are expressed as mean±SEM of log-transformed values.

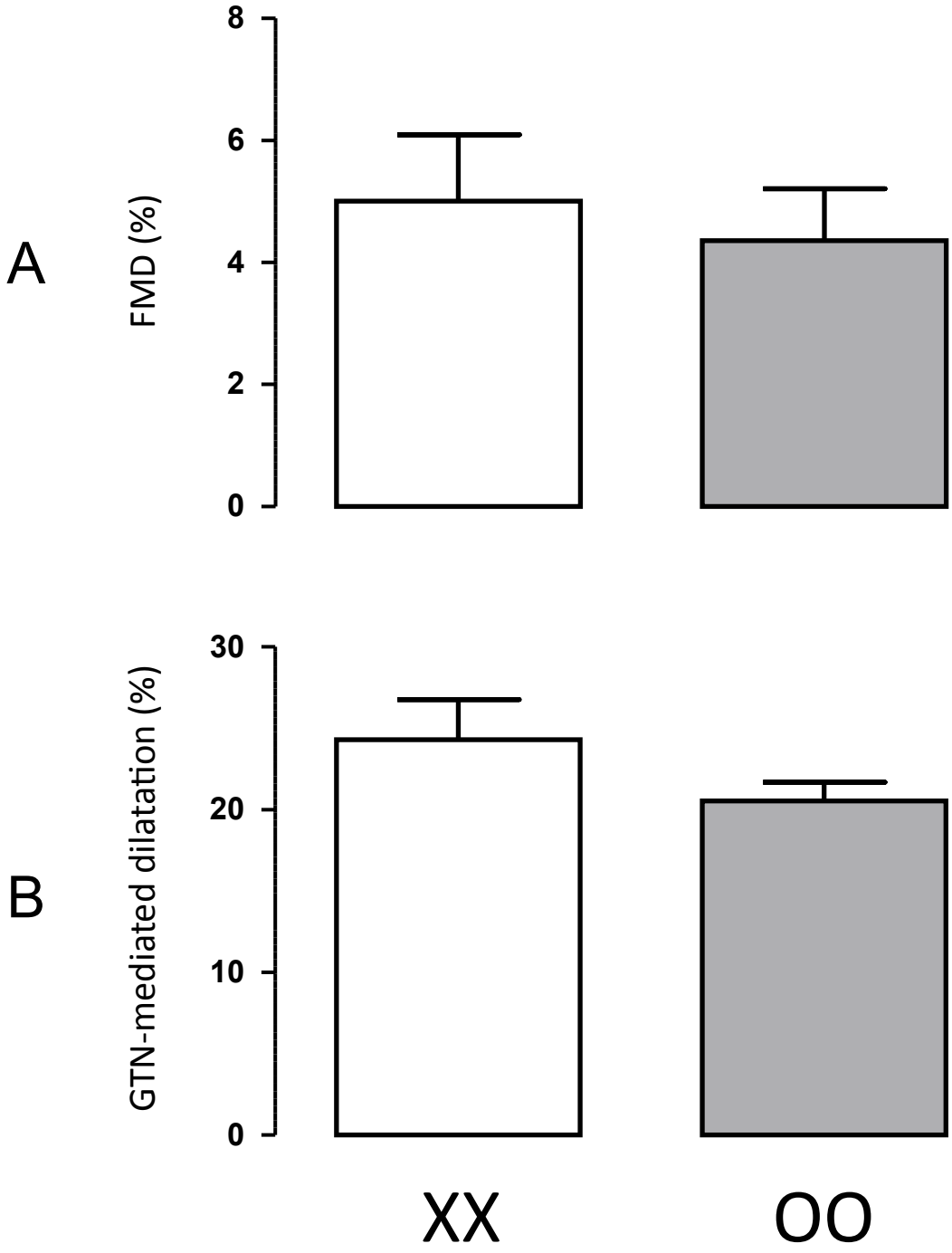


Figure 7-4: Association between *GCH1* genotype and endothelial function
Healthy volunteers with XX (n=18) or OO (n=30) genotype underwent measurement of endothelial function via ultrasound brachial artery flow-mediated dilatation (FMD). There was no significant difference in FMD between genotypes (Panel A). Additionally, there was no difference in endothelium-independent dilatation following 200 mg sublingual glyceryl trinitrate (GTN, Panel B). Values are expressed as mean±SEM.

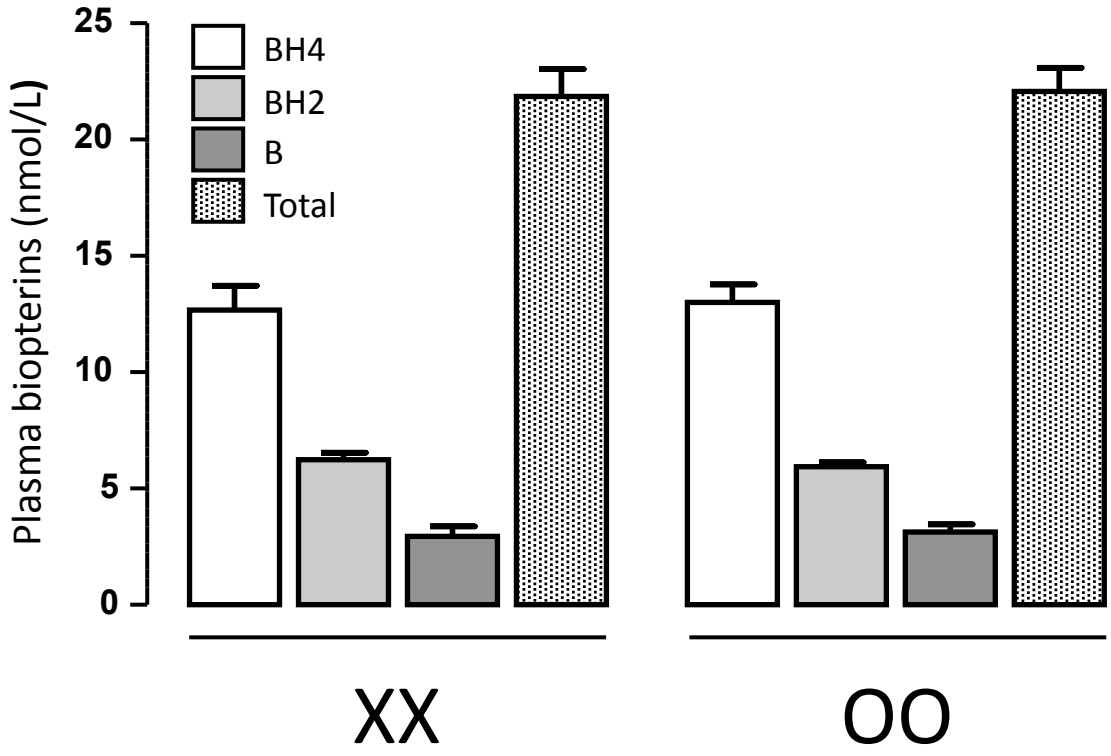


Figure 7-5: Association between *GCH1* genotype and plasma biopterin levels
Blood samples were collected into tubes containing DTE antioxidant from participants following an overnight fast (XX, n=20; OO, n=45). Plasma was stored at -80^{0}C prior to HPLC analysis. There was no significant difference in plasma levels of BH4, BH2 or biopterin (B) between genotypes. Accordingly, there was no difference in total biopterins (the sum of BH4+BH2+B) between genotypes. Data represent mean±SEM.

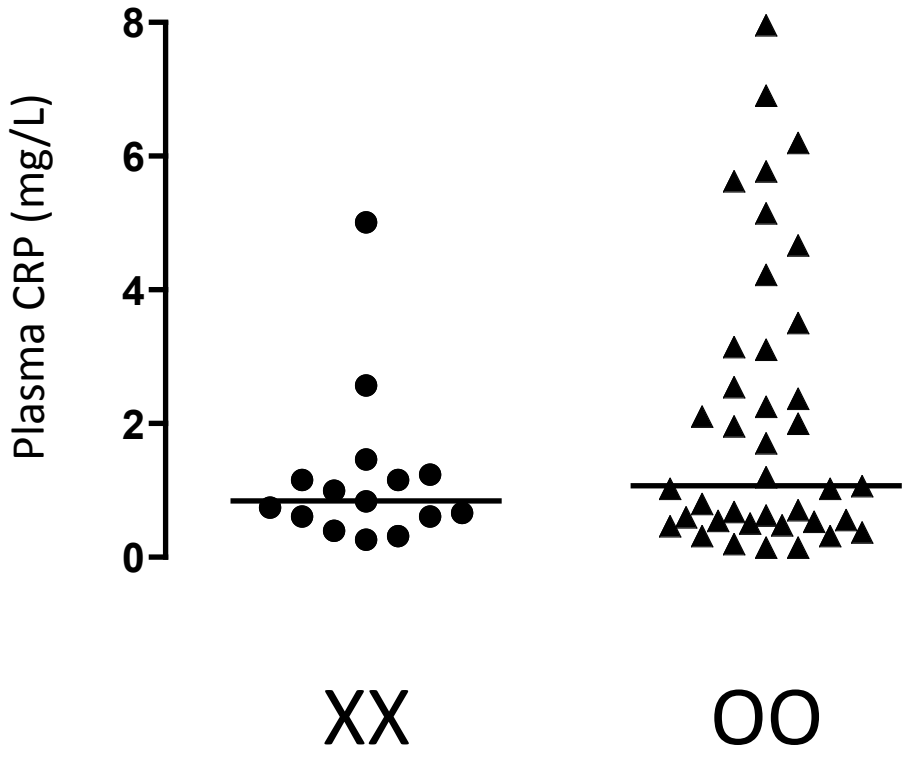


Figure 7-6: Inflammatory status of participants
Plasma C-reactive protein (CRP) was measured by immunonephelometry in all participants. There was no significant difference between XX (n= 15) and OO (n=35) subjects (median CRP 0.85 vs 1.07, p=0.21 for t-test of log-transformed values). Lines represent median values.

following forskolin stimulation, with a downstream attenuation of biopterin synthesis, in leucocytes and whole blood from XX subjects relative to OO individuals. In subjects with CAD, the X haplotype is associated with decreased *GCH1* mRNA in vascular tissue and markedly decreased biopterin levels in both plasma and vascular tissue [95], although the greatest differences between genotypes were observed in individuals with the highest levels of CRP, reflecting a likely involvement of inflammatory processes. The X haplotype was also associated with an altered vascular phenotype characterised by attenuated NO-mediated endothelial function and increased NOS-derived vascular superoxide. In the present study the subjects studied were younger and free of cardiovascular disease, with a lower level of systemic inflammation (median CRP 1.03 vs. 2.20 mg/L, range 0.15–7.85 vs. 0.12-15.50 mg/L). There was no evidence of a difference in plasma biopterins or vascular phenotype in these individuals. Thus, prior inflammatory stimulation of the *GCH1*-GTPCH-BH4 axis – possibly mediated via the development of atherosclerosis itself – may be required before the haplotype exerts a functional effect.

Finally, I did observe one interesting difference between XX and OO subjects which was not one of the main outcome measures of the original protocol. Following five minutes supine rest, XX subjects were noted to have a higher resting heart rate than OO subjects (64 bpm vs 58 bpm, p=0.015) (Figure 7-7). Interestingly, Zhang *et al.* described differences in cardiac autonomic function, with subjects with rs841C>T (one of the four variant SNPs in the current X haplotype) having lower maximum pulse intervals (i.e. higher minimum heart rates) on autonomic function testing [136]. Thus, the X haplotype may exert NO-mediated functional effects in tissues other than vascular tissue, in particular the

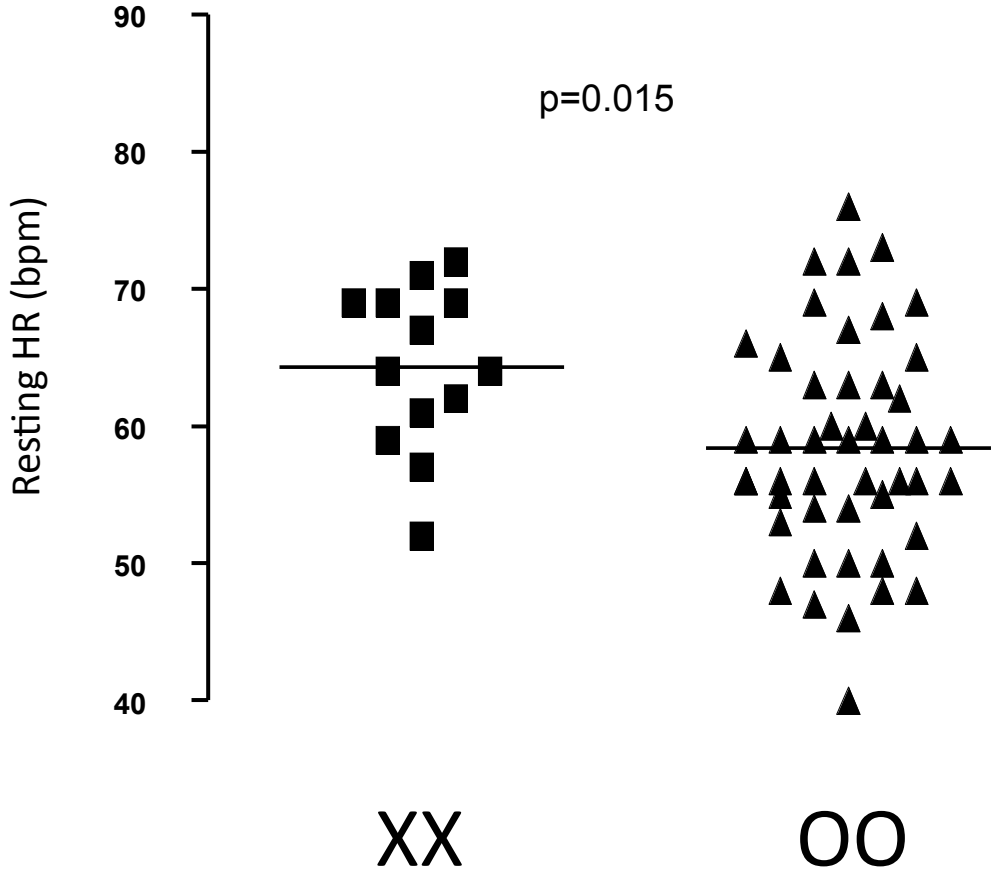


Figure 7-7: Association between resting heart rate and *GCH1* genotype
Healthy subjects with XX (n=13) and OO (n=46) genotype had their heart rate (HR) recorded by automated sphygmomanometer following five minutes supine rest. No subjects were taking rate-limiting medications, e.g. beta-adrenoceptor antagonists. XX subjects had significantly higher resting HR than OO subjects (64bpm vs 58 bpm, p=0.015). Lines represent mean values.

cardiac autonomic nervous system, in which nitric oxide is known to play a role in homeostasis [135], even in subjects with low levels of inflammation.

More recently, patients with rare, dominant *GCH1* mutations that result in the neurological condition DOPA-responsive dystonia (DRD) have been characterised [197]. These mutations are predicted to affect GTPCH expression or function. DRD patients, who exhibited lower plasma total biopterin levels and impaired vascular NO bioavailability (evidenced by a lack of FMD suppression in the presence of pharmacological NOS inhibition) compared to control subjects, also had significantly higher resting heart rates despite having lower circulating levels of adrenaline and noradrenaline [197]. Taken together, these findings add further weight to the hypothesis that the higher heart rates observed in both XX subjects and DRD patients is due to alterations in cardiac autonomic function, and that this aspect of cardiovascular homeostasis is more sensitive to changes in BH4 bioavailability than eNOS-mediated vascular function. Further characterisation of cardiovascular autonomic function in subjects with the *GCH1* X haplotype would be worthwhile.

CHAPTER 8: GENERAL DISCUSSION

8.1 Principal findings of research

The main findings of the research are summarised here:

1. In patients with advanced CAD, oral BH4 therapy, at a dose of 400 mg or 700 mg daily, has no effect on endothelial function or total vascular superoxide production, and more specifically, no effect on eNOS uncoupling, compared with placebo.

2. Pharmacokinetic/pharmacodynamic analysis demonstrates that oral BH4 therapy significantly augments plasma BH4 levels; however, BH2 is similarly elevated, with no net effect on biopterin redox status in plasma.

3. Similar to the findings in plasma, oral BH4 therapy significantly elevates BH4 levels in saphenous veins with a corresponding increase in BH2. Consequently there is no change in BH4/(BH2+B) ratio, implying that this ratio may be a more important determinant of eNOS uncoupling than absolute BH4 levels.

4. Oral BH4 does not significantly increase BH4 in internal mammary arteries, with a tendency towards an increase in arterial BH2 levels. *Ex vivo* experiments indicate that exogenous BH4 is more effective in elevating tissue BH2 than BH4, even in the presence of added antioxidant.

5. Exogenous BH4 is rapidly oxidised to BH2 in blood, even in healthy volunteers. Antioxidant administration has only a small, transient effect on protecting exogenous BH4 from oxidation.

6. The endogenous BH4 and vascular phenotype associated with the 'X' *GCH1* haplotype in patients with CAD is not detected in healthy subjects with no clinical vascular disease. This is likely due to lower levels of inflammatory stimulation of the GTPCH-BH4 axis.

8.2 Future directions

Since BH4 has been proposed as a potential treatment to improve endothelial function in vascular disease states, the results presented in this thesis indicate that strategies to harness BH4-mediated pathways as therapeutic targets in human cardiovascular disease should be reconsidered. The early promise suggested by 'acute' studies of exogenous BH4 administration in humans with established CAD [106] is not likely to translate into an oral therapy based on these results.

Accordingly, future work relating to BH4 as a viable oral therapy should focus on its use in patients at the lower end of the spectrum of atherosclerotic disease, e.g. patients with CAD risk factors but no clinically apparent disease, where there is at least some trial evidence that oral BH4 may be useful in ameliorating surrogate markers of disease progression and prognosis [100]. Furthermore, BH4 may be of use in conditions other than atherosclerosis in which BH4 has been implicated, such as pulmonary hypertension [130], and where exogenous BH4 has been shown to be beneficial in animal models [133].

For patients with established CAD, it is likely to be difficult for new therapies to improve NOS-mediated endothelial function as patients are increasingly likely to be treated with established therapies which are known to improve NO-bioavailability and reduce vascular oxidative stress, such as statins and ACE

inhibitors; furthermore, higher doses and increasingly potent versions of these drugs are being prescribed. Co-administration of oral BH4 with an antioxidant, e.g. vitamin C, remains an unexplored avenue, although the data from my *ex vivo* experiments do not suggest that a clinical benefit is to be expected. Alternatively, strategies to modulate vascular biopterin redox status, e.g using folates [177], may be of clinical use, although data from large-scale clinical trials have so far not shown a significant clinical benefit [198].

Further investigation of the role of endogenous BH4 in humans is certainly warranted. The absence of a functional phenotype associated with *GCH1* haplotypes in healthy individuals with low systemic inflammation raises the possibility that inflammatory stimulation is a prerequisite for the *GCH1* X haplotype to exert a functional effect. Indeed, work that has followed on from the *GCH1* haplotype experiments in this thesis has recently been published [199]. In this paper, we have replicated the observation that differences in endothelial function between *GCH1* genotypes amongst patients with CAD are largest in subjects with high CRP levels, indicating chronic, low-grade inflammation. Furthermore, an acute inflammatory stimulus (using *Salmonella typhi* vaccine) resulted in an increase in circulating plasma BH4 in OO subjects, but not in XX subjects, indicating that *GCH1* is a key determinant of BH4 synthesis in response to acute inflammation [199]. In the same subjects, acute inflammation resulted in a more profound depression in endothelial function in XX subjects compared to OO subjects, suggesting that differences in *GCH1* expression, and BH4 synthesis, are able to modify the vascular response to acute inflammation in humans [199].

As discussed in Chapter 7, there is now growing evidence that endogenous BH4 bioavailability may play a key role in cardiovascular autonomic function. Further

characterisation of the *GCH1* X haplotype with regards to effects on autonomic function would be worthwhile. Given the recent evidence implicating the interaction of acute inflammation with the X haplotype discussed above, autonomic characterisation of XX and OO subjects before and after an inflammatory stimulus would also seem a logical progression.

One final approach to prove a causal role of BH4 in modulating human vascular function, beyond exogenous supplementation and synthetic variation, would be via the use of an inhibitor of GTPCH to deplete rather than augment BH4, as has been used previously in animal studies [50, 53]. To my knowledge, however, a GTPCH inhibitor suitable for clinical use is not currently available. This approach may also be of interest in conditions where excess BH4 may be detrimental rather than beneficial, e.g. septic shock mediated by iNOS.

8.3 Conclusion

In conclusion, I have demonstrated that oral BH4 treatment in patients with CAD significantly elevates BH4 levels in blood, but this effect is significantly limited by systemic oxidation of exogenous BH4 to BH2 which lacks eNOS co-factor activity. Accordingly, the ratio of reduced to oxidised biopterins in blood and vascular tissue is unchanged by exogenous BH4 treatment, resulting in no net effect on eNOS coupling, endothelial function or vascular superoxide production. Targeting BH4-mediated pathways remains a rational therapeutic strategy in several aspects of cardiovascular disease, but future studies should be directed towards interventions that can favourably alter the endogenous BH4/BH2 ratio in human vascular endothelium, either via a selective increase in absolute BH4 levels, prevention of BH4 oxidation, or an increase BH4 recycling. Furthermore,

studies using genetic variation in BH4 synthesis have provided further evidence to support a pivotal role of endogenous BH4 levels in maintaining vascular function in humans.

CHAPTER 9: APPENDICES

9.1 Timing and venue of research

The experiments detailed in this thesis were conducted between November 2006 and July 2010. Participants in the OXBIO study were recruited from the Department of Cardiothoracic Surgery at the John Radcliffe Hospital, Oxford. Magnetic resonance imaging was performed at the University of Oxford Centre for Clinical Magnetic Resonance Research (OCMR). Laboratory experiments were performed initially in Professor Channon's laboratory at the Wellcome Trust Centre for Human Genetics, and later at the University of Oxford Department of Cardiovascular Medicine laboratory at the John Radcliffe Hospital, except where detailed in the text.

9.2 Sources of funding

The OXBIO study was funded by an unrestricted educational grant to the University of Oxford from BioMarin Pharmaceutical Inc., Novato, USA. The *GCH1* haplotype study was funded by a Project Grant from the British Heart Foundation (PG/08/119/26263). Work in the Professor Channon's laboratory is supported by the British Heart Foundation (Programme Grant RG/07/003/23133) and by the NIHR Biomedical Research Centre.

9.3 Attributions

I am extremely grateful for the contributions made to this research by colleagues and collaborators. Apart from the exceptions described below, the author is directly responsible for the planning, execution and analysis of all the experiments and the preparation of this thesis.

The protocol for the OXBIO clinical trial was written and submitted for regulatory approvals by Dr Cheerag Shirodaria. Tissue collection during surgery, and subsequent vasomotor studies and superoxide measurements on fresh tissue, was shared with Dr Tim van Assche. *Ex vivo* vessel incubations were shared with Dr Ruha Cerrato. Genotyping for *GCH1* haplotypes was performed by Dr Matthew Neville on stored samples from the Oxford Biobank study at the Oxford Centre for Diabetes, Endocrinology & Metabolism (OCDEM). Analysis of CRP was performed by Dr Charalambos Antoniades at the University of Athens Medical School. Measurement of brachial FMD by ultrasound was performed by Mr Jonathan Diesch.

9.4 Acknowledgements

I would first like to thank my supervisor, Professor Keith Channon, for giving me the opportunity to undertake this research, and for his generous support, advice, guidance and encouragement during my time in Oxford.

My research would not have been possible without the contributions of many other individuals. In OCMR, I am grateful to Mrs Jane Francis and Dr Justin Lee for teaching me the principles of cardiovascular MRI, and to Dr Alistair Lindsay and Dr Ilias Kylintireas for providing assistance with scanning. I am grateful too for the advice and mentorship of Professor Stefan Neubauer.

In the John Radcliffe Hospital, I am indebted to the cardiac surgeons and their supporting staff in the Department of Cardiothoracic Surgery for allowing recruitment of patients into the clinical trial, and for assistance with tissue harvesting. I am indebted too to Dr Paul Leeson and Mr Jonathan Diesch for their assistance with vascular function assessments.

In the laboratory, I am particularly thankful for the assistance of Dr Tim van Assche who spent many hours working with tissue in the organ bath, amongst other laboratory assays. I am grateful too for the advice and assistance I received from Dr Mark Crabtree and Mr Ashley Hale in learning and performing HPLC analysis. I would also like to thank Dr Felix Böhm and Dr Ruha Cerrato, and laboratory managers Mr Phil Townsend and Dr James Brown, for their contributions and support, and to all members of the Channon Group for providing an enthusiastic and intellectual environment throughout my time in the lab.

In OCDEM, I would like to acknowledge the support of Professor Fredrik Karpe, Principal Investigator of the Oxford Biobank study, and the contributions made by Dr Matthew Neville and Mrs Jane Cheeseman.

Throughout my time in Oxford, Dr Charis Antoniades has been a constant source of advice and inspiration, in many different aspects of my work, including study design, teaching me laboratory techniques, statistical analysis, and manuscript preparation. I am very grateful for his enthusiastic input.

I would like to say a big thank you to my family for their love and support. In particular, Kate – who became my wife during this period – has endured significant hardship and upheaval in order to support my career; for this I am eternally grateful. I must also thank Kate for my three beautiful children – Madeleine, Meredith and Edward – who were all born during my time in Oxford, and to whom this thesis is dedicated.

Finally, I would like to place on record my thanks to all the patients and volunteers who gave up considerable amounts of time to participate in my studies, without whom this work would certainly not have been possible.

9.5 Publications arising from work in this thesis

- **Cunnington C**, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM, Antoniades C, Cerrato R, Francis JM, Sayeed R, Ratnatunga C, Pillai R, Choudhury RP, Neubauer S, Channon KM. Systemic and vascular oxidation limits efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease. *Circulation* 2012; 125: 1356-66.

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9.6 Other research publications during DPhil study

- Kylintireas I, Shirodaria C, Lee JM, **Cunnington C**, Lindsay A, Francis J, Robson MD, Neubauer S, Channon KM, Choudhury RP. Multimodal cardiovascular magnetic resonance quantifies regional variation in vascular structureand function in patients with coronary artery disease: relationships with coronary disease severity. *J Cardiovasc Magn Reson* 2011; 13: 61.

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9.7 Abstracts presented at scientific conferences

- **Cunnington C**, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Ratnatunga C, Pillai R, Choudhury RP, Neubauer S, Channon KM. Chronic oral tetrahydrobiopterin therapy in coronary artery disease elevates total biopterin levels but does not improve redox status or vascular function: a randomised placebo-controlled trial. *Eur Heart J* 2010;31(Abstract Supplement):390.

- **Cunnington C**, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM, Francis JM, Sayeed R, Ratnatunga C, Pillai R, Choudhury RP, Neubauer S, Channon KM. Chronic oral tetrahydrobiopterin treatment in patients with coronary artery disease elevates total biopterin levels but does not improve biopterin redox status or vascular function: a randomised placebo-controlled trial. *Heart* 2010;96(Suppl 1):A1-2. **Runner-Up, British Cardiovascular Society Young Research Worker's Prize.**

- **Cunnington C**, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM, Sayeed R, Ratnatunga C, Pillai R, Choudhury RP, Neubauer S, Channon KM. Chronic oral tetrahydrobiopterin treatment in patients with coronary artery disease elevates total biopterin levels but does not improve biopterin redox status or vascular function: a randomised placebo-controlled trial. BHF Centre of Research Excellence Annual Symposium 2009. **Winner – Best Poster.**

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