The measurement, biological variation and response to acute inflammation of asymmetric dimethylarginine (ADMA)

Dr Scott Blackwell

MBChB MRCP FRCPath

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School of Medicine

College of Medical, Veterinary and Life Sciences

University of Glasgow

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Abstract

Introduction and methods

Asymmetric dimethylarginine (ADMA) is a potent endogenous competitive inhibitor of nitric oxide synthases, which has attracted considerable attention as a marker and mediator of atherosclerotic disease and as a potential mediator of multiple organ failure in critical illness due to endothelial dysfunction. However, data regarding basic aspects of its biology such as biological variation and its response to acute inflammation are lacking. Moreover, significant methodological variability has been a barrier to collating the burgeoning data available. Therefore, this thesis describes the development and validation of a reliable assay for measurement of ADMA and related compounds in plasma, urine and other biological fluids based on isocratic reverse phase high performance liquid chromatography (HPLC). This method was used to determine the biological variation of ADMA in human plasma, and its response to acute inflammation using a model of elective knee arthroplasty. Further HPLC methods for measurement of dimethylamine (DMA), the main metabolite of ADMA, and nitrate were developed and used to determine excretion of these compounds in acute inflammation to complement the observed changes in plasma ADMA concentration.

Results

Complete chromatographic separation of arginine, homoarginine, monomethyl-arginine, ADMA and its structural isomer SDMA was achieved, permitting their accurate quantification using a novel, non-endogenous, internal standard. The intra-individual biological variation of ADMA was found to be low at 7.4%, imposing a tight imprecision goal for analytical methods. Plasma ADMA concentration decreases rapidly during the acute inflammatory response, with a median decrease of around 30%, and a significant change already evident as little as 12 hours following the onset of inflammation. No

similar change was seen in the concentration of the closely related compound SDMA. No significant increase in the urine excretion of DMA was noted during the early phase of the response, with a significant increase seen 5 days following the insult by which point the plasma ADMA concentration had returned to baseline levels. A small, but significant, decrease in nitrate excretion during the inflammatory response was seen, mirroring the observed changes in plasma ADMA.

Conclusion

The low biological variation of ADMA suggests physiological regulation. The rapid and significant decrease in plasma concentration during inflammation does not appear due to increased catabolism, but rather is more likely to represent increased cellular partitioning. This may be associated with an impairment in NOS activity. It is unclear whether this is of pathological significance, or represents a physiological response to regulate NO production in inflammation. Further study is warranted in relevant models, particularly with attention to intracellular concentrations.

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Lastly, I would thank my parents and family for all of their love and support over the years, and Ursula who (mostly) puts up with me uncomplainingly.

Declaration

I declare that the work presented in this thesis is original and has not been presented elsewhere for a higher degree.

The knee arthroplasty study described in chapter 8 was performed in collaboration with Dr David Reid, University Department of Anaesthesia. Dr Reid applied for ethical and management approval. Dr Reid and I shared the responsibility for patient consent and blood specimen collection.

Blood and urine samples were handled by myself or staff in the Biochemistry Departments at the Western Infirmary and Gartnavel General Hospital.

I developed the biochemical methods, in conjunction with Dr Dinesh Talwar, Glasgow Royal Infirmary Biochemistry Department, and performed all of the biochemical analyses described in chapters 2, 5 and 6.

I performed the statistical analysis and wrote all that is in this thesis.

List of abbreviations

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ADMA	Asymmetric dimethylarginine
ALT	Alanine aminotransferase
AUFS	Absorption units full scale
CAT	Cationic amino acid transporter
CHD	Coronary heart disease
CI	Confidence interval
CKD	Chronic kidney disease
CRP	C reactive protein
CV	Coefficient of variation
CV_A	Analytical coefficient of variation
CV_G	Inter-individual biological variation
CV_I	Intra-individual biological variation
DDAH	Dimethylarginine dimethylaminohydrolase
DM	Diabetes mellitus
DMA	Dimethylamine
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESRF	End-stage renal failure

EUFS	Emission units full scale
FE	Fractional excretion
FMOC	9-Fluorenylmethoxycarbonyl chloride
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GFR	Glomerular filtration rate
Hb	Haemoglobin
HCl	Hydrochloric acid
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment – insulin resistance
HPLC	High performance liquid chromatography
ICU	Intensive care unit
II	Index of individuality
IMT	Intima-media thickness
iNOS	Inducible nitric oxide synthase
IV	Intravenous
K_i	Inhibitor constant
K_m	Michaelis constant
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MEA	Monoethylarginine
MI	Myocardial infarction

MMA	Monomethylarginine
NO	Nitric oxide
NOS	Nitric oxide synthase
NPA	N-propylarginine
OPA	Ortho-phthaldialdehyde
PFP	Pentafluoropropionyl
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRMT	Protein arginine methyltransferase
QC	Quality control
RCV	Reference change value
RP	Reverse phase
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SIRS	Systemic inflammatory response syndrome
SPE	Solid phase extraction
SST	Serum separator tube
TCA	Trichloroacetic acid
TE	Total error
TNF-α	Tumour necrosis factor alpha

Publications

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Chapter 1

Introduction

1.1 Arginine and its endogenous methylated derivatives

1.1.1 Biochemistry of arginine

Arginine is a semi-essential amino acid, and is characterised by a guanidino group on its side-chain. With a pK_a of around 12.5 the guanidino group is protonated at physiological pH, giving the molecule a net positive charge. This renders arginine important at the surface of proteins, with a role in receptor-ligand interactions. It is also an important role in several metabolic pathways, including the urea cycle, as a substrate for gluconeogenesis, and, crucially, as the substrate for nitric oxide (NO) production by nitric oxide synthases (NOS).

1.1.2 Methylated derivatives of arginine

In common with other amino acids arginine is subject to various post-translational modifications which contribute to the functionality of different proteins. One or two methyl groups can be added to the nitrogen atoms of the guanidino group to yield mono- and dimethylarginines. Dimethylarginines had been identified in human urine in the early 1970s[1], although it was about two decades before their potential importance in human physiology was realised[2]. The chemical structures of arginine, monomethylarginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are shown in figure 1.1.

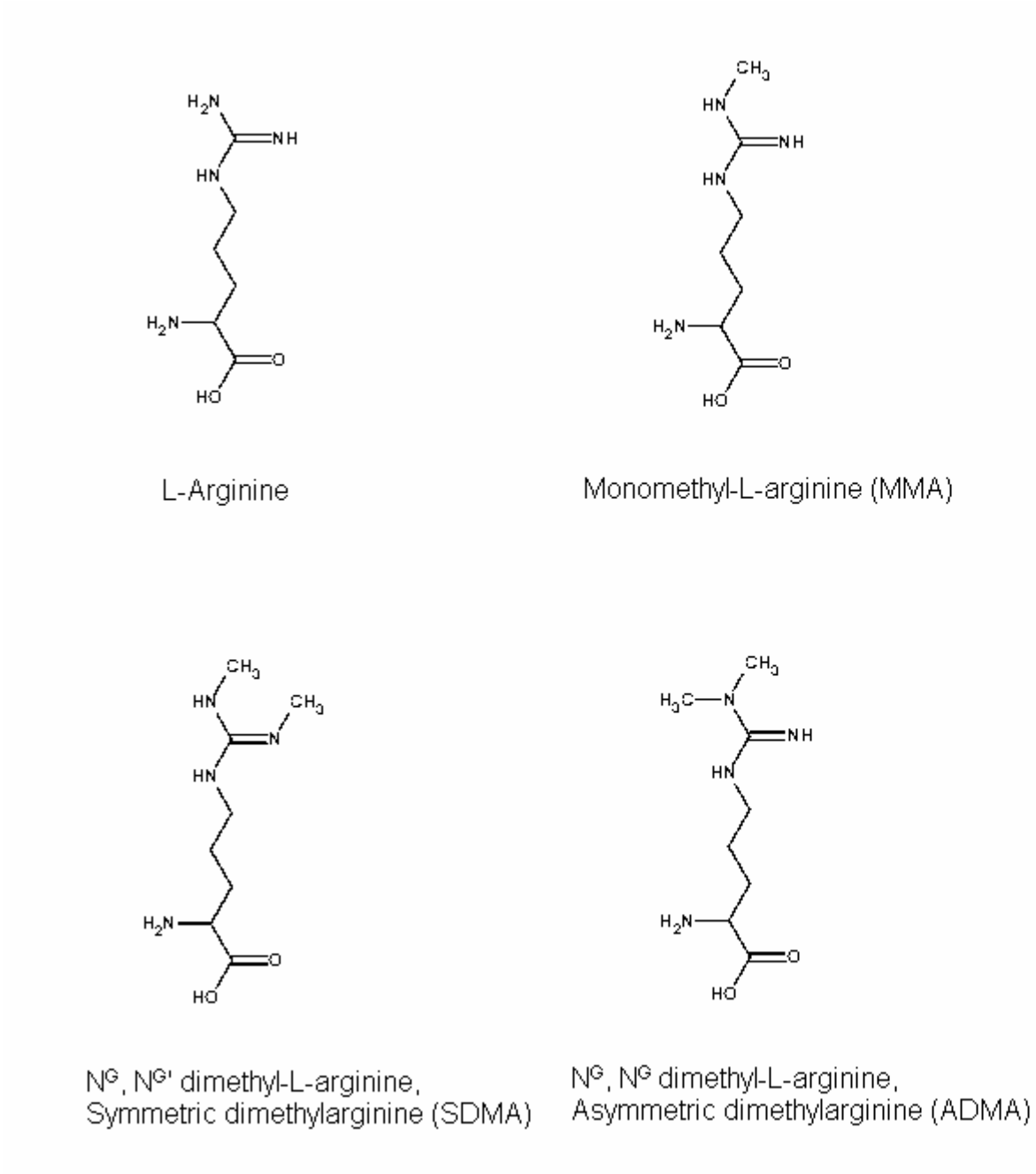


Figure 1.1: Chemical structures of arginine and its methylated derivatives

1.1.3 Synthesis of methylarginines

Endogenous methylarginines are synthesised by a group of enzymes called protein arginine methyltransferases (PRMT), which methylate arginine residues within proteins[3,4]. In mammals there are two main types, both utilising S-adenosyl methionine as a methyl group donor. Type 1 PRMTs have a fairly wide range of substrate proteins, including histones and non-histone nuclear proteins, and largely produce ADMA[4]. Type 2 PRMTs appear specific to myelin basic protein, and largely produce SDMA, a structural isomer of ADMA[4]. Both PRMTs produce MMA. Methylated proteins interact with nucleic acids and thus protein methylation has important roles in processes like transcription and the epigenetic regulation of gene expression. Free MMA, ADMA and SDMA appear on degradation of the proteins containing them. It is generally felt that arginine methylation is an irreversible process. There is evidence that the activity of type 1 PRMTs can be increased by native and oxidised low density lipoproteins (LDL)[5], increased shear stress[6] and anti-double stranded DNA antibodies[7]; this suggests that in certain pathological states increased PRMT activity could contribute to increased ADMA concentrations. It has been estimated that humans generate about 300 µmol of ADMA per day[8].

1.2 Clearance of endogenous methylarginines

1.2.1 Catabolism of ADMA by dimethylarginine dimethylaminohydrolase (DDAH)

Earlier studies in rabbits had suggested the presence of a metabolic pathway for elimination of ADMA many years before the enzymes responsible were first identified[9]. It appears that approximately one-sixth of the ADMA generated in

humans is excreted unchanged into urine[2,8], with the majority metabolised by DDAH[8,10-12] yielding citrulline and dimethylamine[10]. The metabolic activity of DDAH is dependent on a cysteine residue at its catalytic site[12]. It has also been demonstrated that its molecular structure excludes SDMA as a substrate, meaning DDAH is specific for ADMA[12]. SDMA, therefore, appears to undergo clearance predominantly by renal excretion, as suggested by its significant net renal extraction and the strong correlation of plasma SDMA and creatinine concentrations[13]. The Michaelis constant (K_m) of DDAH for ADMA is approximately 180 μM, which is many times greater than typical ADMA concentrations in humans[10]. This means that DDAH should be operating within the linear part of its substrate-velocity curve, theoretically permitting a tight regulatory influence of DDAH activity on ADMA concentration.

1.2.2 Isoforms and distribution of DDAH

There are two isoforms of DDAH. DDAH-1 is expressed widely in tissues expressing neuronal NOS, especially in the liver and renal cortex, but also in diverse tissues such as the pancreas and macrophages[11,14]. The liver and kidney are therefore quantitatively important sites of ADMA catabolism, studies in rats demonstrating fractional excretions of around 30% and 35% respectively[15,16]. In humans the net renal excretion has been calculated as 16.2%[13]. The importance of the liver in ADMA catabolism is further supported by studies showing increased concentrations following major hepatectomy[17], the strong influence of hepatic dysfunction on ADMA concentrations in critically ill patients[18], and reductions in ADMA concentration following liver transplantation[19].

DDAH-2 is expressed widely in foetal tissues, becoming fairly selective for sites expressing endothelial NOS (eNOS) in adults[14,20]. It thus predominates in the vascular endothelium and cardiovascular system, but is also expressed in

immune tissues expressing inducible NOS (iNOS)[20]. While DDAH-1 has also been demonstrated in the endothelium, DDAH-2 appears to exceed it by a factor of 10 or more, suggesting that DDAH-2 is the isoform of major importance in blood vessels[14]. DDAH-2 is also found in the kidney, specifically within its vasculature, and may be involved in NO-mediated homeostatic responses in that organ[21].

Targeted silencing of DDAH isoforms by small interference RNAs has generated data suggesting differential roles of the two isoforms[14]. DDAH-1 silencing is associated with increased plasma ADMA concentration, but little effect on blood vessel NO responses. In contrast DDAH-2 silencing is associated with impaired blood vessel responses, but no significant effect on circulating ADMA conentrations[14]. DDAH-1 has, therefore, been dubbed the "guardian of circulating ADMA" concentrations[20]. However, more recent work using a DDAH-1 knockout mouse showed increased plasma and tissue concentrations of ADMA, with raised blood pressure, and no measurable DDAH activity[22]. The authors of this study speculate that DDAH-1 is the predominant enzyme for ADMA catabolism, and that the role of DDAH-2 is doubtful[22]. It will be important to further delineate isoform-specific roles of DDAH.

Polymorphisms in the genes encoding DDAH enzymes have been increasingly studied because of their potential effects on ADMA concentrations, and certain polymorphisms have been implicated in cardiovascular risk[23], and in the decline in renal function in patients with chronic kidney disease[24].

1.3 Pathophysiological roles of DDAH and ADMA in endothelial dysfunction

1.3.1 DDAH activity impairment in oxidative stress

The critical role of DDAH in vascular health arose from observations using a pharmacological DDAH inhibitor which induced a gradual vasoconstriction in isolated vascular segments that was reversed by the addition of arginine[25]. This suggested that ADMA was produced continuously, DDAH acting to prevent its accumulation. Furthermore, transgenic mice over-expressing DDAH-1 demonstrated a 50% reduction in plasma ADMA concentration compared with wild type, associated with increased urinary nitrate and a 15 mmHg reduction in systolic blood pressure[26]. DDAH over-expression is also associated with protection from exogenous ADMA in the cerebral circulation[27], and DDAH-2 over-expression ameliorates vascular injury in response to ADMA infusion in mice[28].

The cysteine residue at the catalytic site of DDAH is susceptible to oxidative stress with attenuation of enzyme activity[12], thus providing a link between vascular risk factors, oxidative stress, raised ADMA concentration and endothelial dysfunction. Such an effect has been demonstrated with oxidised LDL and tumour necrosis factor α (TNFα)[29], associated with increased ADMA. Similar effects have seen with hyperglycaemia[30], hyperhomocystinaemia[31] and cigarette smoke extract[32]. Furthermore, eNOS uncoupling by ADMA increases production of superoxide with the potential to further impair DDAH and increase ADMA even further in a perpetuating cycle[33]. DDAH can also be inhibited by nitrosylation and in states of excess NO production, especially by iNOS, may provide a mechanism through which accumulating ADMA acts as a "brake" on harmfully excessive levels of NO production[34]. Interactions between oxidative stress, DDAH, ADMA and NOS are summarised and illustrated diagrammatically in figure 1.2.

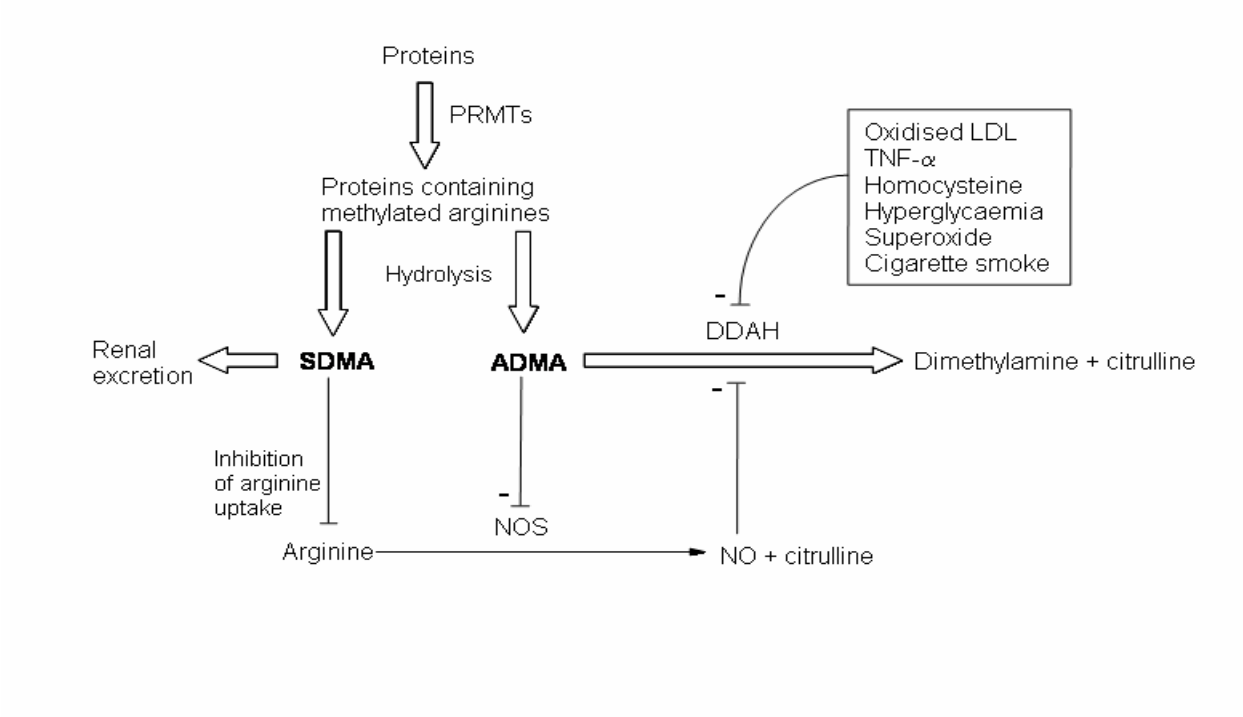


Figure 1.2: Interactions between oxidative stress, DDAH activity, ADMA and NOS

1.3.2 ADMA in NOS inhibition and endothelial dysfunction

The discovery of NO as the endothelial-derived relaxing factor was a major breakthrough in the understanding of the vascular endothelium and its dysregulation in disease[35]. It has several other functions including inhibition of processes involved in atherosclerosis such as smooth muscle cell proliferation, platelet aggregation and adhesion, and monocyte adhesion; it can thus be described as an anti-atherogenic molecule[36]. It is also part of the immune response, and is produced in large quantities in response to sepsis and inflammation[37]. It is synthesised from arginine by NOS[38], of which there are endothelial, inducible and neuronal isoforms[39].

ADMA and MMA are competitive inhibitors of all NOS isoforms[2,5,40,41], although attention has focussed almost entirely on ADMA as the predominant NOS

inhibitor in human plasma. The seminal work of Vallance showed that intra-arterial infusion of ADMA into healthy volunteers caused a dose-dependent decrease in forearm blood flow, an effect that could be partially offset by infusion of arginine[2]. In the same study ADMA injections into guinea pigs resulted in a 15% increase in systolic blood pressure at an ADMA concentration about 9 times that at baseline (9.8 μmol/L). In healthy human males intravenous infusion of ADMA to generate concentrations up to 3 times normal raised systemic vascular resistance by approximately 24% and impaired the cardiac response to exercise[8]. A similar study showed a 14 % decrease in cardiac output[42]. ADMA also appears to reduce renal perfusion and sodium excretion and increase blood pressure[42,43] and inhibit flow/shear-stress-induced vasodilatation in isolated arterioles[44].

The experimental studies described in the foregoing paragraph involve plasma concentrations greater than those seen in health and many disease states (e.g. 2.60 – 5.31 μmol/L compared with a "normal" value of around 0.5 μmol/L[45]). However, observational studies seem to confirm the in vitro relevance of ADMA, with a significant inverse correlation shown between plasma ADMA concentration and brachial flow-mediated dilatation in over 2,000 healthy young adults[46]. Similar associations have been demonstrated in individuals with hypercholesterolaemia[47], type 2 diabetes mellitus[48] and chronic kidney disease[49]. Furthermore, plasma ADMA concentration was significantly associated with progression of carotid intima-media thickness in individuals without overt cardiovascular disease[50].

Another relevant factor in the interpretation of plasma ADMA concentrations is the fact that intracellular concentrations can be approximately 5 – 10 times that found in plasma, with endothelial cells actively able to take up ADMA and concentrate it intracellularly[51]. Given that the inhibitor constant (K_i) of ADMA on eNOS is around 0.9 μmol/L, it is clear that a modest increase in plasma ADMA concentration could have a significant effect on NO production[51].

Further work is needed to elucidate the relationship of intra- and extracellular concentrations of methylarginines *in vivo*.

Although SDMA has no direct effect on NOS, it is able to compete with arginine for transport via cationic amino acid transporters (CAT), thereby potentially reducing substrate availability for NO synthesis[52,53]. In cultured endothelial cells, SDMA reduced NO synthesis in a dose-dependent fashion, an effect that was reversed by arginine supplementation[53].

1.3.3 Associations of ADMA with vascular risk factors

There has been much work seeking associations with traditional and novel cardiovascular risk factors as part of the underlying hypothesis that, through NOS inhibition, ADMA could be a mediator of endothelial dysfunction.

1.3.3.1 Hypercholesterolaemia

The evidence in hypercholesterolaemia is inconsistent. Diet-induced hypercholesterolaemic rabbits and monkeys have an approximately 2-fold increase in ADMA concentration[54,55], and associations have also been demonstrated in humans[47,56,57]. Other human studies have failed to replicate this link[58,59], although one of these studies included only individuals with total cholesterol < 4.6 mmol/L[58] and in the other more than 50% of participants were already taking lipid lowering drugs[59]. Lowering of plasma ADMA concentration on statin treatment has also been an inconsistent finding, with studies showing reductions with rosuvastatin and fluvastatin[60,61], but no changes in other studies despite large reductions in cholesterol[62-66]. It has also been shown that higher ADMA concentrations are associated with a reduced responsiveness in terms of endothelial function to statin treatment[67,68], suggesting that high ADMA concentrations might be able to block the pleiotropic effects of statins, which include up-regulation of eNOS[69].

1.3.3.2 Obesity, insulin resistance and diabetes mellitus

Hyperglycaemia seems to result in oxidative stress which can impair DDAH activity in human cells[30], and plasma ADMA concentrations are raised in individuals with impaired glucose tolerance[70-72], and are associated with impaired myocardial tissue perfusion in this context[72]. In subjects undergoing angiography, ADMA was found to strongly predict glycaemic category (normal, impaired and diabetes)[73], and in women with previous gestational diabetes was found to predict the development of glucose intolerance with an adjusted hazard ratio of 3.94 (1.16 – 13.37) for an ADMA concentration above the median during 3 years of follow up[74]. In obese women, ADMA concentrations are higher in those with associated insulin resistance, and in these individuals weight loss through caloric restriction decreases ADMA concentration and improves insulin sensitivity[75]. Weight reduction surgery in morbidly obese women is also associated with decreases in plasma ADMA[76]. In neither of these studies, however, was weight independently associated with ADMA as such[75,76]; rather it appears that the metabolic abnormalities that can develop in the setting of obesity are what influence the ADMA concentration, and the reduction in ADMA concentration following weight loss occurs in line with improvements in parameters of insulin sensitivity rather than the measured weight[76]. In patients with diabetes mellitus plasma ADMA concentration is associated with albuminuria and microangipathy[77], and independently and strongly predicts the development of retinopathy[78] and the progression of nephropathy[79].

As in hypercholesterolaemia, the effect of pharmacological treatment on ADMA is also variable. In type 2 diabetes, 3 months of treatment with metformin decreased plasma ADMA concentration by about 30%[80], and similar reductions have been seen in women with polycystic ovarian syndrome and insulin resistance[81]. Peroxisome proliferator-activated receptor gamma (PPARγ)agonists have been shown to reduce plasma ADMA and improve endothelial function in subjects with type 2 diabetes[71,82], although negative

results with this therapy have also been reported[83]. DDAH-2 contains a PPARγ binding site in the promoter region, and this provides a possible mechanism for the effect of these drugs[84]. Lastly, intensive glucose control, irrespective of how it is achieved, reduces plasma ADMA concentration and improves vascular function, possibly through anti-cytokine and anti-atherogenic effects[85].

1.3.3.3 Hypertension

The crucial role of NO on vascular tone and function predicts a role for ADMA in hypertension. DDAH-1 knockout mice demonstrate a significant increase in plasma ADMA concentration and blood pressure[22,86,87], although, intriguingly data do not suggest that DDAH-2 is as important, at least under basal conditions[28,88]. In humans with hypertension, plasma ADMA concentrations are increased compared with healthy controls and are related to markers of endothelial dysfunction[89-91] and are associated with mean blood pressure in healthy individuals[70]. Moreover, ADMA infusion into healthy volunteers reduced renal blood flow, increased salt retention and raised systemic blood pressure[42]. Interestingly, plasma ADMA concentration is also raised compared with controls in subjects with so-called "white coat hypertension", perhaps suggesting that this is not an entirely benign phenomenon[92]. It has also been shown that in healthy young men, black Africans have significantly reduced NO and flow-mediated dilatation compared with white Europeans, and this is independently associated with ADMA concentration[93]. It is intriguing to think that this could underlie a fundamental difference in the aetiology of hypertension in these different ethnic groups, a fact which is perhaps already acknowledged by treatment guidelines that reflect a reduced responsiveness to inhibition of the renin-angiotensin system in black African and Caribbean patients[94]. In summary, the role of ADMA in hypertension is likely to involve several factors, including the direct effect of NOS inhibition, and the effect on renal haemodynamics. Moreover, increased shear stress is known to

increase PRMT activity, and this represents another possible mechanism for increased ADMA concentrations[6].

Several studies have demonstrated reductions in plasma ADMA concentration with short-term angiotensin converting enzyme (ACE) inhibitor treatment; in these studies ADMA was reduced by 12 – 20%, and incorporated patients with essential hypertension[95-97], insulin resistance[98], diabetes[99] and chronic kidney disease (CKD)[100]. Angiotensin-receptor blocking drugs produce similar effects on ADMA[95,97,101], and this may be related in part to inhibition of the effects of oxidative stress, such as lipid peroxidation, and so restoration of DDAH activity[102]. This may be a factor in explaining the protective effects of ACE inhibitors on the vascular endothelium, which seem to extend beyond their obvious effects on the renin-angiotensin system[103].

1.3.3.4 Chronic kidney disease

CKD is regarded as a risk factor for cardiovascular disease and mortality, with the excess in mortality not being explained by traditional vascular risk factors alone[104]. Patients with end-stage renal failure (ESRF) were first shown to have a significant increase in plasma ADMA in the landmark study of Vallance[2]. Since then this observation has been repeated in several studies incorporating patients with various stages of CKD and ESRF (reviewed by Jacobi[105]). In CKD, plasma ADMA concentration is increased, on average, around 2-fold over healthy controls, with a wide range of concentrations reported (0.46 – 4.20 μmol/L)[105]. Although plasma ADMA and creatinine concentrations do not correlate well as such, ADMA does increase steadily across the stages of CKD[106,107]. Interestingly, ADMA concentrations are also increased in patients with kidney disease but normal renal function as defined by glomerular filtration rate (GFR), in whom there is also evidence of increased oxidative stress[106,108,109]. This, plus evidence from prospective studies, suggests that ADMA is involved in the progression of renal disease: ADMA independently

predicts progression to ESRF in patients with CKD, diabetic nephropathy and in transplant recipients[110-113]. In one study progression to end point (doubling of serum creatinine or initiation of renal replacement therapy) occurred around 20 months earlier for those with an ADMA concentration above the median[110]. In another a 0.1 μmol/L increase in ADMA was associated with an odds ratio of 1.47 (1.12 – 1.93) for progression[111]. The role of ADMA in renal disease progression may relate in part to peritubular ischaemia, and in an animal model increased expression of DDAH via an adenoviral vector was associated with retardation of kidney damage, in particular tubulointerstitial fibrosis[114]. However, this situation is not clear-cut, given a study of DDAH-1 polymorphisms which showed that increased DDAH-1 mRNA polymorphism was actually associated with a steeper decline in GFR[115].

ADMA may also be a determinant of cardiovascular disease in CKD, being associated independently with carotid IMT and its progression in patients with mild to moderate disease[116] and in those with ESRF[117]. It is also associated with left ventricular hypertrophy and cardiac dysfunction[118], and is an independent predictor of death in CKD[110,119]. As little as a 0.1 μmol/L increase is associated with a 20% increase in mortality[110], and in haemo-dialysis patients ADMA is the second strongest predictor of death after age, with a 2 – 3 fold increased risk of death for an ADMA concentration above the 75th centile[119].

Haemodialysis reduces ADMA concentration and increases vasodilatation, although its clearance is somewhat lower when compared with other low molecular weight compounds such as urea and creatinine: the magnitude of reduction in plasma ADMA concentration is variable, with reductions of 0 – 80% reported[105]. It is possible that plasma concentrations are not always a reliable guide to the more physiologically relevant intracellular concentration. Patients prone to dialysis-associated hypotension have a greater initial reduction in ADMA than normotensive equivalents, and a consequent excess of

NO may be implicated in the blood pressure lability[120]. Following renal transplantation, plasma ADMA concentration decreases and is associated with improved endothelial function[121].

SDMA correlates highly with GFR as determined by inulin clearance (R = 0.85), and appears to be a more sensitive marker of renal function than serum creatinine[122]. Although it could be regarded as the less important dimethylarginine, perhaps as a sensitive and non-invasive marker of GFR it has a future role. However, its potential role in reducing arginine availability has already been mentioned, and in this context it is interesting to note that its concentration is correlated with the angiographic stenosis score in patients with mild CKD[53]. Moreover, SDMA has been shown to independently predict cardiovascular and all-cause mortality in patients referred for coronary angiography[123].

1.4 ADMA in atherosclerotic disease

1.4.1 ADMA in atherosclerotic disease and prediction of events and mortality

Plasma ADMA concentration is associated with the presence and extent of atherosclerotic vascular disease. In a large multi-centre case-controlled study ADMA had the best discriminative power to distinguish cases of coronary heart disease (CHD) from controls, with an odds ratio (95% CI) of 6.04 (2.56 – 14.25) for those in the highest tertile of ADMA concentration versus the lowest (> 0.72 and < 0.58 μmol/L respectively)[124]. Moreover, in men with early stage atherosclerosis ADMA concentration is independently associated with measurable endothelial dysfunction, and in this context is also associated with the presence of erectile dysfunction, a condition directly attributable to reduced NO availability[125]. In patients with established CHD, ADMA is

associated with the severity of lesions at angiography[126]. Patients presenting with acute coronary syndromes (ACS) have higher ADMA concentrations than those with stable angina[127], and these decrease rapidly with medical treatment[128]. The failure of ADMA concentration to reduce to levels seen in stable angina 6 weeks following percutaneous intervention strongly predicted recurrence of a cardiovascular event during the following year[129]. It is also interesting to note that gradients of ADMA concentration have been demonstrated in coronary vessels, with higher concentrations distal to atherosclerotic plaques than proximal to them (2.39 versus 1.52 μmol/L), perhaps reflecting the "local" effects of endothelial dysfunction[130].

It is the evidence from longitudinal and prospective studies which provide arguably the most compelling case for the role of ADMA as not only a marker, but mediator, of atherosclerotic disease, repeatedly showing its predictive value independently of traditional and novel risk factors. This extends to the prediction of cardiovascular events, cardiovascular mortality and all-cause mortality, in individuals with established atherosclerotic disease, in high risk groups and in the general population. Table 1.1 summarises the major findings of these studies, with hazard ratios adjusted for traditional risk factors. Predictive value has been demonstrated in diverse cohorts, including ostensibly healthy individuals in the general population[131-134], high risk groups such as diabetics[112,135,136] and those with CKD[100,137], in patients with established CHD[59,138] and following acute coronary syndromes[139,140], chronic heart failure[141], peripheral vascular disease[142,143] and primary pulmonary hypertension[144]. Generally in high risk patients and in those with a prior history of CHD, it seems that highest tertile ADMA concentrations are independently associated with an apparent doubling of the (relative) risk of cardiovascular events and mortality. This often involves ADMA concentrations which are not markedly increased above what might be regarded as "normal" by many published studies which have reported reference values (discussed later in section on reference intervals), and this partly reflects analytical variation between different assay methodologies. It perhaps also says

something about the utility of reference intervals for describing abnormality, which is another issue discussed later. In this sense a parallel can be drawn with serum cholesterol concentration and its relationship with atherosclerosis. It must also be acknowledged that these studies are not enough to prove a causal relationship between ADMA concentrations and vascular disease, with possible confounding factors remaining even after adjustment for known risk factors.

1.4.2 Arginine supplementation and the "arginine paradox"

As arginine is the substrate for NO synthesis, the provision of supplemental arginine to overcome the effect of ADMA seems an attractive proposition. However, as the inherent plasma concentration is in the region of 60 – 80 μM[145], which is far in excess of the K_m of around 3.14 μM of eNOS for arginine[51], in theory arginine availability should not be a limiting to enzyme activity and NO production. Nevertheless, there have been studies suggesting a benefit from arginine supplementation on NO production and endothelial function. Indeed, in Vallance's landmark study, some of the cardiovascular effects of ADMA infusion could be offset by simultaneous arginine infusion[2]. Other studies in animals and humans have shown similar results[47,54,146-148], and oral arginine supplementation has been associated with increased exercise capacity in patients with stable angina[149] and peripheral vascular disease[150]. The combination of arginine with statin treatment might enhance the effect of statin on endothelial function in patients with higher ADMA concentrations, in whom ADMA has been proposed to offset, or block, the endothelial effects of statins[68,151,152]. A meta-analysis of trials involving arginine supplementation suggested a benefit on vascular endothelial function in patients with impaired flow-mediated dilatation[153]. This apparent benefit of arginine supplementation when, in theory, its concentration shouldn't be rate-limiting, has been termed the "arginine paradox"[154], and might relate to two main factors. First, the competitive nature of its inhibition of NOS, with increases

in the apparent K_m of the enzyme that can be partially overcome by arginine supplementation. Second, by competition with arginine for transport via the cationic amino acid transporter (CAT), for which ADMA has a strong affinity[51,52]. The physical association of the transporter and eNOS in the caveloae of endothelial cells further underlines its importance in delivering the arginine to NOS[155]. The ratio of arginine to ADMA has thus become a parameter which has been increasingly studied, and lower ratios have been associated with severity of heart failure and mortality in patients with cardiomyopathy[156,157] and have been implicated in the link between obesity and late-onset asthma, in which reductions in exhaled nitric oxide metabolites may be important pathophysiologically[158]. Systemic inflammation in a population-based cohort correlated negatively with the arginine:ADMA ratio, and may therefore explain the link between inflammation and reduced NO availability[159]. In patients with inflammatory arthropathies, treatment with TNF-α antagonists was associated with increases in the arginine:ADMA ratio and vascular function[160]. Reference values for the arginine:ADMA ratio have been described in the healthy population[145]. Plasma arginine is a non-essential amino acid, being synthesised largely in the kidney, although this may be insufficient in states of increased demand, i.e. inflammation and catabolic stress[161].

However, the proposed benefits of arginine supplementation have not been demonstrated consistently. Studies in post-menopausal women[162], CHD[163], healthy men[164] and chronic heart failure[165] have returned negative results in terms of its relatively short-term effects on vascular function. Furthermore, there is a possibility of harm, with arginine supplementation being associated with reduced functional capacity in patients with peripheral vascular disease[166], and with a possible increase in mortality following MI[167]. It has been speculated that utilisation of the excess arginine by alternative metabolic pathways might be responsible, for example, with production of ornithine which is a precursor of polyamines that can ultimately act to lessen vascular compliance[168].

The benefits, if any, of arginine supplementation are therefore unclear, and there appears to be insufficient evidence to recommend this as a strategy to improve vascular endothelial function. However, the arginine:ADMA ratio might represent a useful parameter as a reflection of eNOS substrate to inhibitor ratio over and above ADMA concentration alone.

Cohort	*N*	Follow-up	Outcome	ADMA concentration	Hazard ratio (95% CI)	Ref
General population	3320	10.9 years	All cause mortality	Per 0.13 μmol/L increase	1.21 (1.07 – 1.37)	131
Healthy women	880	24 years	MI (fatal & non-fatal) and stroke	Per 0.15 μmol/L increase	1.29 (1.09 – 1.53)	132
Healthy men	256	6.2 years	MI and sudden cardiac death	Highest tertile, > 0.86 μmol/L	2.40 (1.14 – 5.08)	133
Non-smoking men	150	5 years	Acute coronary events	Highest quartile, > 0.62 μmol/L	3.92 (1.25 – 12.3)	134
Type 1 DM & nephropathy	397	11.3 years	Fatal and non-fatal coronary events	Above median, > 0.46 μmol/L	2.05 (1.31 – 3.20)	112
Type 2 DM	125	1.75 years	Fatal and non-fatal coronary events	Highest tertile, > 0.63 μmol/L	2.37 (1.05 – 5.35)	135
Type 2 DM, angiography	163	2 years	All cause mortality	Highest tertile, > 1.05 μmol/L	2.63 (1.13 – 6.11)	136
Chronic kidney disease	131	2.25 years	Progression to ESRD and death	Per 0.1 μmol/L increase	1.20 (1.07 – 1.35)	110
Chronic kidney disease	820	9.5 years	Cardiovascular mortality	Per 0.25 μmol/L increase	1.25 (1.05 – 1.48)	137
Coronary heart disease	2528	5.45 years	Cardiovascular mortality	Highest tertile, > 0.89 μmol/L	1.68 (1.18 – 2.41)	138
Coronary heart disease	1874	2.6 years	Fatal and non-fatal coronary events	Highest tertile, > 0.70 μmol/L	2.62 (1.52 – 4.51)	59
Acute coronary syndromes (men)	193	2 years	Death, coronary events and stroke	Highest tertile, > 1.05 μmol/L	1.81 (1.01 – 3.25)	139
Myocardial infarction	249	1 year	All cause mortality	Highest tertile, > 1.14 μmol/L	4.83 (1.59 – 14.71)	140
Chronic heart failure	253	0.5 years	Coronary events and death	Highest tertile, > 0.64 μmol/L	2.00 (1.01 – 3.97)	141
Peripheral vascular disease	496	1.6 years	Fatal and non-fatal coronary events	Highest quartile, > 0.64 μmol/L	1.70 (1.02 – 2.88)	142
Peripheral vascular disease	133	2.9 years	Major coronary events	Highest quartile, > 0.84 μmol/L	5.10 (2.10 – 12.10)	143

Hazard ratios are presented following adjustment for demographics and known cardiovascular risk factors

Table 1.1: Main findings from longitudinal studies of ADMA concentrations and clinical outcomes

1.5 ADMA in critical illness

1.5.1 ADMA in the pathogenesis of multiple organ dysfunction

Even with best care, admission to the intensive care unit (ICU) is associated with significant mortality, and sepsis is a leading cause of death[169]. Sepsis and tissue injury are associated with a cascade of responses termed the systemic inflammatory response syndrome (SIRS)[170], which is a non-specific reaction to infectious and non-infectious processes. This is mediated by immune cells and inflammatory cytokines, and is involved in the response to infection and tissue repair. Septic shock describes the occurrence of hypotension and impaired tissue perfusion which is often resistant to aggressive fluid resuscitation and vasopressor agents, and seems to result from a dysregulation of the inflammatory response with multiple organ dysfunction resulting from a mixture of excessive inflammation, clotting cascade activation and coagulopathy, and dysfunction of the vascular enodothelium[171]. Septic shock has been termed "warm shock" because of the peripheral vasodilatation which is said to distinguish it from cardiogenic and hypovolaemic shock, at least in its earlier stages. It has been proposed that excessive NO production is an important mediator of septic shock, at least partly explaining the cardiovascular compromise and unresponsiveness to fluid and vasopressors[172]. NO production from the inducible isoform iNOS can lead to highly significant increases in NO [37,172]. While this has a detrimental effect on cardiovascular haemodynamics, it is considered an essential part of the host defence to infection, through the cytotoxic properties of the peroxynitrite ion[172,173]. It has been proposed that activation of DDAH with increased ADMA clearance facilitates the increase in NO from iNOS in vascular smooth muscle cells[174,175].

The role of ADMA in critical illness and shock is not clear. It has been proposed that ADMA is involved in the development of multiple organ failure through its inhibitory effects on constitutive NO production by eNOS[176]. Through a mixture of increased protein turnover, renal and hepatic dysfunction, and the direct effects of oxidative stress, hyperglycaemia and cytokines on DDAH activity, a potent combination of factors could conspire to increase the burden of ADMA, with effects of eNOS impairing organ blood flow, as well as other effects including capillary leakage and thrombocyte aggregation[176]. Moreover, studies in a rat model suggest that in excessive NO generation from iNOS might be directly associated with reduced eNOS activity and renal dysfunction[177]. Therefore, DDAH and ADMA appear well-placed to be an important part of not only the pathogenesis of multiple organ failure, but also as potential therapeutic targets in the treatment of these patients who have a high mortality.

1.5.2 ADMA and mortality in critically ill patients

Significantly increased ADMA concentrations have been reported in ICU patients and have been associated with mortality. In a cross-sectional study in ICU, ADMA was an independent and strong marker of outcome, with a 17-fold increase in mortality for patients in the highest quartile of ADMA[18]. In this study markers of hepatic function were strongly associated with ADMA concentration, suggesting that the liver is an important organ in ADMA handling in this context[15,18]. Indeed, in a rat model of endotoxaemia a significant increase in the hepatic uptake of ADMA was observed, such that the fractional excretion increased to 41% compared with a basal value of around 27%[174]. In another clinical study, plasma ADMA was associated with shock, severity of organ failure and inflammatory markers[178]. However, the situation might be more complex, as significant decreases in the ADMA:SDMA ratio have been demonstrated in septic humans, with significant negative correlations between inflammatory cytokines and ADMA concentrations[179].

This latter observation has been interpreted as reflecting increased ADMA catabolism during inflammation[179], which presumably would be largely mediated by the liver given what has been previously said about studies in rats[174]; furthermore, in septic humans, acute liver failure is associated with a significant increase in plasma ADMA concentration and mortality compared with septic patients with intact liver function[180]. In acute liver failure related to paracetamol toxicity, concentrations of ADMA and inflammatory cytokines are also significantly correlated[181], and plasma ADMA decreases rapidly following liver transplantation[17,19,181].

In light of what has been already been said about the arginine:ADMA ratio, this parameter has been studied in the context of critical illness, though not reported in many studies. In one ICU study, septic patients had a significantly lower arginine:ADMA ratio compared with hospitalised (non-septic) controls, and lower still in septic patients with shock[182]. Moreover, the ratio correlated with the severity of organ failure, and was significantly associated with eNOS dependent microvascular reactivity[182]. The authors of this study speculate that as early sepsis (i.e. without shock) is a hyperdynamic state there is increased hepatic and, to a lesser extent, renal excretion, with increased ADMA concentration as hepatic function falls in shock. A similar ICU study replicated these findings, with the additional observation that a declining arginine:total dimethylarginine (sum of ADMA and SDMA) ratio is independently associated with hospital mortality, with an odds ratio of 1.63 (1.00 – 2.65) per quartile[183]. In addition, arginine:dimethylarginine ratio was significantly associated with urine nitrate excretion, a surrogate marker of overall NOS activity[183]. However, previous trials of arginine supplementation have produced mixed results, with evidence of potential harm[184], therefore while the arginine:ADMA ratio might provide information regarding the potential interaction of substrate and inhibitor on NO generation, its increase by arginine supplementation appears not to be a “quick fix” to improve endothelial function and outcomes in critical illness. This might be related to a diversion of arginine from NOS: human endothelial cells exposed to

lipopolysaccharide (LPS) and TNFα develop increased arginase activity[185], and in humans exposed to intravenous endotoxin the plasma arginine:ADMA ratio decreases significantly within 4 hours of exposure[186]. In paediatric patients with sepsis ADMA concentrations were found to be lower than in febrile and healthy controls, although so too were arginine concentrations, which the authors speculate to be related to arginase activity[187]. This prompted them to dismiss ADMA as a cause of endothelial dysfunction in this patient group, which raises important questions of how plasma and intracellular concentrations are related, given that it is the latter which would be expected to be important. It could also relate to the point in the disease process at which the samples were taken; in adult patients with acute bacterial infections the recovery phase is associated with a significant increase in plasma ADMA concentration, suggesting an initial decrease during the early stages of the inflammatory response[188]. It might be that the significant increases seen in the ICU studies discussed occur at more advanced stages in the disease process, when the inflammatory response and organ dysfunction are more established. Lastly, common polymorphisms in the DDAH-2 gene may be associated with phenotypic variability in the pathophysiology of sepsis and the propensity to haemodynamic disturbance[178,189].

In summary, increases in ADMA concentrations have been associated with organ dysfunction and mortality in critically ill patients, and have been speculated to be causally related through effects on NOS. However, given the potentially harmful effects of excessive NO, the intriguing possibility of a regulatory role of ADMA acting as a "brake" on iNOS remains[34]. Indeed, stimulated macrophages and endothelial cells actively take up dimethylarginines[190,191]. Moreover, while the liver increases ADMA uptake during inflammation there is no definite evidence that this is due to DDAH-mediated catabolism[174]. Therefore it is at least theoretically possible that increased ADMA uptake acts to achieve this regulatory role. The distribution

of ADMA between the intra and extracellular compartments is further considered in relation to the study described later in chapter 8.

1.5.3 Therapeutic manipulation of DDAH and NOS in critical illness

Intensive insulin therapy to maintain normoglycaemia has been associated with improved outcomes in ICU[192]. It has been proposed that modulation of ADMA concentration could account for this beneficial effect[193]: intensive insulin treatment was associated with ADMA concentrations that were lower on day 2 compared with those treated conventionally. Moreover, at the end of the study period intensively treated patients had lower ADMA concentrations and there was an association between total insulin dose and ADMA concentration on the last day[193]. These effects could be explained by preservation of DDAH activity, given the effect of hyperglycaemia on DDAH activity[30,194]. Other explanations potentially include reduced protein catabolism and increased cellular uptake of ADMA via CATs[195].

The potentially harmful effect of excess NO from iNOS has prompted the search for NOS inhibitors to improve cardiovascular stability in sepsis and critical illness. Monomethylarginine (MMA) was first studied in this context, but was actually associated with increased mortality[196]. Similar results were demonstrated in patients with cardiogenic shock following MI[197]. It is probable that the adverse outcomes in these studies related to the non-specific nature of the NOS inhibition, with eNOS inhibition in particular associated with reduced constitutive NO production and organ perfusion. More recently, a pharmacological inhibitor of DDAH-1 has been studied in a rat model of LPS-induced endotoxic shock[198]. In cultured cells, the inhibitor specifically reduced NO production in aorta but not macrophages, thus permitting selective inhibition of iNOS-mediated NO production in blood vessels but not macrophages (which largely express DDAH-2)[198]. In LPS-

treated rats, those treated with the inhibitor demonstrated attenuation of the rate of hypotension. Similar results were seen in heterozygous knockout animals (DDAH1$^{+/-}$), with only modest effects on plasma ADMA concentration[198]. Both DDAH-1 and DDAH-2 are present in blood vessels, and it is now perhaps becoming clear that DDAH-1 is, in fact, of prime importance in blood vessels[22,86,199], despite what has previously been said regarding the tissue distributions of the different isoforms. While these animal studies are promising, it is clear that further work is required to determine whether this is a feasible approach for improving cardiovascular stability in septic humans.

1.6 Measurement of dimethylarginines

1.6.1 General analytical considerations

Measurement of ADMA has been most commonly performed in human plasma and serum, although urinary measurement, and, increasingly, intracellular measurements are being utilised in studies concerned with the physiology and clearance of ADMA. ADMA quantification is rendered difficult by its relatively low, submicromolar, concentration, particularly in the presence of many other amino acids that are present in far greater concentrations. A further analytical challenge is in achieving separation, generally by chromatography, of the structural isomers ADMA and SDMA. Methods such as high performance liquid chromatography (HPLC) and mass spectrometry coupled to gas or liquid chromatography permit the simultaneous measurement of arginine, ADMA, SDMA, and MMA, which is a distinct advantage, given what has been said previously about the increasing use of the arginine:ADMA ratio as a marker of NO substrate availability, and the role of SDMA as a marker of renal function, and possibly as a cardiovascular risk marker in its own right. It is also generally possible to measure the non-essential amino acid homoarginine, which is structurally very similar to arginine, differing only by the length of its

carbon skeleton which is 1 carbon atom longer. Although its role in human metabolism is unclear, homoarginine has been proposed to have a role in the availability of NO, and is strongly correlated with flow-mediated dilatation during normal pregnancies[200], and has been independently associated with cardiovascular events and mortality in patients referred for angiography[202] and in those undergoing haemodialysis[203]. Moreover, a recent report suggests an antagonistic relationship with arginine in connection with blood pressure[201]. Therefore, measurement of endogenous homoarginine concentrations might be of interest. While the concentration of MMA in plasma is many times less that of ADMA, its intracellular concentration can be significantly higher, and thus its measurement might be of interest and relevance in cellular studies[51]. In summary, therefore, methods for dimethylarginine measurement would ideally permit the reliable simultaneous quantification of arginine, ADMA, SDMA, MMA and homoarginine, and be suitable for plasma/serum, urine and intracellular matrices.

1.6.2 High performance liquid chromatography (HPLC)

HPLC has been the most frequently used method for ADMA analysis, the majority of methods employing reverse-phase chromatography with fluorescence detection, and have proved reliable and stable, and capable of high precision. Complete separation of ADMA and SDMA can be achieved under isocratic conditions[205,206,209,201], though some methods have required gradient elution[204,207,208,211]. A universal requirement is sample clean-up given the large number of amino acids and other interfering substances in plasma. Solid phase extraction takes advantage of the basic nature of the amino acids of interest, using cationic exchangers to select the basic amino acids for analysis. However, this adds a potentially laborious pre-analytical sample preparation step to the process, and could thus be considered something of a disadvantage. Furthermore, typical analysis times are in the region of 20 – 40 minutes, which could also be considered a disadvantage compared with the

relatively shorter analysis times using mass spectrometry. That said, HPLC with a monolithic column that permits higher flow rates can reduce analysis times to as little as 10 minutes while retaining excellent separation[212]. Furthermore, use of a programmable auto-injector allows batches of extracted samples to be analysed, for example, overnight. One further requirement is derivatisation, as the amino acids themselves are not fluorescent. This has commonly been achieved with ortho-phthaldialdehyde (OPA) which produces fluorescent adducts that are reliably separated on chromatography[205,206,208,210,211]. One disadvantage of OPA adducts is their relative instability, necessitating derivatisation just prior to analysis to prevent significant reductions in fluorescence during analysis[206].

An internal standard needs to be added prior to sample preparation, and is used essentially to correct for errors during the various pre-analytical processes. An internal standard must, therefore, behave similarly to the analytes of interest and be reliably quantified during analysis. Homoarginine and MMA have been frequently employed as internal standards in HPLC, with the apparent general view that as they are present in low concentrations in plasma, the use of high enough concentrations should avoid large errors from their natural variation in biological samples. However, given what has been said about the emerging biological importance of these compounds, alternative internal standards might be desirable. The ideal internal standard would be one not inherent in biological samples; in this regard N-propyl-L-arginine (NPA) has been used, though its separation from the dimethyl-arginines may not be easily achieved on all columns[45]. Characteristics of the main HPLC methods, including imprecision and reference values obtained using them, are shown in table 1.2

1.6.3 Tandem mass spectrometry

Methods based on tandem mass spectrometry (MS/MS) have become popular principally as they allow discrimination of ADMA and SDMA based on fragmentation pattern, rather than relying solely on chromatographic separation which inevitably prolongs the analysis time. In this way laborious sample preparation steps can often be avoided, save for a simple protein precipitation step for plasma and serum samples. However, the lack of a specific daughter ion for ADMA often necessitates the coupling of liquid chromatography (LC-MS/MS)[213-219], which imposes the need to finds a compromise between adequate chromatographic separation and analysis times that are not too long[45]. Derivatisation is required in gas chromatography MS/MS (GC-MS/MS)[220,221] in order to produce thermally stable and volatile derivatives. In LC-MS/MS, derivatisation is not absolutely required; however, butylation, for example, can lead to characteristic products which increase specificity to such an extent that analysis times are extremely short, making methods suitable for very high throughput of samples[215]. Potential drawbacks of MS/MS methods include the requirement for expensive equipment and technical expertise to develop and optimise methods accordingly. However, when this is available, the advantages of simplified sample preparation and higher throughput makes this an attractive analytical method. Table 1.2 shows characteristics of some of the main methods.

1.6.4 Enzyme linked immunosorbent assay (ELISA)

An ELISA based method which can quantify ADMA only was developed, with the aim of overcoming the drawbacks of HPLC and MS/MS methods as considered above[222]. This has now been used in several large studies. Its major advantage lies in the high throughput of samples possible with its 96

well plate format. A second ELISA, based on much the same principles, is said to offer greater sensitivity[223]. The obvious drawback of ELISA lies in its inability to measure, on the same sample, the concentrations of relevant and related compounds such as SDMA and arginine. It also appears to be relatively imprecise compared with chromatographic methods. There have also been doubts about its accuracy, as discussed in the following section on method comparisons.

1.6.5 Between method comparisons

As can be seen from the reference values in table 1.2, results from different methods display considerable variability, with mean reference values in so-called healthy individuals ranging from around 0.37 to 0.78 µmol/L. It is obvious that this magnitude of variation between analytical methods is a problem when, as previously discussed, small changes in ADMA concentration are associated with significant biological effects. Indeed, it has been cited as a direct hindrance to the assimilation of the large volume of clinical work thus far done on ADMA and a barrier to appreciating the level of risk associated with ADMA[224]. Specifically in renal disease, a review by Jacobi[105] nicely summarises the position by assimilating the range of "control" values for ADMA in renal studies which are meant to represent healthy individuals, finding a range of 0.36 – 1.40 µmol/L. While studies in renal disease have been promising, Jacobi notes that "the variability of ADMA levels currently precludes useful application of this putative biomarker"[105]. The main factors behind this variability are likely to include lack of a reference standard and a lack of specificity in some chromatography methods[45]. Compounding this is the relative lack of any comparative studies, and no agreement on what constitutes acceptable performance for analytical methods. In addition to between-method variation there is the effect of imprecision, which, as Teerlink[225] points out, is to reduce statistical power in clinical trials and under-estimate the strength of associations in epidemiological studies.

Indeed, an experiment simulating the effect of increasing imprecision in the results from a real study shows a rapid falling off of the chance of achieving statistical significance, even in the range of 5 – 10%, a level of imprecision which some might view as being acceptable[225]. It is clear that many of the methods summarised in table 1.2 display relatively high imprecision.

There have been a small number of comparative studies evaluating ELISA with MS/MS, which seem to highlight a specific problem concerning the calibration of the ELISA method. ELISA appears to over-estimate ADMA concentrations up to a factor of 1.5 to 2 in comparison with robust HPLC and LC-MS/MS methods despite good correlation[226-228]. This suggests a problem with calibration, possibly related to matrix-dependent effects, given the calibrators are in an aqueous solution. However, no correlation was noted between the methods in another study[229]. The increasing number of published studies using ELISA highlights the issues of imprecision and bias, and the future of ADMA-based research is arguably dependent on standardisation of analytical methods to allow assimilation of the burgeoning body of research.

1.6.6 Analytical performance goals

European consensus is that goals, or minimal standards, for the satisfactory performance of analytical methods are best defined on the basis of biological variation[230,231]. Biological variation describes the degree of inherent variation both within an individual over time, and between individuals in a population. Desirable imprecision is generally set as less than 0.5 times the intra-individual variation, because at this level analytical variation should have a minimal impact on the total test variation[230,232]. However, there are virtually no data regarding the biological variation of ADMA. Teerlink[225] has estimated a narrow between-subject variation, something which is also suggested by the relatively small standard deviations in the reference data shown in table 1.2.

Method principle	Inter-assay CV, %	Reference values† Mean ± SD, µmol/L	Ref
Gradient RP HPLC, AccQFluor	2.7	0.45 ± 0.07 (n = 30)	204
Isocratic RP HPLC, OPA	2.5	0.58 – 0.64* (n = 292)	205
Isocratic RP HPLC, OPA	2.0	0.47 ± 0.08 (n = 53)	206
Gradient RP HPLC, AccQFluor	6.0	0.44 ± 0.08 (n = 12)	207
Gradient RP HPLC, OPA	< 10.0	0.76 ± 0.12 (n = 35)	208
Isocratic RP HPLC, NDA	2.3	Range 0.38 – 1.30 (n = 50)	209
Isocratic RP HPLC, OPA	Not stated	0.30 ± 0.05 (n = 7)	210
Gradient RP HPLC, OPA	5.6	0.58 ± 0.02 (n = 10)	211
LC-MS/MS, underivatised	4.2 – 5.5	0.46 ± 0.07 (n = 103)	213
LC-MS/MS, underivatised	< 10.0	0.46 ± 0.07 (n = 29)	214
LC-MS/MS, butyl ester	4.4	0.46 ± 0.09 (n = 85)	215
LC-MS/MS, underivatised	Not stated	0.66 ± 0.12 (n = 15)	216
LC-MS/MS, underivatised	< 8.0	0.37 ± 0.06 (n = 14)	217
LC-MS/MS, underivatised	6.0%	95% CI 0.44 – 0.46 (n = 42)	218
LC-MS, OPA	5.7%	0.45 ± 0.13 (n = 15)	219
GC-MS, methyl PFP	1.6%	0.60 ± 0.08 (n = 10)	220
GC-MS/MS, methyl PFP	5.8%	0.39 ± 0.06 (n = 12)	221
ELISA	10.3%	0.65 ± 0.13 (n = 10)	222
ELISA	4.2%	0.78 ± 0.09 (n = 8)	223

* Reference values presented as means in four groups stratified according to age.

† As described in the method validation.

Table 1.2: Summary of major analytical methods for ADMA measurement and associated reference values

1.7 Population reference intervals for ADMA

There have been relatively few population studies encompassing a reasonable number of well-defined "healthy" individuals. Teerlink[233], using a well validated HPLC method, reported a 95% reference interval of 0.39 – 0.63 μmol/L in a large population based cohort (n = 2,311, mean 0.50 μmol/L, age range 50 – 74). The somewhat older average age of Teerlink's cohort should be acknowledged, as well as the presence of impaired glucose tolerance or diabetes in about a third of a cohort, which might influence the results obtained[234]. Schulze[235] reports a 95% reference interval of 0.36 – 1.17 μmol/L using ELISA (n = 500, mean 0.69 μmol/L, age range 19 – 75). Hov[236] reported data from Norwegian blood donors, showing a 95% reference interval of 0.40 – 0.77 μmol/L using HPLC (n = 283, mean 0.59 μmol/L, age range 16 – 69). Schwedhelm[237] reported reference values in healthy non-smoking individuals from the Framingham Offspring Cohort, showing a reference interval between the 2.5 and 97.5 centiles of 0.31 – 0.73 μmol/L, using LC-MS/MS (n = 1,126, mean 0.52 μmol/L, mean age 56).

The higher mean ADMA in study is of Schulze is consistent with what has already been said about the comparison between ELISA and chromatography methods. The relatively wider range most likely represents the higher imprecision of the assay. Each of the four studies in the foregoing paragraph report a weak but significant correlation between ADMA concentration and age. Furthermore, post-menopausal women have significantly higher ADMA concentrations than younger women and younger men[233,235-237]. Indeed hormone replacement therapy is associated with a decrease in ADMA concentrations, which may represent the effect of oestrogen on DDAH[238]. However, these age and gender differences are biologically small, and it has been suggested that the generation of age and gender specific reference intervals would be of little, if any, benefit[235]. Indeed, when this has been

done, the mean concentrations between groups are small, with means ranging from 0.58 to 0.64 μmol/L in a study by Meinitzer[205].

Other studies of greater than 100 individuals have reported results which equate to 95% ranges of 0.30 – 0.82 (mean 0.51) μmol/L and 0.32 – 0.62 (mean 0.50) μmol/L, both using HPLC[70,239]. Considering these with results from well validated methods (see table 1.2), it seems that an ADMA concentration of around 0.50 μmol/L is close to the healthy population value.

1.8 Summary

ADMA is a potent endogenous inhibitor of all NOS isoforms. ADMA is produced ubiquitously following the degradation of protein containing methylated arginine residues, and is largely cleared by the enzyme DDAH. DDAH activity is impaired in a number of oxidative stresses, and this, through NOS impairment, may be a factor in endothelial dysfunction in patients with cardiovascular risk factors. ADMA has also been associated with the extent of cardiovascular disease, and is an independent predictor of cardiovascular events and mortality, both in high risk groups and in the general population. ADMA may also play a role in the pathogenesis of multiple organ failure in critical illness. Analytical methods for ADMA analysis produce results with considerable variability, and this has proven a barrier to the assimilation of the large body of clinical work concerning ADMA and perhaps hindered its recognition as a potent risk factor and therapeutic target.

Data regarding the biological variation of ADMA are absent from the literature, and, consequently, analytical performance goals to guide assay development have not been established. While ADMA has been proposed to

play a role in acute inflammation and the regulation of NO, its early response during the evolution of the inflammatory response, and how this relates to NOS activity, is not known.

1.9 Aims of thesis

1. To establish an analytical method for the reliable and simultaneous measurement of arginine, homoarginine, ADMA and SDMA in biological fluids, based on HPLC and simplified to allow its easy adoption in the clinical laboratory.

2. To establish a laboratory reference interval for plasma dimethylarginines using the developed method.

3. To establish the intra and inter-individual biological variation of dimethylarginines, and from this assess the utility of traditionally defined population reference intervals in detecting “abnormality”.

4. Using biological variation data, to establish analytical performance goals (imprecision, total error, bias) for analytical methods.

5. To develop a method for measuring urine dimethylamine, the excreted product of DDAH-mediated ADMA catabolism, as a marker of whole body DDAH activity.

6. To develop a method for measuring urine nitrate excretion, as a marker of whole body NO production.

7. To design a study using a suitable patient cohort that will allow assessment of plasma dimethylarginine concentrations in the non-inflamed state, and during the evolution of the acute inflammatory response.

8. Using the developed methods, to assess whether significant changes in whole body DDAH and NOS activities occur during the inflammatory response, and relate this to changes in plasma ADMA concentration.

Chapter 2

HPLC analysis of dimethylarginines in biological samples

2.1 Principle of method

HPLC involves separation of compounds based on their relative affinities for two different phases, a solid, or stationary, phase and liquid, or mobile, phase. Reverse-phase HPLC is most commonly deployed, and describes the specific combination of a hydrophobic stationary phase and polar mobile phase. In this type of chromatography analytical columns are often composed of silica bonded alkyl chains, C18 being the most popular. Separation of a mixture of analytes is therefore based on their relative hydrophobicity, with more hydrophobic compounds tending to adsorb more to the column, delaying their progress relative to more polar compounds which have greater affinity for the mobile phase. The composition of the mobile phase, with respect to factors such as pH, ionic strength of the buffer, and concentration of organic solvent can be manipulated to optimise separation of analytes and the overall analytical run time. Isocratic elution refers to HPLC in which the composition of the mobile phase remains constant. Gradient elution is required when some compounds in the mixture are strongly retained on the column, and an increase in the organic solvent component of the mobile phase is needed to encourage elution; this prevents overly long run times and improves the quality and resolution of the peaks obtained.

Once separated on the analytical column, quantitation is based on a detection method in which the output of a detector is proportional to the analyte concentration. Commonly used methods include electrochemical, absorbance of light, fluorescence and mass spectrometry. In amino acid analysis, fluorescence detection following derivatisation is often employed. This has

the advantage of high sensitivity and specificity, the latter from the specific excitation wavelength and wavelength for the re-emission of light. The signals from the detector are captured by data management software, resulting in a chromatogram featuring a series of peaks from which analytes are quantitated based on relative peak heights or peak areas. The major components of a reverse-phase HPLC system are shown in figure 2.1.

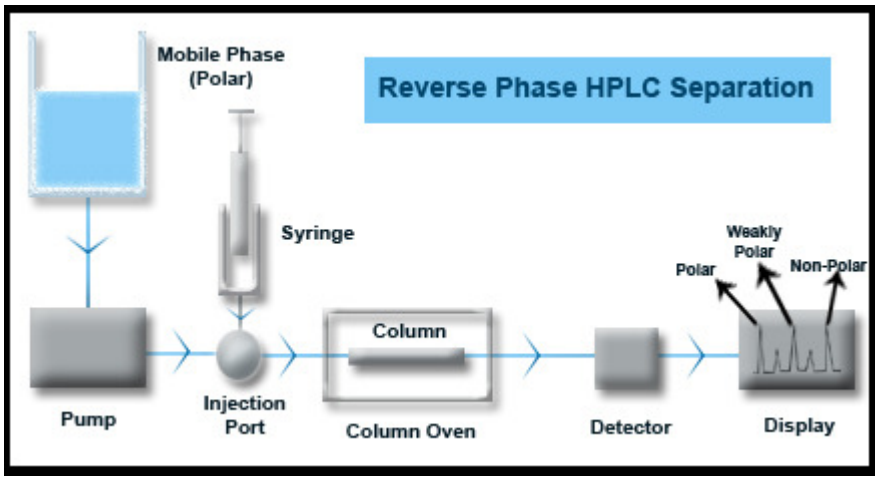


Figure 2.1: Schematic diagram of the HPLC system (from www.lab-training.com)

2.2 Specimen collection and pre-analytical handling

2.2.1 Plasma

Plasma was selected as the preferred specimen type. This was as previous studies have shown measured arginine concentrations to be significantly higher in serum samples compared with plasma[205,225], and that this is artefactual owing to release from blood cells. The choice of anticoagulant, i.e. heparin or EDTA, was considered unimportant as they appear to exert no influence over measured dimethylarginine concentrations[205,225].

The time to separation of plasma from the cellular components of blood has been previously shown to be non-critical for ADMA and SDMA analysis, with no significant change in whole blood after 2 days storage at room temperature[205]. However, arginine does decrease significantly in whole blood, with a reduction in the region of 25% after 2 hours[240]; however, at 30 – 60 minutes the change is minimal. This is most likely due to the effect of arginase from lysed erythrocytes or leucocytes, an effect that can be inhibited by storing the blood on ice[240]. In the studies described in this thesis blood specimens were centrifuged promptly after collection, i.e. generally within 30 minutes, and definitely within 60 minutes. However, if longer processing times were anticipated it would be advisable to store samples on ice to prevent reductions in arginine concentration related to *ex vivo* metabolism.

Specimens were centrifuged (500 g, 4°C, 10 minutes) and the plasma removed into plastic containers. These were stored at -70°C until analysis.

2.2.2 Urine

Urine was collected into plain specimen containers and stored at -80°C until analysis. After thawing, centrifugation was used to remove any insoluble precipitation.

2.2.3 Erythrocyte lysates

After removal of the plasma and buffy coat, packed red cells were stored at -70°C until analysis. Three freeze/thaw cycles were deployed to ensure lysis of the erythrocyte membranes. 100 µL of packed cells were thoroughly mixed with 400 µL of deionised water, and the mixture centrifuged (Eppendorf

microcentrifuge Model 5417C) to remove cellular debris. 200 µL of the supernatant was used for analysis.

2.3 Reagents and buffers

2.3.1 List of chemicals

Asymmetric dimethyl-L-arginine (Calbiochem, Nottingham, UK)

Symmetric dimethyl-L-arginine (Calbiochem, Nottingham, UK)

L-Homoarginine (Sigma chemical company, Poole, UK)

L-Arginine (Sigma chemical company, Poole, UK)

Monomethyl-L-arginine (Sigma chemical company, Poole, UK)

Monoethyl-L-arginine (Calbiochem, Nottingham, UK)

Boric acid (BDH chemicals, VWR international Ltd, Leicestershire)

Ortho-phthaldialdehyde (Sigma chemical company, Poole, UK)

3-mercaptopropionic acid (Sigma chemical company, Poole, UK)

Sodium acetate (BDH chemicals, VWR international Ltd, Leicestershire)

Acetonitrile, HPLC grade (Rathburn chemicals Ltd, Walkerburn, Scotland)

Methanol, HPLC grade (Rathburn chemicals Ltd, Walkerburn, Scotland)

Ammonia (Romil Ltd, Waterbeach, Cambridge)

2.3.2 Preparation of buffers and reagents

2.3.2.1 50 mM borate buffer

3.1 g of boric acid were dissolved in 1 litre of deionised water, and the pH adjusted to 8.5 using 2 M potassium hydroxide. This was prepared every 4 weeks to prevent interferences from contaminating substances which was seen to occasionally affect older solutions.

2.3.2.2 200 mM borate buffer

2.5 g of boric acid were dissolved in 200 ml of deionised water, and the pH adjusted to 8.5 using 2 M potassium hydroxide.

2.3.2.3 Ammonia/methanol for elution from SPE cartridges

A solution containing 10% v/v concentrated ammonia and 50% v/v methanol in deionised water.

2.3.2.4 Derivatising agent

10 mg OPA were dissolved in 200 μL of methanol, then mixed with 1.8 mL of 200 mM borate buffer. Finally 10 μL of mercaptopropionic acid were added. This stock solution was diluted 1:5 with 200 mM borate buffer prior to use. The stock solution stable for approximately 1 week, before a decrease in fluorescence occurred owing to instability of the thiol group.

2.3.2.5 Mobile phase: 50 mM acetate buffer with 9% v/v acetonitrile

A 50 mM sodium acetate buffer was prepared by dissolving 3.73 g of sodium acetate in 910 mls of deionised water, and the pH adjusted to 6.3 using glacial acetic acid. 90 mls of acetonitrile were added. The mobile phase was filtered through a 0.45 μm nylon filter and degassed prior to use.

2.4 Standards

2.4.1 Preparation of stock standards

As no commercially available standards are available, individual stock standards were prepared from pure compounds. These were made in 10mM hydrochloric acid (HCl) at concentrations of 1mM and were stored at -70°C until use.

2.4.2 Working standards

A combined calibration standard was made by preparing in deionised water a solution containing the four main analytes of interest at the following concentrations (table 2.1). These were aliquoted and stored at -70°C until use. Their stability, as assessed by chromatographic peak heights, was determined to be at least 1 year. When a fresh batch was prepared, comparison against the previous lot was used to assign calibrator values and ensure continuity in calibration. As discussed in the section on linearity, a single point calibration was chosen, given the linearity of the detector response across a wide concentration range.

Component	Concentration, µM
Arginine	50
Homoarginine	1.0
ADMA	1.0
SDMA	1.0

Table 2.1: Analyte concentrations in combined calibration standard

2.5 Internal standard

2.5.1 General requirements of an internal standard

The requirement for a sample cleanup technique involving multiple manual steps is a source of random error and thus imprecision. An internal standard is thus added to each standard, QC and sample at the start of the analytical process, and so corrects for random errors especially in the sample cleanup process. The ideal internal standard is chemically similar to the analytes of interest, thus behaving similarly during extraction and analysis, is well resolved from the analytes, and is preferably not inherent in biological samples. MMA and monoethylarginine (MEA) were evaluated, in order to compare the performance of the former commonly used internal standard with the latter which is novel and non-endogenous.

2.5.2 Monomethylarginine (MMA)

MMA is the most commonly used internal standard in HPLC methods for ADMA analysis, and is chosen because of its relatively low concentration in human plasma compared with the other analytes of interest[233]. A stock standard of MMA was prepared in 10 mM HCl. This was diluted in deionised water to obtain a working solution of 5 µM which was aliquoted and stored at -70°C until use.

2.5.3 Monoethylarginine (MEA)

The non-endogenous compound MEA was selected as a candidate internal standard. A stock standard of MEA was prepared in 10 mM HCl. This was diluted in deionised water to obtain a working solution of 5 µM which was aliquoted and stored at -70°C until use.

2.6 Internal quality control material

As there are no commercially available QC materials for ADMA, a large plasma pool was obtained from the blood transfusion service for use as in-house QC. This was stored in aliquots at -70°C until use. It was analysed in duplicate in each analytical run in order to generate analytical variation data.

2.7 Specimen preparation by solid phase extraction

2.7.1 Solid phase extraction (SPE) cartridges and principle

The strongly basic nature of the amino acids of interest make SPE a good method for their extraction from the numerous other amino acids in plasma. At neutral and weakly basic pH, the amino acids of interest are protonated and bind strongly to the cation exchange resin, while other amino acids are washed through. After washing steps, they can be eluted from the column using a strongly basic solution. Isolute PRS columns (1 ml/50 mg cartridges, Kinesis Ltd, Epping, UK) were selected as the SPE medium.

2.7.2 Solid phase extraction procedure

200 µL of plasma, standard or QC material were pipetted into small test tubes. To each of these were added 80 µL of the internal standard and finally 720 µL of 50 mM borate buffer, to create a final volume of 1 mL. For analysis of urine samples, 50 µL of urine were used, with the same quantity of internal standard and 870 µL of borate buffer. This was to bring the higher concentrations of ADMA found in urine into a range ensuring linearity of the detector response. Results were multiplied by a factor of 4 to correct for the smaller volume of urine used in the analysis.

The SPE cartridges were placed on a Vac Elut extraction system, and activated and equilibrated with 2 ml of methanol followed by 2 ml of 50 mM borate buffer. The sample mixtures, as described above, were then run through the cartridges. The columns were then consecutively washed with a further 1 ml of borate buffer, 3 ml deionised water and 3 ml methanol. It was

generally possible to perform these steps under gravity, with vacuum suction only occasionally required if the flow rate through one or more of the columns was sluggish. Following the washing steps, the amino acids of interest were eluted using 3 ml of the 10% ammonia/50% methanol solution and collected into glass tubes. The eluent was then evaporated to dryness at 80°C under air. Using this system, 40 samples could be extracted in approximately 2 hours.

2.8 Derivatisation

The dried extract was reconstituted in 100 µL deionised water and thoroughly mixed. To that was then added 100 µL of the derivatising agent prepared as described above. After thorough mixing, the tubes were left sitting in the dark for 15 minutes in order to ensure completion of the reaction. The derivatised samples were then transferred into auto-sampler vials and placed in the autosampler ready for analysis.

2.9 Chromatography

2.9.1 Chromatographic system and setup

The chromatographic system consisted of a solvent delivery system, programmable autosampler and fluorimeter (Waters, Watford, UK). 20 µL of the derivatised sample was injected by the programmable autosampler onto the column for chromatography. Excitation and emission wavelengths were 340 nm and 455 nm respectively, the optimal wavelengths for OPA adducts. After the elution of arginine the sensitivity was increased, at around 14 minutes, by adjusting the EUFS (emission units full scale) from 32,000 to

8,000. This was necessary as the concentration of arginine is inherently around 100 – 200 times greater than that of the other analytes. Signals from the detector were captured by a data management system (Millennium 2010, Waters, Watford, UK).

2.9.2 Analytical column

The analytical column was a Symmetry C18, 4 μm, 4.6 x 150 mm, protected by a 3 x 4 mm C18 guard column. The ambient temperature in the laboratory was maintained at a constant level in order to ensure stability of retention times.

2.9.3 Mobile phase

The mobile phase, prepared as described, was maintained at room temperature and pumped through the analytical column at a flow rate of 1.5 ml/minute. The mobile phase was recycled in order to allow a larger number of samples to be analysed in a single run without having to prepare overly large volumes of the mobile phase. This was not found to cause any problem with interfering peaks, even in continuous runs of up to 100 sample injections.

2.10 Calculation of results

Quantification was done by the method of internal standardisation on the basis of peak heights as the analytes were all completely resolved at baseline and the peaks consistently sharp and well defined. Analyte concentrations were calculated by dividing the peak height ratio of analyte to internal

standard in the (unknown) sample by that in the calibration standard and multiplying by the known concentration in the standard. A single level calibration was selected owing to the linearity of all analytes over a wide concentration range, as demonstrated in the section on analytical validation.

2.11 Resultant chromatographic profiles

Elution from the analytical column was in the order arginine, MMA, homoarginine, ADMA, SDMA and MEA. All were resolved at baseline and no interference from other peaks was a problem. The retention factors (K') based on the described conditions are given in table 2.2.

Compound	Retention factor (K')
Arginine	12.6
MMA	18.3
Homoarginine	21.0
ADMA	24.0
SDMA	26.2
MEA	35.9

Table 2.2: Retention factors for the analytes of interest

Chromatographic profiles of a combined standard, including MMA, and an extracted human plasma sample using MEA as the internal standard are shown in figure 2.2.

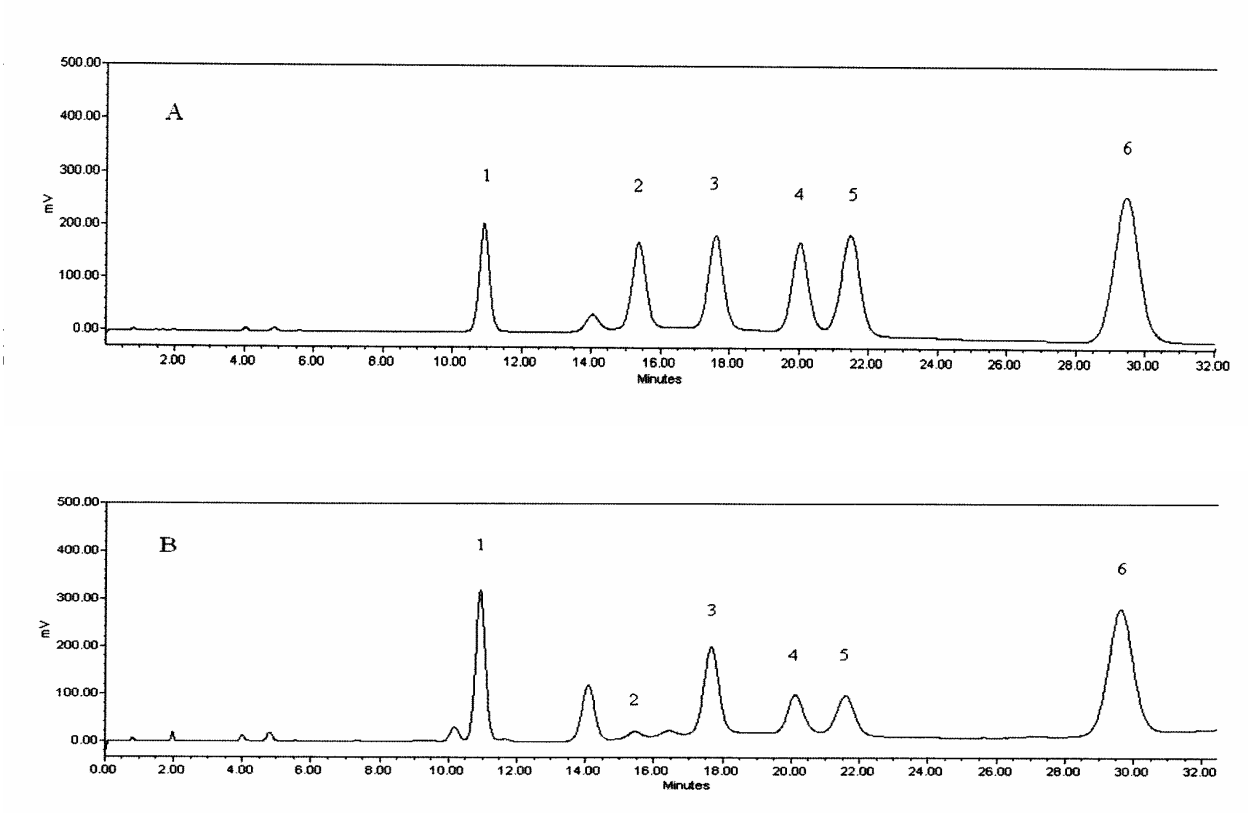


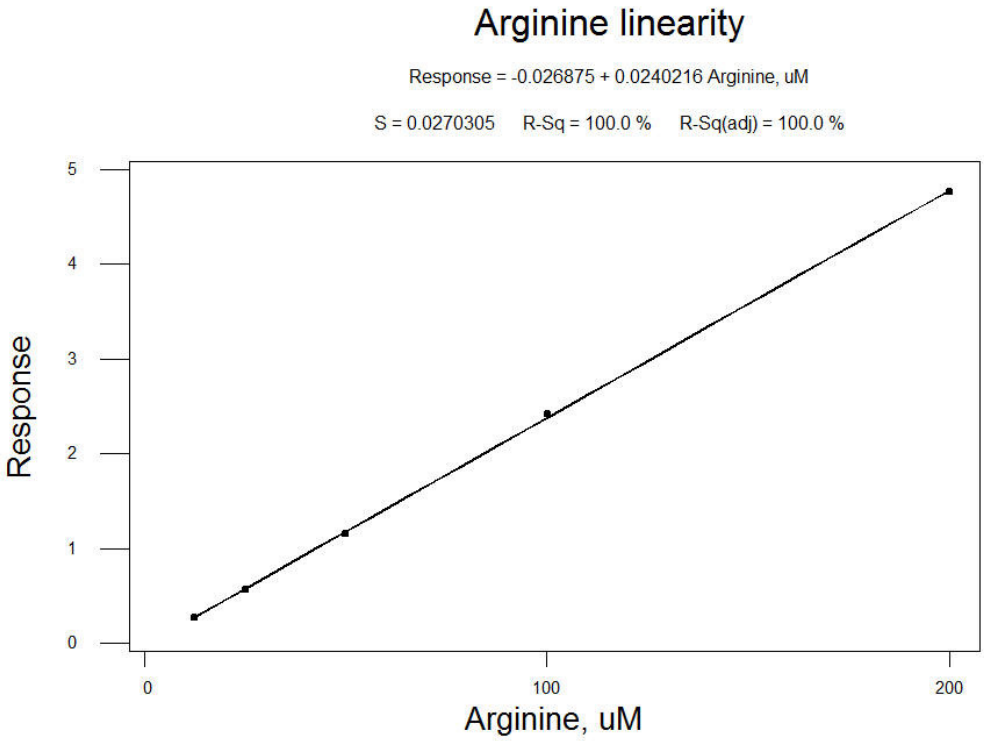
Figure 2.2: Chromatographic profiles of (a) a combined standard and (b) an extracted human plasma sample.

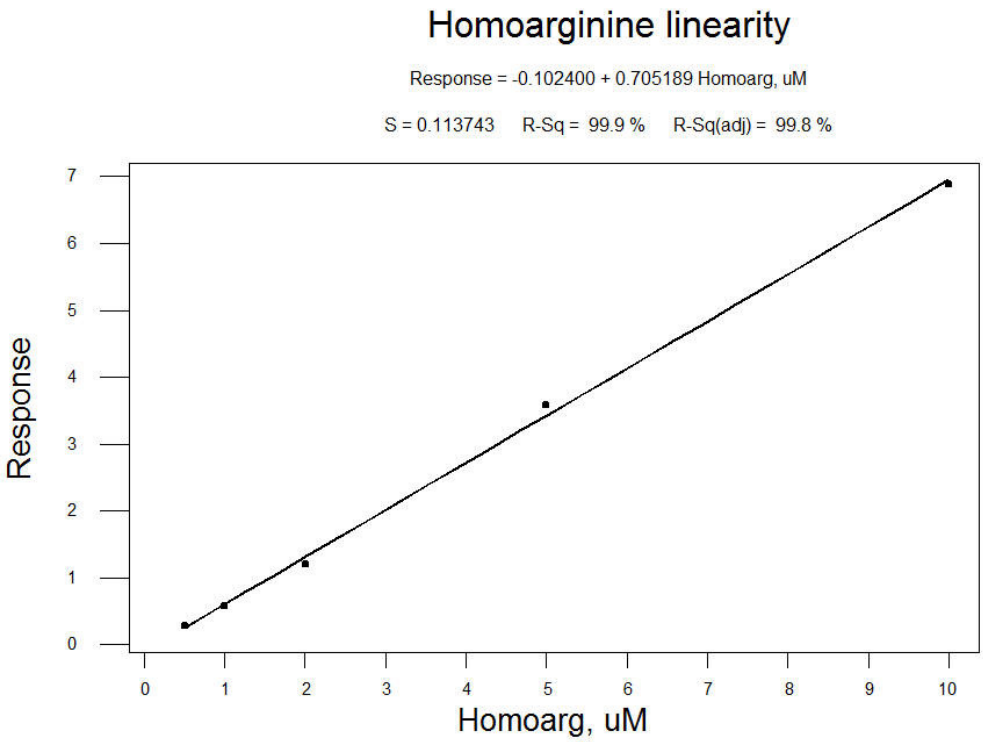
Peak identification: 1) Arginine, 2) MMA, 3) Homoarginine, 4) ADMA, 5) SDMA, 6) MEA

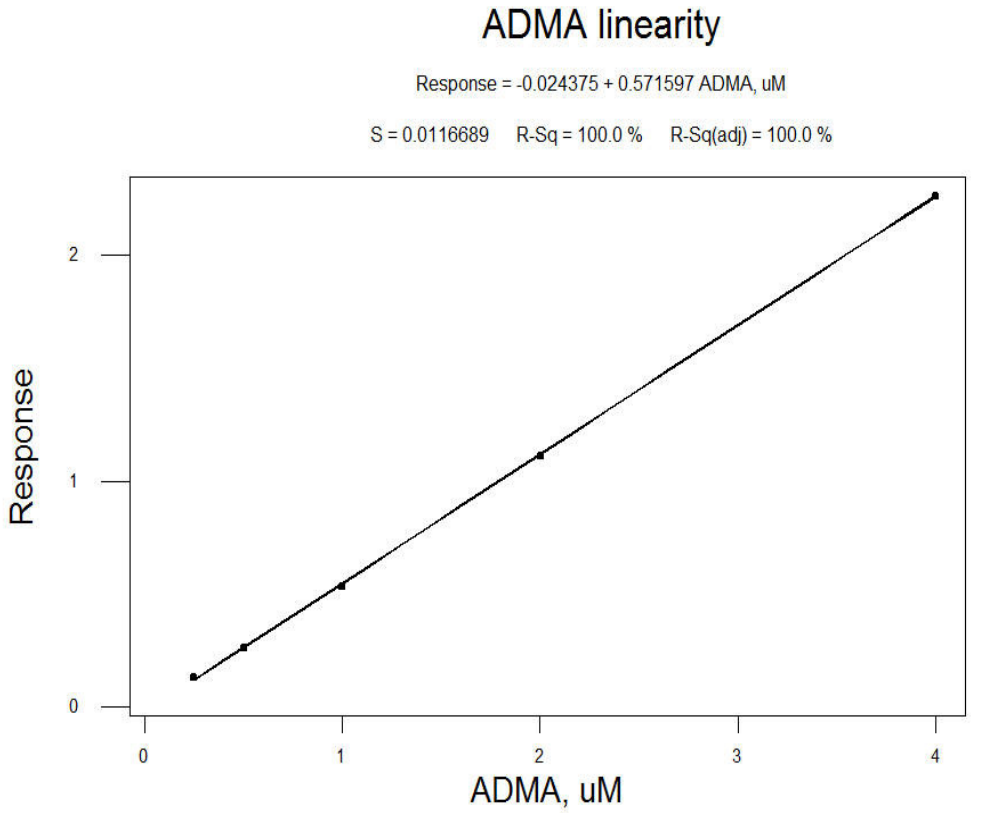
2.12 Analytical validation

2.12.1 Linearity

The detector responses were determined to be linear up to 200 μM for arginine, 10 μM for homoarginine and 4 μM for ADMA and SDMA under the conditions described. Calibration curves and linear regression equations for each of the 4 analytes are shown in figure 2.3. These were determined by preparing sequential dilutions from a stock standard and subjected to the full analytical procedure. The responses on the y axes represent the peak height ratios of analyte to internal standard. The correlation coefficients are > 0.998 for each analyte.




Homoarginine linearity
Response = -0.102400 + 0.705189 Homoarg, uM
S = 0.113743 R-Sq = 99.9 % R-Sq(adj) = 99.8 %
Response
Homoarg, uM
0
1
2
3
4
5
6
7
8
9
10


ADMA linearity
Response = -0.024375 + 0.571597 ADMA, uM
S = 0.0116689 R-Sq = 100.0 % R-Sq(adj) = 100.0 %
Response
ADMA, uM
0
1
2
3
4

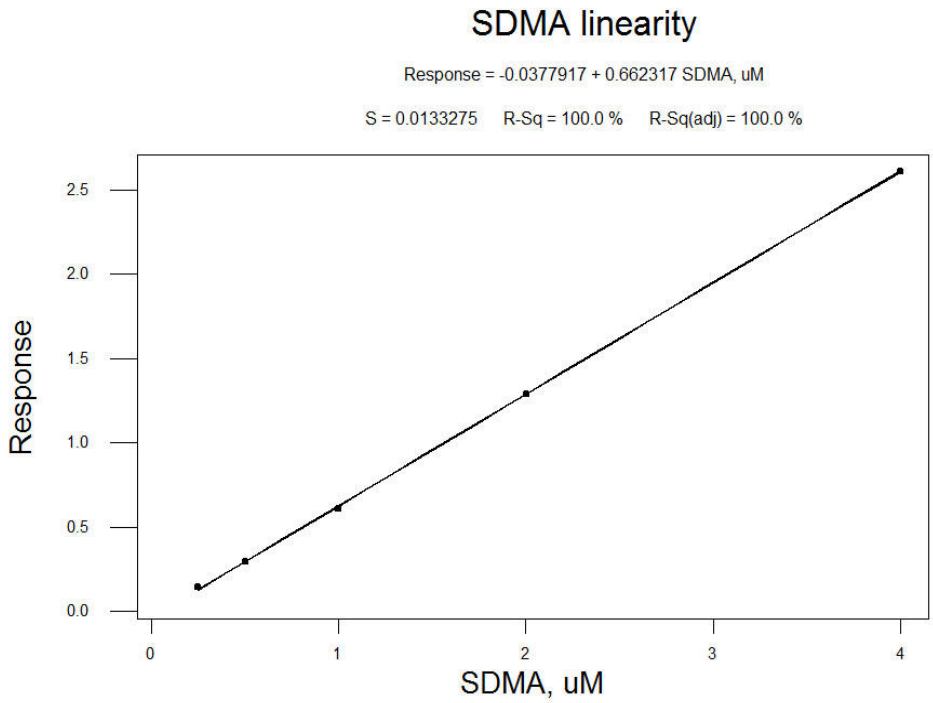


Figure 2.3: Calibration curves and regression equations for arginine, homoarginine, ADMA and SDMA

2.12.2 Limits of detection and quantification

Limits of detection were defined as 3 times the chromatographic baseline noise from a sample blank. The lower limit of quantification was defined as 10 times the signal-to-noise ratio. The results are shown in table 2.3. The lower limit of quantification is significantly lower than the concentrations commonly encountered in biological samples.

Analyte	Limit of detection, μmol/L	Limit of quantification, μmol/L
Arginine	0.01	0.04
Homoarginine	0.001	0.004
ADMA	0.001	0.004
SDMA	0.001	0.004

Table 2.3: Limits of detection and quantification

2.12.3 Recovery

Accuracy was assessed by determining the recovery of analytes from spiked plasma samples. Increasing concentrations of each analyte were added to aliquots of a plasma pool, and the recovery calculated following subtraction of the basal concentration. This experiment was performed in triplicate, and the mean (SD) results are shown in table 2.4. Recoveries were within the range 98.0 – 105.4% for arginine, ADMA and SDMA. For homoarginine, recoveries were within the range 92.1 – 93.5% and linear throughout the concentration range.

Analyte	Added amount µmol/L	Concentration measured Mean (SD), µmol/L	Calculated recovery Mean (SD), %
Arginine (n = 3)	0	79.5 (1.2)	
	50	133.9 (0.8)	103.4 (0.6)
	100	178.6 (0.3)	99.5 (0.2)
Homoarginine (n = 3)	0	1.57 (0.03)	
	2.00	3.46 (0.13)	93.4 (8.3)
	5.00	6.23 (0.08)	92.1 (1.8)
	10.00	10.97 (0.04)	93.5 (0.5)
ADMA (n = 3)	0	0.34 (0.02)	
	0.50	0.84 (0.03)	100.0 (6.1)
	1.00	1.35 (0.02)	100.7 (2.6)
	2.00	2.44 (0.01)	105.4 (0.6)
	4.00	4.46 (0.07)	103.6 (1.9)
SDMA (n = 3)	0	0.27 (0.02)	
	0.50	0.77 (0.03)	99.5 (4.2)
	1.00	1.26 (0.05)	99.0 (3.6)
	2.00	2.26 (0.06)	99.6 (2.5)
	4.00	4.19 (0.06)	98.0 (1.3)

Table 2.4: Recoveries from spiked plasma samples

2.12.4 Precision

2.12.4.1 Intra-assay variation

The intra-assay CV was determined by analysing 10 aliquots of a single plasma pool within a single batch and the results shown in table 2.5. Each aliquot was subjected to solid phase extraction, and so represents the whole analytical procedure.

	Arginine, μmol/L	Homoarg, μmol/L	ADMA, μmol/L	SDMA, μmol/L
1	57.2	1.79	0.44	0.31
2	59.6	1.84	0.45	0.32
3	59.8	1.85	0.45	0.32
4	59.7	1.80	0.44	0.31
5	57.2	1.76	0.45	0.31
6	56.6	1.76	0.43	0.31
7	58.6	1.78	0.43	0.31
8	57.4	1.79	0.44	0.32
9	58.7	1.77	0.44	0.31
10	56.4	1.76	0.43	0.31
Mean	58.1	1.79	0.44	0.31
SD	1.329	0.032	0.008	0.005
CV, %	**2.3**	**1.8**	**1.9**	**1.6**

Table 2.5: Intra-assay variation

2.12.4.2 Inter-assay variation

The in-house QC material was analysed in duplicate in each batch. 50 pairs of QC duplicates were analysed over approximately 1 year. Analytical variance (SD_A^2) was calculated from the difference between each pair of duplicates according to the formula:

$$SD_A^2 = \Sigma\, d^2/2N$$

where d is the difference between each duplicate pair and N is the number of duplicates. Inter-assay CV was calculated from this variance, and is shown in table 2.6.

Analyte	Mean concentration, μmol/L	Inter-assay CV, %
Arginine	37.3	**2.5**
Homoarginine	1.48	**2.9**
ADMA	0.36	**2.5**
SDMA	0.24	**3.2**

Table 2.6: Inter-assay variation

2.12.5 Comparison of internal standards

Comparison of the two potential internal standards was done by analysing plasma samples which had both MEA and MMA added as internal standards. Arginine, homoarginine, ADMA and SDMA results were then calculated using each of the internal standards and the results compared. Data for a group of 70 plasma samples is presented in table 2.7. It can be seen that there are no significant differences, and on linear regression, the correlation coefficients

are all greater than 0.975. These results suggest that either internal standard can be used without a compromise in accuracy or precision.

	Arginine		Homoarginine		ADMA		SDMA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MEA	60.4	22.3	1.93	0.77	0.46	0.08	0.38	0.08
MMA	59.6	21.6	1.90	0.74	0.46	0.08	0.38	0.07

Table 2.7: Comparison of MEA and MMA as internal standards

2.12.6 Stability of analytes in stored plasma samples

Aliquots of the QC material were analysed following 18 months of storage at -70˚C, with no significant differences seen in the measured concentrations from baseline, confirming stability under the described storage conditions. The mean (SD) values from 3 measurements 18 months apart are shown in table 2.8, with no significant differences seen between the two time points (p values all > 0.40).

	Arginine, μmol/L	Homoarg, μmol/L	ADMA, μmol/L	SDMA, μmol/L
Baseline	34 (1.5)	1.41 (0.01)	0.35 (0.01)	0.24 (0.01)
18 months	33 (1.2)	1.40 (0.08)	0.36 (0.02)	0.24 (0.02)

Table 2.8: Stability of analytes in stored plasma samples

2.13 Modification of method for measurements in urine

Minor adjustments were made in order to permit measurement of ADMA and SDMA in urine, where they are present in much higher concentrations, both in the basal state[221] and in certain pathological conditions where they can be even higher, e.g. liver disease[241]. The sample injection volume onto the analytical column was reduced to 12 μL, and the detector response was assessed to its limit of linearity. The net result was linearity up to 20 μM for both ADMA and SDMA. Figure 2.4 demonstrates this, with the y axes showing the detector response in mV.

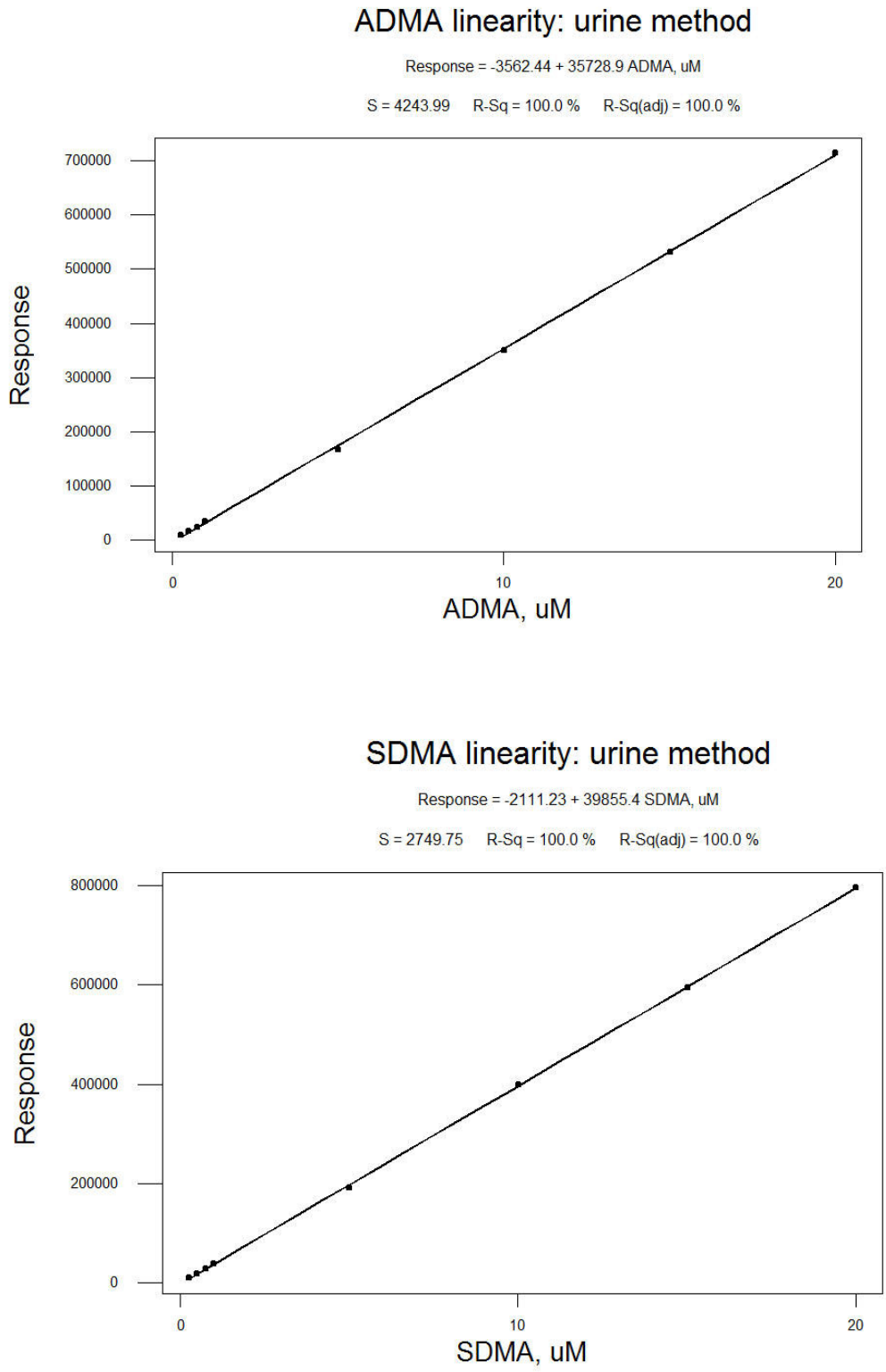


Figure 2.4: Calibration curves and regression equations for ADMA and SDMA in urine

The sample volume taken for solid phase extraction was reduced to 50 μL with results increased by a factor of 4, thereby effectively increasing the measurable range in urine samples to 80 μmol/L. This was felt to be adequate for the overwhelming majority of human urine samples, based on literature values[241]. Any samples above this range were analysed following dilution.

A recovery experiment was performed identically to that described in plasma earlier in the chapter. Urine was spiked with increasing amounts of ADMA and SDMA and the recovery calculated following subtraction of the basal concentration. This was done in triplicate and the mean (SD) results are shown in table 2.9. The recoveries were acceptable, ranging from 95.5 – 99.0% for ADMA and 97.2 – 99.7% for SDMA.

Analyte	Amount added μmol/L	Concentration measured Mean (SD), μmol/L	Calculated recovery Mean (SD), %
ADMA (n = 3)	0	9.10 (0.30)	
	5	13.88 (0.13)	95.5 (2.5)
	10	19.00 (0.01)	99.0 (0.1)
SDMA (n=3)	0	7.44 (0.26)	
	5	12.30 (0.24)	97.2 (4.7)
	10	17.41 (0.06)	99.7 (0.6)

Table 2.9: Recoveries from spiked urine samples

Chapter 3

Reference intervals for dimethylarginines

3.1 Materials and methods

3.1.1 Selection of subjects

Ostensibly healthy volunteers from the laboratory staff were selected to determine reference intervals for arginine, homoarginine, ADMA and SDMA for the current analytical method. Exclusion criteria were smoking and clinical history of a condition known to affect ADMA concentrations, in particular cardiovascular and renal disease. Each individual was informed of the purpose and procedure of the study and the exclusion criteria; individuals who wished to participate and who didn't exclude themselves after being informed of the exclusion criteria provided consent for blood samples to be taken as detailed below. Increased weight, or body mass index (BMI) was not specified as an exclusion criterion, as an independent effect of BMI on ADMA concentrations has not been shown. 100 subjects were recruited (51 female, 49 male), with a mean (range) age of 43 (23 – 65) years.

3.1.2 Specimen collection and handling

Blood was taken by standard venepuncture into tubes containing heparin as an anticoagulant, and the plasma removed following centrifugation and stored at -70˚C until analysis. At the same time a sample was taken into a serum separator tube (SST) for the analysis of serum lipids (total cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol), C reactive protein

(CRP), creatinine and alanine aminotransferase (ALT). This was to exclude subjects with significant dyslipidaemias, active inflammation or renal impairment, all factors which can affect serum dimethylarginine concentrations. Blood sampling was done under non-fasting conditions, with all specimens collected during the early morning after breakfast. The implications of this are discussed in chapter 12.2.

3.1.3 Biochemical analysis

Arginine, homoarginine, ADMA and SDMA concentrations were calculated using the method described in chapter 2. Serum lipids, CRP, creatinine and ALT were analysed on a multi-channel autoanalyser in the routine biochemistry laboratory, using methods described briefly in chapter 7. Low density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation (total cholesterol – HDL – (triglyceride/2.2)). Estimated glomerular filtration rate (eGFR) was calculated using the standard 4 variable modification of diet in renal disease (MDRD) formula, as appropriate to the creatinine method in use in NHS Greater Glasgow and Clyde laboratories[242]:

$$eGFR\ (ml/min/1.73m^2) = 175 \times (Creatinine/88.6)^{-1.154} \times (Age)^{-0.203} \times 0.742 \text{ if female}$$

3.1.4 Statistical methods

The data for arginine, homoarginine, ADMA and SDMA were examined for normality using the Kolmogorov Smirnov test. Non-normally distributed data were logarithmically transformed. Means and 95% reference intervals were constructed from the transformed data. Correlations between ADMA and other parameters was done using Spearman rank correlation where variables

were non-normally distributed. Differences between age and gender stratified groups were examined using the Mann Whitney U test.

3.2 Results

3.2.1 Population distribution

Biochemical data for all of the subjects examined can be seen in appendix 2. The means, medians and SDs from the raw data are presented in table 3.1.

Analyte	Mean, μmol/L	Median, μmol/L	SD, μmol/L
Arginine	68.5	65.7	26.4
Homoarginine	1.99	1.86	0.84
ADMA	0.46	0.45	0.084
SDMA	0.39	0.38	0.077

Table 3.1: Means, medians and SDs of reference group data (n = 100)

The reference values for all four analytes in table 3.1 were found to be non-normally distributed. Therefore logarithmic transformation was employed to normalise the distributions. Probability plots and histograms of the logarithmically transformed data are shown in figures 3.1 (a) – (d).

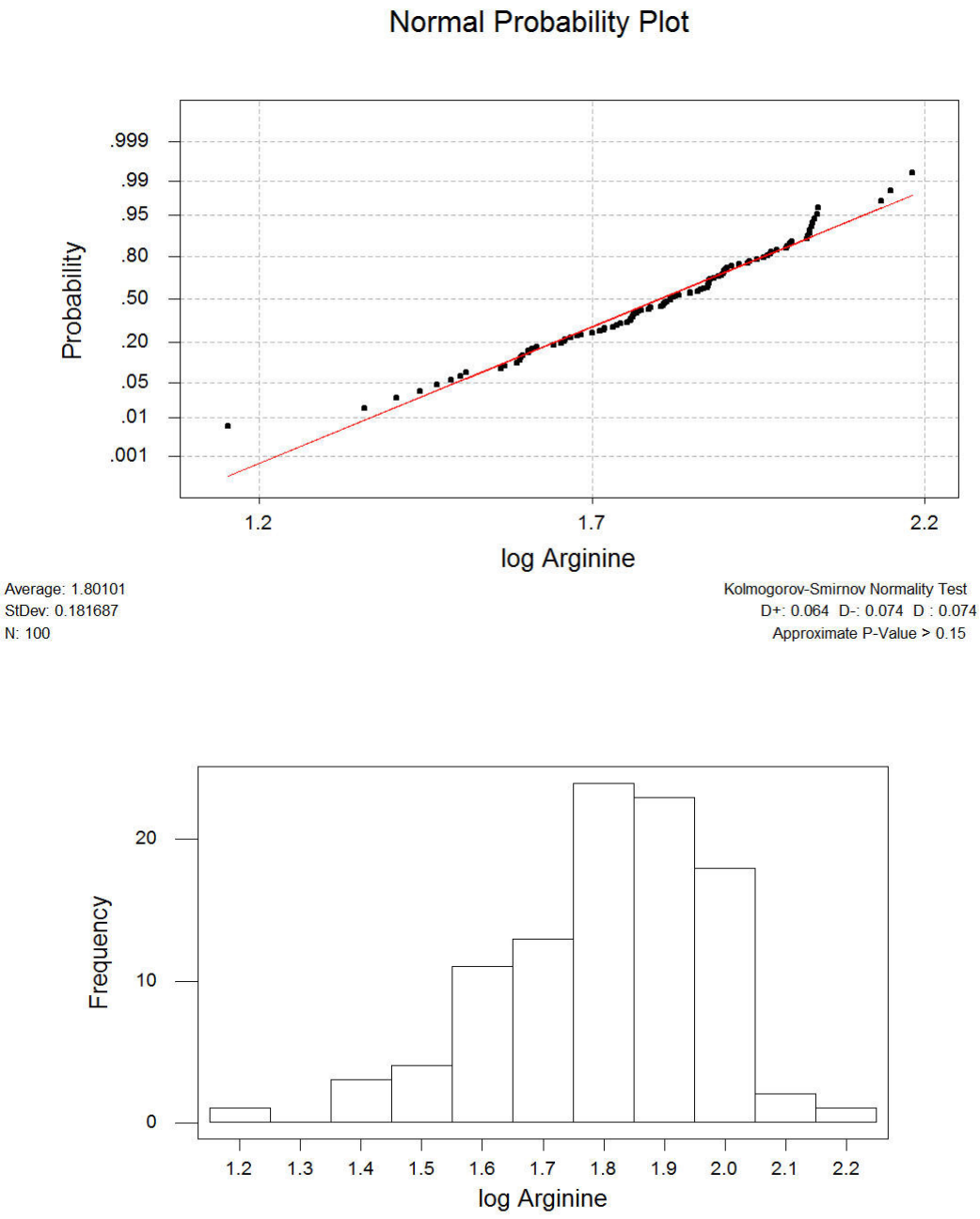


Figure 3.1(a) Probability plot and histogram of reference values for arginine following logarithmic transformation

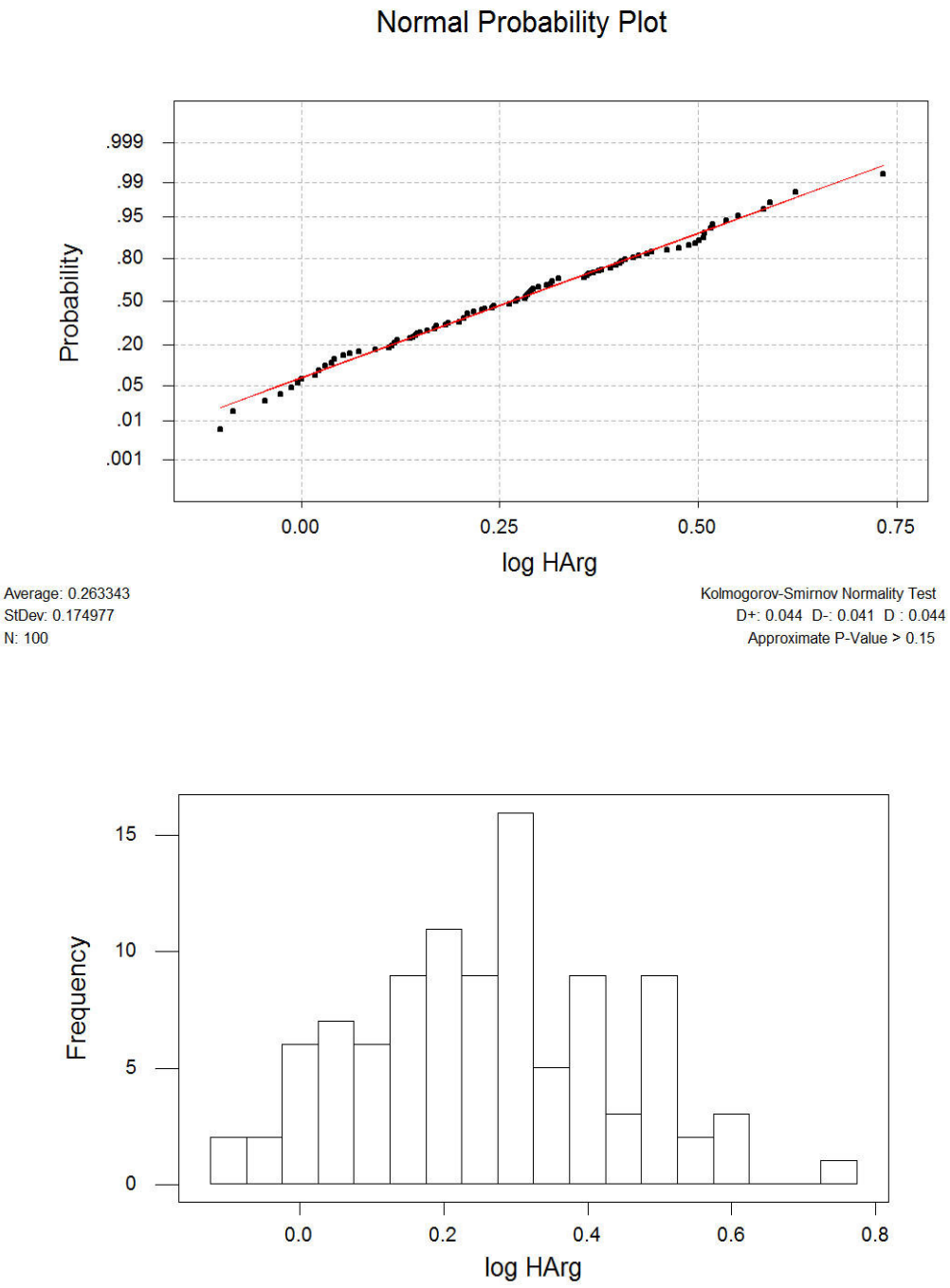


Figure 3.1(b) Probability plot and histogram of reference values for homoarginine following logarithmic transformation

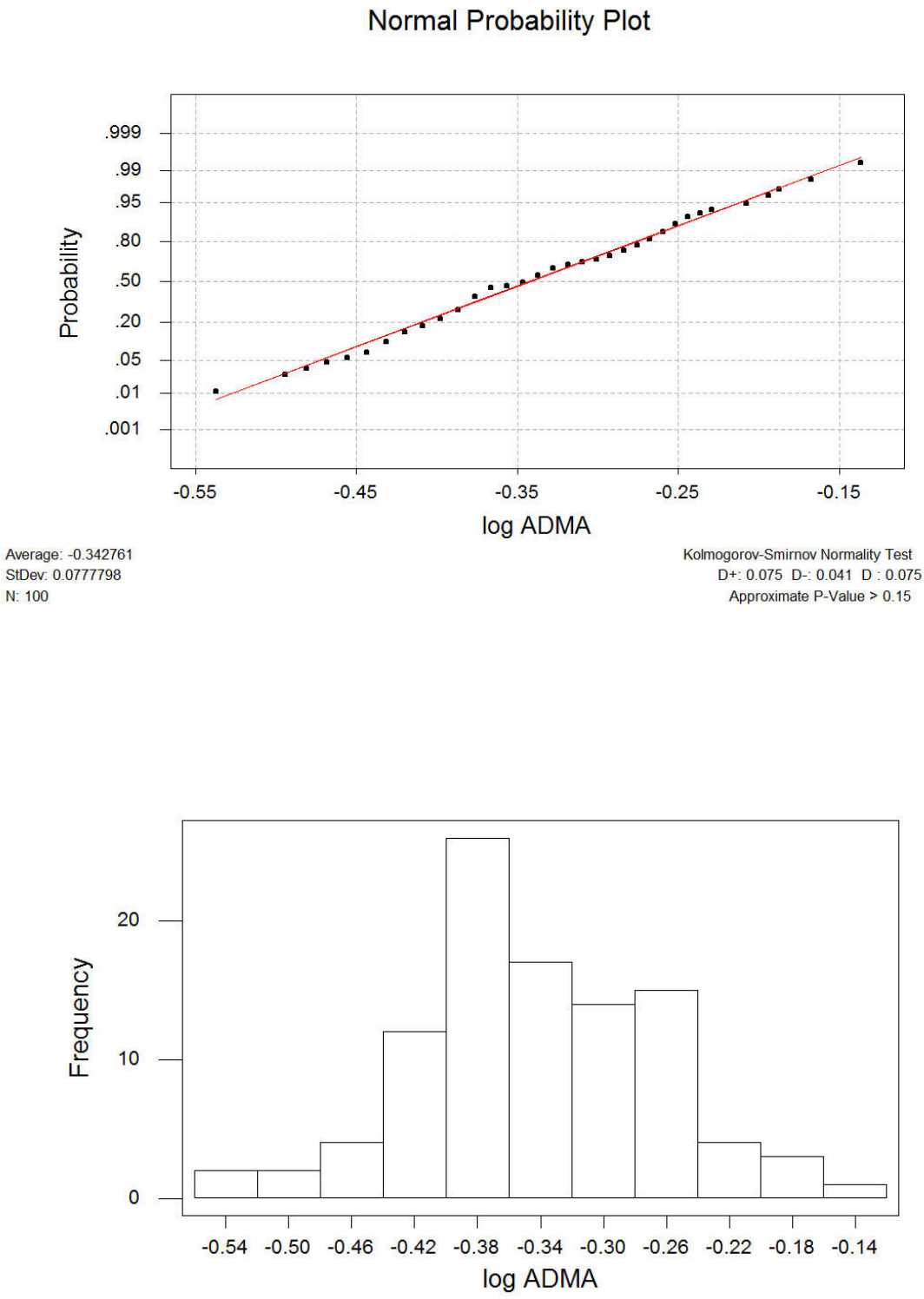


Figure 3.1(c) Probability plot and histogram of reference values for ADMA following logarithmic transformation

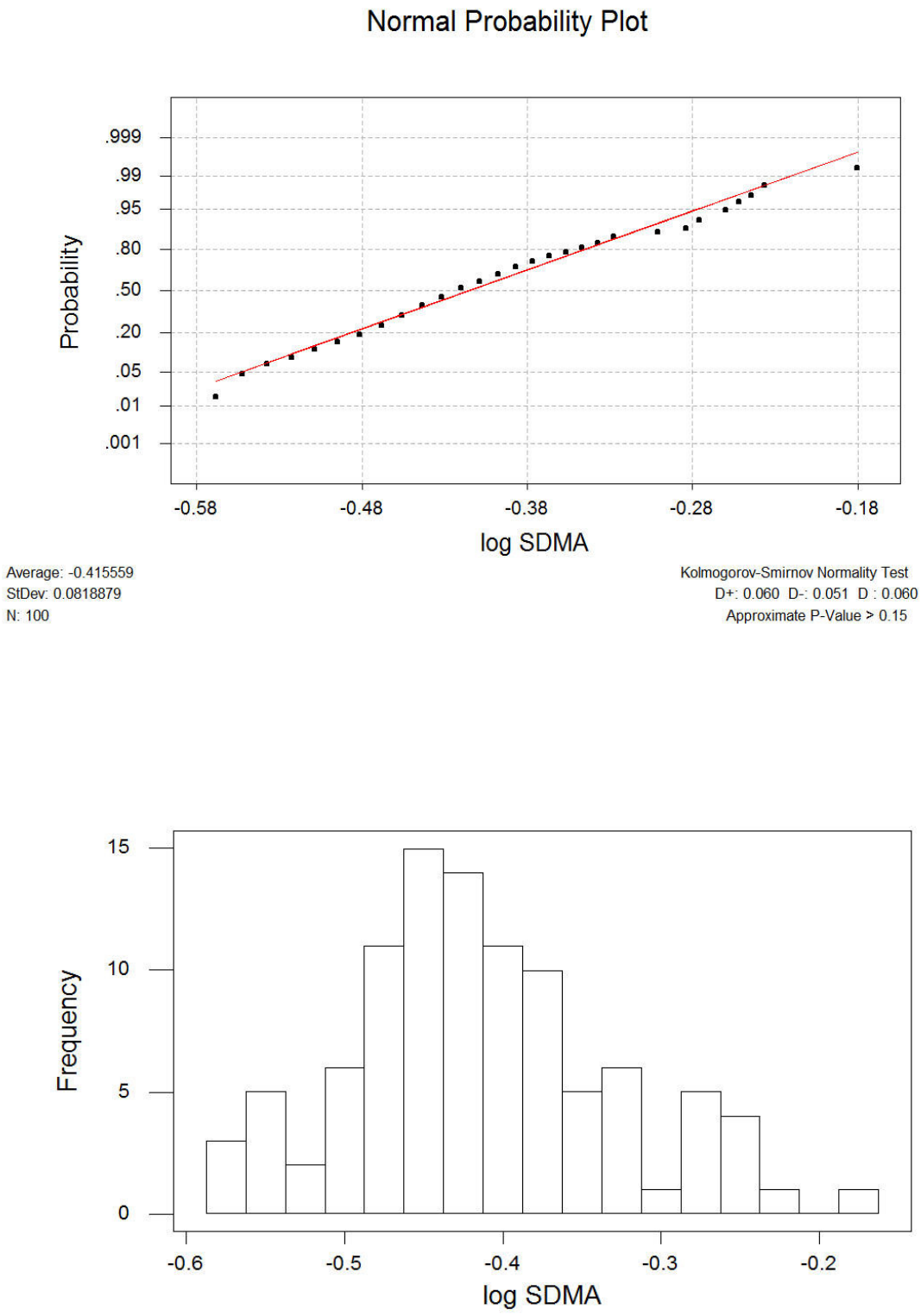


Figure 3.1(d) Probability plot and histogram of reference values for SDMA following logarithmic transformation

3.2.2 Calculation of reference intervals

95% reference intervals were derived conventionally by defining lower and upper limits 2 SDs either side of the logarithmically transformed means. The resultant reference intervals are shown in table 3.2

Analyte	Mean, μmol/L	95% reference interval, μmol/L
Arginine	63	27 – 148
Homoarginine	1.83	0.82 – 4.09
ADMA	0.45	0.32 – 0.65
SDMA	0.38	0.26 – 0.56

Table 3.2: 95% reference intervals following logarithmic transformation

3.2.3 Age and gender influences

3.2.3.1 Comparison of gender stratified groups

The data for the four analytes in question was divided according to gender, and comparisons made between gender groups. The median arginine concentration was higher in men compared with women, but no other gender differences were seen. Table 3.3 shows the median concentrations and p values for inter-group comparisons.

	Arginine	Homoarginine	ADMA	SDMA
Male, μmol/L	75 (53 – 93)	1.86 (1.39 – 2.47)	0.42 (0.38 – 0.52)	0.40 (0.34 – 0.44)
Female, μmol/L	62 (48 – 79)	1.87 (1.37 – 2.49)	0.45 (0.41 – 0.52)	0.37 (0.33 – 0.41)
p value	0.03	0.93	0.26	0.22

Table 3.3: Median (inter-quartile range) values and p values for comparisons of reference group stratified by gender

3.2.3.2 Associations with age

No significant correlations were seen between ADMA and SMDA concentrations and age, both displaying Spearman rank correlations of 0.14 and 0.18 respectively. The female group was divided according to age into two groups of < 50 years and ≥ 50 years in order to investigate the effect of menopausal status. The age cut off of 50 years was selected as it represents the typical age of menopausal transition. No significant differences were seen between the groups ($p = 0.35$).

3.2.4 Correlations of dimethylarginines with other variables

SDMA and ADMA were strongly correlated ($r = 0.53$, $p < 0.001$). There was a borderline weak correlation between SDMA and serum creatinine ($r = 0.19$, $p = 0.08$). These are shown in figures 3.2 and 3.3 respectively. The correlation between SDMA and creatinine was weaker than reported in other studies, but the range of creatinine values encountered was somewhat narrower, which probably accounts for the apparently lesser strength of the association. No significant correlation was seen between SDMA and eGFR ($r = -0.10$, $p = 0.36$), although any potential association between SDMA and renal function could

have been undermined by the use of non-fasting samples which can affect serum creatinine concentrations[243], and the range of encountered eGFRs which are overall in the range where the MDRD equation is known to perform less well[242] (mean eGFR in reference group 78 ml/min/1.73m^2).

No correlations were seen between SDMA and other variables. ADMA demonstrated no correlations with total cholesterol ($r = -0.11$, $p = 0.31$), LDL cholesterol ($r = -0.116$, $p = 0.28$), serum creatinine ($r = -0.10$, $p = 0.334$) concentrations, or eGFR ($r = 0.11$, $p = 0.31$).

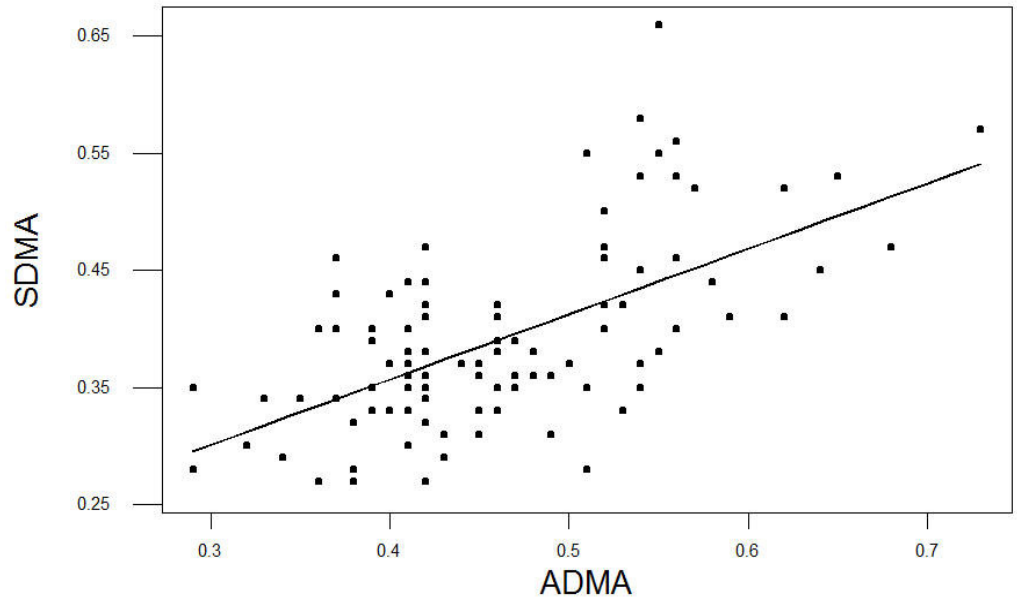


Figure 3.2: Relationship between ADMA and SDMA concentrations in reference group

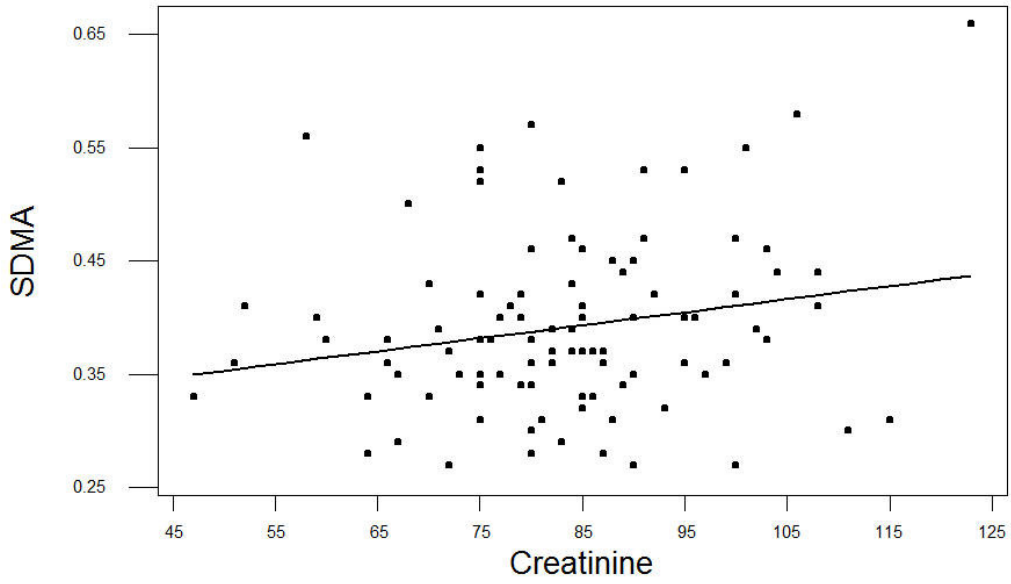


Figure 3.3: Relationship between creatinine and SDMA concentrations in reference group

Chapter 4

Biological variation of dimethylarginines

4.1 Biological variation and its utility

4.1.1 Background to biological variation

Any analyte measured repeatedly will demonstrate inherent variation in the serial results obtained. Broadly speaking this variation consists of two main sources: that related to random variation in the assay, and that which is inherent in the individual and can be considered as random around a homeostatic set point. This is termed the intra-individual biological variation (CV_I). Homeostatic set points differ between individuals, and this is termed the inter-individual variation (CV_G)[244]. A parameter which is homeostatically important, such as blood pH or extracellular potassium concentration, would be expected to have a rather narrow intra-individual variation owing to the action of regulatory mechanisms, while a parameter not subject in itself to regulation, such as urine osmolality, would display a much wider variation[244,245]. Certain analytes have predictable cyclical rhythms which might be diurnal, e.g. serum cortisol, monthly, e.g. female reproductive hormones or seasonal, e.g. vitamin D. Additionally some analytes exhibit changes according to age, or at certain times of life such as puberty, pregnancy or menopause. As has been discussed in the introduction, ADMA has been shown to increase, although by a small absolute amount, with increasing age and after the menopause. However, little is known about its biological variation, although such data is required in order to assess its biological significance, determine the likely significance of changes in serial results, and define quality specifications for analytical methods[244].

In order to determine the components of biological variation, repeated specimens are taken from a group of individuals under standardised conditions. In general it is advised to select a small number of reference subjects who are free from disease or medication known to affect the analyte being measured; they should be sampled under standardised conditions, and samples analysed within a single analytical batch in order to minimise the effect of pre-analytical and analytical variation[230,232].

4.1.2 Reference change value

Biological variation data are also used to determine the reference change value (RCV). Laboratory tests are often measured repeatedly, with changes between measurements used to monitor such factors as disease progression or response to treatment, both in clinical practice and in studies. Since some variation will be expected from biological variation, the RCV is useful in describing the magnitude of difference required to be confident of a biologically significant change at a certain level of statistical significance[232,244].

4.1.3 Index of individuality and the utility of reference intervals

Reference intervals are often regarded as being equivalent to "normal ranges", although clearly there will be, sometimes considerable, overlap between healthy and diseased individuals. Many analytes measured in clinical laboratories exhibit a CV_I which is much less than the CV_G, a prime example of this being serum creatinine[244-246]. In practice this means that the majority of individuals have a range of "normal" values which are contained within a fraction of the overall reference interval, be this in the middle of the range or at either extreme. An individual could, therefore, have a result which is

highly unusual for them but still within the overall reference interval[246]. The index of individuality (II) is derived from the components of biological variation and describes exactly this "biological individuality", and is used to assess the utility of traditionally-defined reference intervals in denoting abnormality in a single measurement on a single occasion[246]. Essentially it is the ratio of CV_I to CV_G. A ratio of > 1.4 generally means that a population derived reference interval can be used to denote "abnormality" in an individual with a reasonable degree of likelihood. In contrast, a ratio < 0.6 indicates that an isolated result has limited diagnostic utility when compared with a reference interval[246].

4.1.4 Analytical performance goals

Performance goals for analytical methods are best based on biological variation[230,231]. These goals are designed to ensure that, at acceptable levels of performance, variation from analytical "noise" does not obscure clinical "signal", and cover both random error, i.e. imprecision, and systematic error, i.e. bias. A desirable goal for imprecision has been set as no more than 0.5 times CV_I, as at this level the impact of analytical variation should be minimal[230,231].

4.2 Methods

4.2.1 Specimen collection and handling

Twelve ostensibly healthy individuals, members of the biochemistry laboratory staff at Glasgow Royal Infirmary, were recruited (6 men, 6 women, age range 20 – 53 years). None had any disease known to affect ADMA

concentrations, and none were on regular medication. All were non smokers, and none consumed alcohol in excess of recommended safe levels. All maintained their usual lifestyles for the duration of the study.

Once per week for 20 weeks blood was collected by conventional venepuncture following an overnight fast. These were collected between 09:00 and 10:00 in the morning with subjects in a sitting position and avoiding venous stasis. Blood was collected into tubes containing heparin as an anticoagulant, centrifuged (500 g, 4˚C, 10 minutes), and the plasma transferred into plastic tubes which were stored at -70˚C until analysis.

Plasma arginine, homoarginine, ADMA and SDMA were measured using the method described in chapter 2. As there were 20 samples from each individual, they had to undergo solid phase extraction in 2 lots, although all were analysed in a single analytical run. The analyses were performed by a single analyst, with a single lot of reagents, QC and calibration standards, in order to minimise analytical variation.

4.2.2 Statistical methods

Statistical analysis was carried out using Minitab statistical software (release 13). Data were examined for normality using the Anderson Darling test, and outliers defined as data points exceeding 3 SD from the mean.

4.2.2.1 Determination of the components of biological variation

For each analyte, the total intra-individual variance was calculated from the data for each participant and transformed into the total intra-individual CV

(CV_{TI}) using the homeostatic mean from each participant. CV_{TI} consists of both biological and analytical components, therefore the intra-individual variation was calculated by subtraction using the general formula:

$$CV_I = (CV_{TI}^2 - CV_A^2)^{1/2}$$

The intra-assay CV, as described in section 2.12.4.1 was used as the CV_A for this calculation, given that samples for a single individual were analysed in a single batch.

The inter-individual variance reflects the difference between means of individuals. It was determined by use of all the individual data points and transformed into the total inter-individual CV (CV_T) by use of the overall mean. The CV_I and CV_A were subtracted from this to give the CV_G, according to the formula:

$$CV_G = (CV_T^2 - CV_I^2 - CV_A^2)^{1/2}$$

4.2.2.2Determination of reference change value and index of individuality

The RCV was calculated with a view toward providing information about significant change at the 95% confidence level. It was thus calculated as follows[232]:

$$RCV = 2.77 \times CV_{TI}$$

The II is simply the ratio of intra and inter-individual variances, as was calculated according to the formula[246]:

$$II = CV_{TI} / CV_G$$

4.2.2.3Determination of analytical performance goals

The desirable goal for imprecision (CV_A)[230,231] was calculated as $\leq 0.5 \times CV_I$.

The limiting goal for bias (B) is one fourth of the group biological variation which is itself made up of intra and inter-individual components[230,231]. The goal was defined as:

$B < 0.25 \times (CV_I^2 + CV_G^2)^{1/2}$

The resultant goal for total error (TE)[230,231] is defined as $< (kI + B)$, where $k = 1.65$ at $\alpha = 0.02$.

4.3 Results

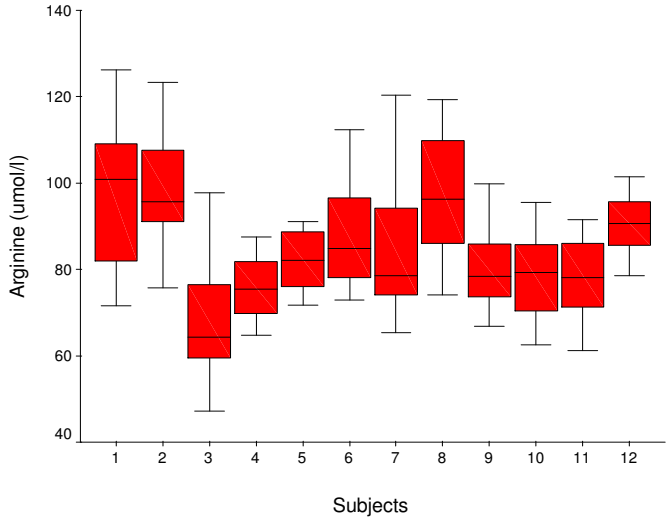
4.3.1 Intra- and inter-individual biological variation

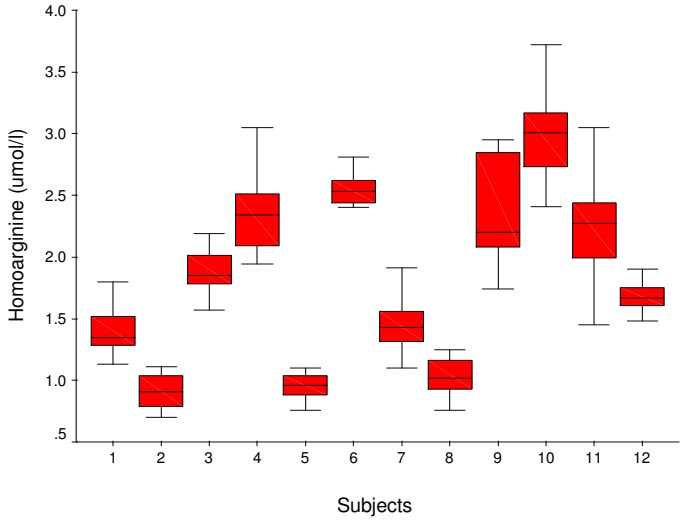
The data for arginine, homoarginine, ADMA and SDMA were all found to be normally distributed in each individual. Two outlying data points were excluded. For each individual participant the mean analyte concentrations, SD and resultant CV_I are shown in appendix 2. The mean CV_I values are shown in table 4.1. As can be seen, ADMA and SMDA exhibit tight regulation in human plasma, with values of 7.4% and 5.8% respectively. Arginine and homoarginine are less tightly controlled, though are much less variable than many commonly measured analytes[245].

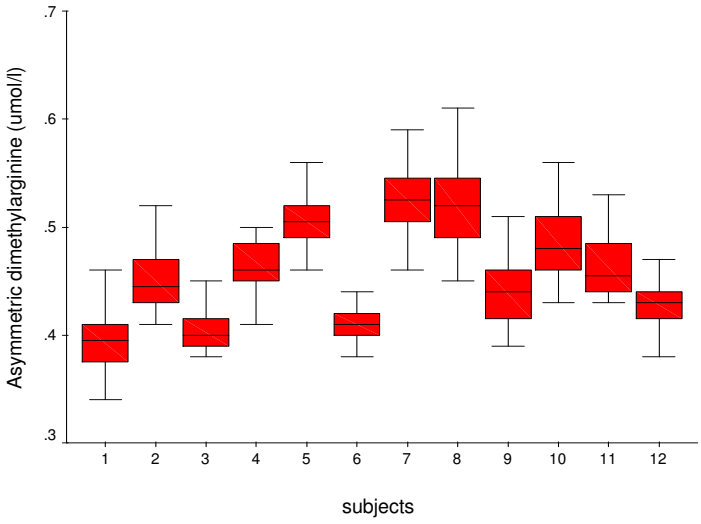
Analyte	Intra-individual variation CV_I, %	Inter-individual variation CV_G, %
Arginine	16.1	13.4
Homoarginine	14.4	37.8
ADMA	7.4	9.6
SDMA	5.8	14.7

Table 4.1: Biological variation of arginine, homoarginine, ADMA and SDMA

The CV_G values are also shown in table 4.1. For ADMA and SDMA the results of 9.6% and 14.7% respectively suggest a spread in the population which is not excessively high, as has been suggested by the data from the various clinical studies examined in chapter 1. However for SDMA in particular it is striking that CV_I is much less than CV_G (5.8% vs 14.7%), suggesting a high degree of biological individuality, analogous to serum creatinine. This can be appreciated visually by examining figure 4.1, which contains box plots depicting the range of values encountered in each individual studied. SDMA, ADMA and homoarginine show this individuality visually, with the individual ranges for arginine demonstrating much more overlap.


Arginine (umol/l)
140
120
100
80
60
40
1
2
3
4
5
6
7
8
9
10
11
12
Subjects


Homoarginine (umol/l)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
1
2
3
4
5
6
7
8
9
10
11
12
Subjects


Asymmetric dimethylarginine (umol/l)
.7
.6
.5
.4
.3
1
2
3
4
5
6
7
8
9
10
11
12
subjects

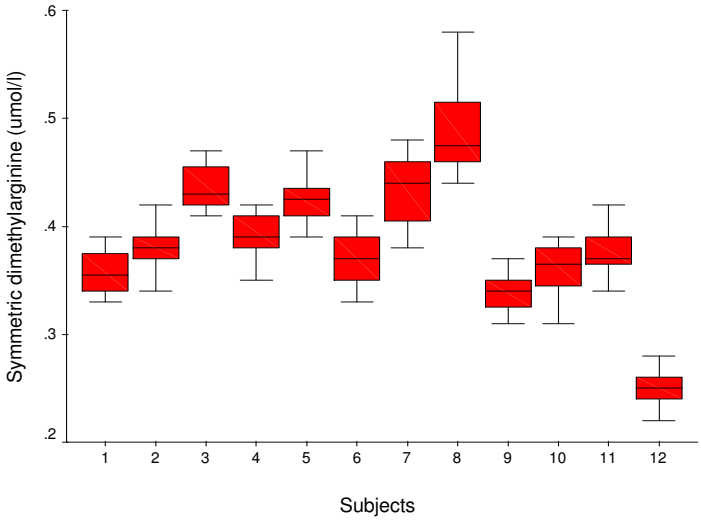


Figure 4.1: Box plots of arginine, homoarginine, ADMA and SDMA concentrations in biological variation study

4.3.2 Reference change value and index of individuality

The RCVs and IIs are shown in table 4.2. The RCVs for ADMA and SDMA are around 20%, indicating that a change of at least this magnitude is required between serial samples to be confident of biological significance. The IIs were < 1.4 for all analytes.

Analyte	Reference change value RCV, %	Index of Individuality II
Arginine	45.7	1.23
Homoarginine	41.0	0.39
ADMA	21.6	0.81
SDMA	19.1	0.47

Table 4.2: Reference change values and indices of individuality

4.3.3 Analytical performance goals

Performance goals for imprecision, bias and total error are shown in table 4.3. The low CV_I of ADMA and SDMA imposes fairly tight imprecision goals, which numerous methods described in the literature fail to meet. The right-hand column gives the performance of the current analytical method as determined in the method validation outlined in chapter 2. This suggests satisfactory performance.

Analyte	Desirable specification			Current method
	Imprecision, %	Bias, %	Total error, %	CV_A, %
Arginine	8.0	5.2	10.3	2.5
Homoarginine	7.2	10.1	16.0	2.9
ADMA	3.7	3.1	7.2	2.5
SDMA	2.9	3.9	10.0	3.2

Table 4.3: Analytical performance goals

4.4 Summary and conclusions

The biological variation of ADMA and SDMA in human plasma is low, suggesting fairly tight regulation. This imposes strict analytical performance goals, something which many methods fail to meet. The indices of individuality of < 1.4 suggest that reference intervals are of limited utility in denoting abnormality from a single result. This is likely to hamper efforts to incorporate ADMA into risk scoring systems or as a diagnostic marker.

Chapter 5

Measurement of dimethylamine in urine using HPLC

5.1 Background and justification

Dimethylamine (DMA) is a short chain aliphatic amine derived from the DDAH-mediated catabolism of ADMA, and is excreted in the urine[247]. It was first identified in human urine several decades ago, and has endogenous and exogenous sources[248]. Theoretically its excretion rate could provide a surrogate measure of overall DDAH activity, provided that two conditions are met: first, that once produced DMA is excreted entirely, or largely, unchanged in urine; and, second, that exogenous, that is dietary, sources of DMA can either be minimised or eliminated to prevent excessive background "noise" obscuring the variation attributable to changes in endogenous production. On the first of these conditions, an experiment has shown that ingested DMA is excreted rapidly into the urine unchanged: 87% of an oral dose of DMA was excreted into the urine during the first 24 hours in healthy male volunteers, with only a small amount (5%) further metabolised[249]. The elimination half-life from plasma was estimated to be 6 – 7 hours[249]. Moreover, as little as 2 hours following IV injection of ADMA, a significant increase in urinary DMA is seen[8]. On the second condition, the dietary sources of DMA have been well characterised. In a study of dietary sources, no foods from the fruit and vegetable, meat, dairy or grain groups produced any significant increases in DMA excretion following ingestion[250]. On the contrary, most fish and shellfish produced significant increases in its excretion, especially squid, coley, whiting, cod and sardines, and appears to be related to the breakdown of trimethylamine N oxide within the fish after being caught[250]. It appears that by restricting access to this specific food group, major fluctuations from diet could be avoided.

The excretion of DMA in human urine has been estimated at around 140 to 400 μmol/day[8,247,251], with a urinary ratio of DMA to ADMA of around 10. The concentrations of DMA and ADMA in urine are significantly correlated (R = 0.84), further suggesting the validity of extrapolating DMA to ADMA catabolism[251], and has a relatively low variation on a stable diet. In patients with coronary disease, urinary excretions of ADMA and DMA are both increased, with an increase in the DMA:ADMA ratio, but retention of their positive correlation[251]. In view of these facts urinary DMA excretion was measured as an indicator of overall DDAH activity.

Published methods for DMA include GC-MS/MS[247,251] and HPLC[252] . The described GC-MS/MS requires pre-analytical sample extraction using toluene and derivatisation to produce pentafluorobenzamide derivatives[251]. Using HPLC, DMA was converted to a stable fluorescent derivative using 9-fluorenylmethylchloroformate (FMOC) which permits reliable and sensitive measurement by fluorescence detection[252]. The HPLC method has the advantage of simpler sample preparation, but requires a heating step to eliminate a prominent interfering peak related to the derivatising agent itself. Both methods demonstrate high sensitivity and acceptable precision, with inter-assay CVs of around 5% or less[251,252]. In the current study, a method was developed using HPLC, taking the method of Teerlink[252] as a starting point, with modifications to allow greater simplicity. The remainder of this chapter describes its development and analytical validation.

5.2 Specimen collection and pre-analytical handling

Urine was collected into plain specimen containers and the samples immediately frozen to ensure stability of DMA. Samples were stored at -70˚C and thawed immediately prior to analysis.

5.3 Reagents and buffers

5.3.1 List of chemicals

Dimethylamine (BDH chemicals, VWR International Ltd, Leicestershire)

Trichloroacetic acid (Sigma chemical company, Poole, UK)

Boric acid (BDH chemicals, VWR International Ltd, Leicestershire)

9-fluorenylmethylchloroformate (FMOC) (Sigma chemical company, Poole, UK)

Glycine (Sigma chemical company, Poole, UK)

Potassium dihydrogen phosphate (BDH chemicals, VWR International Ltd, Leicestershire)

Methanol, HPLC grade (Rathburn chemicals Ltd, Walkerburn, Scotland)

Ethanol, HPLC grade (Rathburn chemicals Ltd, Walkerburn, Scotland)

5.3.2 Preparation of buffers and reagents

5.3.2.1 10% trichloroacetic acid (TCA)

20 g TCA were dissolved in 200 ml deionised water.

5.3.2.2 0.8 M borate buffer

9.92 g boric acid were dissolved in 200 ml deionised water, and the pH adjusted to 9.5 using 2 M potassium hydroxide.

5.3.2.3 Derivatising agent

A 10 mM solution of the derivatising agent was prepared by dissolving 26 mg FMOC in 10 mls acetonitrile. This was prepared freshly prior to each analytical run.

5.3.2.4 100 mM glycine

75 mg glycine were dissolved in 10 ml deionised water.

5.3.2.5 Mobile phase: 50 mM phosphate buffer, 60% v/v methanol, 10% v/v ethanol

A 50 mM phosphate buffer was prepared by dissolving 0.5 g potassium dihydrogen phosphate in 150 ml deionised water, and the pH adjusted to 6.5 using 2 M potassium hydroxide. To this was added 300 ml methanol and 50 ml ethanol. The mobile phase was filtered through a 0.45 μm nylon filter and degassed prior to use.

5.4 Standards

A 10 mM stock DMA standard was prepared in deionised water and stored at -70˚C. From this a 250 μM working standard was prepared for use in each analytical run. The standard was determined to be stable under these conditions by comparing peak heights on chromatography.

5.5 Internal quality control material

Urine obtained from a single volunteer was used as the QC material. This was stored in aliquots at -70˚C until use, and analysed in duplicate within each analytical run in order to generate imprecision data.

5.6 Specimen preparation and derivatisation

300 μL of sample, standard or QC were mixed with 200 μL of 10% TCA, the acidification stabilising the volatile DMA in solution and allowing precipitation of any protein in the samples. Following centrifugation (1000 rpm, 10 minutes), 200 μL of the supernatant were mixed thoroughly with 600 μL 0.8 M borate buffer. 400 μL of this mixture were then added to 300 μL of the derivatising agent, and the reaction allowed to proceed for 1 minute. The excess derivatising agent was then neutralised by the addition of 100 μL 100 mM glycine. The resulting solution was transferred into a glass tube with a screw fitting cap and placed on a heating block at 80˚C for 10 minutes. The heating step was employed to eliminate a peak which eluted on chromatography very close to the DMA peak. This problem was previously described by Teerlink[252], who demonstrated complete removal of the interfering peak with no effect on the DMA peak. The same experience was obtained in the present study. Following heating samples were diluted 1:10 in the mobile phase and transferred into autosampler vials ready for analysis.

5.7 Chromatography

5.7.1 Chromatographic system and setup

The chromatographic system consisted of a solvent delivery system, programmable autosampler and fluorimeter (Waters, Watford, UK). 10 μL of the derivatised sample was injected by the programmable autosampler onto the column for chromatography. Excitation and emission wavelengths were 260 nm and 320 nm respectively, the optimal wavelengths for FMOC derivatives. At 10 minutes the sensitivity was increased by adjusting the EUFS (emission units full scale) from 32,000 to 4,000. The low detector sensitivity at the start of the analytical run was selected as two large peaks elute near the start, namely the hydrolysis product of the derivatising agent, FMOC-OH and the FMOC-glycine derivative[252]. Following their elution, the detector sensitivity was increased to allow reliable quantification of DMA within the linear part of the detector response. Signals from the detector were captured by a data management system (Millennium 2010, Waters, Watford, UK).

5.7.2 Analytical column

The analytical column was a Luna C18, 5 μm, 4.6 x 250 mm, protected by a 3 x 4 mm C18 guard column. The ambient temperature in the laboratory was maintained at a constant level in order to ensure stability of retention times.

5.7.3 Mobile phase

The mobile phase, prepared as described, was maintained at room temperature and pumped through the analytical column at a flow rate of 1.0 ml/minute. The mobile phase was recycled in order to allow a larger number of samples to be analysed in a single run without having to prepare overly large volumes of the mobile phase. This was not found to cause any problem with interfering peaks, even in large runs.

5.8 Calculation of results

Quantification was done by the method of external standardisation on the basis of peak heights as the DMA peak was completely resolved at baseline and the peak consistently sharp and well defined. DMA concentration was calculated by dividing the peak height of analyte in the (unknown) sample by that in the calibration standard and multiplying by the known concentration in the standard. A single level calibration was selected owing to the linearity of DMA over a wide concentration range, as demonstrated in the section on analytical validation.

5.9 Resultant chromatographic profiles

The peak corresponding to DMA was completely resolved from all other peaks. Chromatographic profiles corresponding to an aqueous standard and human urine sample are shown in figure 5.1.

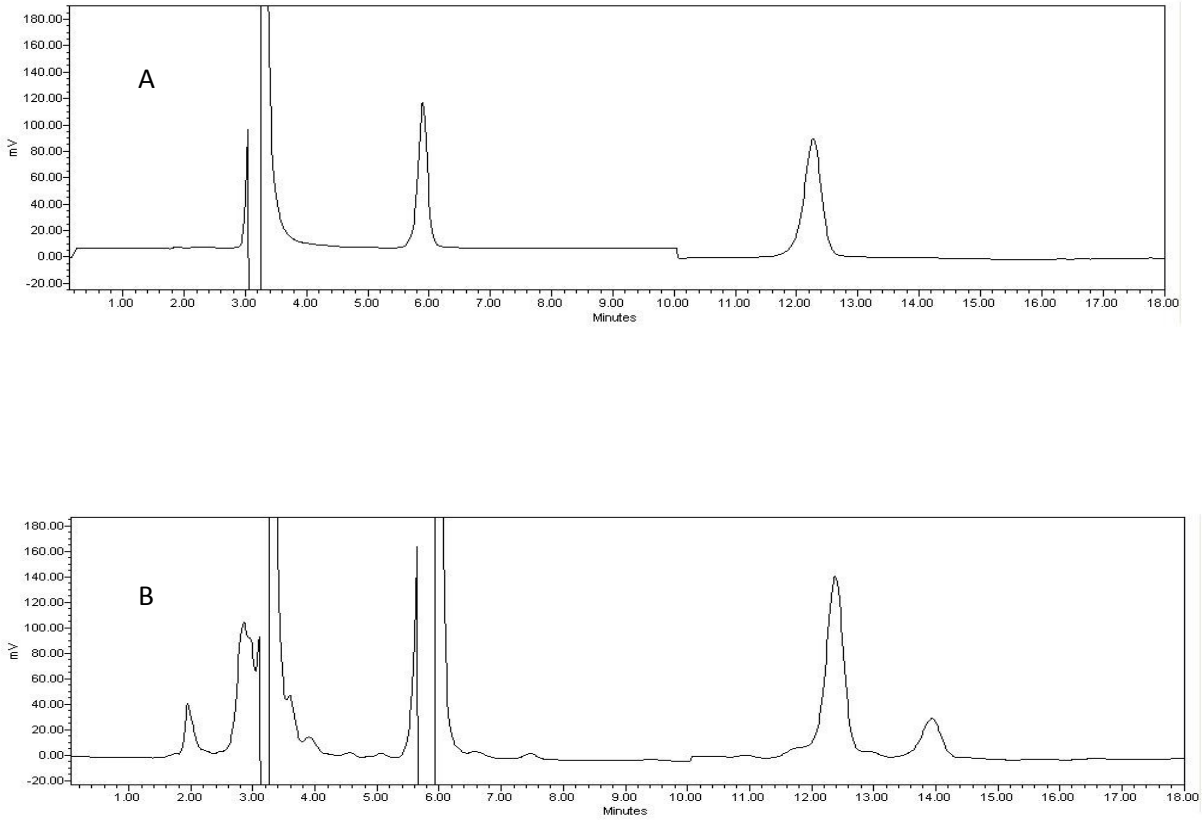


Figure 5.1: Chromatographic profiles of (a) an aqueous standard and (b) a human urine sample. The DMA peak is seen to elute at 12.4 minutes.

5.10 Analytical validation

5.10.1 Linearity

The detector response was determined to be linear up to 2,000 μM for DMA under the conditions described. Calibration curve and linear regression equation for DMA are shown in figure 5.2. These were determined by preparing sequential dilutions from a stock standard and subjected to the full analytical procedure. The values on the y axes represent the detector response. The correlation coefficient was > 0.999.

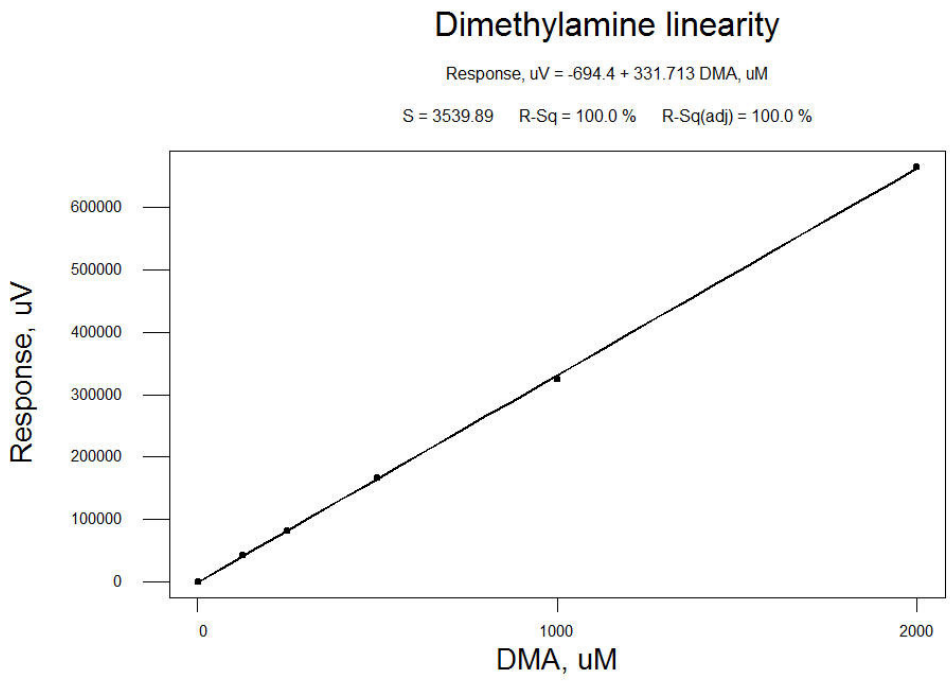


Figure 5.2: Calibration curve and regression equation for dimethylamine

5.10.2 Limits of detection and quantification

Limit of detection were defined as 3 times the chromatographic baseline noise from a sample blank, and was determined to be 2 μmol/L. The lower limit of quantification was defined as 10 times the signal-to-noise ratio, and was determined to be 6.7 μmol/L. The lower limit of quantification is significantly lower than the concentrations commonly encountered in biological samples.

5.10.3 Recovery

Accuracy was assessed by determining the recovery of DMA from spiked urine samples. Increasing concentrations of DMA were added to aliquots of a urine pool, and the recovery calculated following subtraction of the basal concentration. This experiment was performed in triplicate, and the mean (SD) results are shown in table 5.1. Recoveries were within the range 98.4 – 101.5%.

Amount added μmol/L	Concentration measured Mean (SD), μmol/L	Calculated recovery Mean (SD), %
0	351 (12.7)	
250	597 (9.6)	98.4 (3.2)
500	859 (13.3)	101.5 (2.2)
1000	1350 (20.9)	99.9 (1.7)

Table 5.1: Recovery of dimethylamine from spiked urine samples (n = 3)

5.10.4 Precision

5.10.4.1 Intra-assay variation

The intra-assay CV was determined by analysing 5 aliquots of a single urine pool within a single batch. Each aliquot was subjected to the described process, and so the data represent the imprecision of the whole analytical procedure. The results from this are shown in table 5.2.

	DMA, μmol/L
1	526
2	558
3	525
4	526
5	521
Mean	531
SD	15.1
CV, %	2.8

Table 5.2: Intra-assay variation

5.10.4.2 Inter-assay variation

The in-house QC material was analysed in duplicate in each batch. 12 pairs of QC duplicates were used to determine imprecision. The data for these are given in table 5.3. Analytical variance (SD_A^2) was calculated from the difference between each pair of duplicates according to the formula:

$$SD_A^2 = \Sigma\, d^2/2N$$

where d is the difference between each duplicate pair and N is the number of duplicate pairs. Inter-assay CV was calculated from this variance, and was determined to be 3.7%.

Pair no	Sample 1, μmol/L	Sample 2, μmol/L
1	407	401
2	363	372
3	333	344
4	430	416
5	367	380
6	366	381
7	348	382
8	376	349
9	340	358
10	366	392
11	377	353
12	379	362

Table 5.3: QC data for calculation of inter-assay variation

Chapter 6

Measurement of nitrate in urine using HPLC

6.1 Background and justification

NO has a very short half life in the circulation, rapidly reacting with oxyhaemoglobin (oxyHb) to form nitrate which is its major, inactive, metabolite[253]. A small amount undergoes oxidation to nitrite, which can also be oxidised by oxyHb. While NO itself is unstable, nitrate as the major metabolite of NO undergoing urinary excretion provides a relatively simple and non-invasive marker of whole body NO production. The most commonly used methods for measurement of nitrate in biological fluids are based on the Griess reaction, and involve a modification of the original method in which nitrite reacts with the amino group of sulphanilamide under acidic conditions to form a diazonium cation[254]. This couples typically with naphthylethylenediamine to form an azo dye which can be measured by absorption of light at 540 nm. For urinary nitrate measurement, the nitrate must first be reduced to nitrite using nitrate reductase. Therefore total nitric oxide metabolites (sum of nitrate and nitrite) are measured, with the overwhelming majority representing nitrate. A significant problem with these methods is the incomplete reduction of nitrate, which has been estimated at around 30 – 80%[255]. GC-MS and HPLC methods have also been utilised, but suffer in comparison with the Griess methods owing to the equipment required, and sometimes extensive pre-analytical sample preparation to avoid interferences[254].

Nitrate excretion in urine is also contributed to by dietary nitrate, although compared with dietary sources, endogenously formed nitrate has been estimated as being 3 to 6 times greater[254,256]. Dietary sources of nitrate

include green vegetables, processed meats and tap water. Following a high nitrate meal, urinary nitrate peaks after 4 – 6 hours, with the majority excreted during the following 12 hours and a return to near basal levels by 16 hours[257]. When collecting serial samples to examine for changes in endogenous nitrate production, therefore, it is important to standardise the collection time and minimise the variation from changes in the "background" excretion from dietary sources.

The remainder of this chapter describes the development and validation of a simplified isocratic HPLC method for measurement of nitrate in urine and its comparison with a commonly used commercially available Griess colorimetric assay.

6.2 Specimen collection and pre-analytical handling

For patients participating in the knee arthroplasty study described in chapter 8, urine samples were collected in the morning before or around the time of breakfast. The patients' evening meal was at a consistent time around 17:30 to 18:00 hours and was their last main food intake of the day. Urine samples being collected in the morning around 14 hours after this on average. Random samples were collected and the nitrate concentrations expressed as a ratio to creatinine. Urine specimens were collected into plain specimen containers. These were frozen without significant delay, and stored at -70˚C, being thawed only immediately prior to analysis. This was done to prevent bacterial reduction of nitrate to nitrite *in vitro*.

6.3 Reagents and buffers

6.3.1 List of chemicals

Sodium nitrate (Sigma-Aldrich, Gillingham, UK)

Potassium dihydrogen phosphate (BDH chemicals, VWR International Ltd, Leicestershire)

6.3.2 Preparation of mobile phase: *20 mM phosphate buffer*

A 20 mM phosphate buffer was prepared by dissolving 0.4 g potassium dihydrogen phosphate in 300 ml deionised water that was determined to be nitrate free by chromatography. The pH was adjusted to 3.5 using phosphoric acid.

6.4 Standards

A 10 mM stock nitrate standard was prepared in deionised water and stored at -70˚C. From this a 2,000 μM working standard was prepared for use in each analytical run. The standard was determined to be stable under these conditions by comparing peak heights on chromatography.

6.5 Internal quality control material

Urine obtained from a single volunteer was used as the QC material. This was stored in aliquots at -70˚C until use, and analysed in duplicate within each analytical run in order to generate imprecision data.

6.6 Sample preparation

Standards, urine samples and QC material were diluted 1:50 in nitrate free deionised water prior to injection onto the analytical column.

6.7 Chromatography

6.7.1 Chromatographic system and setup

The chromatographic system consisted of a solvent delivery system, programmable autosampler and ultraviolet detector (Waters 2478, Waters, Watford, UK). 100 μL of the diluted sample was injected onto the column for chromatography. Signals were detected at a wavelength of 205 nm, with an AUFS (absorbance units full scale) of 0.8, and captured using a chart recorder.

6.7.2 Analytical column

The analytical column was a Gemini phenyl, 3 μm, 4.6 x 150 mm, protected by a 3 x 4 mm guard column. The ambient temperature in the laboratory was maintained at a constant level in order to ensure stability of retention times.

6.7.3 Mobile phase

The mobile phase, prepared as described, was maintained at room temperature and pumped through the analytical column at a flow rate of 0.75 ml/minute. The mobile phase was recycled in order to allow a larger number of samples to be analysed in a single run without having to prepare overly large volumes of the mobile phase. This was not found to cause any problem with interfering peaks, even in large runs.

6.8 Calculation of results

Quantification was done by the method of external standardisation on the basis of peak heights as the nitrate peak was completely resolved at baseline and the peak consistently sharp and well defined. Nitrate concentration was calculated by dividing the peak height of analyte in the (unknown) sample by that in the calibration standard and multiplying by the known concentration in the standard. A single level calibration was selected owing to the linearity of nitrate over a wide concentration range, as demonstrated in the section on analytical validation.

6.9 Resultant chromatographic profiles

The peak corresponding to nitrate was completely resolved from all other peaks, eluting at 6 minutes. Chromatographic profiles corresponding to a range of aqueous standards and a human urine sample are shown in figure 6.1.

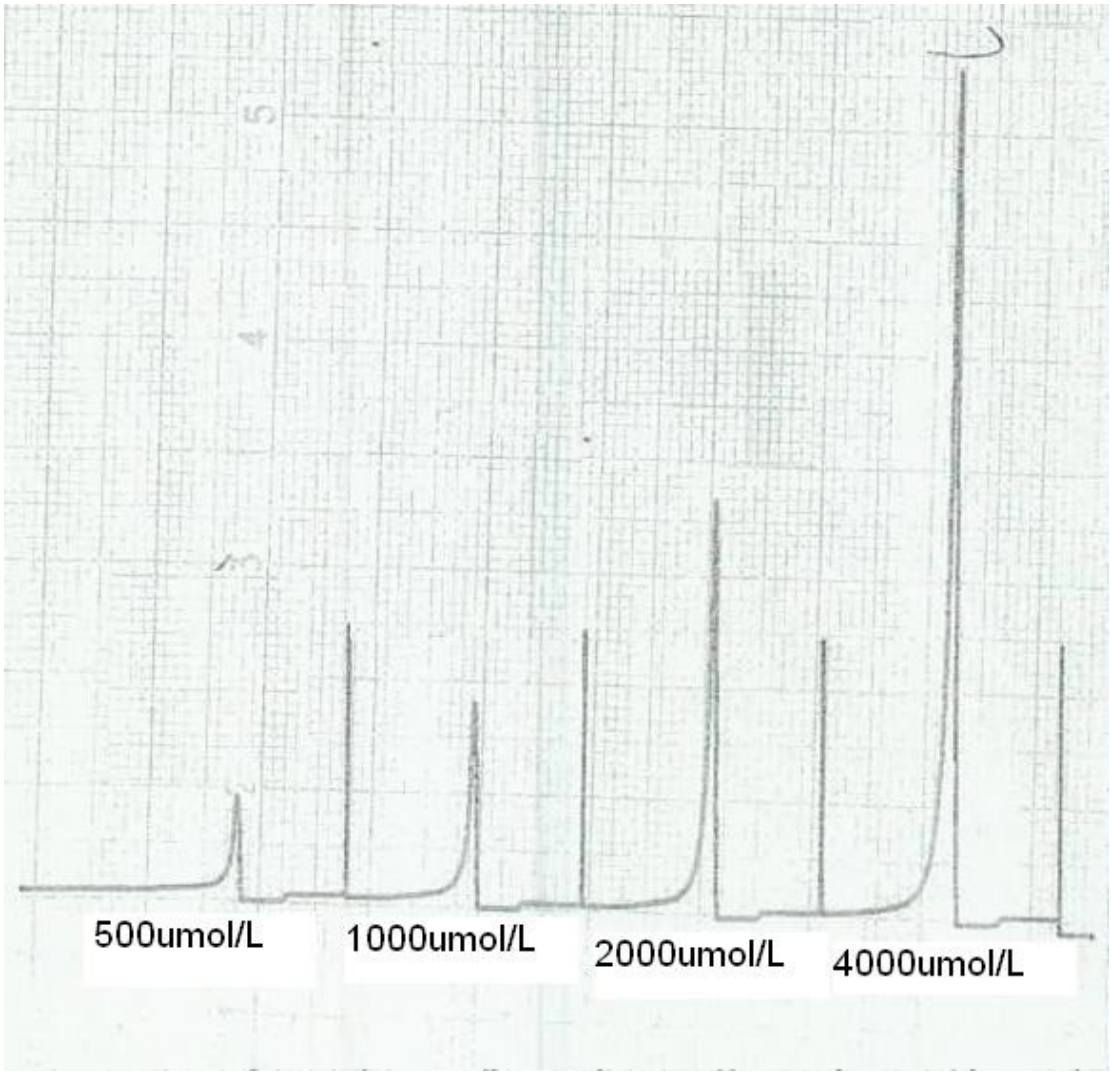


Figure 6.1(a): Chromatographic profiles of aqueous nitrate standards

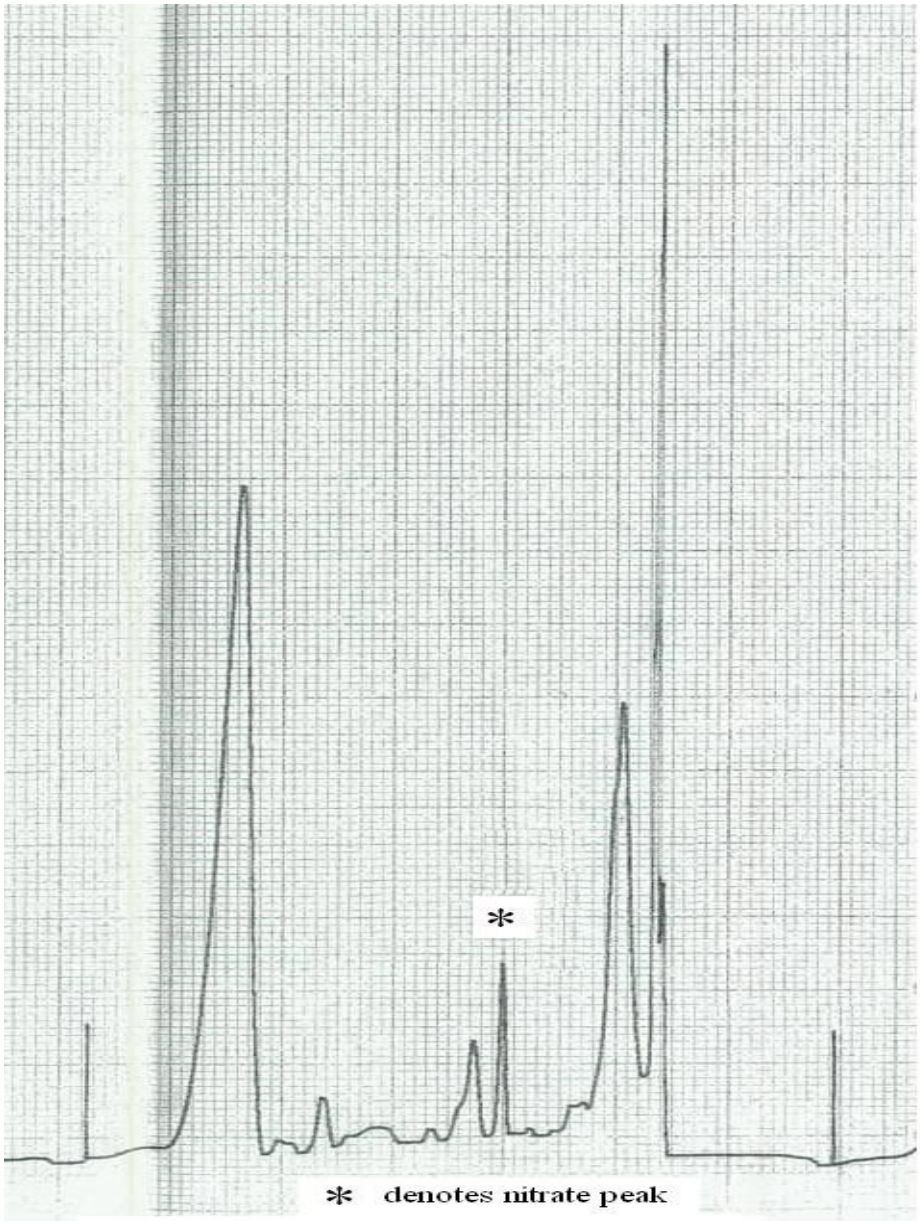


Figure 6.1(b): Chromatographic profile of a human urine sample

6.10 Analytical validation

6.10.1 Linearity

The detector response was determined to be linear up to 4,000 µM for nitrate under the conditions described. Calibration curve and linear regression equation for nitrate are shown in figure 6.2. These were determined by preparing sequential dilutions from a stock standard and subjected to the full analytical procedure. The values on the y axes represent the detector response. The correlation coefficient was > 0.999.

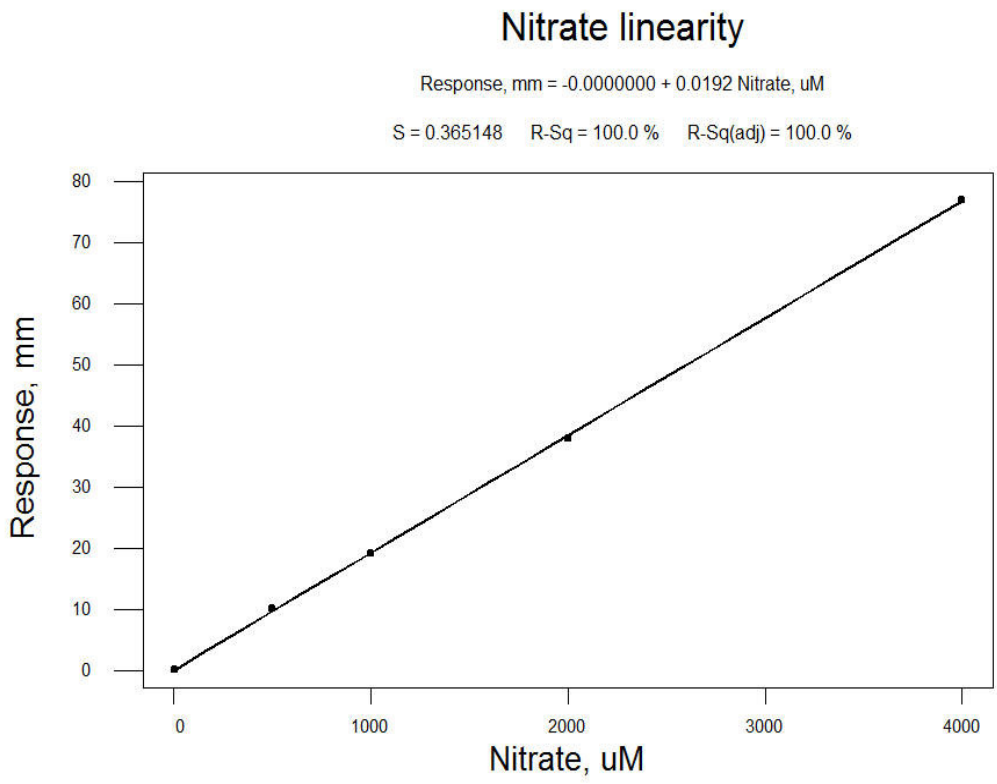


Figure 6.2: Calibration curve and regression equation for nitrate

6.10.2 Limits of detection and quantification

Limit of detection was defined as 3 times the chromatographic baseline noise from a sample blank, and was determined to be 25 μmol/L. The lower limit of quantification was defined as 10 times the signal-to-noise ratio, and was determined to be 83 μmol/L. The lower limit of quantification is significantly lower than the concentrations commonly encountered in biological samples.

6.10.3 Recovery

Accuracy was assessed by determining the recovery of nitrate from spiked urine samples. Increasing concentrations of nitrate were added to aliquots of a urine pool, and the recovery calculated following subtraction of the basal concentration. This experiment was performed in triplicate, and the mean (SD) results are shown in table 6.1. Recoveries were within the range 94.0 – 98.0%.

Amount added μmol/L	Concentration measured Mean (SD), μmol/L	Calculated recovery Mean (SD), %
0	1375 (35)	
864	2222 (59)	98.0 (6.2)
1729	3000 (75)	94.0 (4.3)

Table 6.1: Recovery of nitrate from spiked urine samples (n = 3)

6.10.4 Precision

6.10.4.1 Intra-assay variation

The intra-assay CV was determined by analysing 5 aliquots of a single urine pool within a single batch. Each aliquot was subjected to the described process, and so the data represent the imprecision of the whole analytical procedure. The results from this are shown in table 6.2.

	Nitrate, µmol/L
1	2854
2	3021
3	3063
4	2875
5	2917
Mean	2946
SD	92
CV, %	3.1

Table 6.2: Intra-assay variation

6.10.4.2 Inter-assay variation

The in-house QC material was analysed in duplicate in each batch. 8 pairs of QC duplicates were used to determine imprecision. The data for these are shown in table 6.3. Analytical variance (SD_A^2) was calculated from the difference between each pair of duplicates according to the formula:

$$SD_A^2 = \Sigma\, d^2/2N$$

where d is the difference between each duplicate pair and N is the number of duplicate pairs. Inter-assay CV was calculated from this variance, and was determined to be 2.8%.

Pair no	Sample 1, μmol/L	Sample 2, μmol/L
1	935	887
2	854	879
3	997	934
4	851	889
5	838	866
6	859	846
7	846	850
8	874	856

Table 6.3: QC data for calculation of inter-assay variation

6.11 Comparison with Griess colorimetric assay

6.11.1 Griess assay method

Urine specimens from the knee arthroplasty study described in chapter 8 were analysed for nitrate concentration using both the currently described HPLC method and a commercially available colorimetric kit (Cayman Chemicals Item no 780001, Cambridge, UK). This was performed according to the manufacturer's instructions. The method involves the initial reduction of nitrate to nitrite, the reaction of nitrite with sulfanilamide and subsequent reaction with naphthyl-ethylenediamine to form a purple coloured azo product which is measured spectrophotometrically at 540 nm. The method displays intra- and interassay variations of 2.7% and 3.4% respectively.

6.11.2 Method comparison

108 urine specimens were subjected to analysis by both the HPLC and Griess methods. There was a strong correlation between the methods (R^2 = 88.3%), which is shown in figure 6.3.

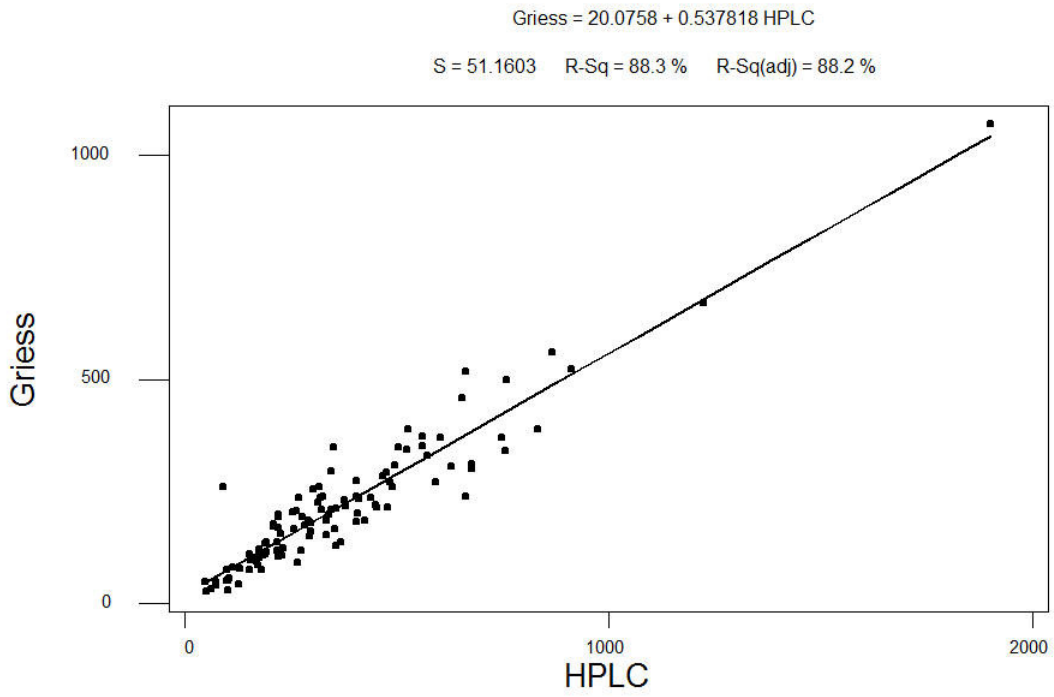


Figure 6.3: Correlation between HPLC and Griess methods for urine nitrate

The Griess assay yielded consistently lower results than those from the HPLC assay, with the Griess results lower by a mean of 37.7%. This constant bias is shown in the Bland-Altman type scatter plot in figure 6.4.

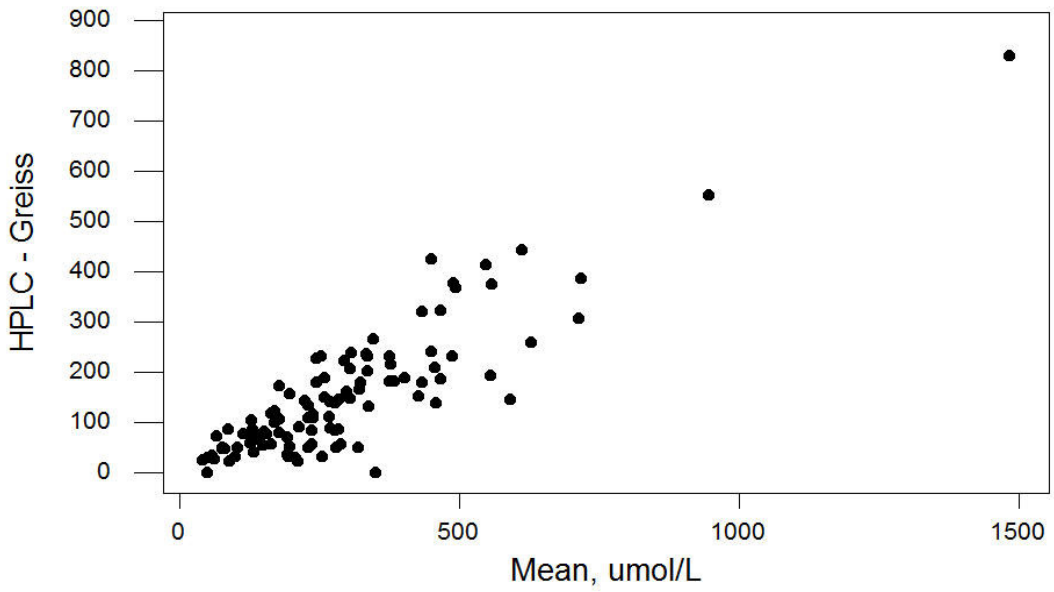


Figure 6.4: Bland-Altman plot of HPLC and Griess nitrate methods

6.12 Discussion

The simplified HPLC method described in this chapter permits quantification of nitrate in urine with very straightforward sample preparation. Interference from strongly retained late-eluting peaks was occasionally found to be a problem, but this was overcome by allowing an approximately 30 minute gap between sample injections after every 10 injections or so. Another approach would be to programme a solvent flush after elution of the nitrate peak, something that would be possible using a programmable solvent delivery system.

In common with other method comparisons between chromatographic and Griess methods, the Griess assay was found to under-recover nitrate compared with chromatography by a magnitude similar to that previously described[254,255]. It has been speculated that this results either from the

incomplete reduction of nitrate to nitrite, or incomplete determination of the reduction recovery rate[254]. Certainly, it suggests a systematic error, possibly resulting from the factors already mentioned or a problem in calibration. Methodological differences are highlighted by examination of supposedly "basal" nitrate concentrations in urine showing marked variability[254]. Creatinine adjusted values in the basal state for patients enrolled in the knee arthroplasty study were in broad agreement with those described in other studies[254].

Chapter 7

Other analytical methods

7.1 Serum C-reactive protein (CRP)

Serum CRP was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The principle of the method is latex agglutination based on an antigen-antibody reaction, agglutination being detected as an absorbance change at 572 nm. The intra and inter-assay CVs were < 1.0% and 2.1% respectively, and the assay performed satisfactorily in the relevant UK National External Quality Assessment Service (UK-NEQAS) scheme (Wolfson Laboratories, Birmingham).

7.2 Serum albumin

Serum albumin was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The principle of the method is the binding of bromocresol purple to albumin to form a coloured complex, with absorbance at 604 nm proportional to the albumin concentration. The intra and inter-assay CVs were all < 1.0%, and the assay performed satisfactorily in the relevant UK-NEQAS scheme.

7.3 Serum urea

Serum urea was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The method is based on a series of enzymatic reactions with consumption of nicotinamide adenine dinucleotide (NADH) being detected as a reduction in absorbance at 340 nm. The intra and inter-assay CVs were < 1.5% and < 2.0% respectively, and the assay performed satisfactorily in the relevant UK-NEQAS scheme.

7.4 Serum and urine creatinine

Serum and urine creatinine were measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The assay is a kinetic Jaffe method, the reaction of creatinine with alkaline picrate being detected as an absorbance change at 500 nm. The intra and inter-assay CVs were < 1.0% and < 4.0% respectively, and the assays performed satisfactorily in the relevant UK-NEQAS scheme. As previously described in chapter 3, eGFR was calculated from the serum creatinine results using the 4 variable MDRD equation[242].

7.5 Serum glucose

Serum glucose was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot

Architect analyser (Abbot Park, Illinois). The method is based on a series of enzymatic reactions starting with hexokinase, the production of NADH being detected as an absorbance change at 340 nm. The intra and inter-assay CVs were all < 1.0%, and the assay performed satisfactorily in the relevant UK-NEQAS scheme.

7.6 Plasma insulin

Plasma insulin was measured in the routine biochemistry laboratory at Glasgow Royal Infirmary on an Abbot Architect analyser (Abbot Park, Illinois). The assay is a one-step immunoassay with chemiluminescence detection. The intra and inter-assay CVs were < 3.5% and < 4.7% respectively, and the assay performed satisfactorily in the Randox International Quality Assessment Scheme (RIQAS).

7.7 Serum alanine aminotransferase (ALT)

Serum ALT was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The assay is based on a series of enzymatic reactions, ALT in the sample catalyzing the deamination of alanine. Ultimately, NADH is consumed, being detected as a reduction in absorbance at 340 nm. The intra and inter-assay CVs were < 2.0% and < 4.8% respectively, and the assay performed satisfactorily in the relevant UK-NEQAS scheme.

7.8 Serum bilirubin

Serum bilirubin was measured in the routine biochemistry laboratories at Gartnavel General Hospital and the Western Infirmary, Glasgow on an Abbot Architect analyser (Abbot Park, Illinois). The principle of the method is the binding of bilirubin to a diazo reagent, the azobilirubin product being detected as an absorbance change at 548 nm. The intra and inter-assay CVs were < 1.0% and < 2.0% respectively, and the assay performed satisfactorily in the relevant UK-NEQAS scheme.

7.9 Haemoglobin (for erythrocyte lysates)

The haemoglobin concentration in erythrocyte lysate preparations was measured in the biochemistry laboratory at Glasgow Royal Infirmary using a Sysmex KX-21N analyser (Sysmex, Canada). The intra and inter-assay CVs were < 2.0%.

Chapter 8

Knee arthroplasty study design

The aim of this study was to investigate the response of plasma dimethylarginines to a significant inflammatory response as seen following surgery, and to relate this to the urinary excretion of dimethylamine (DMA) as a marker of ADMA metabolism, and to the excretion of urinary nitrate as a marker of NO production. The elective surgery model was chosen as it allows examination from a non-inflamed baseline, thus enlightening the temporal relationship between inflammation and changes in ADMA concentration.

8.1 Ethical approval

Approval of the study protocol was granted by the Glasgow Royal Infirmary Research Ethics Committee. Copies of ethical committee and management approval can be seen in appendix 3, along with copies of the patient information sheet and consent form.

8.2 Inclusion and exclusion criteria

Patients undergoing elective knee joint arthroplasty for osteoarthritis at Gartnavel General Hospital were considered suitable for inclusion. Patients with joint disease secondary to inflammatory arthropathies such as rheumatoid arthritis were considered unsuitable as they would be expected to exhibit a baseline (chronic) inflammatory response.

Two patients were subsequently excluded from the study in the immediate post-operative period, one following an acute coronary syndrome and one following a pulmonary embolism.

Suitable patients were identified and their consent for participation obtained during attendance at the pro-operative assessment clinic by Dr David Reid or myself. Copies of the patient information sheet and consent form can be seen in appendix 4.

8.3 Specimen collection

Venous blood and urine specimens were collected according to the protocol outlined below. In general patients had their evening meal around 18:00 hrs, with urine specimens collected between 12 and 15 hours later. This consistency was felt to minimise the variability attributable to DMA and nitrate excretion from ingested food. In addition, patients did not consume food known to contribute significantly to urinary DMA excretion, which, as previously discusses, is largely confined to certain types of fish and shellfish.

<u>Day 0 (baseline)</u>

Urine collected during fasting period into a plain universal container for DMA and nitrate.

Blood collected under fasting conditions into serum separator tube (SST) for routine biochemistry tests and oxalate tube for glucose.

Blood collected by anaesthetist at time of induction of anaesthesia into a heparinised blood tube for dimethylarginines.

12 hours post-operatively

Blood collected into heparinised tube for dimethylarginines.

Day 1 to 5 post-operatively (daily, in ward)

Urine collected during morning following overnight fast for DMA and nitrate.

Blood collected fasting into SST and oxalate tubes for routine biochemistry tests and glucose.

Blood collected into heparinised blood tube for dimethylarginines.

3 months post-operatively (at out-patient clinic)

Blood collected into heparinised blood tube for dimethylarginines.

8.4 Specimen handling

Blood and urine specimens were transported to the biochemistry laboratory within 1 hour of collection. SST and oxalate tubes for routine biochemistry tests were handled according to established procedures in the routine laboratory. Urine specimens were frozen without delay at -70˚C. The heparinised blood tube was centrifuged (500 g, 4˚C, 10 minutes) and the plasma removed and stored at -70˚C until analysis.

8.5 Participant demographics

38 participants were recruited and completed the study. There were 14 males and 24 females with an age range of 55 to 81 years. 9 of these patients had a pre-operative diagnosis of type 2 diabetes mellitus, treated either by dietary control or oral hypoglycaemic drugs. All underwent total knee joint arthroplasty using a standard medial parapatellar surgical approach. Patients underwent anaesthesia either by general anaesthetic or spinal anaesthesia with sedation. Intravenous fluids were administered as part of routine patient care during the first 24 hours post-operatively. Intra-operative blood loss was minimised by use of a tourniquet. Nonetheless, seven patients were judged to require a blood transfusion post-operatively by the surgical team caring for them, based on haemoglobin measurement on the second post-operative day.

10 patients had progressed sufficiently to be discharged home on the fourth post-operative day, and so do not have measurements available for day 5. The remainder stayed to at least the fifth post-operative day. It was possible to obtain specimens from 26 patients 3 months post-operatively, when they were attending the out patient clinic for review.

Participant demographics, diabetes status, details of blood transfusion and available specimens are shown in appendix 4.

8.6 Statistical methods

The Anderson-Darling test was used to assess the distribution of data, with $p < 0.05$ taken to indicate non-normal distribution. Where relevant and possible, logarithmic transformation was used to permit T-testing between paired groups of data. Otherwise the Mann Whitney U test was used, with $p < 0.05$ chosen for statistical significance.

The Friedman test was used to examine for changes in parameters measured repeatedly across the study period, with $p < 0.05$ taken to indicate statistical significance. The Mann Whitney U test was used to compare data from 2 time points to determine at which points the changes occurred.

To determine the association between two variables, Spearman rank correlation (r_s) was used, unless otherwise stated, more or less throughout given that almost all of the data were non-normally distributed. Scatter plots illustrate the data where significant correlations were found.

Chapter 9

Plasma dimethylarginines during the inflammatory response following elective knee arthroplasty

9.1 Changes in acute phase reactants

9.1.1 Serum C reactive protein (CRP)

CRP increased significantly and rapidly in the post-operative period, indicating a significant inflammatory response. The CRP peaked at days 2 – 3 post-operatively, and was still significantly higher than baseline at day 5. The median and inter-quartile CRP concentrations can be seen in table 9.1. The Friedman test was used to establish the significance of the change in CRP, and found this to be highly significant ($p < 0.0001$). The Mann Whitney U test was used to establish the significance of changes between days, finding significant changes between days 0 and 1, and 1 and 2 ($p < 0.0001$), with a plateau between days 2 and 3 ($p = 0.91$). By day 5, CRP was reducing from its peak (day 2 vs 5, $p < 0.01$). By 3 months, CRP had returned to baseline concentrations.

9.1.2 Serum albumin

Serum albumin concentration decreased significantly during the acute phase reaction, with the Friedman test demonstrating a highly significant change ($p < 0.0001$). Mann Whitney U test comparisons showed a significant change between days 0 and 1 ($p < 0.0001$), with a trend to a further decrease between days 1 and 2 ($p = 0.08$), and a plateau thereafter to day 5 (p non

significant). By 3 months, albumin had returned to baseline concentrations. Table 9.2 shows the median and inter-quartile albumin concentrations across the study period.

The observed changes in CRP and albumin were therefore taken to confirm a significant post-operative inflammatory response.

Time point	CRP concentration, mg/L
Day 0	2.7 (1.5 – 4.4)
Day 1	53 (39 – 79)
Day 2	169 (112 – 230)
Day 3	191 (121 – 229)
Day 4	135 (93 – 178)
Day 5 (n = 28)	107 (74 – 136)
3 months (n = 26)	3.0 (1.7 – 5.2)

Table 9.1: Median (inter-quartile range) CRP concentrations during study period

Time point	Albumin concentration, g/L
Day 0	39 (37 – 40)
Day 1	33 (31 – 35)
Day 2	31 (29 – 34)
Day 3	31 (29 – 32)
Day 4	30 (29 – 32)
Day 5 (n = 28)	31 (27 – 32)
3 months (n = 26)	39 (37 – 41)

Table 9.2: Median (inter-quartile range) albumin concentrations during study period

9.2 Fasting glucose and insulin resistance

Patients not known to be diabetic (n = 29) underwent measurements of fasting glucose and insulin pre-operatively and on each morning post-operatively in order to assess the effect of their inflammatory response on insulin sensitivity. As previously discussed, insulin resistance may have adverse effects on DDAH activity and ADMA metabolism.

There was a significant change in fasting glucose across the study period (Friedman $p < 0.0001$). The Mann Whitney U test determined this to be a significant increase between days 0 and 1 (7.7 vs 5.5 mmol/L, $p < 0.0001$). This rise from baseline remained significant to day 4 post-operatively (day 0 vs 4, $p = 0.03$), with a non-significant difference between days 0 and 5 (p =

0.2). The median and inter-quartile range glucose concentrations are shown in table 9.3. Fasting insulin demonstrated a significant change across the study period (Friedman $p < 0.001$). In a similar pattern to glucose, this was evident between days 0 and 1 (13.9 vs 7.2 mU/L, $p < 0.001$), and remained higher than baseline to the end of the study period (day 0 vs 5, $p = 0.01$). The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as a marker of insulin resistance[258], and demonstrated a significant change (Friedman $p < 0.0001$). This was significantly higher at day 1 (4.64 vs 1.64), and remained significantly higher to the end of the study period (day 5 vs 0, $p = 0.02$). Median and inter-quartile range insulin concentrations and HOMA-IRs are also shown in table 9.3.

Spearman correlation showed no significant correlations between the rise in CRP and day 1 glucose concentration or HOMA-IR ($p = 0.56$ and 0.99 respectively).

Day	Glucose, mmol/L	Insulin, mU/L	HOMA-IR
0	5.5 (5.0 – 6.0)	7.2 (5.6 – 10.2)	1.64 (1.32 – 2.68)
1	7.7 (6.4 – 8.2)	13.9 (8.1 – 22.2)	4.64 (2.55 – 7.89)
2	6.5 (5.7 – 7.1)	11.0 (8.5 – 19.9)	3.20 (2.34 – 6.76)
3	6.2 (5.8 – 6.5)	12.0 (9.9 – 13.7)	3.31 (2.60 – 4.26)
4	6.0 (5.3 – 6.6)	13.1 (8.9 – 16.0)	3.25 (2.32 – 4.34)
5 (n = 21)	5.8 (5.3 – 6.4)	10.3 (7.9 – 13.1)	2.56 (1.98 – 3.39)

Table 9.3: Median (inter-quartile range) fasting glucose, insulin and HOMA-IR during study period (n = 29)

9.3 Plasma ADMA during the acute inflammatory response

Plasma ADMA concentration decreased rapidly and significantly during the acute inflammatory response (Friedman $p < 0.0001$). The nadir concentration was reached on day 2 post-operatively, by which point a median reduction of 31% had occurred (0.43 vs 0.68 μmol/L). However, by 12 hours post-op the reduction was already seen to be significant ($p < 0.0001$). The plasma ADMA concentration was recovering by day 4 post-op, with a significant increase seen between days 2 and 4 ($p < 0.0001$). For patients who had samples from both days 4 and 5 available ($n = 28$), a further significant increase was seen between days 4 and 5 ($p = 0.025$), such that by day 5 the ADMA concentration had returned to baseline levels (day 0 vs 5, $p = 0.56$). Table 9.4 shows the median and inter-quartile range ADMA concentrations, and figure 9.1 shows the data as box plots.

No significant correlation between ADMA concentration and eGFR was found either at baseline ($r_s = -0.06$, $p = 0.71$), as shown in figure 9.2, or at the peak of the inflammatory response on day 2 ($r_s = 0.08$, $p = 0.66$).

Time point	ADMA concentration, µmol/L
Day 0	0.62 (0.54 – 0.65)
12 hours	0.48 (0.43 – 0.54)
Day 1	0.43 (0.40 – 0.45)
Day 2	0.43 (0.39 – 0.47)
Day 3	0.49 (0.45 – 0.53)
Day 4	0.55 (0.51 – 0.62)
Day 5 (n = 28)	0.61 (0.57 – 0.64)
3 months (n = 26)	0.58 (0.52 – 0.59)

Table 9.4: Median (inter-quartile range) ADMA concentrations during study period

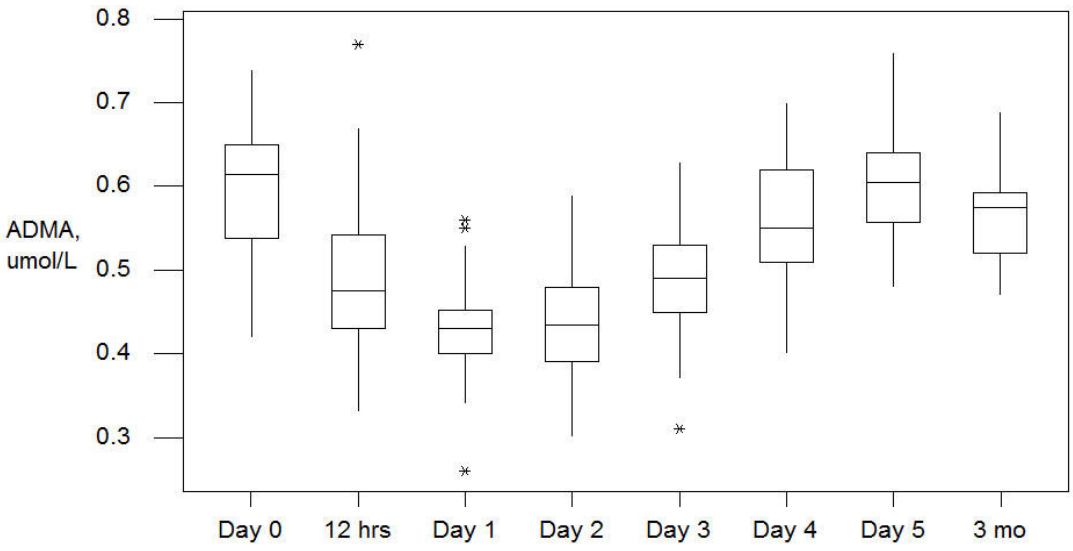


Figure 9.1: Box plots of ADMA concentrations during study period

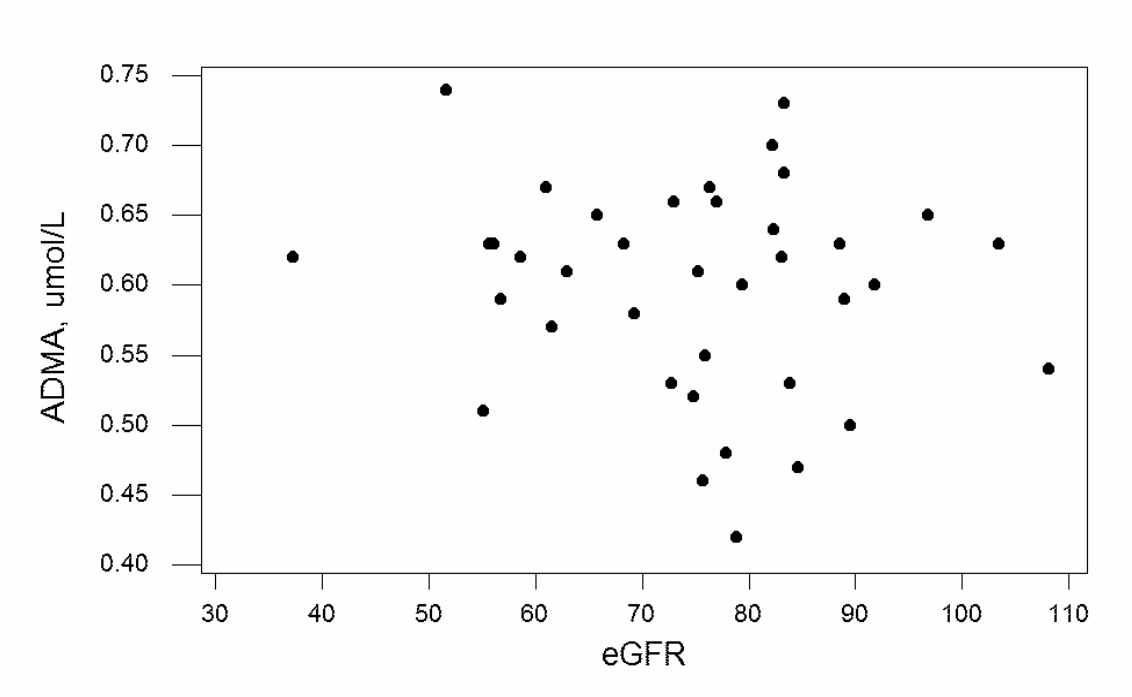


Figure 9.2: Relationship between plasma ADMA and eGFR on day 0

The relationship between the inflammatory response and ADMA concentration was further examined at baseline and at the peak of the inflammatory response, using Spearman rank correlation. No significant correlation was found between ADMA and CRP concentrations at baseline in the non-inflamed state (r_s = -0.22, p = 0.19). To investigate whether the magnitude of the inflammatory response was reflected by the change in ADMA concentration, the relationship between the change in CRP concentration, i.e. the peak minus baseline values, and percent change in ADMA concentration was sought. A relatively weak correlation of borderline statistical significance was found (r_s = 0.28, p = 0.09). These are illustrated in figures 9.3 and 9.4.

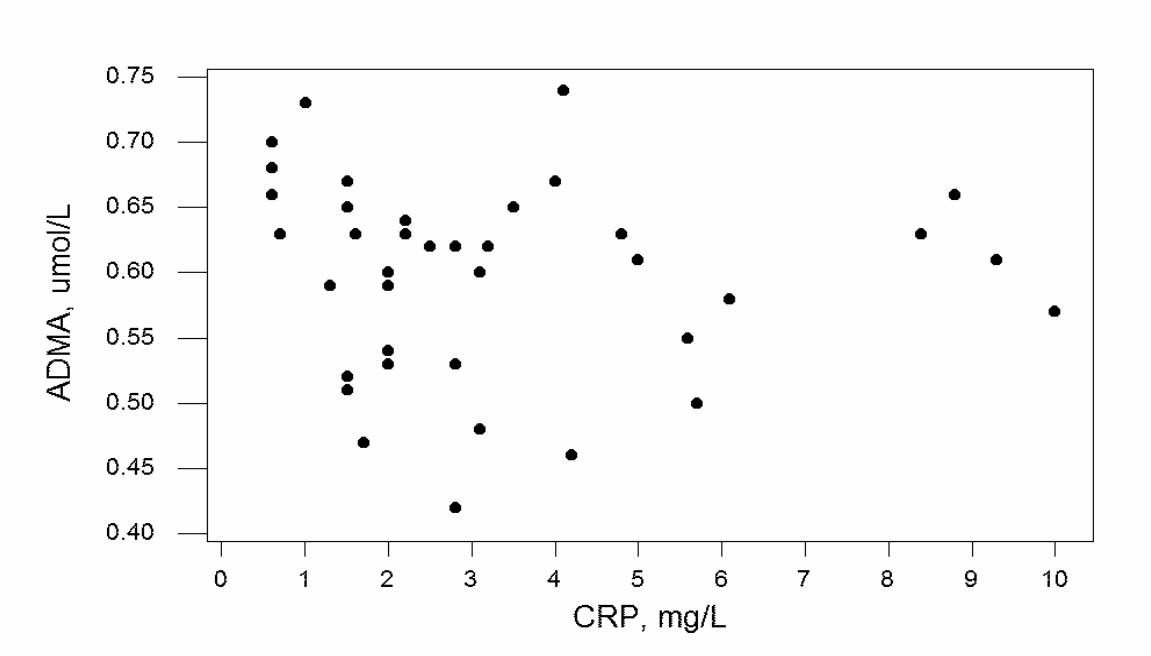


Figure 9.3: Relationship between ADMA and CRP concentrations on day 0

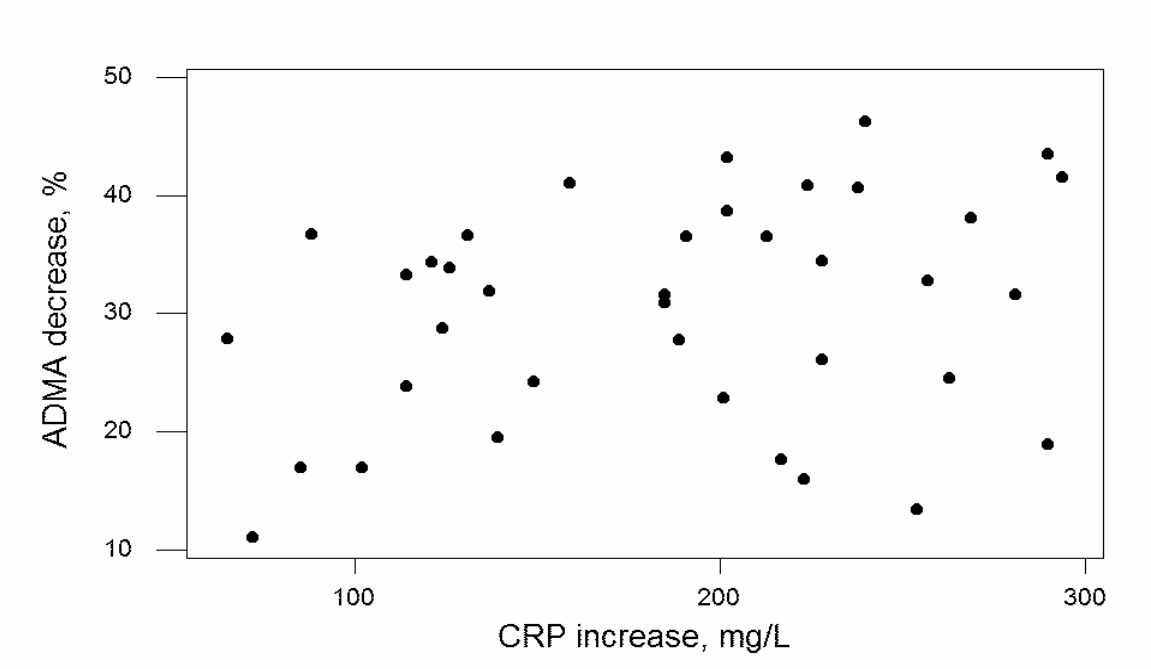


Figure 9.4: Relationship between changes in CRP and ADMA concentrations

9.4 Plasma SDMA during the acute inflammatory response

In contrast to ADMA, the plasma SDMA concentration showed no significant changes during the study period (Friedman $p = 0.638$). The median and inter-quartile range concentrations are shown in table 9.5, with the data illustrated as box plots in figure 9.5. The SDMA concentration was significantly correlated with eGFR at baseline in the non-inflamed state ($r_s = -0.47$, $p = 0.003$), as shown in figure 9.6. This relationship remained unaltered during the peak of inflammation on day 2 ($r_s = -0.59$, $p < 0.001$), as shown in figure 9.7.

The differential responses of the two dimethylarginines is reflected in the ADMA:SDMA ratio which decreases significantly (Friedman $p < 0.0001$) in a pattern similar to that of ADMA. These data are also shown in table 9.5.

Time point	SDMA concentration, μmol/L	ADMA:SDMA ratio
Day 0	0.48 (0.42 – 0.56)	1.28 (1.10 – 1.43)
12 hours	0.49 (0.40 – 0.60)	1.02 (0.82 – 1.16)
Day 1	0.49 (0.42 – 0.55)	0.88 (0.74 – 0.99)
Day 2	0.45 (0.40 – 0.57)	0.89 (0.77 – 1.07)
Day 3	0.48 (0.42 – 0.55)	1.02 (0.89 – 1.16)
Day 4	0.49 (0.42 – 0.55)	1.14 (0.99 – 1.37)
Day 5 (n = 28)	0.48 (0.43 – 0.59)	1.20 (1.03 – 1.40)
3 months (n = 26)	0.45 (0.39 – 0.48)	1.27 (1.14 – 1.45)

Table 9.5: Median (inter-quartile range) SDMA concentrations and ADMA:SDMA ratios during study period

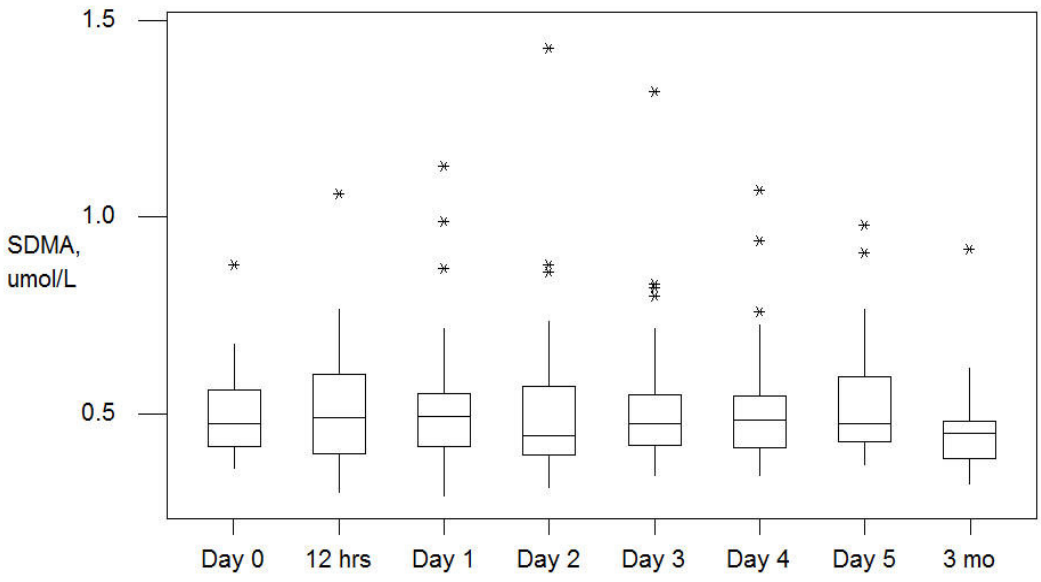


Figure 9.5: Box plots of SDMA concentrations during study period

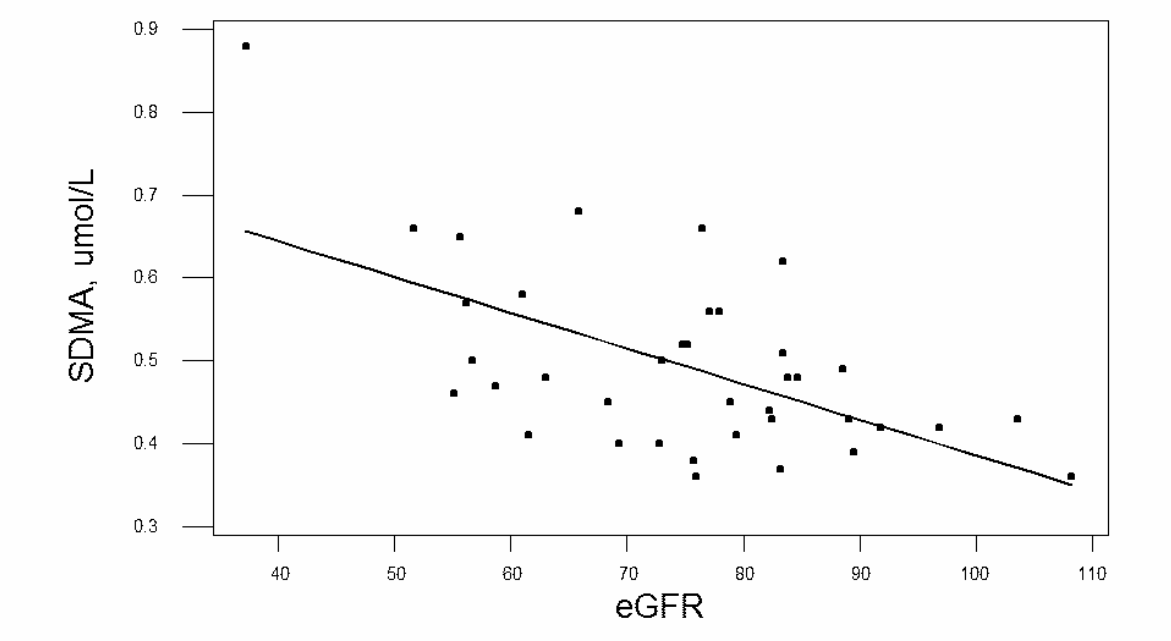


Figure 9.6: Correlation between plasma SDMA and eGFR on day 0

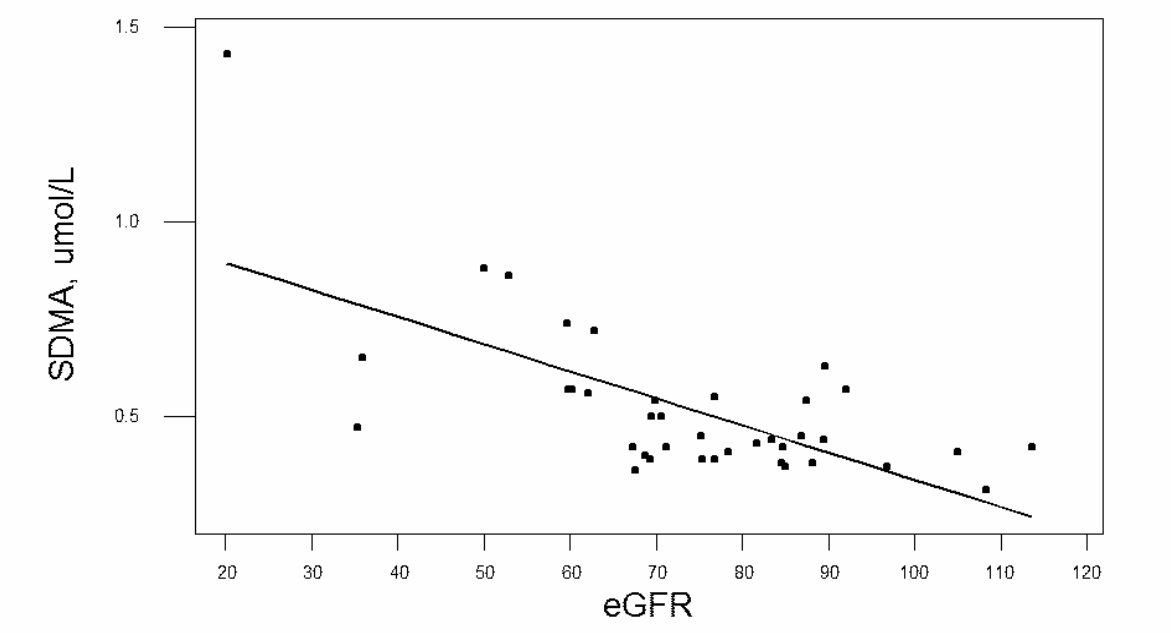


Figure 9.7: Correlation between plasma SDMA and eGFR on day 2

9.5 Serum sodium, urea and creatinine during the acute inflammatory response

In order to investigate the possibility of dilution as a cause for the reduction in plasma ADMA concentration, serum sodium and urea concentrations were measured during the study period. The inflammatory response is associated with promotion of water retention, probably through the enhanced action of the anti-diuretic hormone arginine vasopressin. Significant water retention and dilution would be expected to have effects on the serum concentrations of sodium and urea, with mild hyponatraemia a common finding in hospitalised patients.

Serum creatinine concentrations showed no significant changes across the study period, suggesting no major disturbance in renal function. Serum urea showed a significant change over the study period (Friedman $p < 0.0001$). This was a modest in absolute terms, amounting to a median 12.7% reduction (4.4 vs 5.6 mmol/L, $p = 0.03$). By day 3 this had returned to baseline concentrations. No significant change was evident at day 1 post-op (5.5 vs 5.6 mmol/L, $p = 0.77$). Serum sodium concentration changed significantly during the study period (Friedman $p < 0.0001$), with a modest median 2.2% reduction evident at day 1 (136 vs 139 mmol/L, $p < 0.0001$). This remained reduced from baseline for the duration of the study (day 5 vs 0, $p = 0.02$), returning to baseline concentrations 3 months post-operatively ($p = 0.67$). Median and inter-quartile range sodium, urea and creatinine concentrations are shown in table 9.6.

Time point	Sodium, mmol/L	Urea, mmol/L	Creatinine, μmol/L
Day 0	139 (138 – 140)	5.6 (4.6 – 6.8)	78 (69 – 85)
Day 1	136 (134 – 137)	5.5 (4.4 – 6.8)	77 (67 – 83)
Day 2	137 (135 – 138)	4.4 (3.4 – 6.1)	78 (66 – 87)
Day 3	137 (135 – 139)	4.8 (3.9 – 7.1)	77 (68 – 87)
Day 4	138 (135 – 140)	5.1 (4.6 – 6.7)	77 (65 – 86)
Day 5	138 (136 – 140)	5.5 (4.8 – 6.9)	77 (67 – 83)
3 months	140 (138 – 141)	6.2 (4.9 – 7.6)	81 (75 – 87)

Table 9.6: Median (inter-quartile range) serum sodium, urea and creatinine concentrations during study period

9.6 Plasma arginine and homoarginine during the acute inflammatory response

Arginine concentrations changed significantly across the study period (Friedman $p < 0.0001$). Mann Whitney U test comparisons showed a significant reduction which was evident at 12 hours post-op (33 vs 48 mmol/L, $p < 0.0001$) and remained significantly lower than baseline until day 3 ($p = 0.004$ vs day 0). This was a similar pattern to ADMA, such that there was minimal change in the arginine:ADMA ratio. The arginine:ADMA ratio was lower at 12 hours post-op, achieving borderline significance (70 vs 84, $p = 0.05$); however no significant changes compared with baseline were seen at the other time points.

Homoarginine changed significantly across the study period (Friedman p < 0.0001). Mann Whitney U test comparisons revealed a significant change to have occurred by day 2 post-op (1.05 vs 1.48 μmol/L, p = 0.001). Homoarginine remained significantly lower than baseline until day 5 (p < 0.0001 vs day 0), and had recovered to baseline at 3 months (p = 0.22). Median and inter-quartile range arginine, arginine:ADMA ratio and homoarginine concentrations are shown in table 9.7.

Time point	Arginine, μmol/L	Arginine:ADMA ratio	Homoarginine, μmol/L
Day 0	48 (41 – 60)	84 (68 – 98)	1.48 (1.10 – 1.91)
12 hours	33 (28 – 43)	70 (58 – 90)	1.33 (1.03 – 1.78)
Day 1	32 (24 – 39)	76 (63 – 91)	1.28 (0.96 – 1.73)
Day 2	31 (24 – 43)	76 (52 – 92)	1.05 (0.80 – 1.51)
Day 3	36 (30 – 50)	78 (67 – 97)	0.78 (0.68 – 1.26)
Day 4	49 (40 – 64)	88 (71 – 108)	0.81 (0.65 – 1.23)
Day 5 (n = 28)	46 (36 – 60)	74 (60 – 102)	0.95 (0.66 – 1.15)
3 months (n = 26)	52 (38 – 67)	93 (67 – 126)	1.29 (1.03 – 1.66)

Table 9.7: Median (inter-quartile range) arginine, arginine:ADMA ratio and homoarginine concentrations during study period

9.7 Summary of findings

The post-operative course was characterised by a significant inflammatory response with evidence of an associated insulin resistant state. Plasma ADMA concentration decreased significantly and rapidly during the early phase of the inflammatory response, recovering to baseline concentrations by day 5 post-op. The magnitude of the inflammatory response, as reflected by CRP concentration, was weakly associated with the magnitude of the reduction in plasma ADMA concentration. In contrast, plasma concentrations of the closely related compound SDMA showed no significant changes during the study period, and were closely related to renal function. Although modest changes in serum sodium and urea concentrations were seen, these are unlikely to represent a significant contribution of dilution to the changes in ADMA. Plasma arginine concentration changed similarly to ADMA during the post-operative period. Homoarginine concentration decreased more slowly and progressively, and remained lower than baseline at day 5 post-op at which point a significant inflammatory response was still evident.

Chapter 10

Erythrocyte free ADMA concentrations during the inflammatory response following elective knee arthroplasty

10.1 Introduction

Erythrocytes contain substantial quantities of ADMA, mainly incorporated in methylated proteins. This is largely in proteins other than haemoglobin[259-261], and incubation of erythrocyte lysates at 37˚C yields increases in free ADMA from the action of proteases, something which can inhibited by protease inhibitors and incubation at 4˚C[259-261]. However, erythrocytes also contain a certain concentration of free ADMA within their cytoplasm, which has been determined to be up to 2 times the concentration found in plasma[261]. This concentration is made possible by the expression of system y^+ cationic amino acid transporters (CAT) which have been well characterised on rat erythrocytes[262], and in humans have been implicated in the erythrocyte transport of basic amino acids such as arginine and MMA[263,264]. The relationship between plasma and free erythrocyte concentrations of ADMA is not clear, but Davids *et al* have recently shown a correlation between them in patients with critical illness, but not in healthy controls[261]. The authors speculate that increased expression of CATs in the critically ill patient could account for the correlation, and cite the up-regulation of CATs in other disease states such as heart failure, renal failure and pre-eclampsia in support of this hypothesis[262,264,265]. How this could occur in critical illness is unclear, although CAT-2B transporters can be up-regulated by inflammatory cytokines[266]. Whether human erythrocytes have the capacity to metabolise ADMA is controversial, with mixed results in published studies. Experimental evidence for DDAH-mediated catabolism has been offered from studies in rat and human erythrocytes[259,267,268]; however other studies have failed to demonstrate evidence of DDAH activity[260,261].

In the present study free erythrocyte concentrations were determined in knee arthroplasty participants to determine their relationship to plasma concentrations during the inflammatory response, and therefore to determine their likely relevance to the acute changes in plasma ADMA concentration described in the previous chapter.

10.2 Methods

ADMA concentrations were measured in the lysates of erythrocytes taken pre-operatively and at days 1, 2 and 3 months post-operatively. After exclusion of subjects who underwent blood transfusion, this left 20 subjects with available samples. Erythrocyte ADMA was measured using the method described in chapter 2 and the concentrations expressed as a ratio to haemoglobin concentration in the lysate; this latter step was employed to correct for errors introduced during the pipetting of cellular material.

The Friedman test was used to determine whether significant changes in erythrocyte ADMA occurred over serial samples. Spearman rank correlation was used to examine the relationship between plasma and erythrocyte concentrations where the data were non-normally distributed. Statistical analysis was done using Minitab statistical software.

10.3 Results

Median and inter-quartile range erythrocyte ADMA concentrations, expressed as a ratio to haemoglobin, are shown in table 10.1. Comparisons between pre and post-operative samples, using the Friedman test showed no significant changes ($p = 0.34$).

Time point	Erythrocyte ADMA, nmol/g Hb
Day 0	30.1 (24.1 – 39.4)
Day 1	25.6 (18.9 – 37.3)
Day 2	26.7 (23.6 – 38.3)
3 months	32.1 (23.9 – 37.0)

Table 10.1: Median (inter-quartile range) erythrocyte free ADMA concentrations during the inflammatory response

No significant correlations between erythrocyte and plasma ADMA concentrations were seen, both pre-operatively, in the absence of an inflammatory response or post-operatively at the peak of the inflammatory response ($p = 0.85$ on day 0; $p = 0.28$ on day 2). These are shown in figures 10.1 and 10.2.

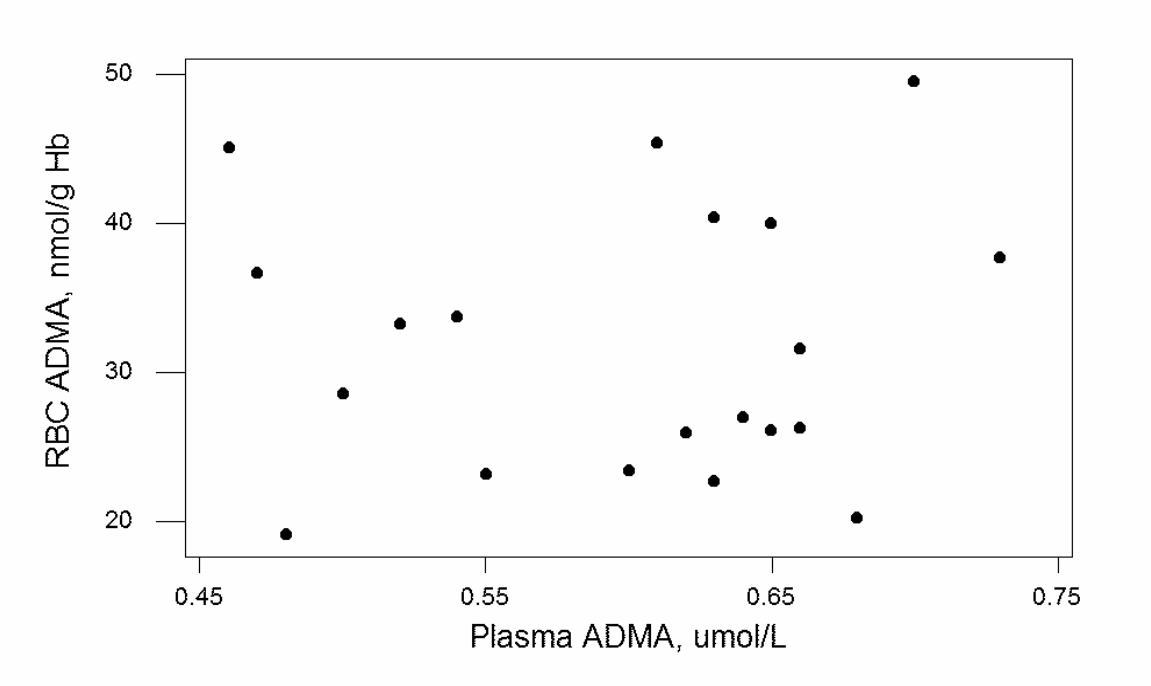


Figure 10.1: Correlation between erythrocyte and plasma ADMA concentrations on day 0

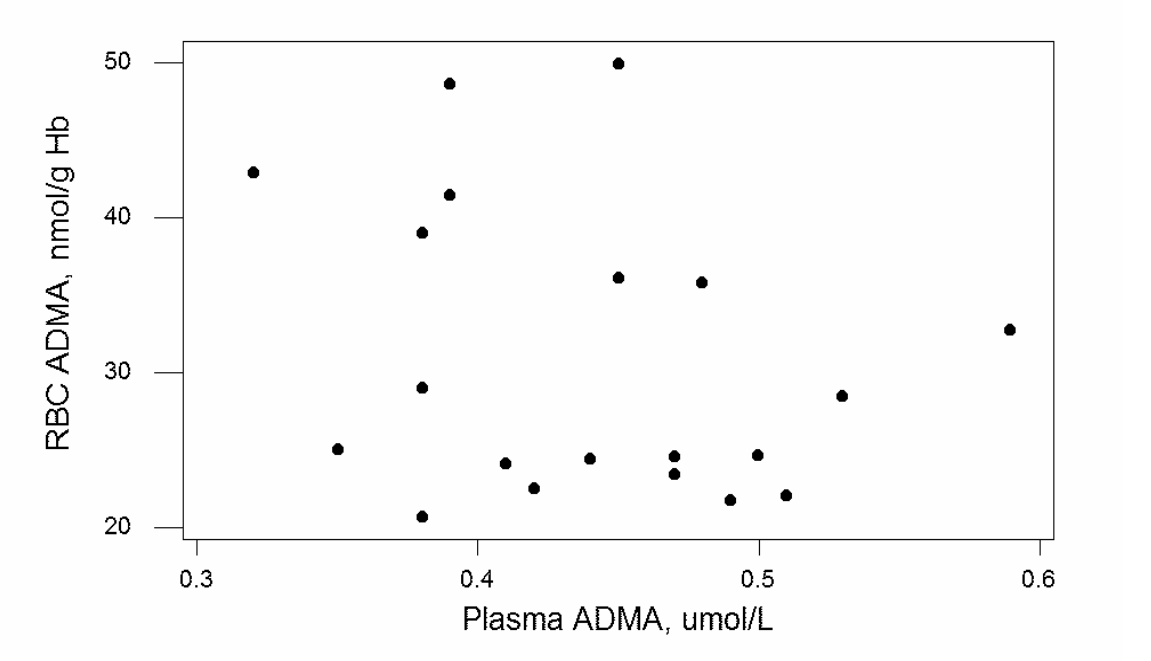


Figure 10.2: Correlation between erythrocyte and plasma ADMA concentrations on day 2

10.4 Summary of findings

Concentrations of free ADMA within erythrocytes did not change significantly on development of the inflammatory response. Pre-operatively and at the peak of inflammation on day 2, no correlations were found between erythrocyte and plasma ADMA concentrations. Therefore no evidence was found to suggest that the abrupt decrease in plasma ADMA concentration which occurred during the inflammatory response was related to increased CAT-mediated uptake of ADMA.

Chapter 11

Urinary excretion of dimethylamine, ADMA and nitrate during the inflammatory response following elective knee arthroplasty

11.1 Introduction

Urine measurements were performed on the subjects who had a full set of urine samples available to day 5 post-op (n = 20). As previously described, these were collected fasting pre-operatively and on each post-op morning. Concentrations of dimethylamine (DMA), dimethylarginines and nitrate were measured using the methods described in the relevant chapters, and expressed as ratios to creatinine.

11.2 Urine DMA during the acute inflammatory response

11.2.1 Urine DMA excretion

Urine DMA concentrations, expressed as ratios to creatinine, across the study period are shown in table 11.1. The Friedman test revealed a significant change over the study period (p = 0.001). Mann Whitney U test comparisons revealed a significant increase in DMA excretion at day 5 compared with baseline (median 105 vs 53 μmol/mmol creat, p = 0.003), but no significant differences between concentrations on days 1 to 4 versus baseline. There was a median increase of 50% in DMA excretion on day 5 compared with baseline.

There was no significant correlation between the magnitude of the inflammatory response and the magnitude of increase in DMA excretion at day 5 (r_s = 0.14, p = 0.54), as shown in figure 11.1.

Time point	DMA, μmol/mmol creatinine
Day 0	53 (44 – 87)
Day 1	59 (41 – 81)
Day 2	46 (38 – 97)
Day 3	59 (48 – 108)
Day 4	60 (44 – 82)
Day 5	105 (63 – 155)

Table 11.1: Median (inter-quartile range) DMA:creatinine ratios during study period

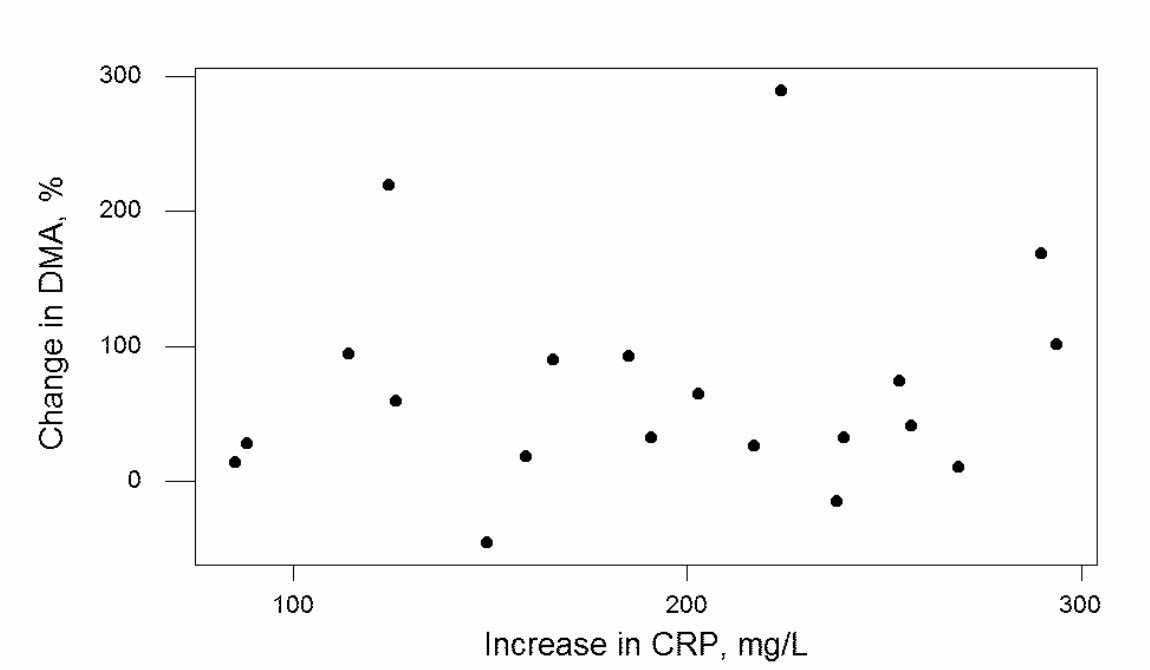


Figure 11.1: Relationship between changes in CRP concentration and DMA excretion

11.2.2 Relationship between concentrations of urinary DMA and ADMA

Urine DMA and ADMA concentrations were significantly correlated at baseline, in the non-inflamed state (r_s = 0.66, p = 0.002), as shown in figure 11.2. This relationship was weakened, though remained significant, on day 1 post-op (r_s = 0.54, p = 0.01), but was lost by day 2, at the peak of the inflammatory response (r_s = 0.08, p = 0.72), as shown in figure 11.3. By day 5 urine DMA and ADMA concentrations were once again correlated (r_s = 0.42, p = 0.07), as shown in figure 11.4

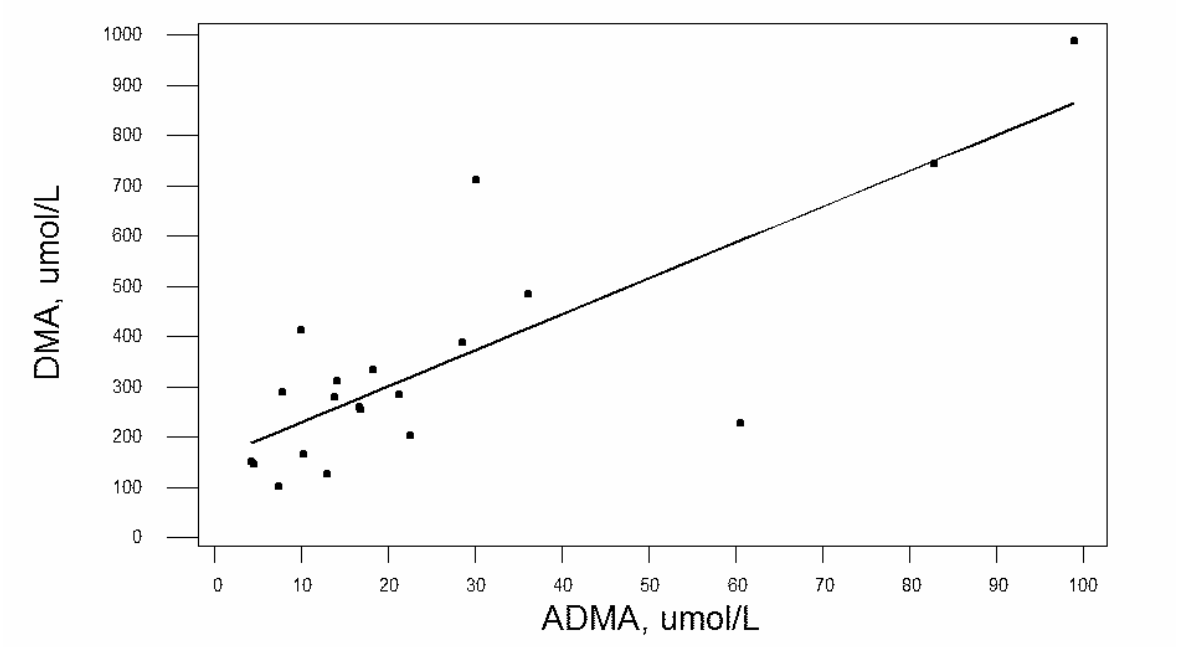


Figure 11.2: Relationship between urinary concentrations of DMA and ADMA on day 0

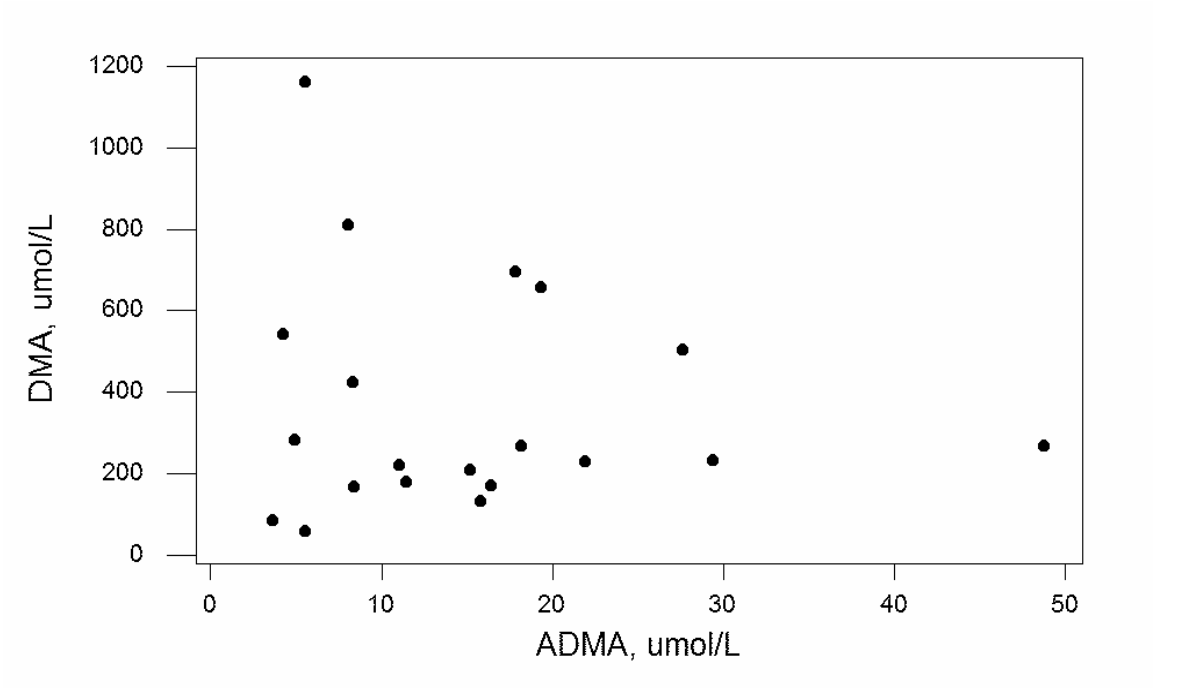


Figure 11.3: Relationship between urinary concentrations of DMA and ADMA on day 2

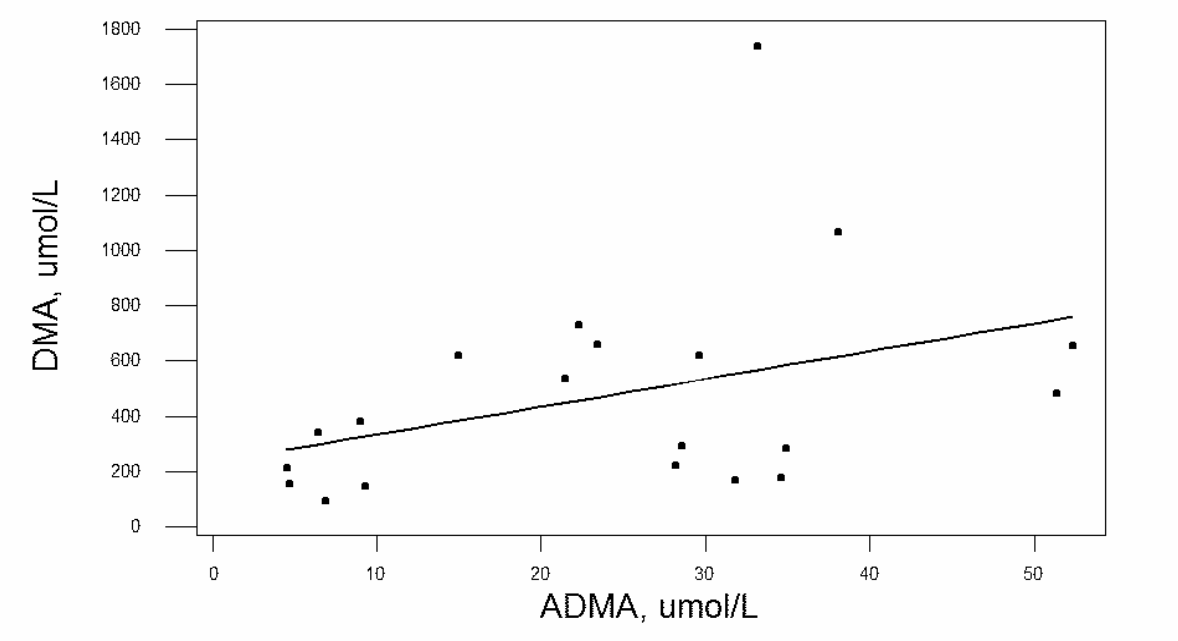


Figure 11.4: Relationship between urinary concentrations of DMA and ADMA on day 5

No significant correlation was seen between plasma ADMA concentration and the urinary excretion of DMA at baseline (day 0: r_s = -0.12, p = 0.62). Similarly, no correlations were noted on any of the post-op days.

11.3 Urine ADMA and SDMA excretion during the acute inflammatory response

11.3.1 Urine ADMA excretion

Urine ADMA concentrations, expressed as ratios to creatinine, across the study period are shown in table 11.2. The Friedman test showed a significant change in ADMA concentrations across the study period (p < 0.0001). Mann Whitney U test comparisons showed a difference between days 0 and 2 which

was of borderline statistical significance (p = 0.07). As ADMA concentrations at day 0 were not normally distributed, the day 0 and 2 results were logarithmically transformed and were normally distributed following transformation (Anderson Darling test, day 0 p = 0.60, day 2 p = 0.22). A paired T test on the log transformed results showed a significant difference (p = 0.008), confirming a significant reduction in urine ADMA at day 2 compared with day 0 (median 2.95 vs 3.95 μmol/mmol creatinine). This represented the nadir of urine ADMA across the study period. The Mann Whitney U test showed a significant increase in ADMA at day 4 compared with day 2 (median 4.95 vs 2.95 μmol/mmol creatinine , p = 0.03), but no significant difference compared with day 0 (p = 0.28). The ADMA on day 5 was significantly higher compared with day 2 (median 5.15 vs 2.95 μmol/mmol creatinine, p < 0.001), and showed a statistically borderline change compared with day 0 (Mann Whitney U test, p = 0.07). Given their non-normal distribution, day 5 results were logarithmically transformed and found to normally distributed following transformation (Anderson Darling test, p = 0.68). A paired T test on the log transformed results showed a significant difference (p = 0.007), confirming a significant increase in urine ADMA at day 5 compared with day 0 (median 5.15 vs 3.95 μmol/mmol creatinine).

At day 0, in the absence of an inflammatory response, plasma and urine ADMA concentrations were moderately, but non-significantly correlated (r_s = 0.33, p = 0.16), as shown in figure 11.5. There was a significant correlation seen on day 2 post-op (r_s = 0.51, p = 0.02), as shown in figure 11.6. However no correlations were seen on any of the other post-op days (days 1, 3, 4 and 5: p values all > 0.35).

Time point	ADMA, μmol/mmol creat	SDMA, μmol/mmol creat	ADMA:SDMA ratio
Day 0	3.95 (3.15 – 4.88)	3.50 (2.63 – 4.30)	1.15 (0.98 – 1.22)
Day 1	3.65 (3.10 – 5.08)	4.90 (3.88 – 5.85)	0.87 (0.67 – 1.02)
Day 2	2.95 (2.02 – 4.55)	3.80 (2.92 – 5.03)	0.77 (0.66 – 1.00)
Day 3	3.65 (2.18 – 4.75)	4.55 (2.88 – 5.40)	0.87 (0.78 – 0.99)
Day 4	4.95 (2.25 – 6.80)	4.75 (3.43 – 6.00)	0.96 (0.89 – 1.15)
Day 5	5.15 (3.53 – 6.43)	4.60 (4.08 – 5.48)	1.10 (0.89 – 1.29)

Table 11.2: Median (inter-quartile range) AMDA and SDMA:creatinine ratios and ADMA:SDMA ratios during study period

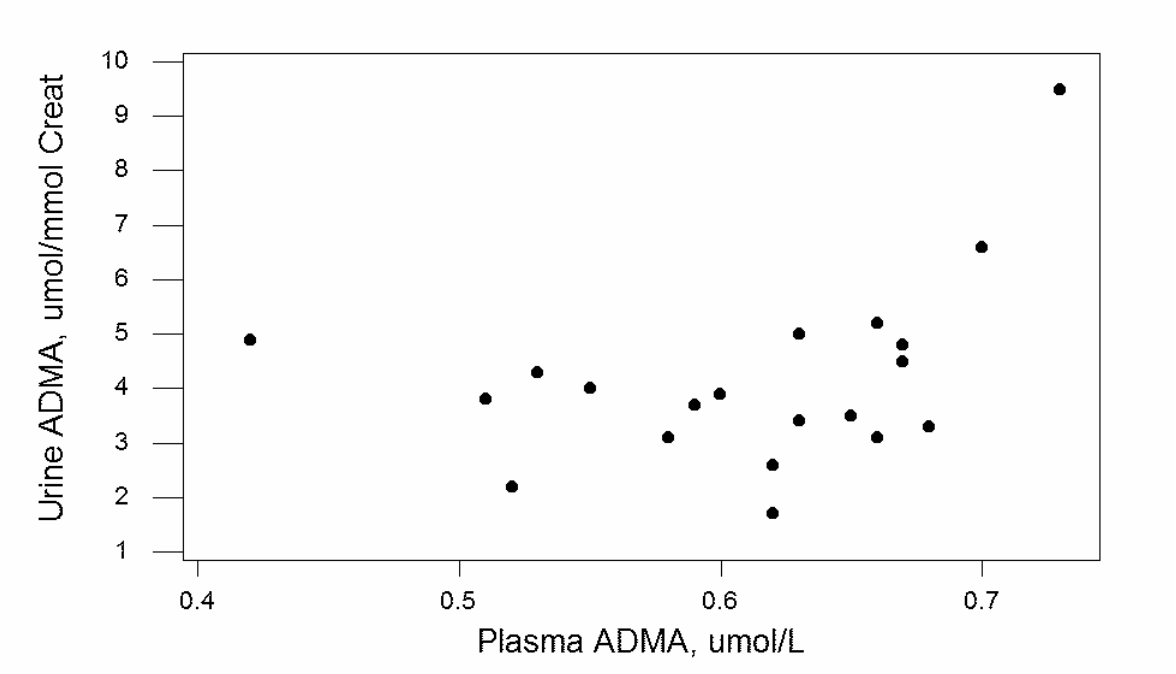


Figure 11.5: Relationship between plasma ADMA concentration and urine ADMA: creatinine ratio on day 0

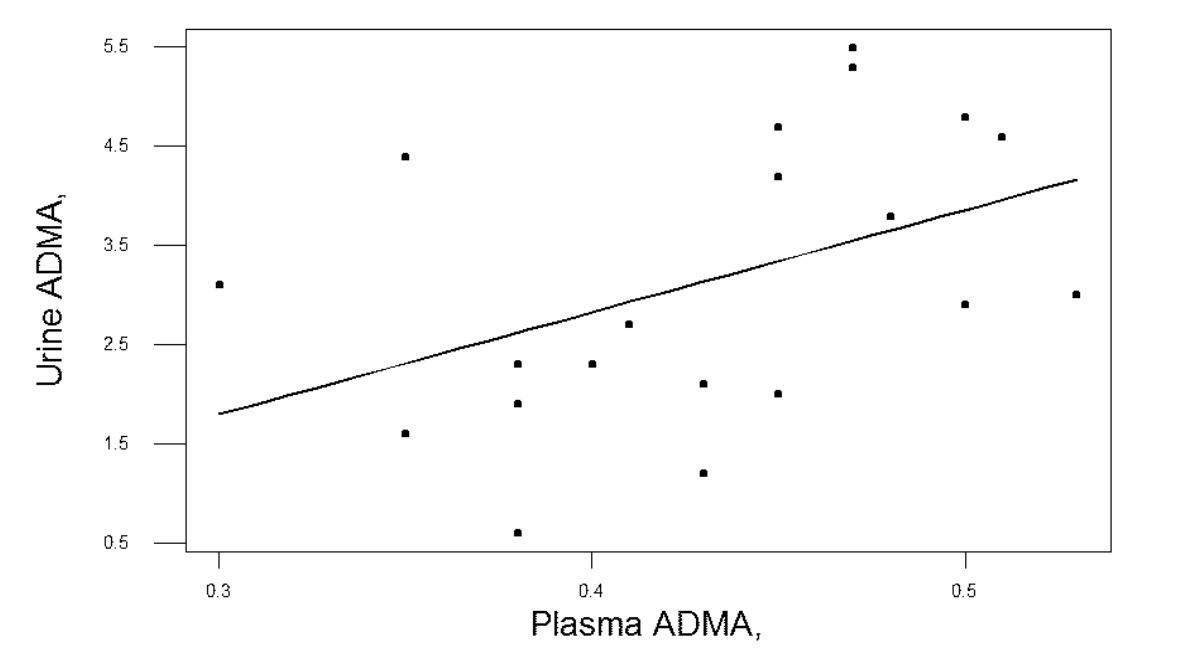


Figure 11.6: Relationship between plasma ADMA concentration and urine ADMA: creatinine ratio on day 2

11.3.2 Urine SDMA excretion

The Friedman test showed a significant change in urine SMDA:creatinine ratio during the study period ($p = 0.008$). Mann Whitney U test comparison showed a significant increase at day 1 (median 4.90 vs 3.50 μmol/mmol creatinine, $p = 0.004$). This difference remained significant at days 3, 4 and 5 post-op compared with day 0 ($p = 0.05$, 0.03 and < 0.01 respectively).

No significant correlations were seen between plasma SDMA concentrations and urine SDMA:creatinine ratios at day 0 ($r_s = 0.18$, $p = 0.45$), as shown in figure 11.7. A similar lack of correlation was seen on each of the post-operative days.

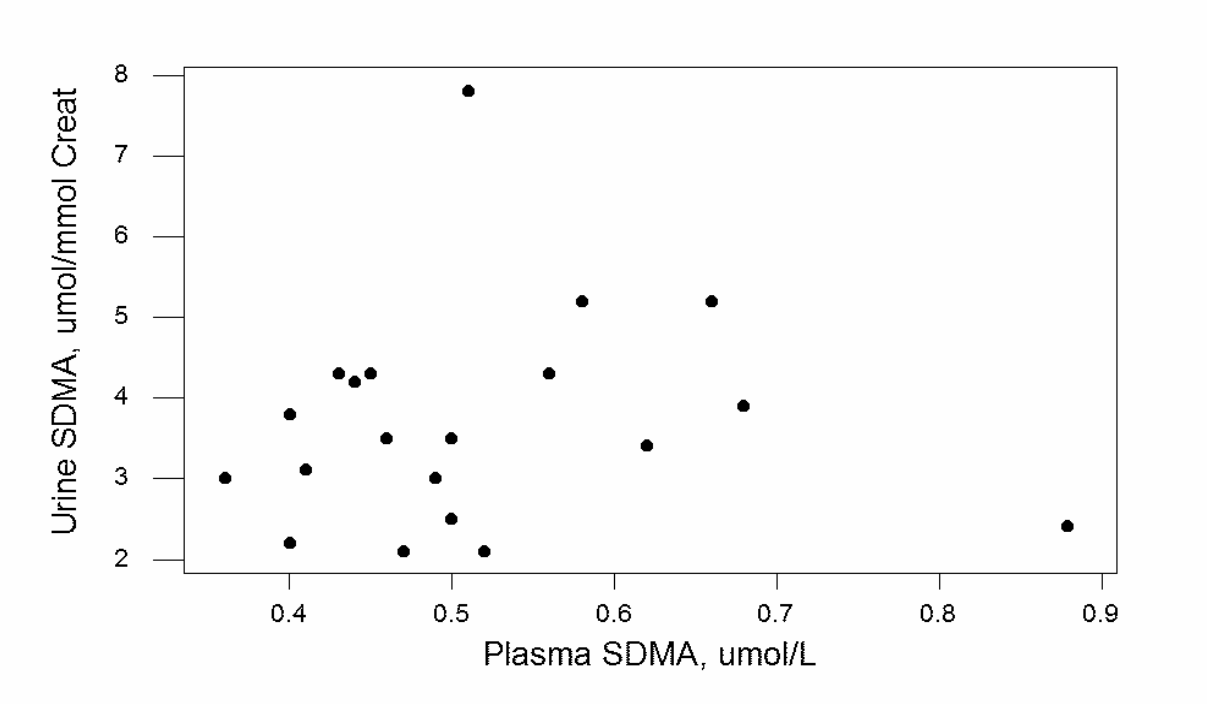


Figure 11.7: Relationship between plasma SDMA concentration and urine SDMA: creatinine ratio on day 0

11.3.3 Relationship between urine ADMA and SDMA concentrations

In spite of the differential changes in urine ADMA and SDMA excretions described, they remained very strongly correlated through the study period. The correlation at day 0 is shown in figure 11.8 ($r_s = 0.95$, $p < 0.001$). The relationship remained significant during the inflammatory response as illustrated by the values on day 2, in figure 11.9 ($r_s = 0.90$, $p < 0.001$). This demonstrates a similar relationship in the non-inflamed and inflamed states, and remains despite no similar correlations between the concentrations of ADMA and SDMA in plasma.

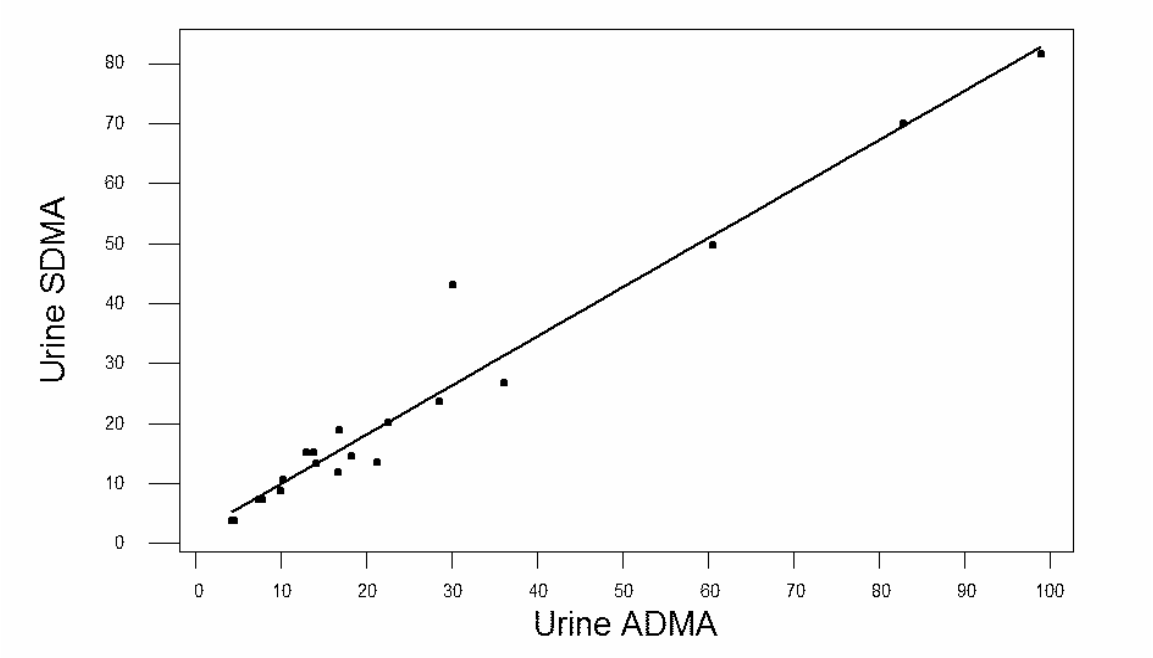


Figure 11.8: Relationship between urinary ADMA and SDMA concentrations on day 0

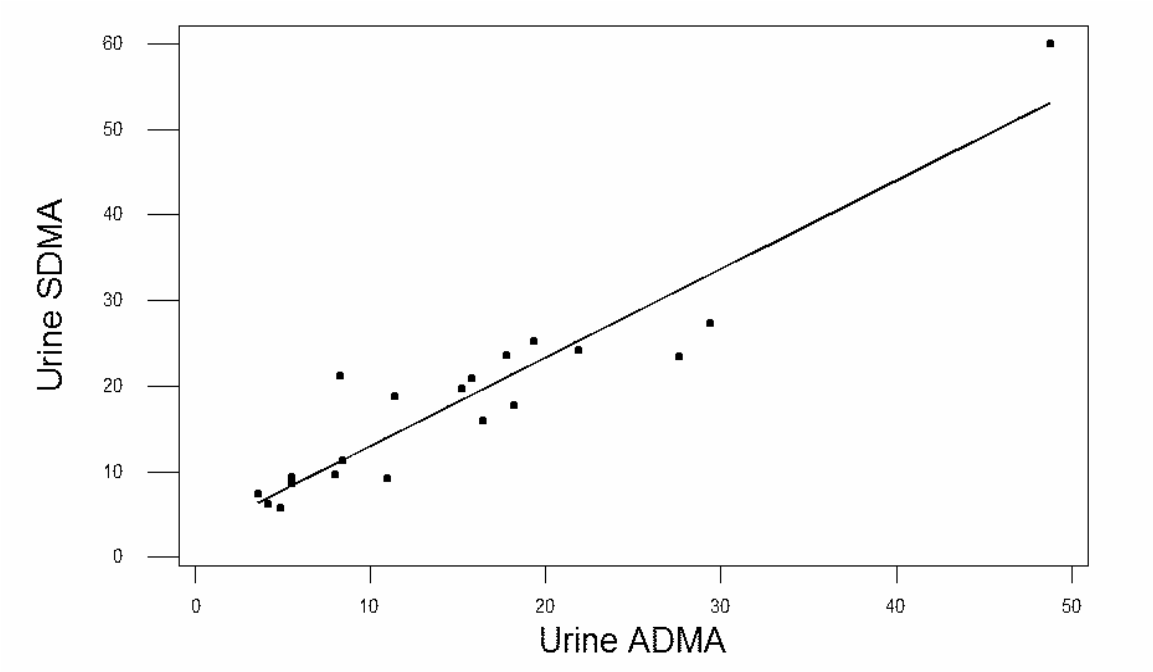


Figure 11.9: Relationship between urinary ADMA and SDMA concentrations on day 2

11.3.4 Urine ADMA:SDMA ratio

The median and inter-quartile range ratio of ADMA to SDMA concentrations in urine are shown in table 11.2. The Friedman test demonstrated a significant change across the study period ($p < 0.0001$). The Mann Whitney U test confirmed a significant decrease at day 1 from baseline (median 0.87 vs 1.15, $p = 0.0002$). The ratio remained lower than baseline at day 3 ($p = 0.002$), and returned to baseline levels by day 5 (median 1.10 vs 1.15, $p = 0.84$).

The ADMA:SDMA ratios in plasma and urine were normally distributed, and so linear regression was used to determine their relationship. A significant correlation was seen at day 0, in the non-inflamed state ($R^2 = 0.77$), as shown in figure 11.10. Similarly significant correlations were seen between plasma and urine on each of the post-operative days, with day 2 illustrated in figure 11.11, representing the peak of the inflammatory response ($R^2 = 0.93$).

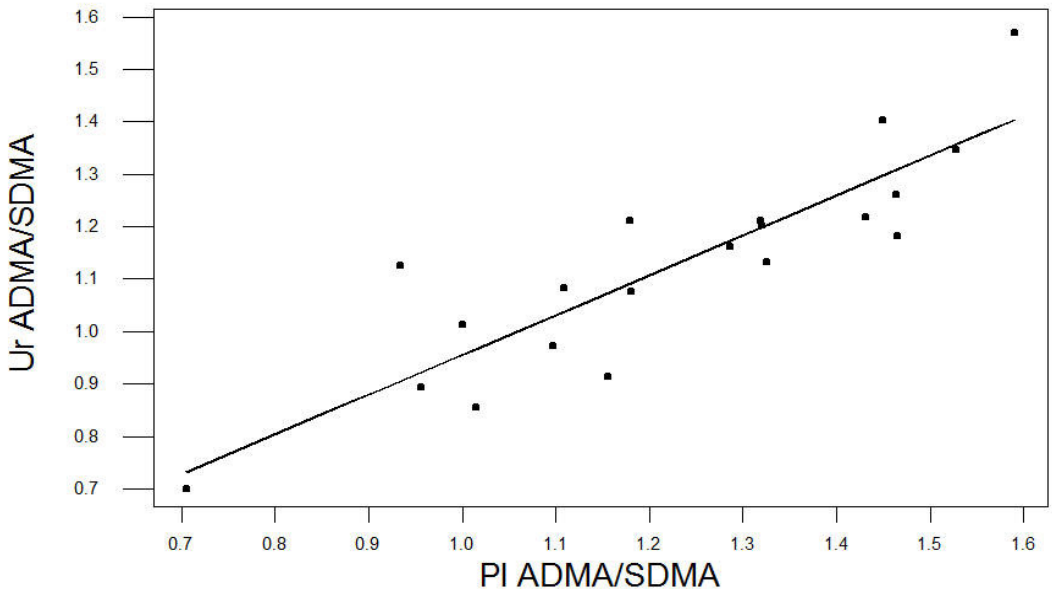


Figure 11.10: Relationship between ADMA:SDMA ratios in plasma and urine on day 0

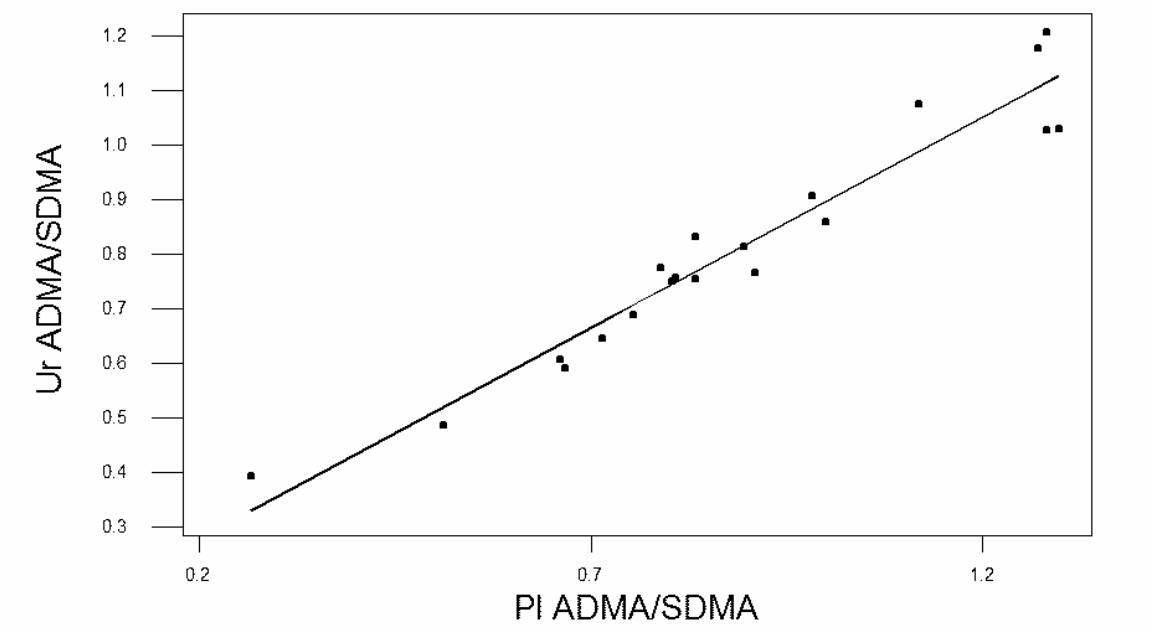


Figure 11.11: Relationship between ADMA:SDMA ratios in plasma and urine on day 2

11.3.5 Urine fractional excretions of ADMA and SDMA

To gain further insight into the renal handling of ADMA and SDMA, their fractional excretions (FE) were calculated. These were done according the formula:

$$FE = (\text{Urine "X"} \times \text{Serum creatinine}) \,/\, (\text{Plasma "X"} \times \text{Urine creatinine} \times 1000)$$

where "X" is ADMA or SDMA in μmol/L, and serum and urine creatinine concentrations are in μmol/L and mmol/L respectively.

FEs are shown in table 11.3. The Mann Whitney test revealed a significant increase in the FE of ADMA was seen on day 1 compared with day 0 (median 0.72 vs 0.49, p = 0.001). On the subsequent post-operative days no statistically significant differences compared with day 0 were noted, until day 5 at which point the increase from day 0 was significant (median 0.58 vs 0.49, p = 0.01).

A very similar pattern was seen for the FEs of SDMA. The Mann Whitney test revealed a significant increase on day 1 compared with day 0 (median 0.79 vs 0.54, p = 0.008). On post-operative days 2 to 4 no statistically significant differences compared with day 0 were noted, until day 5 at which point the increase from day 0 was significant (median 0.61 s 0.54, p = 0.01).

The FEs for ADMA and SMDA were found to be normally distributed, and so linear regression was used to examine their relationship. A strong correlation was noted between the FEs of ADMA and SDMA at day 0 (R^2 = 0.84), as seen in figure 11.12. This association persisted on each of the post-operative days, with day 2 illustrated in figure 11.3 as representative of the inflammatory response (R^2 = 0.89)

Time point	ADMA fractional excretion	SDMA fractional excretion
Day 0	0.49 (0.41 – 0.57)	0.54 (0.44 – 0.64)
Day 1	0.72 (0.55 – 0.94)	0.79 (0.52 – 0.88)
Day 2	0.54 (0.46 – 0.62)	0.59 (0.54 – 0.67)
Day 3	0.59 (0.43 – 0.75)	0.64 (0.50 – 0.81)
Day 4	0.53 (0.45 – 0.76)	0.62 (0.52 – 0.71)
Day 5	0.58 (0.53 – 0.73)	0.61 (0.59 – 0.82)

Table 11.3: Median (inter-quartile range) urinary fractional excretions of ADMA and SDMA during study period

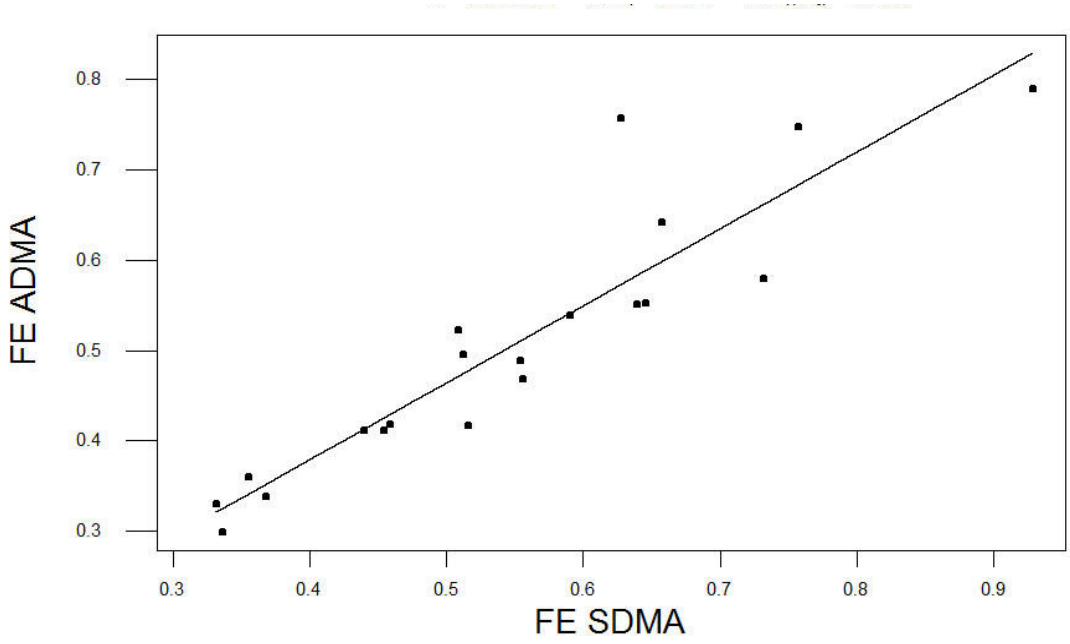


Figure 11.12: Relationship between urinary fractional excretions of ADMA and SDMA on day 0

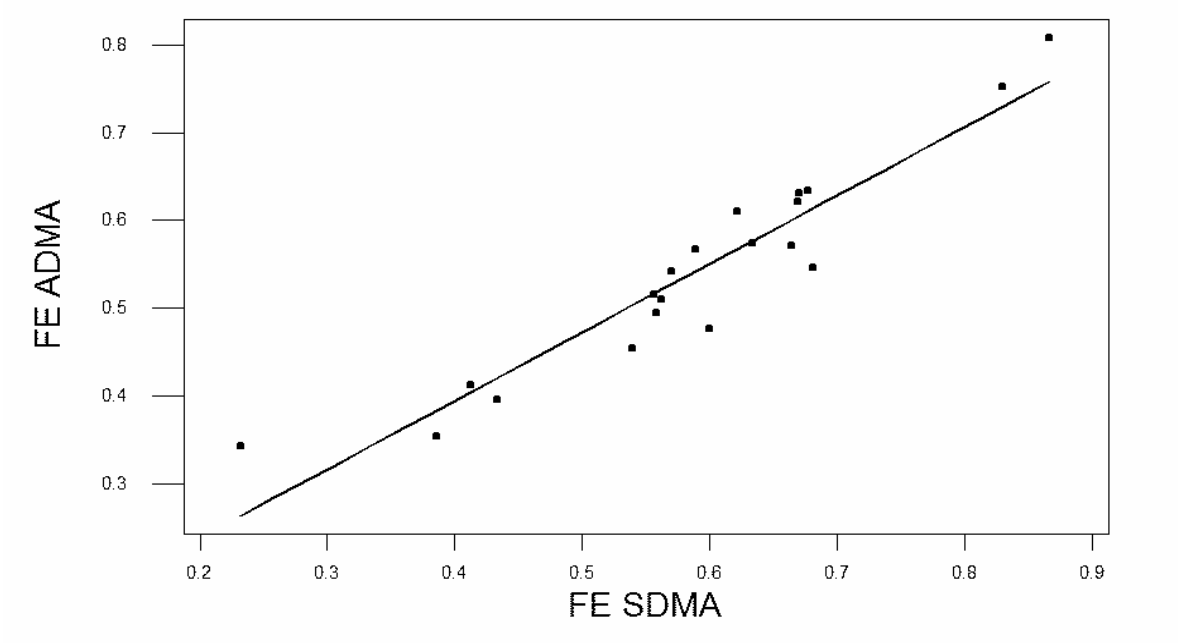


Figure 11.13: Relationship between urinary fractional excretions of ADMA and SDMA on day 2

11.4 Urine nitrate excretion during the acute inflammatory response

Median and inter-quartile range concentrations of urine nitrate, expressed as ratios to creatinine, are shown in table 11.4. The Friedman test suggested a change of borderline significance across the study period ($p = 0.089$). The Mann Whitney U test suggested a decrease in nitrate concentrations at day 2 compared with day 0, which just failed to reach statistical significance (median 55 vs 73 μmol/mmol creat, $p = 0.086$). Closer examination of the data revealed the day 2 concentrations to be non-normally distributed (Anderson Darling test, $p = 0.049$), with normal distribution at day 0 (Anderson Darling, $p = 0.13$). Logarithmic transformation failed to render the day 2 concentrations normally distributed, thus the data were examined for outliers; following the removal of one data point which exceeded the mean by > 3 standard deviations, the data were normally distributed (Anderson Darling, $p = 0.12$). Application of a paired T test to the resultant data

revealed a significant decrease in nitrate concentrations at day 2 compared with day 0 (mean 58 vs 90 μmol/mmol creat, p = 0.002). By day 5 the nitrate excretion had returned to baseline levels (median 78 vs 73 μmol/mmol creat, p = 0.78).

Time point	Nitrate, μmol/mmol creat
Day 0	73 (45 – 124)
Day 1	61 (36 – 102)
Day 2	55 (41 – 74)
Day 3	58 (35 – 75)
Day 4	65 (45 – 90)
Day 5	78 (49 – 110)

Table 11.4: Median (inter-quartile range) nitrate:creatinine ratios during study period

11.5 Summary of findings

The urinary excretion of DMA, the major metabolite of ADMA catabolism, was unchanged during the early phase of the acute inflammatory response, suggesting that increased DDAH-mediated catabolism was not responsible for the early decrease in plasma ADMA concentration. DMA excretion increased significantly at day 5 post-operatively, coinciding with the return of plasma ADMA concentration to baseline values.

The urinary excretion of unchanged ADMA decreased at day 2 post-op, coinciding with the nadir of plasma ADMA concentration. At day 5, on the return of plasma ADMA to baseline levels, the urinary excretion of ADMA was higher than at baseline. The urinary SDMA excretion was increased during the post-operative period, although no changes in plasma SDMA concentration were seen. No direct relationship between plasma and urine ADMA concentrations was found, at least in the non-inflamed state, although there was a strong correlation between the ratios of ADMA:SDMA in urine and plasma. The significant correlation between plasma and urine ADMA concentrations on day 2 was not replicated on any of the other post-op days, but occurred at the nadir of plasma ADMA concentration. The urinary fractional excretions (FE) of ADMA and SDMA were very similar in absolute terms and strongly correlated, both in the non-inflamed state on day 0 and throughout the inflammatory response on the post-operative days. The FEs of both ADMA and SDMA were increased on day 1 compared with baseline, and also at day 5.

The urinary excretion of nitrate decreased during the post-operative acute inflammatory response, reaching a nadir on day 2, coinciding with the peak CRP and nadir ADMA concentrations. This had returned to baseline levels by day 5.

Chapter 12

Discussion

12.1 HPLC method for ADMA measurement

The analytical method described in chapter 2 has been optimised and validated for the simultaneous measurement of arginine, homoarginine, ADMA and SDMA in plasma, urine and other biological fluids. There are certain advantages over previously described methods. First, the novel description of a non-endogenous internal standard offers the obvious advantage of increased precision and accuracy. However, its major advantage lies in permitting the measurement of endogenous homoarginine and MMA, as these two compounds are the usual internal standards employed in HPLC methods for dimethylarginine analysis[204-211]. Although having an unclear physiological role, it is clear that homoarginine is present in concentrations of around 5 – 6 times that of ADMA in human plasma, and has recently attracted interest as a potentially important molecule. In normal pregnancies plasma homoarginine concentration increases in the latter trimesters and is associated with flow-mediated dilatation[201]. Moreover, decreased concentrations have been independently associated with adverse outcomes, including mortality and fatal strokes in patients undergoing angiography[202,269], sudden cardiac death and heart failure in haemodialysis patients[203], and mortality in patients with liver cirrhosis[270]. Thus, the ability to measure homoarginine is likely to be of increasing interest. MMA, though present in small concentrations in plasma, has a concentration 10 fold higher in endothelial cells[51], and measurement of its endogenous concentrations may thus be of interest in cellular studies. Moreover, MMA has been used as part of an NO clamp in experimental studies, thus producing an artificially high concentration in plasma samples[271]. The selection of MEA as the internal standard thus avoids the undesirability of utilising the endogenous compounds homoarginine and MMA for this purpose.

N-propyl-L-arginine (NPA) is another non-endogenous compound that has also been utilised in HPLC analysis of dimethylarginines[209,272]. One of these methods employed phenyl columns[209], which offer less stability and reproducibility than C18 columns. During method development, the use of NPA on a C18 column was investigated, and it was found difficult to achieve separation of NPA from SDMA; moreover chromatographic separation was found to be sensitive to very small variations in mobile phase composition which also affected reproducibility. One published method describes a method using NPA on a C18 column and using 4-Fluoro-7-nitro-2,1,3-benzoxadiazole as a derivatising agent[272]. However, in order to achieve chromatographic separation a complicated gradient elution was required, as was maintenance of the analytical column at 40˚C. Moreover, the run time was 32 minutes, which is not an improvement on the method described in this thesis. It is also notable that the authors did not describe the measurement of arginine and homoarginine, and it is not clear, therefore, whether these can be reliably quantified using that method.

Second, it was possible to achieve baseline resolution of all compounds of interest under isocratic conditions, including arginine, homoarginine, ADMA, SDMA, and MMA if required. It is desirable to measure these compounds simultaneously given the importance of arginine and its interaction with ADMA in determining NO production, and the increasing interest in homoarginine. The inability to measure these compounds is a major drawback of ELISA. Although gradient elution can be utilised with programmable solvent delivery systems there is an advantage in the relative simplicity of an isocratic method.

A disadvantage of the described method is the relatively long analysis time. Under the conditions described attempts to decrease elution times by further manipulating the mobile phase were found to detrimental to the resolution of ADMA and SDMA. However, this could be improved by using monolithic

columns while retaining the advantages already discussed. HPLC columns generally consist of very tightly packed silica particles, with smaller particle sizes used in order to decrease diffusion distances and improve performance. This is at the expense of increased back pressure, which is inversely proportional to the spaces between the particles. Monolithic columns consist of a porous single piece rod, and are much more porous, allowing operation at higher flow rates without excessive pressure. Teerlink has described an HPLC method for dimethylarginine analysis using a monolithic column, allowing isocratic elution and a run time of 10 minutes (albeit using MMA as the internal standard)[212]. It should be possible to employ monolithic columns to the method described in this thesis; use of the internal standard would likely extend the run time to around 15 minutes, which is a significant improvement. Another option would be to employ MMA as the internal standard, which was not found to be detrimental to accuracy or precision, and could shorten the run time to around 25 minutes.

In summary, the described analytical method permits the precise and reliable simultaneous measurement of arginine, homoarginine, MMA, ADMA and SDMA in plasma, urine and biological fluids under isocratic conditions.

12.2 Reference intervals for dimethylarginines and their clinical utility

The sample size of 100 individuals fell slightly short of the 120 recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) which has been calculated with a view to ensuring confidence at the upper and lower reference limits[273]. This could be considered a weakness, although as the group was not sub-divided and the reference intervals obtained were in agreement with other studies involving larger numbers of subjects it was not felt that this slight shortfall would unduly influence the results obtained. As no significant gender-related differences were seen for

homoarginine, ADMA and SDMA concentrations there was felt to need to describe gender-specific reference intervals. While arginine concentrations were overall slightly higher in males in the present study, the biological significance of this was not certain as other studies have shown no significant differences[237].

Blood sampling was performed under non-fasting conditions in the present study. It has been shown that plasma ADMA and SDMA concentrations are not significantly different fasting and post-prandially, which under-pinned the essentially pragmatic decision to collect samples non-fasting[274]. Arginine concentrations increase at 2 and 4 hours post-prandially, which could contribute some variability to the measurements obtained for this analyte[274]. However this wasn't the main aim of the study, and in any case the arginine values are in broad agreement with other published values from fasting samples [237]. However, some caution should be exercised in ascribing biological significance to the apparent gender difference in arginine concentrations.

The reference values for ADMA described here are in broad agreement with other well validated chromatographic methods, which seem to suggest a plasma ADMA concentration of around 0.50 μmol/L as close to the healthy population mean[233,236,237]. The slightly lower mean concentration in the present study (0.45 μmol/L) might represent the overall average younger age of the cohort. Additionally some of the published population studies previously mentioned contain significant numbers of individuals who are not as such "healthy", for example the study of Teerlink[234] in which up to a third of the population had impaired glucose tolerance or diabetes as well as a proportion with chronic kidney disease. These conditions would be expected to be associated with higher ADMA concentrations.

The present data fails to replicate the association of ADMA with age seen in other studies[233,235-237]. This might be related to the size of the cohort, which is arguably too small to allow sub-group comparisons. In particular the number of women in the post-menopausal age range was small (n = 12). Obtaining data from a larger number of subjects to allow sub-group comparisons would be required to further investigate the effects of age and menopausal status, although, as previously noted, the differences are small.

The intra- and inter-individual components of biological variation are of importance in determining the spread of values and hence the likely utility of reference intervals in denoting abnormality or disease in an individual when a single measurement falls outwith the range. In the present study the indices of individuality (II) for ADMA and SDMA are below the threshold of 1.4 which has been proposed as the ratio suggesting utility of reference intervals. This is better understood graphically by examining the box plots of dimethylarginine concentrations encountered in the biological variation study; for most of the individuals studied, their range of values are entirely encompassed within the overall range. This is especially true for SDMA, which is not surprising given its relationship to renal function. Therefore, although the described reference intervals provide something of an index of "normality" in health, it is clear that results within or without them cannot be taken to indicate the absence or presence of clinically significant disease. An alternative approach is to use ADMA concentrations to complement traditional approaches to cardiovascular risk assessment, given what has already been said about its relationship to risk factors and as a prognostic marker. However, at least at the moment, the direct clinical utility of ADMA measurement is uncertain, not least owing to the lack of an intervention to modify its concentration.

12.3 Plasma ADMA concentrations during the acute inflammatory response

The major finding of the knee arthroplasty study was a rapid and significant decrease in plasma ADMA concentration during the early phase of the acute inflammatory response. In order to assess the likely reasons for this it is important to consider the various factors which influence the plasma concentration, namely its rate of production (within cells), metabolism at its site of production, export from cells (via CATs), and uptake (again via CATs) into other organs for clearance. Perturbations in the plasma concentration could, therefore, represent changes in any or all of these processes. Broadly speaking, an abrupt decrease as seen in this study suggests increased clearance from plasma rather than a reduction in synthesis. Previous studies have suggested that inflammation can stimulate DDAH-mediated ADMA clearance. In one, vascular smooth muscle cells were exposed to interleukin-1β, a key cytokine of the inflammatory response, and demonstrated an increase in DDAH expression and activity[175]. In another, endotoxaemia in rats was associated with a significant increase in hepatic fractional excretion of ADMA (41.0 vs 27.7%)[174], which it was assumed reflected metabolism. However, the effects of different factors on DDAH activity make the net effect of inflammation difficult to predict: for example, cytokines have been shown to induce DDAH activity, while factors such as oxidative stress, hyperglycaemia and NO itself have a negative effect[20]. It was anticipated that increased DDAH-mediated catabolism would lead to an increase in urine DMA excretion. In fact, urine DMA excretion remained unchanged during the first 4 post-operative days, while the decrease in plasma ADMA concentration was evident as early as 12 hours post-op. Once produced DMA is expected to appear rapidly in urine given its short elimination half-life of around 7 hours and almost complete excretion of an orally administered dose within 24 hours[249]. The results, therefore, suggest that increased DDAH-mediated catabolism was not responsible for the described changes in plasma ADMA concentration. An increase in DMA excretion was finally seen by day 5, coinciding with the return of plasma concentration to baseline levels.

In the absence of clear evidence for increased metabolism, it could be hypothesised that the decrease in plasma ADMA concentration reflects increased redistribution intracellularly. The data for urine ADMA excretion could provide support for this: although no direct correlation between plasma and urine ADMA concentrations was found, there was a significant decrease in urine ADMA excretion on day 2, and a significant increase on day 5, in absolute terms. The baseline (day 0) ADMA concentrations were very similar to values of 3.4 and 4.1 μmol/mmol creatinine as reported by others in healthy non-inflamed individuals[221,275]. While SDMA is excreted solely by the kidney, only a relatively small fraction of ADMA is excreted unchanged. It is assumed that the urinary excretion of ADMA, in absolute terms, is related to the amount presented to the kidney, and by GFR. This assumption is perhaps justified by the finding urinary fractional excretion (FE) rates of (unchanged) ADMA and SDMA which are numerically very similar and strongly correlated throughout the study period. Although there is an abrupt and transient increase in the FEs of both ADMA and SDMA on day 1, this is unlikely to account for the decrease in plasma ADMA concentration, given the absolute reduction in urine ADMA concentration, and the lack of similar changes in plasma SDMA concentration. Therefore, the described changes in urinary ADMA excretion are likely a simple reflection of a reduction in the circulating ADMA pool on day 2 and a subsequent increase on day 5. It is also notable that the correlation between urinary DMA and ADMA concentrations in the baseline, non-inflamed, state was lost during the inflammatory response, further suggesting a change in the distribution of ADMA between plasma, which is the source of urinary ADMA, and cells, which are the source of DMA. Finally, a decrease in urinary nitrate excretion on day 2 suggested a reduction in NOS production, which could also be consistent with increased cellular partitioning; certainly had a reduction in cellular ADMA concentration occurred, an increase in NOS activity, if anything, might have been anticipated.

There have been very few studies relating plasma and intracellular concentrations of ADMA, most published data focussing on plasma concentrations with the implicit assumption that they reflect concentrations intracellularly. However, it is clear that intracellular ADMA concentrations can be several times higher than plasma in a variety of tissues, and can increase in response to injury and inflammation. In a rabbit model of critical illness, injury was associated with higher concentrations of ADMA in liver compared with healthy control animals[276]; this was especially true in hyperglycaemic animals who had lower liver activities of DDAH. In this same model concentrations of SDMA correlated with concentrations in kidney, liver and myocardium, with no such direct relationship seen between tissue and plasma concentrations of ADMA[276]. Further analysis on pooled data from this same model showed correlations between ADMA concentrations in various tissues, but no strong relationships between ADMA concentrations in plasma and kidney, skeletal muscle or myocardium; plasma and liver ADMA were correlated, but this lost significance in a multivariable model[277]. In this model, it is also interesting to note that DDAH activities are poorly correlated with intracellular ADMA concentrations, but are a much stronger determinant of plasma ADMA concentration, activities in liver, kidney and muscle together accounting for around 50% of the variation in plasma ADMA[277]. Further insights have been gained from studies in models of lung injury. Sheep given a burn injury from smoke inhalation had significantly increased lung tissue concentrations of ADMA 3 weeks following the injury, associated with a significant reduction in DDAH-2 activity; DDAH-1 activity was significantly increased, making it uncertain which isoform of DDAH is of greater importance[278]. Interestingly, in this same model the plasma arginine concentration decreased rapidly following the injury, returning to baseline levels about one week following the injury, similar to the findings in the present study; unfortunately plasma concentrations of ADMA and SDMA were not reported[278]. In a mouse of model of endotoxin-induced lung injury similar findings were reported; as early as 2 hours post-injury a significant increase in lung ADMA occurred (12.13 vs 7.53 nmol/gww), with a further increase at 12 hours (19.10 nmol/gww)[279]. In this study DDAH-1 and DDAH-2 protein

expression was unchanged, but a significant decrease in DDAH activity was measured; moreover, at 12 hours iNOS expression and nitric oxide metabolites were increased[279]. Again, plasma dimethylarginine concentrations were not reported. Even in the basal, non-inflamed, state lung tissue contains significant quantities of protein-incorporated ADMA and free ADMA concentrations similar to that in heart, but significantly lower than liver and kidney[280]. Interestingly, both mice and humans display similar ADMA:SDMA ratios in plasma and fluid from bronchoalveolar lavage, suggesting similar ratios intra- and extracellularly[280]. This study also demonstrated significantly greater expression of DDAH-2 than DDAH-1 in lung tissue.

While the studies described in the foregoing paragraph suggest that intracellular concentrations of ADMA can increase significantly during tissue injury and inflammation, the relationship between plasma and intracellular concentrations is not clear. However, it is known that endothelial cells in the basal state contain up to 10 times the ADMA concentration found in plasma[34,281]. Furthermore, vascular injury results in an almost 4-fold increase in the intracellular concentrations of ADMA and MMA, sufficient to cause eNOS uncoupling and impairment of vascular relaxation[281]. Stimulated peripheral blood mononuclear cells generate a significant increase in ADMA concentration, something which is inhibited by the anti-inflammatory compound salicylic acid[281].

The evidence from the studies discussed suggests that DDAH inhibition in inflammatory states could lead to intracellular ADMA accumulation. However, transport through system y^+ CATs is another potential mechanism for this concentration. CATs permit bidirectional movement of basic amino acids into and out of cells. Methylarginines are good substrates for CATs, with ADMA, MMA and SDMA all capable of transport in competition with arginine[52]. Therefore, in addition to inhibition of NOS, methylarginines, including SDMA, can affect NOS activity by reducing availability of arginine. CATs are widely

distributed although as such do not lead to equilibrium between the intra- and extracellular compartments[163]. They exhibit the phenomenon of trans-stimulation in which high a concentration on one side of the transporter stimulates transport[266,282]. There is distinct tissue distribution, with CAT-1 expressed in virtually all tissues with the exception of the liver; CAT-2A, in contrast, is found mainly in the liver[266,282]. CAT-1 displays a Km for arginine of around 100 – 150 μM, while CAT-2A has a 10-fold lower affinity than CAT-1[266,282]. This may serve to ensure preferential uptake of arginine by cells expressing eNOS, rather than by the liver for degradation by arginase. Indeed, the co-localisation of CAT-1 and eNOS may ensure the efficient delivery of arginine to the enzyme, and extracellular arginine concentrations are probably more important than intracellular concentrations in determining NOS activity in endothelial cells[283]. However, this also ensures access to eNOS by ADMA[155]. The demonstration of DDAH-2 in the cytoplasm and apical vesicles places all of the key players, namely NOS, DDAH and CATs in close proximity[14]. CAT-2B are inducible by inflammatory cytokines and are often found in tissues expressing iNOS, and have lower substrate affinity than CAT-1[190,266,282,284]. Knockout studies have shown that CAT-1 knockout is lethal, while the effect of CAT-2 knockout in mice is to reduce basal NO production by iNOS which leads to lung inflammation in the absence of pathological stimuli, related to reduced NO production in alveolar macrophages[285]. Other stimuli for CAT expression include tissue growth and repair, insulin and starvation, while oxidative stress can have an inhibitory effect[266]. There is evidence in rats that CAT-1 expression is also induced by inflammation in the kidney and lung in particular, and that this induction is rapid, within hours[284]. Human embryonic kidney cells over-expressing CAT-1 were found to be capable of significant increases in ADMA and arginine uptake, which amounted to an almost 9-fold increase in ADMA transport[285]. These studies confirmed the competitive nature of such transport, showing inhibition of ADMA uptake at high, but physiological, concentrations of arginine. It was also shown that these cells demonstrated increased efflux of arginine and ADMA from cells into transport buffer not containing them[285]. The role of CATs is highlighted

by experimental studies showing a rapid removal of arginine from the circulation following infusion, and the rapid clearance of infused ADMA which had a mean plasma half-life of 24 minutes in one human study[42,287]. The effect of insulin is also worthy of consideration in relation to the changes seen in the present study. Patients post knee arthroplasty demonstrated an insulin resistant state and a significant increase in plasma insulin as early as day 1 post-op. Insulin infusions, into both healthy subjects and type 1 diabetics, lead to a rapid and significant decrease in plasma ADMA and arginine concentrations with much less of an effect on SDMA[195,288]. Two further studies in type 1 diabetics showed lower ADMA concentrations compared with healthy controls, with one showing an increase in NO metabolites, and the other showing no relationship between plasma ADMA concentration and forearm blood flow[289,290]. Furthermore, hormone treatment in male to female transsexuals was associated with a reduction in plasma ADMA concentration, with speculation that reduced peripheral insulin sensitivity and consequent hyperinsulinaemia could be responsible for the effect[291]. The observed reductions in ADMA concentrations in critically ill patients receiving intensive insulin therapy has been proposed to be due to preservation of DDAH activity; however, CAT-mediated uptake could potentially be involved[193]. Thus, in the present study stimulation of CAT-mediated transport by inflammation and insulin remain possible contributory mechanisms for the observed changes in plasma ADMA concentration. However, the lack of similar changes in SDMA concentration is more difficult to explain, given that ADMA and SDMA should be equally good substrates for CATs[52]. It would also be difficult to anticipate this effect in isolation, as the hyperglycaemia and oxidative stress would likely also have an inhibitory effect on DDAH and thus ADMA metabolism[30].

There have been relatively few human studies examining plasma dimethylarginines in acute inflammation, and none documenting a sharp decrease from a non-inflamed baseline. It has already been stated that high ADMA concentrations are associated with adverse outcomes in critical illness and may be related to multiple organ dysfunction. In these studies, the

effects of organ failure themselves are likely to a dominant factor in increasing ADMA concentrations, given the importance of liver and kidney function in particular for ADMA metabolism. However, the aim of the current study was to examine the effect of inflammation itself in the absence of significant organ dysfunction. One small study of patients with bacterial infections demonstrated an increase in plasma ADMA concentration on resolution of infection with no changes in SDMA, prompting the authors to speculate increased DDAH activity as a possible cause[188]. Endotoxaemia in humans led to a reduction in plasma arginine concentration and a reduction in the arginine:ADMA ratio, although the study period was only 3.5 hours, possibly too early to see changes in plasma ADMA concentration; however, the reduction in arginine concentration suggests acute uptake or consumption[186]. Further human studies have shown increases in ADMA concentrations during the recovery from sepsis, but not in septic shock[182], and low arginine concentrations in critically ill children which increase during recovery[292]. In another study patients undergoing abdominal surgery demonstrated a modest reduction in plasma ADMA concentration on day 1 post-op, compared with patients undergoing major hepatectomy who showed no change[17]. These findings are largely consistent with those in the present study.

One further possibility to consider is that of metabolic pathways other than DDAH for ADMA degradation. It has been shown that the enzyme alanine-glyoxylate aminotransferase 2 (AGXT2) metabolises ADMA in a mouse model of over-expression using an adenoviral expression vector[293]. Conversely, pharmacological inhibition of AGXT2 in mice was associated with increased concentrations of both ADMA and SDMA[294]. A further study in knockout mice demonstrated increased ADMA concentrations and hypertension[295]. However, the relevance of this pathway in man is uncertain, with DDAH-mediated catabolism likely to account for the majority of enzymatic ADMA clearance[22,296]. However, evaluation of this pathway in different physiological states, including inflammation would be of value in excluding significant non-DDAH mediated catabolism of ADMA in explaining the results from the present

study. Measurement of its metabolic product α-keto dimethylguanidino-valeric acid (DMGV) in urine has been successfully undertaken, providing a possible way of assessing significant changes in its metabolic activity[297].

To summarise, acute inflammation is associated with a rapid, significant and transient reduction in plasma ADMA concentration. While the influence of DDAH on plasma ADMA concentration is not in doubt, the results from the present study mitigate against a significant increase in DDAH activity, at least initially. Neither was increased renal excretion implicated, based on urine concentrations which were felt to simply reflect the ADMA concentration. The likeliest explanation is increased compartmentalisation intracellularly mediated by increased CAT uptake. No evidence was found to suggest increased accumulation within erythrocytes. A decrease in urine nitrate, reflecting reduced NOS activity, is consistent with this hypothesis. The later increase in DMA excretion is likely to represent remobilisation of the intracellular pool and transport to quantitatively important sites of DDAH metabolism: this does not necessarily mean that DDAH activity was increased *per se*, as the Km of DDAH means that increased amounts of ADMA presented to the enzyme will be rapidly metabolised. Certainly it is clear that in the inflamed state the plasma concentration is unlikely to be representative of the intracellular concentration. The differential responses of the dimethylarginines described in the present study raises the possibility of a physiological role for ADMA in regulating NOS activity during the early phase of acute inflammation.

12.4 Suggestions for further work

A large body of work, spanning over 20 years, has been produced providing strong evidence for ADMA as an endogenous inhibitor of NOS, and thus a molecule important in the pathogenesis of disease states characterised by

impairment of NO-mediated responses. This has included large epidemiological and prospective observational studies establishing the independent prognostic value of ADMA for cardiovascular events and mortality, and, increasingly, in the outcome of critically ill patients. Despite this work, however, fundamental questions remain regarding the basic biology of ADMA. Is it simply an "innocent" by-product of protein metabolism which acquires relevance only when some other condition leads to impairment of its metabolism, or is it part of a regulatory system controlling the production of NO under certain physiological conditions such as the inflammatory response? This question is of particular interest as excessive NO production has been proposed to be deleterious and a potential contributor to multi-organ dysfunction in critical illness. This latter proposal has led to trials of the related NOS inhibitor, MMA, in this patient group, unfortunately with adverse outcomes. If the work in this thesis points to intracellular compartmentalisation of ADMA during the acute inflammatory response, it may be speculated that this could potentially be associated with something approaching isoform-specific regulation of NOS, depending on the predominant sites of accumulation. It is suggested that this should be a key question for future research, and the central part of this is the relationship between extracellular, as exemplified by plasma, concentrations and those intracellularly, in various tissues. There is precious little published on this relationship, although that which exists suggests that intracellular concentrations are increased by inflammatory stimuli, although how this relates to the overall distribution of ADMA in these states is not known. Furthermore, most of the published work is in animal models, thus further work in humans is required, although the relative difficulty in obtaining relevant tissue samples, especially in a study involving serial measurements, is acknowledged. A recent publication reporting dimethlyarginine concentrations in peripheral blood mononuclear cells[298] – albeit only in healthy non-inflamed subjects – suggests a way forward, as these could be feasibly assessed serially, perhaps using a model similar to that described in this thesis. These cells would be more relevant than erythrocytes as they contain the necessary cellular machinery to transport and metabolise ADMA,

i.e. CATs and DDAH, as well as the inducible isoform of NOS. This work will be important in elevating ADMA from its status as a marker of disease to that of mediator, and thus its "life-cycle" from production to metabolism as a possible therapeutic target.

Homoarginine is a relative new-comer to the arginine field of research, in terms of possible biological interest in humans. Much fundamental knowledge regarding its basic biology, i.e. origin, metabolism and biological effects, is lacking as its low concentration relative to arginine has seemingly hitherto rendered it of little interest. However, a few recently published studies suggesting a predictive role for mortality following cardiovascular events and an association with blood pressure and vascular haemodynamics have suggested it worthy of further study. This should initially focus on its origin in humans and, along the lines suggested for ADMA, proceed to study of it intra and extracellular distribution and the factors determining this.

Appendix 1

Results from reference subjects (n = 100)

No	Sex	Age	Arginine µmol/L	Homoarg µmol/L	ADMA µmol/L	SDMA µmol/L	Chol mmol/L	LDL mmol/L	CRP* mg/L	Creat µmol/L
1	M	49	39.2	1.94	0.42	0.41	6.6	4.5	2.6	108
2	F	24	31.8	1.92	0.33	0.34	4.8	2.9	2.5	75
3	M	30	56.5	3.17	0.42	0.27	3.9	2.2	0.6	72
4	F	45	57.3	1.60	0.41	0.36	7.4	5.1	1.6	82
5	F	50	45.5	1.74	0.40	0.37	7.0	4.5	3.5	85
6	F	42	29.3	1.29	0.29	0.28	4.8	2.9	1.6	87
7	F	55	80.0	1.96	0.64	0.45	7.4	5.3	3.6	88
8	F	25	50.2	1.96	0.42	0.38	5.1	3.0	4.7	103
9	F	59	52.3	1.13	0.46	0.38	7.9	5.0	0.8	76
10	F	49	65.8	0.82	0.54	0.37	6.5	4.5	3.6	87
11	F	40	57.8	2.52	0.52	0.40	5.1	3.3	1.6	79
12	F	24	32.4	1.62	0.42	0.36	6.0	3.5	3.0	99
13	F	57	64.8	1.05	0.49	0.36	5.3	3.7	1.0	66
14	F	40	65.7	2.49	0.45	0.33	4.0	2.3	0.7	64
15	M	38	51.5	2.07	0.39	0.39	5.4	3.6	0.9	102
16	F	45	72.4	1.37	0.41	0.30	5.2	2.8	1.0	80
17	F	27	61.4	1.83	0.39	0.33	5.2	3.6	1.0	70
18	F	59	75.5	3.14	0.45	0.31	5.7	3.0	5.1	81
19	M	41	58.4	0.79	0.43	0.29	6.4	4.2	0.4	67

20	M	48	70.3	2.33	0.41	0.35	5.9	3.6	0.8	97
21	M	38	72.9	2.27	0.32	0.30	6.6	4.5	1.2	111
22	M	44	57.1	1.70	0.46	0.33	5.5	3.5	0.5	86
23	F	41	64.1	2.06	0.40	0.43	4.7	3.1	5.4	70
24	M	53	25.5	1.09	0.46	0.42	4.6	2.6	0.9	92
25	M	49	55.3	0.94	0.41	0.40	6.1	4.0	0.8	77
26	M	42	108.1	4.20	0.73	0.57	4.6	2.9	1.2	80
27	M	45	105.4	2.89	0.40	0.37	5.2	3.4	0.8	82
28	F	39	53.9	1.62	0.49	0.31	9.0	6.5	4.4	115
29	F	41	100.1	3.55	0.56	0.53	6.0	3.8	1.8	91
30	F	41	78.9	3.30	0.68	0.47	4.7	3.2	0.6	91
31	F	44	58.8	1.48	0.51	0.35	3.7	2.0	3.8	67
32	M	52	99.4	1.92	0.52	0.46	6.7	4.0	1.3	85
33	M	47	36.6	1.75	0.58	0.44	5.7	3.8	0.7	89
34	M	40	81.3	2.62	0.45	0.36	5.7	4.0	2.0	87
35	M	32	30.8	1.04	0.45	0.37	4.3	2.8	0.2	85
36	F	37	14.2	1.60	0.56	0.56	3.5	2.3	0.9	58
37	F	30	75.2	1.07	0.48	0.38	3.7	2.4	1.9	60
38	M	44	93.2	1.65	0.54	0.35	3.1	2.0	0.3	73
39	M	62	48.3	2.07	0.41	0.37	4.0	2.5	5.5	84
40	F	51	75.1	3.22	0.41	0.38	5.3	2.5	< 6.0	80
41	M	61	22.8	1.10	0.43	0.31	4.9	3.0	16.0	75
42	M	57	39.4	2.66	0.46	0.38	5.0	2.7	< 6.0	80

43	F	47	63.6	0.90	0.53	0.42	7.3	4.5	< 6.0	100
44	M	52	79.0	1.00	0.65	0.53	5.8	3.5	8.0	95
45	M	31	92.0	1.53	0.57	0.52	5.7	3.5	< 6.0	83
46	F	47	98.3	2.37	0.47	0.39	4.8	2.4	< 6.0	71
47	M	29	88.8	2.76	0.44	0.37	4.5	1.9	< 6.0	86
48	M	37	92.9	1.47	0.50	0.37	5.6	3.9	< 6.0	72
49	M	42	57.8	2.99	0.53	0.33	6.3	3.3	< 6.0	85
50	F	55	37.0	2.11	0.45	0.31	6.4	4.4	< 6.0	88
51	M	43	47.6	1.87	0.47	0.35	5.8	2.5	< 6.0	75
52	M	43	27.6	1.91	0.46	0.41	6.2	4.2	< 6.0	85
53	M	40	45.0	1.41	0.62	0.52	4.7	2.4	< 6.0	75
54	M	48	64.4	1.95	0.37	0.34	5.4	3.6	< 6.0	80
55	M	49	41.4	1.30	0.34	0.29	6.5	4.2	< 6.0	83
56	M	53	75.1	0.97	0.48	0.36	4.3	2.2	< 6.0	80
57	F	34	73.7	1.47	0.36	0.27	5.2	3.1	< 6.0	90
58	F	29	64.5	1.75	0.38	0.27	6.2	4.3	< 6.0	100
59	F	40	52.1	1.24	0.47	0.36	4.2	2.2	< 6.0	95
60	M	65	43.9	1.60	0.35	0.34	6.1	3.7	< 6.0	75
61	F	27	40.2	2.04	0.29	0.35	5.0	2.7	< 6.0	90
62	F	42	79.6	1.69	0.37	0.40	5.0	2.6	< 6.0	85
63	M	54	70.5	2.45	0.42	0.38	6.2	3.7	< 6.0	75
64	M	33	75.0	1.93	0.39	0.35	5.2	3.1	< 6.0	90
65	M	47	78.4	2.56	0.42	0.35	5.6	3.5	< 6.0	77

66	F	35	40.2	2.30	0.56	0.40	5.3	3.1	< 6.0	90
67	F	23	46.5	1.05	0.37	0.40	6.1	4.3	10.0	95
68	F	43	38.7	3.83	0.46	0.35	5.9	3.8	< 6.0	67
69	M	20	57.7	1.58	0.52	0.42	3.9	2.0	< 6.0	75
70	M	43	76.3	1.86	0.42	0.42	5.1	2.7	< 6.0	79
71	M	23	54.6	1.10	0.55	0.55	5.9	3.7	< 6.0	75
72	F	49	57.4	2.07	0.62	0.41	5.0	2.8	< 6.0	78
73	F	51	74.7	3.21	0.38	0.28	6.8	4.6	< 6.0	80
74	M	52	45.6	3.90	0.56	0.46	4.9	2.7	< 6.0	80
75	F	48	39.0	1.60	0.54	0.45	6.3	3.1	< 6.0	90
76	F	42	75.0	3.22	0.37	0.43	5.2	3.4	0.4	84
77	M	27	109.6	2.73	0.42	0.44	4.7	2.8	0.5	104
78	M	40	106.6	2.49	0.42	0.34	4.8	2.9	0.9	89
79	F	32	77.6	3.08	0.41	0.33	4.0	1.9	0.8	70
80	F	40	106.3	3.43	0.51	0.28	4.6	2.5	0.2	64
81	F	45	61.0	1.18	0.39	0.40	5.7	3.3	0.7	96
82	F	41	151.7	0.99	0.47	0.39	5.0	3.0	0.8	82
83	F	30	107.5	1.44	0.38	0.32	4.7	2.9	0.8	85
84	F	50	90.8	1.52	0.54	0.53	4.1	2.0	3.9	75
85	F	40	83.4	1.32	0.36	0.40	3.8	2.4	0.9	59
86	M	58	107.2	1.15	0.37	0.46	5.7	4.0	0.6	103
87	F	30	136.5	2.53	0.55	0.38	4.4	2.6	0.6	66
88	M	40	109.3	3.28	0.54	0.58	6.1	3.9	0.7	106

89	M	60	105.7	1.94	0.42	0.47	6.8	4.6	0.4	100
90	F	60	40.7	1.40	0.52	0.50	3.4	1.6	0.3	68
91	M	57	59.5	1.31	0.51	0.55	4.6	2.9	2.4	101
92	F	55	66.5	1.38	0.52	0.47	6.4	4.2	5.1	84
93	M	56	85.9	1.39	0.55	0.66	4.7	3.1	1.0	123
94	M	59	95.0	2.39	0.42	0.32	6.2	4.3	1.9	93
95	F	42	79.1	2.29	0.42	0.34	4.5	2.0	0.8	79
96	F	30	66.9	1.60	0.49	0.36	3.0	1.9	0.8	51
97	M	58	141.1	5.41	0.59	0.41	3.9	2.7	0.5	52
98	F	55	67.6	1.31	0.40	0.33	3.5	1.6	0.9	47
99	M	43	98.5	1.86	0.41	0.44	5.2	3.3	1.9	108
100	M	35	86.3	1.99	0.46	0.39	5.9	3.5	3.5	84

*Some CRPs reported as < 6.0 were measured on a less sensitive CRP assay; the remainder were on a high sensitivity assay.

Appendix 2

Demographic information, means, SD and resultant CV_I for subjects enrolled in biological variation study

Subject 1: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	102	1.40	0.40	0.36
SD	17.83	0.21	0.03	0.02
CV_I, %	**17.1**	**14.7**	**7.0**	**3.9**

Subject 2: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	103	0.94	0.45	0.38
SD	19.29	0.18	0.06	0.03
CV_I, %	**17.4**	**19.2**	**12.5**	**5.8**

Subject 3: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	68	1.92	0.40	0.44
SD	11.13	0.21	0.03	0.03
CV_I, %	**16.0**	**10.3**	**7.5**	**5.7**

Subject 4: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	77	2.35	0.47	0.40
SD	17.72	0.30	0.03	0.03
CV_I, %	**22.8**	**12.3**	**6.4**	**6.1**

Subject 5: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	90	0.97	0.49	0.41
SD	19.9	0.14	0.03	0.03
CV_I, %	**21.9**	**13.9**	**6.4**	**6.6**

Subject 6: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	88	2.50	0.42	0.37
SD	10.2	0.23	0.02	0.02
CV_I, %	**11.1**	**8.9**	**5.1**	**5.0**

Subject 7: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	84	1.46	0.52	0.44
SD	16.9	0.24	0.04	0.03
CV_I, %	**19.8**	**15.8**	**8.1**	**6.6**

Subject 8: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	100	1.03	0.54	0.49
SD	17.64	0.16	0.05	0.03
CV_I, %	**17.3**	**15.5**	**9.8**	**6.2**

Subject 9: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	80	2.32	0.44	0.34
SD	9.22	0.42	0.03	0.02
CV_I, %	**11.1**	**17.7**	**6.6**	**3.9**

Subject 10: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	78	3.19	0.49	0.36
SD	9.33	0.52	0.03	0.02
CV_I, %	**11.6**	**16.0**	**6.0**	**5.6**

Subject 11: F	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	83	2.33	0.46	0.37
SD	12.97	0.37	0.03	0.02
CV_I, %	**15.4**	**16.3**	**6.0**	**5.5**

Subject 12: M	Arginine	Homoarginine	ADMA	SDMA
Mean, μmol/L	89	1.73	0.44	0.26
SD	10.75	0.22	0.03	0.02
CV_I, %	**11.7**	**12.0**	**7.0**	**8.1**

Appendix 3

Patient information sheet and consent form

1. Study title

Measurement of cellular oxidative stress following elective orthopaedic surgery.

How does having a knee joint replacement effect the concentration of particular molecules in the blood?

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

3. What is the purpose of the study?

This study's purpose is to give a better understanding of how the human body reacts to inflammation. Inflammation is the normal response to any physical insult. The aim of this study is to measure the concentration in the blood of products of cellular metabolism that may change during inflammation. Increasing our knowledge in this area may help us to understand why individual patients respond differently to similar injuries and disease. Elective knee joint replacement provides a good model to measure this response in normal adults. The data from this study is essential to allow us to measure and understand the same variables in seriously ill hospital patients.

4. Why have I been chosen?

You have been chosen because you are about to undergo a knee joint replacement operation. We intend to include 50 patients in total in the study.

5. Do I have to take part?

It is up to you to decide whether or not to take part. A member of the research team will meet with you to describe the study and go through this information sheet with you. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

6. What will happen to me if I take part?

If you decide to take part, you will have additional blood and urine samples collected. Using a needle and syringe we will take 15 millilitres (about an egg cupful) of blood each time usually from your arm. The first blood sample will be collected in the anaesthetic room, just before the start of your operation. Further blood samples will be collected approximately 6 and 12 hours after the start of your operation and on each morning after the operation for up to 5 days or until you go home, if sooner. We will also collect a urine sample from you each morning during your hospital stay. A final blood sample will be collected when you return to the orthopaedic clinic approximately 6 weeks later.

Where possible, to minimise the number of times that blood is taken from you, samples for this research will be collected with the blood samples that are taken as part of your routine care in hospital.

You will only participate in this study during your normal hospital stay. Participation in this study will neither prolong your hospital stay nor require you to attend the hospital for the purpose of the study alone.

In total you will be involved in the study for approximately 6 weeks during which you will have up to eight additional blood samples taken.

7. What do I have to do?

After your operation, morning blood samples will be collected before breakfast. We require that you don't eat from midnight until the blood sample is taken around 7am. You would be allowed to drink water freely throughout the night. We would also ask you to provide a urine sample each morning that you are in hospital. There are no other lifestyle restrictions other than those explained at the time of surgery. You can carry out your daily activities as normal and take all regular medication.

8. What is the drug or procedure that is being tested?

There is no drug being tested and the procedures used are performed routinely and are safe.

9. What are the alternatives for diagnosis or treatment?

As your operation is not affected by the study, there are no alternatives for treatment.

10. What are the side effects of taking part?

There are no specific side effects of taking part in the study. The operation performed is the same whether you are in the trial or not.

11. What are the possible disadvantages and risks of taking part?

Taking the blood samples may cause some minor brief discomfort. In addition there is a small risk of bruising and local irritation.

Your knee replacement operation itself, has risks and these will be explained to you when you consent for it. The operation performed will be the same as if you were not in the trial.

Very rarely when performing a routine operation or test we find evidence of a disease or condition that is not expected and is causing no symptoms at present. If this occurs we would do what we felt to be in your best interests at the time and then discuss it fully with you subsequently.

12. What are the possible benefits of taking part?

There is no particular benefit to you, over and above that of the operation itself.

13. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about what is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

14. What will happen to the samples I give?

Blood and urine samples collected from you will stored and undergo laboratory analysis at Glasgow Royal Infirmary. No genetic tests are being done. Your blood and urine samples would be labelled with a unique study number allocated to you so that they cannot be identified by laboratory staff.

15. What will happen if I don't want to carry on in the study?

You can withdraw from the study at any time if you wish, and without giving a reason. Information that has already been collected may still be used. Any stored blood samples that can still be identified as yours will be destroyed if you wish.

16. What happens when the research study stops?

When the study stops, patients will attend the hospital for the same routine follow-up as those patients who were not in the study. Any remaining blood samples which have not been used in this study would be destroyed.

17. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

18. Will my taking part in this study be kept confidential?

If you join the study, some parts of your medical records will be looked at by a member of the research team who may or may not be directly involved in your clinical care. All will have a duty of confidentiality to you as a research participant. All information which is collected about you during the course of the research will be kept strictly confidential and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Your G.P will also be made aware of your participation in the study.

19. What will happen to the results of the research study?

The results of the study will be analysed and subsequently published in a scientific journal. You will not be identified in any report/publication.

If you wish to know the results of the study, please contact me through the department of anaesthesia. (0141 211 2069.)

20. Who has reviewed the study?

The study has been reviewed by West Glasgow Research and Ethics Committee.

21. Contact for Further Information

My name is David Reid. If you require any further information, I can be contacted in Gartnavel General Hospital Department of Anaesthesia on 0141 211 2069.

Professor John Kinsella is the Consultant in charge of the trial and he can be contacted via his secretary on 0141 211 4625.

Thank you for taking the time to read this information and for agreeing to take part in the study.

You will be given a copy of the information sheet to keep and a signed consent form.

5th May 2008

Patient Identification Number for this study:

CONSENT FORM

Title of Project: Measurement of cellular oxidative stress following elective orthopaedic surgery.

Name of Researcher: Dr David Reid

1. I confirm that I have read and understand the information sheet dated..... (version.....) for the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
4. I agree to my GP being informed of my participation in the study.
5. I agree to take part in the above study.

_____	_____	_____
Name of patient	Date	Signature
_____	_____	_____
Name of person taking consent	Date	Signature

1 copy for patient, 1 for researcher site file, 1 (original) to be kept in medical notes

West Glasgow Ethics Committee 1

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	08/S0703/84	**Issue number:**	0	**Date of issue:**	03 June 2008
Chief Investigator:	Dr David Reid				
Full title of study:	Measurement of cellular oxidative stress following elective orthopaedic surgery.				

This study was given a favourable ethical opinion by West Glasgow Ethics Committee 1 on 03 June 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Dr David Reid	Clinical lecturer in Anaesthesia	NHS Greater Glasgow and Clyde	West Glasgow Ethics Committee 1	03/06/2008	

Approved by the Chair on behalf of the REC:

Sheila A Lome ..... (Signature of ~~Chair~~/Co-ordinator)

(delete as applicable)

A H Tollie ..... (Name)

Management approval

North Glasgow University Hospitals
NHS Trust

Research & Development
R&D Management Office
1st Floor , Tennent Institute
Western Infirmary
Glasgow G11 6NT
Tel: 0141 232 9447

NHS Greater Glasgow

Our Ref: EP/LR
Enquiries to: Erica Packard
Direct Line: 0141 211 8544
e-mail: *Erica.Packard@ggc.scot.nhs.uk*

20th Aug 2008

Dr David Reid
Specialist Registrar
Department of Anaesthesia
Gartnavel General Hospital
Glasgow
G12 0YN

Dear Dr Reid

REC Ref: 08/S0703/84 **R&D Ref:** WN08AN157
Title: Measurement of cellular oxidative stress following elective orthopaedic surgery

We are pleased to inform you that, based on the information provided, this project has been granted overall Management Approval and may now proceed. This includes Finance, Pharmacy and a favourable Research Ethics Committee opinion.

Under Research Governance, we are required to hold a sponsor file containing the following documents: Protocol, Amendments and Ethics approval. While the study is ongoing you are responsible for updating us with all study amendments.

Further management approval will be required for amendments that increase patient numbers, increase or change the test procedures or bring about a change in pharmacy requirements. Please contact the R&D office if you wish to discuss any future amendments.

Thank you for your current and future collaboration.

Yours sincerely

EPackard

Dr Erica Packard
Academic Research Co-ordinator

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Appendix 4

Demographic details and details of blood transfusion and available specimens for patients enrolled in knee replacement study.

Subject	Sex	Age	Diabetes	Blood transfusion	Available specimens*
1	F	75	N	N	D0 – D4, 3M
2	F	74	N	N	D0 – D4, D5, 3M
3	M	62	Y	N	D0 – D4, D5, 3M
4	F	68	N	N	D0 – D4, D5
5	F	77	N	Day 3	D0 – D4, D5, 3M
6	M	73	N	N	D0 – D4, D5, 3M
7	F	69	N	N	D0 – D4, D5, 3M
8	M	73	N	N	D0 – D4, 3M
9	F	78	N	Day 2	D0 – D4, D5, 3M
10	F	69	N	Day 2	D0 – D4, D5, 3M
11	F	71	N	N	D0 – D4, D5, 3M
12	F	79	N	Day 2	D0 – D4, D5, 3M
13	F	78	N	N	D0 – D4, D5
14	F	75	N	Day 2	D0 – D4, D5, 3M
15	F	64	N	N	D0 – D4, D5, 3M
16	M	74	N	Day 2	D0 – D4, D5, 3M
17	M	65	Y	N	D0 – D4, D5, 3M
18	M	79	Y	N	D0 – D4, D5, 3M

19	F	71	N	N	D0 – D4, 3M
20	F	81	Y	Day 2	D0 – D4, D5
21	M	62	N	N	D0 – D4
22	M	72	Y	N	D0 – D4, 3M
23	M	68	Y	N	D0 – D4, D5, 3M
24	F	55	N	N	D0 – D4
25	F	68	N	N	D0 – D4, D5, 3M
26	F	74	N	N	D0 – D4, D5, 3M
27	M	78	N	N	D0 – D4, D5, 3M
28	F	72	N	N	D0 – D4, D5
29	F	55	Y	N	D0 – D4, D5
30	M	73	N	N	D0 – D4, D5, 3M
31	M	65	N	N	D0 – D4, D5, 3M
32	M	64	Y	N	D0 – D4, 3M
33	F	66	N	N	D0 – D4
34	F	59	Y	N	D0 – D4, 3M
35	F	61	N	N	D0 – D4
36	M	72	N	N	D0 – D4, D5
37	F	63	N	N	D0 – D4, D5
38	F	76	N	N	D0 – D4, D5

References

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