Studies of Biochemical markers of cardiac Fibrosis: The role of Tissue Inhibitors of Matrix Metalloproteinases.

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Declaration.

I declare that the work has been done and the thesis composed by myself, and that the books and papers cited were all consulted by me personally.

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Dr M Mitchell Lindsay.

Presentations and Publications.

Presentations to the American Heart Association.

MM Lindsay, P Timms, FG Dunn.

Biochemical markers of tissue fibrosis predict diastolic dysfunction in patients with hypertension. Presented at the **71st Scientific Sessions of the American Heart Association.** Dallas. November 1998. **Circulation** 1998;Vol98: Suppl I A2338.

Presentations to the American College of Cardiology.

MM Lindsay, P Timms, V Srikanthan, P Maxwell, F G Dunn.

Functional Implications of Elevated Collagen Markers In Untreated Hypertension. Presented at **The 49th Annual Scientific Session of the American College of Cardiology**. Anaheim USA March 2000. **JACC** 2000;vol35,no 2(suppl A) 322A

Presentations to the European Society of Cardiology.

MM Lindsay, P Timms, FG Dunn

Tissue inhibitors of Matrix Metalloproteinases. A serum marker for diastolic dysfunction in essential hypertension.

Presented at the **XXth Congress of the European Society of Cardiology.** Vienna. Septmeber 1998. **Eur Heart J** 1998. Abstract Supplement A1958.

MM Lindsay, P Timms, P Maxwell, FG Dunn.

Biochemical evidence of fibrosis in exercise induced left ventricular hypertrophy.

Presented at the **XXII Congress of the European Society of Cardiology.**

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Publications.

MM Lindsay, P Maxwell, FG Dunn.

Plasma Tissue Inhibitor of Matrix Metalloproteinase type I (TIMP-1)- a Marker of Left Ventricular Diastolic Dysfunction and Fibrosis in Hypertension.***Hypertension.***2002;40:136-141.

Chapter 1

General Introduction.

1.1 Left Ventricular Hypertrophy.

1.1.1 Definition.

Left ventricular hypertrophy is an increase in left ventricular mass in response to stimuli such as hypertension, exercise and high output cardiac states. As this process is an extension of normal growth patterns and initially a protective mechanism an arbitrary cut-off is required. This is best defined by the study of a group of normal volunteers and using the 95th centile as the cut-off. Numerous numerical definitions exist; the Framingham group studied 846 normal volunteers using echocardiographic measurements and found the cut-off to be 131g/m2 in men and 100g/m2 in women. (Levy 1987)

1.1.2 Prevalence.

The prevalence of LVH depends on the cohort studied, the method used and the age and sex of the cohort. The Framingham study demonstrated a prevalence of 2.1% using ECG criteria and 16% using echo criteria in the general population. Additionally the Framingham group demonstrated a progressive increase in prevalence with age, which was independent of confounding variables. (Levy 1990)

Echocardiography identified 7-10 times more cases of increased cardiac mass than ECG. (Savage 1979)

1.1.3 Incidence.

The incidence of LVH is higher than the data for prevalence would suggest due to the high mortality rate of the condition.

Using ECG-LVH the Framingham group demonstrated a close relationship between systolic BP and development of LVH. 50% of patients with a systolic BP of over 180mmHg developed ECG-LVH in the twelve year follow-up of the study. (Kannel 1996)

Using Echo determined LVH the same group demonstrated that a 20mmHg increase in the systolic blood pressure resulted in a 43% increase in men and a 23% increase in women in the development of LVH. Age, myocardial infarction and obesity were also important independent predictors. (Levy 1988)

1.1.4 Diagnosis of LVH.

The diagnosis of left ventricular hypertrophy clinically is difficult and inaccurate. Therefore from any early stage additional means were employed.

Chest radiography.

Chest x-rays have been used to detect LVH however this an inaccurate means of detection that requires left ventricular dilatation to be present. Therefore this technique has been largely abandoned.

Electrocardiogram.

It was appreciated soon after the advent of electrocardiography that the presence of left ventricular hypertrophy resulted in characteristic ECG changes (Einthoven 1906). Specific criteria were first described in the 1940's by a variety of investigators (Sach 1950). In 1949 Sokolow and Lyon published their widely cited paper whose findings were based on the review of ECGs from hypertensive patients thought to have LVH in comparison to normotensive subjects (Sokolow 1949). They reported that the characteristic findings were;

1. A low or biphasic T wave in association with depression of the ST-T segment in leads V5 and V6. In later stages this resulted in inversion of the T wave in the above leads.
2. An increase in the precordial voltages.

They reported that LVH could be detected by abnormalities in the QRS complex in which the R wave in V 5 or V6 exceeded 26mm and/or the sum of the R wave in V5 and the S wave in V1 exceeded 35mm.

Numerous other systems have been described and mostly are a variation on the theme of the above system. Most notably by Romhilt-Estes (Romhilt 1968) and the Cornell voltage (Casale 1985) criteria.

These methods have subsequently been examined using more accurate techniques of assessing the presence of LVH namely echocardiography. This has revealed a high specificity (as high as 100% in some studies) however a low sensitivity (as low as 20%) for almost all ECG criteria.

Echocardiography.

M-mode echocardiography can provide direct measurements of left ventricular chamber size and wall thickness. The first methods using M-mode echocardiography were validated using quantitative angiography (Troy 1972) or assumed geometric models of the left ventricle (Bennet 1975).

Broadly speaking all early echocardiographic techniques were based on the premise that the volume of the myocardium was equal to the total volume contained within the epicardial borders minus the chamber volume. The muscle volume was then converted to mass by multiplying the specific gravity of the cardiac muscle.

These techniques were further advanced by Reichek and Devereux (1977) who applied multiple geometric models and demonstrated the accuracy of these techniques

by comparison with post-mortem measured left ventricular mass. It should be accepted, however, that all these models work best in subjects with normal left ventricular geometry.

Following the advent of 2D echocardiography there was further interest in developing new geometric models. The most accurate model appeared to be Simpson's rule method. In this method the myocardium represented a series of thick-walled, hollow cylinders. The volume of each cylinder is then determined by planimetry of muscle area and section height. The myocardial area of each cylinder is determined by direct measurement of the interval between multiple short axis cross-sections. This method was accurate in vitro but could not be reproduced in vivo (Helak 1981). A simplified version of Simpson's rule was devised which used a single short axis section at papillary muscle level for determination of myocardial area and minor cavity area and uses apical views for determination of left ventricular epicardial and endocardial length. This technique was found to be much more reproducible and Reichek and colleagues demonstrated a high correlation between calculated LV mass and actual weight at post mortem (r=0.93) (Reichek 1983).

Computerised Tomography and Magnetic resonance Imaging.

MRI is probably the most accurate non-invasive method for the assessment of LVH. There are, however, no major studies linking MRI defined left ventricular mass with any cardiovascular outcomes. Limited availability remains this method's major limitation

1.1.5 The Importance of LVH.

Sir Thomas Lewis described graphically in his classic monograph the mode of death in patients with hypertension (Lewis 1940). It was not, as might have been expected, from myocardial infarction or cerebrovascular accident , but from progressive heart

failure consequent upon the unrelenting development of LVH with no effective method of off-loading the ventricle . It is therefore not surprising, if underemphasised, that all major antihypertensive trials have shown a substantial reduction in death from hypertensive heart failure. However despite this reduction in hypertensive heart failure, LVH still confers an adverse risk for coronary artery disease and sudden cardiac death and thus, still constitutes a major cardiovascular risk factor.

Much of the information on LVH as a risk factor comes from the Framingham Study, which was set up in the late 1940s to follow up a large cohort of healthy adults and to identify factors of risk for subsequent cardiovascular morbidity and mortality (Kannel 1970). The authors used the ECG as a means of identifying LVH. In those early years this proved to be an effective screening investigation. The study showed that the presence of definite LVH (defined as ECG voltage criteria plus ST-T change) doubled the risk of cardiovascular disease in comparison to hypertension alone. This increased risk could not be explained on the basis of age, sex, level of blood pressure or other associated risk factors. The importance of LVH as a risk factor for coronary artery disease is even more striking, with a two to three fold increase in the number of myocardial infarctions where LVH is present on the ECG. Sudden death is increased by a factor of five. LVH is an independent risk factor for sudden cardiac death in men independent of the presence of coronary disease, although a less clear relationship was seen in women (Kregger 1987).

The Framingham findings were confirmed in a large study from Glasgow, which assessed the implications of ECG LVH (Dunn 1990). This study showed an increased risk, associated with definite LVH but, in addition, voltage LVH (known in the Framingham study as possible LVH) was shown to carry an increased risk, which

could not be explained on the basis of BP alone. The risk was greater in men at all levels of BP, and was considerably magnified by smoking.

1.2 Pathology of Left Ventricular Hypertrophy.

Left ventricular hypertrophy occurs initially as an adaptive process. When the heart is exposed to a haemodynamic burden it can compensate by one of three mechanisms.

1. Use the Frank-Starling equation to increase crossbridge formation.
2. Augment muscle mass to cope with the extra load.
3. Recruit neurohormonal mechanisms to increase contractility.

The first of these mechanisms is limited and the third is detrimental due to the adverse effects of chronic stimulation of the sympathetic nervous system.

Therefore a key compensatory mechanism is increasing muscle mass. The increase in muscle mass is caused by myocyte hypertrophy and alteration in the extracellular matrix. These mechanisms will be discussed in depth later.

The basis of hypertrophy can be explained by the law of Laplace, which defined the load on any region of the myocardium as a function of (pressure x radius)/ (2 x wall thickness).

Therefore it can be seen that any increase in pressure can initially be offset by an increase in wall thickness. The systolic stress is a major determinant of systolic ejection performance thus by normalising the systolic stress via hypertrophy this maintains normal systolic function. Thus LVH is initially an adaptive process and if this were to be perfectly regulated then a feedback loop would exist ensuring wall stress was constantly normalised however this does not appear to exist and chronic hypertrophy is undoubtedly deleterious.

Pathogenesis.

The pathogenesis of left ventricular hypertrophy can be conveniently divided into myocyte hypertrophy and alterations in the extracellular matrix.

1:2:1 Myocyte Hypertrophy.

Cardiac myocytes account for 80% of tissue volume in the adult heart but only account for 35% of the cell number. In left ventricular hypertrophy the cardiac myocytes increase in length, width and volume.

During "compensated hypertrophy" the myocyte increases in volume however the length width ratio remains constant (Zierhut 1991). However during decompensation and progression to pump failure there is a dramatic increase in length width ratio and prolongation of the myocyte with loss of transverse growth (Gerdes 1996). It is unclear the exact signalling mechanisms that are involved in regulating myocyte growth. It would appear that mechanical signals can be transmitted through collagenous struts to the myocyte (Bloom 1996). However cardiac hypertrophy differs greatly depending on pathogenesis e.g. pressure versus volume overload. With myosin heavy chain synthesis being upregulated early in pressure overload (Imamura T, 1994) whilst in volume overload myosin chain synthesis is decreased (Matsuo T ,1998).

In pressure overload the myocytes hypertrophy results from the parallel addition of sarcomeres. Whilst in volume overload myocyte lengthening occurs by the addition of sarcomeres in series.

Both forms of hypertrophy are accompanied by changes in gene reprogramming (Swynghedauw, 1999).

These include the re-expression of immature foetal cardiac genes including;

1.Genes that modify motor unit composition and regulation.

2.Genes that modify energy metabolism.

3.Genes that encode components of neurohormonal pathways.

The implication from these findings is that mechanical force is transduced into a biochemical event that modifies gene transcription. A candidate for this transducer is the focal adhesion complex.

1.3 The Extracellular Matrix.

Components of the extracellular matrix.

1.3.1 Cardiac Collagen.

Currently eighteen distinct collagen types have been identified. These are coded at 30 separate genes. The relative abundance of the different collagens varies from tissue to tissue. The major interstitial collagens are type I, III and IV, which constitute over 90% of, total cardiac collagen content. The interstitial collagens consist of three polypeptide alpha chains composed of large helical domains. Each chain has a high hydroxyproline concentration that facilitates measurement of hydroxyproline as measure of collagen concentration. The precursor of both types I and III collagen contains three separate domains. Firstly the helical domain described above, secondly an N pro-collagen peptide domain and thirdly a globular C terminal region. On incorporation of the procollagen into the established collagen helix both the N terminal and C terminal are cleaved.

Type I and III collagens have very different structures and functional significance. Type I collagen forms rod like fibres providing tensile strength. Whilst, type III collagen forms a fine network of fibrils. The relative amounts of collagen type I and III vary with development and in the presence of pathological states.

The other collagens present in the cardiovascular system represent a small proportion. Type IV and V are basement membrane proteins whilst collagen type VI is found coating collagen fibres in the interstitium.

Table 1.1 Cardiac Collagens

Type	Composition	Location	Origin	Major function
I		Interstitium, vessels	F,S,E	Structural
III		Interstitium Vessels	F,S,E	Structural
IV		Basement Membrane	F,S,E,M	cell attachment Substrate filter
V		Interstitium Vessels	F,S,E	Structural Cell-Cell signalling
VI		Interstitium Vessels	F,S	Structural

F= fibroblast s=smooth muscle cell, E= endothelial cell, M =myocyte

1.3.2 **The collagen network.**

The normal collagen network consists of collagen type I and III. Types IV, V and VI are present but in far lesser amounts. The cardiac collagen network consists of an epimysium which surrounds large groups of muscle fibres. This is found beneath the endothelial cell lining of the endocardium. From the epimysium arises a perimysium consisting of coiled fibres and weave like structures that surround smaller groups of muscle fibres grouping them into parallel bundles. The perimysium forms a net within

which the muscle fibres are organised which limits cardiac stretch. Finally an endomysium tethers individual muscle fibres together and connects adjacent microvasculature. Individual fibres of the endomysium are connected to myocyte cytoskeletal proteins, which allow transmission of myocyte generated force. Fibrillar collagen preserves muscle fibre orientation and is responsible for maintenance of the cardiac architecture additionally preventing muscle slippage.

The extracellular matrix thus acts as scaffolding for the cardiac myocytes. The myocytes are arranged in series and parallel with elastic elements which are the fibrillar collagens.

Collagen turnover.

1.3.3 Collagen synthesis.

Collagen synthesis is a complex process involving several post transcriptional and post-translational steps. Pro-collagen genes code for the pro-alpha 1 and pro-alpha 2 chains. The pro-alpha chains are then incorporated into triple helical pro-collagen. Pro-hydoxylase then catalyses the hydroxylation of proline thus stabilising the helix. Disulphide bonds form linking the pro-alpha chains to the C terminal. Fibre formation essentially then forms a self-assembly process.

1.3.4 Collagen degradation.

Two main pathways of collagen degradation exist; intracellular degradation and extracellular degradation.

Intracellular degradation occurs in the lysosomes and functions to prevent the secretion of defective molecules and regulates production in response to extracellular stimuli.

Extracellular degradation is largely regulated by a family of zinc and calcium dependent endopeptidases called Matrix Metalloproteinases (MMPs). These

endoproteinases share structural domains but differ in key features; namely, substrate specificity, cellular sources and inducibility. Currently more than twenty mammalian types have been identified. All MMPs share basic features.

1. They degrade extracellular matrix components.
2. They are secreted in a latent form and therefore require activation.
3. They contain an active Zn2+ site.
4. They require calcium for molecular stability.
5. They function at a neutral pH.
6. They are inhibited by a specific group of inhibitors called Tissue Inhibitors of Matrix Metalloproteinase.

The family of MMPs have been classified in several ways. The most helpful method is using substrate specificity and molecular structure. Using this method they can be conveniently be divided into four groups.

1. The Collagenases. This includes MMP1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3). This group can cleave fibrillar collagens (types I, II and III). The fibrillar collagens represent a particularly resistant group due to their apposed and cross linked fibrils and as such are resistant to almost all other proteases. Collagenases cleave fibrillar collagen at a unique site in the triple helix ¾ from the N terminal resulting in ¾ and ¼ collagen fragments. This results in thermal degradation and loss of stability with the fragments losing their characteristic and stable triple helix and forming single chain molecules called gelatins.
2. The Gelatinases. The two main gelatinises are MMP-2 and MMP-9 which are capable of degrading gelatins. In addition gelatinises are capable of degrading basement membrane collagen type IV.

3. The Stromeolysins. This includes MMP-3, MMP-10, and MMP-11. They have proteolytic actions against a broad range of extracellular matrix components including proteoglycans, laminins, fibronectin, vitronectin and some types of collagens. This proteolytic system degrades a wide spectrum of extracellular matrix proteins and is constitutively expressed in a number of cell and tissue types.
4. Membrane bound MMP (MT-MMP). This group has been recently described and constitute a novel class for four main reasons. Firstly, they are membrane bound and hence constitute a focal area for proteolytic degradation. Secondly, MT-MMPs are proteolytically active when inserted into the membrane and do not require further activation. (Murphy 1997, Miyamori 2000) Thirdly, they perform a key role in activation of other non membrane bound MMPs (Murphy, 1997) and finally TIMPs fail to bind effectively to MT-MMP and thus are unable to exert inhibitory control. (Miyamori, 2000)

MMPs play an essential role in normal tissue remodelling however are also expressed and appear to play an important role in numerous pathological processes, for example plaque rupture (Galis, 1994), tumour angiogenesis (Nelson, 2000), metastasis (Nelson, 2000) and rheumatoid arthritis.

Table1.2: The Matrix Metalloproteinases.

Enzyme	MMP	Human Chromosme
Collagenases.		
Interstitial collagenase: Collagenase 1.	MMP-1	11q22-q23
Neutrophil Collagenase: Collagenase 2.	MMP-8	11q21-q22

Collagenase 3	MMP-13	11q22.3
Collagenase 4	MMP-18	NA
Gelatinases.		
Gelatinase A	MMP-2	16q13
Gelatinase B	MMP-9	20q11.2-q13.1
Stromeolysins.		
Stromeolysin 1	MMP-3	11q23
Stromeolysin 2	MMP-10	11q22.3-q23
Stromeolysin 3	MMP-11	22q11.2
Membrane Type MMPs		
Transmembrane.		
MT1-MMP	MMP-14	14q11-q12
MT2-MMP	MMP-15	15q13-q21
MT3-MMP	MMP-16	8q21
MT5-MMP	MMP-24	20q11.2
GPI anchor		
MT4-MMP	MMP-17	12q24.3
MT6-MMP	MMP-25	16p13.3
Others		
Macrophage elastase.	MMP-12	11q22.2-22.3
No trivial name.	MMP-19	12q14
Enamelysin.	MMP-20	11q22.3
XMMP.	MMP-21	NA
CA-MMP.	MMP-23	1p36.3
CMMP.	MMP-27	11q24

Epilysin.	MMP-28	17q21.1

1.3.5 Regulation of MMP activity.

MMPs are potent and their proteolytic activity requires strict control. This can be seen to broadly occur at three levels.

1.Transcriptional regulation. The expression of most MMPs occurs at low levels in most tissue however, is upregulated during certain physiological and pathological processes.

Numerous growth factors, inflammatory cytokines and hormones have been shown to induce or stimulate MMP synthesis. Interleukin I, platelet derived growth factor, Tumour necrosis factor-alpha, epidermal growth factor and basic fibroblast growth factor and CD40 have been shown to have stimulatory effects (Malik 1996, Schonbeck 1997). Whilst TGF beta, heparin and corticosteroid have been shown to have inhibitory effects on MMP gene expression (Wassenaar 1999). Not all MMPs react similarly to these factors.

2.Proenzyme activation. One of the characteristics of MMPs is that they are secreted in an inactive proenzyme form (with the exception of membrane bound MMPs) and so they require activation in order to have any effect on the extracellular matrix. Three different activation mechanisms are present. Firstly, stepwise activation. Secondly, activation at the cell surface by MT-MMPs and thirdly, intracellular activation (Nagase 1997). The first stage in stepwise activation usually involves a proteinase, of which plasmin appears to be the most important (Murphy 1994). Plasmin attacks the proteinase susceptible region of the MMP inducing conformational changes, which renders the activation site susceptible to cleavage by a second proteinase, which is usually a second MMP (Nagase 1997). The generation

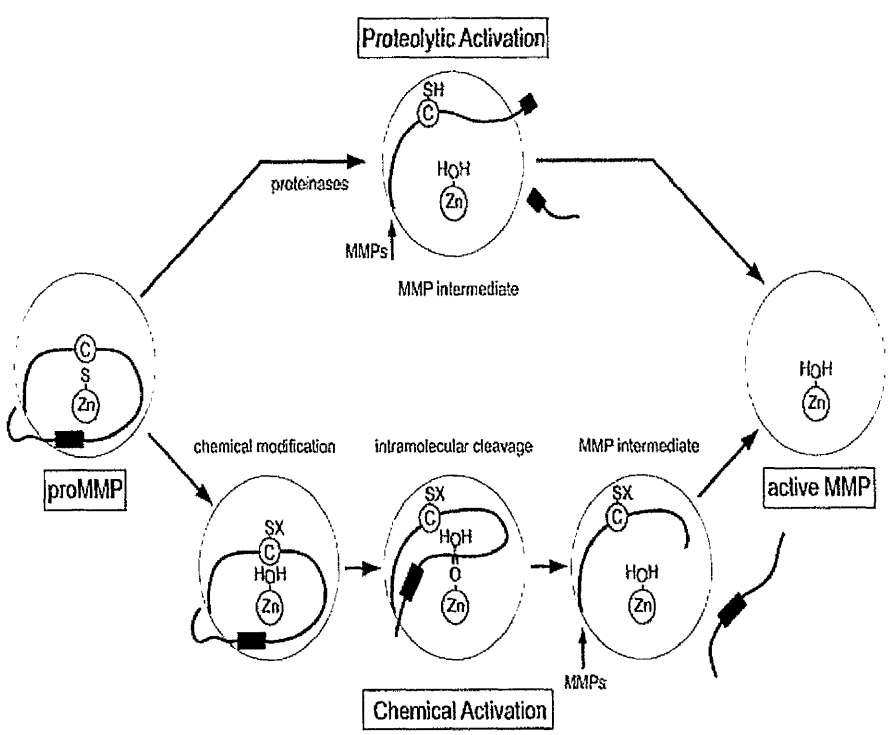
of plasmin from plasminogen activators occurs at the cell surface where both plasminogen and urokinase activators are bound. The roles of urokinase plasminogen activator (uPA) and its specific inhibitor uPA receptor (uPA-r) in addition to Plasmin activator inhibitor I and II have been shown to have central roles. Binding to uPA localises proteolytic activity whilst expression of uPA-r on macrophages and monocytes prevent degradation. (Kircheimer, 1989, Estreicher, 1989) A positive feedback mechanism appears to exist whereby active enzymes activated other proenzymes resulting in a fivefold to eightfold increase in proteolytic activity.

Cell surface activation of MMPs is important in the pericellular degradation of the ECM during cell migration. In addition to the plasminogen system MT-MMPs play an important role in cell surface activation (Sato 1994).

The precise mechanism of intracellular activation has not been identified.

3.Direct Inhibitors of MMPs. The main inhibitors of the MMPs are alpha-macroglobulin, which is restricted by its large size and a family of specific inhibitors called Tissue inhibitors of Matrix Metalloproteinases.

Figure1.1: Activation of Pro-MMPs



1.3.6 Tissue inhibitors of Matrix Metalloproteinases (TIMP).

A balance between the proteolytic activity of MMPs and their inhibitors, TIMPs, is a prerequisite for normal tissue function. The TIMP family consists of at least four distinct members, which possess 12 conserved cysteine residues and express MMP inhibitory activity. TIMPs are expressed by a variety of cell types and are present in most tissues and body fluids. TIMP-1 and TIMP-2 are present in soluble form whilst TIMP-3 is insoluble and bound to the extracellular matrix.

Structure and Function.

The four characterised members of the TIMP family share several structural features.

1. They all possess 12 cysteine residues in conserved regions of the molecule forming six disulphide bonds.
2. The NH2 terminal domain is necessary for the MMP inhibitory activity. All four TIMPs share a consensus sequence (VIRAK) in this domain.
3. A 29 amino acid sequence is cleaved off to produce the active, mature protein.

TIMP-1.

TIMP-1 was originally isolated from rabbit bone and characterised as an inhibitor of collagenase activity. (Sellers A, 1977) Human TIMP-1 was purified and obtained from amniotic fluid (Murphy G, 1981) and skin fibroblasts. (Striklin G 1983) The full protein sequence was ultimately described by Carmichael and co-workers. (Carmichael DF 1986). TIMP-1 is produced and secreted by a variety of cells and widely distributed throughout the body (Cawston 1986). The TIMP-1 molecule consists of 184 amino acids. This includes the 12 cysteine residues, which form six disulphide bonds that hold together the six loops and divide the molecule into three knot-like structures (Docherty 1985). Depending on glycoslylation the molecular

mass extends from 20kDa to 40kDa. The NH2 terminal domain appears to be central to the MMP inhibitory function of the TIMP-1 molecule (Murphy 1991).

TIMP-2

TIMP-2 is a 21kDa unglyosylated protein first described by three separate groups in 1989(DeClerck 1989, Goldberg 1989, Stetler-Stevenson 1989). TIMP-2 shares 40% amino acid identity with TIMP-1 (Stetler-Stevenson 1989) and possesses six disulphide bonds in the same conserved positions (DeClerck 1989). The N terminal domain is once again necessary for the MMP inhibitory action (DeClerck 1993).

TIMP-3

TIMP-3 is an insoluble 21kDa unglycosylated protein, which was, originally purified form chicken embryo fibroblasts (Pavloff 1992). The human molecule was detected in WI-38 fibroblasts (Wick 1994) and shares 30% sequence homology with TIMP-1 and 38% homology with TIMP-2 (Pavloff 1992). TIMP-3 possesses exclusive properties that differentiate it clearly from the other members of the TIMP family. Namely, that it is restricted to the extracellular compartment (Pavloff 1992) and it induces apoptosis when over-expressed in certain cell types (Ahonen 2003, Bond 2002). The mechanism by which TIMP-3 induces cell death is not clear however evidence suggests a link with death receptor-mediated apoptosis(Ahonen 2003, Bond 2002).

TIMP-4

TIMP-4 has been most recently identified by molecular cloning (Greene 1996). It is highly expressed in the heart in comparison with other tissues. It appears to share 37% sequence homology with TIMP-1 and 57% with TIMP-2 and TIMP-3 (Greene 1996).

1.3.7 MMP inhibition and ECM turnover.

TIMPs inhibit MMP activity through high affinity, irreversible, non-covalent binding of the active forms of MMPs at molar equivalence (Murphy 1995). As discussed above, studies have previously shown that the NH2 terminal domain is important in the MMP inhibitory role of the TIMP molecule. A C-terminally truncated form of TIMP-1 comprising only of the N terminal has been shown to inhibit MMP-3 with only a slightly reduced affinity in comparison with the full length TIMP-1 (Murphy 1991). However, more recent work has the demonstrated that the structural relationship between TIMP-1 and MMP-3 is much more complex. Gomis-Ruth et al (1997) described the complex formed between unglycosylated TIMP-1 and the catalytic domain of MMP3 using an X-ray crystal structure analysis. This revealed that TIMP-1 had the shape of an elongated wedge, which occupied the entire length of the active-site cleft of MMP-3. Of the six separate polypeptide segments of TIMP-1, which were involved in the complex, four were provided by the N terminal and two by the C terminal. Demonstration of this unusual architecture will aid the design of future synthetic MMP inhibitors.

TIMPs act on a broad range of MMPs. TIMP-1 potently inhibits the activity of most MMPs with the exception of MMP-2 and MT1-MMP. However the actions of TIMP-1 are more complex than merely the inhibition of active MMP molecules. For example, TIMP-1 can also bind to the proform of MMP-9. The consequent molecule pro-MMP-9/TIMP-1 can inhibit active MMP forming a more stable complex pro-MMP-9/TIMP-1/MMP (Kolekenbrock 1995). TIMP-2 is a potent inhibitor of MMPs with the exception of MMP-9. Additionally TIMP-2 has a dual inhibitory action, binding to progelatinase A (pro-MMP-2) (Goldberg 1989). It has been shown that the

pro-MMP-2/TIMP-2 complex prevents autoactivation of MMP-2 and that the binding of TIMP-2 to the active form of the enzyme has a stabilising effect (Howard 1991). It can also form a complex with MT1-MMP at the cell membrane, which may play a regulatory role in the proteolytic activation of MMP-2.

TIMP-3 is limited to the extracellular compartment and has been shown to bind to MMP1, 2,3,9 and 13 in the extracellular matrix.

TIMP-4 inhibits MMP 1,3,7 and 9 and shows a high level of expression in adult human cardiac tissue.

Regulation.

TIMP-2 expression is largely constitutive, following the pattern of expression of gelatinase A with which it interacts specifically. Whereas, TIMP-1 expression is highly inducible by a variety of cytokines and hormones. TIMP-1 has shown in some studies to be co-ordinately regulated with MMPs and in others reciprocally regulated. Factors such as phorbol esters and interleukin-1B stimulate both TIMP-1 and MMP expression (Overall 1994). Whilst MMPs are downregulated and TIMPs stimulated by factors such as TGF-beta (Overall 1991).

Reported actions of TIMP-1.

Whilst I have concentrated on the role of TIMP-1 in extracellular matrix remodelling TIMP-1 has been shown to be active in numerous conditions listed in tabular format below.

Table 1.3: Reported actions of TIMP-1.

Reported actions of TIMP-1
Role in embryonic bone tissue remodelling. (Hill 1993)
Role in gonadal steroidgenesis. (Boujrad 1995)
Role in ovulation, pregnancy, and parturition. (Jeffrey 1991)

Inhibits tumour cell invasion and metastasis in experimental models. (DeClerck 1992)
Associated with advanced tumour stage of non-Hodgkin's lymphoma. (Kossakowska 1991)
Modulation of vascular injury. (Webb 1997, Dollery 1999)

1.4 Functional significance of the extracellular matrix.

The extracellular matrix has a key role in a normal functioning myocardium.

1. It provides sarcomere alignment and ensures the structural integrity of adjoining myocytes.
2. It provides the means by which myocyte shortening is translated into ventricular pump function.
3. It prevents "myocyte slippage"
4. It is essential for maintaining the alignment of myofibrils within the myocytes.

1.5 Structural Remodelling in Hypertensive Heart Disease.

Myocyte hypertrophy has been described above. In addition to myocyte hypertrophy there is an intensive interstitial fibrosis resulting in a qualitative as well as a quantitative alteration in the myocardial structure. The fibrosis has several distinct morphological presentations. (Anderson 1979, Pearlman 1982, Huysman 1989) It occurs around the intramyocardial coronary arteries ands arterioles where it represents a perivascular fibrosis. From the perivascular space it extends in to the interstitial space producing an interstitial fibrosis. This is a progressive process involving increasing amounts of the interstitium and resulting in distortion of the normal myocardial architecture (Silver 1990). This is a primary reactive process and can be seen throughout the pressure overloaded left ventricle and the normotensive right ventricle (Pearlman 1982). The demonstration of interstitial fibrosis in the

normotensive right ventricle is a key finding and demonstrates that this process is not purely a load dependent phenomena. As the fibrosis progresses individual muscle fibres become encircled by thickened collagen fibres resulting in muscle atrophy (Jalil 1991).

Distinct from this reactive fibrosis a reparative process also occurs resulting in a fibrous "patch" which bridges the gap left by myocyte necrosis. This "patch" consists of thick collagen fibres, which join adjacent muscle fibres, and through this thin collagen fibres can be seen to entwine forming scar tissue. The mechanism responsible for myocyte necrosis is unclear, however there are two proposed mechanisms. Firstly it may be initiated by the initial reactive interstitial fibrosis described above, through direct " strangulation" of the muscle fibres or interference with normal myocyte nutrition by increasing the distance between capillary beds and the myocytes thus affecting oxygen diffusion. Secondly, this phenomena may not represent myocyte necrosis but in fact represents myocyte apoptosis which has been demonstrated to be an important regulatory mechanism in response to pressure overload (Teiger 1996).

1.5.1 Factors controlling structural remodelling.

Haemodynamic Factors

Several clinical observations and some in-vitro work suggest haemodynamic overload plays a role in the development of fibrosis. Tanaka et al (1986) demonstrated that the collagen volume fraction increases from the outer to the inner third of the LV free wall in postmortem hypertensive hearts. Which may reflect transmural gradients of wall stress. Furthermore Querejeta et al (2000) demonstrated some relationship between severity of LVH and severity of fibrosis on myocardial biopsy. Finally, in-

vivo studies have shown that procollagen type I synthesis is stimulated in cardiac fibroblasts submitted to mechanical load (Bishop 1999).

Non-haemodynamic Factors.

There are several features, which demonstrate that non-haemodynamic factors have a key role in the development of interstitial fibrosis. Firstly, fibrosis is seen in the normotensive right ventricle, secondly, conditions that result in hypertension but not activation of the renin angiotensin system do not result in fibrosis (Brilla 1990) and finally regression of fibrosis is independent of antihypertensive effect (Brilla 2000). These concepts will be examined in detail below.

The effector hormones of the renin angiotensin system are central to the control of the development of fibrosis. The animal models described below allow the manipulation of the renin angiotensin system and subsequent pathological examination of the structural myocardial changes.

Using the rat model, stimulation of the renin angiotensin system can be achieved by surgically induced unilateral renal ischaemia or by supra-renal aortic banding. Both these models result in an increase in circulating angiotensin II and aldosterone levels. Studies using these models have demonstrated fibrosis of both the hypertensive hypertrophied left ventricle and the normotensive non-hypertrophied right ventricle. (Doering 1988, Jalil 1988, Brilla, 1990, Lindy 1972) The interstitial fibrosis in this model can be prevented by the administration of Captopril. (Jalil 1991) This implicates a role in the development of fibrosis for circulating effector hormones of the renin angiotensin system.

This is further confirmed when we examine models of hypertensive heart disease which result in sustained hypertension but not in activation of the renin angiotensin system, namely infra-renal banding. Pathological analysis of this model reveals that

neither a reactive or reparative fibrosis occurs despite sustained hypertension (Brilla 1990).

Furthermore models of volume overload hypertrophy such as A-V fistula (Weber 1990), chronic anaemia (Bartosova 1969), atrial septal defect (Marino 1985) and chronic thyroxine administration (Bartosova 1969) which result in hypertrophy but not activation of the renin angiotensin system again do not show a reactive or reparative fibrosis.

The role of AngII and aldosterone was further addressed by studies, which involved the chronic infusion of ang II and aldosterone in subpressor doses. (Sun 1993, Tan 1991) Individually both angII and aldosterone models resulted in fibrosis characterised by both microscopic scar formation and a primary reactive perivascular fibrosis. (Sun 1993)

Other factors are also clearly important in the development of fibrosis. If we look at models of mineralocorticoid excess with suppressed plasma renin and Angiotensin II concentrations then again there is clear evidence of fibrosis (Brilla 1990). This can be prevented by the addition of spironolactone in doses, which did not affect the blood pressure (Brilla 1993). Spironolactone and amiloride have been shown to prevent the formation of microscopic scars and by extrapolation prevent myocyte necrosis in hypertensive rats treated with Aldosterone. (Campbell 1993, Brilla 1992)

Dietary KCL supplementation has been shown to have similar cardioprotective effects probably secondary to the role intramyocardial potassium depletion plays in myocyte loss and subsequent scar formation. (Darrow 1942)

From the findings of the animal models described above we can conclude;

1. Perivascular and interstitial fibrosis is associated with chronic elevations in the effector hormones of the renin angiotensin system.

2. Perivascular and interstitial fibrosis is independent of arterial hypertension and myocyte hypertrophy.
3. Reparative fibrosis following myocyte necrosis is associated with elevations in plasma angII and to intramyocardial potassium depletion secondary to chronic mineralocorticoid excess.

1.5.2 Mechanisms of Fibrosis.

As we have established the central role played by the renin angiotensin system in the development of fibrosis, the mechanisms by which this is effected should be examined. Factors responsible for the appearance of the perivascular and interstitial fibrosis include;

1. Hormonally mediated coronary vascular hyperpermeability.
2. Direct hormonal regulation of fibroblast collagen turnover.
3. Autocrine and paracrine signalling that takes place between cells.

Hormonally mediated coronary hyperpermeability.

Reddy et al demonstrated that an acute elevation in plasma AngII created by a pressor dose of intravenous Ang II was associated with an increase in coronary vascular permeability and the subsequent appearance of macromolecules in cardiac lymph. (Reddy, 1995). This contrasted with a model, which produced hypertension independently of angII (intravenous Methoxamaine), which did not show changes in cardiac lymph. In this study, coronary sinus pressure was increased to create a filtration-independent state in both models. Reedy concluded that angII produces an increase in coronary permeability independent of hypertension or venoconstriction. Sun et al (1993) clarified this situation by using sub-pressor doses of angII and observing the sequence of events that resulted in fibrosis. He reported the appearance

of fibronectin (a macromolecule several times the size of albumin) within the interstitial space within 24-48hours. Proliferating fibroblast were seen in the interstitium within 2-4 days followed by enhanced expression of type I mRNA and increased collagen type I synthesis. This sequence of events was present in both ventricles.

Thus perivascular fibrosis, which occurs in the context of elevations in plasma angII and in the absence of hypertension, is associated with macromolecular hyperpermeability. The contribution of macromolecular hyperpermeability excluding the other pro-fibrotic effects of angiotensin II in the resultant fibrosis cannot be determined by these studies.

Hormonal regulation of fibroblast turnover.

The cardiac fibroblast is responsible for collagen turnover in the myocardium. It is responsible for transcription and deposition of type I and III collagens and also for the transcription of matrix degradative metalloproteinases and their inhibitors. Therefore it plays a key role in the development of fibrosis. Cell surface receptors for angiotensin II, aldosterone and endothelin have been identified on the cardiac fibroblast. The effects of the effector hormones of the renin angiotensin system on cardiac fibroblasts have been studied.

Angiotensin II.

Collagen synthesis of adult rat cardiac fibroblasts has been to shown to increase in a dose dependent manner by the administration of angiotensin II. (Brilla, 1994) This response is mediated through the type I receptor subtype. (Brilla, 1994) The type II receptor subtype appeared to mediate a reduction in collagenase activity in fibroblast

culture. (Brilla, 1994) Furthermore, in mice with aortic banding, the type II receptor subtype mediated an inhibitory effect on remodelling (Akishita 2000).

Aldosterone.

Aldosterone increased fibroblast collagen synthesis in a concentration dependent manner, which could be prevented by the administration of the aldosterone antagonist spironololactone. There was no effect on collagenase activity. (Brilla, 1994)

Endothelins.

Endothelin I and Endothelin 3 have been shown to increase collagen synthesis in a concentration dependent manner. Both Eta and Etb receptors were involved. (Guarda, Katwa, 1993) Collagenase activity was reduced by ET-1 but not ET-3 and this effect could be blocked by an antagonist to the subtype A receptor. (Guarda, Katwa, 1993)

Cell-cell signalling and local regulation of collagen turnover.

Endothelial cell-derived signals.

Endothelial cells produce substances, which can act as promoters or inhibitors of the growth and behaviour of neighbouring non-endothelial cells such as fibroblasts. Guarda et al addressed the effects on fibroblast collagen turnover by endothelial cells. He demonstrated that endothelial cell-conditioned media was found to increase fibroblast collagen synthesis and to increase collagenase activity. This did not appear to be dependent of angII or aldosterone as their respective antagonists did not prevent this response neither did the addition of angII or aldosterone augment the fibrosis. (Guarda,Myers, 1993)

Tissue receptors.

An autocrine or paracrine system requires the presence of receptors in tissue and that theses receptors be functionally active in response to locally generated or circulating signals.

In-situ hybridisation has demonstrated the presence of receptors for angiotensinogen on cardiac fibroblasts

Nonendotheial tissue ACE.

Using in-vitro autoradiography together with an iodinated tyrosyl derivative of Lisinopril Sun et al have demonstrated a nonendothelial tissue angiotensin-converting enzyme (TACE) that was localised to tissues rich in active fibroblasts and fibrillar collagen. TACE binding was found in high density in myocardial scar formation and the fibrosis surrounding intramyocardial vessels. (Johnston, 1991, Sun 1993) TACE binding density was found to be independent of circulating renin and angiotensin. (Schunkert, 1993) TACE appears to be an integral feature of fibrous tissue formation and itself represents a marker for fibrosis. It may regulate local concentrations of angII and bradykinin.

Angiotensin II and aldosterone receptors.

In order to determine whether angII and aldosterone receptors were present in fibrous tissue Sun et al used quantitative in vitro autoradiography. (Sun, Weber, 1993) Myocardial fibrosis was induced with chronic administration of angiotensin or aldosterone. Receptors for both Aldosterone and AT-1 were found in the normal rat myocardium. However the perivascular fibrosis and scarring of the ventricles seen with angII or aldosterone administration was not anatomically coincident with the presence of angII or aldosterone receptors. This may suggest that angII or aldosterone may not directly influence fibrous tissue formation but may in fact act through the release of other mediators such as ET-1 or cytokines such as TGF-beta.

Bradykinin Receptors.

Bradykinin is a substrate for ACE and the presence of bradykinin receptors was examined by Sun et al using a rat model infused with angII or aldosterone. (Sun,

Weber, 1993) Bradykinin receptors were identified in the perivascular fibrosis and endomyocardial scarring seen in each ventricle. These receptors, unlike angII and aldosterone were anatomically coincident with ACE binding. Thus, fibrous tissue ACE appears to function as a kinase regulating local concentrations of bradykinin.

1.6 Biochemical monitoring of myocardial fibrosis.

Cardiac biopsy is undoubtedly the gold standard for assessment of myocardial fibrosis. However this procedure does have appreciable complication rate and is both inconvenient and uncomfortable for the patient. Therefore a non-invasive means of monitoring the process of fibrosis is undoubtedly an attractive one.
Measurement of biochemical markers of collagen synthesis, degradation and inhibition of degradation holds promise in this regard.

1.6.1 Biochemical Assessment of Collagen Type I Synthesis and Degradation.

Collagen type I is synthesised in fibroblasts as a procollagen precursor. This contains an N and a C terminal. When the procollagen is being integrated into the collagen helix propeptides are removed by a variety of proteinases leaving the collagen molecule to be integrated. (Nimmi, 1993) The 100 kDa procollagen type I carboxyterminal propeptide (P1CP) is cleaved from the procollagen type I during the synthesis of fibril-forming collagen type I. A stoichometric ratio of 1:1 exists between the number of collagen type I molecules produced and the number of P1CP released. P1CP is cleared by the liver. (Smedrod, 1990)

The rate-limiting step in degradation is the cleavage of collagen type I fibrils by interstitial collagenase. (Janicki 1995) This enzyme is active at a specific locus and cleaves all three alpha chains of the collagen. This results in a 36kDa and a 12kDa telopeptide. The larger telopeptide spontaneously denatures. The small 12kDa

pyrindinoline cross-linked carboxyterminal telopeptide of collagen type I (CITP) remains intact. This is released in a stoichometric 1:1 fashion. (Ristelli, 1993)

Therefore P1CP can be seen as a potential marker of collagen synthesis and CITP as a marker of collagen degradation.

PICP and CITP can be measured directly using commercially available radioimmunoassays.

Experimental studies.

Diez et al have performed the majority of experimental studies. They studied spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) by means of myocardial biopsy and quantitative analysis of the collagen content. Untreated SHR rats showed an interstitial and perivascular fibrosis and an increase in collagen volume fractions. In addition to the pathological analysis, serum P1CP and CITP were measured. Both P1CP and CITP were elevated and serum P1CP was found to directly correlate with pathological indices namely collagen volume fraction. (Diez, 1996, Varo, 1999, Varo, 2000)

Subsequent experiments compared untreated SHR with SHRs treated with Quinapril (Diez, 1996) and Losartan. (Varo, 1999 and Varo, 2000) There was a marked reduction in fibrosis in the treated rats and this was associated with decreased P1CP and a tendency to increased CITP concentrations. This effect was seen at doses that did not result in blood pressure reduction. (Varo, 2000)

Clinical Studies.

Diez et al have looked at P1CP and CITP concentrations in untreated patients with essential hypertension. (Diez, 1995, Laviades, 1998, Laviades, 2000)

Measurements were repeated after treatment with an ACE-inhibitor (Lisinopril) or an Angiotensin II antagonist (Losartan).

Baseline measurements of P1CP were significantly higher amongst the hypertensive group when compared with a cohort of normal subjects. CITP levels were not significantly different. Serum P1CP levels were found to correlate with left ventricular mass and the severity of ventricular arrhythmias. (Diez, 1995)

Treatment of patients with Lisinopril or Losartan resulted in regression of LVH, normalisation of diastolic filling, reduction in P1CP and elevation in CITP.

Studies utilising transvenous endomyocardial biopsies and estimation of collagen volume fraction (CVF) have demonstrated correlations between P1CP and CVF in an untreated hypertensive cohort (Querejeta 2000) and following treatment (Lopez 2001).

Summary

These studies suggest: 1. Collagen metabolism is abnormal in rats and humans with untreated hypertension. 2.Serum concentrations of P1CP and CITP may be of use for the non-invasive assessment of fibrosis in patients with untreated hypertension. These studies are undoubtedly of interest and lay the foundation for human studies of non-invasive assessment of myocardial fibrosis in untreated hypertension.

1.6.2 Biochemical assessment of Inhibition of Degradation.

As previously described Tissue Inhibitors of Matrix Metalloproteinases provide the central means of regulating MMP activity and consequently collagen degradation. Thus measurement of TIMP-1 levels would provide a non-invasive means of

assessing collagen degradation. TIMP-1 levels can be measured using a commercially available two stage ELISA assay. The technique used is a modification of the original reported by Plumpton (1995).

Laurent et al (1978) proposed that an equilibrium exists between collagen synthesis and degradation in normal tissue. This equilibrium is disturbed during pathological states, which result in fibrosis. Increasingly the role of TIMPs has been assessed in this equilibrium. Clearly, an important mechanism for the development of fibrosis may be inhibition of collagen degradation leading to fibrosis. Several studies have pointed towards this as a potential mechanism. Diez et al (1996) demonstrated in spontaneously hypertensive rats that collagen synthesis is increased however collagen degradation is unchanged. Furthermore Brilla et al (1994) reported that MMP-1 activity is abnormally reduced in hypertensives with LVH. The conclusion reached by these groups was that the intensity of collagen degradation is not sufficient to offset the increment in collagen synthesis and thus results in fibrosis. A possible mechanism for this would be an abnormal increment in TIMP activity.

Laviades et al (1998) attempted to confirm this hypothesis using serum markers of collagen degradation (CITP, MMP-1) and inhibition of collagen degradation (TIMP-1). They studied a small number of patients with essential hypertension before, and after treatment with Lisinopril. They demonstrated increased levels of TIMP-1 in untreated hypertensive patients, reduced levels of MMP-1 and normal levels of CITP. After treatment with Lisinopril MMP-1 levels increased and TIMP-1 levels decreased with treatment. These findings allowed Diez et al to hypothesise that collagen degradation is depressed in untreated hypertension and that elevated TIMP-1 may mediate this process. There are, however, several flaws in this study. Firstly, small numbers of patients were studied in each group. Secondly, the

authors analysed serum levels of TIMP-1, which have been shown to be inaccurate and inferior in comparison to plasma levels (Jung, 1997) probably due to the release of TIMP-1 during platelet activation (Copper 1985). Finally, despite demonstrating a reduction in MMP-1 and an increment in TIMP-1 the authors failed to demonstrate any material difference in actual degradation i.e. CITP levels. Nonetheless we found the hypothesis persuasive and this provided the stimulus to much of our work in patients with untreated hypertensives.

1.7 Clinical Consequences of fibrosis.

1.7.1 Reduced coronary reserve.

It is now recognised that patients with hypertensive heart disease have symptoms and signs of myocardial ischaemia despite having angiographically normal epicardial coronary arteries. This is related to impaired coronary flow reserve. (Houghton, 1990) There are many factors involved in the development of impaired coronary flow reserve however undoubtedly periarteriolar fibrosis plays a role. This has been demonstrated by Schwartzkopff et al who reported that the perivascular collagen fraction correlated with coronary resistance and reduced coronary flow reserve. (Schwartzkopff, 1993) In addition he demonstrated that treatment with ACE-inhibitor resulted in regression of the fibrosis and improvement in the coronary reserve. This was demonstrated in the absence of any change in media hypertrophy. (Schwartzkopff, 2000)

1.7.2 Ventricular Arrhythmias.

LVH is associated with both an increase in sudden death and in ventricular arrhythmias assessed by Holter monitoring (Levy 1987, Malerba 1991, Messerli

1984). This has demonstrated an increased frequency and complexity of ventricular ectopy in patients with LVH (Messerli 1984, McLenechan 1987). However no clear relationship between these dysrhythmias and sudden death has been demonstrated.

Animal studies have revealed multiple electrophysiological abnormalities which may help to explain the incidence of arrhythmias in hypertensive patients. These include increased vulnerability to ventricular arrhythmia (Shechter 1989), dispersion of monophasic action potential duration (Kowey 1991) and refractoriness and action potential prolongation (Aronson 1980). Despite this wealth of laboratory data and observational studies, there is no direct experimental evidence that demonstrable ventricular ectopy leads to complex ventricular arrhythmias and consequent sudden death. Indeed studies using both late potential and invasive electrophysiological stimulation have failed to show any increment in the propensity for the inducement of re-entrant ventricular dysrhythmias (Pringle 1992).

1.7.3 Diastolic Dysfunction.

Diastolic Function.

1.7.3.1 Definition.

Diastole is defined as the time period from the closure of the semilunar valves until the closure of the atrioventricular valves. Put more simply, using the left ventricle as an example, diastole therefore consists of the time period between the aortic valve closure until the mitral valve closure.

1.7.3.2 Physiology of diastole.

Diastole is a complex energy dependent process involving the interplay of numerous factors. Attempts have been made to simplify this process by arbitrary division and further classification.

This is based on the premise that diastole essentially consists of four events.

1. The isovolumic relaxation period. This period extends from the closure of the aortic valve until the opening of the mitral valve. The ventricular pressure declines without any change in the ventricular volume. Using a standard pressure volume curve this period can be seen to coincide with the descending limb.
2. The rapid filling period. This occurs following opening of the mitral valve. Due to the transmitral pressure gradient there is considerable passive filling. In normal circumstances passive filling accounts for up to 80% of ventricular filling.
3. The slow filling period. This period is also known as diastasis and consists of passive filling, which persists despite equalisation of chamber pressures due to momentum of the preceding phase of rapid atrial filling.
4. Atrial filling. This consists of atrial systole and accounts for around 15% of diastolic filling in a normal subject.

Left ventricular filling can therefore be seen to be central to the diastole. The two major determinants of which are 1. Ventricular relaxation. 2 "Effective chamber compliance".

Ventricular relaxation begins mid systole continuing into the first third of diastole. It is an energy dependent process during which the contractile proteins are deactivated and the myofibrils return to their normal length.

The effective chamber compliance can be seen as the passive properties of the left ventricle as blood enters the ventricle during diastole. This description simplifies the complex interplay, which occurs to define the "passive" properties of the ventricles with interaction of ventricular relaxation, diastolic suction, passive filling, pericardial restraint and the viscoelastic forces of the myocardium.

1.7.3.3 Assessment of diastolic function.

The complexity of diastolic function means accurate reproducible measurement will be difficult. Several methods have been employed.

Initially diastolic function was assessed using the measurement of the time constant of left ventricular decay (tau). This required haemodynamic and angiographic measurement at cardiac catheterisation. (Mirsky 1990) However this method is clearly not suitable for serial measurements and does carry a morbidity risk.

Digitised M Mode techniques have been used consisting of left ventricular M modes with simultaneous LV pressure tracings. This allows construction of pressure volume loops and calculation of measures of diastolic filling. (Gorscan 1994) However the major drawback to this technique is that the results are flawed in the presence of any wall motion abnormality.

Radionuclide angiography has also been employed with variable success. (Magorien 1984)

Doppler assessment of diastolic filling.

Doppler assessment of diastolic filling is the most commonly employed method. It concentrates on the mitral flow derived doppler. The mitral flow velocity curves obtained correlates well with accepted methods such as digitised M-Mode echocardiography, radionuclide angiography and left ventricular angiography. (Friedman 1986) (Spirito B 1986)

The mitral valve velocity curves obtained with doppler should be seen as representative of the relative forces across the mitral valve. In the same way measured peak velocity is representative of instantaneous pressure change between the left

atrium and the left ventricle. An understanding of this allows the mitral flow velocity curves to be interpreted in context with diastolic function. When contraction ceases the left ventricular pressure rapidly decays. When this pressure becomes less than the left atrial pressure the mitral valve opens and blood flows from the left atrium to the left ventricle on the pressure gradient created. A process of diastolic suction caused by further LV relaxation further enhances this gradient. This rapid blood flow is seen on the mitral doppler as the E wave. As the left ventricular pressure rises the transmitral gradient falls and ultimately equalises. This results in deceleration in the transmitral flow velocity curve. The rate at which the pressure gradient decreases determines the deceleration rate and deceleration time of the mitral E velocity curve. This is named the E wave deceleration time or DT.

During mid diastole there is equilibration of forces between the left atrium and the left ventricle however flow continues to fill the left ventricle due to inertial forces.

Finally atrial systole occurs with an associated reacceleration on the transmitral velocity curve called the A wave.

It is the assessment of this curve which gives an estimated of diastolic function.

The LA pressure, LA compliance, left ventricular diastolic pressure and the rate of ventricular relaxation, determines the peak E wave velocity. E wave deceleration is dependent on the rate of increase in the left ventricular pressure and is a measure of left ventricular compliance.

The A velocity is dependent on left ventricular chamber compliance as well as the volume and compliance of the left atrium.

The time from the closure of the aortic valve until the onset of mitral flow is called the isovolumic relaxation time (IVRT) and is an additional measure of LV relaxation.

1.7.3.4 Factors affecting normal transmitral flow velocity curves.

1. Age. There are undoubted changes in diastolic filling secondary to the ageing process. These have been well documented and in practical terms consist of i) a reduction in E wave velocity. ii) an increase in A wave velocity iii) a prolongation in the E wave deceleration time and isovolumic relaxation time. (Spritito 1988)
2. Heart rate. With increasing heart rate there is fusion of the E and A waves with a consequent reduction in diastasis. There is an associated increase in A wave velocity. (Yamamoto 1993)
3. Preload. (Choong 1988)
4. Afterload. (Choong 1988)

1.7.3.5 Normal and abnormal filling patterns.

1Abnormal Relaxation.

Abnormalities in left ventricular relaxation result in characteristic changes. Firstly due to impaired left ventricular relaxation there is a reduction in the rate of fall of left ventricular pressure with a consequent fall in transmitral gradient and a consequent reduction in mitral E wave velocity. There is an associated prolongation of the isovolumic relaxation time and the E wave deceleration time. Finally due to the aforementioned abnormalities there is augmentation of atrial contraction manifest by an increase in the A wave velocity

Pseudonormalisation.

Interpretation of the doppler mitral flow velocity curves is made more difficult by the occurrence of pseudonormalisation which means that doppler assessment may on occasion be misleading. As ventricular compliance is reduced the left atrial pressure ultimately rises to compensate for this resulting in an increased early transmitral

gradient. This results in a shortened isovolumic relaxation time, increased E wave velocity, reduced E wave deceleration time and a reduction in A wave velocity. Thus although there is significant impairment of left ventricular relaxation and a reduction in compliance the mitral flow velocity curve can appear essentially normal.

Restrictive pattern.

As impairment of ventricular compliance progresses the left atrial pressure increases as a consequence. This results in a "restrictive" pattern which is characterised by an increased E: A ratio (often greater than 2) and a marked reduction in isovolumic relaxation time and E wave deceleration time.

Differentiating between a normal filling pattern and pseudonormalisation is difficult. Clearly serial measurements will allow monitoring of the development of the patterns described however a one off study will not allow this. Examination of the pulmonary vein flows may help to distinguish. In pseudonormalisation the pulmonary A wave velocity is frequently increased. Additionally a valsalva manoeuvre will result in a reduction in E: A ratio if pseudonormalisation is present.

1.7.3.6 Aetiology.

A number of studies have shown that fibrosis rather than myocyte hypertrophy accounts for the development of diastolic dysfunction in hypertension. Matsubara et al (2000) demonstrated, using isolated papillary muscles from hypertensive rats, that fibrosis rather than hypertrophy increased passive stiffness. This finding has been confirmed in two clinical studies. (Schwartzkopff 2000, Brilla 2000). Therefore it can be seen that measurement of diastolic function will therefore be a non-invasive measure of myocardial fibrosis.

Other important causes besides sustained hypertension include hypertrophic cardiomyopathy, obesity, constrictive pericarditis and conditions that result in a restrictive cardiomyopathy for example amyloidosis, endomyocardial fibrosis and sarcoidosis.

1.7.3.7 Clinical Significance of Diastolic Dysfunction.

Impairment of diastolic function precedes systolic dysfunction in the progression of cardiac disease. Numerous studies have reported that between 30-40% of patients presenting with the clinical syndrome of cardiac failure have normal LV systolic function (Senni 1999). It is hypothesised that this apparent paradox is explained by the presence of diastolic dysfunction. Of more importance perhaps is that even in established left ventricular systolic dysfunction the degree of co-existent diastolic dysfunction determines the degree of cardiac limitation.

1.8 Exercise induced Left ventricular Hypertrophy. (Athletes heart)

1.8.1 Historical perspective.

In 1884 Bergmann first observed that wild animals had larger hearts than similar domesticated animals. Henschen was the first to document exercise-induced changes in humans and in 1889 he described cardiac hypertrophy in cross-country skiers by percussing the cardiac contour (Henschen 1889). Henschen hypothesised that there was symmetrical hypertrophy and dilatation to compensate for the increase in venous return. This was based on the theory of normalising wall stress via the law of Laplace, which has been discussed earlier. The first post-mortem study was reported by Kirsh (1936) who examined the hearts of 35 athletes who died suddenly and reported the finding of hypertrophy. However this did not appear to be symmetrical. The

pathological significance of this finding of hypertrophy has caused considerable debate throughout the later part of the last century and continues to do so.

1.8.2 Cardiovascular adaptation to exercise.

Adaptation occurs when regular physical activity is undertaken. The extent and the nature of the adaptation depend on the frequency, duration and nature of the exercise. There are two pure forms of exercise isotonic (dynamic) and isometric (static) however it should be accepted that almost all sports require a mixture of both.

Isotonic exercise is thought to result in;

1.Increased ventricular stroke volume.

2.Increased left ventricular end-diastolic volume.

3.Increased peak oxygen consumption. (VO2 max)

4.Increased skeletal muscle respiratory enzyme concentrations and capillary muscle density.

5.Mass-to-volume ratio is unchanged.

Isometric exercise on the other hand is characterised by;

1.Increased left ventricular mass.

2.No significant increase in VO2 max.

3.No increase in LVEDD.

4.Increased mass-to-volume ratio.

Physiological ventricular dilatation and hypertrophy occur over a short period. Ehsani demonstrated that LVEDD increases within seven days and increases in left ventricular wall thickness peaks at 3 to 5 weeks (Ehansi 1978). This requires an athlete to exercise for 30-60 minutes three to four times per week at 60-70% of his or her VO2 max. The amount of hypertrophy present depends on the size, age, gender

and numerous other influences. However, rather simplistically, larger, older athletes tend to get more significant hypertrophy.

These changes result in abnormalities in clinical examination, ECG and echocardiography.

1.8.3 Electrocardiographic changes.

Most ECG changes present in athletes are the result of high vagal tone and consist of;

1.Sinus bradycardia is seen in most athletes.

2.33% may have sinus pauses greater than 2 seconds.

3.First, second and third degree block all occur more commonly in athletes.

4.Incomplete right bundle branch block.

5.Vertical axis shift and voltage criteria for LVH.

6.ST-T wave change.

7.QT prolongation.

1.8.4 Echocardiographic changes.

Numerous echocardiographic studies have looked at exercise induced left ventricular hypertrophy. The largest being Pelliccia's study of 947 elite Italian athletes (Pellicia 1991). The average ventricular septal wall thickness was 9.7mm (range extended from 6 to 16mm) with 98% of athletes having a septal wall thickness of less than 12mm. It is noteworthy that only 16 athletes had a septal wall thickness of greater than 13mm which is the range in which hypertrophic cardiomyopathy may be suspected. The left ventricular internal diameter ranged from 40 to 66mm with a mean of 52m in comparison with a sedentary cohort who ranged from 40-52mm. The diastolic filling pattern was found to be within normal range.

An overview of 28 studies performed by Maron (1986) demonstrated an average increase of 46% in left ventricular mass, 10% increase in LV cavity size and a 14% and 18% increase in LV posterior and anterior wall thickness respectively.

Therefore in summary the echocardiographic findings one may demonstrate in elite athletes are;

1. Increased posterior wall thickness (typically does not exceed 12mm)
2. Increased anterior septal thickness (typically does not exceed 15mm)
3. Increased LV cavity size.
4. Increased LV cavity mass (45%)
5. Increased right ventricular end-diastolic diameter.
6. Multivalvular regurgitation.

1.8.5 Pathological versus Physiological.

It remains a major point of discussion whether the changes described above are a physiological or pathological phenomenon. There are well described arguments on both sides which I will discuss.

The hypothesis that athlete's heart is physiological is based on three main points. Firstly it has been well documented that exercise induced left ventricular hypertrophy regresses on cessation of exercise. Ehansi (1978) demonstrated a marked reduction in LVEDD and LV posterior wall thickness within 7 to 14 days of inactivity. A similar finding was demonstrated by Martin (1993) who reported a 20% reduction in LV mass after three weeks deconditioning. The authors suggest that the demonstration of regression on cessation of exercise confirms that exercise induced LVH is physiological.

Secondly the only life expectancy study comparing athletes with sedentary controls reported a prolonged life expectancy within the athletes (Sarna 1993).

Thirdly sudden death in athletes is exceptionally rare and is normally attributable to well described causes of sudden death

These arguments are countered quite simply however. Firstly the fact that athlete's heart regresses on cessation of exercise should give us little comfort. Any form of LVH, for example secondary to hypertension or aortic stenosis regresses on removal of the pathological stimulus for example treatment of hypertension (Dahlof 1992) or valve replacement Kennedy 1977). Therefore it can be interpreted that exercise induced LVH is merely behaving in a fashion similar to established pathological LVH. Secondly the life expectancy study quoted is flawed with no effort made to exclude confounding variables such as smoking. Finally although sudden death is rare post mortem studies have revealed an incidence of " idiopathic LVH" of 18% in this cohort (Maron 1980). Clearly no direct link can be drawn from this but this should raise concern.

1.8.6 Veteran Athletes.

Little evidence on veteran athletes is currently available. However the studies that are available suggest that the cardiovascular adaptation to high intensity exercise in this cohort may be different. A study performed by Nishimura (1980) compared a cohort of veteran athletes with younger subjects looking at echocardiographic variables. The findings reveal that the cohort of veteran athletes developed excessive hypertrophy, left atrial enlargement, ECG changes and also demonstrated some reduction in overall left ventricular function when compared to the younger cohort. A further study by Miki (1994) which was a follow up study involving a cohort of veteran cyclists revealed incomplete regression of LVH on cessation of exercise and impaired

diastolic function amongst the veteran cyclists. These features are not consistent with the overall hypothesis that exercise induced LVH is a physiological phenomena and it may well be that cardiovascular adaptation to prolonged high intensity exercise represents a different entity. A possible and plausible explanation for incomplete regression of LVH and impaired diastolic function would be the presence of interstitial fibrosis occurring as part of the hypertrophic process. Exercise induced LVH is traditionally thought to consist of myocyte hypertrophy alone. However we hypothesise that in veteran athletes the cardiovascular adaptation to prolonged high intensity exercise involves a degree of myocardial fibrosis in addition to the conventional myocyte hypertrophy. Myocardial fibrosis represents an important pathophysiological entity when present in others forms of LVH such as hypertensive heart disease and LVH secondary to aortic stenosis.

1.9 White Coat Hypertension.

White coat hypertension is a term used to describe patients who have persistently elevated office blood pressure but a normal ambulatory blood pressure. The incidence can vary markedly (12- 50%) depending on the definition used and the population studied (Verdecchia 1992,Pickering 1999). More recent work arising from the Pressione Arteriose Monitorate E Loro Associazioni (PAMELA) study describes a incidence of 7% in a random population and a minimum of 12% in untreated patients with stage I or II hypertension (Sega 2001). The pathological significance of "white coat hypertension" has been much debated and has been the subject of several trials. The studies have looked at the conventional markers of target organ damage in sustained hypertension. The results vary significantly which can at least be partly

explained by the varying definitions for " white coat hypertension". Too high a daytime reading will include patients with genuine sustained hypertension and thus demonstrate evidence of end-organ damage whilst too low a cut-off will contain a large proportion of normotensive patients thus leading to negative results. This dilemma illustrates a major persisting problem with the definition of hypertension. Namely providing an arbitrary cut-off in a continuous variable.

Several authors have demonstrated increased LV mass amongst the study population (Kuwajima 1993, Cerasola G 1995, Muscholl 1998, Palatini 1998) whilst authors have failed to demonstrate a significant difference (White WB 1989, Gosse P 1993, Verdecchia 1995). Studies of diastolic filling have shown a similar dispersion of positive and negative results. Palatini et al (1998) demonstrated normal diastolic filling whilst Cerasola et al (1995) and Glen at al (1996) revealed a significant impairment in diastolic filling.

Therefore in the presence of conflicting data on the outcome of "white coat hypertension", experts remain divided on its pathological significance.

A major omission in the current literature regarding white coat hypertension is the absence of any assessment of the collagen equilibrium. Fibrosis occurs at an early stage in the development of hypertension and therefore assessment of fibrosis would provide important additional information on the presence or absence of target organ damage in white coat hypertension and may also provide information about the blood pressure levels at which changes to the extracellular matrix occur. Perhaps giving further information about what can be truly regarded as a normal blood pressure.

Hypothesis.

1.Both collagen synthesis and degradation are abnormal in untreated essential hypertension.

2.Collagen markers will provide a non-invasive measurement of fibrosis in hypertensive heart disease and a means of monitoring this process.

3.Exercise induced LVH in veteran athletes is associated with abnormalities in the collagen equilibrium and fibrosis.

4.Target organ damage in the form of fibrosis occurs in white coat hypertension. Alterations to the collagen equilibrium occur at blood pressure levels previously thought to be normal.

Chapter2

Experimental Methods and Results of Normal Volunteers.

2.1 Investigation of collagen turnover in untreated essential hypertension.

2.1.1 *Ethical Approval*

Ethical approval was obtained from the local ethics committee. All subjects received a full written and verbal explanation of the investigations involved and the aims of the study. Written consent was obtained in all cases prior to any investigations being performed.

2.1.2 *Recruitment.*

Patients were recruited via two main sources. Firstly from the Hypertension clinic run by Dr FG Dunn in Stobhill General Hospital. This clinic is run on a weekly basis and there are on average four new referrals per week. Secondly, direct referrals from local General Practitioners were received. The General Practitioners were made aware of the research project by mail. The project was set up in a manner that offered the GPs a service for assessing patients with suspected hypertension and thus maximised referrals. Subjects were enrolled from August 1997 until July 1999.

2.1.3 *Inclusion criteria.*

Subjects were aged 18 years or over and had never treated essential hypertension. This was defined as three supine blood pressure readings with a mercury syphgmanometer greater than 140/90mmHG and a mean 24hour blood pressure of greater than 140/90mmHG.

2.1.4 *Exclusion criteria.*

Exclusion criteria were based on two main premise. Firstly the exclusion of subjects with conditions, which would result in, left ventricular hypertrophy. Namely the presence of flow limiting aortic stenosis (defined as an aortic gradient calculated by the modified Beroulli equation of greater than 20mmHG). Secondly as our biochemical assays used in this study are not specific for myocardial fibrosis careful screening of the patients to exclude conditions which could result in fibrosis was undertaken. Therefore exclusion criteria were conditions that were known to result in myocardial or tissue fibrosis. These comprised renal impairment (serum creatinine >130μmol/l), coronary artery disease (defined as symptomatic angina, history of previous myocardial infarction or the presence of ECG evidence of ischaemia or previous myocardial infarction), secondary hypertension, malignancy of any site, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction (defined as any hepatic enzyme double the upper limit of normal on routine biochemical analysis), left ventricular systolic dysfunction (defined as echocardiographic calculated ejection fraction of <35% or "eyeball" assessment of impaired left ventricular function),COPD (defined as regular use of inhalers, clinical or radiological hyperinflation or greater than 2 respiratory tract infections per year), asthma(defined as the presence of clinical bronchospasm, history of bronchospasm or the use of inhalers) and recent surgery. These conditions were identified by careful history taking, full clinical examination, urinalysis, Chest X-ray, if required, ECG, Exercise stress testing if appropriate and echocardiography.

2.1.5 *Normal Volunteers.*

Normal volunteers were recruited via a local advertising campaign. All subjects were normotensive as defined by three supine blood pressure recordings at rest of less than 140/90mmHG and exclusion criteria as above applied.

2.1.6 *Study Conditions.*

Clinical investigations were performed on one day at a standard time. Blood pressure measurement was taken in a quiet room after 15 minutes rest. Biochemical assays were taken immediately following this with venesection of 20ml of venous blood. A full cardiovascular history and systemic enquiry was taken. A full clinical examination of the cardiovascular, respiratory and abdominal systems was performed. A random urine sample was obtained and urinalysis performed.

2.1.7 *Baseline Investigations.*

Haematology: haemoglobin, haematocrit and mean cell volume.

Biochemistry: sodium, potassium, chloride, bicarbonate, urea, creatinine, corrected calcium, albumin, Alkaline Phosphatase, Bilirubin, Gamma GT, Alanaine aminotransferase, Aspartate aminotranseferase.

2.1.8 *Electrocardiogram (ECG).*

This was performed with the subjects in a supine resting state using a standard Pagewriter ECG machine with a paper speed of 25mm/sec. Standard skin preparation and lead positions were used. The standard 12 leads were recorded simultaneously. All ECGs were analysed by a single observer (MM Lindsay). The criteria used to diagnose left atrial abnormalities were;

1. Terminal forces in V1 equal to or more negative than –0.04mm sec, as obtained from the product of the depth of the terminal negative deflection and its duration.
2. Bipeak interval in deeply notched P wave wider than 0.04sec in any lead.
3. P wave in lead II higher than 0.3mV or wider than 0.12sec.

These criteria were adapted from those reported and validated by Tarazi (1966). Subjects were classified as having left atrial abnormalities if they had one or more of the above criteria on ECG. The presence of LVH and ST-T abnormalities was

defined using the Glasgow Blood Pressure Clinic criteria. (Dunn, 1990) LVH being defined as SV1 + RV5>35mm or deepest precordial S and Tallest R>40mm. LVH with ST-T changes being defined as (a) LVH as above with ST-T depression >1mm and/or T wave inversion >1mm in leads V5 and V6, or (b) LVH as above with ST depression and/or T wave inversion >1mm in any other lead except III, aVr, avl (if p wave inverted) or V1. Finally ST-T changes only was defined as ST-T changes >1mm as above in the absence of LVH.

2.1.9 *Echocardiography.*

Patients were studied using a Vingmed CFM800 sonos and a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral position. Images were stored on videotape and digital archiving facility (Echopac). A standard two dimensional echocardiographic study was performed. Obtaining the standard views: long axis parasternal, short axis parasternal and apical two, four and five chamber views.

M-Mode measurements were taken in the parasternal long axis view the cursor across the tips of the mitral valve leaflets. Measurements were taken of the left ventricular septum, posterior wall and internal dimensions at the end of diastole using the leading edge technique according to the guidelines laid down by the American Society of Echocardiography. (Sahn 1978) Systolic measurements were taken again using the leading edge technique. An average of at least three measurements was taken. Left ventricular mass was calculated using the formula validated by Reichek and Devereux (1977).

Left Ventricular mass=$1.04(IVS+LVID+PWT)^3-(LVID)^3)-14g$

IVS= Interventricular septum

LVID= Left ventricular internal diameter in diastole

PWT= Posterior wall thickness in diastole.

The figure obtained was then divided by the body surface area to give a value for Left Ventricular Mass Index.

Left ventricular systolic function was assessed by M-mode calculation of fractional shortening and "eyeball" assessment. Fractional shortening was derived from the following equation.

Fractional Shortening = (LVID-LVIDs)LVID

LVID= Left ventricular internal dimension in diastole

LVIDs= Left ventricular internal dimension in systole.

Pulsed wave Doppler was used to assess diastolic filling. To obtain optimal velocity measurements the angle between the ultrasonic beam and the mitral inflow was kept to a minimum and in all cases was less than 20%. Measurement of E: A ratio and the E wave deceleration (E dec) time were made in the apical four chamber view with a cursor at the mitral valve inflow using pulsed wave doppler. E:A ratio was measured as the ratio of the peak velocity of the E wave to the peak velocity of the a wave. E wave deceleration time was measured as the time elapsed from the peak of the E wave to the baseline. An average of three measurements was taken at end expiration. Isovolumic relaxation time (IVRT) was measured with the cursor between the mitral inflow and the left ventricular outflow tract in the apical five chamber using pulsed wave Doppler. Measurement was taken from the completion of the aortic forward flow until the onset of the E wave. An average of three measurements in end expiration was taken. A single observer (M Lindsay) made all measurements. Using digital archiving images intra-observer variability was tested in a blinded fashion.

Intra- observer variability was 3.5%, 4%, 10% and 9% for E: A ratio, E deceleration time, IVRT and calculated left ventricular mass index (LVMI) respectively.

2.1.10 *Blood Pressure Measurement.*

The blood pressure was measured using a standard mercury syphgmanometer. Cuff size was chosen such that the bladder was at least 80% of the circumference of the arm. The bladder was placed over the brachial artery and the cuff inflated. Systolic pressure was measured as when the first sounds were heard and the diastolic pressure when the sounds disappeared completely (Phase V). Measurements were taken to the nearest 2mmHG. The pressure in both arms was taken and the highest pressure recorded.

2.1.11 *24Hour Blood pressure measurement.*

An oscillometric spacelab monitor was used. The bladder of the cuff was situated over the brachial artery. Five measurements were performed prior to allowing the subject home to ensure BP monitor was functioning normally and the subject would to tolerate the monitor. Readings were made every 30 minutes through the daytime period and every hour over the nocturnal period. Patients must have at least 1 reading per hour, 80% of total readings successful and at least a total of 30 readings over the 24-hr period for the blood pressure reading to be accepted. All readings taken during this period were documented and mean daytime; mean nocturnal and mean 24 hr blood pressures were calculated. All taped were analysed by one observer (M Lindsay).

2.1.12 *Biochemical Measurement.*

Routine biochemical measurements were taken and analysed in the standard fashion. All samples were taken at a standard time after thirty minutes in the supine position. Samples were immediately centrifuged and the plasma layer removed. The separated

plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen. All samples were run in duplicate.

We used a commercially available TIMP-1 assay obtained from Amersham. This is a modification of the method developed by Plumpton (1995). Standards and diluted plasma were incubated in a microtitre well which is coated with anti-TIMP antibody. After incubation and washing the bound TIMP-1 is measured by reaction with a second peroxidase labelled antibody and the oxidised substrate read in a spectrometer at 450 nm. We have shown linearity to 100ng/ml, all samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma CITP was measured by radioimmunoassay using a polyclonal antibody direct against CITP. (Ristelli, 1993) All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

Renin and aldosterone were measured using a radioimmunoassay through a routine analyser.

2.2 Studies of biochemical evidence of fibrosis in Veteran Athletes.

2.2.1 *Ethical Approval*

Ethical approval was obtained from the local ethics committee. All subjects received a full written and verbal explanation of the investigations involved and the aims of the study. Written consent was obtained in all cases prior to any investigations being performed.

2.2.2 *Inclusion Criteria.*

We recruited a cohort of participating athletes by contacting the Scottish Veteran Harriers Association. A list of members was obtained from the chairman and individual members were contacted by mail with details and aims of the study provided. We aimed to enrol a population of veteran endurance athletes training on a weekly basis and participating in elite veteran events to ensure the presence of exercise induced cardiovascular alterations in a proportion of these subjects. Inclusion criteria therefore consisted of 1. Age>45 years 2. Subjects had to have been competing for more than 10 years at a competitive level 3. High intensity training defined as running more than 30 miles per week 4. Regularly competing in elite veteran endurance events.

2.2.3 *Exclusion Criteria.*

Exclusion criteria were based on two main premise. Firstly the exclusion of subjects with conditions which would result in left ventricular hypertrophy. Namely hypertension (defined as three supine readings greater than 140/90mmHG) and the presence of flow limiting aortic stenosis (defined as an aortic gradient calculated by the modified Beroulli equation of greater than 20mmHG). Secondly as our biochemical markers of fibrosis were not specific for myocardial fibrosis then subjects with any condition which would result in fibrosis and thus give confounding results were excluded. These comprised renal impairment (serum creatinine >130μmol/l), coronary artery disease (defined as symptomatic angina, history of previous myocardial infarction or the presence of ECG evidence of ischaemia or previous myocardial infarction), secondary hypertension, malignancy of any site, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction (defined as any hepatic enzyme double the upper limit of normal on routine

biochemical analysis), left ventricular systolic dysfunction (defined as echocardiographic calculated ejection fraction of <35% or "eyeball" assessment of impaired left ventricular function),COPD (defined as regular use of inhalers, clinical or radiological hyperinflation or greater than 2 respiratory tract infections per year), asthma(defined as the presence of clinical bronchospasm, history of bronchospasm or the use of inhalers) and recent surgery. These conditions were identified by careful history taking, full clinical examination, urinalysis, Chest X-ray, if required, ECG, Exercise stress testing if appropriate and echocardiography.

2.2.4 *Normal volunteers.*

Normal volunteers were recruited via a local advertising campaign in the local press. Exclusion criteria as defined above applied. In addition normal subject were sedentary defined as participating in less than one hour of regular physical exertion per week.

2.2.5 *Baseline Investigations.*

Haematology: haemoglobin, haematocrit and mean cell volume.

Biochemistry: sodium, potassium, chloride, bicarbonate, urea, creatinine, corrected calcium, albumin, Alkaline Phosphatase, Bilirubin, Gamma GT, Alanaine aminotransferase, Aspartate aminotranseferase.

2.2.6*Blood Pressure Measurement.*

The blood pressure was measured using a standard mercury syphgmanometer. Cuff size was chosen such that the bladder was at least 80% of the circumference of the arm. The baldder was placed over the radial artery and the cuff inflated. Systolic pressure was measured as when the first sounds were heard and the diastolic pressure when the sounds disappeared completely (Phase V). Measurements were taken to the nearest 2mmHG. The pressure in both arms was taken and the highest pressure recorded.

2.2.7*Electrocardiograhy.*

This was performed with the subjects in a supine resting state using a standard Pagewriter ECG machine with a paper speed of 25mm/sec. Standard skin preparation and lead positions were used. The standard 12 leads were recorded simultaneously. All ECGs were analysed by a single observer (MM Lindsay). The criteria used to define the presence of LVH, ST-T abnormalities and atrial abnormalities were identical to those described above

2.2.8 *Echocardiograhic study.*

Patients were studied using a Vingmed CFM800 sonos and a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral position. Images were stored on video tape and digital archiving facility (Echopac). A standard two dimensional echocardiographic study was performed. Obtaining the standard views: long axis parasternal, short axis parasternal and apical two, four and five chamber views. M Mode and Doppler measurements were taken in a standardised fashion as described above. Measurement of LV dimensions in diastole and systole were taken and calculation of LVMI made according to the method described by Reichek and Devereux. (1977). The formula is described above. Doppler measurement of E: A ratio as an estimate of diastolic filling was performed by the method again described above.

2.2.9 *Biochemical assessment of Collagen metabolism.*

Routine biochemical measurements were taken and analysed in the standard fashion. All samples were taken at a standard time after thirty minutes in the supine position. Samples were immediately centrifuged and the plasma layer removed. The separated

plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen. All samples were run in duplicate.

We used a commercially available TIMP-1 assay obtained from Amersham. This is a modification of the method developed by Plumpton (1995). All samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma ITCP was measured by radioimmunoassay using a polyclonal antibody direct against ITCP. (Risteli 1993) All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

2.3 Studies of biochemical evidence of fibrosis in "White Coat" and mild Hypertension.

2.3.1 *Ethical Approval*

Ethical approval was obtained from the local ethics committee. All subjects received a full written and verbal explanation of the investigations involved and the aims of the study. Written consent was obtained in all cases prior to any investigations being performed.

2.3.2 *Recruitment.*

Subjects were recruited from our Hypertension clinic and direct referral from local General Practitioners as described above.

2.3.3 *Normal Volunteers.*

Normal subjects were recruited via a local advertising campaign as described above.

2.3.4 *Inclusion Criteria.*

Subjects were aged 18 or over. And had been provisionally diagnosed but never treated as hypertensive by their general Practitioner. Subjects had three supine blood pressure readings greater than 140/90mmhg. However had mean 24 hour ambulatory blood pressure readings of less than 140/90mmhg. Blood pressure is normally distributed variable and definitions of normal and abnormal therefore have to be made in an arbitrary fashion. Therefore the definition of what is a truly normal 24hour ambulatory blood pressure recording is variable. Therefore this cohort was then subdivided and analysed in three separate cohorts 1. Subjects with mean daytime systolic blood pressure of greater than 140mmhg. 2. Subjects with mean daytime systolic blood pressure of 135-140mmHg. 3. Mean daytime blood pressure of less than 135/85mmhg. This cutoff meets with the definition provided by the British Hypertension Society.

2.3.5 *Exclusion criteria.*

The biochemical assays used in this study are not specific for myocardial fibrosis. Therefore careful screening of the patients to exclude conditions which could result in fibrosis was undertaken. Therefore exclusion criteria were conditions that were known to result in myocardial or tissue fibrosis. These comprised renal impairment (serum creatinine >130μmol/l), coronary artery disease (defined as symptomatic angina, history of previous myocardial infarction or the presence of ECG evidence of ischaemia or previous myocardial infarction), secondary hypertension, malignancy of any site, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction (defined as any hepatic enzyme double the upper limit of normal on routine biochemical analysis), left ventricular systolic dysfunction (defined as echocardiographic calculated ejection fraction of <35% or "eyeball"

assessment of impaired left ventricular function),COPD (defined as regular use of inhalers, clinical or radiological hyperinflation or greater than 2 respiratory tract infections per year), asthma(defined as the presence of clinical bronchospasm, history of bronchospasm or the use of inhalers) and recent surgery. These conditions were identified by careful history taking, full clinical examination, urinalysis, Chest X-ray, if required, ECG, Exercise stress testing if appropriate and echocardiography.

2.3.6 *Study Conditions.*

Clinical investigations were performed on one day at a standard time. Blood pressure measurement was taken in a quiet room after 15 minutes rest. Biochemical assays were taken immediately following this with venesection of 20ml of venous blood. A full cardiovascular history and systemic enquiry was taken. A full clinical examination of the cardiovascular, respiratory and abdominal systems was performed. A random urine sample was obtained and urinalysis performed.

2.3.7 *Baseline Investigations.*

Haematology: haemoglobin, haematocrit and mean cell volume.

Biochemistry: sodium, potassium, chloride, bicarbonate, urea, creatinine, corrected calicum, albumin, Alkaline Phosphatase, Bilirubin, Gamma GT, Alanaine aminotransferase, Aspartate aminotranseferase.

2.3.8 *Electrocardiography.*

This was performed with the subjects in a supine resting state using a standard Pagewriter ECG machine with a paper speed of 25mm/sec. Standard skin preparation and lead positions were used. The standard 12 leads were recorded simultaneously. All ECGs were analysed by a single observer (MM Lindsay). The criteria used to define the presence of LVH, ST-T abnormalities and atrial abnormalities were identical to those described above

2.3.9 *Blood Pressure Measurement.*

The blood pressure was measured using a standard mercury syphgmanometer. Cuff size was chosen such that the bladder was at least 80% of the circumference of the arm. The baldder was placed over the radial artery and the cuff inflated. Systolic pressure was measured as when the first sounds were heard and the diastolic pressure when the sounds disappeared completely (Phase V). Measurements were taken to the nearest 2mmHG. The pressure in both arms was taken and the highest pressure recorded.

2.3.10 *24Hour Blood pressure measurement.*

An oscillometric spacelab monitor was used. The bladder of the cuff was situated over the brachial artery. Five measurements were performed prior to allowing the subject home to ensure BP monitor was functioning normally and the subject would to tolerate the monitor. Readings were made every 30 minutes through the daytime period and every hour over the nocturnal period. Patients must have at least 1 reading per hour, 80% of total readings successful and at least a total of 30 readings over the 24-hr period for the blood pressure reading to be accepted. All readings taken during this period were documented and mean daytime; mean nocturnal and mean 24 hr blood pressures were calculated. All taped were analysed by one observer (M Lindsay).

2.3.11 *Echocardiograhic study.*

Patients were studied using a Vingmed CFM800 sonos and a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral position. Images were stored on video tape and digital archiving facility (Echopac). A standard two dimensional echocardiographic study was performed. Obtaining the standard views: long axis parasternal, short axis parasternal and apical two, four and

five chamber views. M Mode and Doppler measurements were taken in a standardised fashion as described above. Measurement of LV dimensions in diastole and systole were taken and calculation of LVM made according to the method described by Reichek and Devereux. (1977) Measurement of LVM was then indexed for body surface area to provide LVMI. The formula is described above. Doppler measurements of E: A ratio, E wave deceleration time and Isovolumic relaxation time (IVRT) as estimates of diastolic filling were performed by the method again described above.

2.3.12 *Biochemical Measurement.*

Routine biochemical measurements were taken and analysed in the standard fashion. All samples were taken at a standard time after thirty minutes in the supine position. Samples were immediately centrifuged and the plasma layer removed. The separated plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen. All samples were run in duplicate.

We used a commercially available TIMP-1 assay obtained from Amersham. This is a modification of the method developed by Plumpton (1995). All samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma ITCP was measured by radioimmunoassay using a polyclonal antibody direct against ITCP. (Risteli, 1993) All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

Renin and aldosterone were measured using a radioimmunoassay through a routine analyser.

2.4 Studies in Normal Volunteers.

As described above normal volunteers were recruited from a local advertising campaign and from friends and colleagues within the Cardiology department at Stobhill Hospital. 50 normal subjects were enrolled.

Exclusion criteria consisted of hypertension, aortic stenosis, regular aerobic exercise and conditions that may result in tissue fibrosis. A detailed list of these conditions is detailed in previous section.

All normal volunteers underwent detailed history and physical examination to exclude the aforementioned conditions.

2.4.1 *Ethical Approval and consent.*

Ethical approval was obtained from the local ethics committee. Verbal and written consent was obtained in all cases.

2.4.2 *Study Conditions.*

Clinical investigations were performed on one day at a standard time. Blood pressure measurement was taken in a quiet room after 15 minutes rest. Biochemical assays were taken immediately following this with venesection of 20ml of venous blood. A full cardiovascular history and systemic enquiry was taken. A full clinical examination of the cardiovascular, respiratory and abdominal systems was performed. A random urine sample was obtained and urinalysis performed.

2.4.3 *Baseline Investigations.*

Haematology: haemoglobin, haematocrit and mean cell volume.

Biochemistry: sodium, potassium, chloride, bicarbonate, urea, creatinine, corrected calcium, albumin, Alkaline Phosphatase, Bilirubin, Gamma GT, Alanaine aminotransferase, Aspartate aminotranseferase.

2.4.4*Blood Pressure Measurement.*

An average of three supine blood pressure recordings were taken using a mercury sphygmomanometer in a standardised fashion detailed above.

2.4.5*Electrocardiogram.*

Standard 12 lead electrocardiograms were obtained. Each was analysed for the presence of voltage criteria for LVH, the presence of ST-T changes and atrial abnormalities using predefined criteria described above.

2.4.6*Echocardiography.*

Each subject underwent echocardiographic study using a standardised protocol described above.

2.4.7 *Biochemical Measurement.*

Routine biochemical measurements were taken and analysed in a standard fashion. Samples were prepared as described above and measurement of TIMP-1, CITP and P1CP were made using the assays and techniques described above.

All physical examinations, Blood pressure measurement, ECG analysis and Echocardiograms were performed by a single operator. (M Lindsay).

The results of these investigations on normal volunteers are presented below.

2.4.8 Results for Normal Volunteers.

Table 2.1 : Baseline characteristics±SEM

Parameter	Normal Volunteers
Number	50
Sex (male/female)	23/27
Age (years)	52.1±1.5
Smoker (yes/no/ex)	35/7/8
Systolic Blood Pressure (mmHg)	129±2.6
Diastolic Blood Pressure (mmHg)	77±2
Urea	5.1+0.3
Creatinine	97±2.5
Gamma GT	30±5.6

Table 2.2: Electrocardiograhic data.

Parameter	Number
LVH	0
ST-T change	0
Atrial Abnormalities.	4

Table 2.3: Echocardiographic data. (mean ±SEM)

Parameter	Normal volunteers.
Interventricular Septal thickness in diastole (cm)	0.9±0.03
Left Ventricular Internal Diameter in diastole(cm)	4.8±0.07
Left ventricular posterior wall thickness in diastole (cm)	0.91±0.02
Relative wall thickness	0.37±0.01
Left ventricular Mass Index (g/m^2)	97±3
Maximal E wave Velocity (cm/s)	0.73±0.03
Maximal A wave velocity (cm/s)	0.70±0.03
E:A ratio	1.15±0.03
Isovolumic Relaxation Time (ms)	100±0.03
E wave decleration time(ms)	19.3±0.9
Aortic velocity (m/s)	1.07±0.04
Fractional Shortening (%)	40±1.6
Left Ventricular ejection fraction (%)	77±2
Left Atrial Diameter in systole (cm)	3.3±0.8

Table 2.4 Biochemical markers of Collagen metabolism.

Parameter	Mean	Standard Error of Mean	Standard Deviation.	Range.
TIMP-1(ηg/l)	253	11	76	99-421
CITP(μg/l)	2.9	0.15	0.8	1.8-5.1
P1CP(μg/l)	166	9	48	85-262

Table 2.5 Markers of the Renin Angiotensin system. (mean±SEM)

Parmeter	Normal volunteers.
Aldosterone (ηg/100ml)	9.3±1
Renin	15±2

Chapter 3:

Validation and Standardisation of biochemical assays and echocardiographic measurement.

Validation of Biochemical Assays.

3.1 Introduction.

As discussed in the introduction, biochemical assessment of the collagen equilibrium has increasingly been used for non-invasive monitoring of end-organ fibrosis. This has been demonstrated in numerous conditions. For example, pulmonary fibrosis (Montano, 1989), hepatic fibrosis (Li, 1994), bone metabolism and renal disease.

The measurement of these markers forms a major part of this thesis and therefore requires careful validation. To achieve this we performed intra-assay variability on three assays used. This was not a longitudinal study therefore inter-assay variability was not performed for the purposes of this thesis. However, inter-assay and batch variability has been separately assessed within our laboratory with satisfactory results, 4.9% for TIMP-1, 6.9% forP1CP and 6.4% for CITP. Every effort was made to standardise sample collection and measurement. The venous blood samples were taken in standard conditions and at a standard time of day to exclude any effect diurnal variation may have on measurement. The samples were immediately centrifuged at 3000rpm for seven minutes and the plasma layer was removed. This was then divided into equal aliquots and stored at –80oC. The samples were not thawed and refrozen on any occasion. Several preliminary analyses of normal samples were performed prior to analysis of the study subjects to ensure adequate reproducibility for each of the assays. All samples were analysed in duplicate.

The assays were purchased as commercially available kits. Every effort was made to maintain quality control of the assay. Inter-assay and batch variability was tested as described above with limited variability.

3.2 Methods

3.2.1 Assay variability

To adequately assess assay variability we chose samples from our cohort of normal volunteers, hypertensive subjects, white coat hypertensives and athletes. This allowed assessment of variability across the full range of absolute values for each of the three biochemical assays used. Our laboratory technician who had no prior knowledge of the source of the samples randomly selected samples to be analysed. Intra-assay variability was tested by running the samples in duplicate (which was our normal practice throughout the study).

3.3 Results

3.3.1 Assay variability.

The results for each of the three assays are expressed in the form of absolute and percentage difference. The raw data is presented in tabular format and the results are also presented in graphical format as a Pearson's correlation and in a Bland-Altman graph.

3.3.2 TIMP-1

Forty samples were analysed for intra-assay variability of TIMP-1. These are presented in tabular format (Table 3.1). The absolute values of the samples studied ranged from 107nanograms/ml to 764 nanograms/ml and therefore was a representative selection. The intra-assay variability of TIMP-1 was 4.6% (95% CI 3.9-5.2). The correlation co-efficient was 0.998. There was no variation in variability across the range of absolute values.

Table 3.1: Intra-assay variability for Plasma TIMP-1. Absolute and percentage differences are shown.

TIMP Sample 1 (nanograms/ml)	**TIMP Sample 2 (nanograms/ml)**	**Absolute Difference**	**% difference.**
205	213	8	3.8
118	125	7	5.8
146	152	6	4
108	115	7	6.2
241	256	15	6
121	126	5	4
162	168	6	3.6
117	129	12	9.8
110	118	8	7
152	158	6	3.9
111	117	6	5.3

135	146	11	7.8
102	104	2	1.9
248	257	9	3.6
146	153	7	4.7
102	103	1	0.9
286	296	10	3.4
121	126	5	4
356	364	8	2.2
764	774	10	1.3
243	250	7	2.9
114	122	8	6.8
186	194	8	4.2
110	110	0	0
165	170	5	3
250	263	13	5.1
147	158	11	7.2
133	139	6	4.4
107	111	4	3.7
153	165	12	7.5
191	203	12	6.1
128	136	8	6.1
107	109	2	1.9
194	202	8	4
136	145	9	6.4

123	131	8	6.3
141	146	5	3.5
356	367	11	3
308	323	15	4.7
113	121	8	6.8

Figure 3.1: Bland Altman graph illustrating intra-assay variability of plasma TIMP-1.

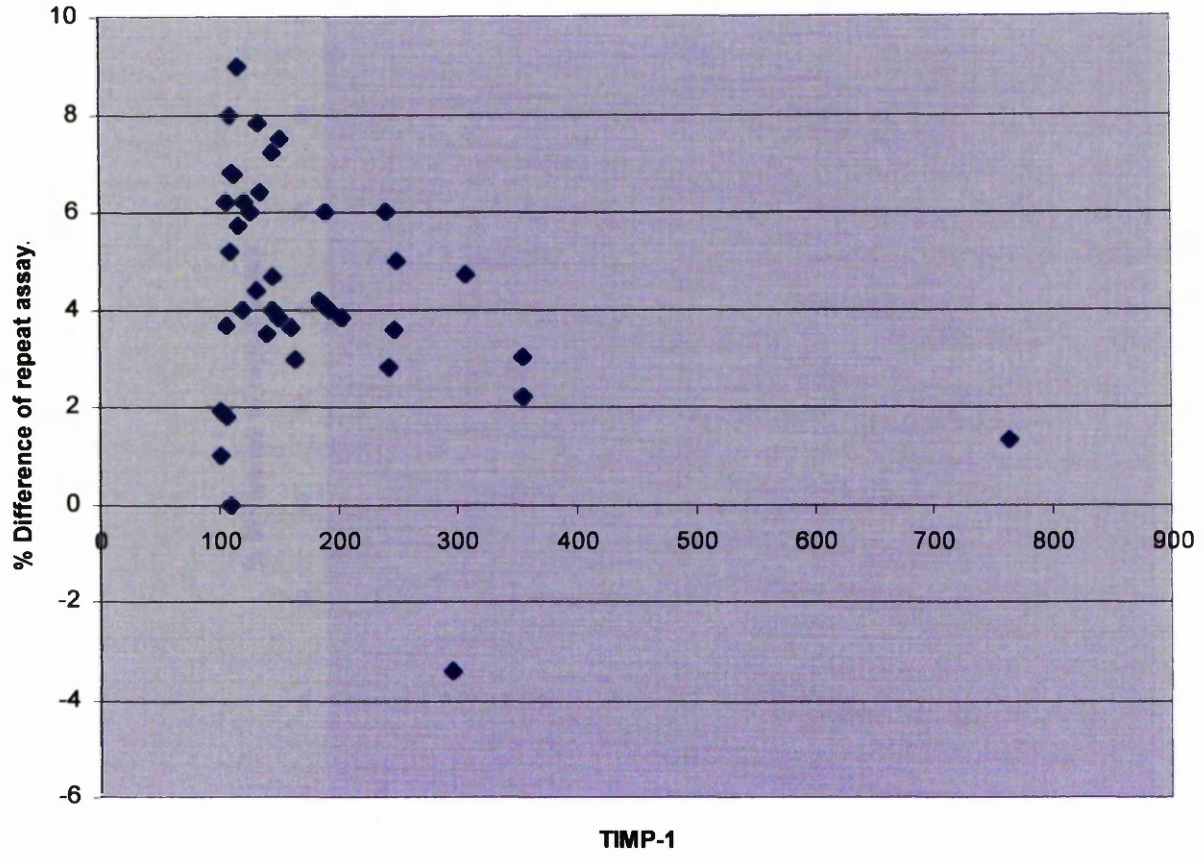
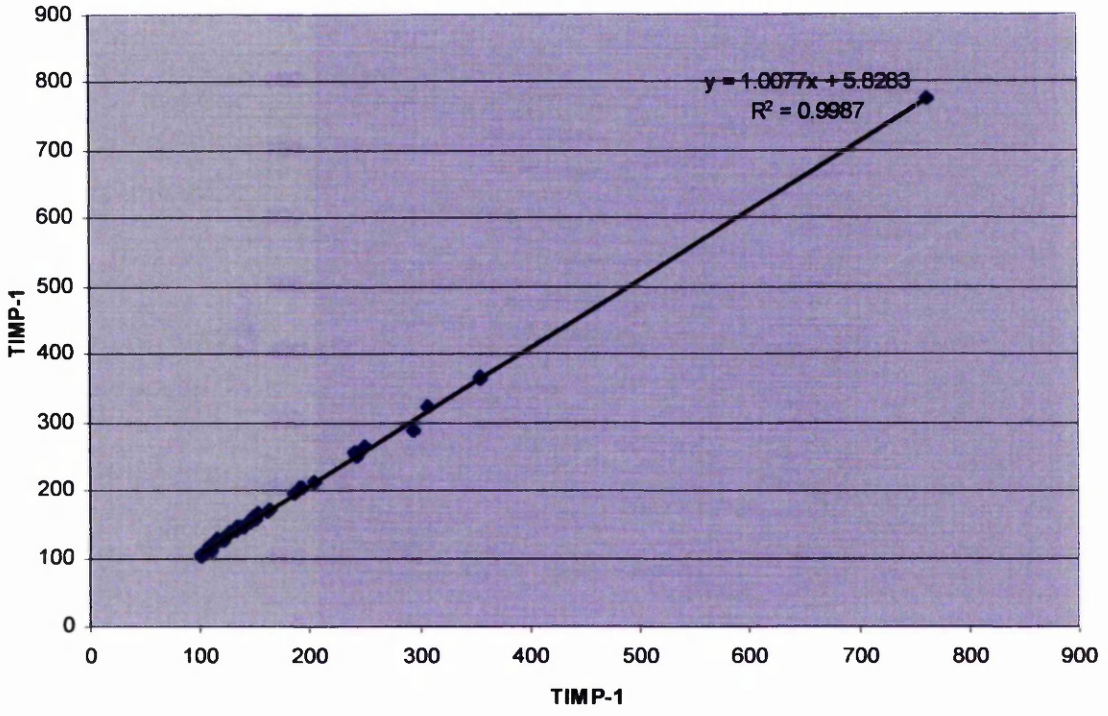


Figure 3.2: Scatterplot graph illustrating intra-assay variability of plasma TIMP-1.



3.3.3 CITP

Forty samples were analysed to assess intra-assay variability of the CITP assay. The range studied was 2.4μg/l to 8.9μg/l and therefore again was a representative sample of all our study populations. The raw data is presented in tabular format below in Table 3.2. The intra-assay variability of CITP was 4.4% (95% CI 3.2-5.6). The correlation co-efficient was 0.95. There was no variation in variability across the range of absolute values.

Table 3.2. Intra-assay variability for plasma CITP comparing samples 1 and 2. Absolute and percentage differences are shown.

CITP Assay 1	***CITP Assay 2***	**Absolute Difference**	**% Difference.**
3.4271	3.4164	0.0107	0.312701
4.9664	4.9993	0.0329	0.660285
3.8824	3.8403	0.0421	1.090306
3.962	4.557	0.595	13.98158
4.1611	3.9908	0.1703	4.178526
4.2453	3.8279	0.4174	10.34525
2.4355	2.416	0.0195	0.802932
3.2678	3.5521	0.2843	8.3392
3.414	3.5654	0.1514	4.33873
3.4967	3.1794	0.3173	9.508256
4.4027	4.391	0.0117	0.266103

4.2979	4.3069	0.009	0.209186
3.5147	3.5027	0.012	0.342007
3.5267	3.269	0.2577	7.585435
4.6563	4.5176	0.1387	3.023961
5.2914	5.0254	0.266	5.157438
4.8089	4.9555	0.1466	3.002868
4.6616	4.9132	0.2516	5.256231
3.5533	3.6819	0.1286	3.55504
3.3782	2.9888	0.3894	12.23643
4.4261	4.643	0.2169	4.900477
3.8601	4.2146	0.3545	8.783449
3.9421	3.87	0.0721	1.845878
3.9109	3.8973	0.0136	0.348352
3.2218	3.0564	0.1654	5.269362
3.9271	3.71	0.2171	5.686074
4.7109	4.6191	0.0918	1.967888
6.8633	6.1342	0.7291	11.23196
8.8998	8.8766	0.0232	0.26102
6.4913	5.9706	0.5207	8.361703
7.6106	7.3361	0.2745	3.673617
4.5768	4.557	0.0198	0.433554
5.8742	5.7921	0.0821	1.407485
6.0513	5.5559	0.4954	8.540938
4.6178	4.7056	0.0878	1.883473

4.7056	4.7189	0.0133	0.28224
4.2979	4.3069	0.009	0.209186
4.3936	4.4027	0.0091	0.206908
5.9419	6.3987	0.4568	7.406687
6.3844	5.8413	0.5431	8.890453

Figure 3.3: Bland Altman graph illustrating intra-assay variability in Plasma CITP.

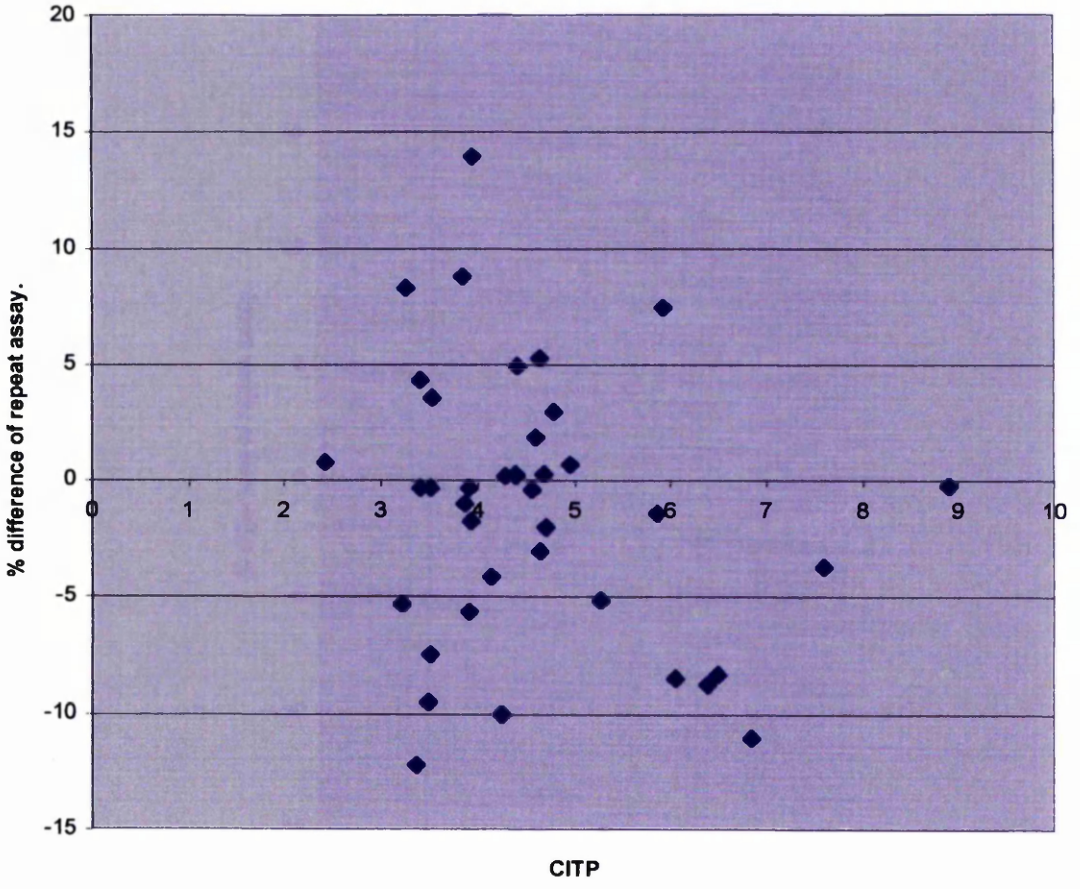
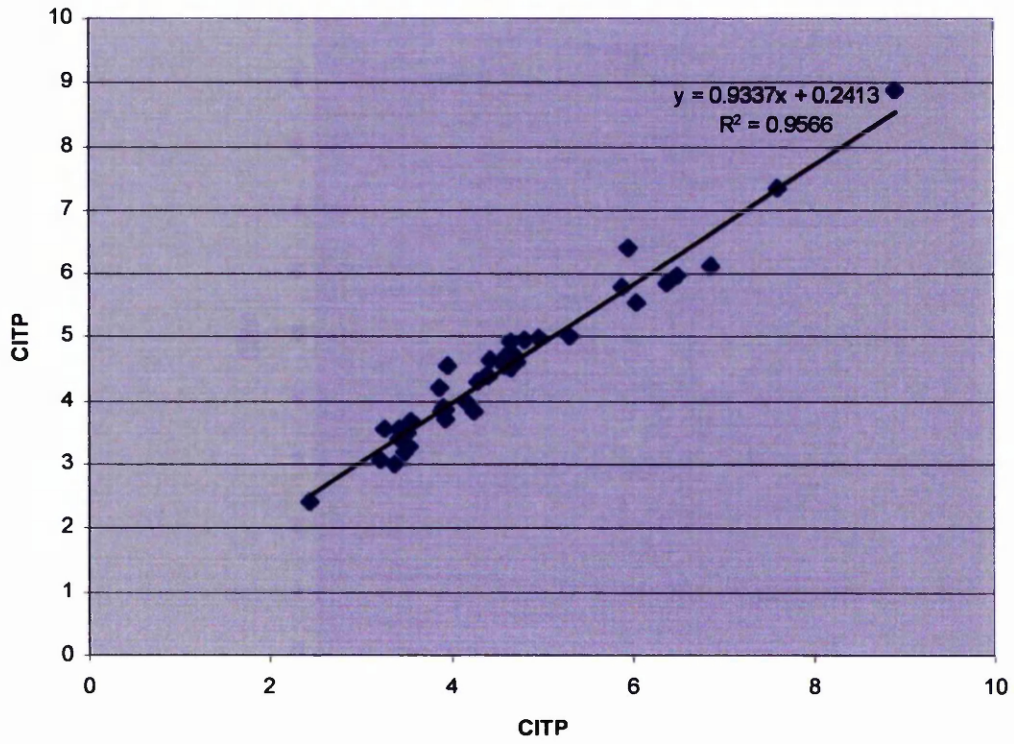


Figure 3.4: **Scatterplot graph illustrating intra-assay variability in Plasma CITP.**



3.3.4 P1CP

Forty samples were analysed to assess intra-assay variability of the plasma P1CP assay. The range of absolute values studied was 82μg/l to 216μg/l and therefore again was a representative sample. The raw data is presented below in tabular format in Table 3.3. The intra-assay variability of the P1CP assay was 4.2% (95% CI 3.3-5.3%). The correlation co-efficient was 0.93. There was no variation in variability across the range of absolute values.

Table 3.3: Intra-assay variability for plasma P1CP comparing sample 1 and 2. Absolute and percentage differences are shown.

P1CP assay 1	P1CP assay 2	Absolute Difference	% Difference.
113.32	118.18	4.86	4.112371
111.67	102.76	8.91	7.978866
143.49	147.61	4.12	2.832199
144.69	144.04	0.65	0.449267
158.96	155.58	3.38	2.126321
199.85	190.73	9.12	4.563423
124.2	130.89	6.69	5.247882
106.15	105.31	0.74	0.794476

108.43	99.723	8.707	8.376142
160.17	147.73	12.44	8.091057
190.18	199.78	9.6	4.926361
216.63	206.29	10.34	4.892359
131.02	126.29	4.73	3.6775
151.49	132.98	18.51	12.21863
149.65	139.04	10.65	7.386088
132.95	127.23	5.72	4.398647
107.51	109.6	2.09	1.92538
118.16	117.26	0.9	0.764591
151.53	149.25	2.28	1.516159
148.64	163.11	14.47	9.299486
158.2	138.39	19.81	13.40597
160.72	155.07	5.65	3.579347
148.92	149.29	0.37	0.248456
134.78	131.08	3.7	2.783839
130.23	143.69	7.46	5.456806
117.07	112.72	4.35	3.787219
115.91	128.52	12.61	10.33861
130.56	119.83	10.73	8.582627
104.49	104.23	0.26	0.249138
103.6	101.55	2.5	2.43736
111.7	116.43	4.73	4.148031
114.93	111.26	3.67	3.245777

103.5	103.71	0.21	0.202683
98.368	100.01	1.642	1.655509
113.67	116.07	2.4	2.0895
112.74	112.51	0.23	0.204208
158.38	155.5	3.12	1.988148
82.76	79.949	2.811	3.455822
105.43	101.49	3.94	3.80934
110.29	110.75	0.46	0.416214

Figure 3.5: Bland Altman graph illustrating intra-assay variability in plasma P1CP.

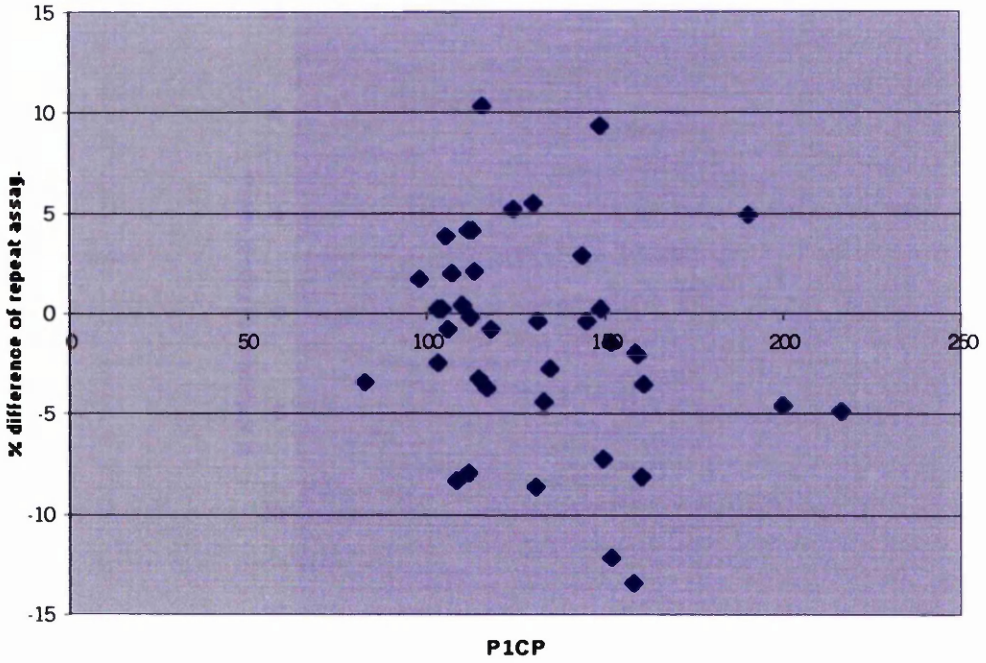
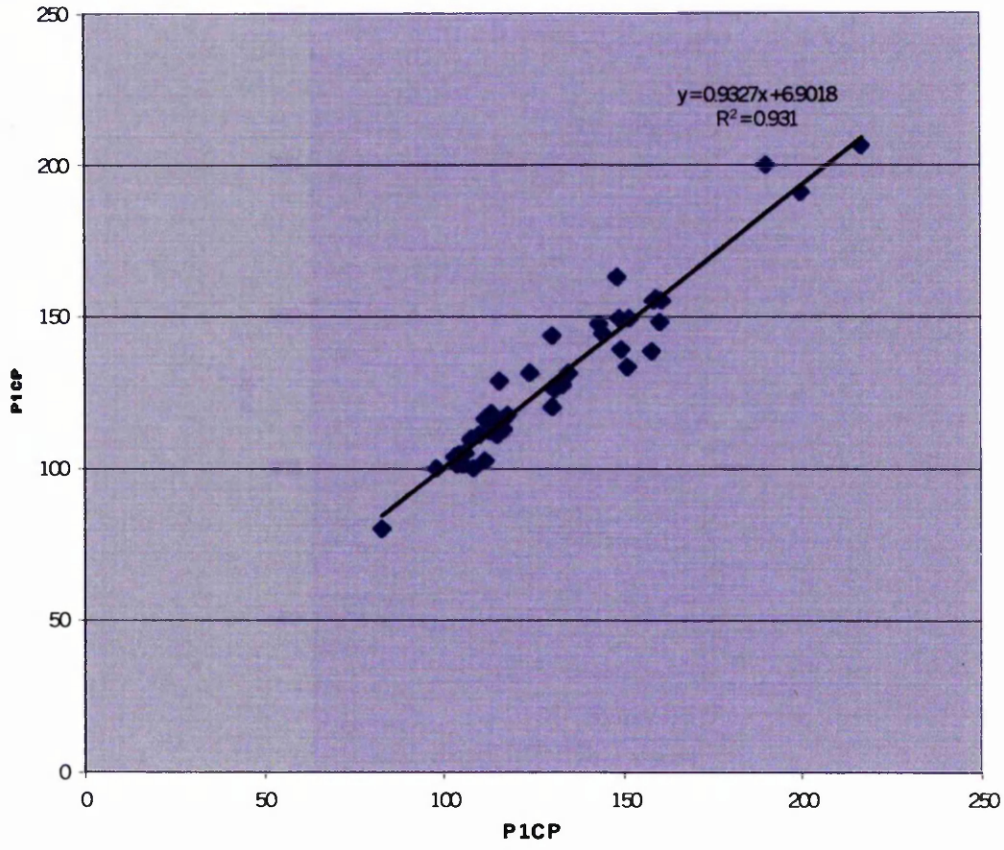


Figure 3.6: Scatterplot illustrating intra-assay variability of Plamsa P1CP.



3.3.5 Conclusions.

We have demonstrated that all three assays are robust and have limited intra-assay variability. This compares favourably with results published by other centres using similar techniques. (Laviades, 1998) (Diez, 1995).

Standardisation of Echocardiographic Measurement.

3.4.1 Introduction.

It should be accepted that variability is inherent in the technique of echocardiography. Therefore every effort was made to limit variability and improve reproducibility.

Patient position was standardised as much as possible allowing for some variation in achieving optimal imaging.

Respiration has been shown to have a significant effect on echocardiographic measurement. Therefore all measurements were taken at end expiration.

All measurements were taken as a mean of a minimum of three readings.

All echocardiographic studies and interpretation was performed by one observer (M Lindsay)

3.4.2 Methods

30 subjects from both the hypertensive and normal cohorts were selected at random to test intra-observer variability. M Mode and doppler images stored on a digital archiving facility (Vingmed Echopac) were re-examined and repeat measurements were made by M Lindsay who was blinded to the original results. The digital archiving facility allowed re-analysis without access to the original measurements. The results for the M Mode and Doppler indices are presented below. The results are presented in tabular format as a mean of the first and second measurements, the mean of the absolute difference between individual measurements, the mean percentage difference and the standard error of mean difference.

Results.

3.4.3 M-Mode Measurement.

Table 3.4: M-mode variability.

Parameter	Mean of Measurement 1	Mean of measurement 2	Mean of difference of Measurements	Mean of % difference In measurements	Standard error of mean difference.
IVSd (cm)	0.958	0.963	0.049	4.5	0.01
LVPWd (cm)	0.917	0.878	0.057	6.2	0.01
LVDd (cm)	4.86	4.76	0.136	2.8	0.02

IVSs (cm)	1.48	1.44	0.09	5.2	0.016
LVPWs (cm)	1.57	1.53	0.13	9.2	0.02
LVDs (cm)	2.88	2.91	0.16	5.4	0.03
EF (%)	78	76	4	4.6	0.5
FS (%)	40	39	3.5	8.6	0.5
LVM (g)	207	197	14	7	1.7
LVMI (g/m^2)	104	99	8	8	1

3.4.4 Doppler measurement.

Table 3.5: Doppler Variability.

Parameter	Mean of Measurement 1	Mean of measurement 2	Mean of difference of Measurements	Mean of % difference In measurements	Standard error of mean difference.
E velocity	0.74	0.73	0.02	3	0.003
A velocity	0.70	0.69	0.02	3	0.003
E: A ratio	1.08	1.07	0.03	3	0.006
E Deceleration time (ms)	20	20	2.5	5	0.13

IVRT (sec)	0.11	0.11	0.01	10	0.001

3.4.5 Conclusions.

Despite echocardiography being a technique with inherent variability by using high quality imaging machines and standardised techniques we have demonstrated limited variability in all measurements.

Chapter 4

Investigation of Collagen Turnover in Untreated Essential Hypertension.

4.1 Abstract

This study was designed to document non-invasively the pathological mechanisms responsible for myocardial fibrosis and to assess the clinical utility of plasma markers of collagen synthesis and degradation as screening tools for the assessment of fibrosis in hypertension. We studied 100 never treated hypertensive patients and 50 normal subjects. Echocardiographic assessment was made of LV mass and diastolic filling using measurement of E: A ratio, E wave deceleration time (E dec) and isovolumic relaxation time (IVRT). The presence of diastolic dysfunction was taken as a surrogate marker for the presence of myocardial fibrosis. Plasma carboxy-terminal propeptide of collagen type I (PICP), carboxy-terminal telopeptide of collagen type I (CITP) and tissue inhibitor of matrix metalloproteinases type I (TIMP-1) were measured as markers of collagen synthesis, degradation and inhibition of degradation respectively. Plasma TIMP-1 was significantly elevated in the hypertensive cohort (358ηg/l Vs 253ηg/l $p<0.001$) as were CITP (5.2μg/l Vs 2.9μg/l $p<0.001$) and PICP (200μg/l Vs 166μg/l $P<0.05$). TIMP-1 was significantly elevated in patients with diastolic dysfunction (421ηg/l Vs 283ηg/l $p<0.01$) and correlated with markers of diastolic filling namely E: A ratio ($r=0.26$ $p<0.05$) and E Dec ($r=0.41$ $P<0.01$). A plasma TIMP-1 level of >500ηg/l had a specificity of 97% and a positive predictive value of 96% in predicting diastolic dysfunction. In patients with untreated hypertension there is evidence of increased collagen synthesis, degradation and inhibition of degradation resulting in fibrosis. Our results demonstrate that Plasma TIMP-1 correlates with markers of LV diastolic filling, is predictive of LV dysfunction and is a potential non-invasive marker of fibrosis.

4.2 Introduction.

Left ventricular hypertrophy when defined both by ECG and Echo criteria (Devereux 1993, Kannel 1970, Kregger 1987) confers an increased cardiovascular risk in patients with hypertension independent of blood pressure level. (Dunn 1990)

The two key pathological processes in LVH comprise hypertrophy of the myocyte and fibrosis of the interstitium. The fibrosis results from an increase in the extracellular matrix and more specifically an increase in the concentrations of collagen type I and III (Jalil 1989). This is both a primary fibrosis and a secondary reparative fibrosis following myocyte necrosis (Weber 1995). These findings have been demonstrated in both post-mortem studies (Rossi 1998, Tanaka 1986) and studies utilising endomyocardial biopsies (Ciulla 1997).

This accumulation results in a distortion of tissue structure and an increase in myocardial stiffness. This has pathological significance in the development of diastolic dysfunction, as a substrate for ventricular arrhythmias and ultimately in the development of systolic dysfunction (Weber 1993).

Due to the pathological significance of fibrosis an understanding of the mechanisms involved and a means of accurately monitoring this process is the subject of considerable research. Cardiac biopsy is undoubtedly the gold standard in this regard but has limitations being an invasive procedure with an appreciable morbidity rate in a patient group who do not obviously benefit form this procedure. Thus the possibility that the pathophysiological mechanisms central to this process can be demonstrated and the process can be monitored non-invasively using markers of

collagen synthesis and degradation is particularly attractive. Preliminary data is now available in this field, most notably from Diez et al who have demonstrated that Procollagen type I carboxy-terminal propeptide (P1CP), a marker of collagen synthesis, correlates significantly with fibrosis in endomyocardial biopsies (Querejeta 2000). This work demonstrates a potential role of these markers as surrogates of fibrosis and requires further investigation.

We studied a large cohort of never treated hypertensives to achieve two main aims. The first was to document non-invasively the pathological mechanisms responsible for fibrosis and the second to assess the clinical utility of a number of non-invasive markers of fibrosis in this group of patients.

This was achieved firstly by a full cardiac assessment of each patient with ECG, echo and ambulatory blood pressure studies. Secondly all patients had a full non-invasive assessment of cardiac fibrosis facilitating a full assessment of the pathological mechanisms responsible for fibrosis in hypertension.

Studies have shown that interstitial fibrosis results in LV diastolic dysfunction (Brilla 1991, Conrad 1995, Thiedeman 1983) and subsequent regression of this fibrosis with ACE-inhibitors results in improvement in parameters of LV diastolic filling (Brilla 2000). We therefore took the presence of diastolic dysfunction using conventional echo parameters as non-invasive evidence of interstitial fibrosis in our study.

4.3 Methods.

Subjects.

The study population consisted of 100 untreated hypertensive patients with hypertension diagnosed on 24-hr blood pressure monitoring (mean 24hr BP greater

than 140/90 mmHG). Patients were enrolled via referral to our hypertension clinic or by direct referral from local general practitioners. All patients gave consent and the local research ethics committee approved study protocols.

Exclusion criteria comprised conditions that are known to result in myocardial or tissue fibrosis. These comprise renal impairment (serum creatinine >130mmol), coronary artery disease, secondary hypertension, malignancy of any site, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction, left ventricular systolic dysfunction and COPD.

The control subjects were enrolled through adverts in local press. The normal subjects were determined to be normotensive on three standard BP readings and the above noted exclusion criteria were applied to this group.

Study conditions.

Patients were studied at a standard time in the morning on one study day. A full history and clinical examination was made to identify exclusion criteria and suitability for the study.

ECG

All patients received a standard twelve lead ECG. ECGs were analysed for the presence of LVH and ST-T changes using standard criteria (Dunn 1990). Additionally the presence of atrial abnormalities was assessed using pre-defined criteria (Tarazi 1966). All ECGs were analysed by a single observer. (M Lindsay).

24hr Blood Pressure Monitoring.

24 hr BP recordings were taken using a standard oscillometric Spacelab BP monitor. Readings were made every 30 minutes through the daytime period and every hour over the nocturnal period. Patients must have at least 1 reading per hour, 80% of total readings successful and at least a total of 30 readings over the 24-hr period for the blood pressure reading to be accepted. All readings taken during this period were documented and mean daytime; mean nocturnal and mean 24 hr blood pressures were calculated. All taped were analysed by one observer (M Lindsay)

Echocardiographic study.

Patients were studied using a Vingmed CFM800 sonos and a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral position. Measurements were taken according to the guidelines laid down by the American Society of Echocardiography (Sahn 1978). Left ventricular mass was calculated using the formula validated by Reichek and Devereux (Devereux 1977) and indexed for body surface area. An average of at least three measurements was taken. Measurement of E: A ratio, E deceleration time and isovolumic relaxation times (IVRT) were made in the apical view with a cursor at the mitral valve inflow. An average of three measurements was taken at end expiration. A single observer (M Lindsay) made all measurements. Using digital archiving images intraobserver variability was tested in a blinded fashion. Intra- observer variability was 3.5%, 4%, 10% and 9% for E: A ratio, E deceleration time, IVRT and calculated left ventricular mass index (LVMI) respectively.

Biochemical Measurements.

Routine biochemical measurements were taken and analysed in the standard fashion. All samples were taken at a standard time after thirty minutes in the supine position. Samples were immediately centrifuged and the plasma layer removed. The separated plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen.

We measured plasma P1CP as a marker of collagen synthesis, carboxy-terminal telopeptide of collagen type I (CITP) as marker of extracellular collagen type 1 degradation and tissue inhibitor of matrix metalloproteinase type I (TIMP-1) as a marker of inhibition of collagen degradation.

Plasma TIMP-1 was measured using a commercially available two stage ELISA assay specific for TIMP-1available from Amersham Pharamaceuticals. All samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma CITP was measured by radioimmunoassay using a polyclonal antibody direct against CITP (Ristelli 1993). All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

Renin and aldosterone were measured using a radioimmunoassay through a routine analyser.

Statistical analysis.

The distribution of the collagen markers was tested for normality using the Anderson Darling test. CITP was normally distributed whilst both TIMP-1 and P1CP were non-parametric and thus were log transformed prior to analysis. These continuous

variables were then analysed using an unpaired two-tailed student's T test. Data is presented in the non-logarithmic format. Correlations were tested using Pearsons correlation co-efficient in a logarithmic format as required. Non-continuous variables were analysed using a chi-squared test.

4.4 Results.

Baseline characteristics.

I recruited 100 never treated hypertensive subjects and 50 normal subjects. 10 hypertensive subjects were excluded during screening prior to recruitment and analysis (1 subject due to renal impairment, 1 subject due to chronic pulmonary disease, 4 subjects due to poor echo views, 2 subjects with LV dysfunction and 2 subjects with probable angina) and 4 normal subjects were excluded prior to recruitment and analysis (2 subjects with hypertension and 2 subjects with poor echo views).

The baseline characteristics are presented in *table4.1.*±SEM

As would be expected the hypertensive group had a significantly higher blood pressure. There was no significant difference in the age, sex distribution, renal function or hepatic parameters between the two groups.

Table 4.1: Baseline criteria ± 1 SEM.

Characteristic	*Hypertensive*	*Normal*	*P value*
Number	100	50	
Age	52	51	***NS***
Smoker (yes/no/ex)	65/20/13	35/7/8	NS

Sex M/F	49/51	23/27	NS
Office systolic BP mmHG	167±2.3	129±2.6	P<0.001
Office diastolic BP mmHG	104±1.4	77±2	P<0.001
24Hr Systolic BP mmHG	150±1.2		
24Hr Diastolic BP mmHG	93±0.87		
Urea (μmol/l)	5±0.17	5.2±0.3	NS
Creatinine (μmol/l)	96±2.6	97±2.5	NS
Gamma GT (μ/l)	30±3.6	30±5	NS

ECG

Using the defined criteria above 16% of hypertensives and no normals were found to have LVH ($p=0.002$). 14% had ST-T changes and no normals ($p<0.01$), 5% had LVH and strain compared with no normals and 9% had atrial abnormalities compared with 8% of normals in one of the three criteria used.

Ambulatory Blood Pressure.

Readings were on average 90% successful with a mean total of 40 readings over the 24-hr period. Mean 24 hr blood pressure was 151/93 within this group with an average 24hr heart rate of 76/min. Analysis of the daytime readings revealed a mean daytime systolic BP of 155mmHG and diastolic of 97mmHG. Daytime heart rate was averaged at 77/min. Nocturnal mean systolic BP was 140mmHG and diastolic was 84mmHG with a mean nocturnal heart rate of 71/min. there was no relationship between blood pressure measurements and collagen markers.

Echocardiography.

Patients were excluded if poor quality imaging prevented analysis of m-mode and Doppler studies.

Results are summarised in the* table4.2±*SEM

Table 4.2: Echocardiographic Parameters ± 1 SEM.

	HBP	*Normals*	*P Value*
Number	100	50	
IVSd(cm)	1.14±0.02	0.9±0.03	P<0.001
LVPWd (cm)	1.18±0.09	0.9±0.02	P=0.004
LVMI(g/m^2)	132±4	97±3	P<0.001
IVRT(sec)	0.123±0.003	0.099±0.004	P<0.001
E:A Ratio	0.968±0.03	1.16±0.03	P<0.001
E wave Deceleration(ms)	22.5±0.8	19.3±0.9	P=0.02
% With LVH	56%	2%	P<0.01

There was a significant increase in the septal (1.14cm Vs 0.9cm p<0.001) and posterior wall dimensions (1.18cm Vs .9cm p=0.004) within the hypertensive group. Calculated left ventricular mass index was also elevated in the hypertensive cohort (132g/m^2 Vs 97g/m^2 p<0.001). All the markers of diastolic filling i.e. IVRT (0.123sec Vs 0.099sec p<0.001), E: A ratio (0.97 Vs 1.16 p<0.001) and E wave deceleration times (22.5ms Vs 19.3ms p=0.02) were all significantly prolonged in the hypertensive cohort. These measurements reveal our hypertensive cohort had demonstrable evidence of hypertensive heart disease. The control group had echo parameters within the normal range.

Table 4.3. Collagen markers in Hypertensive subjects.

Parameter	Mean	Standard Error of Mean	Range	Standard Deviation
TIMP-1 (ηg/ml)	385	29	83-1704	276
CITP (μg/l)	5.2	0.2	1.8-10.2	1.7
PICP (μg/l)	200	11	72-518	94

TIMP-1

Timp-1 levels were significantly elevated in the hypertensive group as a whole in comparison to normal subjects. (385ηg/l Vs 253ηg/l p=0.0007) (95% C.I. 71,194) (*Figure 4.1.*)

There was no significant difference in TIMP-1 level in the groups with (n=56) and without LVH (n=44)(350ηg/l Vs 450ηg/l p=ns) (95% CI –20,232). Further analysis of the hypertensive group revealed that patients with evidence of diastolic dysfunction as defined by an E: A ratio of less than one (n=63) had a significantly higher TIMP-1 level than patients with hypertension and normal diastolic filling (n=37) (421ηg/l vs283ηg/l p=0.005) (95% CI 44,232). Furthermore there was no significant difference in TIMP-1 levels between normal subjects (n=50) and hypertensive patients with normal diastolic filling (n=37)(253ηg/l Vs 283ηg/l p=ns) (95%CI –83,23)(*Figure 4.2*). TIMP-1 levels correlated with indices of diastolic filling namely E: A ratio(r=0.26 p<0.05)(*Figure4.3*) and E wave deceleration time (r=0.41 p<0.01)(*Figure 4.4*). There was no relationship between TIMP-1 levels and indices of left ventricular mass (*Figure 4.5*). These findings would suggest that TIMP-1 is a marker of fibrosis and is independent of both blood pressure and markers of left ventricular mass

In regard to the ECG; TIMP-1 levels were found to be higher in patients with LVH (n=16) (475ηg/l Vs 337ηg/l p=0.05)(95% CI 2,273) and patients with ST-T changes (n=14) (530ηg/l Vs 335ηg/l p<0.05) (95% CI 7,396). There was no relationship between P1CP or CITP levels and the presence or absence of ECG changes.

To assess whether an elevated TIMP-1 was truly predictive of diastolic dysfunction and hence fibrosis we analysed hypertensive patients with a TIMP-1>500ηg/l. We chose this figure as it represented one standard deviation above the maximum TIMP-1 level within the normal group and this constituted approximately 20% of the cohort (19 patients). This cohort had an elevated E wave deceleration time and an abnormal E: A ratio confirming the presence of diastolic dysfunction within this cohort. This was independent of the confounding variables of age, heart rate and LVMI. Thus reflecting a group with genuinely impaired diastolic filling. Indeed a TIMP-1 level of over 500ηg/l in our study showed a specificity of 97% and a positive predictive value in predicting diastolic dysfunction of 96%. (*Table 4.4*)

Table 4.4: Comparison of hypertensive patients with plasma TIMP>500ηg/l and <500ηg/l.± 1 SEM.

Variable	*Timp>500ηg/l*	*Timp-1<500ηg/l*	*P value*
Number	19	81	
Age (years)	52±1.5	53±1.5	NS
Heart Rate (/min)	78 ±1.1	75±1	NS
LVMI g/m^2	123±3.5	134±4.4	NS
E: A ratio	0.84±0.03	1±0.03	<0.01

E wave deceleration	27±2.3	20±0.8	<0.05
IVRT	0.13±0.003	0.12±0.003	NS

PICP

Plasma P1CP was elevated in the hypertensive group as a whole compared with normal subjects. (200μg/l Vs 166μg/l p=0<05) (95% C.I 4,62) There was no demonstrable relationship between plasma P1CP and any echocardiographic findings or blood pressure parameters.

CITP

Plasma CITP was elevated in the hypertensive group compared with normal subjects. (5.2μg/l Vs 2.9μg/l p<0.001) (95% C.I 1.73,2.79) There was no relationship between CITP and echocardiographic findings or blood pressure measurements.

Relationships between Markers.

There was no significant relationship seen between TIMP-1 and CITP or P1CP. However a significant positive correlation was demonstrated between CITP and P1CP as one would expect. (r=0.35, p<0.01)

Renin/aldosterone

Aldosterone levels were significantly elevated in the hypertensive cohort (13.7ηg/100ml Vs 9.3ηg/100ml p<0.01). Renin levels were also elevated but not significantly so. The average Aldosterone/Renin ratio was 1 and no subject had an elevated ratio consistent with primary aldosteronism. There was no relationship between renin or aldosterone levels with collagen markers or echo parameters.

4.5 Discussion.

We set out to non-invasively delineate the pathophysiology of fibrosis in hypertensive heart disease and to assess the clinical feasibility of using collagen markers in this setting. This is by far the largest study to date in this field. We enrolled a large cohort of well-characterised never treated hypertensives. We used robust assays in standard conditions, which we demonstrated to have acceptable reproducibility within our laboratory. It should be accepted that none of the markers used in this study are exclusive to the myocardium. Indeed previous studies have demonstrated elevation in many fibrotic conditions including pulmonary fibrosis (Montano 1989) and hepatic cirrhosis (Li 1994). However we made strenuous efforts to exclude confounding conditions enrolling a well characterised cohort without co-existent conditions leading to fibrosis.

To allow a full assessment of the collagen equilibrium we measured markers of collagen synthesis, degradation and inhibition of degradation. We measured PICP as a marker of collagen synthesis. P1CP would appear to be the most accurate marker of collagen synthesis as it is reliably cleaved during the extracellular processing of collagen type I unlike other markers such as procollagen type III amino terminal peptide (Nimni 1993). As this is excreted via a hepatobiliary route, patients with hepatic dysfunction were excluded from this study.

Collagen degradation was assessed by measurement of CITP which is a cross-linked telopeptide (Laurent 1987) released in a 1:1 stoichometric fashion when

collagen type I fibrils are degraded (Ristelli 1993) thus giving an accurate measurement of collagen degradation.

Matrix metalloproteinases (MMP) are a family of zinc and calcium dependent endopeptidases which play a key role in the degradation of collagen. MMPs have potent proteolytic activity, which is controlled in a major part by the production of specific naturally occurring inhibitors called tissue inhibitors of matrix metalloproteinases (TIMP) (Denhardt 1993) .The best characterised is TIMP-1. Therefore the measurement of TIMP-1 allows an estimation of inhibition of degradation.

We demonstrated an elevation in P1CP confirming that collagen synthesis is elevated in hypertensive heart disease. These findings are in concordance with previous published data (Diez 1995, Querejeta 2000).

We demonstrated a significant increase in TIMP-1 in the hypertensive cohort taken as a whole. However, only patients with echocardiographic evidence of diastolic dysfunction were found to have a significant elevation in plasma TIMP-1. The remaining patients with normal diastolic filling had TIMP-1 levels comparable to normal subjects. Furthermore, there was no relationship between TIMP-1 and any index of systemic blood pressure or left ventricular mass. Therefore TIMP-1 levels are not elevated in hypertension per se but only in patients with diastolic dysfunction and fibrosis. This suggests that the synthesis and release of TIMP-1 is independent of blood pressure and likely to be dependent on a variety of neurohormonal factors. Additionally we have demonstrated that an elevation in measured Timp-1 of greater than 500ηg/l is strongly associated with the presence of diastolic function.

One would expect an elevation in TIMP-1 would result in a reduction in collagen degradation. However plasma CITP was in fact elevated.

It is impossible to be sure using non-invasive assessment of the relative roles played by fibrosis/degradation and there is no doubt that the interplay between TIMP-1 and MMPs is a complex situation. However we have demonstrated that degradation of collagen is increased in hypertension. Whether this elevation is sufficient to offset the increase in synthesis cannot be determined in this study.

The elevation in TIMP-1 would seem at odds with our finding of increased collagen degradation. This need not necessarily be the case. We hypothesise that the elevation in measured plasma TIMP-1 although not sufficient to offset the increment in collagen synthesis is modulating or limiting collagen degradation and thus contributing to the development of fibrosis.

Our results are at odds with the only previous work published in this field. In this study Diez et al (Laviades 1998) demonstrated an elevation in TIMP-1 levels and normal CITP levels. This allowed the authors to conclude that collagen degradation is in fact inappropriately low. Differences may be explained by larger numbers in our study, our use of plasma rather than serum (plasma now being regarded as preferable (Jung 1997, Lein 1997)) and our characterisation of patients by 24hour BP rather than solely office blood pressure.

The other aim of our study was to assess each of the collagen markers as possible non-invasive markers of fibrosis in hypertensive heart disease. Abnormalities in diastolic filling have been shown to precede left ventricular hypertrophy in mild to moderate hypertension (Diazumba 1986, Inouye 1985). In addition animal and in vivo studies have shown diastolic dysfunction is predominately secondary to fibrosis (Matsubara 1997) and is independent of myocyte hypertrophy (Kaito 1996). We therefore used diastolic dysfunction as a marker of cardiac fibrosis. CITP and P1CP do not appear to be accurate predictors. However, we have shown TIMP-1 to be

independent of blood pressure, to correlate with indices of diastolic filling and have a specificity and positive predictive value for diastolic dysfunction of over 95%. A measured TIMP-1>500ηg/l is an accurate and robust predictor of diastolic dysfunction and hence end-organ damage.

Finally we have demonstrated that TIMP-1 levels were significantly elevated in patients with ECG ST-T changes. This may suggest that ECG ST-T changes in hypertension are indicative of fibrosis.

4.6 Perspectives.

We have demonstrated using non-invasive markers of the collagen equilibrium that untreated essential hypertension is characterised by an increase in collagen synthesis, degradation and inhibition of degradation. The relative roles played by these processes in the resultant fibrosis cannot be determined by this study. Secondly, we demonstrated an association between biochemical markers of fibrosis and LV diastolic dysfunction in patients with untreated hypertension. In particular we identified TIMP-1 as a potential non-invasive marker of fibrosis. Further studies to investigate these markers as predictors of risk are needed and if positive will provide an important additional marker in the risk assessment of patients with hypertension.

Figure 4.1: Data Points show plasma TIMP-1 concentrations (nanograms/ml) in hypertensive patients(HBP)n=100 and normal volunteers (normal)n=50.

Figure 4.1.

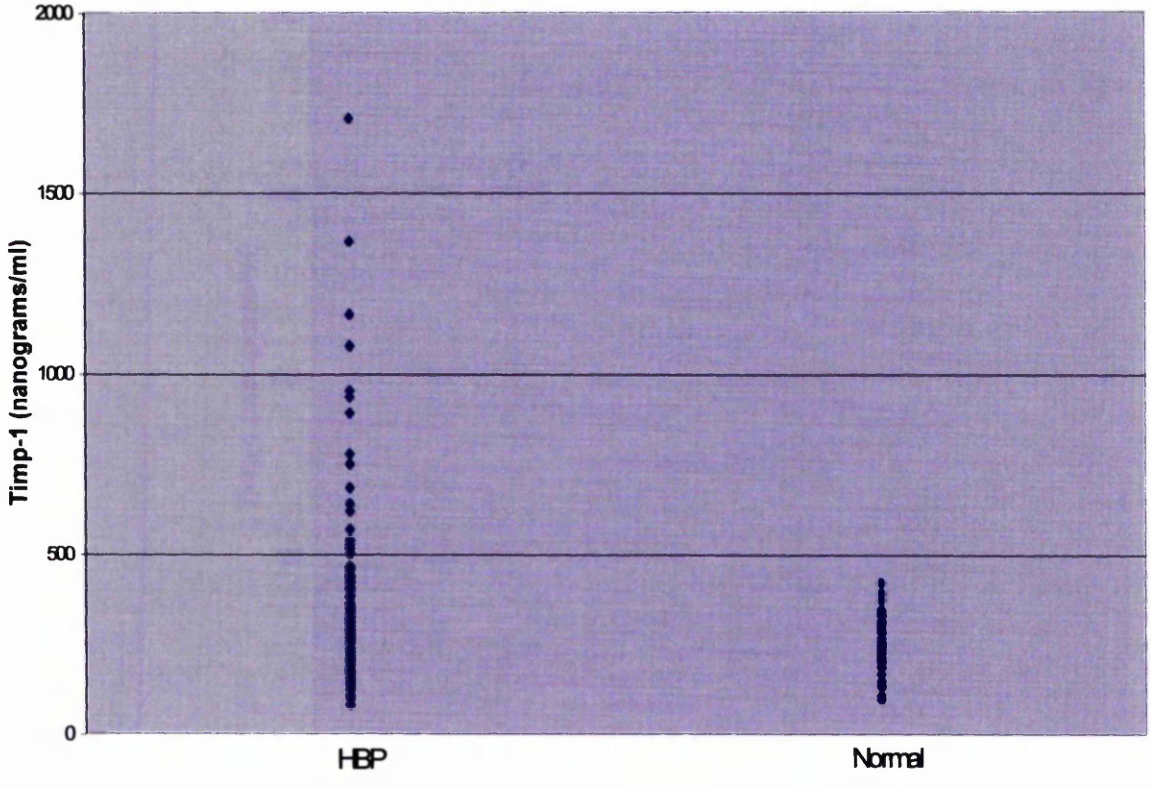


Figure 4.2: Data points show plasma TIMP-1 concentrations (nanograms/ml) in hypertensive patients with E:A greater than 1(n=37), hypertensive patients with E:A ratio of less than 1 (n=63) and normal subjects (n=50).

Figure 4.2

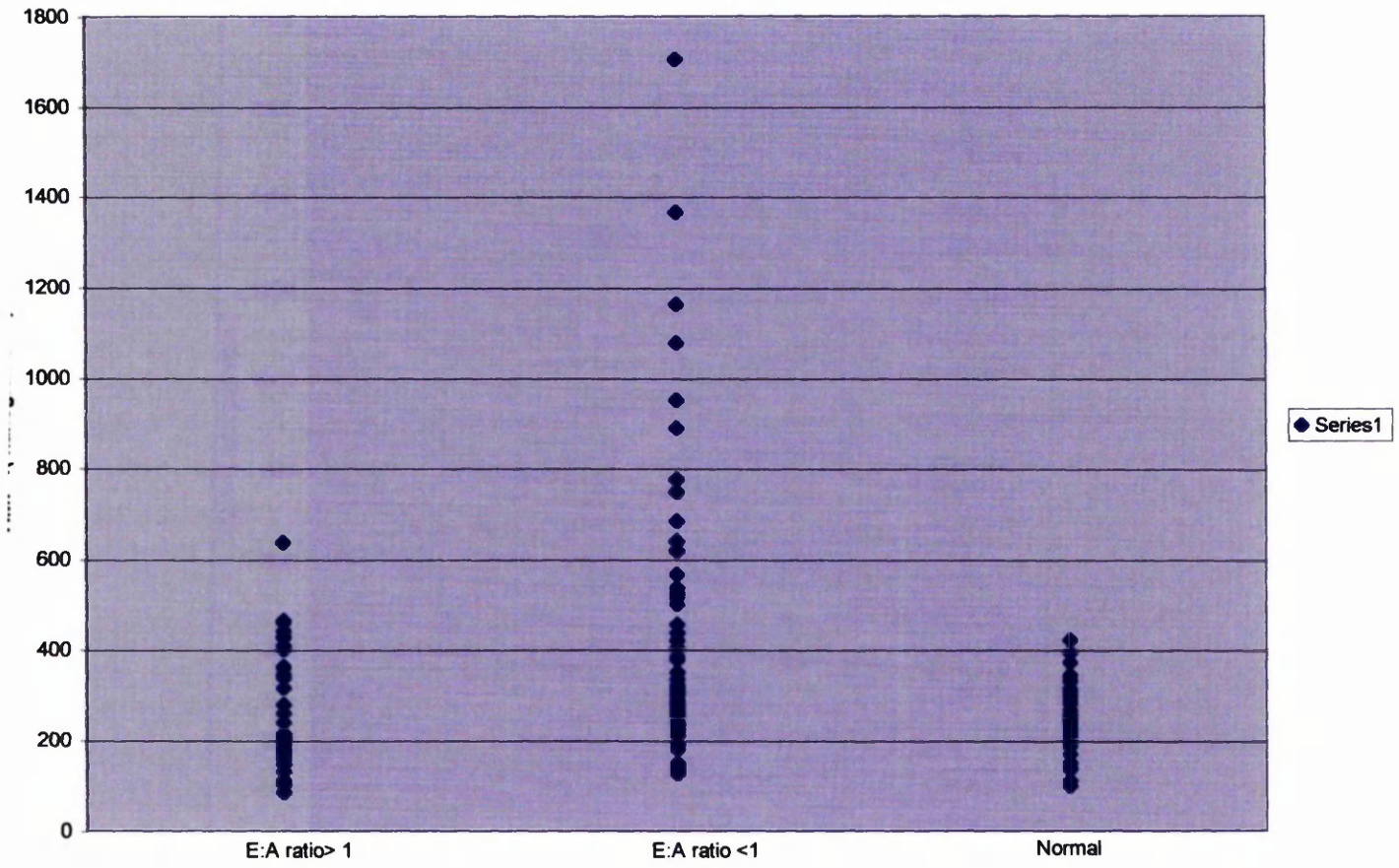


Figure 4.3: Scatter plot shows inverse correlation between Log plasma TIMP-1 concentration and E:A ratio in hypertensive patients.(r=0.26, p<0.05).

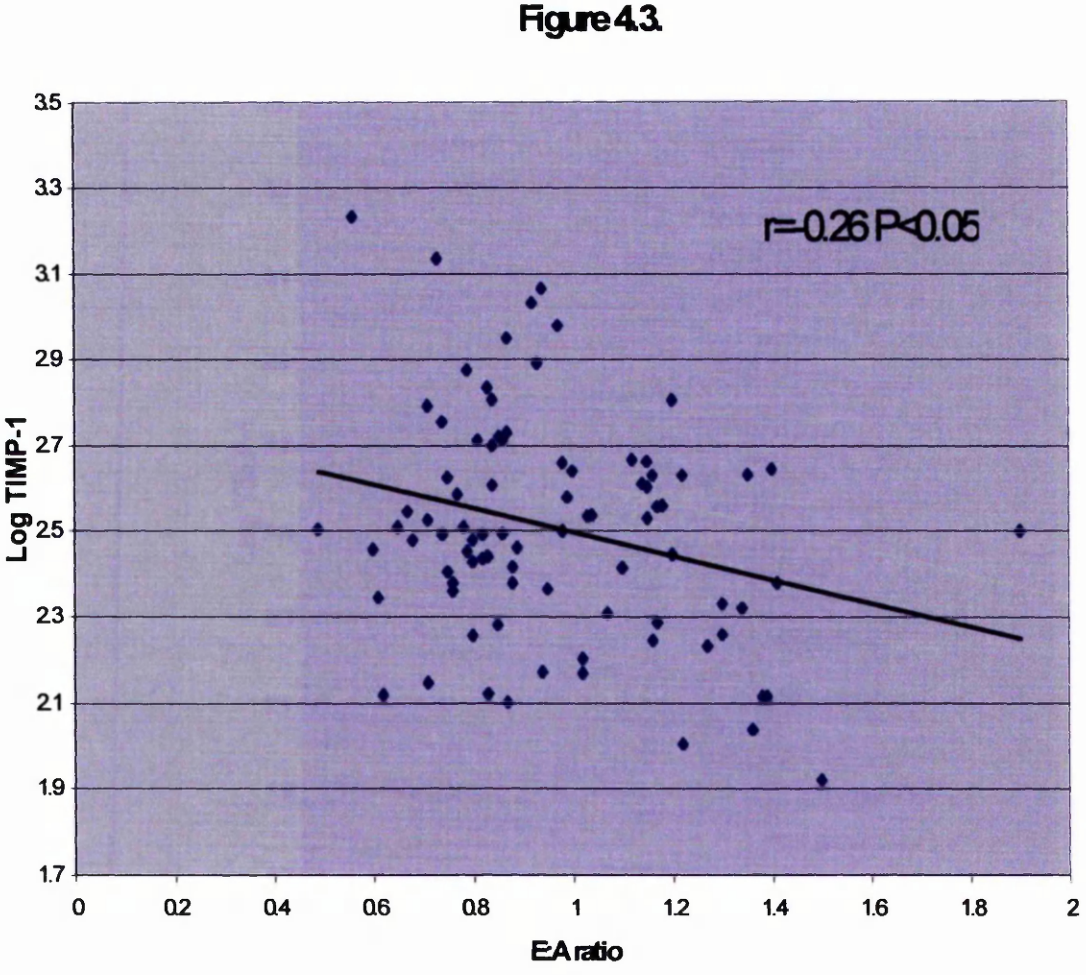


Figure 4.4: Scatter plot shows a positive correlation between Log plasma TIMP-1 concentration and E wave deceleration time (E Dec) in hypertensive patients. (r=0.41 p<0.01)

Figure 4.4.

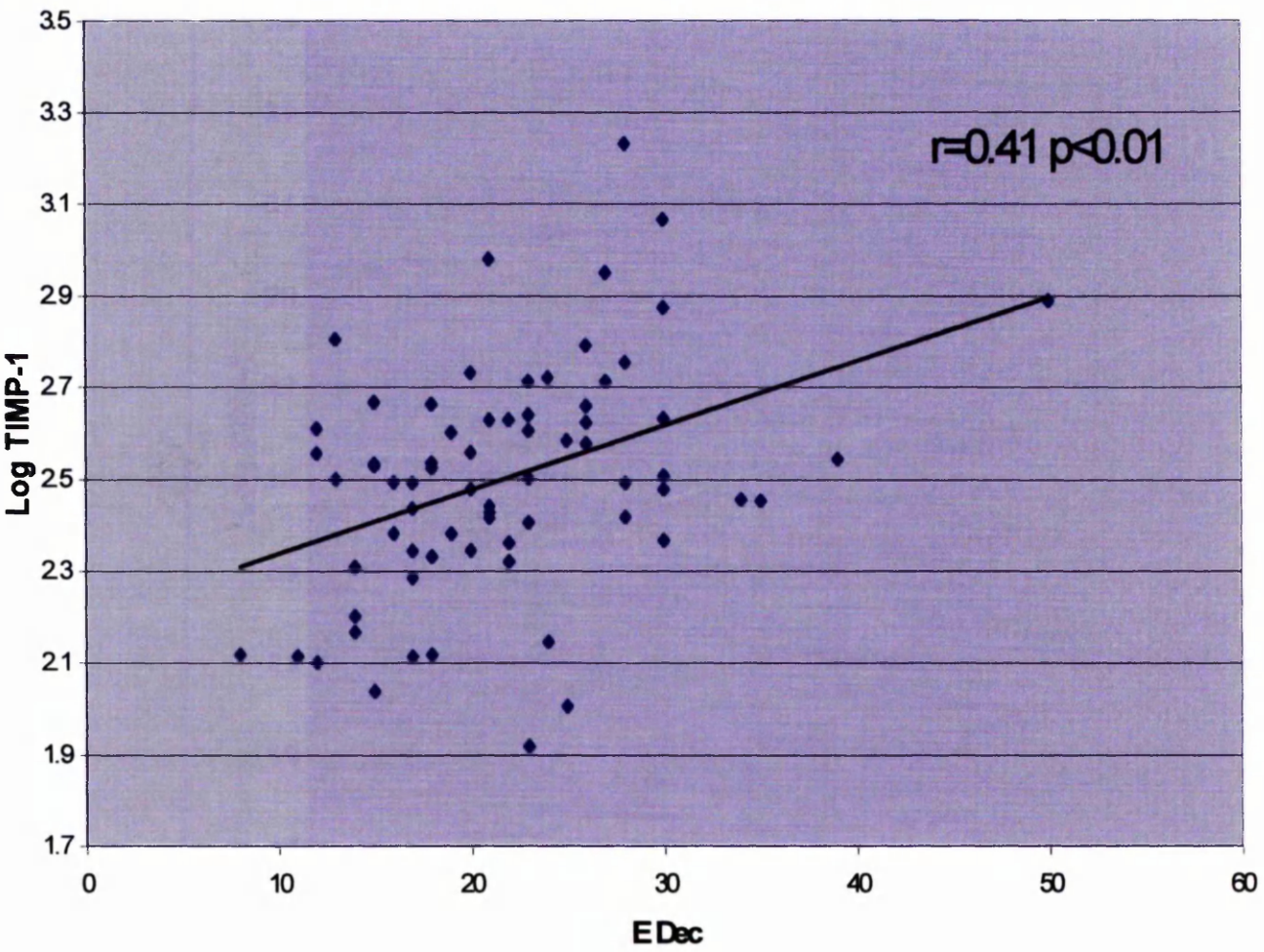
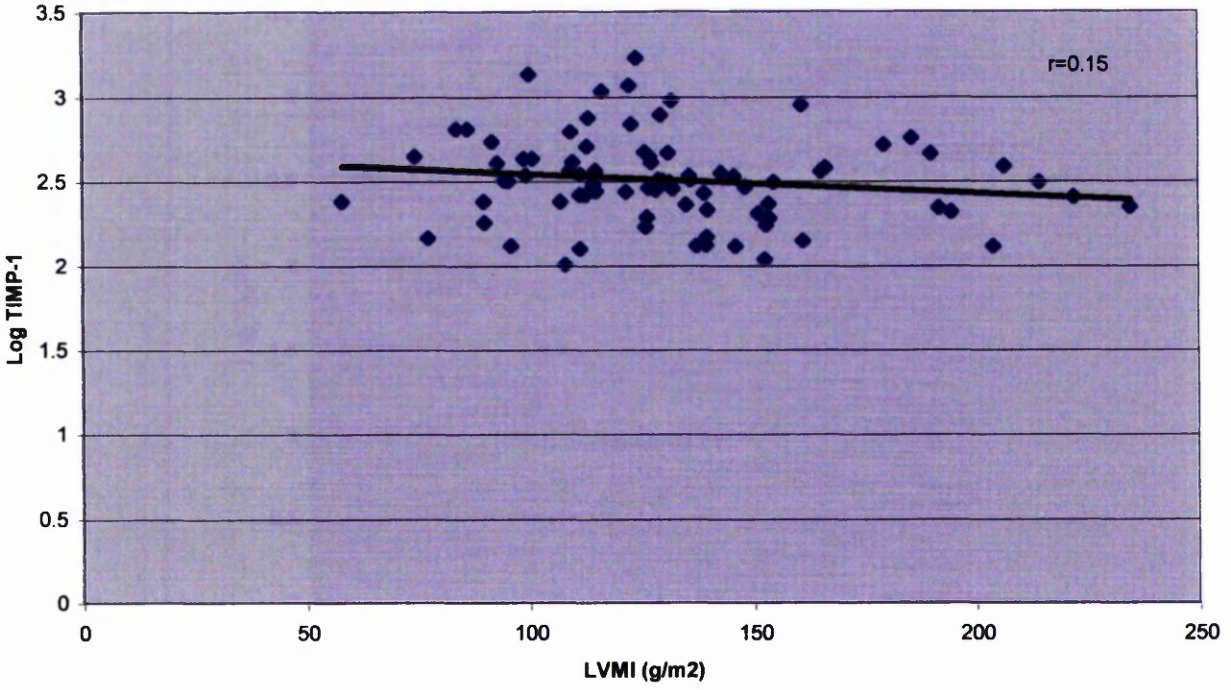


Figure 4.5: Scatter plot shows no correlation (r=0.15 p=ns) between Plasma Log TIMP-1 and LVMI (g/m2) in hypertensive patients.



Chapter 5

Studies of Biochemical Evidence of Fibrosis in Veteran Athletes.

5.1 Abstract

Background

Studies of exercise induced left ventricular hypertrophy (LVH) in veteran athletes suggest the presence of abnormal diastolic filling and incomplete regression of LVH on cessation of exercise. We hypothesise that this is secondary to the presence of fibrosis. This study was therefore designed to document non-invasively the presence of fibrosis in veteran athletes with exercise induced LVH.

Methods and Results.

We recruited a cohort of 45 normotensive elite veteran athletes and 50 normal sedentary subjects. Echocardiographic assessment was made of LV dimensions, LV mass index, LV systolic and LV diastolic function. Plasma carboxyterminal propeptide of collagen type I (PICP), carboxy-terminal telopeptide of collagen type I (CITP) and tissue inhibitor of matrix metalloproteinase Type I (TIMP-1) were measured as markers of collagen synthesis, degradation and inhibition of degradation respectively. Veteran athletes had significant elevation in LV dimensions and calculated LV mass index confirming the presence of exercise induced LVH in this cohort. Diastolic and systolic function was normal. Plasma PICP was significantly elevated in the cohort of veteran athletes (259μg/l Vs 166μg/l $p<0.001$) as were CITP (5.4μg/l Vs 2.9μg/l $p<0.001$) and TIMP-1 (350ηg/mlvs253ηg/ml $p=0.01$). There was a further significant elevation of TIMP-1 in athletes with echocardiographic evidence of LVH defined as a LVMI>130g/m^2 (417ηg/ml Vs 266ηg/ml $p=0.02$)

Conclusion.

There is biochemical evidence of disruption of the collagen equilibrium favouring fibrosis in veteran athletes with LVH. This may suggest that fibrosis occurs as part of the hypertrophic process in veteran athletes.

5.2 Introduction

Chronic high intensity exercise results in structural cardiac changes in the human heart. Henschen first described this at the turn of the last century (Henschen 1889). The changes are characterised by an increase in left ventricular chamber dimensions and an increase in left ventricular wall thickness (Maron 1986). This is traditionally thought to be non-pathological as the hypertrophy regresses on cessation of exercise (Ehsani 1978, Maron 1993), it is thought to consist purely of myocyte hypertrophy and doppler indices of diastolic filling are on the whole within age matched limits (Pellicai 1991).

This situation may however be different in veteran athletes. The limited data which is available within this cohort suggests that diastolic function is abnormal and more importantly that incomplete regression of LVH occurs on cessation of exercise (Miki 1994, Nishimura 1980). Our hypothesis was that these findings could be explained by the presence of co-existent fibrosis.

Fibrosis occurring in the context of LVH has important pathological consequences (Weber 1993) and therefore the demonstration of fibrosis present in exercise induced LVH would be a key finding in our understanding of the clinical significance and potential long-term consequences of this process.

The biochemical assessment of fibrosis using collagen markers represents a practical, validated and non-invasive method for the assessment of fibrosis in this cohort. The clinical utility of this technique in the context of left ventricular

hypertrophy has been demonstrated by our group (Lindsay 2002) and also most notably by Diez et al (Diez 1995, Laviades 1998, Querejeta 2000).

The aim of this study was to demonstrate, using biochemical markers, the presence of fibrosis in exercise induced LVH in a cohort of veteran athletes.

5.3 Methods

Subjects

We enrolled a cohort of veteran athletes. All subjects were active members of the Scottish Veteran Harriers club. The subjects were contacted directly and invited to attend for the study. All subjects were over the age of 45, had been running for more than 10 years at a competitive level, ran more than 30 miles per week and regularly competed in elite veteran endurance events.

Exclusion criteria were firstly, conditions that result in left ventricular hypertrophy namely hypertension and aortic stenosis. Secondly, conditions that result in fibrosis were excluded thus preventing spurious elevation in collagen markers of synthesis and degradation. These conditions included renal impairment (serum creatinine >130mmol), coronary artery disease, malignancy, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction, left ventricular systolic dysfunction and smoking related airways disease.

A cohort of sedentary normal subjects was recruited via a local advertising campaign.

Ethical approval was obtained from the local ethics committee. All subjects received a full written and verbal explanation of the investigations involved and the aims of the study. Written consent was obtained in all cases prior to any investigations being performed.

Study conditions.

Patients were studied at a standard time in the morning on one study day. A full history and clinical examination was made to identify exclusion criteria and suitability for the study. Blood pressure was recorded with a mercury syphgmanometer in the supine position and an average of three readings was taken.

Echocardiographic study.

Patients were studied using a Vingmed CFM800 sonos or a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral position. Echo measurements were taken in the standard parasternal and apical positions. M-Mode measurement was taken in a perpendicular parasternal long axis view with the cursor through the tips of the mitral valve leaflets. Measurements were taken according to the guidelines laid down by the American Society of Echocardiography (Sahn 1978). Left ventricular mass was calculated using the formula validated by Reichek and Devereux (Devereux 1977) and indexed for body surface area. An average of at least three measurements was taken and images were stored on super VHS videotape and on a digital archiving facility (Vingmed EchoPac). Ventricular function was assessed via calculation of fractional shortening and left ventricular ejection fraction from a left ventricular m-mode. Measurement of E: A ratio was made in the apical view with a cursor at the mitral valve inflow. An average of three measurements was taken at end expiration and images were stored on digital archive.

Standard assessment of valvular function was made.

A single observer (M Lindsay) made all measurements. Using digital archiving images intraobserver variability was tested in a blinded fashion. Intra- observer variability was 3.5% and 9% for E: A ratio and calculated left ventricular mass index (LVMI) respectively.

Biochemical Measurements.

Routine biochemical measurements were taken and analysed in the standard fashion.

All samples were taken at a standard time after thirty minutes in the supine position. Samples were immediately centrifuged at 3000 rpm for seven minutes and the plasma layer removed. The separated plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen.

Plasma Tissue Inhibitor of Matrix Metalloproteinase type I (TIMP-1) was measured as a marker of inhibition of collagen degradation. Plasma carboxyterminal telopeptide of collagen type I (CITP) was measured as marker of collagen degradation and plasma carboxyterminal propeptide of collagen type I (PICP) was measured as a marker of collagen synthesis.

Plasma TIMP-1 was measured using a commercially available two site ELISA assay specific for TIMP-1available from Amersham Pharamaceuticals. This technique is a modified version of that described by Plumpton (1995). All samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma CITP was measured by radioimmunoassay using a polyclonal antibody direct against CITP (Ristelli 1993). All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

ECG

All patients received a standard twelve lead ECG with a paper speed of 25mm/sec. This was recorded in the supine position on the same study day as the other recordings. ECGs were analysed for the presence of LVH and ST-T changes using standard criteria (Dunn 1990). Additionally the presence of atrial abnormalities was assessed using pre-defined criteria (Tarazi 1966). All ECGs were analysed by a single observer. (M Lindsay).

Statistical Analysis.

The distribution of the collagen markers was tested for normality using the Anderson -Darling test. All markers were not normally distributed. Therefore results were log transformed prior to analysis. Results are presented in the non-logarithmic format. All continuous variables were analysed using unpaired student's T test. Non-continuous variables were analysed using a chi-squared test.

5.4 Results.

45 athletes (5 athletes were excluded prior to recruitment and analysis due to hypertension (1 subject), renal impairment (1 subject) and poor echo views (3 subjects)) and a matched population of 50 normal subjects were recruited. Four normal subjects were excluded prior to recruitment and analysis (2 subjects with hypertension and 2 subjects with poor echo views).

Baseline criteria

The two groups were well matched for age, and blood pressure. (*Table 5.1*)

Table 5.1: Baseline Criteria. (+/- 1 SEM)

	Athletes	Normal subjects
Number	45	50
Weight (Kg)	68±0.4	71±3.4
Systolic BP (mmHg)	129±2.4	129±2.6
Diastolic BP (mmHg)	77±1.2	77±2
Duration of training (years)	20±1.9	0
Weekly training (miles)	30±2.4	0
Age (years)	52±1.7	52±1.5

ECG findings.

Using the above criteria 36% of athletes and no normal subjects were found to have LVH ($p<0.001$). 11% of athletes and no normal subjects had ST-T abnormalities ($P<0.01$). There was no difference in the incidence of atrial abnormalities There was no relationship between any of the collagen markers and ECG findings. (*Table 5.2*)

Table 5.2: ECG findings in athletes and normal volunteers.

ECG Finding	*Athletes*	*Normal subjects*	*P value*
LVH	*36%*	*0%*	*P<0.001*
ST-T changes	*11%*	*0%*	*P<0.01*
Atrial abnormalities	*9%*	*9%*	*NS*

Echocardiography

The cohort of athletes (n=45) had a significant increase in inter ventricular septal thickness in diastole (IVSd) 1.17cm Vs 0.9cm ($p<0.01$), posterior wall thickness in diastole (LPWd) 1.1cm Vs 0.91cm $p=0.04$, left ventricular internal dimension in diastole (LVIDd) 5.2cm Vs 4.8cm $p<0.001$ and left ventricular mass index (LVMI) 141g/m^2 Vs 97g/m^2 $p<0.001$. There was no difference in indices of diastolic filling. 57% of athletes had an LVMI >130g/m^2, demonstrating a high incidence of exercise induced LVH amongst our cohort. Relative wall thickness (RWT) was significantly elevated amongst athletes 0.42cm Vs 0.37cm $p=0.002$ suggesting the hypertrophy present was concentric in nature. (*Table 5.3*)

Table 5.3: Echo parameters in athletes and normal volunteers. (+/- 1SEM)

Parameter	*Athletes*	*Normal subjects*	*P value*
Number	45	50	
IVSd (cm)	1.17±0.03	0.9±0.03	P<0.01
LVIDd (cm)	5.2±0.06	4.8±0.07	P<0.001
LVPWd (cm)	1.1±0.02	0.91±0.02	P=0.04
RWT(cm)	0.42±0.01	0.37±0.01	P=0.002

LV Mass Index (g/m^2)	141±5	97±3	P<0.001
E: A ratio	1.25±0.07	1.15±0.03	NS
Max e wave vel (m/s)	0.73±0.03	0.73±0.03	NS

Collagen markers.

Table 5:4: Collagen Markers in Athletic cohort.

Parameter	**Mean**	**Standard Error of mean**	**Range**	**Standard Deviation**
TIMP-1 (ηg/ml)	350	35	156-1225	233
CITP (μg/l)	5.4	0.25	2.8-8.7	1.5
P1CP (μg/l)	259	14	152-498	86

TIMP-1

Plasma TIMP-1 was significantly elevated in the cohort of athletes (n=45) compared with the normal control group (n=50) (350ηg/ml Vs 253ηg/ml p<0.01) (95%C.I 20,174). When the cohort of athletes was dichotomised depending on the presence (n=25) or absence (n=20) of exercise induced LVH (defined as LVMI greater than $130g/m^2$) then the athletes with LVH (n=25) were found to have significantly higher plasma TIMP-1 levels (417ηg/ml Vs 266ηg/ml p=0.01) (95% C.I 27,276) *Figure 5.1.* In fact the athletes without LVH (n=20) had plasma TIMP-1 levels comparable to the cohort of normal subjects (n=50) (266ηg/ml Vs 253ηg/ml p=ns) (95% C.I –61,34). No relationship was seen with duration or intensity of training (*Figure 5.2*).

The cohort of athletes with exercise induced LVH were then further characterised to exclude confounding factors, which could explain the elevation in TIMP-1. *Table 5.5* demonstrates that there is no significant difference in any of the baseline criteria between the two groups. Therefore, the elevation in TIMP-1 appears to reflect a genuine increase in inhibition of collagen degradation in athletes with exercise induced LVH.

Table 5.5: Athletes with LVMI>130g/m Vs athletes LVMI<130. (± 1 SEM)

Parameter	LVMI>130g/m2	LVMI<130g/m	P Value
Number	25	20	
Age (years)	52±2.5	53±3	Ns
BSA	1.9±0.02	1.8±0.05	Ns
Systolic BP (mmHg)	129±3	129±5	Ns
Diastolic BP (mmHg)	77±1	76±3	Ns
Max E wave velocity (m/s)	0.75±0.04	0.73±0.03	Ns
E: A ratio	1.4±0.08	1.3±0.13	Ns
TIMP-1 (nanog/ml)	417±57	266±20	0.01
CITP (μg/l)	5.6±0.4	5.2±0.3	Ns
PICP (μg/l)	258±19	273±23	Ns

Duration of training. (Years)	19±2.7	21±3	Ns
Weekly training. (Miles)	26±2.7	37±4	0.06

CITP

Plasma CITP levels were significantly elevated in the cohort of athletes (n=45) compared with the normal volunteers (n=50) (5.4μg/l Vs 2.9μg/l p<0.001) (95% C.I 1.9,3.1) *Figure 5.3*. This elevation was demonstrated in athletes with and without LVH. No relationship was demonstrated between CITP and LV dimensions, LV mass or the duration or intensity of exercise *(Figure 5.4 and 5.5)*.

P1CP

Plasma P1CP levels were also elevated within the cohort of athletes (n=45) compared with normal volunteers (n=50) (259μg/l Vs 166μg/l p<0.001)(95%C.I 60,126) *Figure 5.6*. This elevation was demonstrated in athletes with and without LVH. Again no relationship was demonstrated between P1CP and LV dimensions, LV mass and duration or intensity of exercise (*Figures 5.7 and 5.8*).

5.5 Discussion.

Exercise induced LVH is widely thought to be a benign physiological process. However in the absence of prospective controlled studies this cannot be certain. There is evidence available that should make us re-examine conventional thinking. Firstly, the fact exercise induced LVH regresses on cessation of exercise does not confirm its physiological basis. Pathological forms of LVH namely hypertensive heart disease and LVH in the context of aortic stenosis all regress on removal of the pathological

stimulus as does exercise induced LVH. Secondly, follow up studies purporting to confirm that athletes have increased a life expectancy are flawed, as these studies have not been controlled for major cardiovascular risks such as smoking (Sarna 1993). Finally, post-mortem studies have revealed an incidence of 18% of idiopathic LVH in sudden cardiac death in athletes (Maron 1980). Clearly this limited data cannot confirm a causative role of LVH however should raise some concern.

The issue of the possible pathological role of exercise induced LVH in veteran athletes is even less well defined. The studies that are available reveal some concerning findings, namely, that middle-aged athletes are more prone to developing ECG abnormalities, prominent hypertrophy and may have slightly depressed LV function (Nishimura 1980). In addition diastolic function may be abnormal and regression of LVH after 2 years of cessation of exercise may be incomplete (Miki 1994).

It was this finding of incomplete regression of LVH in veteran athletes which was the stimulus to our study. If regression was incomplete than this may reflect pathological abnormalities. Namely, that in addition to the accepted myocyte hypertrophy there may in fact be a degree of interstitial fibrosis. Interstitial fibrosis occurring in the context of left ventricular hypertrophy is an important pathological entity resulting in the development of diastolic dysfunction and acting as a substrate for ventricular arrhythmias (Weber 1993). Therefore the demonstration of fibrosis would be an important finding in allowing us to understand the clinical significance and consequences of exercise induced LVH.

Biochemical assessment of the collagen equilibrium is now possible. PICP, which is cleaved on incorporation of procollagen type I into the collagen helix and has been shown to be a non-invasive measure of fibrogenesis (Nimini 1993, Savolainen

1984). CITP which is cleaved when collagen type I is degraded has been shown to be a measure of collagen degradation (Ristelli 1993, Eriksen 1993). Finally TIMP-1 which is a naturally occurring specific inhibitor of Matrix Metalloproteinases, which are the rate limiting step in collagen degradation has, been shown to be a measure of inhibition of collagen degradation (Dernhardt 1993). Therefore by measurement of PICP, CITP and TIMP-1 we achieved an accurate biochemical assessment of both sides of the collagen equilibrium. The clinical utility of biochemical markers of fibrosis in the context of LVH has been previously demonstrated by our group (Lindsay 2002). These markers are not specific to the myocardium therefore care was taken to exclude individuals with conditions that may result in fibrosis through other means.

Care was taken to enrol a cohort of elite athletes who would have exercise induced cardiovascular changes. This was achieved using strict inclusion criteria. All echo parameters of LV mass were significantly increased and 57% of our cohort had a LVMI greater than $130g/m^2$.

Our results demonstrate that in elite veteran athletes there is biochemical evidence of disruption of the collagen equilibrium in comparison with a matched cohort of sedentary control subjects. Taking the cohort of athletes as a whole we have demonstrated a significant increase in collagen synthesis as assessed by an elevation in PICP, an increase in collagen degradation as assessed by an elevation in CITP and an increase in inhibition of degradation as assessed by an elevation in TIMP-1. Of more significance is the finding that athletes with echo evidence of exercise induced LVH ($LVMI>130g/m^2$) had a further statistically significant elevation in TIMP-1. Indeed TIMP-1 was only elevated in athletes with LVH; the remaining athletes having TIMP-1 levels comparable to normal. Therefore, there only appears to be an elevated

inhibition of degradation (TIMP-1) amongst athletes with LVH. This disruption of the collagen equilibrium amongst athletes with LVH, characterised by inhibition of collagen degradation, would favour the development of fibrosis. This contrasts with P1CP and CITP, which were elevated throughout the cohort of athletes irrespective of the presence of LVH.

These findings suggest that increments in collagen synthesis and degradation occur at an early stage in the development of exercise induced cardiovascular adaptation. Whereas inhibition of collagen degradation occurs only in more advanced stages of cardiovascular adaptation associated with established exercise induced LVH. Within the setting of exercise induced LVH, increments in collagen degradation would favour the development of fibrosis. The finding of elevated TIMP-1 amongst athletes with LVH cannot be explained by confounding factors and represents a genuine elevation. We can therefore conclude that in veteran athletes with LVH there is a disruption of the collagen equilibrium characterised by an increased inhibition of collagen degradation favouring the development of fibrosis.

It should be noted that the cohort of veteran athletes studied did not demonstrate evidence of diastolic dysfunction which one might expect in the context of myocardial fibrosis. There are several possible explanations for this. Diastolic dysfunction is a complex process, which has been best described in the context of hypertensive left ventricular hypertrophy. However, the factors involved in the development of fibrosis in hypertensive heart disease are clearly different from those important in exercise induced LVH. Namely, in hypertensive heart disease there are chronic elevations in the effector hormones of the renin angiotensin system, which are central to the development of fibrosis and independent of myocyte hypertrophy (Weber 1994). Fibrosis, if present in exercise induced LVH, is likely to be mediated

by different factors and may relate to the chronic loading of regular high intensity exercise. In addition, due to the complex cardiovascular adaptive changes which occur in exercise induced LVH the development of diastolic dysfunction secondary to fibrosis may occur much later in this process than is seen in hypertensive heart disease. Therefore the absence of diastolic dysfunction in this cohort of athletes does not mean the absence of fibrosis.

Other possible explanation for our findings would be, firstly, that the changes in the collagen equilibrium demonstrated, merely reflects remodelling associated with increments in LV mass. However if this were the case then one would expect a direct correlation between increasing LV mass and increments in PICP, CITP and TIMP-1. This is not seen. There are no correlations with markers of the collagen equilibrium and any echo assessment of left ventricular mass. Furthermore TIMP-1 levels are only elevated in the presence of documented LVH. Therefore, we feel this explanation can be discounted. Secondly, it must be accepted that the markers used have wider biological roles. It is conceivable that the elevations demonstrated may relate, in part, to these roles. For example TIMP-1 was first described as a haemopoetic-stimulating factor and, as such, may be stimulated by aerobic training (Gasson 1985).

In conclusion, our results demonstrate that there is biochemical evidence of disruption of the collagen equilibrium favouring fibrosis in veteran athletes with LVH. This suggests fibrosis may occur as part of the hypertrophic process of exercise induced LVH in veteran athletes. This would explain incomplete regression of LVH in this cohort and may suggest that this process is pathological.

Figure 5.1: Data points show plasma TIMP-1 concentrations (nanograms/ml) in athletes with LVMI>130g/m2 (n=25), athletes with LVMI<130g/m2 (n=20) and normal subjects (n=50).

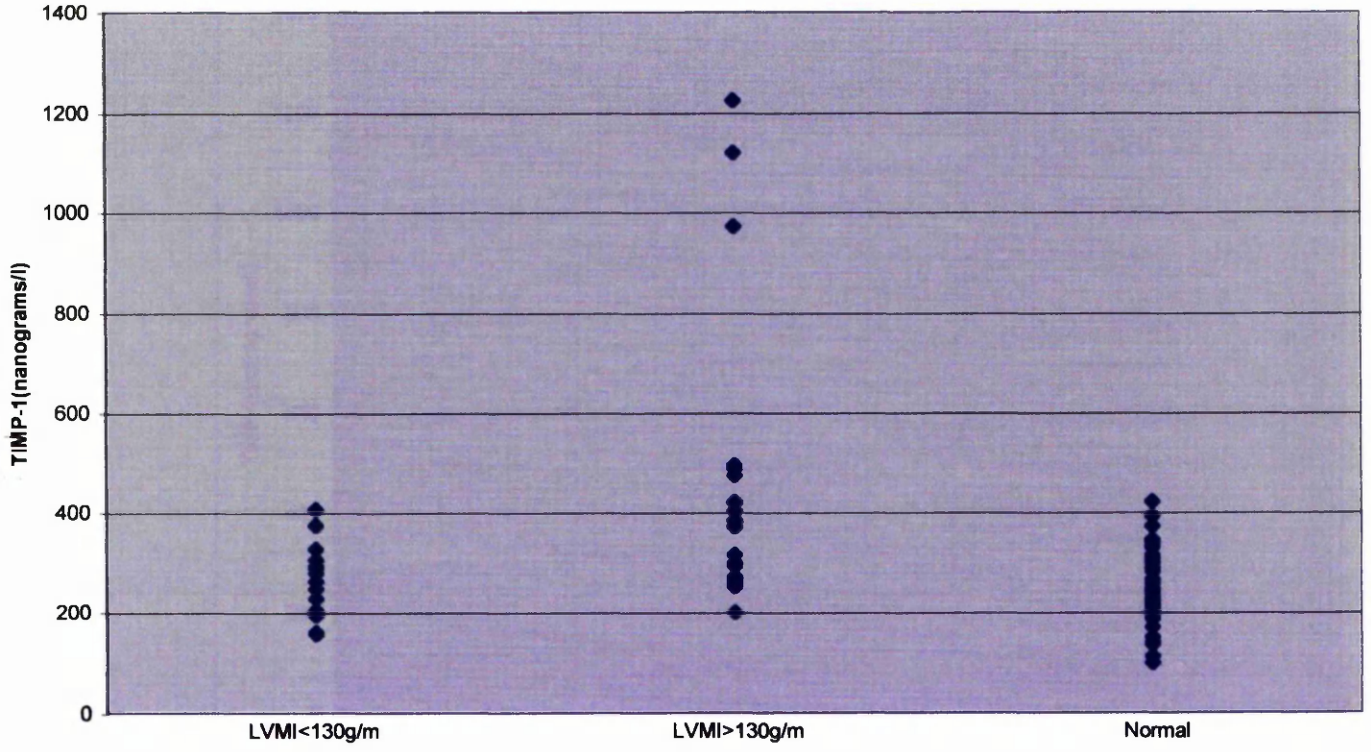


Figure 5.2: Scatter plot show no significant correlation between Log Plasma TIMP-1 and intensity of weekly training (miles per week)(r=0.12 p=ns) in the athletic cohort.

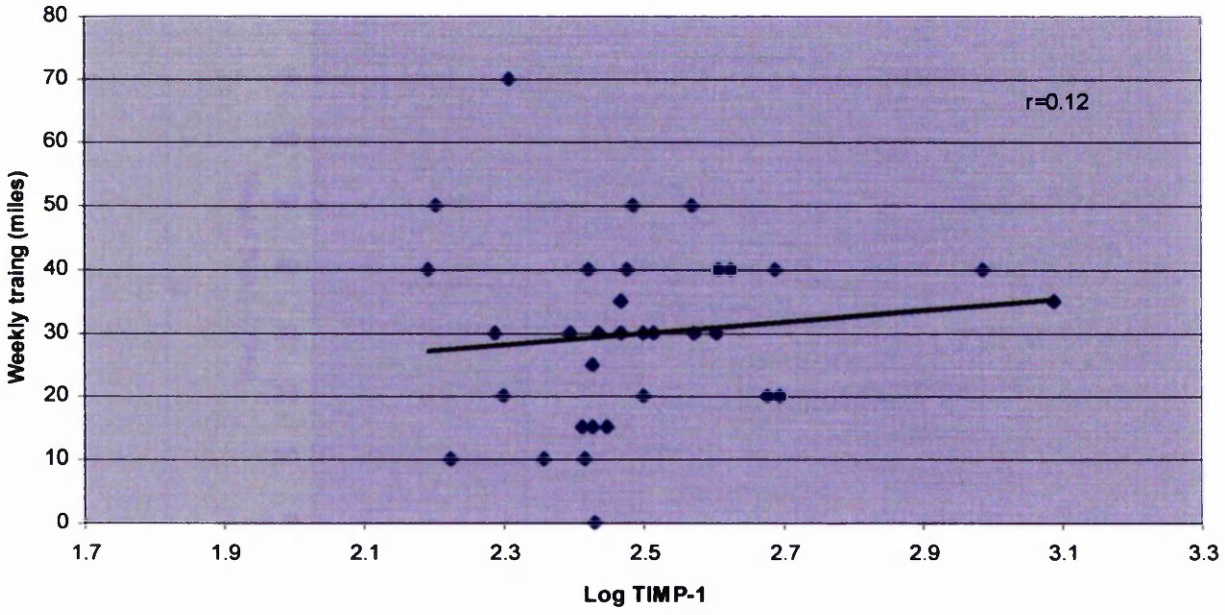


Figure 5.3: Data points show plasma CITP concentrations (micrograms/l) in Athletes(n=45) and normal volunteers (normals).

Figure 5.3

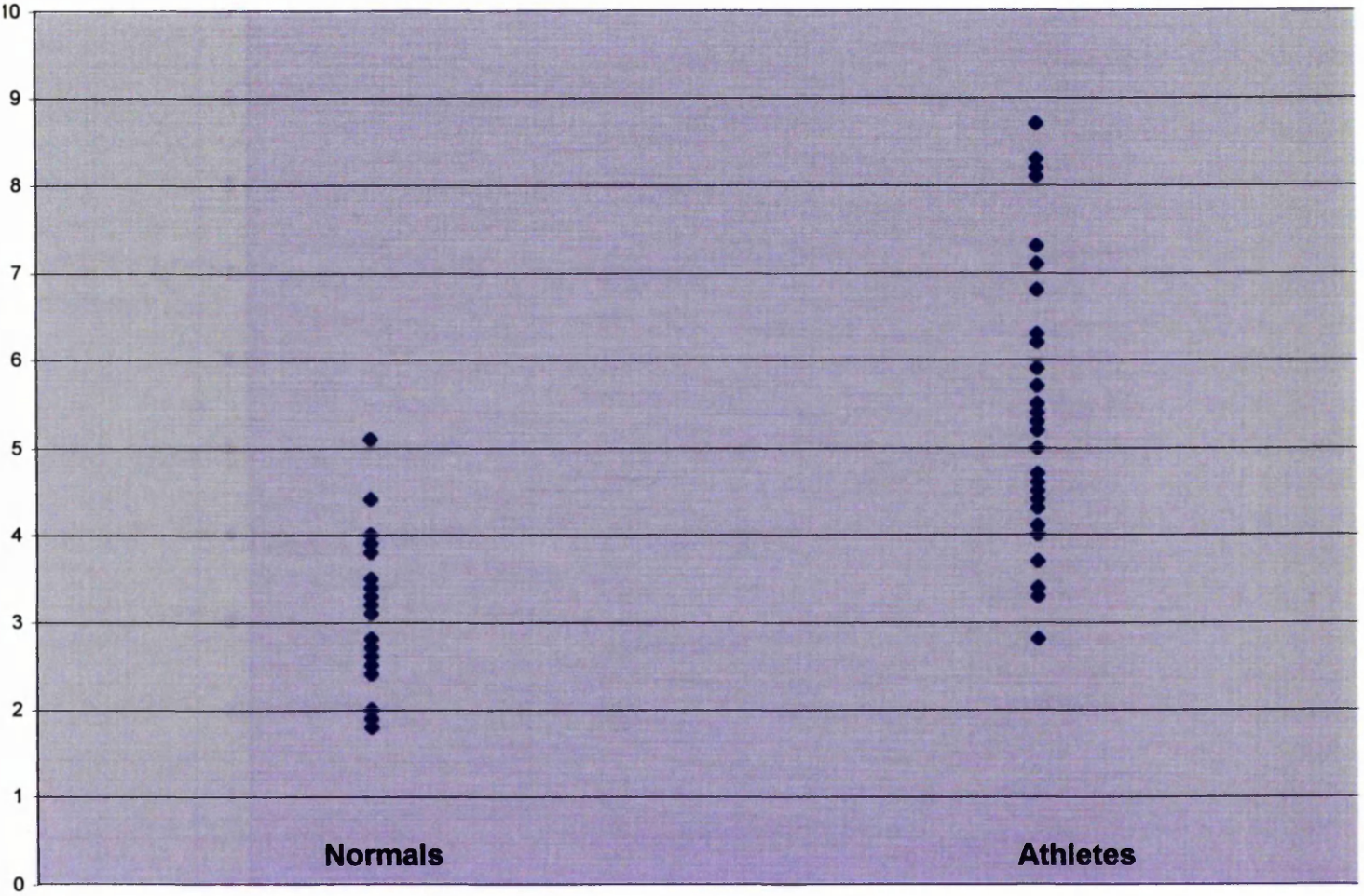


Figure 5.4: Scatter plot show no significant correlation between Log Plasma CITP and LVMI (g/m^2) (r=0.02 p=ns) in the athletic cohort.

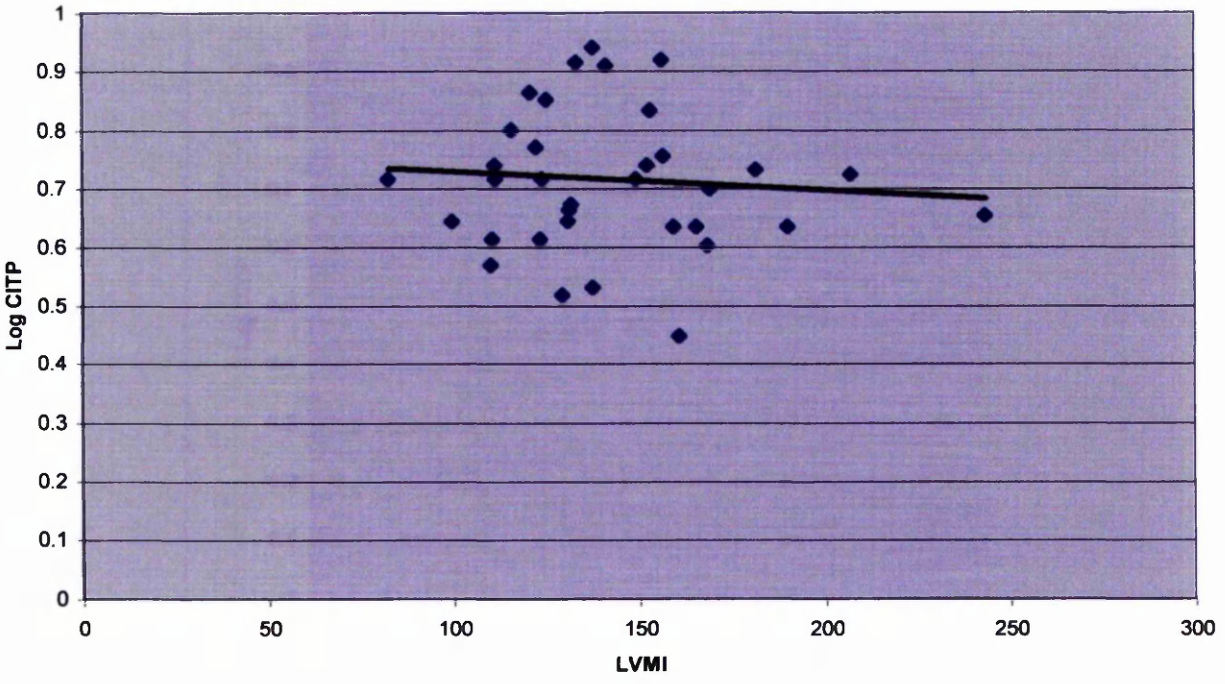


Figure 5.5: Scatter plot shows no significant correlation between Log plasma CITP and intensity of weekly training (miles per week) (r=0.1 p=ns) in the athletic cohort.

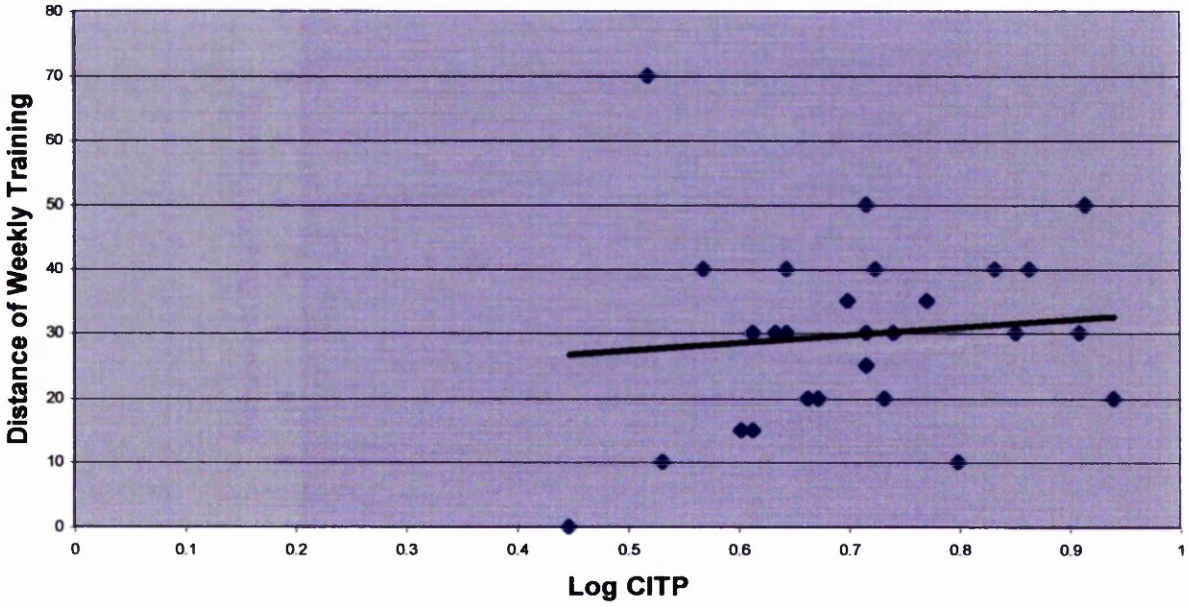


Figure 5.6: Data points show plasma P1CP concentrations (micrograms/l) in Athletes (n=45) and normal volunteers(normals).

Figure 5.6

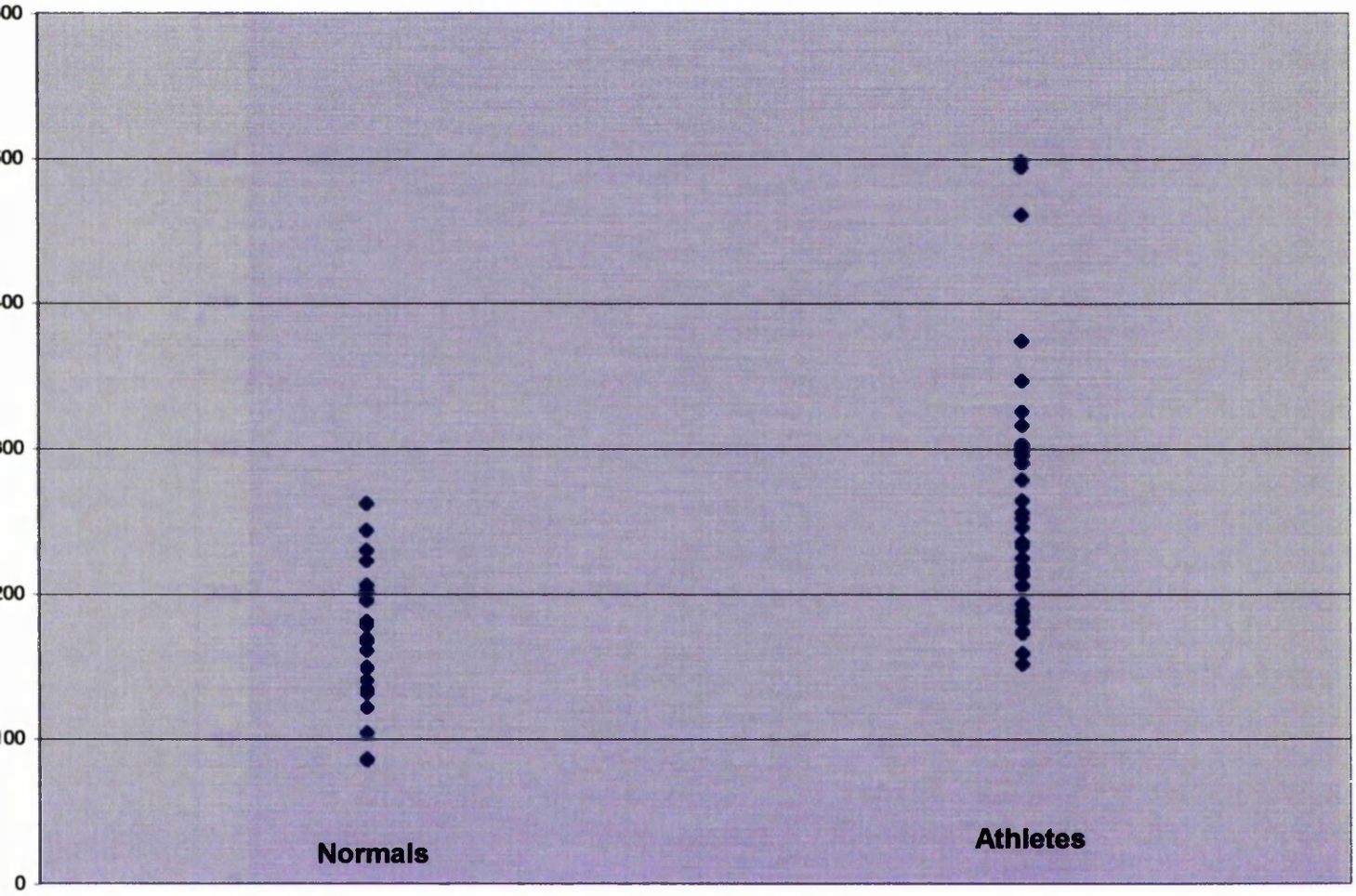


Figure 5.7: Scatter plot shows no significant correlation between Log plasma P1CP and LVMI (g/m^2) (r=0.2 p=ns) in the athletic cohort.

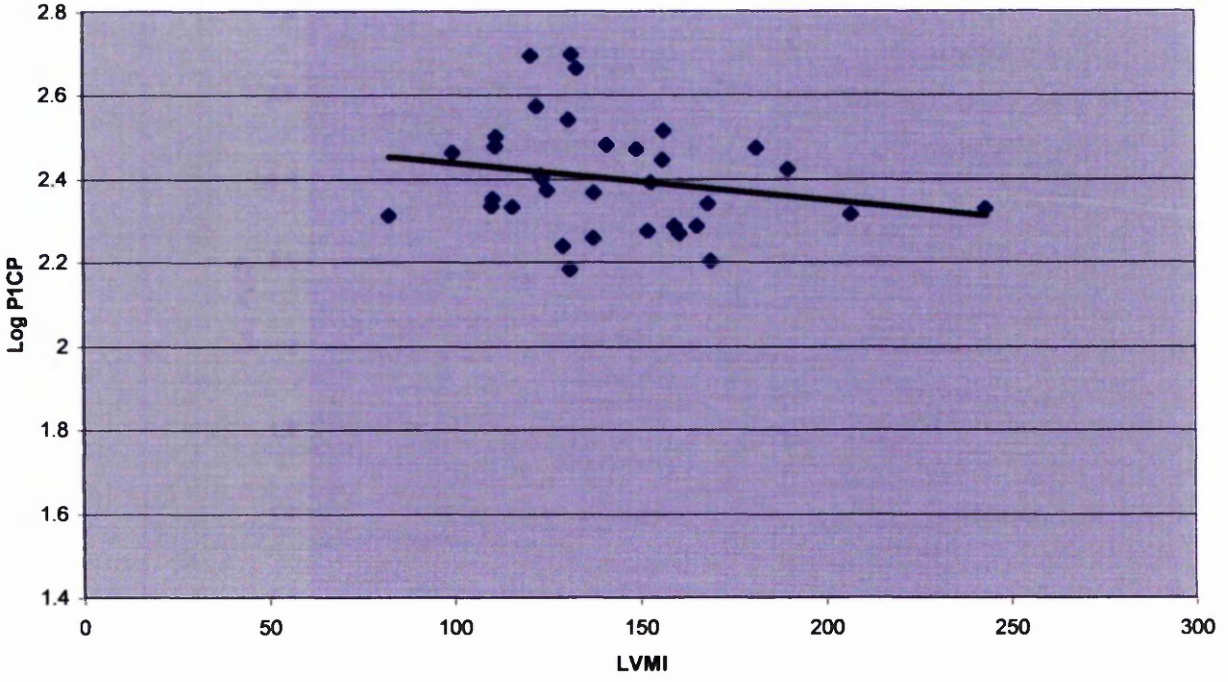
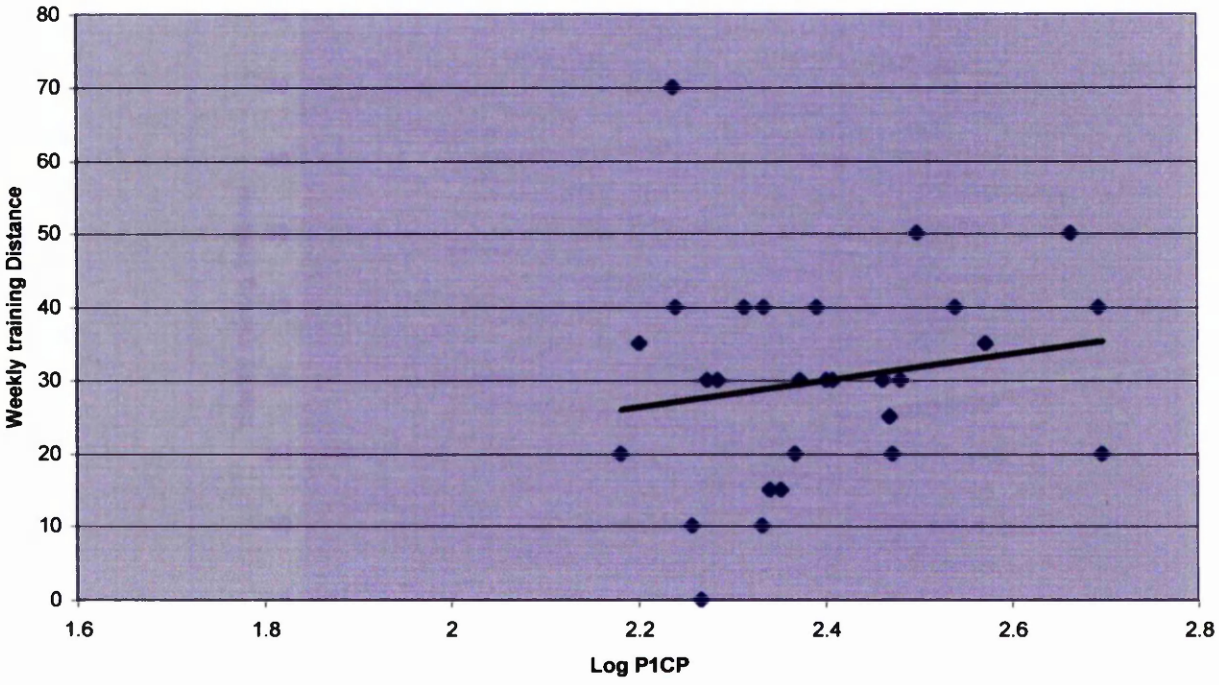


Figure 5.8: Scatter plot show no correlation between Log plasma P1CP and intensity of weekly training (miles per week) (r=0.18 p=ns) in the athletic cohort.



Chapter 6:

Studies of biochemical evidence of fibrosis in "White coat" and mild hypertension.

6.1 Abstract.

The pathological significance of white coat hypertension and the BP level target organ damage occurs remains unclear. We attempted to further this debate by studying the presence of fibrosis, using biochemical markers, in patients with stage I and white coat hypertension. We enrolled 50 never treated hypertensives with an office BP >140/90mmHg and a mean ambulatory BP <140/90mmHg. The cohort was divided into three groups according to ambulatory BP. Group 1(n=25): mean daytime systolic BP > 140mmHg, Group 2 (n=13) mean daytime systolic BP 135-140/90mmHg and Group 3 (n=12): mean daytime BP < 135/85mmHg. Echocardiographic and electrocardiograhic assessment was made. Plasma carboxy-terminal propeptide of collagen type I (P1CP), carboxy-terminal telopeptide of collagen type I (CITP) and tissue inhibitor of matrix metalloproteinases type I (TIMP-1) were measured as markers of collagen synthesis, degradation and inhibition of degradation respectively. Significant elevations in LV mass index were demonstrated in group 1 and 2. TIMP-1 was not significantly elevated in any of three groups. P1CP was elevated in group 1(217μg/l Vs 167μg/l $P<0.05$) and CITP was significantly elevated in group 1(4.5μg/l Vs 2.9μg/l $p<0.01$), group2 (5μg/l Vs 2.9μg/l $p<0.01$) and Group3 (5.1μg/l Vs 2.9μg/l $p<0.05$) when compared to normal volunteers. Therefore, we have demonstrated abnormalities in collagen synthesis and degradation in patients with white coat hypertension. This suggests that early qualitative changes in the extracellular matrix of the cardiovascular system occur in patients with white coat hypertension including subjects with a normal ambulatory blood pressure recording by current definitions.

6.2 Introduction.

White coat hypertension is term now commonly used to describe individuals with persistently elevated office blood pressure readings but who have a normal ambulatory BP (Mancia 1987). The incidence of this condition varies markedly depending on the definition used. Pickering et al (1988) estimated that 20% of individuals thought to have mild or stage 1 hypertension had "white coat hypertension". Data from the Italian PAMELA study revealed a prevalence of between 7% to 12% in a randomly selected population and 12% upwards in untreated individuals with stage I or II hypertension (Sega 2001).

The prognosis of patients with white coat hypertension is not clear despite several studies having addressed this issue directly. The results of theses studies have varied considerably with some authors reporting that white coat hypertension is associated with an adverse outcome (Palatini 1988, Pierdomenico 1995, Glen 1996) whilst others report no positive findings (Gosse 1993, Hoegholm 1993, Cavallini 1995). The variations in results can be explained to some extent by variations in definition of white coat hypertension. Namely a cohort with a higher blood pressure will by definition have a higher incidence of end-organ damage.

Most of the published studies have attempted to demonstrate the presence of left ventricular hypertrophy as an end-point (Gosse 1993, Hoegholm 1993, Cavallini 1995), however, none have addressed the presence of myocardial fibrosis in this cohort. Despite the fact that it has become increasingly apparent that it is not only the quantity i.e. left ventricular mass but also the quality of the left ventricle structure which is important. Myocardial fibrosis occurs as part of the hypertrophic process in hypertension (Ciulla 1997) and has a key pathological role in the development of

diastolic dysfunction and subsequent systolic dysfunction in hypertension and also as a substrate for ventricular arrhythmias (Weber 1993).

Therefore the demonstration of fibrosis or a disruption in the normal collagen equilibrium within a cohort of white coat hypertensives would be an important finding in the ongoing work to determine the pathological nature and clinical consequences of white coat hypertension. A large study involving myocardial biopsies of an otherwise well population of white coat hypertensives would not be feasible. However increasingly biochemical markers of collagen metabolism have been used in the context of hypertension to help us determine the mechanisms of responsible for fibrosis and perhaps provide a biochemical means of diagnosis. This has been demonstrated in published work by our group and others (Laviades 1998, Lindsay 2002).

Therefore the aim of this study was to demonstrate evidence of fibrosis or disruption of the collagen equilibrium within a well-defined cohort of patients with white coat hypertension.

6.3 Methods.

Subjects.

The study population consisted of 50 never treated hypertensives who had three office blood pressure measurements greater than 140/90mmHg with a subsequent mean 24hr ambulatory blood pressure reading of less than 140/90mmHg. Patients were enrolled via referral to our hypertension clinic or by direct referral from local general practitioners. All patients gave consent and the local research ethics committee approved study protocols.

Prior to analysing our results, we chose to divide and analyse our cohort according to predefined blood pressure parameters. The reasons for this were twofold. Firstly, as the cohort would contain a spectrum of blood pressures ranging from stage I hypertension to blood pressures currently defined as normal. We thought it important, for the validity of our results, to ensure that any positive result obtained was not merely driven by subjects with stage I hypertension. Secondly, by dividing the cohort into pre-defined blood pressure parameters we were able to define at what blood pressure levels changes in the collagen equilibrium occur.

The cohort was therefore divided and analysed in three groups. Group 1 consisted of subjects with a mean daytime systolic BP of >140mmHg (n=25). Group 2 consisted of subjects with a mean daytime systolic BP of 135-140/90mmHG (n=13). Group 3 consisted of all subjects with a mean daytime BP of less than 135/85mmHG (n=12).

As the collagen markers used are not specific for myocardial collagen; conditions, which result in fibrosis of other organs, or conditions, which result in left ventricular hypertrophy of other causes, were excluded. These comprise aortic stenosis, renal impairment (serum creatinine >130μmol/l), coronary artery disease, secondary hypertension, malignancy of any site, pulmonary fibrosis, connective tissue disorders, significant hepatic dysfunction, left ventricular systolic dysfunction and chronic obstructive pulmonary disease.

50 control subjects were enrolled through adverts in local press. The normal subjects were determined to be normotensive on three standard BP readings (<140/90mmHG) and the above noted exclusion criteria were applied to this group.

Six hypertensive subjects were excluded prior to analysis and recruitment. Four had poor echo views, one patient had renal impairment and one patient had symptomatic coronary disease. Four normal subjects were excluded prior to

recruitment and analysis (2 subjects with hypertension and 2 subjects with poor echo views).

Study conditions.

Patients were studied at a standard time in the morning on one study day. A full history and clinical examination was made to identify exclusion criteria and suitability for the study.

ECG

All patients received a standard twelve lead surface ECG which were analysed for the presence of LVH, ST-T changes and atrial abnormalities using standard criteria (Dunn 1990, Tarazi 1966). All ECGs were analysed by a single observer. (M Lindsay).

24hr Blood Pressure Monitoring.

24 hr BP recordings were taken using a standard oscillometric Spacelab BP 90217 monitor. The monitor was attached to the non-dominant arm and three test readings were performed to ensure satisfactory recording. The monitor was set to make a recording every 30 minutes through the daytime period and every hour over the nocturnal period. Patients must have at least 1 reading per hour, 80% of total readings successful and at least a total of 30 readings over the 24-hr period for the blood pressure reading to be accepted. All readings taken during this period were documented and mean daytime, mean nocturnal and mean 24 hr blood pressures were calculated. All tapes were analysed by one observer (M Lindsay).

Echocardiographic study.

Patients were studied using a Vingmed CFM800 sonos and a Vingmed System 5 echo machine. Examinations were made in a darkened room in the standard left lateral

position. Measurements were taken according to the guidelines laid down by the American Society of Echocardiography(Sahn 1978). Left ventricular mass was calculated using the formula validated by Reichek and Devereux and indexed for body surface area (Devereux 1977). An average of at least three measurements was taken. Measurement of E: A ratio, E wave deceleration time and isovolumic relaxation times (IVRT) were made in the apical view with a cursor at the mitral valve inflow. An average of three measurements was taken at end expiration. A single observer (M Lindsay) made all measurements. Using digital archiving images intraobserver variability was tested in a blinded fashion. Intra- observer variability was 3.5%, 4%, 10% and 9% for E: A ratio, E deceleration time, IVRT and calculated left ventricular mass index (LVMI) respectively.

Biochemical Measurements.

Routine biochemical measurements were taken and analysed in the standard fashion. All samples were taken at a standard time after thirty minutes in the supine position in a darkened room. Samples were immediately centrifuged at 3000rpm for seven minutes and the plasma layer removed. The separated plasma was divided into three equal aliquots and frozen at minus 80°C. Samples were not thawed and refrozen.

To obtain an accurate biochemical assessment of the collagen equilibrium three markers were used. We measured plasma carboxy-terminal propeptide of collagen type I (PICP) as a marker of collagen synthesis, carboxy-terminal telopeptide of collagen type I (CITP) as marker of extracellular collagen type 1 degradation and tissue inhibitor of matrix metalloproteinase type I (TIMP-1) as a marker of inhibition of collagen degradation.

Plasma TIMP-1 was measured using a commercially available two stage ELISA assay specific for TIMP-1available from Amersham Pharmaceuticals. This

technique is a modified version of that described by Plumpton (1995). All samples were analysed in duplicate and intra-assay variability was 4.5%.

Plasma CITP was measured by radioimmunoassay using a polyclonal antibody direct against CITP (Ristelli 1993). All samples were run in duplicate with the intrassay variability calculated as 4.3%.

Plasma PICP was measured using a radioimmunoassay. Samples were run in duplicate with the calculated intrassay variability of 4.2%.

Statistical analysis.

The distribution of the collagen markers was tested for normality using the Anderson Darling test. CITP was normally distributed whilst both TIMP-1 and P1CP were non-parametric and thus were log transformed prior to analysis. These continuous variables were then analysed using an unpaired two-tailed student's T test. Data is presented in the non-logarithmic format. Correlations were tested using Pearson's correlation co-efficient in a logarithmic format as required. Non-continuous variables were analysed using a chi-squared test.

6.4 Results.

Baseline Characteristics.

The results are presented in *Table 1*± 1SEM.

Table 6.1: Baseline Criteria ± 1 SEM.

Variable	HBP	Normals	P value
Number	50	50	
Age (years)	42	52	<0.01
Sex (male/female)	26/24	23/27	NS
Smoking (yes/no/ex)	12/36/2	35/7/8	NS
Systolic BP (mmHg)	148±1.5	129±2.6	<0.01
Diastolic BP (mmHg)	96±1	77±2	<0.01

Urea (μmol/L)	4.6±0.2	5.2±0.3	NS
Creatinine (μmol/L)	87±2	97±2.5	NS

There is no difference in sex distribution, smoking habits or renal function. The group of white coat hypertensives is however significantly younger. We do not feel that this affects the validity of our results. As one would expect office blood pressure readings are significantly higher amongst the cohort of subjects with white coat hypertension.

ECG.

ECG were analysed using the above referenced criteria. 2% of white coat hypertensives and no normals had LVH. 14% of white coat hypertensives had ST-T change and no normal subjects ($P<0.01$). The presence of ST-T change was not related to blood pressure level. 8% of both groups had atrial abnormalities.

Ambulatory Blood Pressure.

Readings were on average 85% successful with 37 individual readings taken over an average of a 25hr period. Mean 24hr blood pressure for the cohort of white coat hypertensives was 131/81mmHG. Mean daytime blood pressure was 136/87mmHG with an average daytime heart rate of 80/min. Mean nocturnal blood pressure was 119/73mmHG with an average nocturnal heart rate of 70/min. The strict definition as to what represents normal blood pressure is variable. We therefore analysed our cohort of white coat hypertensives at three pre-defined blood pressure levels as described above.

Echocardiography.

Patients were excluded if poor quality imaging prevented analysis of m-mode and doppler studies.

Results are summarised in the* table 2±*SEM

Table 6.2: Echocardiographic parameters ± 1 SEM.(* p<0.01 and ♦p<0.05 Vs Normal Volunteers.)

Parameter	*Group 1*	*Group 2*	*Group 3*	Normal Volunteers
Number	25	13	12	50
IVSDd (cm)	1.08±0.05*	1.09±0.06♦	1.00±0.05	0.90±0.03
LVPWd (cm)	1.05±0.03*	1.09±0.04*	1.00±0.04	0.90±0.02
LVMI (g/m2)	112±5♦	121±8♦	103±5	97±3
E: A ratio	1.24±0.07	1.1±0.08	1.15±0.10	1.15±0.03
E Dec (ms)	17.8±1.0	16.1±1.4	18.1±1.0	19.3±1.0
IVRT (sec)	0.1±0.004	0.1±0.01	0.1±0.004	0.09±0.003

The echo data was analysed at three pre-defined blood pressure cut-offs as defined above. Group 1 (n=25) had a significant increase in interventricular septal thickness in diastole (IVSd) (1.08cm Vs 0.90cm p<0.01), left ventricular posterior wall thickness in diastole (LVPWd) (1.05cm Vs 0.90cm p<0.01) and left ventricular mass index (LVMI) (112g/m2 Vs 97g/m2 p<0.05) when compared with the group of normal volunteers (n=50). There was no difference in any of the indices of diastolic filling, namely, E: A ratio (1.24 Vs 1.15 p=ns), E Dec (18ms Vs 19ms p=ns) and IVRT (0.1sec Vs 0.1sec p=ns). Group 2 (n=13) again demonstrated an increase in IVSd (1.09cm Vs 0.90cm p<0.05), LVPWd (1.09cm Vs 0.90cm p<0.01) and LVMI (121g/m2 Vs 97g/m2 p<0.05) in comparison with the group of normal volunteers (n=50). There was again no difference in the indices of diastolic filling. However the cohort with a mean daytime blood pressure of less than 135/85mmHg (n=12) demonstrated no significant difference in IVSd (0.98cm Vs 0.90cm p=ns), LVPWd (0.99cm Vs 0.91cm p=ns) or LVMI (103g/m2 Vs 97g/m2 p=ns) when compared to the normal volunteers (n=50). There was no difference in indices of diastolic filling.

Collagen markers.

TIMP-1

TIMP-1 was not significantly elevated in group 1 (n=25)(307ng/ml Vs 253ng/ml p=ns) (95%C.I –32,140), group 2 (n=13)(282ng/ml Vs 253ng/ml p=ns) (95%C.I – 42,100), group 3 (n=12)(315ng/ml Vs 253ng/ml p=ns) (95% C.I –67,192) or when the cohort was analysed as a whole (n=50) (290ng/ml Vs 253ng/ml p=ns) (95% C.I – 13,88) when compared with normal volunteers (n=50). There was no relationship between TIMP-1 and blood pressure, markers of LV mass or indices of diastolic filling.

CITP

CITP was significantly elevated in group 1 (n=25)(4.5μg/l Vs 2.9μg/l p<0.01)(95% C.I 0.41,2.7), group 2 (n=13)(5μg/l Vs 2.9μg/l p<0.01)(95% C.I 0.69,3.35), group 3 (n=12)(5.1μg/l Vs 2.9μg/l p<0.05)(95% C.I 1.09,2.41) and when the cohort was analysed as a whole (n=50) (4.8μg/l Vs 2.9μg/l p<0.01)(95%C.I 1.04,2.64) when compared with normal volunteers (n=50). These findings are demonstrated on *Figures 6.1,6.2,6.3,and 6.4*. This elevation was consistent amongst all the groups and independent of any blood pressure variable. There was no relationship between CITP and any of the markers of LV mass or indices of diastolic filling.

P1CP

P1CP was significantly elevated in group 1 (n=25)(217μg/l Vs 166μg/l p<0.05)(95% C.I 1,98.7) (*figure 6.5*) and when the cohort was analysed as a whole (n=50) (214μg/l Vs 166μg/l p<0.01) (95% C.I 12,81.7). However no significant elevation was observed in group2 (n=13) (214μg/l Vs 166μg/l p=ns)(95%C.I –32,127.8) or group 3 (n=12)(205μg/l Vs 166μg/l p=ns)(95%C.I –14,91) when compared with normal

volunteers (n=50). There was no relationship between P1CP and blood pressure, markers of LV mass or indices of diastolic filling.

6.5 Discussion.

As described above the pathological significance of white coat hypertension remains uncertain. The first and perhaps the key issue is the strict definition of what classes as white coat hypertension remains variable. There is now a consensus that an office blood pressure greater than 140/90mmHg is abnormal. The subsequent definition of a normal ambulatory blood pressure remains contentious. Almost all the large studies have used different definitions and therefore have different results regarding incidence of long-term sequelae and incidence of target organ damage. For this reason, in addition to analysing our cohort as a whole, we chose to analyse our cohort at three pre-defined blood pressure levels. The first being a mean daytime systolic BP of greater than 140mmHg. Most experts would agree that this group would consist predominately of subjects with stage I hypertension. The second cut-off was a mean daytime systolic BP 135-140mmHg and finally a cut-off of a mean daytime of <135/85mmHg, which most consensus statements would class as normal (JNC VI).

The aim of our study was to demonstrate the presence of target organ damage among this cohort and, if possible, to define the level of blood pressure at which this became demonstrable. Most previous studies have looked for echocardiographic evidence of an increase in left ventricular mass or impairment in diastolic filing for this purpose (Gosse 1993, Hoegholm 1993, Cavallini 1995). However no previous work has addressed the issue of qualitative change in left ventricular structure in the form of fibrosis. This is recognised as a key pathological component of hypertensive left ventricular hypertrophy. We therefore undertook to study the presence or absence

of fibrosis in this cohort. We achieved this by the use of biochemical markers of collagen metabolism.

To allow a full assessment of the collagen equilibrium we measured markers of collagen synthesis, degradation and inhibition of degradation. Plasma P1CP was measured as marker of collagen synthesis. This peptide is the most accurate marker of collagen synthesis as it is reliably cleaved during the extracellular processing of collagen type I unlike other markers such as procollagen type III amino terminal peptide (Nimni 1993). As this is excreted via a hepatobiliary route, patients with hepatic dysfunction were excluded from this study.

Collagen degradation was assessed by measurement of CITP, a cross-linked telopeptide released in a 1:1 stoichometric fashion when collagen type I fibrils are degraded thus giving an accurate measurement of collagen degradation (Ristelli 1993).

Matrix metalloproteinases (MMP) are a family of zinc and calcium dependent endopeptidases, which play a key role in the degradation of collagen. MMPs have potent proteolytic activity, which is controlled in a major part by the production of specific naturally occurring inhibitors called tissue inhibitors of matrix metalloproteinases (TIMP) .The best characterised is TIMP-1. Therefore the measurement of TIMP-1 allows an estimation of inhibition of degradation (Dernhardt 1993).

The limitations of this study should be accepted. Firstly, none of the above markers are specific for myocardial collagen introducing potential confounding results. However, care was taken to exclude any conditions that would result in myocardial fibrosis of another aetiology or end-organ fibrosis elsewhere. Secondly, the blood pressure parameters used do not represent a strict definition of "White coat"

hypertension under current British Hypertension Society Guidelines. However, guidelines have changed since this study was conceived and recruited and hence the variance with current recommendations.

Our results demonstrate some very illuminating findings. Firstly it is notable that even within the cohort of subjects that a mean daytime systolic BP of 135-140mmHg there is still a significant increase in left ventricular dimensions and calculated left ventricular mass index when compared with the cohort of normal subjects. The demonstration of an increment in LV mass at such a low level is surprising. There was no abnormality in diastolic filling in any of three groups.

Analysis of the collagen markers allows us further insight. There was no significant elevation in inhibition of collagen degradation (TIMP-1) in any of the cohorts. This would be consistent with our previous published work on collagen markers in sustained hypertension, which demonstrated that TIMP-1 was a marker of diastolic dysfunction and only elevated in the presence of abnormal diastolic filling (Lindsay 2002). However, the positive and important findings from this study are twofold. Firstly was the finding of elevated collagen synthesis in Group1. As noted above this group had a mean 24hr BP of less than 140/90mmHg and a daytime systolic BP of greater than 140mmHg. Most experts would agree that blood pressure at this level is abnormal and represents stage I hypertension but nonetheless it is a notable finding that collagen synthesis is stimulated in the context of mild hypertension. The second important finding was the demonstration of elevated collagen degradation amongst all groups. This may not have been too surprising if this were limited to group1, where we have demonstrated elevated collagen synthesis and which contains subjects with stage I hypertension. However, even the cohort with a daytime BP of less than 135/85mmHg there was a significant elevation in collagen

degradation when compared to the normal cohort (5.1μg/l Vs 2.9μg/l $p<0.01$). Therefore, using a strict definition of white coat hypertension, namely, an office blood pressure of greater than 140/90mmHg and a mean daytime ambulatory blood pressure of less than 135/85mmHg there is still demonstrable evidence of disruption of the collagen equilibrium.

Clearly, this study using non-invasive biochemical markers does not prove definitively that fibrosis is present in white coat hypertension. However, we have demonstrated a disruption of the collagen equilibrium with an increments in collagen degradation at all levels and an increment in collagen synthesis within group 1 of our cohort. These findings echo those demonstrated in sustained hypertension of which fibrosis is an accepted part. Therefore this allows us to hypothesise that changes in extracellular matrix leading to fibrosis occur in white coat hypertension.

Furthermore, this study allows us to hypothesise about the temporal relationship of collagen synthesis, degradation and inhibition of degradation in hypertension and the resulting fibrosis. It would appear that abnormalities in collagen degradation are the initial pre-cursor to the development of fibrosis occurring at modest elevations in blood pressure previously regarded as normal, collagen synthesis is stimulated in borderline and stage I hypertension and inhibition of degradation is only demonstrated when fibrosis is established and demonstrable by non-invasive means for example diastolic dysfunction on echo.

Therefore, in conclusion, we have demonstrated disruption of the collagen equilibrium with increased collagen synthesis and degradation in patients with white coat hypertension. We have shown these changes occur at blood pressure levels currently considered to fall within the normal range. These findings suggest that qualitative changes occur in the myocardium/vasculature in white coat hypertension at

a very early stage. This has important implications in the ongoing debate regarding the clinical consequences of white coat hypertension, at what levels should hypertension be treated and also raises questions about what truly represents a normal ambulatory blood pressure in patients with elevated office readings.

Figure 6.1. Data points show CITP concentrations (micrograms/l) in the cohort of hypertensives (n=50) as a whole and normal volunteers.

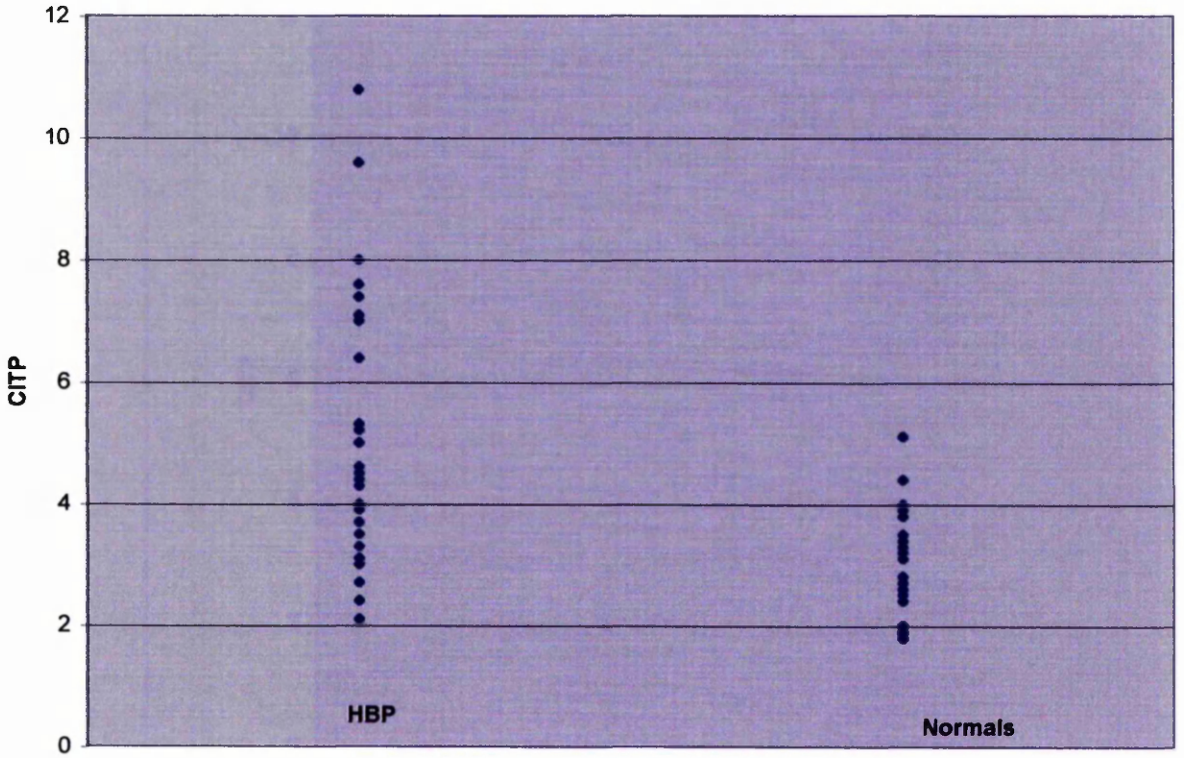


Figure 6.2: Data points show plasma CITP (micrograms/l) in subjects with a mean daytime systolic BP of >140mmHg (group 1) (n=25) and normal subjects.

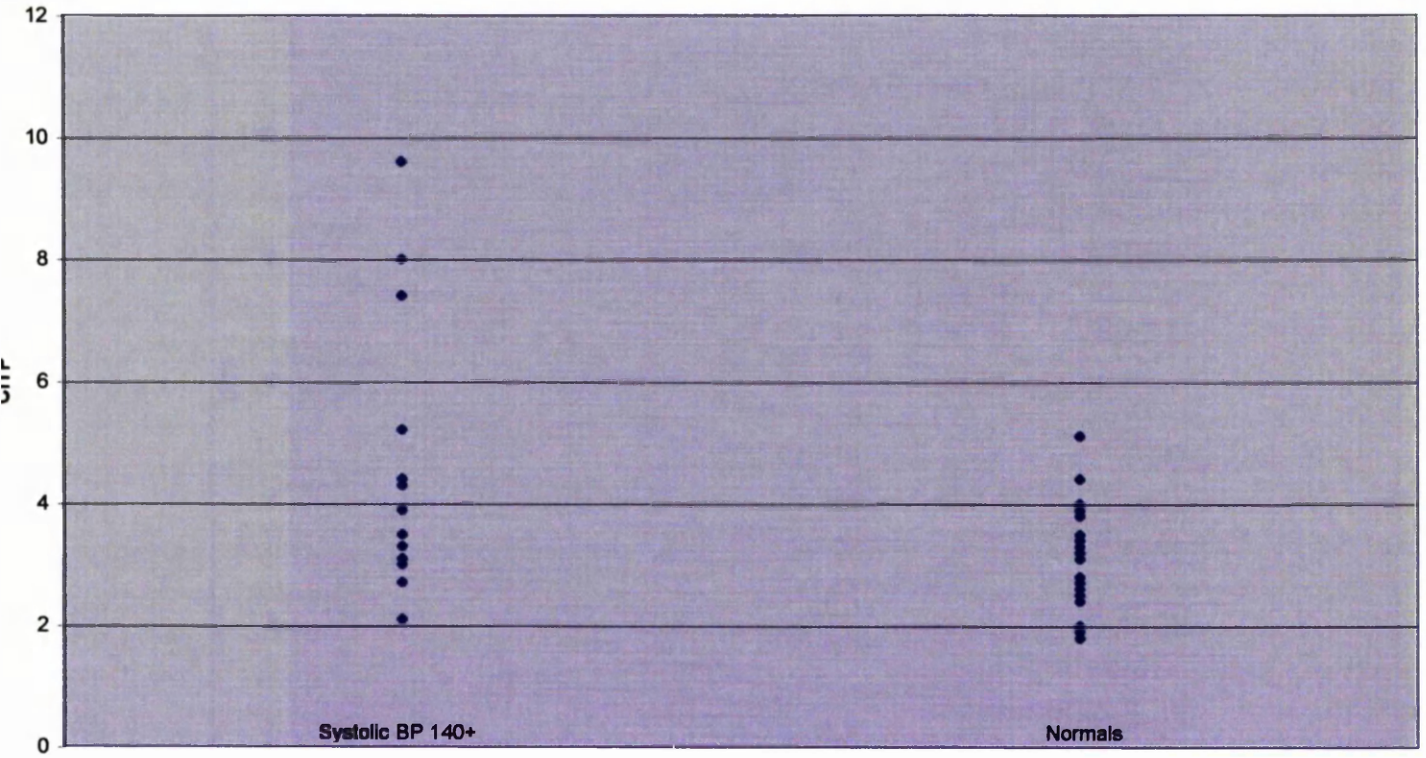


Figure 6.3: Data points show plasma CITP levels (micrograms/L) in subjects with a mean daytime systolic BP of 135-140mmHg (group 2)(n=13) and normal subjects.

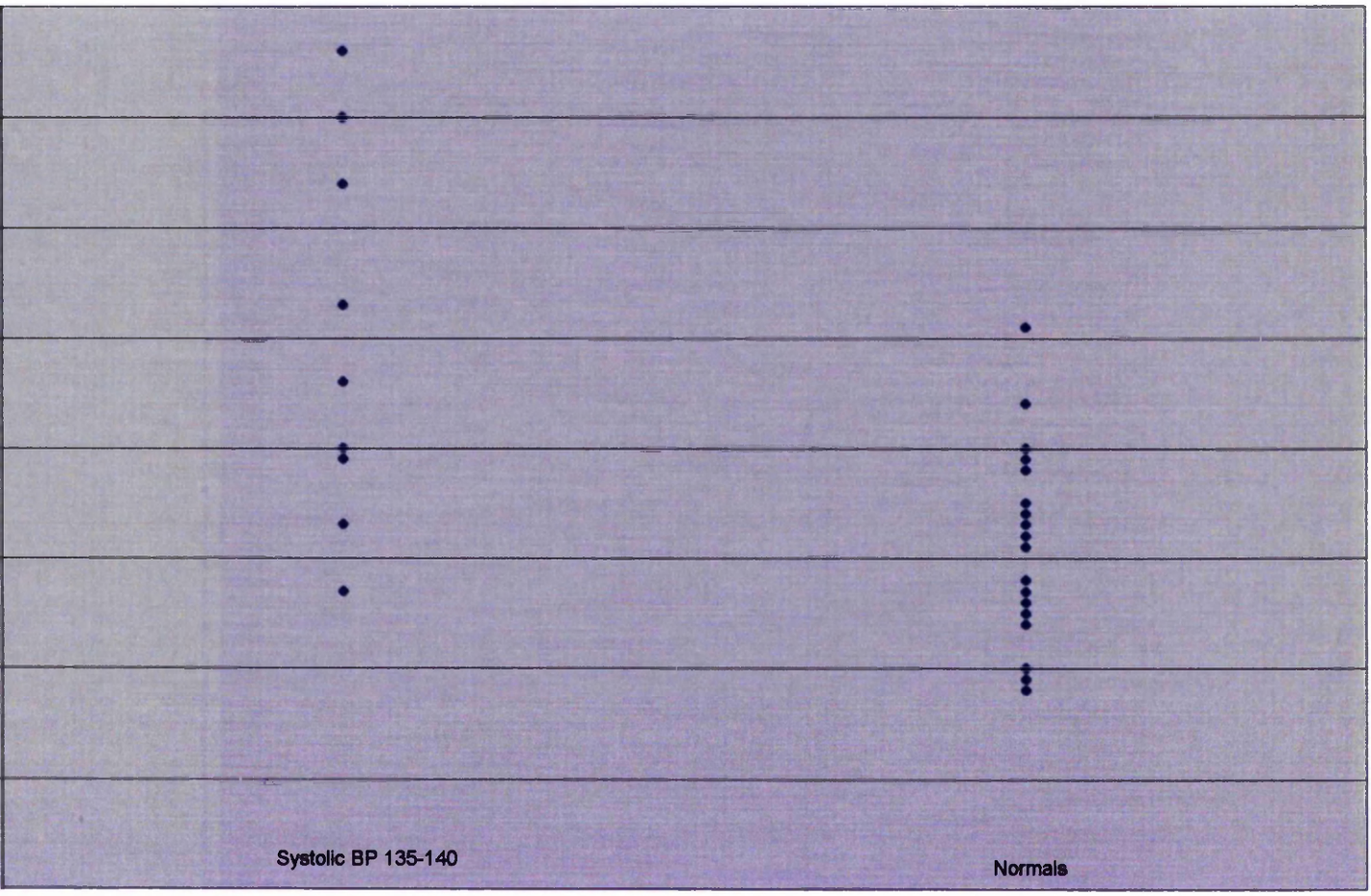


Figure 6.4: Data points show plasma CITP (micrograms/l) in subjects with a mean daytime BP <135/85mmHg (group3) (n=12) and normal subjects.

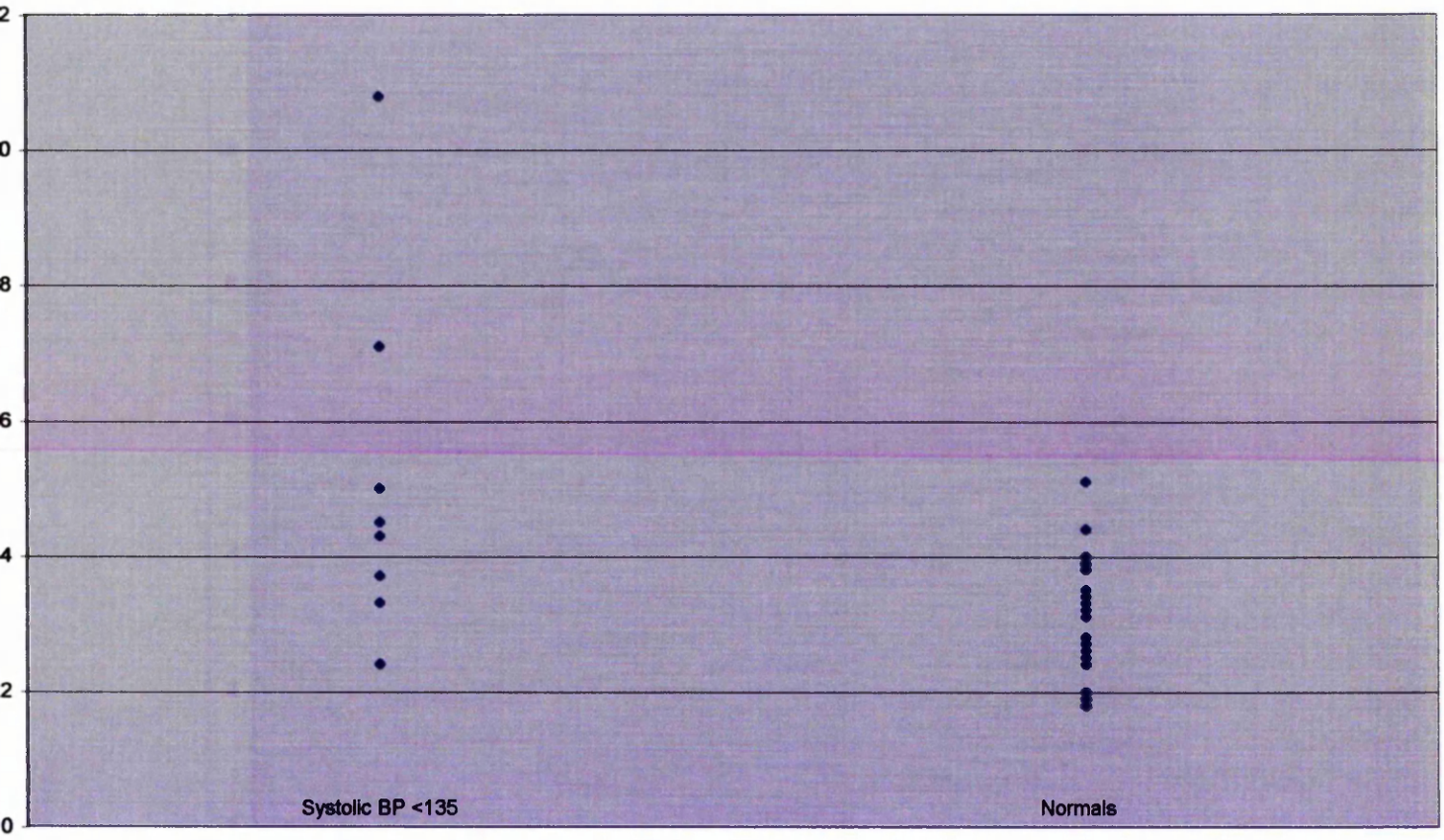
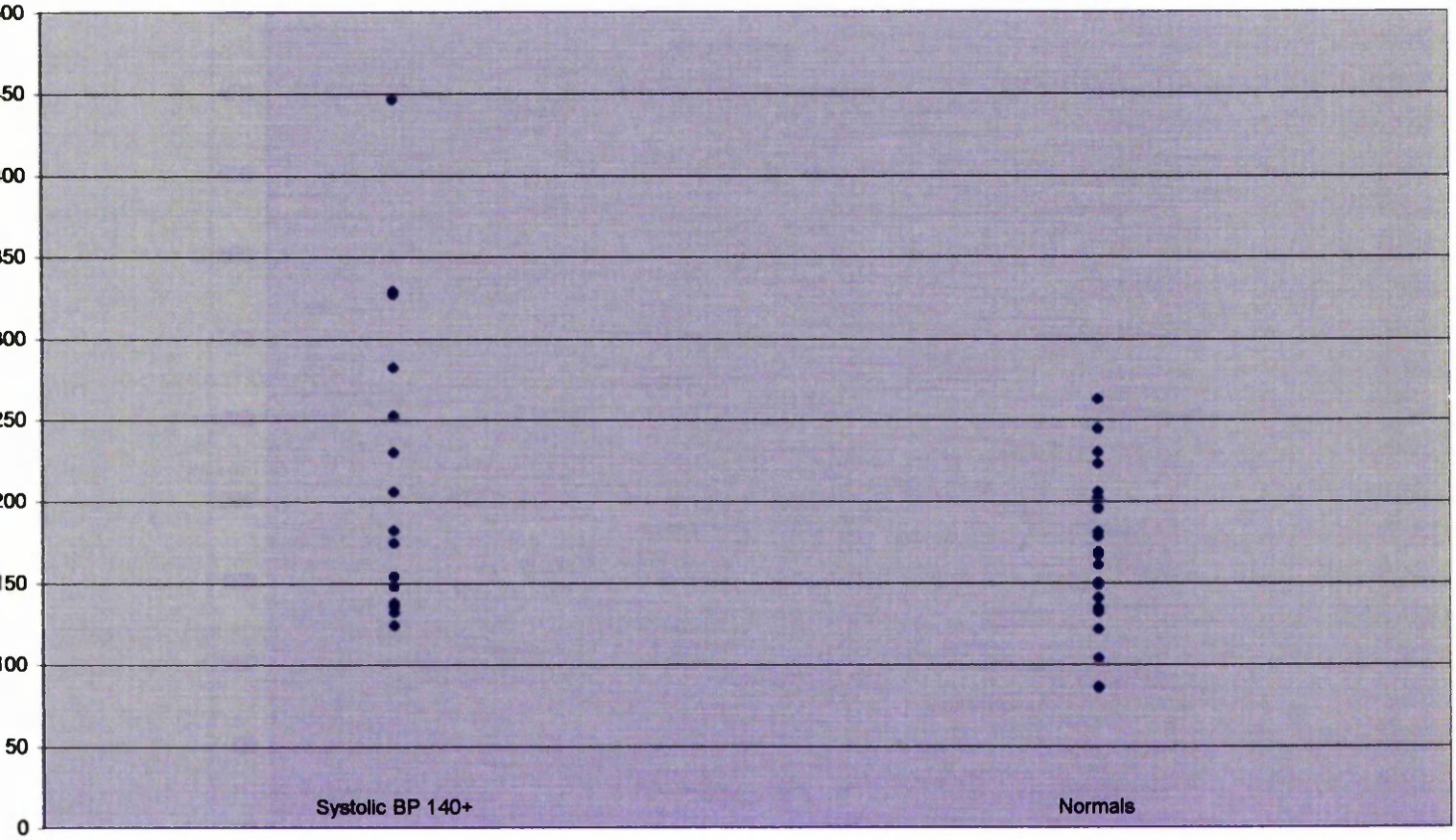


Figure 6.5: Data points show plasma P1CP(micrograms/l) in subjects with a mean daytime systolic BP of > 140mmHg (n=25) and normal subjects.



Chapter 7

Summary and conclusions.

The results of any clinical investigations rely on the validity and reproducibility of the methods used. It was therefore key to this thesis that the assays used and techniques employed were reliable and reproducible. To ensure this, we performed extensive validation and reproducibility experiments on all three assays prior to any clinical studies. The results are documented in Chapter 3 and reveal intra-assay variability of less than 5% for all three assays, which compares very favourably with previous published work.

Echocardiography is a central technique in this thesis and has undoubted inherent variability. Echo studies were performed by one operator (M Lindsay) using a standardised protocol in an effort to limit variability. Furthermore, blinded variability studies for both Doppler and M-mode measurements were conducted to ensure the results were reproducible and that meaningful comparisons could be drawn. The results are presented fully in chapter 3 and compare very favourably with any previously published data.

In Chapter 4 I set out to non-invasively delineate the pathophysiology of fibrosis in hypertensive heart disease and to assess the clinical feasibility of using collagen markers in this setting. This is by far the largest study to date in this field. I enrolled a large cohort of well-characterised never treated hypertensives and used robust assays in standardised conditions. Measurement of P1CP, CITP and TIMP-1 allowed full assessment of the collagen equilibrium. My results demonstrate an increase in collagen synthesis, degradation and inhibition of degradation in untreated essential hypertension. Collagen synthesis and degradation appeared to be elevated throughout the cohort; however, TIMP-1 was only elevated amongst patients with

established diastolic dysfunction. The remaining patients with normal diastolic filling had TIMP-1 levels comparable to normal subjects. Furthermore, there was no relationship between TIMP-1 and any index of systemic blood pressure or left ventricular mass. Therefore TIMP-1 levels are not elevated in hypertension per se but only in patients with diastolic dysfunction and fibrosis.

The other aim of this study was to assess the clinical utility of collagen markers as a non-invasive assessment of fibrosis. Diastolic dysfunction has been shown in animal and human models to be secondary to fibrosis and independent of myocyte hypertrophy. The presence of diastolic dysfunction was used as a surrogate for fibrosis in this study. Our results show TIMP-1 to be independent of blood pressure, to correlate with indices of diastolic filling and have a specificity and positive predictive value for diastolic dysfunction of over 95%. A measured TIMP-1>500ηg/l is an accurate and robust predictor of diastolic dysfunction and hence end-organ damage.

In summary, I have demonstrated using non-invasive markers of the collagen equilibrium that untreated essential hypertension is characterised by an increase in collagen synthesis, degradation and inhibition of degradation. The relative roles played by these processes in the resultant fibrosis cannot be determined by this study. Secondly, I demonstrated an association between biochemical markers of fibrosis and LV diastolic dysfunction in patients with untreated hypertension. In particular I identified TIMP-1 as a potential non-invasive marker of fibrosis. Further studies to investigate these markers as predictors of risk are needed and if positive will provide an important additional marker in the risk assessment of patients with hypertension.

In chapter 6, I enrolled a cohort of never treated patients with "white coat hypertension". This was diagnosed on strict ambulatory blood pressure criteria. This

was a particularly attractive cohort to study as the issue of interstitial changes in "white coat " hypertension had never been addressed. Furthermore, by dividing our cohort into three groups according to 24hr blood pressure measurement we were able to assess at what blood pressure levels changes in the interstitium occur. The first notable finding was that TIMP-1 was normal throughout the cohort. This was consistent with our work in sustained hypertension, which shows TIMP-1 to be only elevated in the presence of diastolic dysfunction. Group 1 (mean daytime systolic BP>140mmHg) demonstrated an increment in collagen synthesis (P1CP) and degradation (CITP). This group would contain patients with stage I hypertension however it is nonetheless notable that increased collagen synthesis and disruption to the equilibrium is occurring at the mild spectrum of disease. P1CP was normal in groups 2 and 3.

An additional finding of this study was that in groups 2 (mean daytime systolic BP 135-140mmHg) and group 3 (mean daytime systolic BP<135/85mmHg) there was a significant elevation in collagen degradation (CITP). Thus, even using the strictest definitions of normal ambulatory blood pressure (mean daytime systolic BP<135/85mmHg) there is evidence of disruption of the collagen equilibrium in patients with elevated office BP recordings.

Clearly, this study using non-invasive biochemical markers does not prove definitively that fibrosis is present in white coat hypertension. However, I have demonstrated a disruption of the collagen equilibrium with an increments in collagen degradation at all levels and an increment in collagen synthesis within group 1 of our cohort. These findings echo those demonstrated in sustained hypertension of which fibrosis is an accepted part. Therefore this allows us to hypothesise that changes in extracellular matrix leading to fibrosis occur in white coat hypertension.

Therefore I have demonstrated disruption of the collagen equilibrium with increased collagen synthesis and degradation in patients with white coat hypertension. These changes occurred at blood pressure levels currently considered to fall within the normal range. These findings suggest that qualitative changes occur in the myocardium/vasculature in white coat hypertension at a very early stage. This has important implications in the ongoing debate regarding the clinical consequences of white coat hypertension, at what levels should hypertension be treated and also raises questions about what truly represents a normal ambulatory blood pressure in patients with elevated office readings.

Taking my results as a whole I have demonstrated that collagen markers are useful, robust non-invasive assessment of fibrosis. Within the context of hypertension we have demonstrated that TIMP-1 is a marker of fibrosis. We now have enough data to hypothesise about the temporal relationship and pathogenesis of fibrosis within hypertension. It appears that the first alteration to the collagen equilibrium is increment in degradation, presumably via up-regulation of MMPs, followed by increments in collagen synthesis. Inhibition of degradation, appears to play a central role only in more advanced stages, when fibrosis is present. These finding are consistent throughout the cohort of 150 never treated sustained hypertensives and "white coat" hypertensives.

Finally, in chapter 5, I studied left ventricular hypertrophy occurring outwith the context of hypertension. Namely exercise induced left ventricular hypertrophy and in particular veteran athletes. The pathological significance and clinical consequences of exercise induced LVH is unclear and this is particularly relevant amongst veteran endurance athletes where studies have suggested incomplete regression of LVH on

cessation of exercise. Using strict inclusion criteria I was able to enrol a population of veteran athletes with structural cardiac changes and without any co-morbidity. The aim of this study was to demonstrate, using biochemical markers, the presence of fibrosis in exercise induced LVH in a cohort of veteran athletes.

My results demonstrate that in elite veteran athletes there is biochemical evidence of disruption of the collagen equilibrium in comparison with a matched cohort of sedentary control subjects. Taking the cohort of athletes as a whole, there was a significant increase in collagen synthesis as assessed by an elevation in PICP, an increase in collagen degradation as assessed by an elevation in CITP and an increase in inhibition of degradation as assessed by an elevation in TIMP-1. Of more significance is the finding that athletes with echo evidence of exercise induced LVH ($LVMI>130g/m^2$) had a further statistically significant elevation in TIMP-1. Indeed TIMP-1 was only elevated in athletes with LVH; the remaining athletes having TIMP-1 levels comparable to normal. Therefore, there only appears to be an elevated inhibition of degradation (TIMP-1) amongst athletes with LVH. This disruption of the collagen equilibrium amongst athletes with LVH, characterised by inhibition of collagen degradation, would favour the development of fibrosis. This contrasts with P1CP and CITP, which were elevated throughout the cohort of athletes irrespective of the presence of LVH.

These findings suggest that increments in collagen synthesis and degradation occur at an early stage in the development of exercise induced cardiovascular adaptation. Whereas inhibition of collagen degradation occurs in more advanced stages of cardiovascular adaptation associated with established exercise induced hypertrophy.

In conclusion, my results demonstrate that there is biochemical evidence of disruption of the collagen equilibrium favouring fibrosis in veteran athletes with LVH. This may suggest fibrosis occurs as part of the hypertrophic process of exercise induced LVH in veteran athletes and would provide a possible explanation for incomplete regression of LVH in this cohort.

Limitations

Many of the potential limitations of this study arise from the use of non-invasive markers of fibrosis. A potential limitation relates to the reliability of the assay. However, as described in Chapter 3, the three assays used were robust and reproducible.

A further limitation arises from the fact that the assays are not specific to cardiac tissue or the vasculature. The three markers used, have been shown to be elevated in other conditions characterised by fibrosis, for example, hepatic cirrhosis. Therefore, every effort was made by a careful screening procedure to exclude subjects with conditions that may confound our results. I have listed the patients that were excluded prior to any analysis and were not recruited. This is a relatively small number as the General Practitioners referring the patients were given an exhaustive list of exclusion criteria which they kept to rigidly.

The collagen markers have been shown to have additional biological properties. I have listed some of the effects of TIMP-1 in the introduction. It must be accepted that some of these properties may have produced a confounding effect on my results. For example, TIMP-1 has haemopoetic-stimulating effects and therefore may have been

stimulated by high intensity aerobic training and thus potentially influencing the results of the athletic cohort.

Finally, in the absence of cardiac biopsies the conclusions I have drawn have to be inferred from the non-invasive data available to me rather than presenting "gold standard" pathological evidence. However, the collagen markers used have been validated against cardiac biopsy findings and a biopsy study of this size would not have been feasible.

Further study is required to confirm and advance the findings of this study and to further delineate any potential clinical role for these collagen markers.

The study of a larger cohort of " White coat" hypertensives would be appropriate allowing valid assessment of subgroups and allowing us to explore the continuous relationship between collagen metabolism and blood pressure.

A further study of a hypertensive cohort investigating vascular fibrosis rather than merely cardiac involvement and also investigating the effect, if any, of the common cardiovascular genetic polymorphisms on the collagen equilibrium would advance our knowledge and understanding further.

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