Modulation of Plasma Matrix Metalloproteinase 9 and its Inhibitors by Vitamin D.

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This work is submitted in 2012 for a PhD degree at

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Acknowledgements

Many thanks to my previous supervisor, Professor Chris Price and to my current

supervisor, Professor Graham Hitman, for their help and unflagging enthusiasm. Thanks to the late Dr Ray Edwards, for his assistance in validating the recalcitrant TIMP-4 method and Dr Barbara Boucher for help in statistical analysis. In addition, my thanks to Tim Venton for his pithy humour and scientific advice.

Many thanks to my wife, not only for her support but also for reading the many drafts.

Among the numerous people at the Homerton Hospital who helped me along the way, special mention must be made of Dr Shad Hussain and Dr. John Anderson for their guidance.

I dedicate this thesis to my late parents

Mary McLean Beat and Ernest Edgar Timms.

Statement of Acknowledgement of Donation of Samples

I confirm this is my work alone, with the exceptions stated below, which used the resources of several clinicians:

The samples for “The Vitamin D Supplementation Trial in Bangladeshis” were the generous gift of Dr Barbara Boucher

Samples for the TIMP-4 assay, from normal patients, were kindly donated by Dr. Muzhair and samples from patients undergoing angioplasty were a resource from Professor Rothman.

Signed Date

Abbreviations

AMI	Acute myocardial infarction
Ang II	Angiotensin II
AP1	Activated protein 1
BSA	Body surface area
CYP	Cytochrome P450
DEQAS	Vitamin D external quality assurance scheme
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
HDL	High density lipoprotein
Hg	Mercury
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
Internal Standard	I.S
IVSd	Intraventricular septal width in diastole

kD	Kilo Daltons
LDL	Low density lipoprotein
LVMI	Left ventricular mass index
LVPWd	Left ventricular posterior wall diastole
MI	Myocardial infarction
ml	millilitre
MMP	Matrix metalloproteinase
NF-κβ	Nuclear factor kappa B
ng/ml	nanogramme/millilitre
nmol/L	nanomole/litre
Ox LDL	Oxidised LDL
PEA 3	polyomavirus enhancer activator 3
PERE	phorbol ester-responsive element
pg/ml	picogrammes/ millilitre
PIIINP	Pro-collagen III N terminal peptide
PMA	Phorbol 12- myristate 13 acetate
PTH	Parathyroid hormone
RAR	Retinoic acid receptor

RIA	Radio Immunoassay
TRE	Transcriptional responsive elements
RXR	Retinoid X receptor
SMC	Smooth muscle cells
STAT	Signal transducers and activators of transcription
STP	Steroid transport protein
TATA	Promotor sequence rich in adenine (A) and thymidine (T)
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
TPA	Tetradeconoyl phorbol acetate
UA	Unstable angina
UK	United Kingdom
25(OH)vitD	25hydroxyvitamin D
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Abstract

Introduction: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are upregulated in a variety of diseases.

Hypothesis: As TIMP-1 levels are elevated in liver fibrosis, might a similar process occur in essential hypertension driven left ventricular hypertrophy and furthermore may TIMP-1 be a marker of vascular disease? If TIMP-1 levels are a potential marker of cardiovascular disease could their levels be modulated by vitamin D?

Methods: Plasma TIMP-1 levels and aldosterone were measured a) in patients with essential hypertension who had never been on treatment or had been off treatment for 1 month and b) healthy controls. All participants underwent echocardiography. To assess whether TIMP-1 was a marker of vascular disease insulin, sCRP, fibrinogen, homocysteine, PAI-1 were measured in Bangladeshis pre supplementation with vitamin D. TIMP-1, MMP2, 9 and 25 hydroxyvitamin D 25(OH)vitD were also measured pre and post supplementation. Subsequent studies included measurements of MMP2, 9 and TIMP-1and 4 in submariners pre and post patrol and MMP9 and 25(OH)vitD in patients who re-stenosed post angioplasty. TIMP-4 was validated using a radioimmunoassay, 25(OH)vitD measured using a triple quad MS and other assays using ELISAs.

Results: Plasma TIMP-1 was higher in hypertensive patients than in the controls ($p<0.0001$) and was correlated with left ventricular hypertrophy and with aldosterone. In the Bangladeshi study,. TIMP-1 was not correlated with other markers of vascular disease. TIMP-1 was correlated with systolic blood pressure ($p<0.007$) There was an inverse correlation of 25(OH)vitD with MMP9 ($P<0.001$) and TIMP-1 ($p<0.05$) and sCRP ($p<0.05$). The inverse relationship between MMP9 and 25(OH)vitD was also repeated in the submariner and restenosis studies.

Conclusions: Plasma TIMP-1 may be an important determinant in essential hypertension and 25(OH)vit D may have a positive effect in reducing the inflammatory response as measured by MMP9. The increased 25(OH)vitD may also act by reducing aldosterone levels and thus suppressing TIMP-1 levels.

CHAPTER 1 INTRODUCTION

1.0.0 General introduction

Vascular disease is a major cause of morbidity and mortality. The incidence of ischaemic heart disease (IHD) varies dramatically, depending on predisposing pathologies, such as essential hypertension, diabetes, hypercholesterolaemia and possibly, dietary factors.

The incidence of essential hypertension is 25%. The definition of essential hypertension is arbitrary but normotensive blood pressure levels of greater than 140/90 or, in a diabetic, 130/80, are considered unacceptable. The risk associated with a particular blood pressure for cardiovascular disease (CVD) is dependent on a variety of factors, including hypercholesterolaemia, diabetes and most importantly, left ventricular hypertrophy (LVH). LVH in Caucasians is associated with ischaemia of the left ventricle and small infarctions. Repair of these lesions is associated with a collagen deposition, rich in angiotensin converting enzyme. This pathological repair process results in increased intra- cardiac levels of angiotensin II, a cardiotoxic hormone.

Diabetes is associated with an increased risk of cardiovascular disease. The mechanism for this increased risk is associated with hypertension, dyslipidaemia, insulin resistance and obesity (Rosengran et al., 1989).

Hypercholesterolaemia is a major risk factor in IHD (Khot et al., 2003). Atherosclerosis appears within the second decade of life as a patchy disease, consisting of fatty streaks. Within these streaks, macrophages ingest chemically modified LDL producing foam cells. Matrix metalloproteinases (MMPs) cause migration and proliferation of smooth muscle cells and result in a lipid layer surrounded by a cap of collagen, that is, the mature atherosclerotic lesion.

More recently, one of the factors involved in IHD, is inflammation of the coronary vessel. The inflammatory response has several effects, one of which is upregulation of cytokines, which increase MMPs activity and destabilise plaque.

A further factor is the role of nutritional supplements in the aetiology of cardiovascular disease. Red wine consumption (high in antioxidants) may explain the paradox of a low incidence of IHD (yet high fat intake) in France, in contrast to the high incidence of ischaemic heart disease (and high fat intake) in the UK, particularly Scotland (Artaud-Wild et al.,1993).

More recently there has been evidence that low vitamin D levels may be an independent factor associated with an increase in IHD (Edward 2009, Gouni-Berthold et al., 2009).

1.1.0 Vitamin D and its metabolism

Vitamin D arises from two sources:

a) Firstly, it is ingested. The foods containing the highest amounts of vitamin D are herring and pilchards. Dietary sources of vitamin D are limited, with vitamin D intake reduced in many vegetarians, particularly Asians living in the UK. In inner cities and deprived areas of the UK, vitamin D intake is likely to be sub-optimal. Margarine and breakfast cereals are supplemented with more vitamin D in the USA, than in the UK. The only source of vitamin D for vegetarians, is called ergocalciferol or vitamin D_2 and is not as potent as vitamin D3.

b) The other major source of vitamin D_3 is from the action of sunlight on the skin, where the UV component splits the B ring of cholesterol, producing vitamin D. The efficiency of UV light action on the skin, in producing vitamin D, is decreased in dark skinned people and in all races, as the subject becomes older.

Vitamin D is transported via a carrier protein to the liver where it is metabolised.

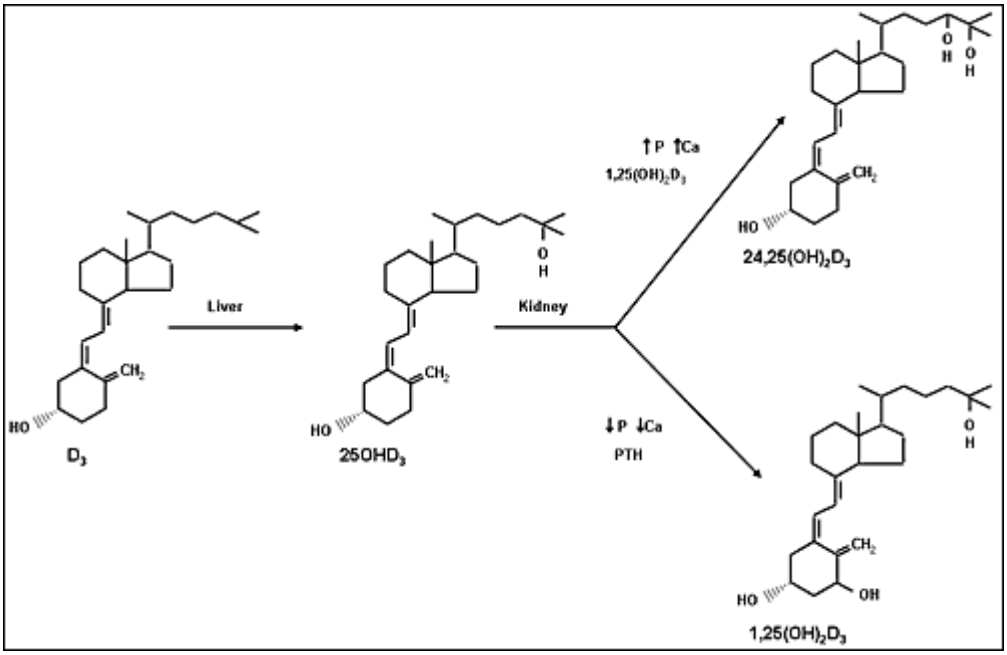


Figure 1. Metabolism of vitamin D to its active metabolite, 1,25(OH)2D
modified (D Bikle, 2009)

Historically there has been significant controversy as to the hepatic enzyme which hydroxylates vitamin D on the 25 position. The mitochondrial enzyme CYP27A1, so called because it also hydroxylates bile acid metabolites, was thought the likely candidate but this enzyme does not 25 hydroxylate vitamin D2. The microsomal enzyme, the hepatic CYP 2R1, hydroxylates both vitamin D2 and D3 and is the major enzyme involved in 25 hydroxylation of vitamin D (Bikle, 2009).

The second hydroxylation step produces the active metabolite of vitamin D (calcitriol) by hydroxylation of 25(OH)vitD by CYP27B, the mitochondrial 1α hydroxylase in the kidney. The renal 1α hydroxylase is the main rate-limiting step in calcitriol synthesis. The activity of the 1α hydroxylase is dependent on many factors, including elevated plasma PTH, low calcium and phosphate. The CYP24A1 is probably involved in the inactivation of 25(OH)vitD, although it may also be important in bone mineralisation (Hollick, 2007). The receptor for calcitriol is the vitamin D receptor (VDR) which is a transcription factor.

There are still many issues to be resolved regarding vitamin D metabolism:

- Firstly, cellular uptake by cubilin and megalin and endocytosis of the vitamin D binding protein with its tightly bound 25(OH)vitD into the renal cell and other cells, such as the macrophage.
- Secondly, the mechanism whereby the 25(OH)vitD is translocated across the cytoplasm to the mitochondria, prior to the final hydroxylation step, is unclear and

requires clarification. Exciting research into these two areas is still waiting to be developed.

1.1.1 Biological actions of calcitriol

Twenty years ago, it was thought that calcitriol acted on only four tissues, namely, the gut, kidney, bone and parathyroid. However, VDR receptors have been located in many tissues, including the immune system, the heart, vasculature and the brain. VDR has been located in 80 different cells and is involved in the activation of over 200 genes (Dusilová-Sulková et al., 2009).

1.1.2 The Macrophage 1α hydroxylase

The control of macrophage 1α hydroxylase and that of the renal 1α hydroxylase is significantly different. The macrophage 1α hydroxylase is not rate-limiting and unlike the renal 1α hydroxylase, is not affected by plasma calcium, phosphate, PTH or calcitriol. Macrophage production of calcitriol may be a reflection of circulating plasma 25(OH)vitD levels that are dependent on vitamin D intake and more importantly, on sunlight exposure (Verstuyf, 2010).

Theoretically, it seems possible that when vitamin D levels are low, macrophage production of calcitriol will be reduced. This causes IL10 levels to fall, thereby resulting in an increase in pro-inflammatory cytokines, which stimulate macrophage MMP production and in turn, cause destabilisation of arterial plaque. Conversely, when subjects are exposed to more UV light or an increased intake of

vitamin D, 25(OH)vitD levels rise, resulting in an elevation of macrophage calcitriol. This increases IL10 levels, which will reduce MMPs production, thereby enhancing plaque stabilisation.

1.1.3 Mechanism of action of 1, 25 dihydroxyvitamin D

Calcitriol acts on a variety of tissues by two different mechanisms:

1) By a fast non-genomic response involving cell membrane receptors in bone, gut and liver. 1, 25 dihydroxyvitamin D regulates calcium and chloride channel activity, protein kinase C activation and distribution and phospholipase C activity.

2) By a slower genomic response, (Figure 2) in which the effects of 1, 25 dihydroxyvitamin D occur through the actions of the vitamin D receptor protein (VDR). VDR binds with a high affinity to 1, 25 dihydroxyvitamin D, which forms a heterodimer with 9 cis retinoic acid receptor (RXR). The heterodimer binds to specific DNA sequence elements, which are called the vitamin D response elements (VDRE). The binding of the VDR/RXR complex to VDRE results in the migration of coactivators. One of these co-factors is a group of proteins called VDR interacting proteins which span the gap between the VDRE and the site for RNA polymerase II activity. Histone acetyl transferase opens up the structure, permitting RNA polymerase II-mediated transcription.

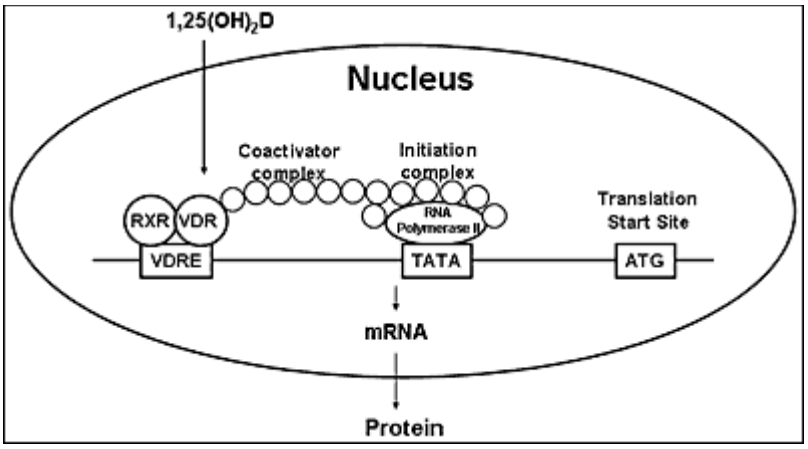


Figure 2 1,25 Dihydroxyvitamin D3 on the vitamin D response elements.
The gap between the RXR VDR complex and the TATA box is bridged by a coactivator complex (Bikle, 2009)

1.1.4 The activated protein (AP1) promoter and its relationship to MMP, TIMP-1 and vitamin D

Activated protein (AP1) is an important factor in the regulation of MMPs, TIMPs and vitamin D. It consists of a dimer, consisting of several oncoproteins including c fos and c jun which bind to a TRE site. Most MMPs and TIMPs, with the exception of MMP2, 11 and TIMP-2 have similar cis acting regulatory elements. These include at least one AP1 site, (5´–TGAG/CTCA-3´) which lies about 70 base pairs from the TATA box. In MMP1, 3, 7, and 11, there is an additional AP1 site further upstream of the gene, at about – 180 base pairs, which has a different consensus 5´ **–**TTAATCA-3´ (Crawford and Matrisian,1996). MMP9 also has a second AP1 site but this is in perfect consensus (5´–TGAG/CTCA-3´) with the proximal AP1 site, adjacent to the TATA box.

1.1.5 Pathological non-calcaemic actions of vitamin D

Until recently, the major role of calcitriol was thought to be control of calcium homeostasis and bone remodelling. Epidemiological studies show that higher serum 25(OH)vitD concentrations or environmental ultraviolet light exposure are associated with lower rates of breast, ovarian, prostate, and colorectal cancers and multiple sclerosis. The prevalence of hypertension increases with increasing latitude north of the equator (Rostand et al.,1997). Additionally, hypertension becomes less severe in subjects whose 25(OH)vitD concentrations are increased to >100 nmol/L through ultraviolet exposure (Krause et al.,1998). There is evidence that sunlight (and by inference, increasing levels of vitamin D) may reduce the incidence of ischaemic heart disease. Tuberculosis (TB) is associated with an increase in MMPs and recent work has shown a higher incidence of TB in Asians living in West London, when the levels of plasma 25(OH)vitD were low. Vitamin D status may have a role in controlling MMP activity, which may have a role in the development of TB (Hollick, 2007).

1.2.0 Modifiable and non-modifiable factors which affect heart disease.

Vascular and heart disease can be subdivided into those factors which are amenable to treatment and those where lifestyle changes make no difference to morbidity or mortality. Non-modifiable risk factors include age, sex, gender, family history and personal history. Modifiable risk factors include smoking, hypertension, serum cholesterol level, diabetes, obesity and fibrinogen. Of these

factors, there is evidence that modification of smoking, hypertension and cholesterol levels will attenuate the risk of IHD.

Historically, it was perceived that conventional risk factors could predict less than 50% of patients with ischaemic heart disease (IHD). However in a meta analysis Khot et al., (2003) reported that 80-90 percent of patients with IHD had at least one conventional risk factor. An observation not made by the authors was that several of these risk factors have been reportedly associated with vitamin D deficiency (Hollick, 2007).

1.2.1 Dietary factors and the incidence of ischaemic heart disease

In America, Stamler (1981) showed that the incidence of IHD fell in the seventies and this was associated with a dramatic change in food products. During the period, 1950-78, the amount of margarine consumption in America increased by 100%. This was associated with a number of dietary changes, including that of beef, by a reduction of 90% and an increase in consumption of turkey and chicken by 132%. The consumption of fish increased by only 8%. According to this data, the increase in fish consumption does not adequately explain the fall in IHD, although changes in beef, turkey, chicken and margarine may do so. Interestingly, margarine is a relatively good source of vitamin D due to vitamin D supplementation.

There is a legal requirement in Britain and America that margarine is supplemented with vitamin D. Also, vitamin D is added to milk and to cereals in America but not generally added to these in Europe. Stamler (1985) showed that amongst American physicians living in California, there was a marked drop in the incidence of IHD within both the smoking and non-smoking groups, in comparison to age-matched white males. This observation suggests that in addition to smoking, other factors may be involved in the cause of IHD, one of which may be the level of vitamin D intake.

1.2.2 Effect of sunshine on the incidence of ischaemic heart disease

There are several papers (Fleck, 1989, MacPherson and Basco 2000, Pilz et al., 2010) which suggest a relationship between sunshine and incidence of ischaemic heart disease. Initially, the seasonal relationship of vitamin D and the increased incidence of myocardial infarction (MI) related to season, will be discussed. This topic will be developed further by discussing some epidemiology studies that relate to IHD directly or indirectly, exposure to sunshine and the association between plasma levels of 25(OH)vitD or calcium and IHD.

1.2.3 Seasonal variation in 25hydroxyvitamin D levels and the incidence of MI

There is a well-established relationship between time of year and 25(OH)vitD. Levels of 25(OH)vitD reach a nadir in the winter and a peak in the summer, in the northern hemisphere. In the southern hemisphere, levels of 25(OH)vit D are the

opposite, with lowest levels occurring during September and peak levels during early spring. There is a correlation (0.747 ($p<0.005$) between plasma levels of 25(OH)vitD and hours of sunshine measured each month throughout the year (Stryd et al.,1979).

Seasonal mortality statistics show IHD increases in the northern hemisphere in the first few months of the New Year but in the southern hemisphere, this increase occurs in the months June to September. The variation in IHD in the northern and southern hemispheres parallels that of 25(OH)vitD.

These seasonal fluctuations in MI could be explained by a reduction in temperature, known to be associated with an increase in blood pressure of 5 mm Hg. However these observations are at variance with the data from subjects living at altitude, where the incidence of IHD falls with decreasing temperature (Pell et al.,1999). Pollution and cloud reduce the intensity of UV light at low altitude, in comparison with the intensity of UV light found at higher altitude. This increase in UV light at a higher altitude would be associated with increased amounts of 25(OH)vitD, possibly explaining the reduction in IHD with increasing altitude.

1.2.4 Association of IHD and sunshine

Several studies have shown that sunshine may be an important factor in the incidence of IHD. (Fleck, 1989) reported that the incidence of IHD dropped in

Europe with reducing latitude. Grimes et al., (1996) assessed the relationship between the number of hours of sunshine and the increase in IHD in the UK and showed that the incidence of IHD fell from the north to the south and from the west to the east of Britain.

1.2.5 Differences in IHD within Europe

There is a marked difference in the incidence of IHD in different parts of Europe which cannot be easily explained on the basis of the classic risk factors associated with IHD. There is a marked difference in the mortality between Belfast and Toulouse or France and Finland. Both countries have similar intakes of dietary fat but the incidence of IHD in Belfast and Finland is four times greater than that of France (Artaud-Wild et al., 1993). This discrepancy might be explained by other factors. In France, wine is widely consumed. Wine has antioxidant properties, which would decrease free radical production. Additionally, a component of red wine contains resveratrol that can stimulate sirutin levels which in turn decrease MMP9 levels which are implicated in plaque destabilisation (An et al., 2010).

1.2.6 IHD and the Asian population

The incidence of IHD in Asians worldwide, which is three times as high as that of Caucasians living in Britain, may be due, in part, to an absolute or relative vitamin

D deficiency. This deficiency in vitamin D may arise due to religious reasons, in that the women may totally cover up their bodies and also, their increased dermal pigmentation will further reduce production of 25(OH)vit D. I am unaware if there is per se, a difference in 25(OH)vitD between men and women.

1.2.7 The relationship between hair calcium and 25 hydroxyvitamin D and IHD

There is more direct evidence on the association between hair calcium and plasma 25(OH)vitD and IHD. Similarly, as with serum, the level of calcium in hair is dependent on UV light. Work by MacPherson and Basco (2000) in the UK, has shown that the incidence in the UK of IHD, is inversely related to the number of hours of sunshine and hair calcium concentration. Patients who have a history of MI have reduced plasma 25 hydroxyvitamin D 25(OH)vit D in comparison to controls (Scragg et al.,1995). The above data suggests that levels of 25(OH)vitD may be important in attenuating cardiovascular risk.

1.2.8 Difficulties associated with relating 25 hydroxyvitamin D and ischaemic heart disease (IHD)

A major difficulty associated with the relationship between vitamin D deficiency and IHD is that the incidence of IHD should be high in the early part of the last century, because industrialisation is associated with high density housing and pollution. Both of these variables should reduce 25(OH)vitD production from

sunshine. The incidence of IHD at the start of the last century was low and only started to increase in the 1920s. However, since average life expectancy was very low during this period, patients may have died due to other diseases, before symptoms of IHD became apparent. Alternatively, levels of activity may have been higher then, whilst levels of smoking and consumption of refined foods would be reduced, in comparison to lifestyle habits of the twenty-first century.

In contrast to vitamin D acting as a protective factor against IHD, excessive vitamin D intake has a toxic effect on the vasculature. Thus, pigs fed on diets 12.5 times their normal dose of vitamin D, developed extensive lipid deposits, six months after supplementation. Evidence of vitamin D toxicity on the vasculature is only evident with excessive ingestion of vitamin D. This vascular toxicity is of potential importance, as many foods (dairy products, bread, cereal, margarine and pasta) are supplemented with vitamin D (Kumerow, 1979). However, this degree of toxicity is unlikely to occur in the human, as it would require pharmacological ingestion of vitamin D.

There is now a portfolio of evidence derived from epidemiology and other sources consistent with the role of vitamin D, in reducing the incidence of IHD (Verstuyf et al., 2010). The mechanism, by which this might arise, is described below.

1.3.0 Inter-relationship of vitamin D interleukin 10 (IL10) and MMPs

IL10 is found in Th2 cells, which are responsible for antibody production and it inhibits cytokine responses from Th1 lymphocytes. Human epidermal keratinocytes (HaCaT cells) treated with 25(OH)vitD, calcitriol or calcipotriol showed an increase in IL10 receptor expression of 2, 11, and 12 fold respectively. Low levels of 25(OH)vitD, in the sub-physiological range, also cause an increase in IL10 receptor (Michel et al.,1997).

The addition of IL4 to human cultured lung macrophages reduce MMP9 levels. On addition of IL10 to the same culture, MMP9 levels fall further and TIMP-1 concentrations increase, via a transcription mechanism (Lacraz et al., 1995), which favour matrix deposition?

It can be hypothesised that elevated levels of IL10 could stabilise unstable plaque, as IL10 acts as a break on the inflammatory cascade, causing a reduction of inflammatory cytokines One of the factors affecting IL10 is calcitriol, which can be synthesised by the macrophage, by a non-rate limiting reaction, thereby raising an interesting speculation. Could increased plasma levels of 25(OH)vitD (by raising macrophage production of calcitriol with the subsequent elevation in IL10) reduce intra-macrophage production of MMP9? This hypothesis suggests a mechanism for the observation that the incidence of IHD is higher in areas where vitamin D levels are low, due to reduced dietary intake or sunshine. However, this

hypothesis requires a detailed understanding of the role of MMPs and their tissue inhibitors in matrix degradation.

1.4.0 Extracellular matrix

The extracellular matrix (ECM) contains a variety of structural proteins which include collagen, proteoglycans and glycoproteins. These proteins act as a cellular “skeleton" providing structure, form, protein-cell and cell-cell interactions. Normal matrix levels arise by a controlled balance between synthesis and degradation by MMPs (Matrisian et al., 1990). The activity of these MMPs is inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs). When this balance of synthesis and degradation is uncontrolled, inadequate or increased, amounts of ECM are deposited (Stetler-Stevenson, 1996).

This alteration in levels of tissue matrix can have a major impact on organ function. In vascular pathology, increased matrix deposition is associated with a reduction in vessel elasticity, whereas in plaque, lower levels of matrix cause instability. Elevated matrix levels are associated with LVH, cirrhosis and pulmonary fibrosis, whereas reduced matrix levels are linked with cardiomyopathy, unstable plaque and aortic aneurysms (Timms et al., 2002).

1.4.1 Matrix metalloproteinases

The first description of the role of an MMP was in the early 1960s, where MMP1 was described as important in the reabsorption of the tadpole tail, as it developed into a frog. From this first observation, there has emerged an ongoing and detailed understanding of the genetic control, control of activity and the importance of MMPs and their inhibitors both in terms of physiology and pathology. It is in this last area where therapeutic intervention although problematical, holds the greatest promise, as several pathologies which have high morbidity and mortality. are associated with inappropriate levels of MMPs or their inhibitors.

To date, 23 MMPs have been described (Greenwald, 1999) - Table 1
Historically, they were assigned a common descriptive name in the belief that each was specific for the hydrolysis of a particular matrix protein. This view has now been superseded. A numbering system was introduced when it was appreciated that each MMP could hydrolyse a variety of substrates, including the degradation of other MMPs. There are other classification systems, one of which divides the MMPs into three groups depending on the spatial inter-relationship between the TATA box and the AP1 site, those with a TATA box but no AP1 site and those without a TATA box (Clark et al., 2007)

MMP	Common	Known Substrate
1	Collagenase 1	Collagens, IGF-BP3, IGF-BP5 MMP3,9 Proteoglycan link protein gelatin aggrecan
2	Gelatinase A 72kDa Gelatinase	Collagens, gelatin, laminin, MMP1,9,13 Proteoglycan link protein
3	Stromelysin 1	Collagens III, IV, V, IX, MMP2/TIMP2, MMP7,8,9,13 and activation of MMP1
7	Matrilysin	Collagen IV, X, MMP2,3,9, MMP/TIMP-1
8	Collagenase 2	Collagens, aggrecan, gelatin
9	Gelatinase B 92-kDa gelatinase	Collagens III, IV, V elastin
10	Stromelysin 2	Collagens III, IV, V, gelatin casein
11	Stromelysin 3	Laminin, collagen IV, fibronectin
12	Macrophage	Collagen IV, gelatin elastin
13	Collagenase 3	Collagens, gelatin,
14	MT1-MMP	Collagen I,II,III, MMP2 and 13
15	MT2-MMP	Fibronectin, large tenascin C laminin
16	MT3-MMP	Collagen III
17	MT4-MMP	
18	Collagenase 4	
19	No trivial name	gelatin
20	Enamelysin	Amelogenin

Table1. 1 The Matrix Metalloproteinases.
In the order they were discovered, their common name and their most important substrates

1.4.2 MMP domains

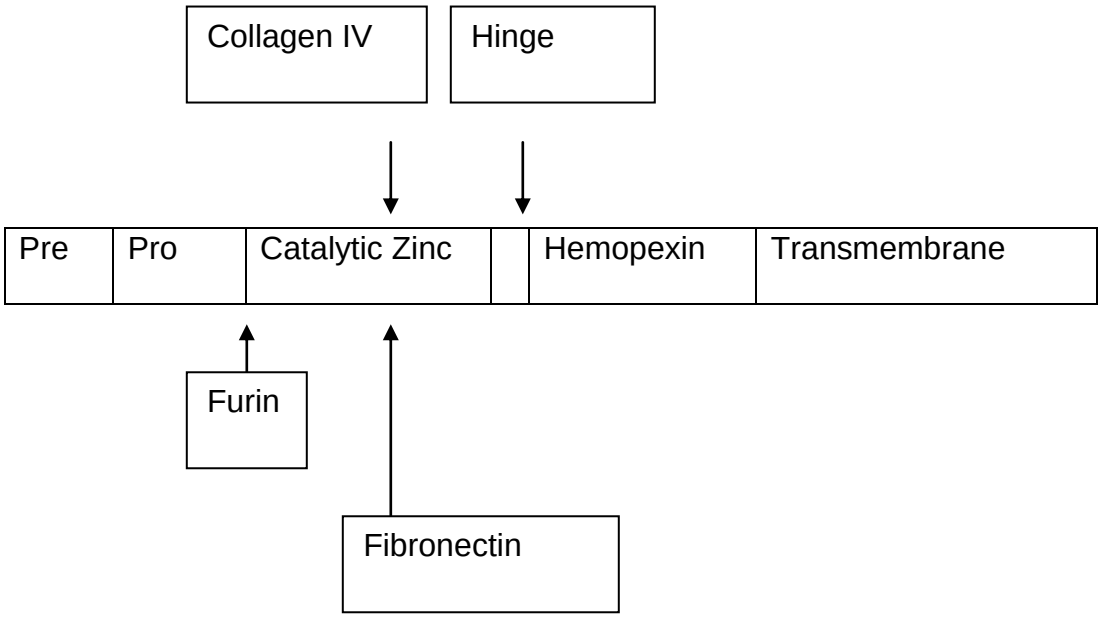


Figure 3 Major domains of the intact MMP molecule
The pre, pro and catalytic domains are common to all the MMPs. The collagen IV and fibronectin domain are important in substrate recognition and the hemopexin domain is the binding site of the substrate for the enzyme

MMPs have three common domain structures (Figure 3). Firstly, there is a pre-domain which targets the MMP for extracellular excretion, secondly, there is a pro-domain which maintains the MMP in an inactive form (called the proMMP) and thirdly, there is a zinc dependent catalytic domain, which becomes active on hydrolysis of the pro-domain. The catalytic domain contains an additional zinc atom and up to three calcium atoms, plus a five-stranded beta sheet containing three helices.

MMP7 is structurally the least complex MMP. MMP7 only has the pre, pro and catalytic domains. Other MMPs have additional domains within the catalytic domain, as well as at the C terminal portion of the MMP (Nagasa, 2006).

Within the catalytic domain, there are two further domains that are involved in substrate recognition. These are the collagen domains that recognise collagen IV and the fibronectin domain. The fibronectin domain, which confers substrate specificity, is repeated thrice in both MMP2 (gelatinase A) and MMP9 (gelatinase B). An additional domain found on all MMPs except MMP7, is the hemopexin domain. The hemopexin domain is involved in the binding of substrates and inhibitors and is essential for collagenase activity.

The importance of the hemopexin domain has been shown by Sanchez-López et al., (1993) who compared the substrate specificity of normal (native) MMP1 with that of a C terminally truncated MMP1, that is, the MMP1 lacking the hemopexin domain. Native MMP1 hydrolyses collagen into its ¼ - ¾ ratio, whereas the C terminally truncated MMP1 does not hydrolyse collagen, although both the native MMP1 and its C terminally truncated form, hydrolyse gelatin. Both the N and C terminal portion of MMP1 are required for hydrolysis of native collagens.
MMP3 (stromelysin 1) has a similar domain to MMP2 and MMP9, except it lacks the fibronectin type 2 repeats. The C terminally truncated MMP3 is very similar to MMP7. Baragi et al., (1994) demonstrated that native MMP3 bound TIMP-1 more tightly than its C terminal truncated form (which had similar binding affinities to

MMP7). This suggests that in at least some MMPs, the C terminal end is important for TIMP-1 binding and subsequent inhibition of MMP activity.
Other domains include the transmembrane domain, which is associated with membrane bound MMPs (MT-MMP) MMP14, MMP15, MMP16 and MMP17. MMP14 activates proMMP2 into its biologically active form. This is not common amongst all MT-MMP as MMP17 shows little activation of proMMP2 (Murphy et al., 1999). In the activation of proMMP2, TIMP-2 binds to the C terminal portion of proMMP-2, thus bringing the proMMP2 into a spatially optimal position, whereby MT1- MMP can hydrolyse the proMMP-2 into the active MMP-2 (Woesser,1999). Other domain sites have been described, including a furin domain, which is an alternative intracellular site for activation of MMPs

1.4.3 Hydrolysis of non-collagen proteins by MMPs

The non-matrix hydrolytic actions of MMPs include hydrolyses of receptors and binding proteins. Active MMP2 and MMP7 hydrolyse the ß4 integrin receptor and MMP2 hydrolyses the fibroblast growth factor (FGF) type 1 receptor. MMP2, MMP3 and MMP9 activate interleukin 1ß (normally produced as an inactive precursor) into its biologically active form (Schönebeck et al.,1998).

One of the most important non-matrix hydrolytic actions of MMPs is the release of insulin-like growth factors (IGF). The biological effect of IGF is expressed via an IGF receptor. IGF is bound to insulin growth factor binding proteins (IGFBP3) but

when complexed, the IGF is not biologically active, since the interaction of IGF with the receptor is reduced. MMP1, MMP2 and MMP3 can hydrolyse this IGF/IGFPB3 complex, increasing levels of unbound IGF, which then interact with the receptor, increasing the bio-availability of IGF (Fowlkes et al.,1994).

In the mouse, MMP9 is important in the development of the first stages of kidney development. In the MMP9 $^{-/-}$ knockout mouse there is an increase, histologically, in renal apoptotic nuclei and a reduction in bud formation in comparison with wild type MMP9 mice. This is no direct effect on collagen degradation but this is due to the release of a stem cell factor which inhibits apoptosis (Arnould et al., 2009) in wild type mice.

1.4.4 Control of MMPs

Because of their potency and importance in ECM remodelling, MMP activity is controlled at three points in the production of the active MMP (figure 4). MMP activity is dependent on:

1) transcriptional regulation of the gene

2) activation of the proMMP to the MMP

3) inhibition of the active MMP, by a family of antiproteinases called tissue inhibitors of metalloproteinase (TIMP).

This schematic (Fig 4) represents a skeletal outline of the control of MMPs. Factors such as methylation and acetylation of chromatin, in addition to factors which affect stability of the transcribed MMP RNA (Clark, 2008) are not included in

this outline. However, as a model to develop a hypothesis to explain the pathophysiology of matrix associated disease, it has proven useful.

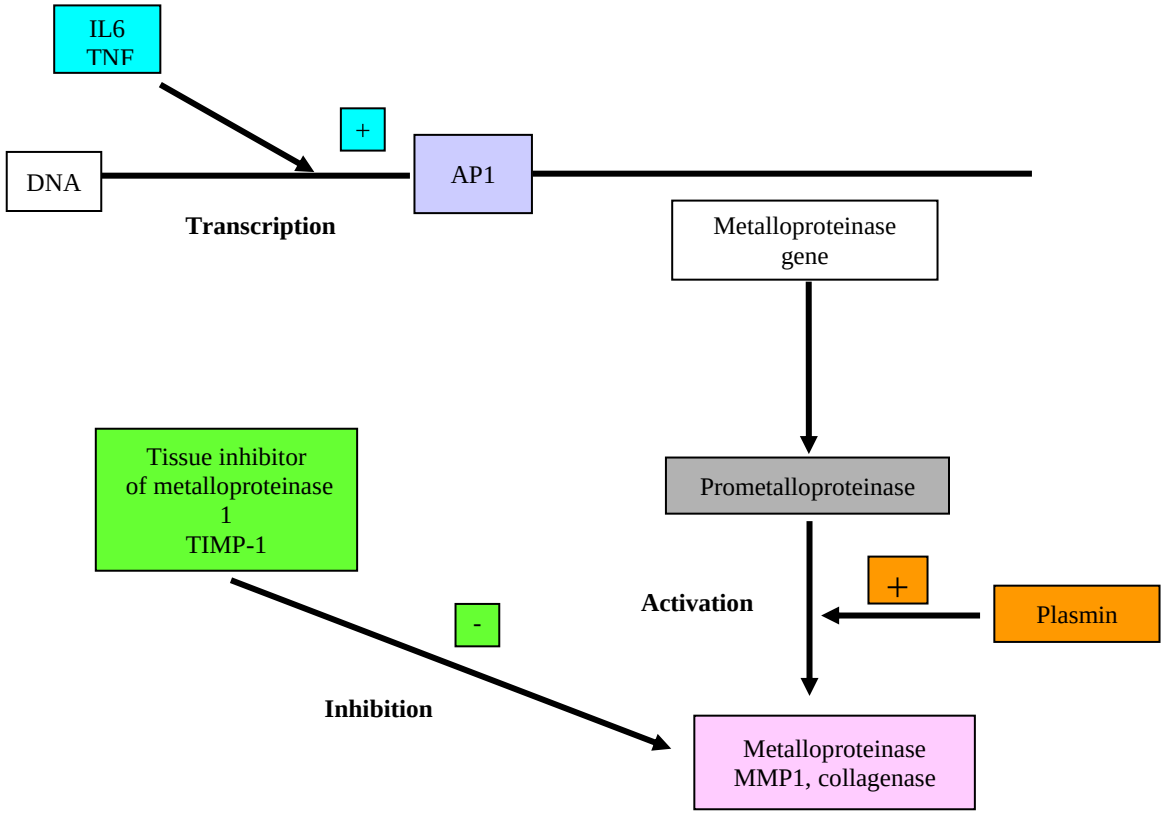


Figure 4 The three important control points in MMP regulation.
Upregulation of the gene by inflammatory cytokines, activation of the proMMP and finally inhibition by TIMP-1

1.5.0 Structure of MMP and TIMP genes

MMPs and TIMPs can be subdivided into constitutive or responsive types. MMP2, and TIMP-2 are generally constitutively expressed, in that they are not upregulated by 12-O-tetradecanoylphorbol-13-acetate (TPA) and cytokines. There is a marked difference in the responses of MMPs and TIMPs to different

cytokines. Elucidation of these mechanisms may provide the impetus for new drugs.

1.5.1 Gene structure of MMP9

MMP9 gene structure is one of the most complicated of all the MMPs. It contains two AP1 consensus sites. In addition to an NF-κB site, there are numerous PEA sites that enhance the transcription of the AP1.

TNF α treated immortalised keratinocytes showed a decrease in MMP9 on the addition of calcitriol. The calcitriol reduced levels of C Jun N terminal kinase, lowering the levels of NF κβ, both of which reduced the activity of the AP1 site on MMP9 (Bahar-Shany et al., 2010). This model may not reflect the mechanism occurring in human pathology but it offers a potential mechanistic approach. What is the mechanism of calcitriol's induced reduction of MMP9? Sirutin 1 is a family of anti-ageing enzymes which deacetylase. The removal of the acetyl groups from histone, in general, is a process associated with a reduction in gene expression (Nakamaru et al., 2009).

Fox O is a group of transition factors, the target genes of which are regulated by 1,25 hydroxyvitamin D via the VDR (Am et al., 2010). Additionally, this group demonstrated there was a 1, 25 dihydroxyvitamin D induced VDR-Sirulin-FoxO

inter-relationship, suggesting a mechanism whereby vitamin D supplementation may suppress MMP9.

There is a variety of further factors affecting the regulation of MMP9 levels, including demethylation of histones, in addition to quercetin, a flavenoid which suppresses TNFα induced MMP9 (St. Pierre et al., 2004).

1.5.2 Gene structure of TIMP-1 and –2

TIMP-1 contains three AP1 sites, one in the first exon, the second, similar in position to the AP1 site on MMP9 and the third at – 802. A hypoxic response element has also been reported close to the initiation site. Again, like MMP9 there are several PEA sites along the length of the gene. MMP9 and TIMP-1 can be co-excreted but these proteins can be differentially expressed. The TIMP-2 gene like that of the MMP2 gene has little detail. It has an AP1 site at –288 and 2 PEA sites that are not adjacent to the AP1 site. The regulatory sequences of MMP2 and TIMP-2 are similar. The AP1 site is not associated with the initiation site and there is little opportunity for ‘cross talk’ between the PEA sites and the AP1 site. These factors explain the constitutive expression of MMP2 and TIMP-2.

1.5.3 Activation of pro-MMP

Plasminogen activating factor, which is inhibited by plasminogen activator inhibitor-1 (PAI-1) increases the concentration of plasmin (Roberts et al., 1995).

Plasmin (with the exception of MMP2) converts the inactive prometalloproteinase to the active metalloproteinase. Angiotensin II increases the concentration of plasminogen activator inhibitor -1 (PAI-1) that reduces plasmin concentrations. Consequently, high angiotensin II levels decrease MMP activity and increase tissue collagen deposition (Diez et al., 1997).

Until recently, the importance of membrane bound MMPs was activation of proMMP2 to MMP2. Membrane bound metalloproteinases (MT-MMPs) control cell migration through tissue matrix but soluble MMPs seem to have little effect on tissue morphogenesis. Knockout MMP2 mice show only mild growth retardation, whereas knockout MMP9 mice display compensated vascular deficiencies associated with the growth plate. However MT-MMP1 knockout mice have severe defects in angiogenesis, bone formation and collagen defects that result in premature death (Quaranta et al., 2000).

1.6.0 Inhibition of MMPs

Alpha 2 macroglobulin accounts for 95% of the plasma inactivation of MMPs in vitro (Cawston, 1986). However, at the tissue level, inhibition of MMP activity is mediated by TIMP (Sticklin et al.,1983, Nagase et al, 2006) resulting in MMP/TIMP complexes appearing in blood, synovial fluid, broncheo-alveolar lavage (Zucker et al., 1999), vitreous humour and blood.

1.6.1 Structure of TIMPs

To date, four members of the TIMP family have been described (Woessner, 1991, Gomaz et al., 1997). They are called TIMP-1 (Sticklin et al.,1993), TIMP-2 (Stetler-Stevenson et al.,1992) TIMP-3 (Uria et al.,1994) and most recently TIMP-4 (Greene et al.,1996). All of these TIMPs share a similar structure, although there are significant differences in amino acid sequences and the degree of carbohydrate substitution. TIMPs are low molecular weight proteins containing 12 sulphydryl groups (Woessner et al.,1991) that are conserved throughout the TIMP family (Greene,1996). The sulphydryl groups form 6 disulphide bonds, which divide the molecule into 6 loops.

Loops 1, 2 and 3 are associated with the N terminal region where TIMP binds to the MMP. Loops 4, 5 and 6 of the TIMP molecule are located at the C terminal end of the molecule. In the truncated form of TIMP, which lacks the C terminal peptide, inhibitory activity is still present (Douglas et al., 1997) albeit at a lower activity. Inhibition of MMPs by TIMP is dependent, initially, on binding at the N terminal region of the TIMP molecule but the C terminal portion of this molecule determines the rate of inhibition.

TIMP-1, molecular weight 28 KD, contains mannose and sialic acid sugars (Birkedal-Hansen et al., 1993) and is glycosylated at two sites. TIMP-2, like TIMP-4, is not glycosylated and has a molecular weight of around 24 KD. While TIMP-1,

2 and 4 are found in plasma TIMP-3 is not, as it is bound to the extracellular matrix (Douglas et al., 1997).

1.6.2 Structural Functionality and Inhibitory Function

By definition, each TIMP will inhibit all the MMPs but do so at differing rates. At the N terminal region, TIMP-1 and 2 bind tightly to MMPs, with a very high affinity (KI^{-9} M) and with a 1:1 molar ratio of enzyme to inhibitor (Nagase et al., 2006). The mechanism for the inhibition of collagenase by TIMP-1 is a non-competitive two-step process (Taylor et al., 1996). During the first reversible stage, there is an initial rapid inactivation of MMP activity while during step 2, a tighter MMP/TIMP complex slowly forms (Woessner, 1991). Inhibition of MMP3 by TIMP-1 requires the amino acids around Cys1-Cys70 (Woessner, 1999). Additionally, TIMP-1 binds specifically to proMMP9 and TIMP-2 binds to proMMP2 via the C domain of the enzyme. Brigg et al., (1997) reported binding of TIMP-4 to the C domain of proMMP2.

1.6.3 Non inhibitory functions of TIMPs

TIMP-1 and 2 have been shown to stimulate a T lymphoblast cell line (Docherty et al., 1985, Stetler-Stevenson et al., 1992) and erythropoiesis, by a mechanism independent of metalloproteinase inhibition. TIMP-1 also upregulates the growth of keratinocytes (Bertaux et al., 1991) by binding specific TIMP-1 receptors on the keratinocytes and not by modification of extracellular matrix turnover.

Hayakawa et al., (1992) demonstrated that TIMP-2, at less than ten times the concentration of TIMP-1, increased cell growth of Raji cells, which are derived from a patient with Burkitt's Lymphoma. Raj cells do not secrete MMPs. Alkylated TIMPs, which have no metalloproteinase inhibitory activity, stimulate Raji cell growth. TIMP-depleted foetal calf serum or the addition of TIMP antibodies, resulted in a marked reduction of growth stimulation, which was reversed on the addition of TIMP-1 or TIMP-2 to the medium. TIMP-1 bound to proMMP-9 or TIMP-2 bound to proMMP-2 had no growth stimulating activity (Hayakawa et al., 1994).

Chesler et al., (1995) studied growth enhancement of mutated forms of TIMPs, the binding of TIMP-1 to MMP, and inhibitory activity of TIMP-1, using modified and truncated forms of the protein. Altered amino acids in positions 3 to13 in TIMP-1 had minimal MMP inhibitory activity but normal binding to MMP and growth stimulation. Substitution of histidine for tyrosine at amino acid 35, markedly reduced growth stimulation but had no effect on the binding or inhibition of TIMPs on MMPs, suggesting that the varying functions of TIMP-1 are located in different parts of the molecule.

1.6.4 Upregulation of TIMPs

This is a profoundly complex area in that most of the work on regulation of the TIMP family has been done using immortal cell lines or from studies, which

although are of theoretical interest, may have little direct relationship to human pathophysiology.

TIMP-1 is regulated by a variety of cytokines and hormones. Chua et al., 1996 demonstrated, in a tissue culture of rat heart endothelial cells, that angiotensin II (ang II) increased mRNA TIMP-1 expression and showed by Western Blot a band corresponding to TIMP-1 with a molecular weight of 28kDa. This effect was blocked by Losartan, an AT1 receptor antagonist or actinomycin C. Although this work utilised rat cells, it is potentially relevant to human pathophysiology, in that it suggests that endothelial cells which line the arteries may be important in upregulation of TIMP-1, which is involved in collagen deposition in the left ventricle and the arteries.

Steroids have been implicated in the control of TIMPs. Patients with acute exacerbations of Multiple Sclerosis, had CSF taken pre and post methyl prednisolone. Post administration of the steroid, the CSF levels of MMP9 levels fell but those of TIMP-1 increased (Rosenberg et al., 1996). The ramifications of this work are of possible importance in other aspects of neurological disease, where the blood brain barrier may be disrupted as a fall in MMPs with an increase in TIMPs may result in stabilising the blood brain barrier.

The groups, Orphanides et al., (1997) and Norman et al., (2000), showed, in tubular epithelial cells, that hypoxia caused an upregulation in TIMP-1 mRNA and collagen I and IV. Hepatic hypoxia has been demonstrated when alcohol is

withdrawn acutely. This observation is clearly potentially important in tissues such as lung, heart and kidney, all of which can develop significant degrees of fibrosis secondary to a hypoxic episode.

Timms et al., (2002) showed that plasma TIMP-1 was significantly reduced by the supplementation of vitamin D to Bangladeshi subjects. This vitamin also suppressed MMP9 levels but to a greater extent, thus favouring collagen deposition.

Shapiro et al., (1992) suggested a possible novel function of TIMP-2 in alveolar phagocytes. TIMP-2 production was not related to MMP production and was downregulated by lipopolysaccarides, unlike TIMP-1, which was upregulated. This observation may suggest that TIMP-2 is more constitutively expressed or that the TIMP-2 may have potentially an additional function, which may be a growth factor.

The above factors demonstrate the variety of effects which growth factors can have on TIMP-1 activity. The relevance of these observations are related to the effect of TIMP-1 in reducing collagen degradation, abnormal concentrations of which have deleterious effects on organ function, with the inevitable increase in morbidity and mortality.

1.6.5 Multiple forms of TIMPS

Multiple forms of TIMP-1 were described in scleroderma cell lines by Kirk et al., (1995) whose team demonstrated electrophoretically on SDS polyacrylamide gel,

two bands corresponding to immunological TIMP-1 activity. The major band had a molecular weight of 28kDa and the minor band, a molecular weight of 30kDa.

Miyazaki et al., (1993) separated TIMP-1 into 6 multiple forms, using isoelectric focusing. Some of these multiple forms reflect post transcriptional modification of the protein but may be a consequence of multiple transcription sites of the TIMP-1 gene. The physiological or pathological role of these multiple forms of TIMP-1 awaits clarification.

1.6.6 Tissue localisation of TIMP

TIMP-1 is located in a wide variety of tissues, including vascular endothelial tissue (Chua et al., 1996) bone, ovary, liver and heart (Chadwick et al., 1998). Placenta and lung alveolar macrophages (Hammani et al., 1996) are amongst the major sources of TIMP-2, whereas TIMP-3 is located mostly in kidney, brain (Edwards et al., 1996) and in developing epithelia, cartilage and muscle (Gordon et al., 1993). TIMP-4 is found predominantly in heart tissue (Greene et al., 1996) but it is also located in the ovary, brain, and in rat arteries (Dollery et al., 1999).

1.7.0 Methods for measuring MMPs and TIMPs

MMP activity can be measured using zymography. This technique is labour intensive but by incorporating different substrates into the gel, the specificity for groups of MMPs can be enhanced. Polyacrylamide gels containing gelatin will

measure gelatinase activity (MMP2 and MMP9). TIMP levels in various fluids can be assessed using reverse zymography (Fabunmi et al., 1996). In reverse zymography, a sample of cell extract undergoes electrophoresis on a gelatin-coated gel. This gel is then treated with interstitial collagenase, and the gelatin is digested, except in areas where there is metalloproteinase inhibition by TIMPs. The gel is stained to show areas where gelatin persists, indicating inhibition of MMPs by TIMPs. This inhibitory activity can be compared to purified TIMPs extracts, run on the same gel. Reverse zymography lacks specificity and most of the current assays use a sandwich ELISA method.

Although there is disagreement (Kodama et al., 1990) on normal TIMP-1 levels in serum and plasma TIMP-1, most authors (Clark et al., 1991,Plumpton et al., 1995, Capper, 1995, Gehrmann et al., 1997, John and Jung, 2006) as well as our own results (Timms et al., 2002) indicate a mean ± (2 sd) concentration of 614 ± (190) ng/ml for serum and a (mean ± 2 sd) of 190 ±(60) ng/ml for plasma TIMP-1. Holten-Andersen et al., (1999) reported much lower values of plasma TIMP-1 but it is likely that the variation in normal range reflects different methods and the use of a variety of calibration materials. Higher values of TIMP-1 found in serum in comparison to plasma, are probably due to its release on clotting from platelets (Cooper et al., 1985). Plasma is the preferred specimen for the analysis. Levels of plasma TIMP-2 are mean ± (2sd) 100±(40) ng/ml. There is little or no data on plasma levels of the other MMPs or TIMP-4. Neither the half-life of MMP9 nor TIMP-1 is known.

1.8.0 Physiological actions of TIMPs

The physiological roles of the MMPs and TIMPs are pivotal in maintaining normal matrix turnover. MMP and TIMPs act in tandem during tissue differentiation, which is important in normal, wound healing (Seifert, 1996). TIMP1, TIMP2 and TIMP3 independently fluctuate in cycling endometrium (Salamonsen, 1998) and are important in implantation of the fertilised egg (Ghosh et al., 1998).

TIMP-1 stimulates steroidogenesis (Boujrad et al., 1995) by an FSH dependent protein, called steroidogenesis stimulating protein (STP) which is produced by the Sertoli cells. STP is a heterodimer consisting of TIMP-1, 28KDa and cathepsin L 38KDa. TIMP-1 is essential for bioactivity of STP, whereas cathepsin L maximises this activity. TIMPs are important in the formation of ductules in the salivary gland and breast, as well as extravasation of leukocytes through matrix (Liotta et at., 1991). Additionally, TIMPs are involved in anti-angiogenesis (reduction in the formation of new blood vessels).

1.9.0 Pathology of MMPs and TIMPs

The pathological role of abnormal matrix deposition (with the exception of inherited metabolic defects) has, until the last 25 years, been largely ignored, in part due to the view that these proteins have long half-lives. The study of the factors controlling matrix in relation to disease, provides a focus for understanding morbidity and mortality in terms of deficient or excessive matrix deposition.

The role of MMPs and TIMPs has been established in several diseases, which include cancer (Liotta et al., 1991, Wan et al., 1997) Sorbsy's dystrophy (Weber et al., 1994), neurological diseases (Cuzner et al., 1999) and rheumatoid arthritis (Malemud, 2006)

Three diseases - psoriasis, liver and cardiac disease, will now be discussed in more detail. In psoriasis, hepatic and breast cancer, there is an important interaction with MMPs and calcitriol. They illustrate the importance of MMPs and TIMPs in diagnosis, or as a marker for disease progression, in addition to a potential modulatory role of vitamin D.

1.9.1 Psoriasis

Psoriasis a chronic skin disease affects about 2% of the population and may be an immune mediated disorder. Kang et al., (1998) studied patients with psoriasis who were subdivided into a control and a calcipotriene (an analogue of calcitriol) intervention group. Patients were scored on the basis of erythema, thickness and scaling and of psoriatic plaques. At baseline, 3 and 7 weeks post treatment, punch biopsies were taken for IL8 and IL10 measurements. The psoriatic lesions improved within 2 weeks and in the skin biopsies, IL10 increased by 57%, with IL8 levels falling by 70% in the calcipotriene but not in the placebo treated group. The addition of an active metabolite of vitamin D suppresses the inflammatory response and enhances healing of the lesion.

1.9.2 The role of MMPs and TIMPs in liver disease

Regardless of the aetiology, a final common event in chronic liver disease is the progression of hepatic fibrosis to cirrhosis. Once cirrhosis has developed, the liver architecture is so disrupted that hepatocytes are unable to function normally, and a return to normal liver function is not clinically possible without transplantation. The cirrhotic liver contains approximately six times the amount of collagen (mainly types I and III) of normal liver (Rojkin et al., 1979). TIMPs and MMPs have been studied in an effort to understand the underlying mechanism(s) of hepatic fibrosis, their possible use as clinical markers of disease and their therapeutic potential. A normal extracellular matrix is necessary to maintain the hepatic stellate cells (also called lipocytes or Ito cells) in their normal phenotype. The activity of hepatic collagenase (MMP1) decreases as hepatic fibrosis increases in severity. Liver injury, causing inflammation, stimulates stellate cells to release various factors, including TGF α (Arthur, 1995) which causes activation, proliferation and transformation of the normally quiescent Ito cells. Hepatic damage without inflammation (for example, in haemochromatosis) may also activate stellate cells, probably by a factor released from damaged hepatocytes.

Activated stellate cells secrete both collagenous and non-collagenous extracellular matrix proteins and these cells are also involved in regulation of collagenous matrix via production of MMP 2 and MMP3 and TIMP-1 (Iredale et al., 1992). In an elegant series of experiments, Benyon et al., (1996) showed that in fibrosis, there is hepatic upregulation of TIMP-1, TIMP-2 relative to MMP2 mRNA

levels and their increased protein synthesis was demonstrated by reverse zymography. The elevation in TIMP-1 was localised to lipocytes. Upregulation of MMP1 mRNA did not occur in fibrosis (Benyon et al., 1996, Arthur et al., 1992). Benyon et al., (1996) showed an increase in both MMP2 and TIMP-1 and TIMP2 following lipocyte activation.

The increase in TIMP-1 and 2 expression was relatively much greater than that of MMP2 resulting in an increase ratio of TIMP/MMP. Furthermore the correlation between TIMP-1 and TIMP-2 mRNA and tissue hydroxyproline content ($r = 0.65$ and 0.80, $p<0.05$ and <0.01) respectively demonstrate an association between gene expression and collagen accumulation. The net effect of this process resulted in inhibition of collagen degradation, promoting fibrosis.
There is now persuasive data that, at least in part, excess hepatic collagen deposition arises as the result of decreased collagen degradation, mediated by increased TIMPs.

The natural progression of cirrhosis involves upregulation of the stellate cell, with consequential inflammation and aberrant collagen deposition. Since the stellate cell is a component of the recticulo-endothelial system it may be downregulated by administration of vitamin D or 25(OH)vitD. Target tissues for 25(OH)vit D and its active metabolite have been located in targets cells through the gut and liver. Levels of radioactive labelled 25 and 1,25 dihydroxyvitamin D are located in the stellate cells, the hepatic equivalent of a macrophage (Stumpf, 2008). Terrier et

al., (2011) in a group of patients with hepatitis C, demonstrated that fibrosis was significantly worse in patients with low levels of 25(OH)vitD. Theoretically, this is not unexpected, as decreasing levels of 25(OH)vitD are inevitable in liver disease and the stellate cell may be upregulated by decreasing 25(OH)vitD. However, this issue will not be resolved until a formal trial in which vitamin D and 25(OH)vitD are administered as two intervention arms in a control study.

1.9.3 The role of plasma TIMP-1 as a marker of fibrosis

There is a good correlation between serum and hepatic levels of TIMP-1 in a range of liver diseases (Murawak et al., 1997) and encouraging studies on the role of plasma TIMP-1, as a non-invasive marker of histological damage in alcoholic liver disease, hepatitis C (Walsh et al. 1999, Kasahara et al., 1997) and other chronic liver diseases (Murawaki et al.,1993). Li et al. (1992) studied 44 alcoholics versus eight controls. Alcoholics had elevated TIMP-1 levels, which could distinguish patients with septal fibrosis from controls. TIMP-1 was not successful at discriminating steatosis from periventricular fibrosis or cirrhosis in this group of patients.

These studies suggest that there is a correlation between increasing histological damage and increasing levels of plasma TIMP-1, although they have not so far demonstrated a clear clinical usefulness.

Routine tests of liver function are inadequate indicators of hepatic fibrosis. Liver biopsy is obviously invasive and is not without risk. Perhaps the most promising potential role for TIMP-1 as a marker of fibrosis, would be in monitoring disease progression and response to treatment. TIMP-1 may be a useful plasma marker of hepatic fibrosis (Flisiak et al., 2002) but more useful when used in conjunction with other potential plasma markers of fibrosis for example, hyaluronic acid, bilirubin and gamma gutamyl transferase, as shown by Leon et al. (2005).

Plasma TIMP and MMP levels may also provide useful information for research into disease pathogenesis, in situations where biopsy would not normally be performed. For example, in alcoholic subjects without clinically overt liver disease, we found that TIMP-1 levels were markedly elevated (Campbell et al., 2001). Abrupt alcohol withdrawal increases PIIINP plasma levels, which are a marker of collagen synthesis. Whether this is due to altered collagen production or merely reflects changes in clearance of these metabolites, is unclear. The clinical promise of TIMPs and MMPs would be the development of new therapies to prevent or reverse cirrhosis. Traditionally, the cirrhotic process is thought to be irreversible, and for practical purposes a reversal of cirrhosis is almost never observed clinically in chronic liver disease. However, early work suggests that histologically some reversal of cirrhosis might be possible, especially if not fully developed (Perez-Tamayo, 1979). Iredale et al., (1998) suggested that carbon tetrachloride-induced fibrosis (which resolves after removal of the toxic insult) is driven by increasing TIMP-1 and TIMP-2 levels, whilst MMP levels are maintained. No work

has yet attempted to alter the MMP/TIMP ratio to modify fibrosis in a clinical setting in humans.

1.9.4 Role of MMPs and TIMPs in Left Ventricular hypertrophy

Left ventricular hypertrophy (Srikanthan, 1997) most commonly caused by essential hypertension, is diagnosed by electrocardiography or echocardiography. LVH has a very poor prognosis (Cruickshank, 1992).

In general, LVH can be subdivided into physiological LVH, which occurs in athletes and in which the collagen myocyte ratio in the left ventricle is normal and pathological LVH, which is associated with an absolute increase in ventricular collagen (Weber et., 1992). The increase in collagen within the left ventricle is associated with a reduction in its elasticity, an increase in the number of ectopic beats and a reduction in coronary arteriole dilatation.

1.9.5 Mechanism of left ventricular hypertrophy

Two mechanisms are involved in the pathophysiology of LVH. Firstly, the myocyte, which is a terminally differentiated cell, hypertrophies due to increased blood pressure. Secondly, Brilla et al., (1990) described, in a series of elegant experimental models of hypertension, that upregulation of angiotensin II (the one clip model) was associated with increased fibrosis in the left ventricle. Rats rendered hypertensive by infra-aortic banding (although having similar elevations

in blood pressures to the one clip model) had normal angiotensin II levels and no evidence of ventricular fibrosis. This suggests that the fibrosis associated with LVH is angiotensin II dependent and the myocyte hypertrophy is primarily driven by pressure.

1.9.6 Effect of drugs on cardiac fibrosis, in rats with essential hypertension

Further experiments showed that treatment with suppressor doses of the angiotensin converting enzyme inhibitor (ACEI) Lisinopril to spontaneously hypertensive rats (SHR) had no effect on blood pressure and did not affect the development of LVH but did reduce ventricular fibrosis (Brilla et al., 1995). In contrast, treatment of SHR with a diuretic or alpha blocker would reduce blood pressure but had no effect on cardiac fibrosis. Treatment with an ACEI at pressor doses reduced blood pressure, ventricular stiffness and ventricular fibrosis. In the left ventricle of the hypertrophied heart, metalloproteinase activity was reduced. These studies show that renin-driven hypertension increases TIMP-1 levels, thereby increasing collagen deposition in the left ventricle.

1.9.7 Mechanism of cardiac remodelling

Cardiac remodelling may occur by the following mechanism:
ACEI reduces angiotensin II levels, which cause a fall in both TIMP-1 and PAI-1 concentration, resulting in an increase in MMP activity (Lavaides et al., 1998) and consequent increased degradation of matrix. Angiotensin II has several actions on the heart, including the increase of collagen deposition.

Human cardiac fibroblasts were isolated from explanted human hearts and incubated with 100nmol/L of angiotensin II. At this dose, angiotensin II has several effects, including an increased c-fos and the early growth response gene-1 and a profibrotic action. Angiotensin II increases TGF-β levels which increase collagen 1 synthesis and TIMP-1 levels. Angiotensin II increased expression of PAI-1 results in reduced levels of the active MMPs from their inactive precursor (Kawano et al., 2000). Angiotensin II (Chua et al.,1996) indirectly increases collagen synthesis and inhibits collagen degradation by increasing TIMP-1 levels, possibly via a signal transducer and activators of transcription (STAT) (Wang et al., 2006) and reduces degradation of the proMMP to the active form by inhibition of plasmin.

These multiple profibrotic effects of angiotensin II explain the mechanism for the increase of collagen deposition in hypertensive induced LVH.

1.9.8 The aetiology of cardiac ischaemia

IHD continues to be the commonest cause of death in the western world. Patients with diabetes have a threefold increased risk of IHD, compared to controls, in addition to the traditional risk factors (smoking, hypertension and hypercholesterolaemia).

The hallmark of coronary artery disease is the atheromatous plaque, which begins as a fatty streak and is seen almost ubiquitously in adolescence and early

adulthood. At this early stage, the lesion consists of intimal thickening, secondary to accumulation of extracellular matrix (ECM) and smooth muscle cells (SMC) that have migrated from the media. As the plaque enlarges, it compromises the lumen of the coronary artery, becoming clinically significant once the lumen has been reduced by 70-80%.

Prospective angiographic studies show that it is not the most severe lesions (in terms of lumen reduction) which present with acute coronary syndromes, acute myocardial infarction (AMI) and unstable angina (UA) but rather those which are less than 50% occluded. The development of an acute coronary syndrome is initiated by fissuring of the fibrous cap with subsequent thrombosis, which is either occlusive AMI or sub-occlusive UA. Many mechanisms have been suggested for plaque fissuring, one of which is the upregulation of matrix MMPs (Dollery et al., 1995).

1.9.9 Plasma IL6 as a risk factor for myocardial infarction

MMPs are important in maintaining plaque stability and several studies on heart disease have looked at levels of plasma cytokines, which upregulate MMPs. IL6 was measured on 202 male patients who had a history of myocardial infarction and 202 healthy subjects, who were matched for age and smoking habits. The risk of an MI increases with increasing IL6 levels ($p<0.001$) with men in the

highest quartile for IL6, having an increased relative risk 2.3 times higher than the control group. IL6 was positively correlated with CRP (Ridker et al., 2000a).

1.9.10 Plasma levels of TNF α as a predictor of re-infarction

In an extension to the Cholesterol and Recurrent Events Trial (CARE) the TNFα levels were 2.57 pg/ml in those who remained event-free but were 2.84 pg/ml (p=0.02) in those who re-infarcted. Those patients with the highest levels of TNFα had a threefold increase in AMI recurrence. The cause of the increased events is multifactorial but may, in part, be due to the action of TNFα increasing macrophage production of MMPs (Ridker et al., 2000b).

It is not clear whether the relationship between IL6, TNFα and heart disease are cause and effect or an epiphenomenon. These markers may be useful for epidemiological studies but like sCRP, are poor indicators of individual risk.

1.9.11 Role of lipids in destabilising atheromatous plaque

Plasma LDL and HDL are independent risk markers for ischaemic heart disease. Macrophages incubated with oxidised low-density lipoprotein (ox LDL) showed upregulation of MMP9. This increase in MMP9 was attenuated by the addition of HDL (Xiao-Ping et al., 1999). When IL8 was incubated with human-derived macrophages, TIMP-1 levels fell but MMP9 levels increased in the culture fluid (Moreau et al., 1999). If results from both these studies could be extrapolated to

plaque, an increase in MMPs and a fall in TIMP-1 levels within the atheroma would destabilise plaque by reducing the thickness of the fibrous cap.

Statins are the treatment of choice in reducing plasma cholesterol in patients with primary hypercholesterolaemia. The statin, Fluvastatin, has been shown to reduce MMP activity and increase collagen content in rabbit atheroma (Aikawa et al., 1998). In both mouse and human monocyte-derived macrophages, incubation with Fluvastatin was associated with a dose-dependent reduction in MMP9 in the culture medium. The MMP9 levels were reduced within the culture medium, due to defective secretion (Bellosta et al., 1998). This data suggests that statins not only reduce cholesterol levels but also may stabilise plaque by a reduction in MMP9 levels by a class effect.

Elevation of arterial TIMP-4 has been associated with balloon- induced vascular injury in the rat (Dollery et al., 1999) demonstrating TIMP-4 is not cardiac specific but is also located in the arterial wall.

Several studies have shown, that within unstable plaque, MMPs are elevated, being modulated by inflammatory cytokines (Galis et al., 1994, Galis et al., 1995., Libby, 1995). That MMP2 is elevated in unstable plaque is surprising, as this MMP is usually constitutively expressed. However, it was recently shown that MMP14, the membrane bound enzyme involved in activation of MMP2, was increased by inflammatory cytokines (Rajavashisth et al., 1999).

1.9.12 Plasma MMPs and TIMPs in vascular disease

There is increasing evidence that during acute plaque disruption, there is a significant increase in MMPs with a resulting imbalance between proteolytic degradation and the lack of inhibition (Loftus et al., 2002).

Plasma MMP2 and MMP9 have been measured in patients with coronary syndromes. Patients with AMI or UA, within three days, had differing levels in both MMP2 and MMP9 after the episode. The levels of MMP2 and MMP9 were higher in those patients with UA than in those with the AMI (Kai et al., 1998). Plasma levels are also elevated in patients with peripheral arterial disease (PAD) (Tayebjee et al., 2004) showing that MMPs and TIMP-1 have a role in plaque remodelling. Maxwell et al., (2001) demonstrated that plasma TIMP-1 levels are elevated in well controlled Type 1 diabetics. Work by Ebihara et al., (1998), showed plasma MMP9 levels are elevated in diabetic patients, several years prior to the development of microalbuminuria.

Smoking is one of the modifiable risk factors in IHD. Plasma MMP9 levels were significantly higher in 50 smokers versus 50 non smokers (52 ± 27, versus 34 ± 17 ng/ml respectively). MMP9 levels were also higher in those who had smoked longer. Furthermore, stopping smoking for six months, reduced the levels of plasma MMP9. The higher levels of MMP9 may be a reflection of the greater risk of IHD associated with smoking (Tsukasa et al., 1998).

In patients who were successfully re-perfused immediately post infarction, Hirohata et al., (1997) showed that plasma TIMP-1 levels were low. This is an appropriate response, if plasma TIMP-1 levels reflect those found in the heart, since MMP activity must be maximal to degrade ischaemic muscle and connective tissue. However, this illustrates an interesting but as yet unexplained, down regulation of TIMP-1 in presumably hypoxic conditions.

Vitamin D deficiency is very common in the UK and from the works of McPherson et al., (2000) and Grimes et al., (1995) this vitamin D deficiency may be associated with cardiovascular disease raising the interesting speculation, 'Does vitamin D suppress MMPs?'

1.10.0 Summary of Introduction

Recent work on MMPs and TIMPs has raised some important questions, including the control of MMP levels in plaque and restenosis. Studies in patients with psoriasis suggest treatment with calcitriol, or one of its analogues, reduces inflammation via an IL10 mediated mechanism. New data on the importance of the inflammatory response in destabilising plaque in patients with IHD (in addition to the apparent relationship between low levels of vitamin D and IHD) raises the intriguing possibility that vitamin D may increase IL10 levels within plaque and down regulate MMP levels. MMP inhibitors reduce the incidence of restenosis due to inhibition of MMPs, which, theoretically, might be achieved by an increase in vitamin D consumption.

1.11.0 Hypotheses: There are four hypotheses in this study.

1. The first addresses whether plasma TIMP-1 is an acute phase protein.

2. The second hypothesis, using parallels with the elevation of TIMP-1 in liver fibrosis, concerns a potential relationship between TIMP-1 and LVH..As some causes of LVH may be driven by the RAAS system, it is a possibility that TIMP-1 levels may be elevated by aldosterone.

3. In the third hypothesis, it is suggested that plasma MMPs and TIMPs which represent the production from many tissues, may be produced in the vasculature.

4. In the fourth hypothesis, it is suggested that MMP2, MMP9 and TIMP-1 may be related to established serum markers of cardiovascular risk. As this concept developed, the association of 25(OH)vit D with TIMPs and MMPs was investigated.

1.12.0 Aims and Objectives

The clinical studies were divided into three groups.

In the first group, we wished to determine whether plasma TIMP-1 was an acute phase protein, as suggested by previous work on non invasive markers of liver fibrosis. This question was addressed by measuring several specific proteins, (prealbumin, albumin, orosomucoid, transferrin, CRP and TIMP-1 in patients with liver disease.

In the second group, the role of plasma TIMP-1 measurement as an indicator of enhanced collagen deposition in LVH secondary to hypertension, was accessed by measuring TIMP-1 plasma levels in normotensive and hypertensive patients in whom echocardiograph status had been established. Any potential relationship between TIMP-1 and aldosterone was assessed in patients treated with an angiotensin converting enzyme inhibitor (ACEI).

The second group of experiments arose from a continuation of the work from the study which addressed TIMP-1 as a potential marker for fibrosis in patients with essential hypertension. The inter-relationships between MMP2, MMP9 and TIMP-1 levels with other established plasma markers of vascular disease insulin, sCRP, fibrinogen and PAI-1 were evaluated.

1. MMP2, MMP9 as well as TIMP-1 relationships with 25(OH)vitD were studied in a group of Bangladeshi subjects.

2. On the basis of the above experiment, the relationships between MMP9, TIMP-1, TIMP-4, sCRP and 25(OH)vitD in submariners was studied.

3. Finally, the theoretical concept of whether levels of 25(OH)vitD or MMP9 may be important in predicting restenosis post angioplasty in naïve patients was studied.

4. A total TIMP-4 (tTIMP-4) assay was validated and measured in normal subjects and submariners.

Chapter 2

ELISA assays

2.0.0 Principle of the ELISA method

In ELISA assays, a sample containing a specific protein antigen is first incubated in a microtitre plate well pre-coated with a specific antibody directed against the protein antigen. The microtitre plate is washed with assay buffer to remove unbound antigen and a ‘sandwich’ is formed by reacting the antibody-antigen complex with a second, enzyme-labelled antibody, raised against a different epitope of the antigen. Any excess peroxidase antiserum is removed by further washing. The amount of peroxidase bound to the plate is determined using tetramethylbenzidine (TMB). TMB, when oxidized, forms a blue colour, which on the addition of sulphuric acid, becomes yellow. The absorbance of this yellow colour 3, 3' 5, 5' tetramethyl diphenoquione 4-4' diammonium is produced by incubating 3, 3' 5 5' tetramethylbenzidine with hydrogen peroxide. The absorbance of the final colour is measured at 405 nm. The specific protein present in the original specimen is quantified by comparing this absorbance to a standard curve.

2.1.0 Amersham Biotrak TIMP-1 assay

2.1.1 Reagents

Details of the Amersham kits are in Appendix 1

Assay buffer: This was supplied as a powder and 90 millilitres (ml) of distilled water were added to give a final concentration of 0.1M sodium phosphate buffer

pH 7.5, 0.9 % {w/v) NaCl, 1% (w/v) bovine serum albumin (BSA) and 0.1 % Tween
20. The master standard supplied with the assay kit was supplied as a powder and was reconstituted with 1 ml of assay buffer, to give a final concentration of 100ng/ml of TIMP-1. Working standards were prepared by serial dilution. Hence, 400 µl of master standard (100 ng/ml) was added to 400 µl of assay buffer and mixed to give a 50 ng/ml standard. 400 µl of the 50 ng/ml standard was added to 400 µl of assay buffer to give 25 ng/ml and so forth to give a range of 3.13 ng/ml – 50 ng/ml.

Peroxidase reagent: This was supplied as a powder and was reconstituted immediately prior to use, by the addition of 12 ml of distilled water, to obtain the working second antibody- peroxidase conjugate reagent in assay buffer.

Wash Buffer: This was supplied as a liquid and was reconstituted by addition of 500ml of distilled water to give 100 mM/L phosphate buffer pH 7.5 in 0.05% Tween 20. TMB substrate solution 3, 3, 5, 5 tetramethylbenzidine (TMB/hydrogen peroxide in 20% dimethylformamide) was supplied ready to use.
Sample preparation: The specimens were diluted 1:100 and dispensed into the microtitre plate using a Quattro dilutor.

2.1.2 Method

100 µl of blank (assay buffer) was added into the zero standard wells followed by the standards in duplicate, in columns 1A to 1G and column 2A to 2G. The diluted samples were dispensed into the remaining wells of the microtitre plate. The microtitre plate was incubated for 2 hours at 20 degrees centigrade. The plate was then "flicked" to remove the unbound contents of the well and plunged into the wash buffer. This procedure was repeated 3 more times. The microplate was then inverted over a pad of paper towels and blotted. 100 µl peroxidase conjugate was added to all wells using a multichannel pipette. After incubation for 1 hour, the plate was "flicked", washed three times and blotted as before. 100 µl of TMB was then added to all wells and the microplate was incubated in the dark for 30 minutes. 100 µl of stop solution was added to each well and the optical density read at primary and secondary wavelengths of 405 nm and 570nm respectively.

2.2.0 MPP2 assay system Amersham Biotrak

2.2.1 Principle previously described in section 2.3.1

2.2.2 Reagents

Assay buffer: This contained 0.03M phosphate buffer pH 7.0, 0.1MNaCl, 1% (w/v) bovine serum albumin and 0.01M EDTA, when reconstituted with 90 ml of distilled water.

Master Standard: This was supplied as powder. 1ml of distilled water was added to give a master standard of 48ng/ml MMP2 in assay buffer.

Preparation of working standards.

Working standards of 1.5, 3.0, 6.0, 12.0, 24.0 and 48 ng/ml were prepared by serial dilution of the master standard, as described for TIMP-1.
Peroxidase reagent: This reagent was supplied as a powder and was reconstituted immediately prior to use, by the addition of 12 ml of distilled water to obtain the working second antibody reagent in 0.03 M phosphate buffer pH 7.0 with 0.1 sodium chloride, 2% bovine serum albumin and 0.01M EDTA.
Wash Buffer: This reagent was supplied as a liquid and was reconstituted with 500ml of distilled water to obtain a solution of 0.01M phosphate buffer pH 7.0 in 0.05% Tween 20.

TMB substrate solution in 20% dimethylformamide is ready to use.
Sample preparation: The specimens were diluted fifty-fold automatically and dispensed into the microtitre plate, using a Quattro dilutor.

2.2.3 Method

100 µl of blank assay buffer was added into the zero standard wells followed by the standards in duplicate in columns 1A to 1G and column 2A to 2G. The samples are as previously described, diluted and dispensed into the microtitre plate using the Quattro dilutor. The microtitre plate was incubated for 2 hours at

20 degrees centigrade. The plate was "flicked" to remove the contents of the wells and plunged into the wash buffer. This procedure was repeated 3 more times. The microplate was inverted over a pad of paper towels and blotted, to ensure the wells were drained. Using the multichannel pipette 100 µl of peroxidase conjugate was added to all wells. After incubation for a further hour, the plate was "flicked" to remove the contents of the well and plunged into the wash buffer. This procedure was repeated 3 more times. The microplate was inverted over a pad of paper towels and blotted to ensure the wells were drained. Using the multichannel pipette 100 µl of TMB was added to all wells. The microplate was incubated in the dark for 30 minutes. 100 µl of stop solution was added to each well and the optical density read at primary and secondary wavelengths of 405 nm and 570 nm respectively.

2.3.0 Measurement of MMP9 (R&D) assay

2.3.1 Principle previously described in section 2.3.1

Details of the kit are in appendix 1

2.3.2 Reagents

Assay diluent: 11 ml of a buffered solution containing albumin.

Master standard: The powder on reconstitution with 1 ml of de-ionised water had a concentration of 20ng/ml.

Preparation of working standards: These were prepared as for the TIMP-1 assay to provide a range of standards of 0.312, 0.625, 1.25, 2.5, 5.0 and 10.0 ng/ml.

Second antibody- peroxidase conjugate reagent 21 ml is ready to use.

Wash Buffer: Prepared by adding 500 ml of water to 20 ml of wash buffer.

Reagent 1: Hydrogen peroxide ready to use

Reagent 2: TMB ready to use.

Working colour reagent: 15 minutes prior to use, equal volumes of Reagents 1 and 2 must be added and mixed.

2.3.3 Method

100 µl of blank assay buffer was added into all the wells, followed by the standards in duplicate in columns 1A to 1G and column 2A to 2G. The samples were as previously described, diluted and dispensed into the microtitre plate using the Quatrro dilutor. The microtitre plate was incubated for 2 hours at 20 degrees centigrade. The plate was "flicked" to remove the contents of the well and plunged into the wash buffer. This procedure was repeated 3 more times. The microplate was inverted over a pad of paper towels and blotted to ensure the wells were drained. Using the multichannel pipette, 100 µl of peroxidase conjugate was added to all wells. After incubation for a further hour, the plate was "flicked" to remove the contents of the well and plunged into the wash buffer. This procedure was repeated 3 more times. The microplate was inverted over a pad of paper

towels and blotted to ensure the wells were drained. Using the multichannel pipette 100 µl of TMB was added to all wells. The microplate was incubated in the dark for 30 minutes. 100 µl of stop solution was added to each well and the optical density read at primary and secondary wavelengths of 405nm and 570nm respectively.

3.0.0. Comments on the ELISA assays.

The ELISA assays for MMP2 and MMP9 were robust and satisfactory. However, there was a significant difficulty with the TIMP-1 assay. Some of the following is speculation regarding the history of the Amersham TIMP-1 assay. In 2004. suddenly, the TIMP-1 assay failed to perform in terms of recovery experiments which showed recovery increasing, as more standard was added to the serum. Furthermore, the assay failed to differentiate as well between healthy and hypertensive and cirrhotic subjects. The explanation from Amersham was that their mouse monoclonal antibodies had become infected and Amersham had changed the kit formulation without informing anyone. The R & D kit was tried but the characteristics of this kit were similar to those of the new Amersham method. I discovered that Professor Cawston, one of the major workers, developing the original TIMP-1 assay, still had an assay available with the original mouse monoclonal antibodies and he kindly sold me several kits. However, even this source of the TIMP-1 assay became unavailable and so I could no longer use the plasma TIMP-1 measurements in further studies.

Another kit from Oncogene was investigated and this had satisfactory recovery and a satisfactory clinical differentiation between healthy, hypertensive and cirrhotic patients. We are now measuring the interaction between TIMP-1 and aldosterone using this assay in subjects pre and post vitamin D supplementation.

Chapter 3

Development of a new total TIMP-4 assay

Prior to developing this total TIMP-4 assay (tTIMP-4) its potential suitability in the clinical context was assessed using Western Blotting.

3.1.0: Principle of TIMP-4 Immunoblot

The serum sample was electrophoresed in polyacrylamide gel to separate out the proteins, on the basis of their molecular weight. The gel was removed from the cartridge and a “sandwich” made between the gel and the nitrocellulose. This sandwich was loaded into a cartridge and by electrophoresis the proteins were transferred from the gel to the nitrocellulose. The nitrocellulose is carefully removed from the gel and incubated with 3% skimmed milk. This solution blocked all the protein binding sites, not occupied by the transferred protein, with milk proteins. After incubation overnight, the sample was incubated with an antibody against TIMP-4. A second antibody with HRP bound to it, reacted with the first antibody, permitting localisation of the protein band of interest. The enhanced chemiluminescent substrate (ECL) reagent is a sensitive method for HRP stimulated oxidation of luminol, thereby enhancing chemiluminescence.

3.1.1 Reagents

3.1.1.0 TIMP-4 antibody

The TIMP-4 antibody was obtained from the antibody production unit of the West of Scotland Blood Transfusion Service (WSBTS). Antigen for inoculation was

prepared by synthesis of the last C terminal 7 amino acids of TIMP-4. The polypeptide with adjuvants was injected into a sheep and an IgG fraction was prepared.

3.1.1.1. Reagents for the Western blot

Details of reagent supplier are in Appendix 1

Electrophoresis buffer: Tris glycine buffer pH8.3 from Bio-rad. Diluted 1x10 to prepare a working solution.

Transfer Buffer: 600ml of methanol, 15.15 g TRIS, and 72g glycine were added and made up to three litres with deionised water and mixed and stored at 4 degrees centigrade.

Phosphate buffered saline: 0.01 M phosphate buffer pH 7.4 made up in 0.9% NaCl.

Working buffer: Glycine TRIS buffer 40 ml was diluted to 400 ml. and stored at 4 degrees centigrade.

Working Laemilli buffer: In a fume cupboard, 50 µl of mercaptoethanol was added to 950 µl of working buffer.

Secondary antiserum for TIMP-4 : HRP anti sheep

Enhanced Chemiluminesence (ECL) reagent from Amersham was prepared immediately prior to use, by adding 1ml of reagent A and B and mixing.

3.1.2.0 Method for Western Blot

3.1.2.1 Pre-treatment of sample

100 µl of plasma was added to 400 µl of Laemilli buffer in a glass tube, which was placed in boiling water for 5 minutes, after which the tube was removed and allowed to cool to room temperature.

3.1.2.2 Preparation of gel cartridge

The outer cover was removed, as was the plastic at the bottom of the gel and the unit was assembled in the electrophoresis cartridge, ensuring the open gel was facing the inside of the cartridge. 200 ml of working buffer was added, until the level was 4 mm from the top. Using both hands, the comb was carefully removed, revealing the sample wells.

3.1.2.3 Application of samples and initial electrophoresis

8 µl of samples or coloured markers were added to each well. The coloured markers had a molecular weight range from 10 to 250 kD. The electrophoresis tank was connected to the power pack and run at constant voltage 200 V for 30-35 minutes.

3.1.2.4 Removal of gel from the plastic cartridge

The cartridge was rinsed in distilled water and the plastic holding the two plates together, was cut with a scalpel. The plastic plates were squeezed hard and were gently separated, yielding the gel, which was placed onto the equilibrated filter paper.

3.1.2.5 Western Blotting

Plastic gloves were worn during the following procedure. All the units of this cartridge were equilibrated in blotting buffer which is used ensure transfer of the proteins from the gel to the nitrocellulose. The blotting buffer was kept at 4 degrees centigrade, except for the membrane, which was initially rinsed in methanol. The sandwich was assembled in the following order:
The blotting cartridge (black side down) was placed in a plastic container. After the addition of a nylon pad, the filter paper (with the gel uppermost) was added to this pad. The membrane was nicked on the left hand side and carefully laid on top of the gel, ensuring all air bubbles were excluded.

The sandwich was assembled in the blotting cartridge, the chiller inserted and the chilled blotting buffer added, until it was near the top of the sandwich. The Western blot was run at 100 volts for 1 hour.
The sandwich was taken apart. Any gel still attached to the membrane was removed by a short wash in phosphate buffer.

----------red (+)
----------pad
----------filter paper
----------membrane
----------gel
----------filter paper
----------pad
----------black (-)

Figure 5 Details of the sandwich assembly.
The bottom of the cartridge was covered by an nylon pad, onto which was placed the filter paper gel and membrane. The sandwich was completed by additional of a further filter paper and pad.

3.1.2.6 Antibody antigen reaction on membrane

The gel was incubated for at least 3 hours or overnight, in 3% non-fat milk (from Tesco supermarket). It was imperative that only this brand of dried milk was used as other brands eg Asda Supermarket, in my experience, are useless, giving rise to numerous analytical problems, which include a high background and non specific binding, which cannot be removed by washing. This solution was poured over the membrane and incubated overnight at 4 degrees centigrade, in a fridge and the membrane was washed for 15 minutes in 50 ml of phosphate buffer. This wash step was repeated three times.

The membrane was incubated and rotated at room temperature for 3 hours, or overnight in a fridge, in a universal tube, in 5 ml of 10% non fat milk, to which was added 50 µl of TIMP-4 antibody. The membrane was washed, using 20 ml of phosphate buffer for 15 minutes, on four occasions. 5µl of the antisheep HRP was added to 5 ml of phosphate buffer for 30 minutes. The membrane was extensively washed, as previously stated.

Excess buffer was removed from the membrane, which was placed on cling film – procedure as follows:

1) ECL mixture was added for 1 minute and then excess reagent removed. The membrane was wrapped up in cling film, ensuring the surface (which would come into contact with the X ray plate) was smooth and not wrinkled.

2) In the dark room, the wrapped membrane was placed on top of the x-ray plate, initially, for 30 seconds. This process was repeated on different parts of the film for 1, 2, 5 and 8 minutes. The film was kept on the plate until the film was developed.

3.1.3.0 Results of the Western Blot

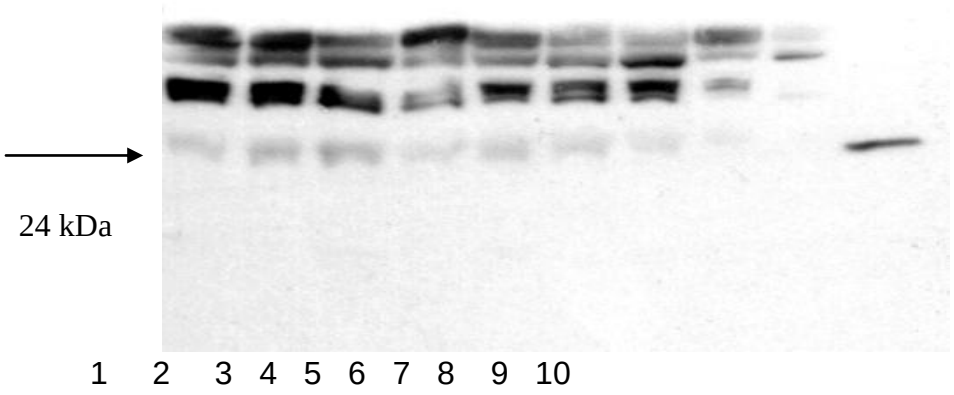


Figure 6 Western blot of plasma TIMP-4.
Samples were electrophoresed blotted onto nitrocellulose and treated with sheep anti TIMP-4. There is a single free TIMP-4 band at 24 kDa and a series of TIMP-4 and MMP complexes with higher molecular weights.

The pattern of staining in figure 6 shows a lower molecular weight band corresponding to 24kD and higher molecular weight complexes of up to 90kD.

3.1.4.0 Discussion on the Western Blot

The lower molecular band matches that of the TIMP-4 marker protein at 24kD and corresponds to free or unbound TIMP-4 (Greene et al., 1996). The other multiple

bands probably reflect the heterogeneity of TIMP-4 binding to a variety of MMPs and their inactive precursors proMMPs (Bigg et al., 2001 and Liu et al., 1997). This Western Blot provided evidence that this antiserum may be a suitable component for an ELISA.

3.2.0 Validation of an immunoassay for serum total TIMP-4 (tTIMP-4)

3.2.1 General Introduction

Probably the most robust immunoassay design is the sandwich technique called enzyme linked immunosorbent assay (ELISA). This method avoids the use of radioactive iodine assays which have a short life span and they also improve assay specificity, as they have, by definition, a capture antibody and a signal antibody. Such an assay design permits increased specificity due to antibody binding at two different locations

3.2.2.0 Optimising the TIMP-4 assay.

3.2.2.1 Introduction

In this experiment preliminary evidence of the TIMP-4 antiserum dilution used in the sandwich and an approximate concentration of the three different antiserum, each of which will be used as the signal antibody.

3.2.2.2 Reagents

Three antibodies were purchased form Chemicon

Rabbit anti human antiloop1

Rabbit anti human antiloop2

Rabbit anti human anti loop3

Anti human sheep raised against the last seven amino acids of TIMP-4 was obtained from the Scottish Blood Transfusion Service.

TIMP-4 standard was purchased from R&D.

Priming buffer phosphate buffered saline PBS, 1% bovine serum albumin and 2% Bronidox which is added as a preservative.

Assay buffer 0.1% bovine serum albumin, 0.01 % Triton X in phosphate buffered saline.

Wash solution 0.01 % Triton X 100 in PBS.

Glazing solution 2% mannitol and 1% hydrolysed gelatin dissolved in water and freshly prepared prior to use.

Borate buffer pH8 for passive coating

3.2.2.3 Method

The micro-titre plates which had previously been coated with a donkey anti-sheep antibody (which reduces background noise) were now additionally coated with 200 µl of three dilutions of the sheep anti TIMP-4; a 1/1000 (20 µl up to 20ml) a 1/5000 (4 µl up to 20ml) and a 1/10,000 (2 µl up to 20ml). The plates were incubated

overnight and placed in a humidifier. The next morning these plates were washed five times with wash solution and using a multichannel pipette, 300 µl of glazing solution was added to each well for 30 minutes. The contents of each well were aspirated but not washed. The plates were freeze dried and stored in aluminum foil with a desiccant.

For each dilution of the sheep antibody, a blank and 1000 ng/ml standard was set up in duplicate and incubated for four hours. The wells were aspirated and washed for 5 cycles.

Two dilutions of each of the rabbit antisera to loop1, loop2, and loop3 were prepared; 1/1000 (7µl up to 7ml) and a 1/20,000 (0.5 ml of the 1/1000 dilution added to 9.5 ml of assay buffer). 200 µl of each anti serum was added with 200 µl of assay buffer to the appropriate wells and the plates left overnight in a humidified chamber. The next morning the plates were washed and aspirated for 5 cycles.

Rabbit HRP antiserum was made up in a 1000 dilution (20 µl up to 20ml in assay buffer) and a 2000 dilution (10 µl made up to 20ml in assay buffer) and added to the appropriate wells. The plates were mixed for two hours and again washed for 5 cycles. 100 µl of acid was added to each well and the plate was read at 450nm.

3.2.2.4 Results

Unfortunately there was no difference between the blank and the 1000ng/ml standard: a total failure!

3.2.2.5 Discussion

Despite utilising several permutations of incubation times and utilising the rabbit anti human loop1, 2 and 3 as the capture antibody and the sheep C terminal as the second antibody, there was absolutely no difference between the blank and the standard absorbance. The reason for this lack of binding is described in Section 3.2.3.0

3.2.3.0 Iodination of rabbit antiserum loop1, loop2 and loop3

3.2.3.1 Introduction

Iodination of the loop1, loop2 and loop3 by ^{125}I provides an indication of antibody robustness since denatured antibody will not demonstrate a symmetrical peak when passed through a Sephadex column.

3.2.3.2 Method

3.2.3.3 Reagents

Wash buffer: phosphate buffered saline pH 7.4 and 0.1 % Triton X 100.
Assay buffer: 50ml PBS with 1ml of bronidox L, (which is a preservative) made up to 1 litre with water.

Standard buffer: 3 g of BSA was added to 100 ml of assay buffer.

Preparation of stock: TIMP-4 standard, obtained from R&D. The powder was reconstituted with 10 µl of 1M sodium chloride and vortexed to ensure adequate mixing. The vial was spun down and 3 µl added to 3.0 ml of assay buffer to make a solution of 1000 ng/ml.

Working TIMP-4 standard: Tubes were labelled from 12.5 to 500 ng/ml. and these standard values were obtained by doubling dilutions in standard buffer.

The remaining 7 µl of the TIMP-4 standard was use for radio iodination.

Reagents for iodination.

2mg/ml of chloramine T

2mg /ml of sodium metabisulphite

1M KI in 1 % BSA

1 M phosphate buffer

1% BSA in 0.01% bronidox L

Sodium iodine tracer ^{125}I: purchased from Amersham

Diluent buffer: 50mmol/L phosphate buffer in 1 % BSA and 2 % mannitol

Elution buffer: 50mmol/L phosphate buffer in 1% BSA and 0.01% bronidox L

3.2.3.4 Method for iodination of TIMP-4

5 µl of phosphate buffer, 5 µl of ^{125}I, using a Gilson micropipette, were added to a vial containing 7 µl of TIMP-4. 5 µl of chloramine T was added and the material vortexed for 30 seconds. The oxidation was stopped by the addition of 5 µl of

metabisulphite and re-vortexed for 30 seconds. 100 µl of KI and 100 µl of elution buffer were added to the vial, prior to loading the solution onto the column.
The iodinated TIMP-4 and free iodine were separated using a column 20 cm long with 1cm internal diameter, which was filled with Sephacryl G-300 and equilibrated with elution buffer. Fractions of 1ml were then collected at 5 minute intervals. The column inlet tube was removed from the elution buffer and placed in the vial, whereupon the contents were slowly aspirated on to the column.

3.2.3.5 Result

The traces from the Sephadex column did not show the expected two peaks which is ^{125}I bound antibody and free ^{125}I Iodine. Instead several very small non-symmetrical peaks appeared early but there was the typical late peak associated with free ^{125}I non antibody bound iodide.

3.2.3.6 Discussion

This distribution of peaks is consistent with an antibody which has become denatured and can no longer bind antigen (ref 3.3.1.2). This is the explanation as to the lack of dose response between the blank and the standard.

Due to the failure of the ELISA assays, it was decided to utilise the sheep C terminal anti TIMP-4 antibody bound to polished polystyrene beads in a radioimmunoassay.

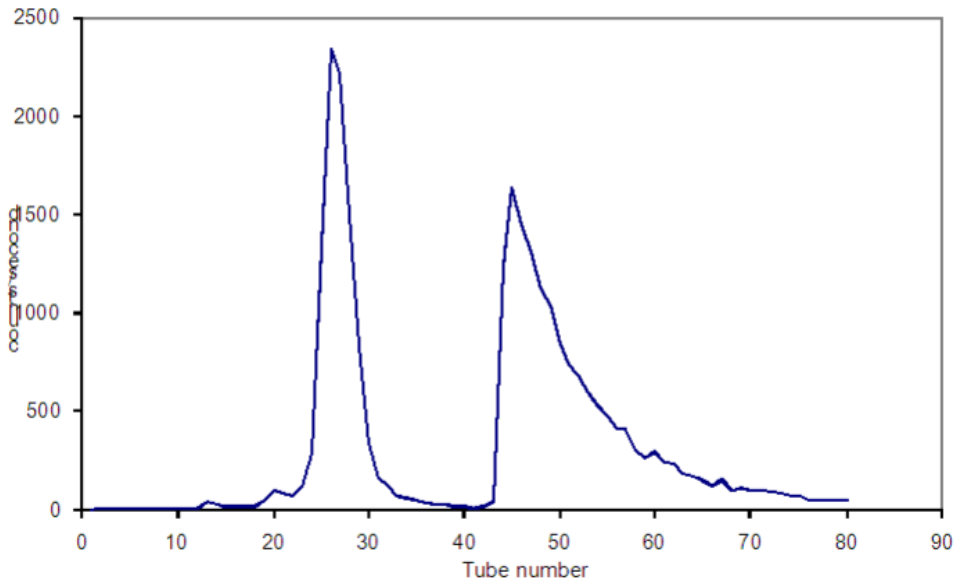
3.3.0 Validation of a radioimmunassay for tTIMP-4

The TIMP-4 antiserum was absorbed onto polystyrene beads, resulting in competition between ^{125}I TIMP-4 and the serum samples containing TIMP-4, for the binding sites on the antibody-coated bead. Post incubation, the polystyrene beads were washed in phosphate buffered saline (PBS) and counted.

3.3.1.0 Reagents

For details see section 3.2.3.2

3.3.1.2 Results of the iodination



Graph 3.1 Iodination of TIMP-4
-separation of bound and free 125I using a sephacryl G-300 column. The first peak is 125I bound to antigen TIMP-4, and the second peak free 125I

The empty vial was counted (148 counts per minute) having been washed once, in a radioactive sink and recounted (88 counts per minute).The column was left running overnight and the vials counted the next day off line on a NE1600 counter. The vials were counted and Graph 3.1 shows the counts per tube. Two peaks were found. The first was due to ^{125}I TIMP-4 (tubes 25-29) and the second was due to free ^{125}I.

3.3.1.3 Discussion on the iodination of TIMP-4

Counting the empty vial provided an indication of the likely success of radioactive iodination. High counts before and after washing suggest that the radioactive iodine had not been incorporated into the antigen but had reacted with the glass. The counts in this assay pre and post washing suggest that iodination of the antigen was successful and it was worth subjecting the material to column purification.

The peak at fractions 25-29 was symmetrical and had a relatively high activity, suggesting that the material was pure. Impure antigen or breakdown products due to oxidation were associated with multiple small peaks, which have poor symmetry.

Having achieved a satisfactory iodination, the method was then validated. The first experiment was optimisation of the antibody concentration

3.3.2.0 Optimal Antibody concentration

3.3.2.1 Introduction

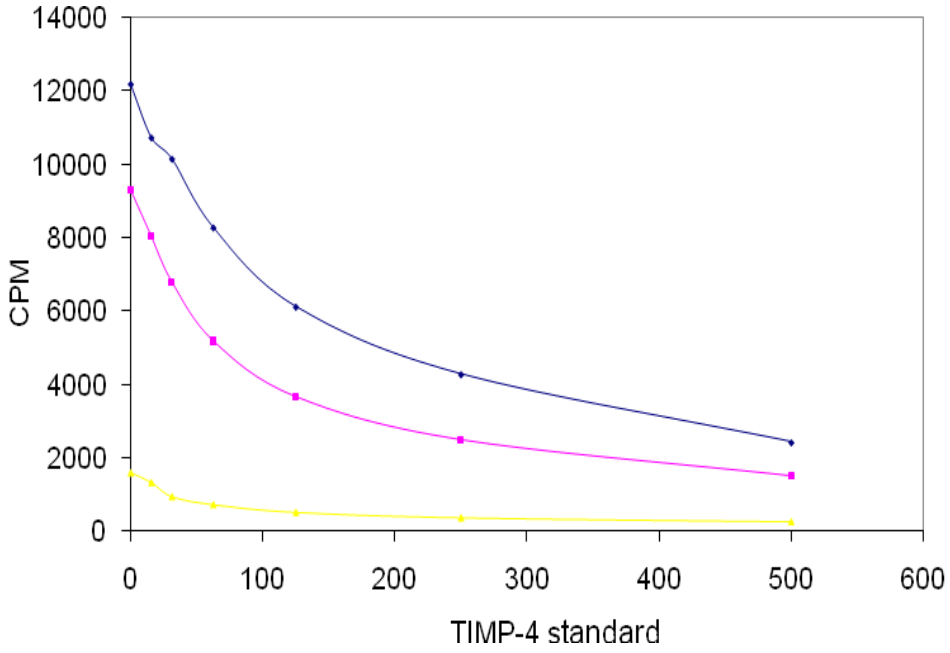
Antibody concentration is one of the major factors which affect assay sensitivity. In general, the higher the antibody concentration, the less sensitive the assay, the more expensive the test and the increased likelihood of cross reaction. Conversely, at lower antibody dilutions, the greater the sensitivity, the cheaper the assay and results in a reduction in cross reaction.

3.3.2.2 Method

Polystyrene beads were coated with the TIMP-4 IgG anti-sheep at a dilution of 1x100, 1x1000, and 1x10,000 overnight. The beads were subsequently washed with a solution of 0.1% triton in PBS. The contents were aspirated and this wash cycle repeated twice.

For each antibody dilution, 50 µl of blank or standards 12.5-200ng/ml in duplicate

were added to each antibody dilution and incubated overnight, with 200µl of TIMP-4 iodinated tracer and 200µl of PBS.



Graph 3. 2 Standard curve at three different antibody concentrations:
-1x 100 blue line, 1x 1000 pink line and 1x 10,000 yellow line. Antibody diluted 1x1000 was used in constructing the assay.

The TIMP-4 antibody dilution demonstrated poor resolution between the low and high standards (Graph 3.2) at a dilution of 1x 10,000, whilst percentage binding was satisfactory for either the antibody diluted x 100 or x 1000 (Table 3.1). The percentage of non-specific binding was low in all dilutions.

Antibody dilution	Percentage binding	Percentage/Non Specific Binding
1x 100	48	0.4
1x 1000	37	0.4
1x 10,000	6	0.4

Table 3. 1 Percentage binding and NSB at three antibody concentrations.

1x 100, 1x 1000 and 1x 10,000 demonstrating minimal non specific binding at all levels of antibody dilution.

3.3.3.0 Discussion of antibody dilution

The antibody dilution at 1x1000 was used, as the percentage binding was satisfactory and at this dilution, non-specificity would be reduced in comparison to the 1x 100 dilution. In the next experiment, the time to equilibration for the antibody antigen reaction was studied.

3.3.3.1 Introduction Optimising the time for the antigen antibody reaction

This study will optimise the time required to achieve the best antigen antibody complex, in addition to ensuring maximal assay throughput.

3.3.3.2 Method: Optimising the time for the antigen antibody reaction

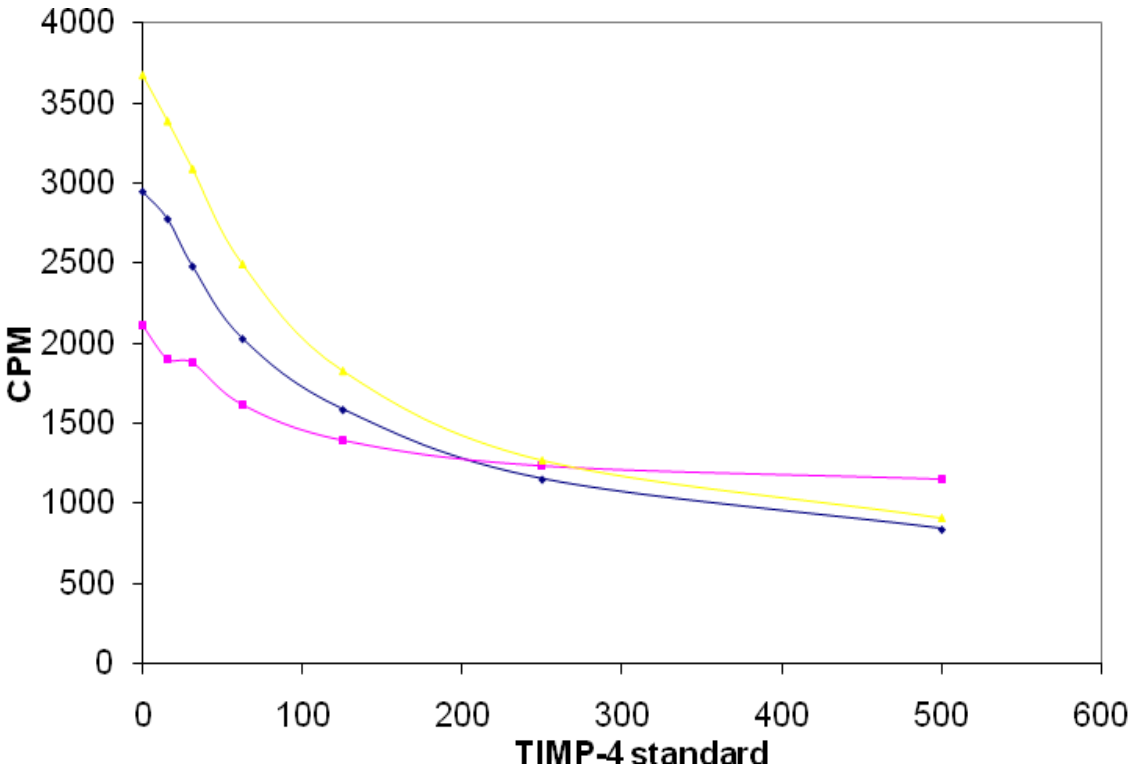
50 µl of blank or standards 12.5-500 ng/ml in duplicate, were added to polystyrene beads, containing antibody against TIMP-4, diluted x 1000 and incubated, as described, with 200 µl of TIMP-4 iodinated tracer and of 200 µl PBS. Three experiments were set up in duplicate:

- A one step assay, incubated for 2 and 4 hours

- A two step assay, in which the first incubation for 2 hours included the addition of blank or standards to the polystyrene beads and a second incubation for 2 hours, with the addition of the radioactive TIMP-4 label.

3.3.3.3 Result: Optimising the time for the antigen antibody reaction

The one step assays provided an adequate dose response curve; however, the response of the two step assay showed no dose response above a TIMP-4 standard of 100 ng/ml graph (Graph 3.3)



Graph 3. 3 Calibration of TIMP-4 at 2 different time intervals:
Yellow line - one step incubation for 4 hours. Blue line - one step incubation for 2 hours. Pink line - 2 step assay, incubation for a total of 4 hours. The one step incubation for 4 hours was used in assay design.

3.3.3.4 Discussion: Optimising the time for the antigen-antibody reaction

To optimise the time and sensitivity of the assay, a one step assay, incubated for four hours, was used for the final method. In order to determine ideal conditions to store the standards, the stability of the calibrators (under different conditions) was investigated.

3.3.4.0 Stability of the TIMP-4 standards

3.3.4.1 Introduction

Establishing standard stability is essential in the design of a robust assay. Standard instability will adversely affect the assay accuracy

3.3.4.2 Method: Stability of the TIMP-4 standards

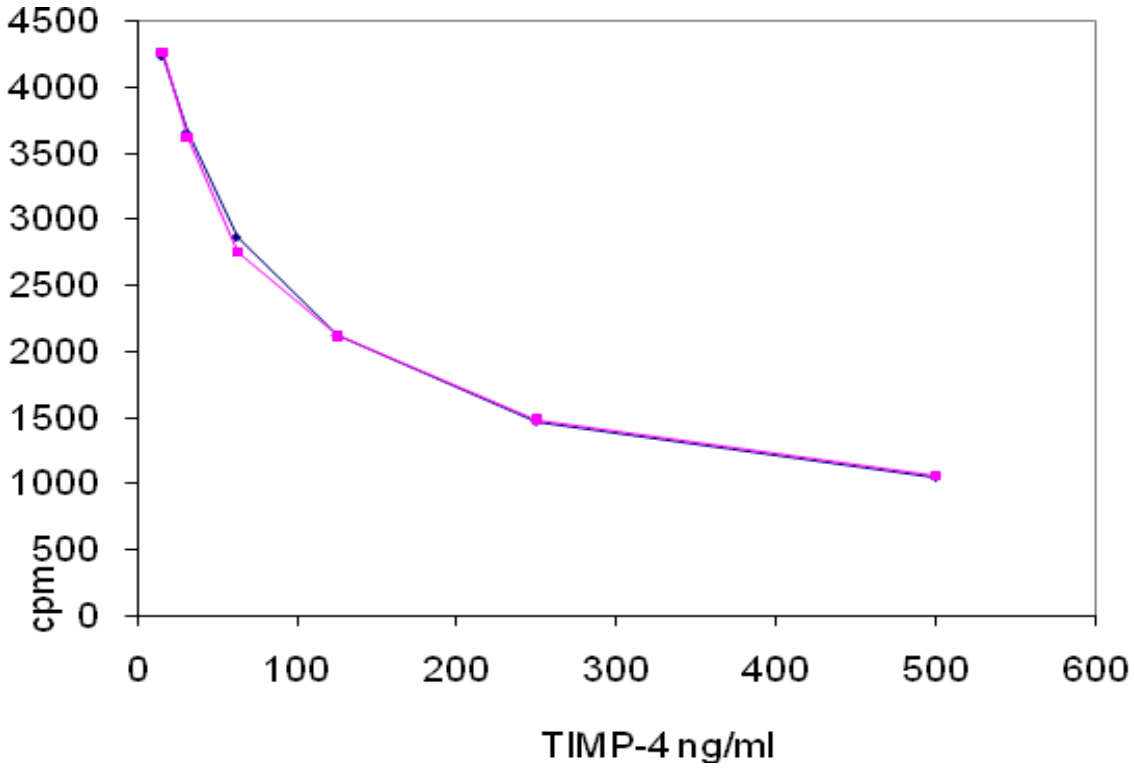
50 µl of blank or standards 12.5-500 ng/ml in duplicate, were added to the polystyrene beads, containing antibody against TIMP-4 diluted x 1000 and incubated for 4 hours, with 200 µl of TIMP-4 iodinated tracer and 200 µl of PBS. One set of standards was freshly reconstituted; the other had been used the previous week and was stored frozen at -20°C.

3.3.4.3 Results: Stability of the TIMP-4 standards

There was no difference between the freshly re-constituted standard and that remaining frozen for a week at -20° C (Graph 3.4).

3.3.4.4 Discussion: Stability of the TIMP-4 standards

The standards are stable for one week, when frozen and this information permitted economic use of the expensive calibration material. The method appeared satisfactory but the differences in dose response curves between aqueous and serum based standards now required investigation.



Graph 3. 4 Stability of TIMP-4. Pink line - standards prepared fresh, in buffer.
Blue line - standards prepared in buffer and stored for one week at – 20°C centigrade. The working standards were stored frozen at – 20°C.

3.3.5.0 Investigation of matrix effects

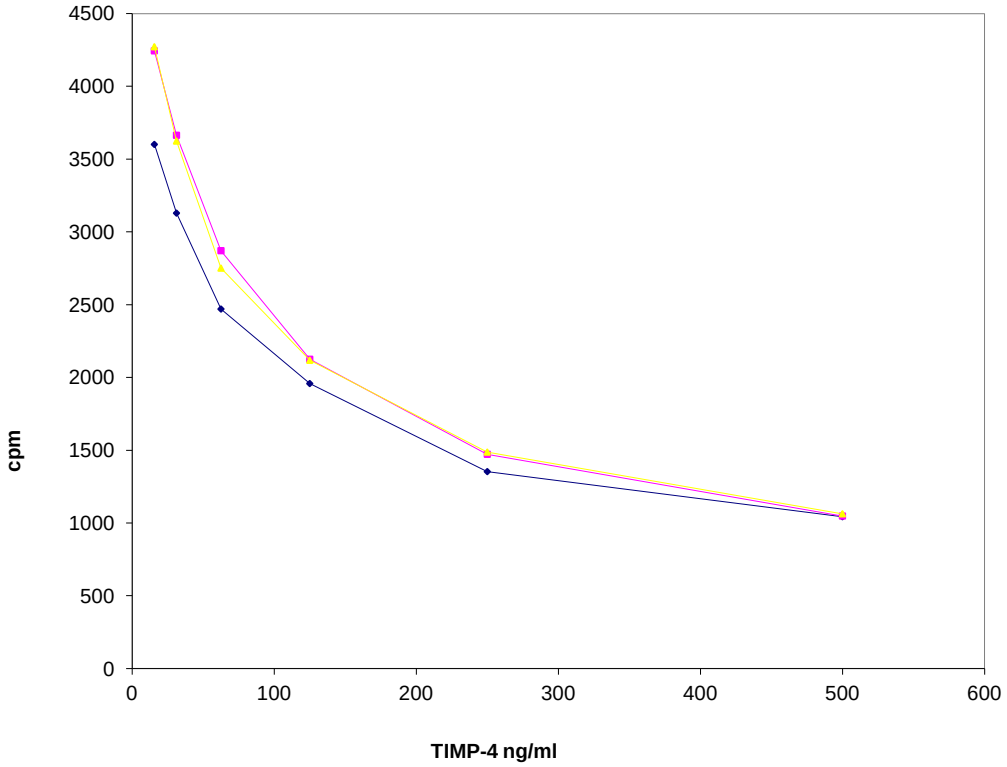
3.3.5.1 Introduction: Investigation of matrix effects

In general, the standard response curve is dependent on the matrix in which the standards are made. As serum or plasma samples will be used in the assay,
It is important to use the correct matrix, so that there will be equivalent responses between standards and serum.

3.3.5.2 Method: Investigation of matrix effects

50 µl of blank or standards 12.5-500 ng/ml in duplicate, were added to the polystyrene beads coated with the antibody diluted 1x1000 and incubated for 4 hours with 200 µl of TIMP-4 iodinated tracer and 200 µl of PBS. One set of standards was diluted with 4 % BSA, the other set diluted with horse serum and finally a set made up in saline.

3.3.5.3 Results: Investigation of matrix effects



Graph 3. 5 Effect of three differing matrix on the response curve for TIMP-4.
The matrix was prepared in horse serum - blue line, BSA - pink line, Saline - yellow line. The standards were made up in BSA.

The dose response curves for the BSA and serum standards were similar. The serum standards gave slightly lower results (Graph 3.5).

3.3.5.4 Discussion: Investigation of matrix effects

The matrix effect appears small, becoming minimal, at higher concentrations of TIMP-4. Standards prepared in BSA were used for the assay. Having established

the basic assay parameters, the method had now to be validated in terms of accuracy, precision, interference and detection limit.

A more robust method to assess matrix interferences would be to utilise the method of standard additions. Using this technique, small volumes of TIMP-4 standard, at differing concentrations are added to virus-free serum. The intercept on the y-axis would reflect the TIMP-4 value of the blank serum, without any standard addition. This potentially would be one of the best methods for eliminating matrix effects between the standard and the sample.

3.3.6.0 Calibration of the Optimised TIMP-4 Assay

Using the above data, the method selected for TIMP-4 assay was:

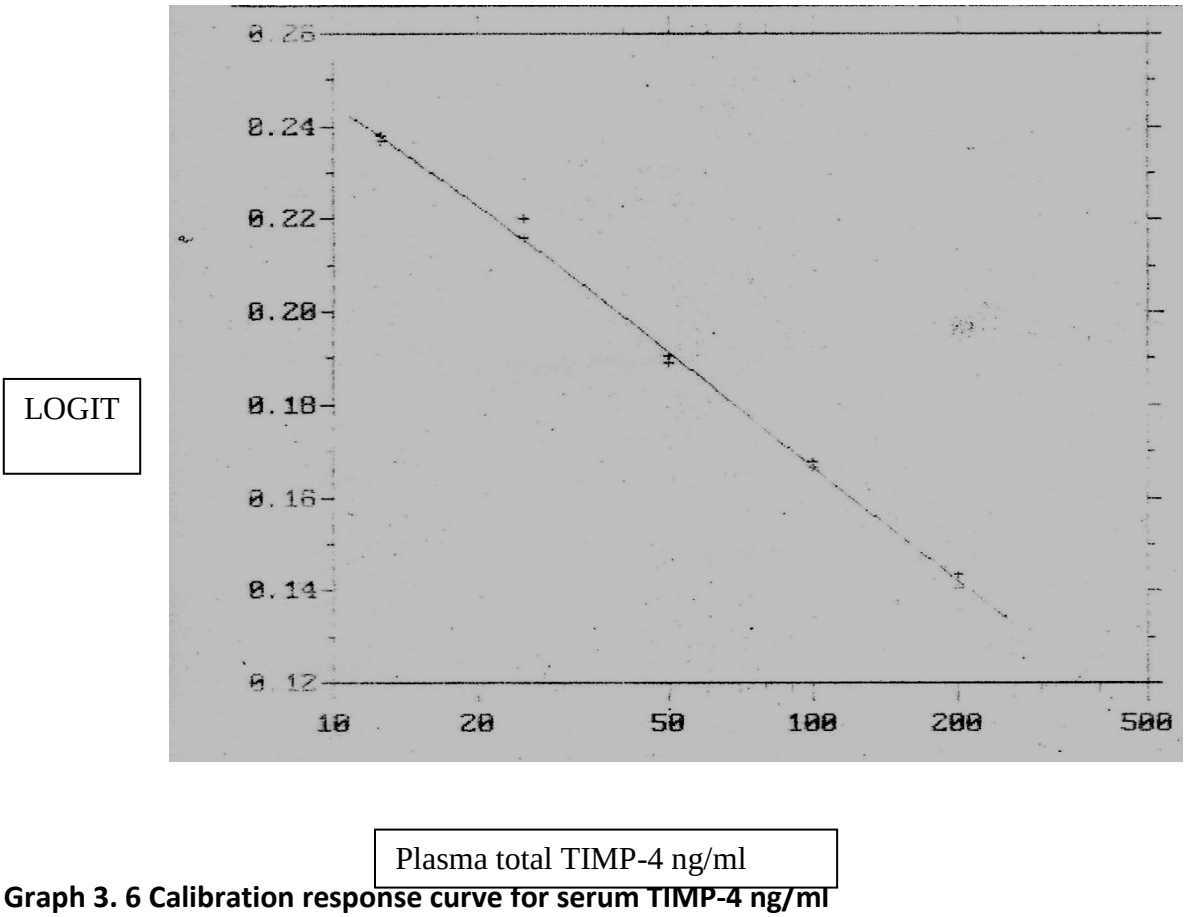
TIMP-4 antibody diluted 1x 1000 and bound to the polystyrene beads. In duplicate tubes containing polystyrene beads coated with TIMP-4 antibody, 50 µl of blank, standard or sample were added to 200µl of PBS and ^{125}**I** TIMP-4 antiserum. The tubes were incubated at room temperature for 4 hours, aspirated and then washed twice with PBS. The samples were counted in a NE 1600 gamma counter. The results of the calibration of serum TIMP-4 assay, using the partially validated method, are shown in Graph 3.6.

3.3.6.1 Result of calibration

There was a linear inverse response with concentration (graph 3.6)

3.3.6.2 Discussion on the calibration

There was good dose response between the low and high standards. This method can now be further validated in terms of accuracy, precision and interferences.



Graph 3. 6 Calibration response curve for serum TIMP-4 ng/ml

For standard range 12.5-200 ng/ml in BSA demonstrates an adequate dose response curve

3.3.7.0 Assessment of recovery

3.3.7.1 Introduction: Assessment of recovery

Establishing the accuracy of a method is fraught with difficulties. Where the assay is established, then there are likely to be quality control schemes, which will

provide a consensus mean. In the case of tTIMP-4 there are no such schemes available and so the conventional approach is to undertake recovery experiments. Two approaches were used to assess accuracy.

3.3.7.2 Method: Assessment of recovery

a) TIMP-4 standard was added to different samples; the specimen was mixed and assayed, as previously described. The calculated result was compared with the assayed result and expressed as a percentage. 40 µl of a sample was added to 10 µl of a 500ng/ml standard, in duplicate.

b) 25 µl of a sample was added to 25µl of each standard. The samples were analysed in duplicate and assayed, as previously described.

3.3.7.3 Results a) Assessment of recovery using the same standard

The results in Table 3.2 for three samples show the recovery was between 94% and 104%, when a different sample was added to the same standard. When different calibrators were added to the same sample (table 3.3) the percentage recovery was again satisfactory.

Sample	40 µl sample + 10 µl 500 ng/ml standard	Theoretical result ng/ml	%Recovery
10 ng/mL	108	108	100
21 ng/mL	113	116	97
25 ng/mL	113	120	94

Table 3. 2 Percentage recoveries on adding three different sample concentrations to the same standard 100ng/ml of TIMP-4.

This experiment demonstrated satisfactory recovery.

3.3.7.4 Results b) Assessment of recovery using the calibrators

Sample result 20 ng/ml 1+1 dilution with standard	25 µl standard +25 µl of patient's specimen assayed result ng/ml	Theoretical result ng/ml	Percentage recovery
S_o + sample	10	10	100
S_1 (25) sample	21	22.5	93
S_2 (50) sample	31	35	86
S_3 (100) sample	55	60	92

Table 3. 3 Percentage recoveries throughout the calibration range of tTIMP-4 based on adding the same sample to a standard range of 12.5-200ng/ml.
This experiment demonstrates satisfactory recovery.

3.3.7.5 Discussion: Assessment of recovery experiments

These recovery experiments showed a satisfactory recovery, both at low and high levels. Adding different samples to the same standard gave similar levels testing recovery only, at the level of 120 ng/ml. However, the range of values in the

recovery experiment using different calibrators covered the range 10-55 ng/ml. There were minimal matrix effects. The assay has a good recovery, is not matrix dependent and there was no concentration related bias.

There was a good recovery but this does not mean that the assay is accurate. Accuracy depends upon a standard which has international acceptability. Such a standard does not exist for TIMP-4. Therefore the accuracy of this assay is not established.

3.3.8.0 Assessment of interferences

3.3.8.1 Introduction: Assessment of interference

Interference occurs in most assays but simple competitive assays are likely to be

more adversely affected than “sandwich assays”. There is a significant molecular similarity between TIMP-1, TIMP-2, TIMP-3 and TIMP-4 but as only TIMP-1 and TIMP-2 are found in serum, the interference experiment was directed at establishing whether there was any cross reactivity with TIMP-1and TIMP-2.

3.3.8.2 Method: Assessment of interference

40 µl of a 1000 ng/ml (equivalent to 800ng) of standard for TIMP-1 or TIMP-2 was mixed with 10 µl of a serum sample of TIMP-4, which had previously been

analysed at 21 ng/ml. Additionally, 25 µl of the 1000 ng/ml of TIMP-1 or TIMP-2 standard (equivalent to 500 ng) was added to the sample.

3.3.8.3 Result: Assessment of interference

The theoretical result on adding 800 ng/ml standard to the serum sample should have been 4ng/ml for both TIMP-1 and TIMP-2.and the theoretical result on adding the 500ng/ml standard should have been 10.5ng/ml (Table 3.4).

Sample	Volumes of TIMP standard and sample assayed result ng/ml	Theoretical result ng/ml	Predicted recovery %
40 µl std +10 µl of 21 ng/ml	40 µl TIMP-1std + 10 µl of samples sample Result 3 ng/ml	4	75
25 µl std +25 µl 21 ng/ml	25 µl TIMP-1 std + 25 µl sample Result 9.5 ng/ml	10.5	90
40 µl + 10 µl of 21 ng/ml	40 µl TIMP-2 + 10 µl sample Result 3.5 ng/ml	4	88
25 µl std + 25 µl 21 ng/ml sample	25 µl TIMP-2 + 25 µl sample Result 10 ng/ml	10.5	96

Table 3. 4 Result of TIMP-1 and TIMP-2 interferences on TIMP-4 levels using a recovery experiment.

800ng/ml of either TIMP-1 or TIMP-2 was added to serum samples of different values and the percentage recovery calculated. There is no interference of TIMP-1 or TIMP-2 in the tTIMP-4 assay.

3.3.8.4 Discussion: Assessment of interference

A high concentration was used for the TIMP-1 and TIMP-2 in the recovery experiment because TIMP-1 and to a lesser extent, TIMP-2, may reach very high concentrations in pathological states, for example, in cirrhosis and metastatic cancer. The recovery experiment showed no interference between TIMP-1 or TIMP-2 in the TIMP-4 assay. The result of the recovery experiment was the same as the result that would have been expected, had saline been added to the serum samples. In terms for cross reactivity between TIMP-1 and TIMP-2, this TIMP-4 method is robust.

3.3.9.0 Assessment of Precision

3.3.9.1 Introduction: Assessment of Precision

Assessment of precision is an important parameter of analytical performance. Precision is assessed using ‘within run’ and ‘between run’ criteria.

3.3.9.2 Method for assessing precision

Two previously assayed serum levels for TIMP-4, at concentrations of 12 and 22 ng/ml were run 15 times in the same batch, generating within batch imprecision data. Between batch imprecision was generated by running these same samples on 12 different occasions over 12 working days.

TIMP-4	Mean ng/ml	Standard deviation ng/ml	Coefficient of variation %
Control I Within batch	11.8	± 0.8	6.7
Control II Within batch	21.5	± 1.25	5.8
Control I Between batch	12.4	±1.3	10.4
Control II Between batch	22.2	± 2.8	12.6

Table 3. 5 'Within batch' and 'between batch' imprecision
Serum samples were analysed in the same run (within batch) and analysed on different days (between batch). The imprecision is satisfactory for both controls within and between batch.

3.3.9.3 Discussion on precision data

The data on precision was satisfactory for an RIA assay. It may have been expected that the precision results would have been lower on the higher sample. This was not the case but may have occurred, had the sample been more concentrated 100 ng/ml (table 3.5).

3.3.10.0 Introduction: Assessment of sensitivity

3.3.10.1 Introduction to assay sensitivity

The sensitivity of the assay provides an important indicator between assay noise and the level that can be reliably measured.

3.3.10.2 Method for assessing sensitivity of the method

Using further serial dilutions of the 12.5 ng/ml standard a 1.5 ng/ml standard was run within batch using 10 within run duplicates.

3.3.10.3 Results: sensitivity of the method

The sensitivity at 1.5ng/ml was judged satisfactory, as the CV was 18%.

3.3.10.4 Discussion: sensitivity of the method

This was not an ideal method for assessment of sensitivity as ideally, a serum sample run several times between batch should be used to asses this parameter.

However, on the basis of normal samples (see data on normal range section 3.3.11.3) the method has adequate sensitivity for serum assays. Should TIMP-4 be measured on tissue culture fluid, then it would be possible to improve sensitivity by increasing the sample size or by increasing the antiserum dilution.

3.3.11.0 The normal range for tTIMP-4

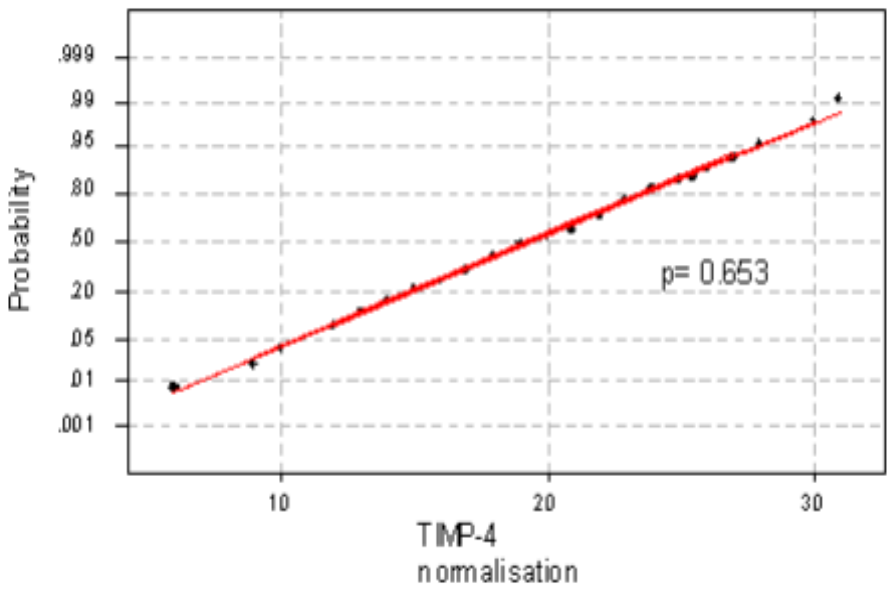
3.3.11.1 Assessment of the normal range for TIMP-4.

Having established the TIMP-4 assay characteristics, the next step was to determine the normal range for this assay.

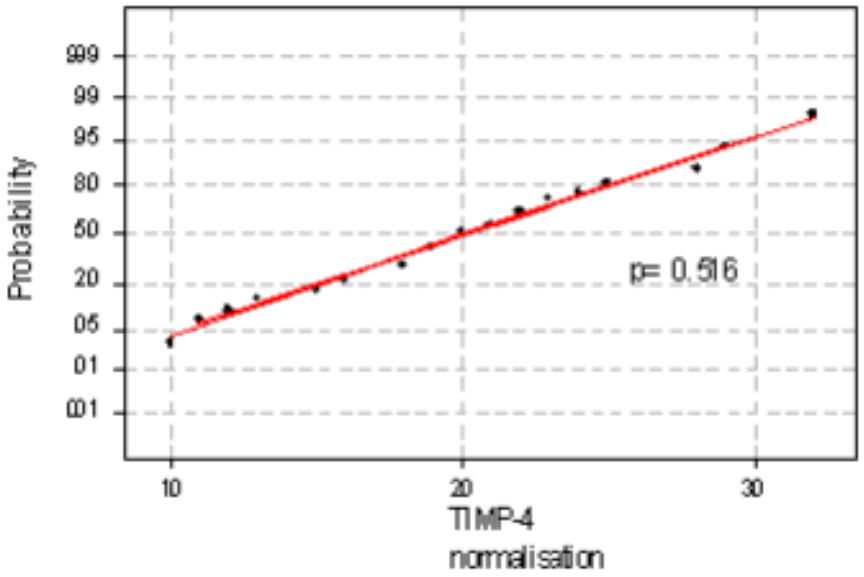
3.3.11.2 Method used to establish normal range.

There is significant agreement, as to how normal range should be established. Other than age, sex and ethnicity, other factors are important: one of the major factors being should the range be derived from healthy subjects or from hospital in-patients?

In the first instance, serum TIMP-4 levels were analysed in patients without any major disease, in submariners, whose health was well documented and in a group of subjects without evidence of cardiovascular disease (CVD). The latter group was chosen as it was hypothesised that tTIMP-4 levels may be increased in patients with heart disease.



Graph 3. 7 Anderson- Darling plot (normal probability)
Plot for serum TIMP-4 in subjects with no chest pain. There is no deviation from the normal distribution (p>0.05).



Graph 3. 8 Anderson- Darling plot (normal probability) plot for serum TIMP-4 in submariners pre and post patrol.
There is no deviation from the normal distribution (p>0.05).

Variable	N	Mean ng/ml	Median ng/ml	St/Dev ng/ml
Jan patrol	37	20.1	20	5.6
April patrol	35	21.5	21	4.1
TIMP-4 No CVD	78	19.3	19	5.2

Table 3. 6 Descriptive statistics for serum TIMP-4
The mean and median and the standard deviation are similar consistent with Gaussian distribution.

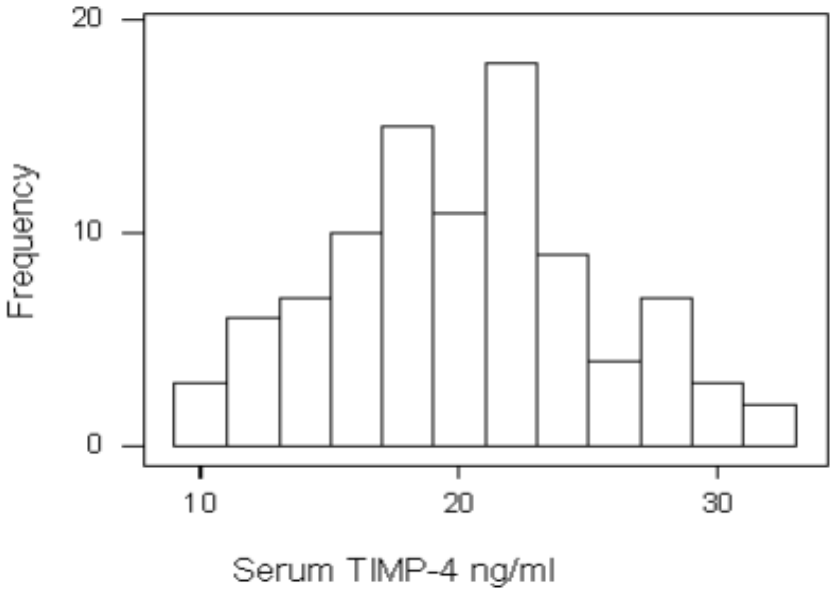
The first blood sample from the submariners and controls were taken immediately before boarding the submarine prior to the patrol. The second

sample was taken just before the end of the patrol and prior to the submarine resurfacing. The second samples from the shore based controls were also taken at nearly the same time. The results for TIMP-4 are similar in submariners' blood samples pre and post patrol in January and April (Table 3.6). As with the submariners, the control subjects had similar results, with similar standard deviations. The data has a Gaussian distribution (graphs 3.7 and 3.8). The two sample t test p>0.05 showed there was no difference between the serum TIMP-4 levels in the specimens taken in January or April. The one way unstacked ANOVA (Table 3.7) showed no significant differences (p>0.05) between the submariners group and the control subjects.

Level	N	Mean ng/ml	St/Dev ng/ml		--------+---------+---------+--------
Jan	37	20.1	5.6		(----------*---------)
April	36	21.5	4.0		(----------*---------)
TIMP-4 No chest pain	58	19.6	4.9		(-------*-------)
					--------+---------+---------+--------

Table 3. 7 Serum TIMP-4 One way ANOVA: January, April for submariners and patients without chest pain.
The means are very similar as are the standard deviations. The data is not statistically significant.

Accordingly, a combination of the January data from the submariners TIMP-4 with the TIMP-4 results from the control group, was used to derive the normal range. The resulting histogram (graph 3.9) is shown below and the range for serum TIMP-4 using this non-competitive RIA method is 10-30ng/ml.



Graph 3.9 Combined histogram distribution of serum TIMP-4.
In submariners and subjects without cardiovascular disease n=140

3.3.11.3 Discussion: Normal Range for Total TIMP-4

The data for TIMP-4 gave a Gaussian distribution, as evidenced by the mean and medium having very similar values (Table 3.6). Furthermore, the data in the Anderson Darling plot (Graphs 3.7 and 3.8) does did differ from a normal distribution at a probability of ($p<0.05$).

There was no difference in the means or SD of serum tTIMP-4 levels between the submariners, who were all male and under 50 years old, and the group of patients without CVD who were both male and female and whose age was a mean 50 ±17 SD years. This data suggests that TIMP-4 is not dependent on sex or age.

Other than TIMP-3, which was undetectable in serum, tTIMP-4 has the lowest concentration of the tissue inhibitors of matrix metalloproteinases. The mean TIMP-1 plasma level is 100 ng/ml and that of TIMP-2 is 60 ng/ml, whilst that of tTIMP-4 is 20 ng/ml. tTIMP-4 is bound to a variety of MMPs. There is only one established reference range for serum TIMP-4 and that is not total TIMP-4 but the MMP9/TIMP-4 complex. The mean normal result of the MMP9/TIMP-4 complex is 2 ng/ml (Papadopoulos, 2005) which is 10% of the total TIMP-4 result. However, our antibody was raised against tTIMP-4, not only the MMP9/TIMP-4 complex. TIMP-4 inhibits a variety of MMPs. In an interesting inhibitory mechanism, TIMP-4 inhibits MMP2 activation by inhibiting MT1 MMP (Bigg et al., 2001) and MMP26 (Zing et al., 2002) in addition to MMPs 1,2,3,7,9, (Liu et al., 1997) so it is not unexpected that the range for tTIMP-4 is much higher than that previously reported for the MMP9/TIMP-4 complex. This assay is the first to describe the concentration of total TIMP-4 in serum.

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Chapter 4

Correlation between TIMP-1 and acute phase proteins and TIMP-1 and PIIINP in patients with essential hypertension

4.0.0 General Introduction

Prior to studying the role of MMPs and TIMPs in various pathologies, the question arose as to whether TIMP-1 was an acute phase protein.

4.1.0 TIMP-1 and acute phase proteins

4.1.1 Background and aims

The amount of collagen in tissues is tightly controlled by the rate of collagen synthesis and the rate of collagen degradation. Collagen degradation is governed by the hydrolytic actions of matrix metalloproteinases (MMPs). The activity of MMP is inhibited by a group of antiproteinases called TIMPs (Li et al., 1994). TIMP-1, the best characterised of these antiproteinases, has a molecular weight of 28 kD and is glycosylated at two sites.

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is an important factor controlling the development of hepatic fibrosis. The activity of TIMP-1 is upregulated by IL6 (Arthur, 1995), which is also involved in the acute phase response. Since an acute phase protein response (Lippi,1992) was also found in patients with alcoholic liver disease, it was decided to investigate a group of alcoholic patients, who were undergoing acute alcohol withdrawal, to determine if TIMP-1 correlated with a variety of established acute phase proteins.

4.2.0 Overview of methods

Immunoturbidimetric methods were used to measure the specific proteins - orosomucoid, prealbumin, transferrin and CRP. Albumin was measured using the Olympus 600 bromocresol green dye-binding method. A number of different techniques were employed in the assay of 25-hydroxyvitamin D within the department. These included (initially) an RIA commercial assay marketed by IDS and subsequently, a liquid chromatography mass spectrometer - mass spectrometer technique (LC-MS/MS).

MMP9 was measured using ELISA assays from R&D Systems and MMP2 and TIMP -1 levels were assayed using an Amersham kit. The MMP9 assays correlated well between the Amersham and R&D assays and the Amersham TIMP-1 assay was extensively validated by Cawston's team at Cambridge (Plumpton et al., 1995)

4.2.1 Immunoturbidimetric methods

4.2.1.1 Principle of the method

A solution of a specific protein is mixed and incubated with a specific antiserum to the protein, resulting in the formation of insoluble antibody-antigen aggregates, which increase the turbidity of the solution. This increase in turbidity causes a

decrease in the intensity of an incident beam of light, as it passes through the solution. Turbidimetry is the measurement of this decrease in intensity at 180 degrees from the incident beam. The result is proportional to the concentration of the specific protein (Tietz, 1986).

4.2.1.2 Reagents

The immunoturbidimetric assays used two reagents.
Reagent R1 was common to all the immunoturbidimetric assays and consisted of phosphate-buffered saline containing "co-polymers", which enhance assay performance by increasing the reaction rate, stabilising the suspension of the immune complexes and allowing the use of lower concentrations of reactants
Reagent R2 was specific to the protein being measured and consisted of anti human (orosomucoid, prealbumin, transferrin or CRP) antibody. No reconstitution of the reagents was required.

4.2.1.3 Method protocol

The Olympus immunoturbidimetric assays were performed on an Olympus 600 Analyser according to the manufacturer’s protocol. In brief, the sample was diluted in reagent R1, according to the volumes in Table 4.1 and incubated for the required number of cycles. The resulting turbidity was read at the appropriate primary and (if necessary) secondary wavelengths.

Method	**Sample** Volume µl	**Reagent volume R1 R2 µl**	**Wavelength Primary/Secondary**	**Measuring point *** Point 1 Point 2
Prealbumin	3	150 65	340 800	10 27
Transferrin	3	150 65	340 800	10 27
Orosomucoid	3	165 45	600 none	10 27
CRP	2	150 150	570 none	8 27

Table 4. 1 Parameters for the measurement of acute phase proteins on the Olympus 600.
All assays required a small serum sample, were two reagent additions and were blanked at a secondary wavelength. One measuring point is 18 seconds*.

4.2.2 Dye-binding method

4.2.2.1 Principle

Dye-binding methods require the specific binding of a dye to the protein being measured (in the presence of other proteins) such that there is a substantial shift in the absorption wavelength of the dye in the bound form. It is spectrally distinct from the free (unbound) form of the dye, which is present in excess. The absorbance of the dye-protein complex is proportional to the concentration of the specific protein in the sample. (Tietz 1986)

4.2.2.2 Reagents

The reagent utilised in the Olympus 600 dye binding method for albumin consisted of bromocresol green 0.2mmol in succinate buffer pH4.2.

4.2.2.3 Principle

The Olympus 600 BCG method for albumin followed the manufacturer's protocol. Briefly, 2 µl of serum/std or quality control solution was added to 300 µl of reagent and incubated for 4 minutes. The colour resulting from the formation of the bromocresol green albumin complex was read at a primary wavelength of 600 nm and a secondary wavelength of 800nm.

4.2.2.4 Patients

This study was passed by the West of Scotland Ethics Committee and was part of the study on the biochemical affects of acute alcohol withdrawal. Twenty-four alcoholic patients (22 males and 2 females) were recruited consecutively to an alcohol and drug rehabilitation unit. All had previously consumed more than 150 g of alcohol daily for more than one year. The patients had varying degrees of liver disease, as judged by clinical and biochemical criteria. Screening tests for other causes of liver disease (auto-immune hepatitis, hepatitis A, B or C and haemochromatosis) were negative.

4.2.2.5 Methods

There is no data on the half-life of TIMP-1 so we compared TIMP-1 levels with the acute phase proteins: prealbumin, albumin, transferrin, orosomucoid, ferritin and CRP, all of which have different half-lives.

Plasma samples were obtained within 3 days of admission and stored at -20°C

Plasma TIMP-1 was assayed using an ELISA sandwich assay (Amersham). The acute phase proteins were assayed using immunoturbidimetric assays. Data was normalised either by log transformation (CRP and orosomucoid) or by taking the square root of TIMP-1.

4.2.2.6 Statistics

All statistical calculations were performed using Minitab Statistical Software Minitab, Pennsylvania (United States of America). The data was either Gaussian or log-converted to render it Gaussian. Associations were analysed using Pearson Correlation.

4.2.2.7 Results

Most alcoholic subjects had TIMP-1 levels elevated above the normal range. The normal range for TIMP-1 was derived from 17 controls from the hypertensive study (Table 4.4). The reference ranges for the other parameters were derived from the manufacturer's quoted ranges. Table 4.2 shows the levels of TIMP-1 and

acute phase proteins found in this study. There is no significant correlation between TIMP-1 and the other acute phase proteins measured (Table 4.3). However, the acute phase proteins did correlate with each other significantly. There were significant correlations between log_{10} orosomucoid and albumin (r = -0.45), log10 orosomucoid and log10 CRP (r = 0.54) log10 CRP and prealbumin (r = - 0.61).

Protein	Range in 24 alcoholic subjects 95% confidence median range Median Range	Reference Range (Normal range for TIMP-1)
TIMP-1 ng/ml	1100 208 – 2349	61 – 320*
Ferritin µg/L	276 13 – 1181	6 - 260
CRP mg/L	4 0 – 102	<10
Orosomucoid g/L	1.27 0.83 - 2.24	0.55 - 1.4
Albumin g/L	43 34 – 49	36 - 52
Prealbumin g/L	0.32 0.12 - 0.44	0.2 - 0.4
Transferrin g/L	2.50 1.77 - 3.36	2 - 4

Table 4. 2 TIMP-1 and acute phase proteins in the alcoholic subjects

* Normal values for TIMP-1 are derived from the control group in the hypertensive study (table 4.4). The other reference ranges were derived from manufacturer's quoted range.

Acute phase protein	Ferritin μg/L	Albumin g/L	Log CRP mg/L	*Log Oros g/L	Transferrin g/L	Prealbumin g/L
▽ TIMP ng/ml	r = 0.013 p = 0.95	r = - 0.12 p = 0.57	r = 0.35 p = 0.11	r = 0.29 p = 0.16	r = - 0.03 p = 0.89	r = - 0.12 p =0.59

Table 4.3 Pearson correlation coefficients between TIMP-1 and the acute phase proteins.
Each acute phase protein has a different half -life but there was no correlation with TIMP-1.

4.2.2.8 Discussion

TIMP-1 concentration is increased in fibrosis associated with liver disease.. The acute phase response is associated with an increase in interleukin 1, 6 and tumour necrosis factor release, which stimulate TIMP-1 release. We assayed CRP, prealbumin, transferrin, ferritin and orosomucoid because they cover a wide range of different half-lives (from hours for CRP to 20 days for albumin). The lack of correlation between TIMP-1 and CRP observed in this study has previously been reported by Plumpton et al.(1995) who studied patients with rheumatoid arthritis. No correlation between any of the measured acute phase proteins and TIMP-1 was found (Timms et al., 1999) This study had small numbers which had a significant sex bias but there were correlations between CRP and prealbumin,

CRP and orosomucoid and orosomucoid and albumin, suggesting that the lack of correlations seen with TIMP-1 and the acute phase proteins, was not just due to lack of power of the study. The elevated TIMP-1 and the correlations between the established acute phase proteins suggest that there is a different control mechanism governing TIMP-1 and acute phase protein modulation in these patients. Theoretically, the release of TIMP-1 as part of a generalised acute phase response, would be physiologically undesirable, since an acute phase response could then be associated with increased collagen deposition.

Plasma levels of TIMP-1 can be elevated due to damage from several tissues. The increase in liver collagen is in part due to a reduction in collagen degradation caused by an increase in TIMP-1 levels. Using a similar argument, might the potential role of TIMP-1 be important in the aetiology of LVH secondary to essential hypertension?

4.1.0 Plasma TIMP-1 levels in patients with essential hypertension

4.1.1 Introduction

Hypertensive patients have an increase in the amount of collagen in the heart and arteries (Weber et al., 1992). Left ventricular hypertrophy (LVH) is an established risk factor for all the sequelae of coronary artery disease with a threefold to fivefold fold increase in cardiovascular mortality. The pathological basis for LVH is

a combination of myocyte hypertrophy and increased collagen deposition within the myocardium. Theoretically, elevated collagen levels may be due to an increase in collagen synthesis (Lavaides et al.,1994) or a decrease in collagen degradation or both. The synthesis of collagen III can be monitored by measurement of plasma N terminal pro-collagen III peptide (PIIINP). Alternatively, the increased collagen levels within the left ventricle could reflect decreased degradation, due to reduced levels of MMPs because of an absolute reduction in MMPs or because the MMPs were inhibited by an increase in TIMP-1. Brilla (1994) showed, in cultured fibroblasts, cardiac levels of MMP1 were reduced post incubation with angiotensin II.

Markers of left ventricular mass were determined using echocardiogram and plasma levels of TIMP-1 and PIIINP were measured. The hypertensive patients, some of whom had LVH, were compared with a control group in order to determine whether the elevated tissue levels of collagen in hypertension are associated with increased plasma TIMP-1 concentrations.

4.1.2 Patients

After informed consent, 31 patients with essential hypertension and 17 normotensive controls were recruited. The hypertensive patients had either never been treated with antihypertensives (n=18) or had been off treatment for one month (n=13). Hypertension was confirmed by elevated supine blood pressure

above 140/90 on at least 3 separate clinic visits. Patients with the following conditions were excluded:

- those with secondary causes of hypertension;
- with a clinical history or biochemical evidence of renal disease;
- chronic liver disease or alcohol excess;
- lung or connective tissue diseases and previous major surgical procedure, including injury resulting in scars in the previous year.

Seventeen normotensive controls, satisfying the above inclusion criteria, were age and sex-matched for the hypertensive group.

A 2-D echocardiogram and Doppler study was performed on all patients by a cardiologist or a trained echocardiographer, whose accuracy and precision was comparable to that of the cardiologist, using a Vingmed CFM 800 with Echopac analysis package. 2-D guided M mode measurements were made with left ventricular dimension being recorded at the level of the tips of the mitral valve leaflets. At least three measurements were made and the average of these measurements was used for analysis. LV mass was calculated by the formula of Devereux and Reichek (1977).

4.1.3 Methods

Heparinised blood samples taken after the patient was supine for 15 minutes were used in the analysis. TIMP-1 was assayed using a sandwich ELISA kit modified by

Amersham Biotrak (as previously described), PIIINP was measured using a radio immunoassay technique (CIS) (Rhode et al.,1979) and serum aldosterone was measured using the Coat-a-Count method (Siemens).

4.1.4 Statistics

The results were analysed using the Minitab statistical package. The data were either Gaussian or log-converted to render them Gaussian. Associations were analysed using the Pearson Correlation and p values <0.05 were significant. On the basis that the left ventricle may increase by 30%, theoretically the TIMP-1 value in the hypertensive group may increase by 100 ng/ml. To achieve a $p< 0.05$ between the normotensive and hypertensive patients, 40 samples would be required.

4.1.5 Results

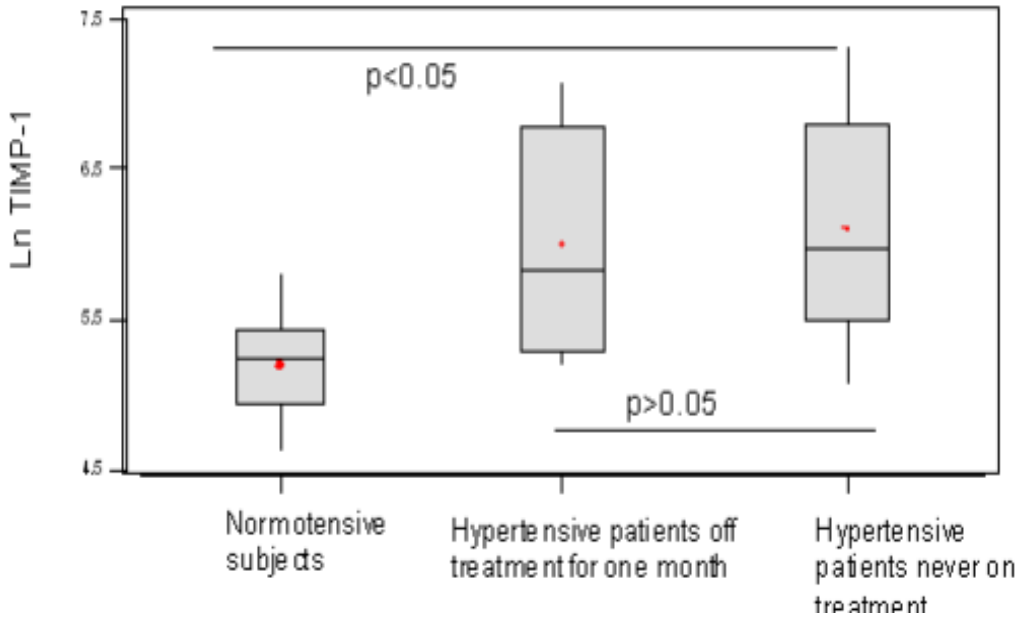
Table 4.4 shows the clinical and biochemical data for the normotensive and hypertensive groups of patients. As expected, the results for blood pressure, LVMI, IVSd, and LVPWd are significantly higher in the hypertensive group than in the controls. There was no significant difference in PIIINP levels between the hypertensive and normotensive groups. There was no correlation between TIMP-1, systolic or diastolic blood pressure, nor was there any correlation between LVMI, IVSd or LVPWd and TIMP-1 in the control subjects and the previously treated hypertensive patients.

There was no significant difference between TIMP-1 levels in those who had never been treated and those off treatment for one month but there was a very significant difference between the normotensive and these two hypertensive groups (Table 4.4, Graph 4.1)

Factor	Normal (n=17) mean (±2SD)	Hypertensive (n=31) mean (±2SD)	p value
Age(range)	47.1 (14)	48.7 (24)	p= ns*
Sex(M:F)	5:12	11:20	
BSA ????????? (m Sq)	1.79 (0.46)	1.88 (0.56)	p= ns*
BP Diastolic mmHg	78.1 (19)	99.1 (23)	p<0.001
BP Systolic mmHg	123.5 (35)	151.5 (32)	p< 0.001
LVMI g/m^2	105.2 (30)	125.6 (74)	p< 0.01
IVSd cm	0.85 (0.30)	1.01 (0.48)	p< 0.01
LVPWd cm	0.89 (0.30)	1.02 (0.4)	p<0.01
TIMP-1 ng/mL	Median 178 Range 99 to 320	Median 380 Range 160-1560	p<0.0002
PIIINP (U/ml)	0.52± 0.2	0.56± 0.3	p= ns*

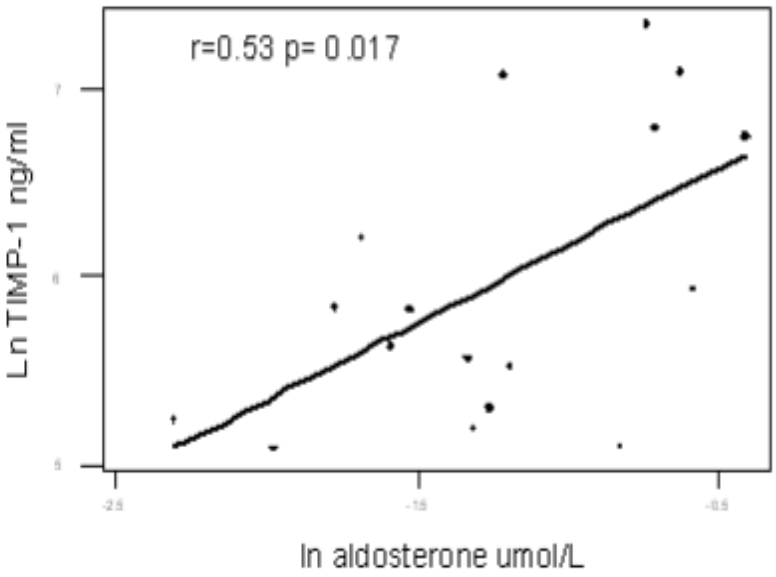
Table 4. 4 Comparison of clinical and echocardiography data in patients with and without hypertension

*ns = not significant. Other than age, sex and BSA there was a significant difference between the normotensive and hypertensive patients.



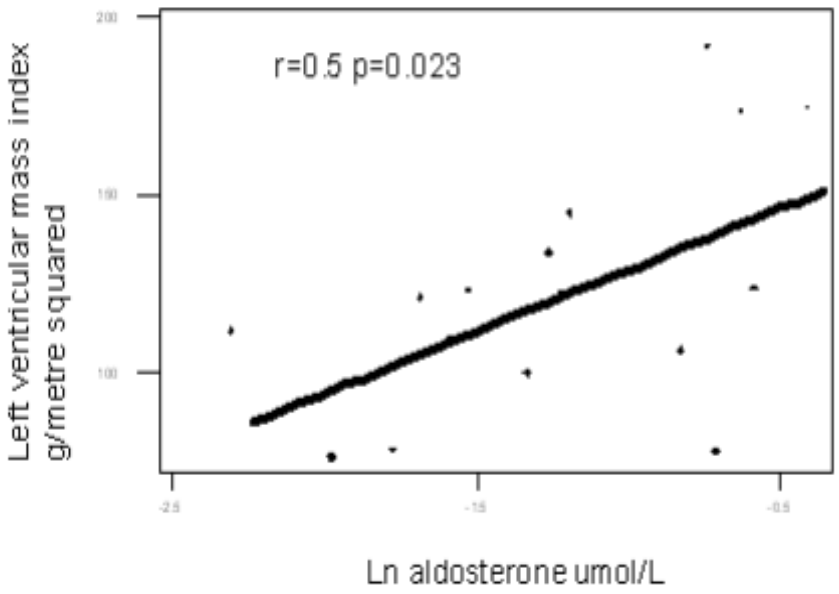
Graph 4. 1 Box plot of plasma TIMP-1 in normotensive and hypertensive patients.

Plasma TIMP-1 levels were the same in patients off treatment for one month and naive hypertensives. The median is the centre line while the lower and upper ends of the box are the 25th and 75th percentiles and the lower and upper lines of the box the 10th and 90th percentiles. Other points above or below the whiskers are outliers.

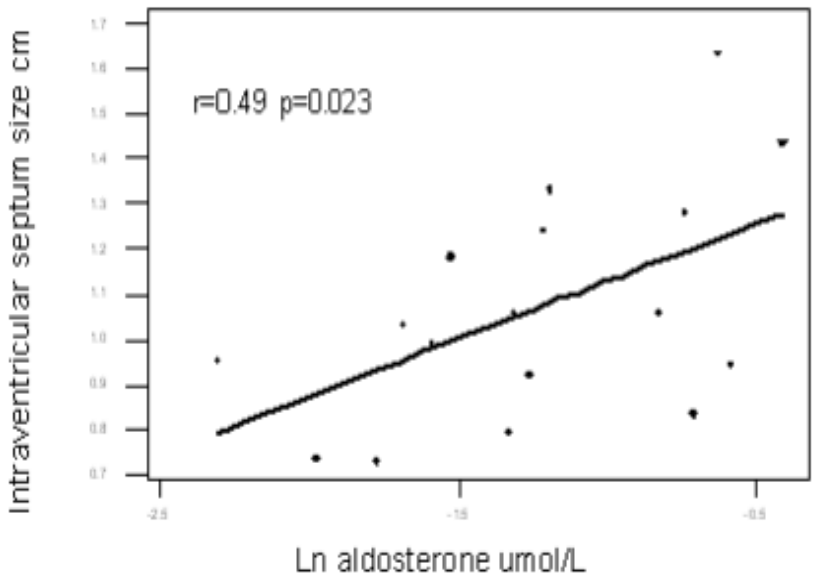


Graph 4. 2 Regression plot: Plasma Ln TIMP-1 versus serum Ln aldosterone
Patients with essential hypertension never treated with antihypertensive drugs Loge (Ln), there is a positive and significant correlation between Ln TIMP-1 and Ln aldosterone.

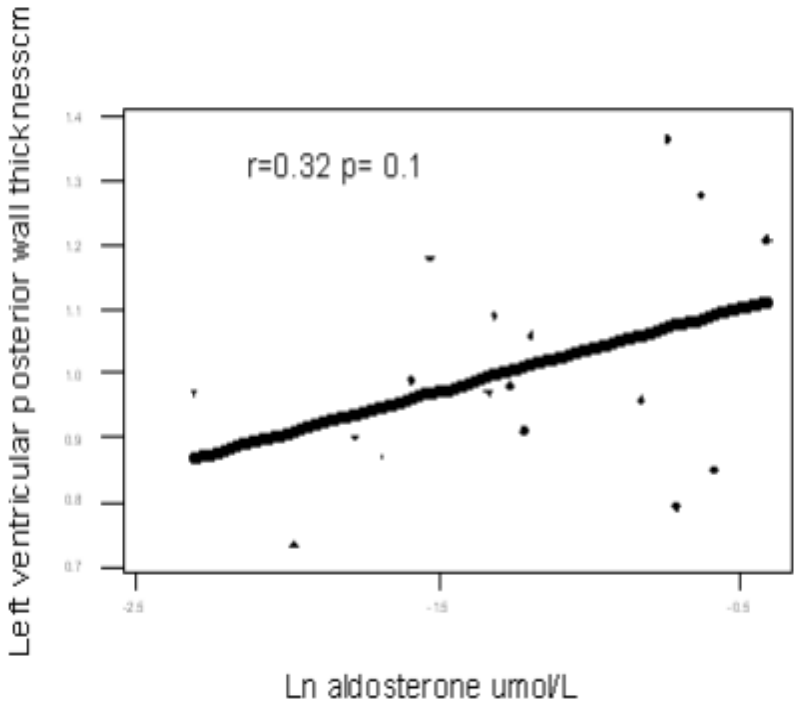
There was a significant association between plasma Ln TIMP-1 and aldosterone (Graph 4.2) and an association between aldosterone and LVMI and IVSd but not interestingly LVPWd (Graph 4.3, 4.4, and 4.5 respectively).



Graph 4. 3 Regression plot: left ventricular mass index versus serum Ln aldosterone. Patients with essential hypertension never treated with antihypertensive drugs Loge (Ln). There is a positive and significant correlation between Ln TIMP-1 and left ventricular mass index.

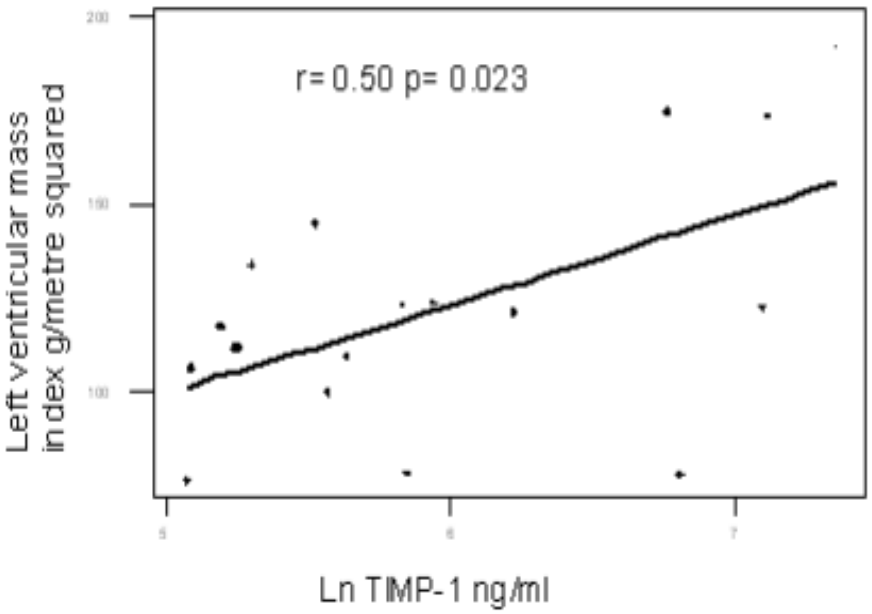


Graph 4. 4 Regression plot: Intraventricular septum size versus serum Ln aldosterone
Patients with essential hypertension never treated with antihypertensive drugs. Loge (Ln). There was a positive and significant correlation between Ln TIMP-1 and intraventricular septum size.

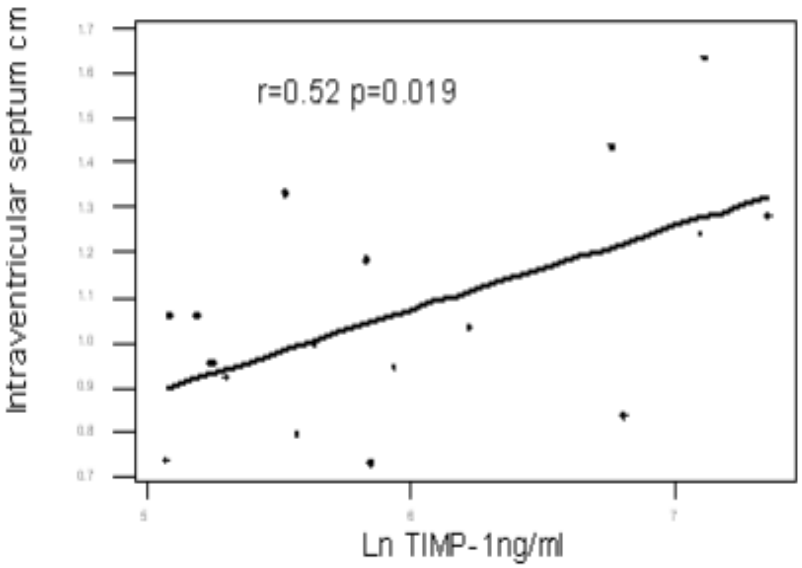


Graph 4. 5 Regression plot: Left ventricular posterior wall thickness versus serum Ln aldosterone.

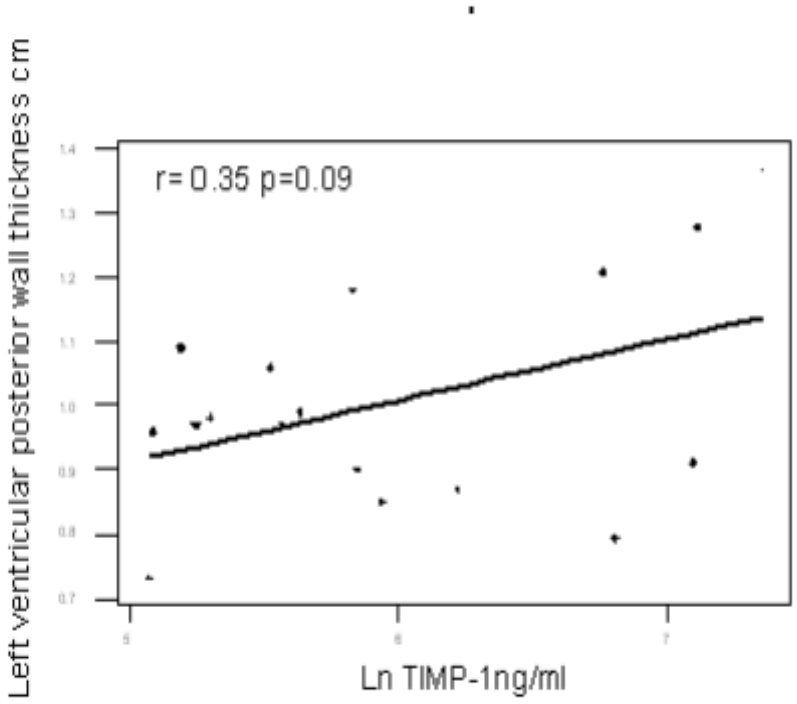
Patients with essential hypertension never treated with antihypertensive drugs. Loge (Ln). There is no correlation between Ln TIMP-1 and left ventricular posterior wall thickness.



Graph 4. 6 Regression plot: Left ventricular mass index versus plasma Ln TIMP-1.
Patients with essential hypertension never treated with antihypertensive drugs. Loge (Ln). There was a positive and significant correlation between Ln TIMP-1 and left ventricular mass index.



Graph 4. 7 Regression plot: Intraventricular septum versus plasma Ln TIMP-1.
Patients with essential hypertension never treated with antihypertensive drugs Loge (Ln). There is a positive and significant correlation between Ln TIMP-1 and intraventricular septum size.



Graph 4. 8 Regression plot : Left ventricular posterior wall thickness versus plasma Ln TIMP-1.
Patients with essential hypertension never treated with antihypertensive drugs Loge (Ln). There is no correlation between Ln TIMP-1 and left ventricular posterior wall thickness.

There was no association between aldosterone and TIMP-1 in all normotensive and all hypertensive patients, who had previously been on treatment. There was an association in all hypertensive patients who had never been treated between Ln TIMP-1 and LVMI and IVSd (Graph 4.6 and 4.7 respectively). The association between Ln TIMP-1 and LVPWd was not significant (Graph 4.8 p = 0.09).

4.1.6 Discussion

The present study has shown a significantly higher level of TIMP-1 in a selected group of hypertensive patients in comparison with normotensive controls and this is in agreement with the data reported by Lavaides et al., (1998). However, this data has shown better discrimination between the hypertensive and normotensive groups than that of Lavaides, (1998) who measured serum TIMP-1, which unlike plasma, is contaminated with platelet TIMP-1 cross-reacting protein (Cooper et al.,1985). Unlike Lavaides et al., (1994), there was no significant difference in PIIINP between the normotensive and hypertensive patients.

In hypertensive patients not on treatment (but not in normotensive patients) there was a relationship between aldosterone and TIMP-1. Castoldi et al., (2002) showed that angiotensin II up-regulated TIMP-1 (assessed by Western Blotting) in the aorta of rats and angiotensin II increases TIMP-1 levels in cultured rat endothelial heart cells (Chua et al.,1996) Ang II increases aldosterone levels which in turn may upregulate TIMP-1. Additionally, in this study, there was a significant association between aldosterone and LVMI and IVSd. The lack of statistical significance between LVPWd and aldosterone and LVPWd and TIMP-1 is unexpected and the explanation unclear. This mechanism may be explained by the profibrotic action of aldosterone. This data is broadly in agreement with that of Lazurova et al., (2003) whose group did demonstrate an association between aldosterone and LVMI, IVSd and LPWd.

There was no correlation between previously treated hypertensives and TIMP-1 but there was an association, in never treated hypertensives, between TIMP-1 and LVMI and IVSd, implying the increase in ventricular size may be due, at least in part, to inhibition of MMPs.

The highest predicted plasma TIMP-1 result (assuming the left ventricle increased by 30% due to LVH) should be approximately 400 ng/ml. However, the plasma TIMP-1 concentration in hypertensive patients was at least twice and up to five times the TIMP-1 levels present in the controls. Plasma TIMP-1 results in alcoholics (Campbell et al., 2001) with severe fibrosis are similar to the TIMP-1 levels reported here in hypertensives. This suggests that cardiac tissue cannot be the only source of plasma TIMP-1 in hypertensive patients.

Angiotensin II is elevated in essential hypertension, suggesting that the plasma TIMP-1 elevation may be from two sources - cardiac and the vascular bed. In agreement with Lavaides et al., (1998) our unpublished data showed (section 4.2.0), that angiotensin converting enzyme inhibitors (ACEI) reduce plasma TIMP-1 levels significantly. This observation emphasises the important role of angiotensin II in increasing TIMP-1 levels in hypertensive patients and suggests that the endothelium may produce significant levels of TIMP-1.

That there was a correlation between TIMP-1 and LVMI and IVSd in the never treated hypertensives group but no correlation in the previously treated

hypertensives may be due to different half-lives of connective tissue versus TIMP-1. The withdrawal of antihypertensive treatment for one month is clearly sufficient to change TIMP-1 to levels similar to those found in the never treated group (Graph 4.1) but it will be months before collagen levels change to a new steady state. The suggestion is that enhanced collagen deposition in the vascular bed and heart is due not only to an increase in collagen type III synthesis but to a reduction in collagen degradation because of inhibition of matrix metalloproteinases by TIMP-1. This hypothesis is supported by the work of Funck et al., (1997) who showed that MMP1 activity was low in angiotensin II stimulated cultured human cardiac fibroblasts. Additionally, I investigated the inter-relationship between plasma TIMP-1 and aldosterone.

4.2.0 Plasma levels of TIMP-1 in patients treated with ACEI

4.2.1 Introduction

Having shown that, in never treated hypertensives increasing TIMP-1 was associated with higher concentrations of aldosterone, I hypothesised that in patients on ACEI, this relationship should be maintained.

4.2.2 Patients

Ten patients with essential hypertension were treated only with the ACEI, Lisinopril. These patients attended the cardiology clinic to participate in a drug trial. Ethical permission was obtained and blood taken, after written informed consent.

4.2.3 Method

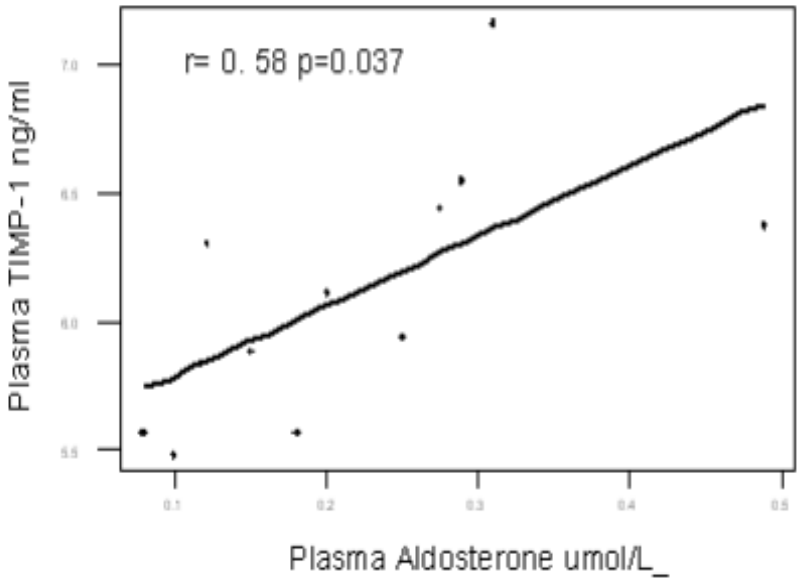
TIMP-1 was measured using the original Amersham assay and aldosterone measured using an antibody coat a count (Siemens)

4.2.4 Statistics

The results were analysed using the MiniTab statistical package. The data was either Gaussian or log-converted to render them Gaussian. Associations were analysed using Pearson Correlation.

4.2.5 Results

There is a significant relationship between TIMP-1 and aldosterone in patients who are both normotensive and hypertensive (graph 4.9).



Graph 4. 9 Regression plot: Plasma TIMP-1 versus serum aldosterone
Patients with essential hypertension treated with Lisinopril. There was a positive correlation between TIMP-1 levels and aldosterone.

4.2.6 Discussion

Since the potentially direct interaction of angiotensin II and TIMP-1 could not be measured, aldosterone was used as a surrogate. There was a significant positive correlation between aldosterone and TIMP-1 in patients who were treated with ACEI. The higher levels of TIMP-1 and aldosterone were associated with those patients whose blood pressure remained high on ACEI, presumably due to poor compliance. This study does not prove that angiotensin II / aldosterone was modulating TIMP-1 levels but it suggests that the RAAS is involved in altering TIMP-1 levels and agrees with the data in the never treated patients, where there was a correlation between TIMP-1 levels and aldosterone. In the work of Nerea

(2000) spontaneously hypertensive rats (SHR) treated with Losartan had lower mRNA TIMP-1 levels than the placebo treated SHR. This is an observation, at the level of gene expression, which agrees with our data that TIMP-1 may be important in renin driven hypertension, although this view has been disputed by Onal et al. (2009) who showed that post Losartan therapy, TIMP-1 levels increased in SHR. These differing results may be explained by the TIMP-1 assay used in Onal's work.

The studies to date show that TIMP-1 is not an acute phase protein and that TIMP-1 is elevated in essential hypertension. The associated LVH is probably driven by a component of the RAAS, either Ang II, aldosterone or both. This study has focused on fibrotic deposition in the vascular tree and the left ventricle. Now I wish to assess whether plasma levels of TIMP-1 are related to other markers of vascular disease.

Chapter 5

Inter-relationship between 25 hydroxyvitamin D, matrix metalloproteinases and their tissue inhibitors 1 and 4

5.0.0 Introduction and Aims

Data from Chapter 4 showed that plasma levels of TIMP-1 were elevated in essential hypertension and correlated with LVMI. However, it was unknown whether TIMP-1 was related to other markers of vascular disease, for example, blood plasminogen inhibitor factor 1 (PAI-1), homocysteine, insulin and lipids. Plasma TIMP-1, MMP2 and MMP9 and serum levels of CRP was assayed in healthy Bangladeshi subjects, in whom PAI-1, homocysteine, insulin and lipids had previously been measured.

5.0.1 Patients

171 British adults of Bangladeshi origin mean age 43 range 35-65 years, with no history of diabetes, ischaemic heart disease, hypertension or any other current illness, but who had blood glucose (mmol/L) results > 6.4 <2hours after a meal or >4.4 >2hours post prandially, gave written informed consent. Details of betel nut and cigarette consumption were also noted. These subjects were recruited for a cross sectional study at their GP surgeries, whilst attending for routine appointments. Forty-seven of these patients, were consented into the interventional trial. The Ethics Committee refused permission unless both arms of the trial were given vitamin D supplementation. Accordingly, a low dose of 500IU IM cholecalciferol with a high IM dose of cholecalciferol 50,000 IU was administered every three months for one year. (Timms et al., 2002). Except for PTH, all other routine bone markers were not significantly different between the

two groups, which were then compared pre and post vitamin D supplementation by measuring 25(OH)vitD, TIMP-1 MMP2, MMP9 and CRP.

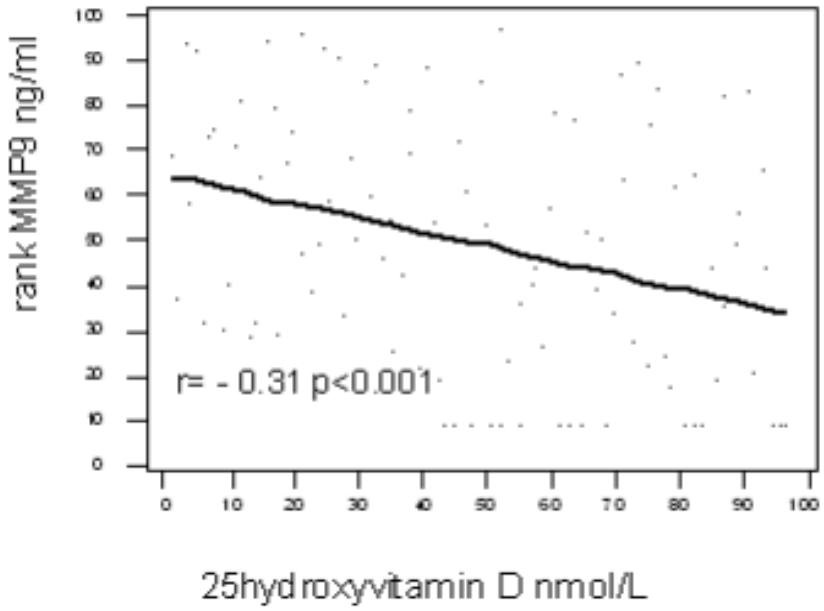
5.0.2 Methods

The assays measured were MMPs and TIMP-1, which have previously been described and the sensitive CRP assay was measured, using a DPC kit. The data from Dr Boucher's resource previously measured, insulin, proinsulin, 32:33 split proinsulin, PAI-1 and homocysteine, the latter being measured by HPLC. Fasting serum lipid profiles and a clinical chemistry profile were measured by standard automated techniques. PAI-1 was measured by ELISA and plasma fibrinogen by a modified Clauss technique (Immuno A.G. Vienna, Austria). Serum 25(OH)vitD was measured by immunoassay (IncStar, Minnesota, USA) and concentrations < 28nmol/L defined vitamin D deficiency (Malaban et al.,1998)

5.0.3 Results of Cross Sectional Study

These samples from the Boucher study were stored for 5 years prior to analysis. Of the 171 subjects 97, (40 men and 57 women) had plasma TIMP-1, MMP2 and MMP9 measured. There is a negative association between 25(OH)vitD and MMP9 (graph 5.1) and sCRP table 5.1. Neither plasma TIMP-1, MMP9 or MMP2 concentration were related to age or body build. TIMP-1, MMP9 and MMP2 was not correlated with other markers of vascular dysfunction from this study such as

homocysteine, fibrinogen, glycaemia, lipid or insulin profiles, diabetic status or blood pressure directly (personal communication from Dr Boucher). Additionally, neither MMP2 nor MMP9 related to any of these factors on multiple regression analysis. On multiple regression analysis, a major independent determinant of TIMP-1 was systolic blood pressure (p = 0.007); however, TIMP-1 levels were not correlated with 25(OH)vitD (table 5.1).



Graph 5. 1 Spearman rank regression of the cross sectional data: Plasma MMP9 versus serum 25hydroxyvitamin D
97 Bangladeshi subjects who had vitamin D deficiency but no overt evidence of essential hypertension, diabetes or heart disease,

5.0.4 Discussion of Cross Sectional Study

In Table 5.1, only plasma TIMP-1 was not correlated to 25(OH)vitD. The significant negative correlations between CRP and MMP9 with 25(OH)vitD suggest that MMP9 may be a marker of vascular disease in this population. This is the first time this association has been described.

Correlates	**r p**
TIMP-1v betel nut	+0.29 0.019
TIMP-1 v cigarette smoking	+0.41 0.009
MMP9 v 25(OH)vitD	-0.41 0.0001
TIMP-1 v 25(OH)vitD	-0.06 ns
CRP v 25 (OH)vitD	-0.22 0.031
TIMP-1 v MMP9 pre vitamin D supplementation	0.87 <0.0001
TIMP-1v MMP9 post vitamin D supplementation	0.63 <0.0001

Table 5. 1 Cross sectional study: correlates of MMP2, 9, TIMP-1 and 25(OH)vitD and lifestyle factors in 97 Bangladeshi subjects.
They had vitamin D deficiency but no overt evidence of essential hypertension, diabetes or heart disease. Of all the correlates only TIMP-1 v 25(OH)vitD was not significant.

This finding has other significant clinical implications, as MMP9 levels are elevated in a wide variety of pathologies including malignancy, CVD and rheumatism (Section 1.9.0). In the next part of this study, the intervention arm, we hoped to determine whether this relationship between MMP9 and 25(OH)vitD is a "true" association.

5.0.5 Interventional study

5.0.6 Results

The mean of 25 (OH)vitD levels pre supplementation were 20nmol/L and post supplementation the values for the mean for the high supplemented group was 32nmol/L and for the low supplemented group, 30nmol/L. As the change in 25(OH)vitD is not significantly different between the low and the high supplemented group, the data from the two groups were combined. The results of this study are presented for TIMP-1, MMP2, MMP9, and CRP in Table 5.2
The major finding in Table 5.2 is that vitamin D supplementation of Bangladeshis resulted in a 67 % fall in MMP9, where the mean value of MMP9 fell from 27.7 pre and 9.2 post vitamin D supplementation. There was a 40% decline in TIMP-1, from a mean of 263.8 pre to a mean of 161.6 post vitamin D supplementation. Finally, a 23% fall in CRP corresponded to a mean level of 4.7 pre to 3.8 post vitamin D supplementation and a 14% fall in MMP2. With the exception of MMP2, the falls in MMP9, TIMP-1 and CRP were significant at $p<0.01$.

Test	Pre-supplementation Mean SD	Post-supplementation Mean SD	% change	p value
25(OH)vit D (1) nmol/L (2)	20 [6] 20 [7] (n= 47)	34 [10] 32 [13]	70 80	<0.001 <0.001
MMP9 (ng/ml)	27.7 [32.6] (n = 34)	9.2 [12.1] (n = 34)	- 67	<0.0001
TIMP-1 (ng/ml)	263.8 [174] (n = 38)	161.6 [84.8] (n = 38)	- 40	<0.002
MMP2 (ng/l)	322.1 [70.2] (n = 28)	278.4 [78.9] (n = 28)	-14	<0.04
CRP (mg/l)	4.7 [5.6] (n=24)	3.83 [3.6] (n=24)	-23	<0.01

Table 5. 2 Serum circulating 25hydroxyvitamin D, and plasma MMP9, MMP2, TIMP-1 & CRP in the intervention arm.

Mean (SD) in deficient serum 25hydroxyvitamin D < 28 nmol/L subjects before and after supplementation (1) high supplemented group (2) lower supplemented group n = number analysed for each assay cross sectional study. The Bangladeshi subjects who had vitamin D deficiency had no overt evidence of essential hypertension, diabetes or heart disease. There was a significant fall in MMP9, TIMP-1, MMP2 and CRP in response to vitamin D supplementation.

5.0.7 Discussion

There are two major observations in this study. Firstly, the positive correlation between plasma TIMP-1 and systolic blood pressure (BP) which is in agreement with our previous work, demonstrating an increase in TIMP-1 in Caucasians with essential hypertension (Timms et al., 2002). The second major finding in this study was the negative correlations between 25(OH)vitD and plasma MMP9 and 25(OH)vitD and TIMP-1. Supplementation caused only a small increase in 25(OH)vitD, but this resulted in a marked fall in plasma MMP9 levels, even in those whose levels remained <20ng/ml. According to this data, it appears that plasma levels of MMP9 would be reduced by increasing 25(OH)vitD levels possibly below that which is the accepted recommended range for 25(OH)vitD.

Serum CRP levels as an established risk marker for ischaemic heart disease provide robust data in Caucasians but are less reliable in other ethnic groups (Anand et al., 2004). In addition, MMP9 levels are increased in unstable plaque and MMP9 and MMP2 are elevated in acute coronary syndromes (Kai et al., 1998). Possibly MMP9 levels, like CRP, may become validated as markers of risk assessment in ischaemic heart disease (IHD).

The role of vitamin D in suppressing the inflammatory response has been documented in rheumatoid arthritis (Merlino et al.,2004). Low levels of 25(OH)vitD have been implicated in an increased incidence of TB in East London (Martineau et al.,2011).

The above work is clearly interesting but a major issue is whether the converse relationship between 25(OH)vitD and MMP9 can be translated into a different ethnic group, the health of whom is well documented. Levels of 25(OH)vitD levels fall in submariners on a 3 month patrol. I contacted the Hospital for the Armed Forces near Portsmouth and this resulted in the next project.

5.1.0 25 Hydroxyvitamin D and MMP9 levels in Submariners.

5.1.1 Introduction

Ideally, we wished to investigate a group of people who had little exposure to ultraviolet light. Prisoners and miners were considered as potential groups of subjects who might be expected to have low levels of 25(OH)vitD: certainly they would have low exposure to ultraviolet light. But it was felt that these two groups would cover a wide age span and would have multiple pathologies which would make the interpretation of any relationship between 25(OH)vitD and MMP9 difficult.

Due to these issues, the Royal Navy at Portsmouth was approached to recruit submariners into a study to access the inter-relationship between 25(OH)vitD and MMP9. Submariners were chosen because it is known, over a 3 month period, that levels of 25(OH)vitD fall (Gilman et al.,1982) and I hypothesised that this may increase blood levels of MMP9 and sCRP. All the crew was male and white Caucasian, with the exception of one submariner, so there were no confounding factors e.g. the menstrual cycle in females. The subjects were relatively fit and their health had been thoroughly documented. There is no source of ultraviolet light aboard the nuclear submarine, neither is there a formal Royal Naval policy on vitamin D supplementation for submariners at the time of the study. There would be variability in food intake but this would be limited by food stocks aboard the submarine and of course, there is the potential confounder of vitamin supplementation. Accordingly, levels of 25(OH)vitD, CRP, TIMP-1, TIMP-4 and MMP9 were assayed on samples taken immediately prior to the start of the patrol and at the end of the patrol, whilst the submarine was still submerged.
Since, low serum 25(OH)vitD levels are associated with worsening glycaemia (Pittas et al., 2007) we also measured fructosamine as a measure of glycaemic control.

5.1.2 Subjects

Subjects were 47 volunteers (who had given informed written consent) were recruited from a ballistic submarine that was going to be deployed for about three

months. About 40 male personnel aged between 18 and 35 years, serving in the submarine base port during the months of January/February were also recruited as shore based controls. Both controls and submariners had a sample of blood taken pre and post patrol (about 3 months). Prior to deployment, the submariners underwent a medical examination.

5.1.3 Method

Twenty mls of blood were collected into three lithium heparin tubes and a 5ml sample into an EDTA tube. Lithium heparin samples are taken first, as EDTA may contaminate the vacutainer needle and interfere with the assays planned for the lithium heparin plasma. The samples were spun at 3000 revolutions per minute for 10 minutes, within 90 minutes of collection. The supernatant from the lithium heparin tubes was divided into 3 aliquots, transferred to cryogenic tubes and frozen at -20°C. These samples were tested for 25(OH)vitD, MMP9, TIMP-4, and CRP. After the 3 months deployment, a further lithium heparin and clotted sample was collected and treated as above. This second blood sample was collected whilst the submarine was deployed (i.e. had not surfaced). The specimens were separated as before and stored in a -20°C freezer until the submarine docked, whereupon the samples were transferred, still frozen, to a -20°C freezer at Gosport Naval Hospital.

5.1.4.0 Measurement of 25 hydroxyvitamin D using an in-house high performance liquid chromatography mass spectrometer mass spectrometer method.

5.1.4.1 Principle

The sample was precipitated using methanol containing hexa-deuterated hydroxyvitamin D and was extracted into hexane. The sample was aspirated onto an ultra pressure liquid chromatography (UPLC) column and the eluate aspirated into the LCMSMS. In the mass spectrometer, the molecule was ionised within the ionisation chamber. The ion was separated on the first quadropole, on the basis of its mass to charge ratio. The sample then entered the collision cell and the subsequent daughter ions were detected in the third quadropole. The daughter ions were separated on the third quadropole and expressed as a ratio of 25(OH)vitD3 or 25(OH)vitD2 to internal standard, which was then directly related to the concentration of 25(OH)vitD3 or 25(OH)vitD2 in the sample.

Separation of ions occurs along the z axis of two pairs of rods one positively charged, the other negatively charged, at 10 volts. Superimposed in this charge is an A/C voltage oscillating at (10,000 Hz) which is tuned to permit the ion of the required M/Z ratio.

5.1.4.2 Equipment

Please refer to Appendix for details of consumables and reagents

Waters Acquity UPLC system and the mass spectrometer.

UPLC system consists of:

- Waters Acquity binary pump
- Waters Acquity autosampler
- Waters Premier MS/MS detector
- Waters Mass Lynx & Quanlynx software.
- Vortex mixer
- Millipore 1L quickfit vacuum filter system
- PTFE 47mm 0.2um filters:
- GHP 47 mm 0.2 um filters,
- Varian Captiva 96 well filter plate 0.2 um filter
- Varian Captiva 96 well, 1mL collection plate
- Varian vacuum manifold and pump for Captiva filter & collection plates.

5.1.4.3 Consumables

- De-ionised water; volumetric pipette, adjustable pipettes
- Analytical UPLC column- Waters Acquity UPLC – BEH C18, 1.7um, 2.1 x 50mm.

- Stainless steel in line filter for Acquity columns -
- Frits for in line filter:

5.1.4.4 Reagents, Calibrants / Standards & Controls

5.1.4.5 Reagents

Methanol Hypersolve grade.2.5 L– catalogue number 152507P

Ammonium Acetate 250g – catalogue number 09689

Formic Acid 0.5L

Deuterated 25 Hydroxyvitamin D3, 5 mg,

All HPLC solvents were of Hypersolve grade

5.1.4.6 Calibrator / Standard

The mobile phase reagent was freshly prepared before each run. The Internal Standard, quality control and serum calibration standard were stored frozen.

Calibrator: Chromsystems: 25 Hydroxyvitamin D2 & D3 calibrator, 5 x 2.0mL. The date and lot number of the calibrator was recorded, when used. The signal to noise ratio of the calibrator was recorded each day.

5.1.4.7 Quality Controls

Chromsystems 25-OH-Vitamin D3 bi-level control (I+II) both 2x 5 mL - catalogue number 0028, was used.

Each lot number of quality control solution was recorded, when changed.

External Quality Assessment

The laboratory is enrolled with Charing Cross External Quality Control Schemes (DEQAS) for 25 hydroxyvitamin D. This external quality control scheme is used to validate assay performance.

5.1.4.8 Preparation of internal standard (I.S.) solution

10 µL of the working deuterated 25 hydroxyvitamin D3, which had been stored at – 80° C in a freezer, was added to 10 mL of methanol (Hypersolve grade). The internal standard was added to correct for any analytical loss, in addition to any variation in injection.

5.1.4.9 The Acquity UPLC settings

Flow rate: 0.50 mL/min

Column temperature: 40 degrees centigrade

Mobile phase A2: 20 mmol/L Ammonium Acetate + 0.1 % Formic acid

Mobile Phase B2: 20 mmol/L Ammonium Acetate + 0.1 % Formic acid in Methanol

The mobile phase was circulated for 20 minutes before starting the run.

5.1.4.10 Ionisation settings

Source temperature 120° C

Desolvation gas temperature 450° C

Desolvation gas flow 800 L/hr

Cone gas flow 50 L/hr

Capillary voltage 3.39 kV

Argon, the collision gas, flow rate 0.35 mL/min, pressure 5.14^{e-003}.

5.1.4.11 Assay Procedure

150 µl of calibrator, quality control or sample was pipetted into a microtitre plate containing 10µl of internal deuterated standard. 150 µl of 0.2M zinc sulphate and 300µl of methanol was added and the plate was vortex mixed for two minutes. After the addition of 750 µl of hexane, the plate was mixed for a further 2 minutes and centrifuged for 5 minutes at 1500 g. The hexane layer was aspirated into a fresh microtitre plate and evaporated to dryness. The residue was re-dissolved in 200µl of 80% methanol buffer and the plate was loaded onto the Acquity UPLC.

5.1.4.12 Optimisation LCMSMS tuning

25-hydroxyvitamin D3 or the 25-hydroxyhexadeuterated 25-hydroxyvitamin D3 was aspirated directly into the mass spectrophotometer.

For the deuterated ion:

25 (OH)vitD3, the transition was 407.4>159

25(OH)vitD3 401.4>159

25(OH)vitD3 401.4>365.2

25(OH)vitD2 413.4>83

5.1.4.13 Precision data

Between batch imprecision was determined by analysing two quality control samples over 30 runs.

5.1.4.14 Results of imprecision studies

25(OH)vitD	Mean nmol/L	Standard deviation nmol/L	Coefficient of variation %
Between Batch Control I	87.3	2.4	2.8
Between Batch Control II	184	5.3	2.8

Table 5. 3 Results of the imprecision studies for 25 hydroxyvitamin D.
n=30 shows excellent imprecision for both low and high controls.

The between batch precision for 25(OH)vitD is less than 3% (table 5.3).

5.1.4.15 Discussion of precision studies

The major problems which beset the measurement of 25(OH)vitD by LCMSMS are the difficulties associated with variable ionisation. However, this precision data is excellent for both the control I and II levels.

5.1.4.16 Accuracy of the LC-MSMS method

Samples from the DEQAS external quality control scheme were compared with the ALT mean. Samples were also analysed using an ELISA method and these results compared with our method.

5.1.4.17 Results of the accuracy experiment

The results for the comparison between the LC-MSMS method and the consensus mean of the DEQAS scheme are shown as a correlation (Graph 5.2) and a

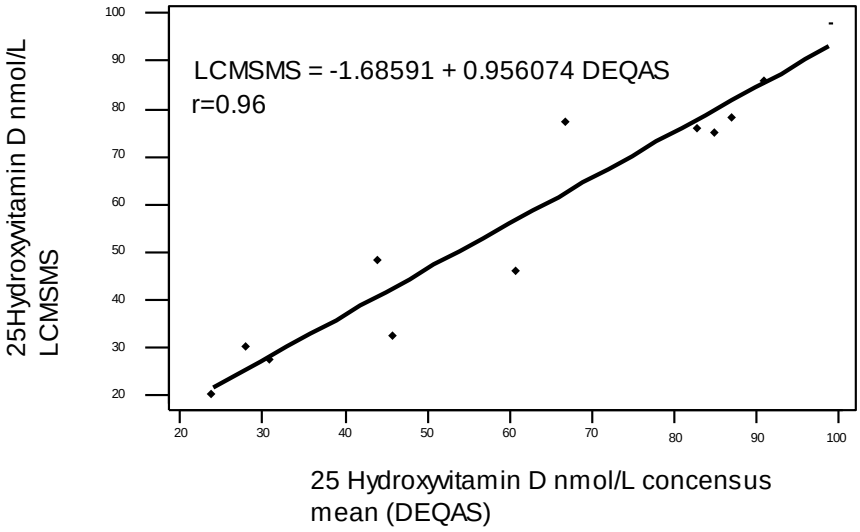
difference plot in Graph 5.3. 25(OH)vitD results from the in-house method by LCMSMS were compared against the ELISA method again as a correlation (Graph 5.4) and as a difference plot (Graph 5.5).

The data demonstrates a satisfactory agreement, in that the gradient of the line is greater than 0.94. Although the Bland Altman plot (Graph 5.5) shows little concentration related bias, the same plot for the DEQAS scheme has a negative bias with a degree of imprecision. Although not apparent in Figure 6, as a rule LCMSMS methods tend to be lower than other methods in the DEQAS scheme.

5.1.4.18 Discussion accuracy of the 25 hydroxyvitamin D method

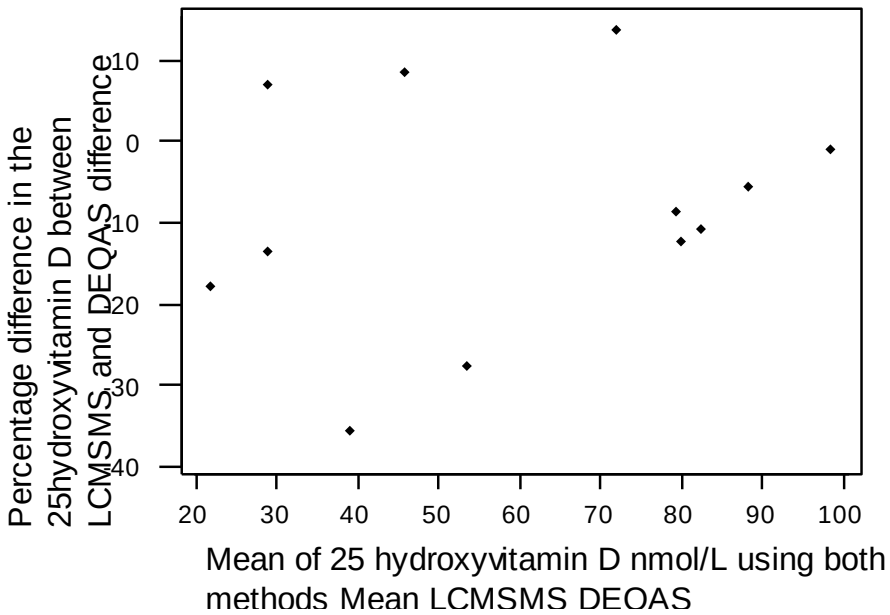
There is no established method for measuring 25(OH)vitD, despite its clinical importance. Neither is there an agreed international standard for calibration of vitamin D, although consensus will be greatly enhanced once the NIST standard is commonly introduced.

Other factors affecting inter-laboratory variation in the 25(OH)vitD assay include the type of matrix used for the calibration, the extraction itself, which may be liquid to liquid or solid phase extraction cartridges and the amount of formic acid added to the buffer.

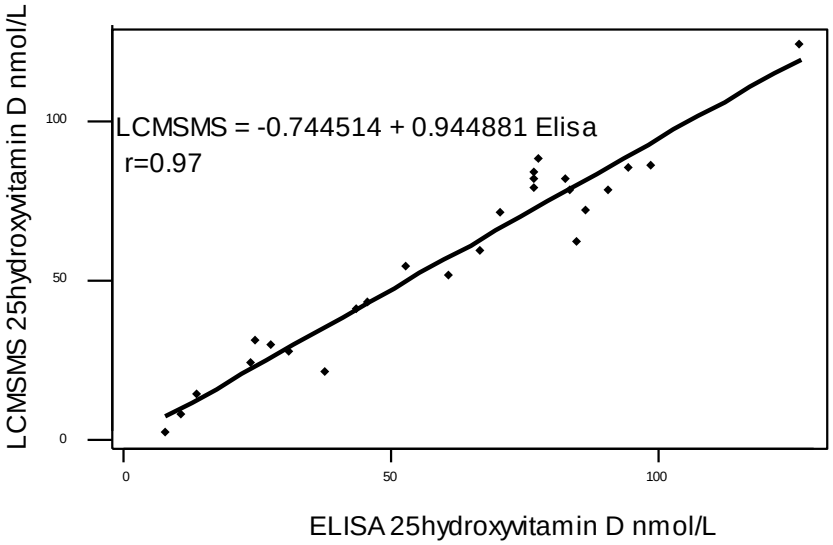


Graph 5.2 Regression Plot: 25Hydroxyvitamin D LCMSMS method versus DEQAS consensus mean result.

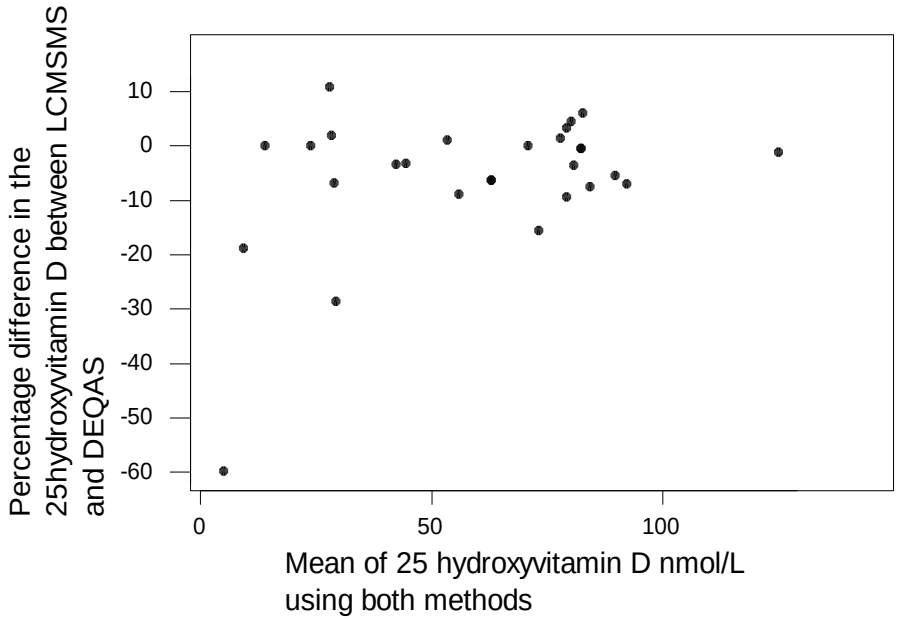
There is a good linear equation between the LCMSMS and the DEQAS mean. The LCMSMS method is about 5% lower than the DEQAS mean. The correlation coefficient is satisfactory.



Graph 5. 3 Percent-difference plot:25 hydroxyvitamin D3 DEQAS.
Comparison between the percent difference y axis (LCMSMS-DEQAS) *100/mean and the mean of in house LCMSMS and DEQAS results. The data suggests that at the lower mean levels of 25(OH)vitD, there is a significant negative bias.



Graph 5. 4 Regression Plot: 25hydroxyvitamin D measured by LCMSMS versus ELISA
There is a good linear equation between the two methods although the LCMSMS method is 5% lower than the ELISA technique. The correlation coefficient is satisfactory.



Graph 5. 5 Percent-difference plot:25 hydroxyvitamin D3 ELISA.
Comparison between the percent difference y axis (LCMSMS-ELISA) *100/mean and the mean of in- house LCMSMS and ELISA results. The data suggests there is a small negative bias over the assayed range.

The problems in calibration are reflected in a report from DEQAS (Figure 7), which showed the quality of the 25(OH)vitD results were appalling. The all trimmed mean (ATM) for sample 321 is 119.8 nmol/L - a result that should not stress any method. However, the range of results submitted, varies from less than 30 to more than 200 nmol/L. In clinical terms, that means an interpretation varying from vitamin D deficiency to over replacement. Clearly, some clinical chemistry departments are generating “random numbers” in terms of 25(OH)vitD results.

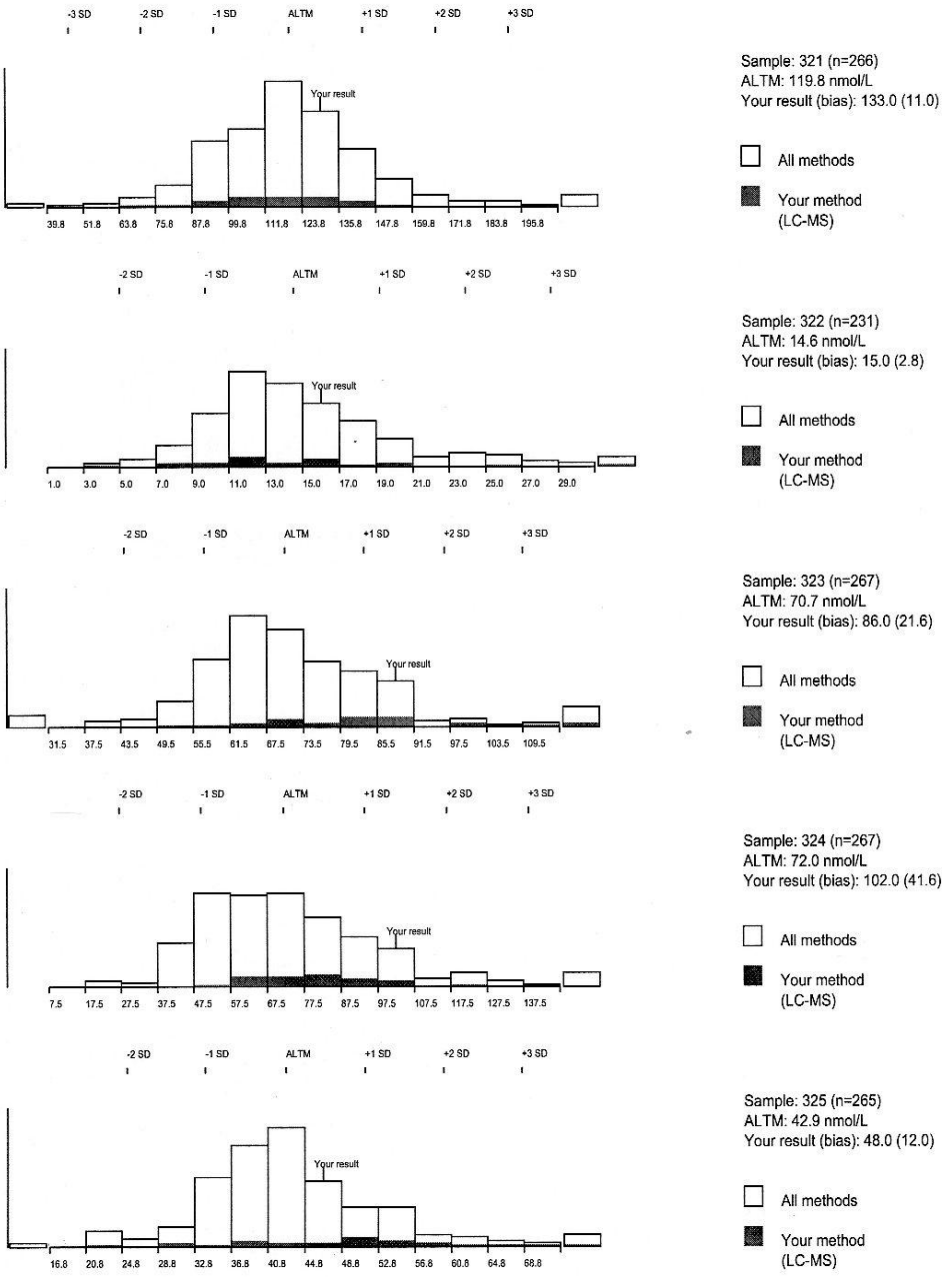
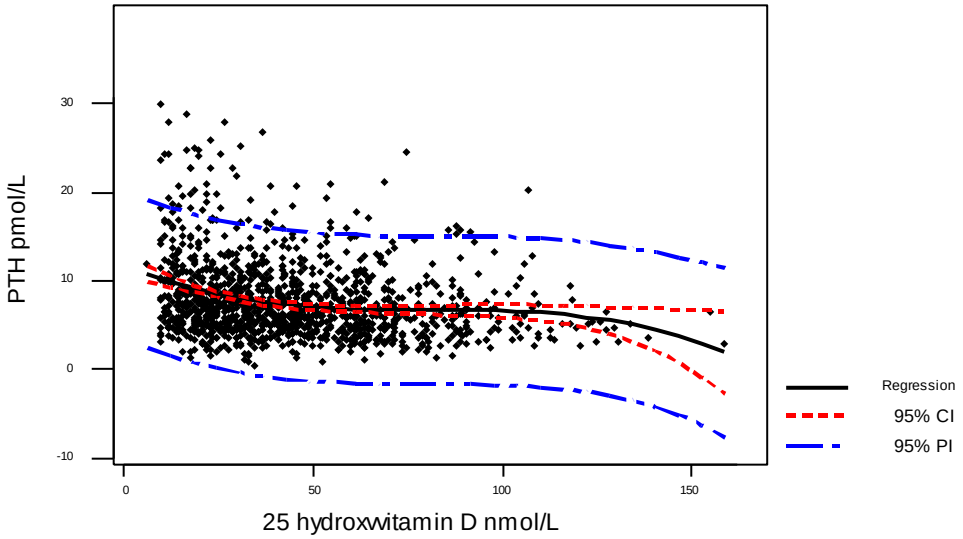


Figure 7 Print out from DEQAS scheme showing the results from over 250 laboratories analysing the same sample. The clear columns represent the frequency of results which laboratories achieved using all the different methods. The black sections represent the results obtained using all the LCMSMS methods. This data shows very poor agreement in laboratories participating in the DEQAS scheme.

5.1.4.19 A reference range for 25 Hydroxyvitamin D based on suppression of PTH

Data was extracted for the laboratory WinPath system over 2006 - 2007 for corrected calcium, PTH and 25(OH)vitD from patients' samples requested by general practitioners. PTH levels greater than 20pmol/L were excluded, as were all samples with a urea greater than 10.0mmol/L and all corrected calcium levels outwith the established Reference Range 2.2-2.6 mmol/L. All 25(OH)vitD results analysed in the department over a year, were analysed to determine whether there was a relationship between 25(OH)vitD and age.

5.1.4.20 Results : A reference range for 25 hydroxyvitamin D using suppression of serum PTH



Graph 5. 6 Polynomial Regression Analysis: Serum PTH versus serum 25(OH)vitD n=3508

The level of serum 25(OH)vitD where the serum PTH levels plateau, provides an indication of the appropriate serum 25(OH)vitD level in terms of PTH suppression.

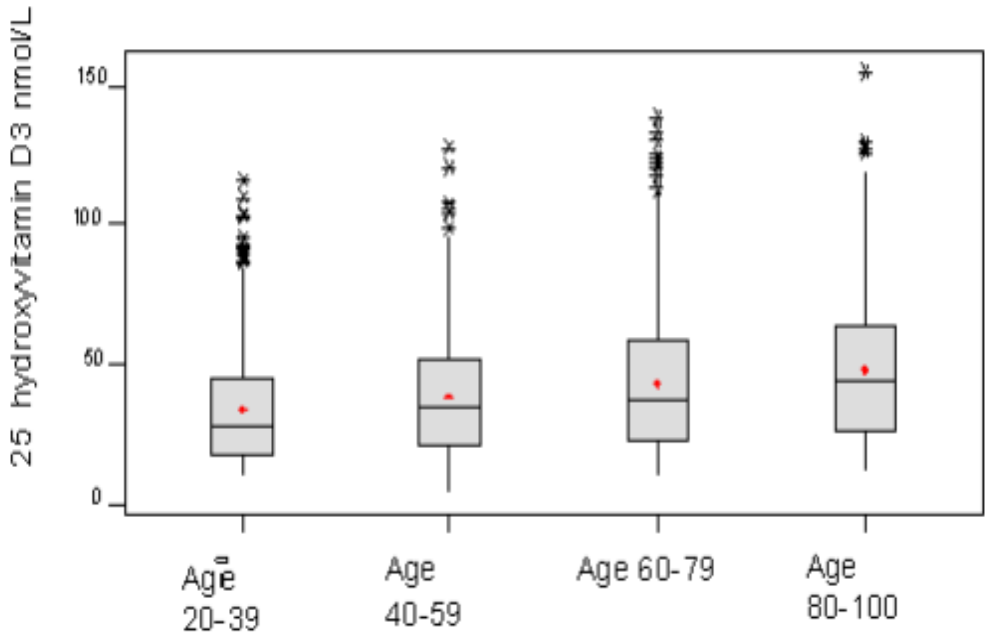
There is a significant relationship between PTH and 25(OH)vitD using the polynomial regression analysis (Graph 5.6). In relation to PTH and 25(OH)vitD, there is a small plateau around 50 nmol/L, as described by Mosekilde (2005) but interestingly, there is a more dramatic fall in the relationship between PTH and 25(OH)vitD at a 25(OH)vitD level of 125 nmol/L.

One way analysis of variance for age related to changes in 25(OH)vitD shows an increase in the mean 25(OH)vitD with age (Table 5.4 Graph 5.7). Using Tukey's comparison of the means, only the 25(OH)vitD in groups aged 20-39 years and those aged 40-59 years were not statistical significance.

Level	N	Mean	St/Dev	--------+---------+---------+--------
vit20-39	277	34.18	22.57	(--*---)
vit40-59	513	38.74	22.39	(---*--)
vit60-79	641	43.11	25.55	(--*--)
vit80-100	206	48.53	28.48	(-----*----)
				--------+---------+---------+--------
		36.0	42.0	48.0

Table 5. 4 ANOVA (stacked) Results for 25 hydroxyvitamin D sorted by age p<0.000

The criteria for the ANOVA was satisfied, as the standard deviations are similar in all age groups which were all Gaussian distributed. This data show that with advancing age serum 25(OH)vitD levels increase.



Graph 5. 7 Box plots of 25 hydroxyvitamin D related to age
The median is the centre line, while the lower and upper ends of the box are the 25th and 75th percentiles and the lower and upper lines of the box the 10th and 90th percentiles. Other points above or below the whiskers are outliers. This data show that with advancing age serum 25(OH)vitD levels increase.

5.1.4.21 Discussion on reference intervals of 25hydroxyvitamin D

The “normal” range for 25(OH)vitD is now defined in terms of functional activity This new concept defined an appropriate 25(OH)vitD on the basis of suppression of plasma PTH. In general, 25(OH)vitD at a level of 50 nmol/L caused near

maximal suppression of PTH. However, this simple relationship is not applicable in a general population (Graph 5.7) possibly because PTH may act as an acute phase protein (Fisher and Southcott, 2005) and may be a marker of all causes of mortality. The work of Fisher and Southcott, in elderly women presenting with hip fracture, did demonstrate an association between PTH and troponin and MI post surgery. More data is required on a wider population before any definitive statement can be made on the role of vitamin D deficiency and hyperparathyroidism in risk stratification of cardiovascular disease.

However, by default, rather than good science, the consensus view is that a level of 25(OH)vitD greater than 50 nmol/L (Holick 2007) is consistent with an adequate vitamin D intake, although the idea that a more appropriate minimal level should be 75 nmol/L is gaining credibility.

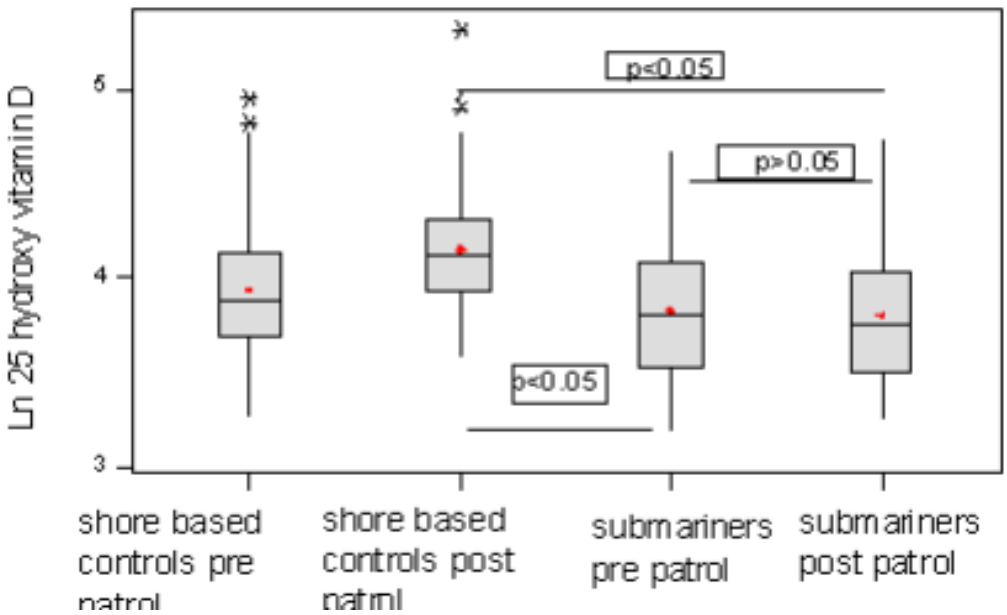
The recommended levels in serum or plasma for 25hydroxyvitamin D3 for men and women in summer or winter is 75 -150 nmol/L.

The obvious criticism of this work would be levels of 25(OH)vitD are higher in the elderly because they take supplements. However, elderly patients as a group are given 800 IU/day in a form that is unpalatable resulting in very poor compliance. Even if compliance was good, it is very difficult to achieve levels of over 50nmol/L particularly in a group which has little access to UV light. An intriguing idea arises “Might high levels of 25(OH)vitD be a factor in those elderly patients longevity”?

5.1.22 Statistics

The results were analysed using Minitab. The data was either Gaussian or log-converted to a Gaussian distribution. Associations were analysed using Pearson Correlation. Statistical significance was determined using a student t test or Mann Whitney test. Significance was established if $p<0.05$. For multi comparisons of the means, a stacked one way ANOVA was used.

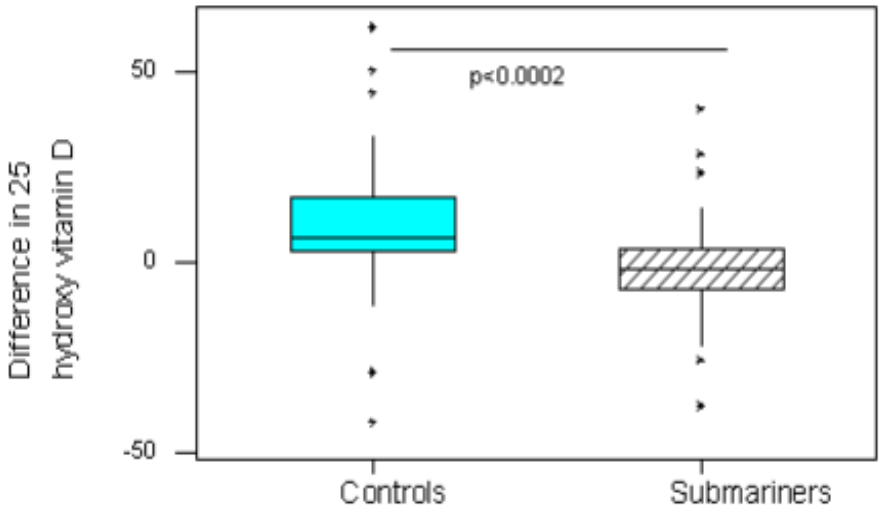
5.1.23 Results:



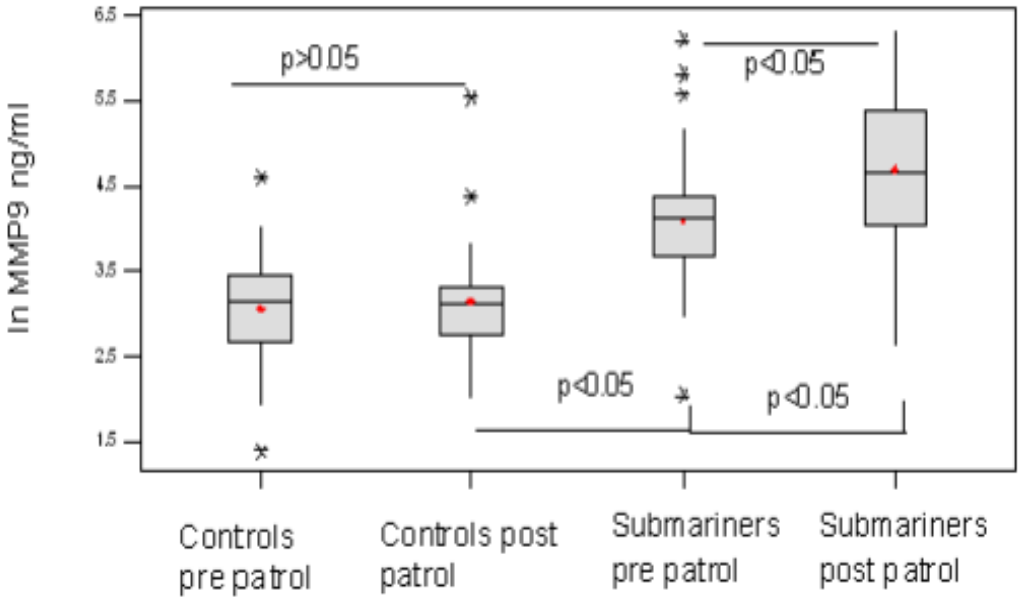
Graph 5. 8 Box plots: serum 25 hydroxyvitamin D in submariners and controls pre and post patrol.
The median is the centre line while the lower and upper ends of the box are the 25th and 75th percentiles and the lower and upper lines of the box the 10th and 90th percentiles. Other points above or below the whiskers are outliers. The serum levels of 25(OH)vitD in the submariners post patrol were not significantly lower from those pre patrol.

Using a stacked one way ANOVA (Graph 5.8) it was found that the groups which were significantly different for 25(OH)vitD were the control results post patrol and the submariners post patrol. There was no significant difference in 25(OH)vitD in the submariners pre and post patrol. The differences in 25(OH)vitD for the controls and submariners pre and post patrol (Graph 5.9) showed a highly significant difference between the two groups.

Using stacked one way ANOVA, Ln MMP9 levels were significantly different between the control group and the submariners pre and post patrol. In the control group there was no difference in MMP9 pre and post patrol but the submariners post patrol had higher levels of MMP9 than the controls and the submariners pre patrol ($p<0.05$) (Graph 5.10). Differences in MMP9 between the control and submariner group (Mann Whitney Test) were significant (Graph 5.11).

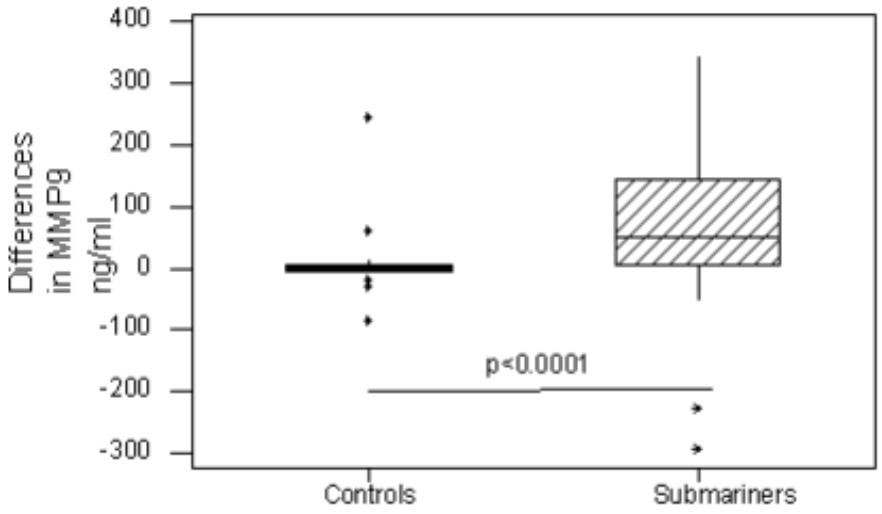


Graph 5. 9 Serum 25 hydroxyvitamin D: difference between controls and submariners. There were significant differences in 25(OH)vitD between the controls and submariners post and pre patrol. For explanation of the boxplots see Graph 5.8.



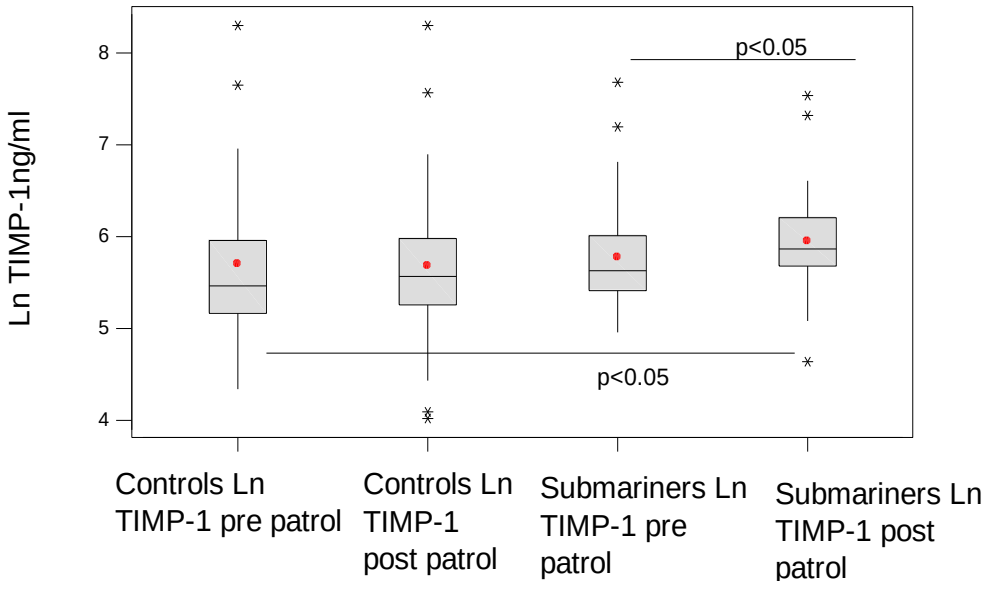
Graph 5. 10 Boxplots: Plasma Ln MMP9 levels in controls and submariners pre and post patrol.

MMP9 levels post patrol were higher than those pre patrol, which were more elevated than the shore based controls. For explanation of the Boxplots please see Graph 5.8.



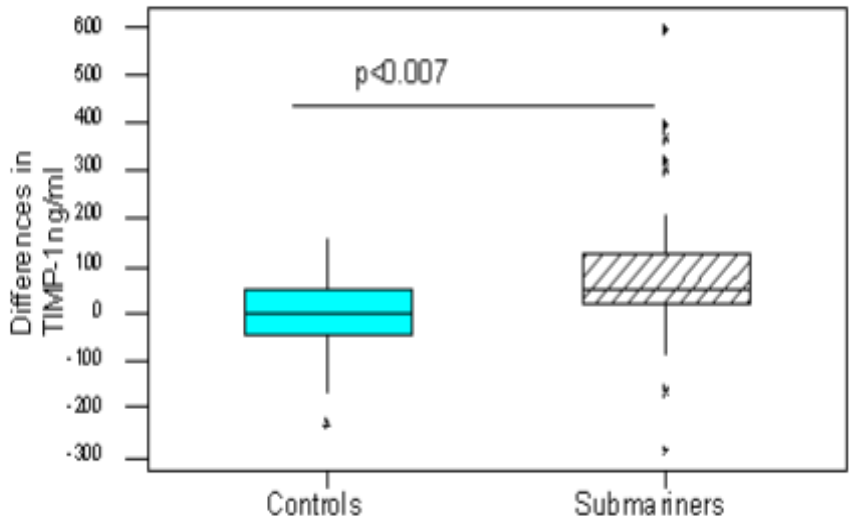
Graph 5. 11 Box plots Plasma MMP9 pre and post patrol in controls and submariners

For explanation of the Boxplots see Graph 5.8. This data demonstrates a marked increase in plasma MMP9 levels in submariners post patrol.

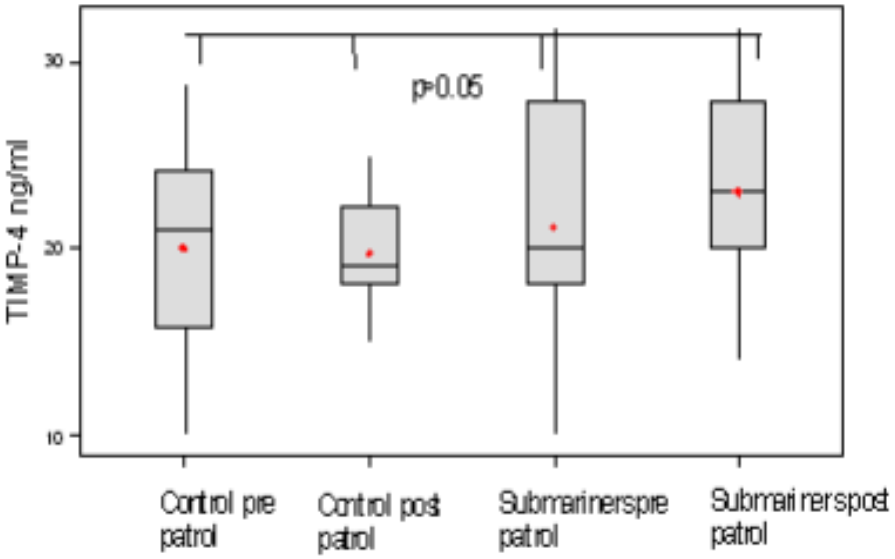


Graph 5.12 Box plots of plasma Ln TIMP-1 pre and post patrol in controls and submariners.
For explanation of the Boxplots see Graph 5.8. There is no difference in controls pre and post patrol but there is an increase in TIMP-1 in submariners post patrol.

There was a slight but statistically significant difference in TIMP-1 between the controls and the submariners post patrol (Graph 5.12). However, differences in plasma TIMP-1 levels between the control and patrol group ($p<0.007$) were highly significant (Graph 5.13).

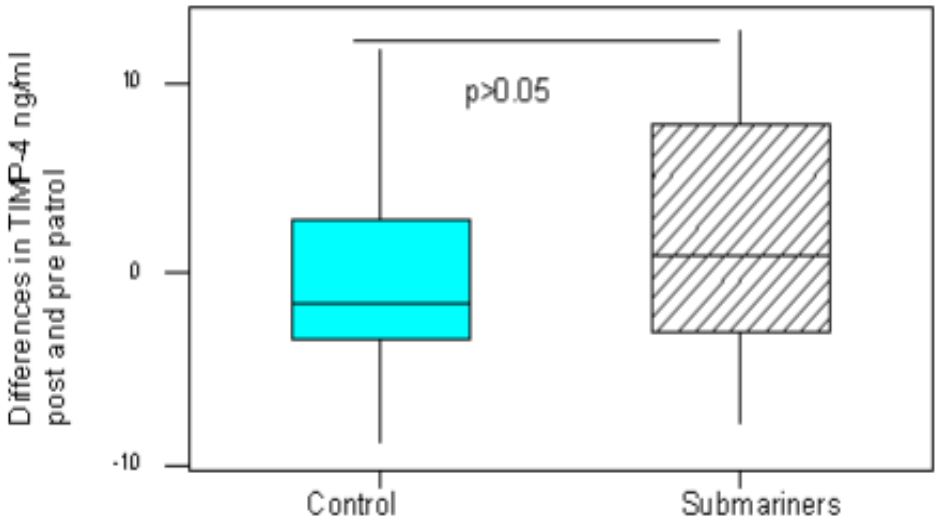


Graph 5. 13 Box plots: Differences in plasma TIMP-1 pre and post patrol in controls and submariners.
For explanation of the Boxplots see Graph 5.8. This data demonstrates a marked increase in plasma TIMP-1 levels in submariners post patrol.



Graph 5. 14 Box plots of serum tTIMP-4 results pre and post patrol in controls and submariners.

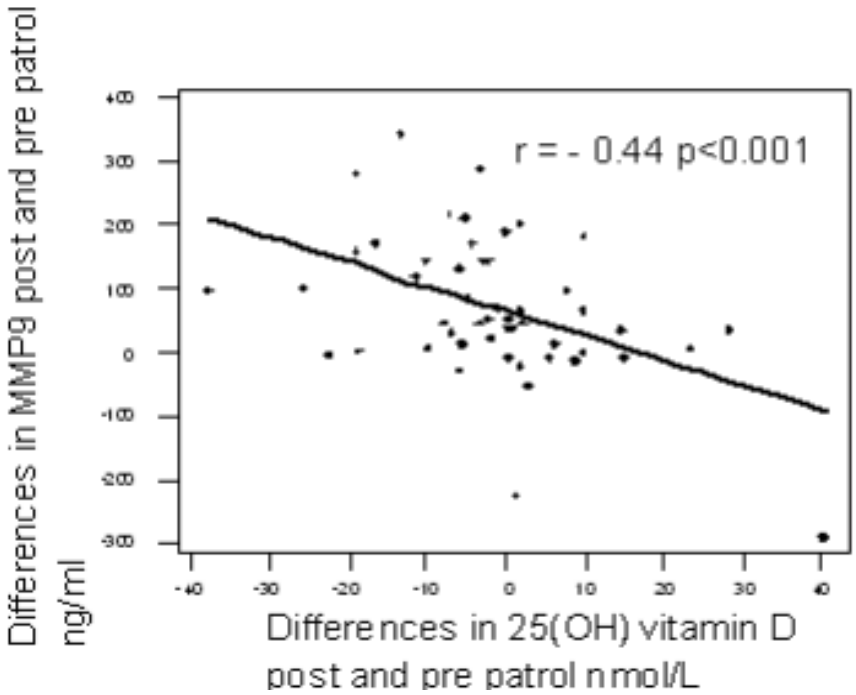
For explanation of the Boxplots see Graph 5.8. There is no change either between the controls or the submariners pre and post patrol.



Graph 5. 15 Box plots: Differences in serum TIMP-4 between pre and post-patrol in both controls and submariners.
For explanation of the Boxplots please see Graph 5.8. There is no difference in tTIMP-4 between the controls or the submariners post and pre patrol.

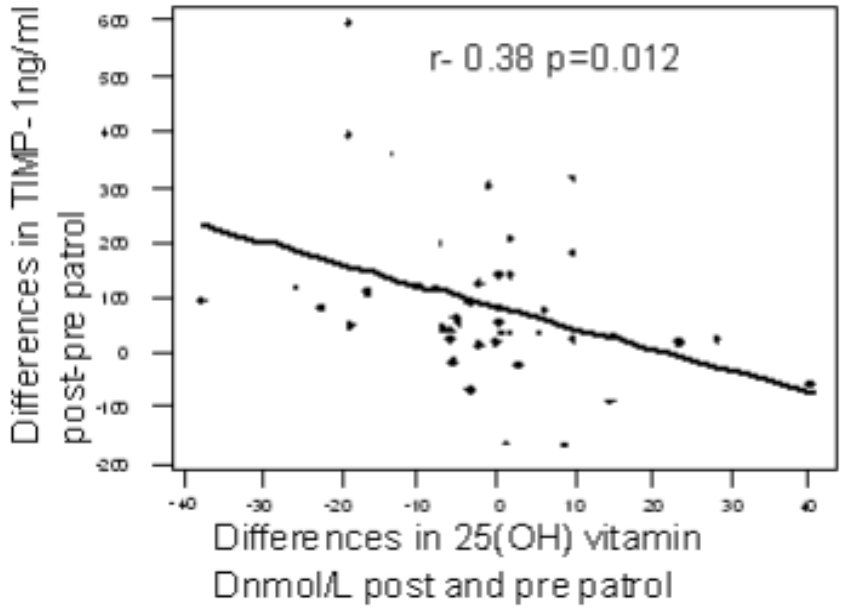
There was no difference in serum TIMP-4 levels pre and post patrol in the controls or the submariners, neither as an absolute figure or as a difference pre and post patrol (Graphs 5.14, 5.15).

In terms of inter-relationships, there was a significant correlation between differences both in MMP9 (Graph 5.16) and TIMP-1 (Graph 5.17) with differences in 25(OH)vitD only in the submariner group. As might be predicted from the Bangladeshi study, differences between MMP9 and TIMP-1 (Graph 5.18) were positively correlated in the submariners.



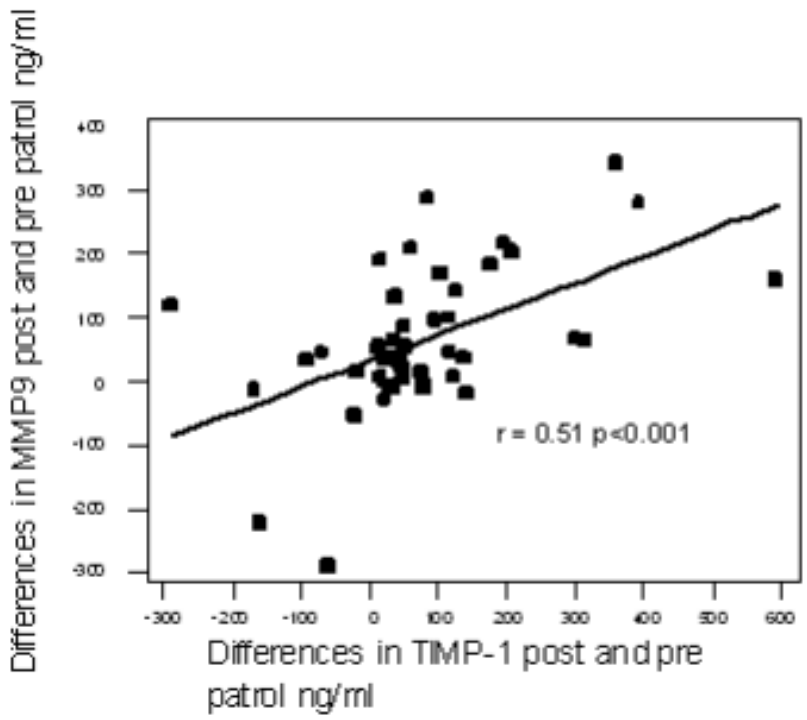
Graph 5. 16 Regression plot: Differences in MMP9 and 25(OH)vitamin D in submariners post and pre-patrol.

This data demonstrates a significant negative correlation between differences in MMP9 and 25(OH)vitD. The higher the positive difference in 25(OH)vitD, the lower the difference in MMP9.



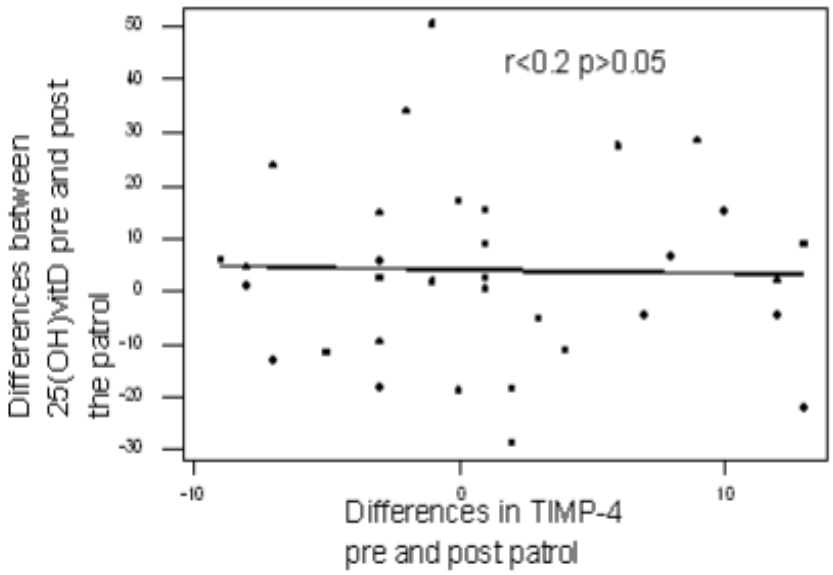
Graph 5. 17 Regression Plot: Differences in TIMP-1 and 25(OH)vitD pre and post-patrol in submariners.

This data demonstrates a significant negative correlation between differences in TIMP-1 and 25(OH)vitD. The higher the positive difference in 25(OH)vitD, the lower the difference in TIMP-1.



Graph 5. 18 Regression plot: Differences in TIMP-1 and MMP9 post and pre-patrol in submariners.

The data shows a positive correlation between differences in MMP9 and TIMP-1. The good association suggests that similar factors may cause upregulation of both the metalloproteinase and its inhibitor.



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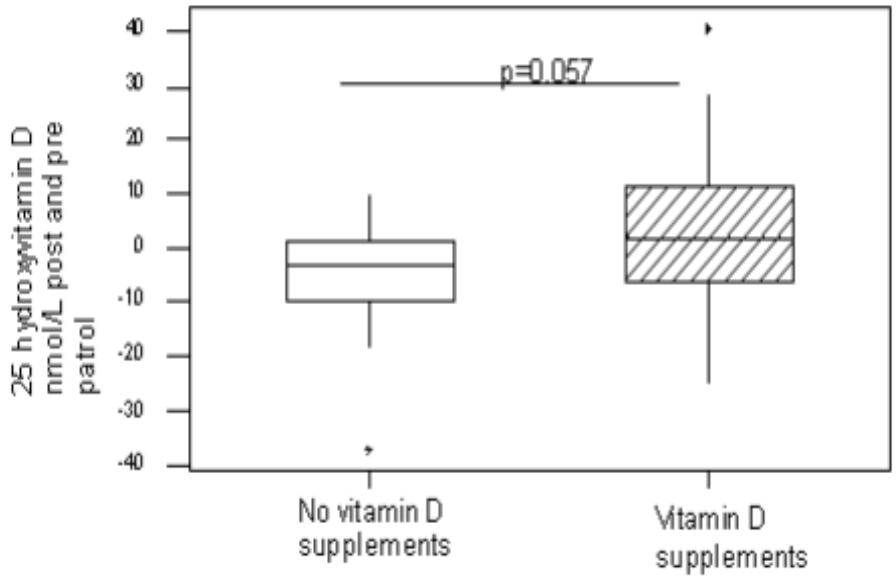
Graph 5. 19 Regression Plot: Differences in 25(OH)vitD and TIMP-4 pre and post-patrol.

There is no correlation between differences in 25(OH)vitD and TIMP-4 implying vitamin D is not involved in the regulation of tTIMP-4.

There was no association between differences in TIMP-4 and 25 (OH)vitD (Graph

5.19) and differences in TIMP-4 and differences in Ln MMP9.

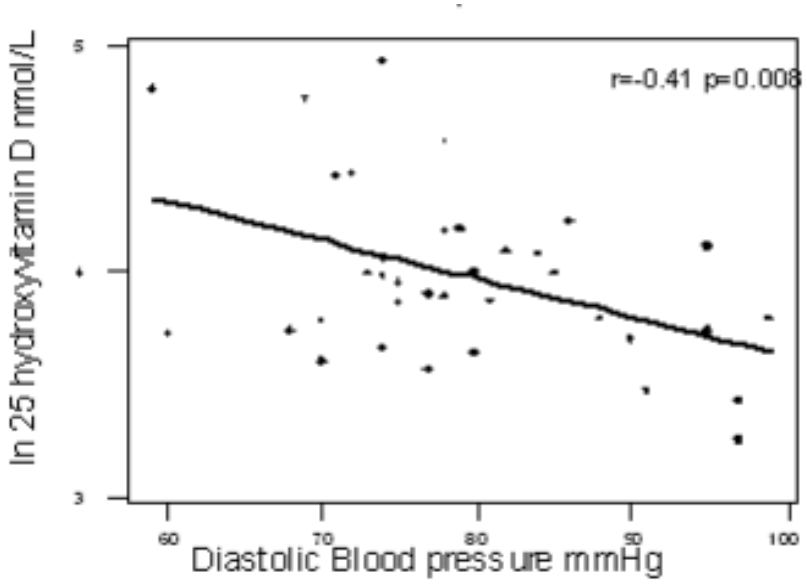


Graph 5. 20 Box plots: Differences in serum 25hydroxyvitamin D pre and post-patrol in submariners who were and were not taking supplements.
There is no statistical difference in 25(OH)vitD in submariners taking supplements and those not consuming supplements, suggesting in this environment vitamin D supplementation of 400IU has no effect in increasing blood levels of vitamin D. For explanation of the Boxplots see Graph 5.8.

Using the Mann Whitney Test, there was no significant difference in 25(OH)vitD levels in those submariners who took supplements of vitamin D and in those subjects who had no vitamin D supplementation (Graph 5. 20, $p = 0.057$). However on univariate analysis, the 25(OH)vit D levels post patrol was dependent on the pre patrol 25(OH)vitD levels ($p<0.001$) and whether the submariner was consuming vitamin D supplements ($p<0.001$).

Between these subjects not consuming and those consuming vitamin D supplements, there was no significant difference in CRP (p = 1.00) and MMP9 (p = 0.7).

Blood pressure was only measured pre patrol. In submariners there was no significant association with either systolic or diastolic blood pressure and TIMP-1 but there was a statistical finding (r= - 0.41 p=0.008) with a negative correlation between 25(OH)vitD and diastolic blood pressure in shore based controls (graph 5.21). There was no correlation with 25(OH)vitD in systolic or diastolic blood pressure in the submariners nor in the controls between systolic blood pressure and 25(OH)vitD.



Graph 5. 21 Regression plot: 25hydroxyvitamin D versus diastolic blood pressure in shore based controls pre-patrol.
There is an inverse correlation between 25(OH)vitD and diastolic blood pressure suggesting low 25(OH)vitD levels may be associated with an increase in diastolic pressure.

5.1.4.24 Discussion

The higher levels of MMP9 in the submariners pre patrol in comparison to the control group, is possibly due to multiple patrols by the submariners. On average, the submariners had been deployed on three patrols. However, it has not been investigated whether multipatrols on a nuclear submarine for stretches of 3 months may raise MMP9 levels by a non-dependent vitamin D mechanism. There was no significant difference in 25(OH)vitD between submariners pre and post patrol. This may be because in January, the levels of 25(OH)vitD would be at their nadir but by April, levels of 25(OH)vitD would have starting increasing in the control group.

Irrespective of the rather small non significant fall in 25(OH)vitD in the submariners post patrol relative to pre patrol levels, there was a significant increase in plasma MMP9 levels between the submariners post and pre patrol. Additionally, there was an inverse correlation between the differences in 25(OH)vitD and MMP9 ($r= -0.44$ $p<0.001$) and an inverse correlation between 25(OH)vitD and TIMP-1 (r - 0.38 $p=0.012$) in the submariner group. Validating the inverse relationship between 25(OH)vitD and MMP9 found in the Bangladeshi subjects in a healthy white Caucasian group strengthens these novel observations. The mechanism of this inverse relationship between MMP9 and 25(OH)vitD is not clear. Increasing concentrations of 25(OH)vitD entering the

macrophage may increase intra-macrophage levels of calcitriol, thereby upregulating IL-10 levels, which, in turn, reduce MMP9 concentrations. That tTIMP-4 levels showed no correlation with TIMP-1, MMP9 or 25(OH)vitD, suggests that if tTIMP-4 is modulated at all, this is not via the action of 25(OH)vitD. It is possible that similar to TIMP-2, tTIMP-4 is constituently expressed and in general, is not upregulated.

The lack of a significant difference, between controls and submariners, in the sCRP results might be due to the relatively high level of fitness and health, as compare to the Bangladeshis.

Eighteen of the submariners voluntarily took a vitamin supplement which included 400IU of vitamin D. The results (graph 5.20) were not significant, demonstrating that even in the environment of the submarine, 400IU is inadequate in increasing 25(OH)vitD levels in the healthy adult. Univariant analysis showed that the serum levels of 25(OH)vitD were dependent both on the pre patrol levels of 25(OH)vit D and the consumption of vitamin D supplements. That inflammatory markers sCRP were the same in submariners on supplements and those not on supplements was predictable if, as suggested, levels of 25(OH)vitD are important in modulating the inflammatory response.

The lack of correlation of both diastolic and systolic blood pressure with TIMP-1 was not surprising, as the subjects were generally normotensive. However, there

was a negative inter-relationship between diastolic (p=0.008) but not systolic blood pressure and 25(OH)vitD in the controls. The significance of this observation is uncertain but there have been reports in the literature suggesting that vitamin D levels may be important in modulating the RAAS.

5.2.0 Plasma levels of MMP9 and 25Hydroxyvitamin D in patients who re-stenose after coronary angioplasty

5.2.1 Introduction

A further area of interest is that of restenosis, which is due to upregulation of MMPs causing migration of smooth muscle cells within the arterial wall. The hypothesis would be that inadequate levels of 25(OH)vitD would upregulate MMP9 causing restenosis post angioplasty.

As atherosclerosis plaques progress, they ultimately block the arterial lumen, which can result in angina if the plaque is stable, or an acute coronary syndrome if the plaque is unstable. Although the pathogenesis of these events is different, the treatment involves angioplasty, during which a balloon is inflated within the blocked section of the artery and a stent inserted. Fifteen percent of patients who undergo intervention re-stenose post angioplasty. An inflammatory cellular response is one of the major components in this pathology, where leucocytes and T-cells are attracted to the site of the angioplasty (Toutouzas, 2004). During angioplasty, the vessel is damaged due to the balloon being inflated. The resulting

injury gives rise to the migration of inflammatory cells, which excrete cytokines and MMPs, causing smooth muscle cell proliferation.

5.2.2 Patients

Seventy-five venous blood samples were obtained from anonymised patients, undergoing coronary angioplasty, at the London Chest Hospital, who had given informed written consent, prior to their first angioplasty. Thirty-one patients were not treated with statins and forty-four were on various statins. All patients were followed up after a year and those with cardiac symptoms had a repeat angiogram.

5.2.3 Methods

Blood samples were collected in heparinised tubes, and the plasma was separated at 805g for 10 minutes, into 3 aliquots. The samples were then stored at -70 degrees centigrade until used. MMP9 was measured, as previously reported and 25(OH)vitD assayed using the method described below

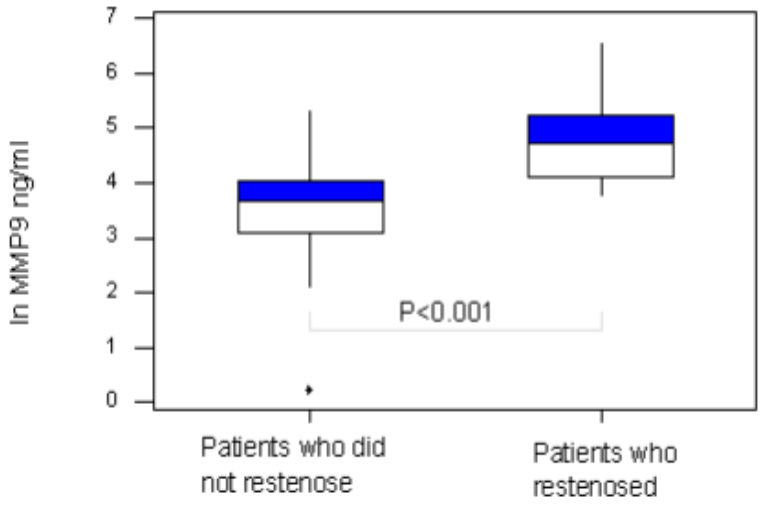
5.2.4 Statistics

The results were analysed using a Minitab statistical package. The data was either Gaussian or log-converted to a Gaussian distribution. Associations were analysed using Pearson Correlation. Statistical significance was determined using a Student t Test or Mann Whitney Test. Significance was

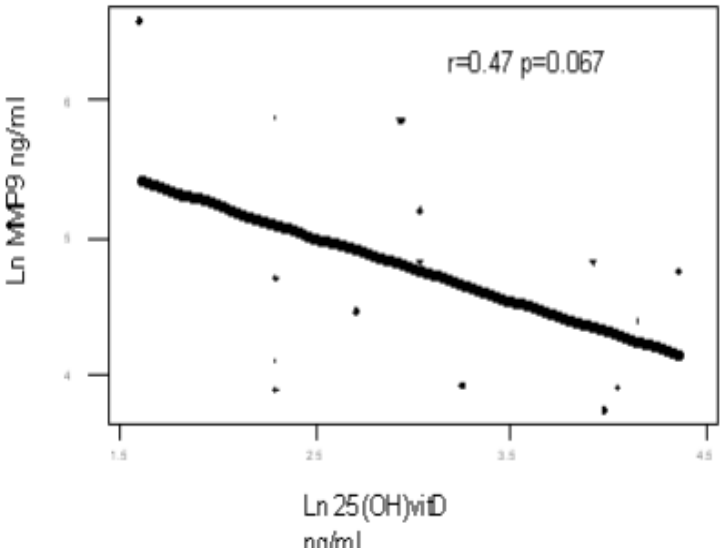
established if p<0.05. Using a difference in MMP9 levels of 40ng/ml, 40 samples were adequate to achieve an α 0.05.

5.2.5 Results

Of the 75 patients, 10 re-stenosed.

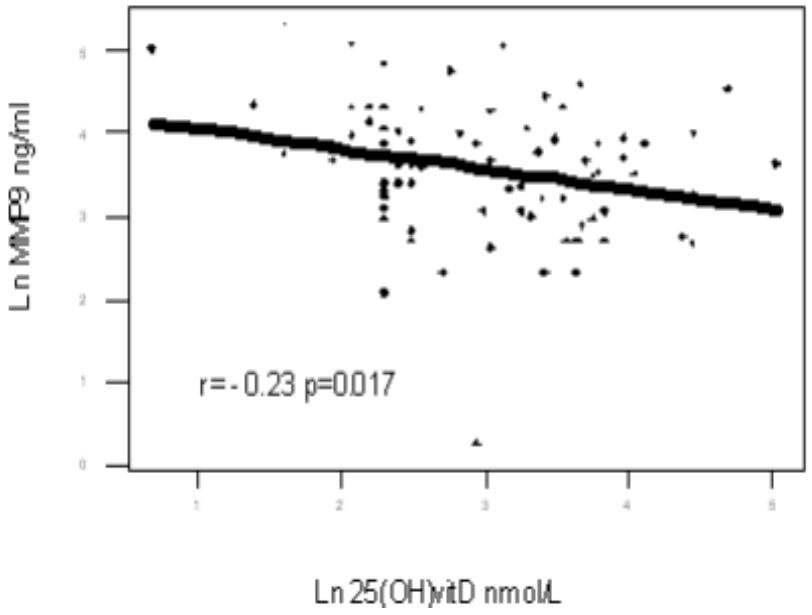


Graph 5. 22 Plasma Ln MMP9 levels in patients with and without re-stenosis post-angioplasty.
The level of plasma MMP9 is higher in patients who re-stenose in comparison to those who do not. For explanation of the Boxplots see Graph 5.8.



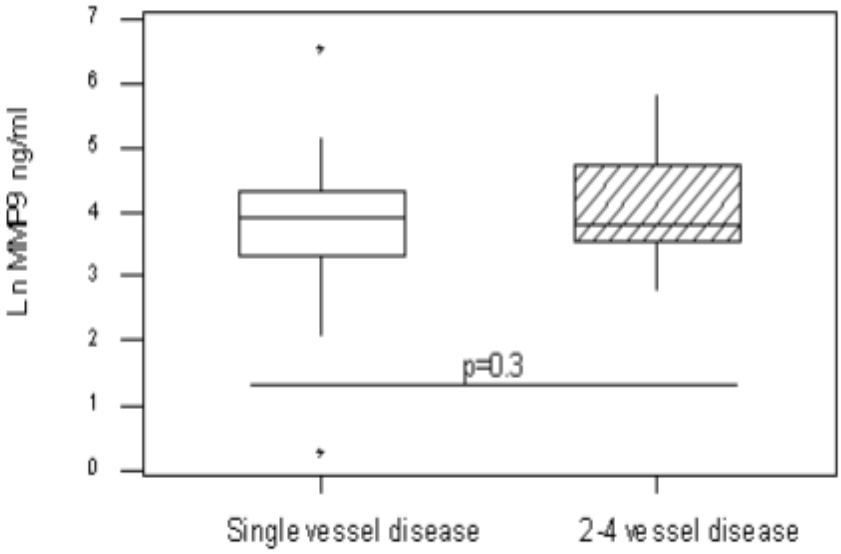
Graph 5. 23 Regression plot: Plasma LnMMP9 versus serum Ln 25hydroxyvitaminD in patients who re-stenosed.

There was no statistical inverse relationship between Ln MMP9 and Ln 25(OH)vitD



Graph 5. 24 Regression plot: Ln MMP9 v Ln 25 hydroxyvitamin D in subjects who did not re-stenose.

There is a significant inverse relationship between Ln MMP9 and 25(OH)vitD, predicting that even in patients on statins, increasing levels of 25(OH)vitD are associated with a fall in MMP9.



Graph 5. 25 Box plots: Plasma LnMMP9 in patients who had single vessel disease and those who had 2-4 vessel disease.
There was no difference in MMP9 levels in patients with 1 or 2-4 coronary vessel disease. For explanation of the Boxplots see Graph 5.8

There is a significant difference in the median plasma MMP9 between those patients who did and those who did not re-stenose p<0.001(Graph 5.22). Additionally there was no significant difference in 25(OH)vitD (p=0.078) between those patients who had and who had not re-stenosed.

In the re-stenosis group, there was a trend for a negative correlation between Ln25(OH)VitD and LnMMP9, although this did not reach statistical significance (Graph 5.23). A negative correlation between LnMMP9 and Ln25(OH)vitD was found in the group who did not re-stenose (Graph 5.24, r= - 0.23, p=0.017). There

was no significant difference in the medians between those patients with one vessel disease and those with 2 - 4 vessels diseased (Graph 5.25). Additionally, there was no significant difference in both MMP9 (p=0.3) or 25(OH)D (p=0.43) levels in patients treated with or without a statin.

The re-stenosis data was analysed using binary logistic regression with re-stenosis (yes or no) as the dependent variable and smoking, statins, MMP9 and 25(OH)vitD as the independent variable. Only MMP9 was significantly correlated with the risk of re-stenosis (odds ratio = 1.009, 95% CI 1.002 to 1.016, p=0.008).

5.2.6 Discussion

Plasma TIMP-1 levels were not measured in this study, as Amersham's monoclonal antibodies for TIMP-1 were no longer were available. Neither Amersham's new kit nor the R & D assay were satisfactory, as they preferentially measured free TIMP-1 rather than the MMP/TIMP-1 complex which gave rise to very poor recoveries.

The data demonstrates that for every ng/ml increase in MMP9 there is a 0.9% increase in the risk of re-stenosis. The ability of this simple model classified correctly 96% of patients who did not re-stenose.

High levels of plasma MMP9 pre-angioplasty are, however, not a reliable indicator of re-stenosis. MMP9 levels are a good negative predictor for those who do not re-stenose but a poor positive predictor of re-stenosis.

Ln25(OH)vitD was negatively correlated with LnMMP9 and in the group which did re-stenose, this relationship just failed statistical significance, probably due to lack of power. However, it is unlikely that there is a difference in the relationship between MMP9 and 25(OH)vitD in either group in this study. The MMP9 levels are predicated on the 25(OH)vitD concentration in both groups, not whether or not they re-stenosed. The observation of an inverse relationship between MMP9 and 25(OH)vitD is in agreement with that found in the Bangladeshi and submariner studies. However, these cardiac patients were treated with a variety of drugs including statins, which are known, as a class, to reduce MMP9 levels (Bellosta et al., 1998, Aikawa et al., 2001) although this was not documented in this study. This work showed that there is an association between 25(OH)vitD and MMP9s, which is unaffected by statins.

Hypertension and glucose intolerance are potential confounding factors in this study but due to the patients' anonymity, these factors could not be investigated further.

Chapter 6

Discussion

6.0.0 Introduction

The role of TIMPs and MMPs is fundamental to many disease processes. The pathology of many disorders, for example, cardiovascular disease, cancer, and fibrosis of the lung and liver (irrespective of the initiating insult) results in aberrant matrix deposition. This may be due to an increase in collagen synthesis or at the tissue level, derangement of TIMPs or MMPs synthesis. Changes in tissue matrix levels are one of the pivotal factors in causing morbidity and mortality in elderly patients and this explains the commercial impetus to modulate MMPs and TIMPs activity.

It has been the goal of pharmaceutical companies to develop a drug to inhibit MMPs, as upregulation of these enzymes is common in a variety of pathologies. The major difficulty in designing such a drug, is that in inhibiting one MMP, there is an upregulation of the other members of the MMP family and to date, there has been no drug which has received FDA approval, (except a derivative of Tetracycline), which is used in the treatment of severe gum disease.

6.1.0 Validation of the TIMP-4 assay

The assays for MMP2 and MMP9 and TIMP-1 assays were not extensively validated, as they were established methods. Due to the role of TIMP-4, which is

relatively specific for cardiac tissue, I wished to establish an assay for this inhibitor.

At the outset, an ELISA format for tTIMP-4 was investigated. However, this proved impossible, despite months of work. After iodination of the captured antibodies, post sephadex separation did not demonstrate two clear peaks - in fact, all that was present were small irregularly and asymmetrically shaped peaks. Clearly, the Chemicon antibodies were inactive. The next step was validation of an RIA assay for tTIMP-4.

The antibody for TIMP-4 was raised against the last seven C terminal amino acids of the TIMP-4 molecule and as such, measured total TIMP-4. The assay appeared satisfactory in that there was good precision, accuracy and no interference with TIMP-1 and TIMP-2. The reference range for TIMP-4 had a mean of 20 ng/ml with a 95% confidence interval of 10-30 ng/ml. This assay was used to measure TIMP-4 levels in the Submariner Study.

6.1.1 Validation of 25hydroxyvitamin D assay.

NIST have now established a standard for 25(OH)vitD and the team are participating in evaluating a 25(OH)vitD free serum. The existing methods for the measurement of 25(OH)vitD are associated with a liturgy of analytical problems.

Most methods utilise an antibody reaction with a pre-extraction step. The immunoassays rarely have equal affinity with both 25(OH)vitD2 and D3. This is a major source of concern, particularly in America where vitamin D2 is a common source of vitamin D supplementation. There are two immunoassay methods but each is labour intensive and lacks robustness. But recently automated immunoassay methods have been marketed by several manufacturers though it is always a case of Caveat Emptor ('buyer beware') and it is essential to fully validate these new methods The ELISA assay correlates very poorly at 25(OH)vitD greater than 100nmol/L with the LCMSMS assay. The method of choice is LCMSMS or HPLC, the latter being more likely to be subject to interference than the LCMSMS method. This in-house LCMSMS assay for 25(OH)vitD is analytically simple and has good precision and accuracy. In addition to 25(OH)vitD3, it also measures 25(OH)vitD2.

In comparison to the young, the skin in the elderly population is less efficient at producing 25(OH)vitD, (Mosekilde, 2005). In general, the elderly spend more time indoors and their diet can be inadequate. All these factors would suggest that the levels of 25(OH)vitD in the senior population would be lower than that for the younger age group. However, in our GP population group, the levels of 25(OH)vitD increased with age, raising the question, "Are 25(OH)vitD levels an indicator of longevity and if so what might be the mechanism?".

Two possible explanations spring to mind. Firstly, those with high 25(OH)vitD levels are unlikely to succumb to major fractures including fracture of the femur,

which in the elderly is associated with a significant increase in mortality and morbidity. Secondly, the other major reason is that the high levels of 25(OH)vitD suppress the anti-inflammatory response, resulting in a reduction in cardiovascular disease and possibly malignancy, the major causes of mortality (excluding Alzheimer's disease) in developed countries (Verstuyf et al., 2010)

6.2.0 Correlation between TIMP-1 acute phase proteins, diabetic-induced retinopathy, and essential hypertension.

One of the first questions to be answered was:

"Is TIMP-1 an acute phase protein?"

There is little data regarding the half-life of TIMP-1, so TIMP-1 was correlated with a variety of proteins, including those with short half-lives - prealbumin and CRP and those with longer half-lives, albumin and orosomucoid. There was no correlation between TIMP-1 and any of the proteins studied but there was a correlation between CRP and prealbumin and orosomucoid and albumin, proving internal consistency between the known acute phase proteins. TIMP-1 is not an acute phase protein. Ideally, in this study, relationships between specific proteins and other acute illnesses, for example, infection, should have been investigated.

Studies on plasma levels of the MMPs and TIMPs have until recently concentrated on liver disease and rheumatoid arthritis. Utilising a similar logic as to why increased hepatic fibrosis could, in part, be explained by enhanced

collagen deposition due to upregulation of TIMP-1 levels, triggered the question, “Could a similar mechanism exist in vascular and cardiac tissue?”

The patients in this study were divided into those who were naïve, in terms of hypertension therapy, or who had been off treatment for one month prior to the study. The plasma levels of TIMP-1 were significantly increased in patients with essential hypertension in comparison to normotensive controls, irrespective of whether they were naïve or had previously been treated for hypertension. Possible explanations are

1) In the early stages of essential hypertension, the vessels stretch and as part of the repair mechanism, TIMP-1 is released, resulting in increased vascular collagen deposition and reduction in elasticity.

2) In those patients who have never been treated for essential hypertension, there was an association between aldosterone and TIMP-1 and markers of left ventricular size, suggesting renin- driven hypertension could be associated with an increase in TIMP-1 (possibly even prior to a measurable increase in blood pressure). This observation of an elevation in TIMP-1 may explain the down-regulation of MMP activity in the heart of spontaneously hypertensive rats and in part, the mechanism for the angiotensin II (ang II) and aldosterone induced cardiac fibrosis associated with essential hypertension (Brilla et al.,1990). Essential hypertension and LVH are associated with an increase in collagen deposition in the heart and blood vessels and the elevation of TIMP-1 suggests that, at least in part, this collagen deposition is due to a defective degradation of collagen.

The elevation in TIMP-1 and its subsequent fall in rat endothelial tissue after treatment with an ACEI, suggests a mechanism whereby this class of drugs could modulate collagen levels.

In a group of patients on ACEI, TIMP-1 and aldosterone were measured. There was an association between TIMP-1 and aldosterone, which was used as a surrogate for the RAAS. This observation is similar to that found in the rat, in that aldosterone modulates TIMP-1 and suppression with ACEI is associated with an attenuation of TIMP-1 levels. In my recent work on samples, from Scragg in New Zealand there is a positive correlation between plasma aldosterone and TIMP-1, yet again demonstrating a causal relationship between TIMP-1 and aldosterone.

Not all workers have shown a similar relationship between aldosterone and TIMP-1. Data from Yan et al.,(2008) in patients with heart failure, did not show a correlation between aldosterone and TIMP-1 but interestingly showed a correlation between levels of MMP9 and TIMP-2 with brain naturetic peptide. This finding is unusual in that in the literature and in my experience, TIMP-1 is bound to MMP9 but not to TIMP-2, which is constitutively expressed. However, the results of Yan et al., (2008) may reflect this patient group, all of whom had heart failure.

The RAAS system may be important in modulating TIMP-1 levels in other tissues. Work by Ren and colleagues (2011) has shown, in cultured hepatic stellate cells,

that the aldosterone upregulation of TIMP-1 and MMP2 RNA levels is suppressed by Spironolactone. There is now a significant body of data from tissue and clinical work, showing that the stellate cell, when activated, can produce the components of the RAAS system.

Similarly the RAAS is important is wound healing. Experimental wounds in rats are associated with an increase in ang-II receptors and local production of the RAAS is important in a variety of tissues brain, gut and the eye. (Paul et al., 2006).

One of the most important criticisms of the hypertensive study is the number of subjects involved. Fortunately, there was a very significant difference between the medians of TIMP-1 in the control subjects and the hypertensive patients. Recruiting a large numbers of naïve patients with essential hypertension in one centre is very difficult, as most patients are prescribed an antihypertensive drug by the GP. In the ACEI experiment, levels of TIMP-1 pre treatment would have been a useful adjunct for comparison.

6.3.0 Discussion on Chapter 5

One of the most novel observations arising from my studies in Bangladeshis was the dramatic fall in plasma TIMP-1 and MMP9, in Bangladeshi subjects supplemented with 25(OH)vitD. Additionally, vitamin D supplementation was associated with a decline in sensitive CRP (sCRP).

Another interesting finding of the Bangladeshi study was that levels of TIMP-1 correlated with systolic hypertension ($p<0.007$). There are now two studies from two differing populations suggesting that TIMP-1 may be involved in essential hypertension. There was no correlation with TIMP-1 in the essential hypertension study with either systolic or diastolic blood pressure but that may merely reflect lack of power. There was simply, as a group, a highly significant difference in the medians between the TIMP-1 of the control versus the group with essential hypertension. The association of TIMP-1 with systolic hypertension in the Bangladeshis is counter-intuitive because theoretically TIMP-1 should be related to diastolic blood pressure. A colleague has shown that in patients with Type 1 diabetes (without evidence of essential hypertension) plasma TIMP-1 levels were raised and that these levels approached but did not reach statistical significance (probably due to the small numbers in the study) when correlated with blood pressure (Maxwell et al., 2001).

Both the Bangladeshi and Submariner studies demonstrated a reduction in TIMP-1, with increasing 25(OH)vitD. Vitamin D status may be an important factor in hypertension.

The question arises as to the appropriateness of extrapolating a “generic” 25(OH)vitD range for other ethnic groups. Currently, no work has been published in this field, although Scragg and his team are studying this topic (as yet

unpublished). By default, the range remains 75-150nmol/L, irrespective of ethnicity.

An animal model demonstrates the importance of vitamin D in hypertension. Li et al., (2002) showed, in wild type mice, that suppression of 1,25 dihydroxyvitamin D levels were associated with an increase in renin levels but injection of 1,25 dihydroxyvitamin D caused suppression in renin concentration. Using VDR knockout mice, the upregulation of the RAAS system can be attenuated by administration of Captopril, which reduced cardiac hypertrophy. Anti renin antibody staining was associated with increased renin synthesis in the left ventricle of the VDR knock out mice (Xiang et al., 2005).

Wang et al., (2008) subdivided his cohort from the Framingham Offspring study into those whose 25(OH)D was less than 15ng/ml and those whose 25(OH)vitD was less than 10ng/ml. The risk of developing cardiovascular disease increased as the level of 25(OH)D fell. Clinically, Forman et al., (2007) showed that increasing 25(OH)vitD levels were associated with a fall in blood pressure, another major factor associated with cardiovascular disease.

The other major observation in the Bangladeshi study, was in the cross sectional study, where plasma TIMP-1 levels were significantly related to VDR polymorphisms Taq1 (tt) (Timms et al., 2002). In Martineau's et al., (2011) study, patients with TB were treated in the intervention arm with conventional treatment and vitamin D supplementation with the control group of TB patients being treated

with the conventional régime. The primary endpoint of the study was the time required to produce TB negative sputum. The patients on vitamin D supplementation did not achieve TB sputum negativity faster than the control group but when compared with those who had the VDR polymorphism Taq1, those with the tt genotyope achieved a negative culture faster than the TT genotype.

“Two swallows do not a summer make”, but as vitamin D deficiency has been implicated in an increasing number of pathologies it may well be that associations could be related to particular VDR polymorphisms in an attempt to increase clinical significance between 25(OH)vitD levels and disease state.

In the Bangladeshi study there was a marked fall in MMP9 levels in both the cross sectional study and the intervention arm where the subjects were given vitamin D supplementation. sCRP is an accepted marker for cardiovascular disease and the decrease in both sCRP and MMP9 might reflect a reduction in the anti-inflammatory response of the vascular tree, in addition to other tissues..

This inverse relationship between 25(OH)vitD and MMP9, may only be applicable to Asians.. It was important to investigate this association further, by studying this important relationship of 25(OH)vitD and MMP9 in other populations. To address this question, The Gosport Naval Hospital was contacted. This call resulted in the Submariner Study, which most importantly showed, like the Bangladeshi work, an

inverse correlation between 25(OH)vitD and MMP9. The Submariner Study was conducted on a group who were male and (with the exception of one submariner, Caucasian). This inverse relationship between 25(OH)vitD and MMP9 may now be a general observation that is not dependent on ethnicity.

Another important observation in the Submariner Study was that the levels of 25(OH)vitD in submariners not taking vitamin D supplements and those consuming vitamin D supplements (400 IU) daily were not significantly different. The conclusion is inevitable. In order to correct vitamin D deficiency, oral supplements greater than 400 IU daily must be consumed. Daily supplements of 1000 -2000 IU are now recommended for vitamin D supplementation by more conservative workers (Holick, 2007).

In the last study, I wished to determine whether the plasma level of MMP9 could predict the likelihood of a patient developing re-stenosis post angioplasty. The hypothesis was that upregulation of MMP9s would increase smooth muscle cell migration across the artery, enhancing collagen deposition and that in the re-stenosed group, there would be an inverse relationship between 25(OH)vitD and MMP9.

There was a significant difference in plasma MMP9 levels between those who did and those who did not re-stenose ($p<0.001$) but the differences in 25(OH)vitD between the groups were not significant. That MMP9 pre angioplasty was an

excellent marker for patients who did not re-stenose, may be useful in these patients, as it offers the opportunity to provide treatment with less sophisticated and less expensive stents. However, the numbers in the re-stenosed group were small. A much larger study is required.

There was no significant inverse relationship between 25(OH)vitD and MMP9 in those who re-stenosed, possibly due to a lack of power. However, in all patients undergoing angioplasty, there was an inverse relationship between 25(OH)vitD and MMP9. That there was an inverse relationship in this group is surprising, as 80% these patients were treated with a statin, which is known to reduce MMP9 levels in blood. I was unable to demonstrate a difference in plasma MMP9 for those treated and not treated with a statin. This may be due to the fact that the concentration of 25(OH)vit D has a greater effect on plasma MMP9 levels than statins.

Wasse et al., (2011) recently demonstrated, in patients undergoing dialysis, that there was an inverse relationship between MMP9 and 25(OH)vitD. In both the Wasse's group and that of Timms et al (2002) roughly 8% of the patients did not demonstrate any fall in MMP9 levels as the concentration of 25(OH)vitD increased. However, the explanation for this observation is unclear. But it is reassuring that there is another study documenting lack of MMP responsiveness to vitamin D in a minority of patients.

Coussens et al., (2008) showed, in TB infected moncytes, the addition of 1,25 dihydroxy vitamin D3 increased levels of IL10. This elevated IL10 may attenuate the inflammatory response by several mechanisms but Smallie et al., (2010) suggested that vitamin D induction of IL10 may indirectly affect transcription of inflammatory cytokines.

There is now a portfolio of evidence demonstrating that there is an inverse association of MMP9 and 25(OH)vitD. On the basis of the intervention arm of the Bangladeshi Study, it can be stated that 25(OH)vitD levels suppress MMP9 concentrations.

The one common feature of the analytes measured in this thesis, vitamin D, RAAS and TIMP-1, is their involvement in the early stages of evolution. This issue is important to address, as it may explain the role of these systems in diseases of middle and old age.

Hollick, (2003) described the presence of VDR receptors in plankton. The role of this receptor will result in an increase in vitamin D responsive gene expression in this unicellular organism. Hollick, (1972) described the formation of the 5, 6 trans vitamin D in lizards, rather than the classic 5, 6 cis vitamin D. In comparison to the cis form, the trans isomer is rotated around the A ring, bringing the hydroxyl group into the one position, which makes the compound act as an active vitamin D

metabolite. When administered to rats without kidneys, trans vitamin D increases calcium reabsorption. The further implication from this paper suggests that vitamin D “activity” may differ depending on whether it was derived from ultraviolet light or an oral supplementation.

The changes in skin pigment between dark and fair skin may also be an important evolutionary factor, so as to maximise the cutaneous production of vitamin D.

One of the factors in ensuring survival of multicell organism is the ability to control the excretion of water. In uni and multi cellular organisms, control of intracellular fluid was achieved via the process of osmotic pressure. This primitive system lacked flexibility and so these organisms generally lived in an environment of relatively constant osmolality.

During the evolutionary process, as biological structures become more complex, controlling water loss became more important and by necessity, more complex. The interaction of the RAAS system and ADH controlled water loss and permitted survival in the tropics. The other major function of the RAAS was as a repair mechanism, thus ensuring survival. When attacked and injured, two factors dictate survival. Firstly, maintenance of intravascular volume and secondly, repair of the wound. Both of these factors are functions of the RAAS which in turn increases TIMP-1 levels thereby increasing collagen deposition and further enhancing wound healing. Interestingly, the non inhibitory actions of TIMP may also be

important in wound healing, as TIMP has been implicated in increasing the growth of keratinocytes (Bertaux et al.,1991).

This is all well and good if you die at the likely survival age of 25-30, typical, one suspects, of Stone Age Man. What happens when you are 60, or older? The ability to generate vitamin D cutaneously from sunlight is reduced, increasing inflammation, the RAAS system is upregulated increasing blood pressure and reducing vascular elasticity mediated by an increase in TIMP-1 levels.
The above scenarios result in a marked level of mortality and morbidity which may be reduced by modulating levels of vitamin D, the RAAS and TIMP-1

A further role of vitamin D may be its association with longevity. Our data surprisingly showed an increase in 25(OH)vitD in advancing age. In addition to vitamin D action in increasing gene expression recent work An et al., (2010) suggested that the interaction between the VDR, sirtuin and FOXO modulates deacetylation of nuclear proteins, causing suppression of MMP9 and other genes. Sirtuin also suppresses AT1 receptor mRNA expression, whereas an inhibitor of sirtuin upregulated AT1 receptor expression (Miyazaki et al., 2008). This work also potentially describes a role for sirtuin in modulating the cardiotoxicity of ang II. An inter-relationship between vitamin D and the RAAS has been previously described: it would be interesting to speculate on the role of sirtuin and FOXO in this morbid fibrotic process.

6.4.0 Summary of this work where there is data from at least two studies

1. Plasma TIMP-1 is related to blood pressure.

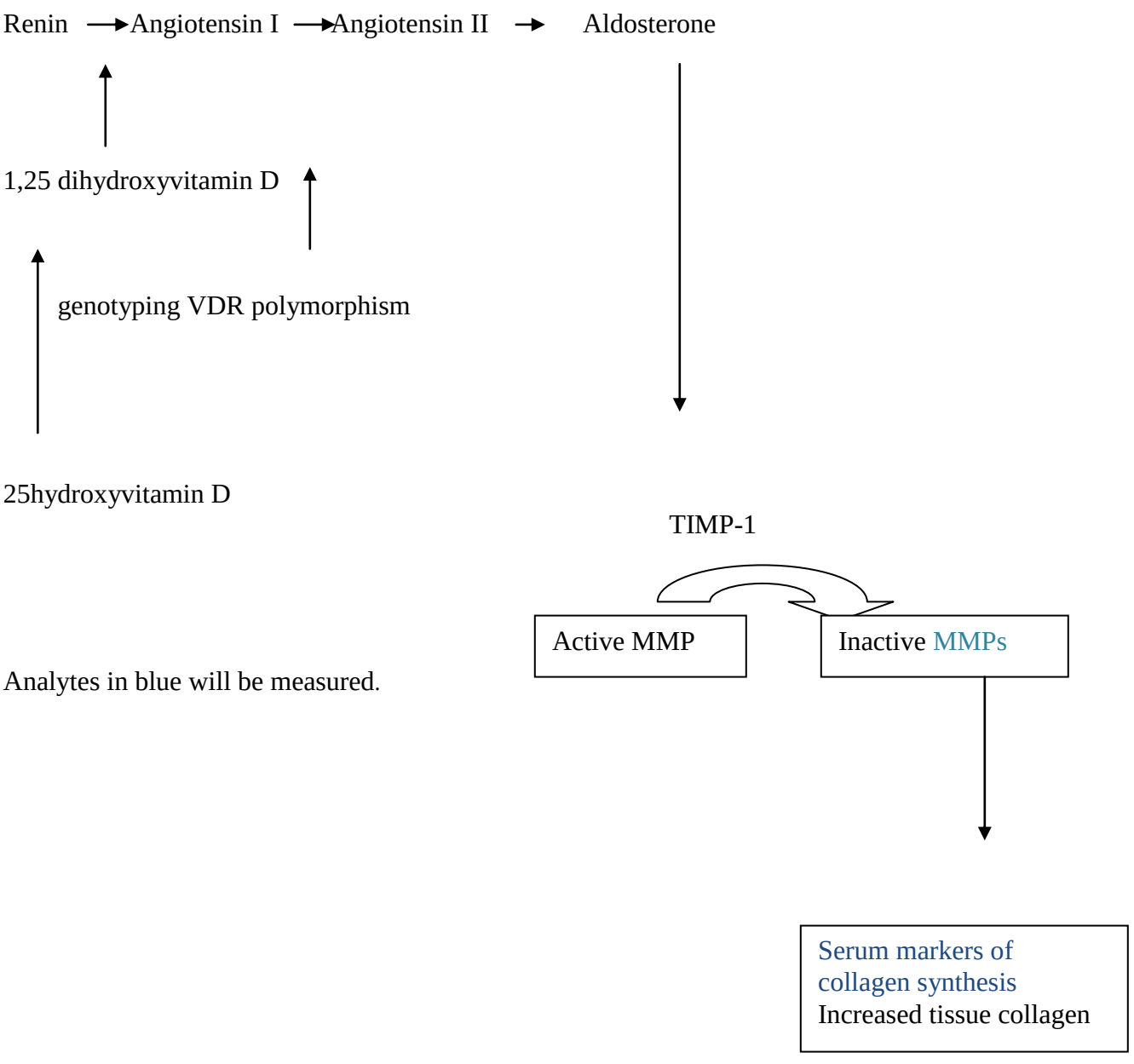
2. Plasma MMP9 and TIMP-1 levels are reduced by 25(OH)vitD.

6.4.1 Relationship between the aims of the thesis and the experimental observations.

In my thesis, in two different studies, there is an negative association between 25(OH)vitD and TIMP-1 and MMP9. The fall in MMP9 is greater than that of TIMP-1, resulting theoretically in a tendency to enhance collagen deposition as the 25(OH)vit D increases. However, other factors are involved. Downregulation of the RAAS is associated with a reduction in TIMP-1. Alas, TIMP-4 had no effect on 25(OH)vitD

6.5.0 Further developments on this thesis

From the observations in this thesis a further hypothesis involving the understanding of the following compounds, which are shown in the diagram below, in control patients and those with hypertension and heart disease. can be developed.



Analytes in blue will be measured.

The hypotheses for such a study are:

1. Are levels of TIMP-1 and MMP9 predicated on VDR polymorphism?

2. Is pulse pressure responsive to an increase in vitamin D levels and a reduction in plasma TIMP-1 levels?

3. Does angiotensin or aldosterone drive TIMP-1 expression: if so what are the factors involved in intracellular signalling?

4. About 40% of patients with 25(OH)vit D less than 20 nmol/L have a normal serum PTH. Do these patients have a reduced risk of cardiovascular disease, in comparison to patients with an elevated PTH and 25(OH)vitD less than 20 nmol/L?

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Campbell S, Timms PM, Maxwell P, Docherty E, Danesh BJ. Effect of alcohol withdrawal on hepatic transamine levels and markers of fibrosis. J Gastrol Hepatol. 2001;16:1254-9

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Chapter 7

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Appendix: Source of reagents used in this thesis

Assay	Reagent	Source
ELISA and RIA for TIMP-4	Triton X-100 :	Sigma Aldrich - T8787
	Phosphate Buffer :	Sigma Aldrich – P3619
	NaCl:	Sigma Aldrich –S3014
	Bronidix L :	Henkel & Cie Gmbh Henkelstrasse 67 Dusseldorf DE
	BSA:	MPBio -11BSASG100
	TIMP-4 standard	R@D 974-TSF-010
	Chloramine T	Sigma Aldrich - 31224
	sodium metabisulphite Sigma Aldrich - 31448	Sigma Aldrich - 31448
	KI	Sigma Aldrich – P9541
	^{125}I	Amersham Pharmacia IMS-30
	Mannitol	M1425
Western Blot TIMP-4	Laemmli buffer	(BioRad #161-0737
	Beta-mercaptoethanol	BioRad #161-0710
	Molecular weight markers	BioRad #161-0324
	Molecular weight markers	BioRad #161-0324
	ECL	Amersham Pharmacia RPN 2109
	TIMP-1	Amersham Pharmacia RPN 2611
	MMP9	Amersham Pharmacia RPN 2614
	MMP9	R and D DMP 900
25hydroxy vitamin D	Analytical UPLC column- Waters Acquity UPLC BEH C18, 1.7um, 2.1 x 50mm	BEH - catalogue number 186002350
	· Stainless steel in line filter	catalogue number 205000343
	Frits for in line filter	catalogue number 700002775
	Methanol Hipersolve grade	VWR catalogue number 152507P
	Ammonium Acetate	Sigma Aldrich catalogue number 09689
	Formic Acid	Sigma Aldrich catalogue number 33015
	Deuterated 25 Hydroxyvitamin D	www.Synthetica.no
	25 Hydroxyvitamin D2 & D3 calibrator	catalogue number 38033
	Chromsystems 25-OH-Vitamin D3 bi-level control	catalogue number 0028