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ABBREVIATIONS

ACEI – Angiotensin Converting Enzyme Inhibitor

ACR – Albumin Creatinine Ratio

AGE – Advanced Glycation End Products

ALT – Alanine Transaminase

ARB – Angiotensin Receptor Blocker

BAP – Bone specific Alkaline Phosphatase

BMP – Bone Morphogenic Protein

BMI – Body Mass Index

CHF – Chronic Heart Failure

CKD – Chronic Kidney Disease

CRP – C - reactive protein

CTI – Chronic Tubulointerstitial Injury

CVC – Calcifying Vascular Cells

CVD – Cardiovascular Disease

DM – Diabetes Mellitus

DN – Diabetic Nephropathy

DV – Diabetic Vasculopathy

EC – Endothelial Cell

ECD – Endothelial Cell Dysfunction

EDHF – Endothelium Derived Hyperpolarizing factor

ELISA – Enzyme Linked Immunosorbent Assay

EPO – Erythropoietin

EPOR – Erythropoietin Receptor

ESRD – End Stage Renal Disease

ET – Endothelin

GDM – Gestational Diabetes Mellitus

GGT – Gamma Glutamyl Transferase

GFR – Glomerular Filtration Rate

HIF – Hypoxia Inducible Factor

HLA – Human Leucocyte Antigen

HRE – Hypoxia Responsible Element

ICAM – Intercellular Adhesion Molecule

IDDM – Insulin Dependent Diabetes Mellitus

IFG – Impaired Fasting Glucose

IGT – Impaired Glucose Tolerance

IL – Interleukin

IR – Insulin Resistance

Macrovasculopathy – Large Vessel (Artery) Disease

MAC – Medial Arterial Calcification

MD – Macula Densa

MDRD – Modification of Diet in Renal Disease

MI – Myocardial Infarction

Microvasculopathy – Small Vessel (Artery) Disease

MODY – Maturity Onset Diabetes of the Young

MRDM – Malnutrition Related Diabetes Mellitus

Msx2 – Homeobox, msh-like 2 gene

NAG – N - acetyl- β -D-glucosaminidase

NICE – National Institute of Clinical Excellence

NIDDM – Non Insulin Dependent Diabetes Mellitus

NO – Nitric Oxide

Ntx – N- linked telopeptide of Collagen

OPG – Osteoprotegerin

PDGF – Platelet Derived Growth Factor

PPi – Inorganic pyrophosphate

PTC – Peritubular Capillaries

PTH – Parathyroid Hormone

PVD – Peripheral Vascular Disease

RAGE – Receptor for Advanced Glycation End Products

RANKL – Receptor Activator for nuclear factor kappa B ligand

RAS – Renin Angiotensin System

RBC – Red Blood Cells

RBP – Retinol Binding Protein

ROS – Reactive Oxygen Species

TGF – Tubulo-Glomerular Feedback

TGF- β – Transforming Growth Factor Beta

TNF – Tumour Necrosis Factor

TRAIL – TNF - related apoptosis inducing ligand

VC – Vascular Calcification

VCAM – Vascular Adhesion Molecule

VDR – Vitamin D Receptor

VEGF – Vascular Endothelial Growth Factor

VSMC – Vascular Smooth Muscle Cells

vWF – Von Willebrand Factor

Wnt – Signalling pathway

ACKNOWLEDGEMENTS

I dedicate this thesis to the memory and blessings of my late father Shri. Kailashnath Nageshwar Singh, who died of diabetic vascular complications.

This thesis owes a great deal to the patience of my whole family especially my mother, wife and brother who have been my pillars of support in this endeavour. Several people have helped me to realise this thesis. First and foremost I take this opportunity to pay my humble respects to my principal supervisor and Guru - Prof. Ken Farrington, without whose patience, support and motivation, this thesis would not have seen the light of the day.

I owe a great deal to my supervisors Dr. Peter Winocour and Dr. David Wellsted who spent a great deal of their time and energy to formulate the ideas and statistical robustness of the clinical studies, apart from their persistent motivation.

Special thanks to my research colleague Bev Summerhayes who provided crucial support for organising patient visits, blood sampling, and collection of data. Thanks are due to my research colleagues Joe Chilcot, Dr. M. Sivalingam and Dr. Enric Vilar for their suggestions on the clinical and statistical aspects of the research studies. Thanks are due to G. Sivakumar and Dr. Adie Viljoen for their help with analysis of the samples and Dr. Sunil Kaniyur for the help with radiological assessments. The assistance of the nursing and secretarial staff at the renal research unit, Lister hospital, Stevenage is much appreciated.

I would like to thank the Lister Renal Research Fund, *Eli Lily*, Diabetes UK and Roche for the funding provided for various studies.

My humble thanks to all the participants of the studies.

AUTHOR'S DECLARATION

The concepts and hypotheses submitted in this thesis were formulated during several rounds of discussion between Prof. Ken Farrington, Dr. Peter Winocour and I. Statistical guidance for these studies was provided by Dr. David Wellsted. The reported studies were personally undertaken and written by me under the guidance of my supervisors. The contributions of other individuals have been stated above. The various sources of information have been acknowledged and permission for replication of figures was sought, where required.

I hereby confirm that this thesis or part of this thesis has not been submitted or accepted as a previous degree to either University of Hertfordshire or elsewhere. However, individual components in form of abstract or research papers have been submitted to various scientific meetings or peer-reviewed journals. Request for details of the study or data can be made to me on dsingh4@nhs.net

Dr. Dhruvaraj K. Singh

SUMMARY

Diabetic vasculopathy (DV) is the most important consequence of chronic hyperglycemia in patients with diabetes mellitus (DM). This thesis explores the interaction of blood, bone and kidney in the pathogenesis of DV by i) reviewing the current understanding of pathogenesis of macrovascular and microvascular diseases in DM to identify gaps in literature and generate hypotheses relating to various facets of DV ii) undertaking a series of prospective studies to examine these hypotheses iii) analysing the findings and integrating any new information obtained from the clinical studies into the current knowledge base and iv) generating hypotheses upon which future work might be based.

The literature search was carried out with the aim of understanding current concepts of pathogenesis of DV and its potential modulators. The original reviews resulting from this process are presented in chapters 2 to 4. A series of pilot studies reported in chapters 7 to 11, were then carried out to interrogate hypotheses originating from this process. The first study was carried out in healthy individuals to define the biological variation of potential modulators of DV, namely erythropoietin (EPO), parathyroid hormone, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D to facilitate the design and interpretation of subsequent studies. It revealed a wide biological variation of these modulators in the healthy population thus, emphasizing the need to have a control group in the subsequent study population.

To examine whether tubulointerstitial dysfunction occurs before the onset of microalbuminuria, a measurement of the above mentioned parameters was carried out along with markers of tubulointerstitial injury in patients with type 1 and type 2 DM without microalbuminuria and in non-diabetic controls. It was found that tubulointerstitial dysfunction with low levels of EPO and 1, 25-dihydroxyvitamin D and higher excretion of tubular injury markers, occurs before the onset of microalbuminuria. Subsequently, diabetic and nondiabetic chronic kidney disease (CKD) patients with EPO deficiency anaemia were examined to study the effects of EPO therapy on the excretion of tubular injury markers.

However, in these patient groups, we were unable to demonstrate an effect of EPO therapy on the markers of tubular injury in spite of a beneficial haematological response.

To examine whether vascular calcification (VC) and bone mineral density (BMD) were linked in patients with diabetes mellitus and to explore their relationship to modulators of DV, an assessment of VC and BMD was undertaken in patients with type 2 DM with different degrees of proteinuria and normoalbuminuria. VC was assessed by CT scan and BMD by a DEXA scan. Modulators of DV were measured including serum Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa- β -ligand (RANKL). The findings were i) a high prevalence of VC and osteopenia in normoalbuminuric type 2 DM patients with normal serum creatinine ii) a weak inverse relationship between VC and osteopenia iii) proteinuric patients had worse VC but not osteopenia iv) weak relationships between OPG levels and both VC and osteopenia, masked by age in multivariate analysis. The final study examined the relationship between modulators of DV, including OPG and RANKL, and the degree of CKD. It was found that abnormalities of OPG and RANKL occur before the onset of microalbuminuria and progress with deterioration of renal function. Compared to non-diabetics, DM patients have higher OPG levels in the predialysis phase and lower levels in haemodialysis phase, a phenomenon that might indicate endothelial exhaustion in dialysis patients with DM.

The derangements associated with DV seem to occur earlier than previously thought. Further work is required to untangle these complexities and to define the contribution of factors such as the adverse blood milieu, the vasculature, abnormal bone and mineral metabolism, and early tubulointerstitial damage. The findings from the studies reported here may help in the formulation of new hypotheses, which might contribute to future work in this area.

SECTION I

CHAPTER 1

DIABETES MELLITUS AND ITS COMPLICATIONS

DIABETES MELLITUS AND ITS COMPLICATIONS

1.1 Introduction

Diabetes Mellitus (DM) is a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.¹ DM has been known to physicians since antiquity. Initially, the disease was described as Diabetes (greek- *syphon*) literally meaning passing huge amounts of water, by Aretaeus, the Greek physician. Mellitus (latin- *honey*) was added by Thomas Willis, an English physician, to signify ‘sweet urine’ passed by these patients.

The prevalence of DM is increasing worldwide with estimated projection of approximately 300 million patients worldwide by year 2025.² My own country, India has been recognised as the diabetes capital of the world by the international diabetes federation with approximately around 40.9 million people affected by diabetes as of 2006. This number is projected to rise to 69.9 million by 2025.³ In the Indian diabetes population, the prevalence of macrovascular complications such as coronary artery disease and peripheral vascular disease has been reported as 21.4 and 6.3% respectively.⁴ In the same population, the prevalence of microvascular complication such as microalbuminuria and diabetic nephropathy was reported as 26.9 and 2.2% respectively.⁵

In ancient times, DM was recognised as incurable and the prognosis was poor. The history of investigation into the pathology and treatment of DM is as long as the

disease itself. Aretaeus described the disease as "a melting down of the flesh and limbs into urine."⁶ For centuries, was thought to be a disorder of the kidneys and bladder. The ultimate cause and source for 'sweet urine' kept eluding clinicians and researchers until the late nineteenth century. It was left to Joseph von Mering and his colleague Oskar Minkowski, who, successfully demonstrated development of diabetes mellitus in a dog, following pancreatectomy.⁷

When the pancreas was identified as source of the 'substance' that was vital for controlling the sweetness in the blood, the search then focused on isolating this 'substance'. The efforts of researchers finally paid off in the first half of twentieth century, with discovery of Insulin, isolated from a pancreatic extracts by Frederick Banting and Charles Best. The crude insulin was purified by JJR Macleod and James Collip and was finally ready for use in 1921.⁸ Banting and Macleod received the Nobel Prize for the discovery of insulin in 1923, which they shared with their colleagues, Best and Collip.

With the discovery of Insulin, DM was no more perceived as an imminent death sentence. Whereas, Insulin provided a new and rational treatment for DM, it did not take long for the physicians to realise that Insulin was not the cure for the disease but only a means of reducing blood glucose levels. Furthermore, the disease had a progressive course with patients ending up with complications of diabetes not previously appreciated. This was due to the fact that previously, patients with diabetes died early even before developing complications. Insulin prolonged the lives of patients, which resulted in patients ending up with increased morbidity, as a result of complications of diabetes.

The appreciation of these new complications gave an impetus to new research to look into the pathophysiology of diabetes. There were suggestions that the disease may not be a singular entity, based on the observations that, not all patients required insulin to maintain their blood glucose levels. These observations lead to characterisation of the disease by Himsworth in 1936, who proposed the terminology of Insulin sensitive and Insulin insensitive types,⁹ to denote the need of insulin for glucose control.

1.2 Classification of Diabetes Mellitus

With increased understanding of the pathophysiology of DM, new variants of the disease were noticed over time. To incorporate the new understanding about the disease and to overcome the chaotic situation about classification, the World Health Organisation (WHO) proposed and published the first widely accepted classification of DM in 1980¹⁰ and, in modified form, in 1985.¹¹ The 1980 WHO Expert Committee proposed two major classes of DM and named them, insulin dependent diabetes mellitus (IDDM) or type 1 DM, and non-insulin dependent diabetes mellitus (NIDDM) or type 2 DM. In the 1985 Study Group Report the terms type 1 and type 2 were omitted, but the classes IDDM and NIDDM were retained, and a class of Malnutrition-related DM (MRDM) was introduced. In both the 1980 and 1985 reports other classes of DM included Other Types and Impaired Glucose Tolerance (IGT) as well as Gestational DM (GDM).

The WHO classification of DM was further revised in 1997 by ADA (American Diabetes Association).¹² The new classification incorporates both, the staging of DM

based on clinical descriptive criteria (Figure 1.1) and a complementary aetiological criteria (Table 1.1).

Table 1.1 Etiologic classification of Diabetes Mellitus

1. Type 1 diabetes (β -cell destruction, usually leading to absolute insulin deficiency)
 - A. Immune mediated
 - B. Idiopathic
2. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
3. Other specific types
 - A. Genetic defects of β -cell function
 2. Chromosome 12, HNF-1 α (MODY3)
 3. Chromosome 7, glucokinase (MODY2)
 4. Chromosome 20, HNF-4 α (MODY1)
 5. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
 6. Chromosome 17, HNF-1 β (MODY5)
 7. Chromosome 2, *NeuroD1* (MODY6)
 8. Mitochondrial DNA
 9. Others
 - B. Genetic defects in insulin action
 - a. Type A insulin resistance
 - b. Leprechaunism
 - c. Rabson-Mendenhall syndrome
 - d. Lipoatrophic diabetes
 - e. Others
 - C. Diseases of the exocrine pancreas
 - a. Pancreatitis
 - b. Trauma/pancreatectomy

- c. Neoplasia
- d. Cystic fibrosis
- e. Hemochromatosis
- f. Fibrocalculous pancreatopathy
- g. Others

D. Endocrinopathies

- a. Acromegaly
- b. Cushing's syndrome
- c. Glucagonoma
- d. Pheochromocytoma
- e. Hyperthyroidism
- f. Somatostatinoma
- g. Aldosteronoma
- h. Others

E. Drug- or chemical-induced

- a. Vacor
- b. Pentamidine
- c. Nicotinic acid
- d. Glucocorticoids
- e. Thyroid hormone
- f. Diazoxide
- g. β -adrenergic agonists
- h. Thiazides
- i. Dilantin
- j. α -Interferon
- k. Others

F. Infections

- a. Congenital rubella
- b. Cytomegalovirus

- c. Others
 - G. Uncommon forms of immune-mediated diabetes
 - a. “Stiff-man” syndrome
 - b. Anti-insulin receptor antibodies
 - c. Others
 - H. Other genetic syndromes sometimes associated with diabetes
 - a. Down's syndrome
 - b. Klinefelter's syndrome
 - c. Turner's syndrome
 - d. Wolfram's syndrome
 - e. Friedreich's ataxia
 - f. Huntington's chorea
 - g. Laurence-Moon-Biedl syndrome
 - h. Myotonic dystrophy
 - i. Porphyria
 - j. Prader-Willi syndrome
 - k. Others
4. Gestational diabetes mellitus (GDM)

1.3 Type 1 Diabetes Mellitus

Type 1 DM, which was previously recognised as IDDM (insulin dependent diabetes mellitus), based on absolute insulin requirement for survival, is now classified on the basis of aetiopathogenesis of the disease. It is an autoimmune disorder characterized by loss of the insulin-producing beta cells in the islets of Langerhans, in the pancreas, resulting in deficiency of insulin. The autoimmune destruction of the beta cells is induced by CD4+ and CD8+ T cells and macrophages infiltrating the islets.¹³

Type 1 DM accounts for about 10% of the total occurrence of DM,¹⁴ while the majority of DM comprises of type 2 DM. It typically affects young children and generally manifests before the age of 40, although there may be exceptions. Due to the frequent occurrence in children, previously it was also termed as "juvenile diabetes", although the term is now obsolete. Among the various racial groups, type 1 DM is most common in caucasians. The overall incidence of type 1 DM is on a rise with current rate of 3% and is expected to be much higher in future.¹⁵

Genetic studies have provided newer insights in the aetiopathogenesis of type 1 DM. An association of HLA (human leucocyte antigen) on chromosome 6 in patients with type 1 DM has been implicated in the susceptibility of these individuals for DM. A vast majority of type 1 DM patients may have either of the combinations of HLA genes, DR4-DQ8 or DR3-DQ2.¹⁶ It is widely believed that environmental factors such as enteroviruses,¹⁷ rotavirus¹⁸ and rubella¹⁹ may play an important role in precipitation of the disease in genetically susceptible patients, however, this is far from proven.

The autoimmune reaction in type 1 DM patients encompasses the binding of antibodies to sections of pancreatic islets. These antibodies are termed as islet cell antibodies. These antibodies may bind to either of the three major autoantigens, namely glutamic acid decarboxylase (GAD 65),²⁰ a protein tyrosine phosphatase-like molecule (IA-2)²¹ and insulin.²² Up to 90% of the patients with new onset type 1 DM have autoantibodies directed against at least one of these three autoantigens.¹⁴ There may be variable humoral response in these patients. Young children with new onset type 1 DM have higher prevalence of autoantibodies directed against insulin.²³ The

levels of IA-2 antibodies may gradually taper after diagnosis;²⁴ however, antibodies to GAD may persist longer.²⁵

The rate of pancreatic beta cell destruction as a result of autoimmune reaction is highly variable.²⁶ The rapidly progressive form is more commonly seen in children,²⁷ whereas the slow onset form usually occurs in adults, where it is sometimes referred as latent autoimmune diabetes in adults (LADA).²⁶ Type 1 DM patients may also have associated other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis and Addison's disease.²⁸

Stages	Normoglycemia	Hyperglycemia		
Types	Normal glucose regulation	Impaired Glucose Tolerance or Impaired Fasting Glucose (Pre-Diabetes)	Diabetes Mellitus	
Type 1*			Not insulin requiring	Insulin requiring for control
Type 2				Insulin requiring for survival
Other Specific Types**				
Gestational Diabetes **				

The diagram illustrates the progression of glycemia stages across different types of diabetes. Type 1, Type 2, and Other Specific Types all begin at the 'Normoglycemia' stage and transition to 'Hyperglycemia'. Gestational Diabetes begins directly at the 'Hyperglycemia' stage. Horizontal arrows indicate the progression from Normoglycemia to Hyperglycemia for each type.

Figure 1.1 Disorders of glycemia: Etiologic types and stages

Legend

- *Even after presenting in ketoacidosis, these patients can briefly return to normoglycaemia without requiring continuous therapy (i.e., "honeymoon" remission);
- **in rare instances, patients in these categories (e.g., Vacor toxicity, type 1 diabetes presenting in pregnancy) may require insulin for survival.

1.4 Type 2 Diabetes Mellitus

Type 2 DM is a metabolic disorder characterized by hyperglycemia as a result of insulin resistance and/or relative insulin deficiency. Up to 90% of patients with DM have type 2 DM. It was formerly recognized as non insulin dependent diabetes mellitus (NIDDM) or adult-onset diabetes. Type 2 DM may remain undiagnosed for long periods because the hyperglycemia in these patients may not be severe enough to precipitate severe symptoms of DM.²⁹

The aetiopathogenesis of type 2 DM is multifactorial and often associated with strong familial and genetic predisposition.³⁰ The incidence of type 2 DM differs in various racial/ethnic groups.³¹ The relative risk of developing type 2 DM increases with family history of diabetes,³² obesity,³³ lack of exercise or sedentary lifestyle,³⁴ prior history of gestational diabetes (GDM) in woman,³⁵ hypertension,³⁶ smoking³⁷ dyslipidemia³⁸ and age.³⁹ A vast majority of patients with type 2 DM are obese or have higher abdominal distribution of fat. Obesity promotes insulin resistance and this may play a crucial role in the pathogenesis of type 2 DM in obese individuals.⁴⁰

The pathogenesis of type 2 DM is characterised by peripheral insulin resistance and/or impaired regulation of hepatic glucose production and declining β-cell function,

eventually leading to pancreatic β -cell failure.⁴¹ Unlike type 1 DM where there may be absolute deficiency of Insulin, patients with type 2 DM may have inadequate insulin response or hyperinsulinemia, which may make these patients insulin resistant.⁴² The other contributing factors in the pathogenesis of type 2 DM are i) increased hepatic glucose production (e.g. from glycogen degradation),⁴³ decreased insulin-mediated glucose transport in the muscle and adipose tissues (receptor and post-receptor defects)⁴⁴ and loss of early phase of insulin release in response to hyperglycemia.⁴⁵

1.5 Complications of Diabetes Mellitus

From the initial understanding of DM being an incurable condition, improved medical care and scientific advancements in treatment of diabetes has helped in prolonging the lifespan of diabetics like never before. Ironically, increased survival, potentially, predisposes a patient with diabetes to long-term vascular complications of diabetes. The onset of these complications reduces the overall quality of life in a diabetic patient.⁴⁶ There has been an increasing awareness of the magnitude of the problem presented by diabetic complications in the last few decades.

Diabetic vascular complications or diabetic vasculopathy (DV) broadly comprises of microvascular and macrovascular complications. The microvascular complications of diabetes include diabetic neuropathy, diabetic nephropathy (DN) and diabetic retinopathy (DR). The macrovascular complications of diabetes are cardiovascular disease (CVD), cerebrovascular disease and peripheral vascular disease (PWD). Cardiovascular Disease (CVD) is the leading cause of death worldwide.⁴⁷ Patients with DM have two-fold increased risk of cardiovascular mortality compared to

nondiabetics.⁴⁸ Results from recent studies^{49, 50} provide ample evidence for the beneficial effects of early intervention to improve outcomes in people with diabetes and given the high prevalence of CVD in this population, an estimation of absolute cardiovascular risk evaluation of all patients with DM is desirable.

Various models for estimation of cardiovascular risk such as Framingham,⁵¹ Prospective Cardiovascular Munster (PROCAM),⁵² Lipid Research Clinic⁵³ and UKPDS⁵⁴ have been proposed. Whereas the other models are more applicable to the general population, the UKPDS model is diabetes specific model which takes into account several known risk factors such as glycaemia, systolic blood pressure, lipid levels, age, sex, ethnic group, smoking status and time since diagnosis of diabetes, and hence more appropriate for people with diabetes.

Chronic hyperglycemia in Type 1⁵⁵ and Type 2 DM⁵⁶ is an important predictor of development of premature cardiovascular disease. DM is the leading cause of patients requiring renal replacement therapy, blindness under the age of 65 and non-traumatic amputation worldwide and reduces life expectancy by about 5-10 years in these patients.⁵⁷ The onset of diabetic complications puts an extra burden on the individual and society in terms of reduced quality of life and the cost of treatment.

1.6 Conclusions

There has been a worldwide impetus on diabetes research, given the high cost of treatment and increased morbidity and mortality in these patients. Vascular complications of DM are progressive in nature and a major cause of concern in DM.

One of the important aspects of containing the pandemic of DM is to prevent and/or arrest the progression of DV. A better understanding of the pathophysiology of DM and DV may help us to devise methods for early intervention and treatment to prevent the progression and hopefully reduce morbidity and mortality.

The aim of this thesis is to study the relationship between blood, bone and kidney in the genesis and progression of Diabetic Vasculopathy. To achieve this, a detailed literature search was undertaken to comprehend the basic concepts and the current understanding about DV. Various lacunae in the literature were identified about the genesis, progression and complications of DV. The relationship of the triad (blood, bone and kidney) is complex and there is not enough literature on the combined role of these components in the genesis and progression of DV. In an attempt to address some of these issues and to fill some of the gaps in the current literature, several hypotheses were generated, which were subsequently tested.

Chapters 2 to 5 encompass the literature review of DV. Chapter 6 discusses the general methodology applied to all the clinical studies. Chapters 7-11 discuss the clinical studies carried out to examine the various aspects of DV. Chapter 12 comprises of general discussion, conclusions from the clinical studies and the future directions for research in this area.

The following chapter discusses the current understanding of the pathogenesis of diabetic nephropathy, one of the major microvascular complications of DM.

CHAPTER 2

DIABETIC NEPHROPATHY: GLOMERULAR OR TUBULAR DISEASE

DIABETIC NEPHROPATHY: GLOMERULAR OR TUBULAR DISEASE?

2.1 Introduction

The aim of this review is to summarize the role of chronic hypoxia in the tubulointerstitium as a major factor in the pathogenesis of diabetic nephropathy (DN) and to explore possible future interventions. Diabetic Nephropathy is the commonest cause of end stage renal disease (ESRD) and in the UK accounts for around 20% of all patients requiring renal replacement therapy.⁵⁸ Five stages of DN have been defined, the first of which involves the development of glomerular hyperfiltration. This is followed by silent stage, incipient nephropathy with microalbuminuria, overt proteinuria and finally ESRD.⁵⁹ Incipient nephropathy is first manifested by the onset of persistent microalbuminuria. Over a period of time, progressive microalbuminuria may lead to persistent proteinuria, also termed as overt diabetic nephropathy.⁵⁸ The cumulative risk of renal failure after 5 years of persistent proteinuria has been estimated around 60% in both type I and type II diabetes.⁶⁰

2.2 Histology of Diabetic Nephropathy

The histological changes in DN encompass various structural changes in all the compartments of the kidney. The earliest morphological change in DN is glomerular basement membrane (GBM) thickening as a result of mesangial matrix deposition and hypertrophy of mesangial cells.⁶¹ With the progression of the disease, there is enhanced deposition of the normal extracellular matrix of types IV and VI collagen, laminin and fibronectin as a result of enhanced production and/or decreased

degradation.⁶² This results in a diffuse mesangial expansion also termed as diabetic glomerulosclerosis, which can be associated with nodular lesions consisting of areas of marked mesangial expansion with a fencing of mesangial nuclei surrounding the nodule and compression of the associated glomerular capillaries (Kimmelstiel-Wilson nodules).⁶¹

The changes in the tubulointerstitium such as thickening of tubular basement membrane intimately parallel that of GBM expansion, suggesting that glomerular hemodynamic disturbances are not required for these perturbations in the tubules.⁶³ In contrast to the mesangium, initial tubulointerstitial expansion is primarily due to an increase in the cellular component of this renal compartment.⁶⁴ The mesangial expansion encroaches upon the afferent and efferent arterioles leading to hyalinosis, which may contribute to glomerular sclerosis, through severe compromise of the glomerular blood flow. Efferent arteriolar hyalinisation is pathognomonic for DN.⁶⁵ In the tubulointerstitium, thickening of the tubular basement membrane, tubular atrophy, interstitial fibrosis and peritubular capillary rarefaction is seen.⁶⁶ In addition, DN is characterised by presence of fibrin caps, capsular drops and capillary microaneurysms.⁶⁵

The pathogenesis of DN is still not clear. It is traditionally considered to be a primarily glomerular disease. However, the typical findings of glomerulosclerosis are present in only one-third of type 2 diabetics with microalbuminuria, while another third demonstrates normal renal structure. The remaining third has minor or absent glomerular changes but disproportionately severe tubulointerstitial lesions.⁶⁷ It has been widely accepted,⁶⁸ and histological studies have confirmed, that the

deterioration of renal function in chronic renal diseases correlates better with tubular and interstitial changes than with glomerular changes.⁶⁹

2.3 Tubular Handling of Proteins

An understanding of renal handling and excretion of various proteins in normal and diseased kidneys is vital for diagnosis and monitoring the progression of proteinuria in patients with kidney disease. Renal excretion of any substance is a balance between glomerular filtration, tubular reabsorption and tubular secretion. Albumin with a molecular size of 65 kDa constitutes about 60 % of the plasma proteins and is a major protein molecule excreted in urine.⁷⁰ The amount of urinary albumin excretion is influenced by glomerular filtration rate, size and electrical charge of the molecule, with more than 95% of the filtered albumin being reabsorbed by the tubules.⁷¹

In healthy individuals daily albumin excretion is about 25 mg/day or less. The endocytic uptake of albumin and other proteins is subjected to a complex hormonal and enzymatic regulation in the tubular milieu. Two major cellular pathways: the degradation pathway and the retrieval pathway are recognised in the handling of albumin. Most of the albumin is returned to the blood supply by the retrieval pathway, which works through the process of endocytosis.⁷⁰ This process is carried out by two major protein-binding receptors such as Megalin and Cubulin along with other low-molecular-weight binding proteins, on the tubular surface. These receptors bind to various proteins in the glomerular ultrafiltrate such as albumin, transferrin and vitamin carrier protein molecules.⁷² The remaining albumin in the ultrafiltrate is excreted through the degradation pathway.

N-acetyl-beta-glucosaminidase (NAG) is a large protein of the size between 130 -150 kDa and is present in the serum in very low concentrations. Its presence in the urine invariably signifies tubular damage as due its large size, it is not filtered by the glomerulus, but is released in the tubular lumen with proximal tubular damage.⁷³ Urinary NAG levels reflect the activity of the lysosomal system, which is modulated by the process of endocytosis, in the tubular lumen. Hence higher excretion of NAG is considered as a reflection of tubular damage and dysfunction.⁷⁴ RBP is a 21 kDa carrier protein for Retinol (Vitamin A) in the serum. It is bound to pre-albumin and retinol, but once retinol is delivered to the target cells, it is filtered through the glomerulus and rapidly reabsorbed by the process of endocytosis in the tubular lumen. Higher excretion of RBP reflects tubular dysfunction.⁷⁵

2.4 Tubular Dysfunction in Diabetes

The early proteinuria seen in patients with DN comprises of glomerular and tubular components.⁷⁶ Tubular dysfunction has been reported in patients with diabetes in the absence of microalbuminuria. Urinary excretion of NAG, a tubular enzyme, is elevated in type 2 diabetic patients.⁷⁴ In addition the excretion rate of NAG and RBP has been reported to be higher in normotensive, normoalbuminuric type 1 diabetic subjects than in normal controls.

These findings suggest a pivotal role of the tubulointerstitium in the development of diabetic nephropathy.⁷⁷ Tubular hypertrophy, reduced organic ion transport, and other tubular changes are already apparent before the onset of proteinuria in diabetes. Indeed, increased tubulo-glomerular feedback, defective uptake and lysosomal

processing may independently contribute to hyperfiltration and urinary protein loss, respectively.⁷⁸

2.5 Tubulointerstitial Injury and Hypoxia

Tubular cells may be directly damaged by hyperglycemia. Glucose uptake by tubular cells is independent of insulin, so there is a direct relation between plasma glucose concentrations and glucose levels within tubular cells.⁷⁹ Glucose-dependent metabolic pathways such as advanced glycation, increased formation of polyols, activation of the enzyme protein kinase C, and of vasoactive hormones such as angiotensin II along with systemic and intra-glomerular hypertension may directly influence tubular and interstitial cells, leading to renal dysfunction caused by non-glomerular mechanisms.⁸⁰

Chronic tubulointerstitial injury (CTI) encompassing tubular atrophy and interstitial fibrosis represents one of the major determinants of progression of chronic renal disease regardless of cause. Peritubular capillaries (PTCs) are essential to maintain the normal structure and function of renal tubules and CTI may result from ischemia induced by PTC loss.⁸¹ Ischemia may occur as a result of several mechanisms including, intra-renal vasoconstriction (secondary to increased local levels of angiotensin II and/or endothelin 1, or due to local deficiency of nitric oxide production), and structural lesions relating to arteriolar damage in conditions such as diabetes or hypertension.⁸²

2.6 Oxidative Stress and Interstitial Fibrosis

Diabetes is associated with increased production of reactive oxygen species (ROS)⁸³ and decreased renal oxygen tension.⁸⁴ ROS directly interacts with nitrite oxide (NO) to form peroxynitrite, resulting in reduced bioavailability of NO.⁸⁵ Reduced NO results in increased vascular tone and increased oxygen consumption.⁸⁶ Decreased renal oxygen tension along with reduced bioavailability of NO may increase free radical damage in the tubulointerstitium. Chronic hyperglycemia may precipitate alterations in the intra-renal blood flow regulation.⁸⁷ In addition, chronic hyperglycemia increases the lactate concentration in the renal medulla with decrease in pH,⁸⁸ which may result in reduction of oxygen delivery to this region⁸⁹ and hamper the degradation of hypoxia inducible factor-1 (HIF-1)⁹⁰

HIF-1 is one of the main molecular mediators enabling cell adaptation to hypoxia by facilitating vasculogenesis, increased oxygen delivery as well as alteration of cell metabolism.⁹¹ HIF-1 regulates pathways involved in glucose utilization,⁹² cell proliferation and apoptosis,⁹³ production of EPO, angiogenic growth factors, such as vascular endothelial growth factor (VEGF), iron metabolism, cellular proliferation, differentiation, and viability, pH regulation and extracellular matrix metabolism.⁹⁴ It has been suggested that HIF-1 may regulate genes involved in fibrogenesis. A deregulation of gene expression in response to hypoxia within the kidney may contribute to the development of interstitial fibrosis.⁹⁵

Interstitial fibrosis may lead to local ischemia by impairing diffusion from the capillary to the tubule. The juxtamedullary region and outer medulla are uniquely susceptible to

hypoxia. The tubules in this region are normally in a borderline hypoxic state due to the countercurrent circulation and high oxygen demands of the medullary thick ascending tubules and the S3 segments of the proximal tubules.⁸² Hence, even modest reductions in the renal blood flow may lead to worsening hypoxia in this region.⁹⁶

2.7 Abnormal Red Blood Cells in Diabetes

The life span of red blood cells is reduced in Diabetes Mellitus (DM).⁹⁷ In animal models, metabolic and functional abnormalities of red blood cells have been described in diabetes and in presence of hyperglycemia. These abnormalities include, increased sorbitol levels,⁹⁸ decreased activity of Na/K-ATPase⁹⁹ and altered activity of acetyl cholinesterase.¹⁰⁰ Cell size and osmotic fragility is increased and there is reduced filterability of red blood cells. Red cell membrane viscosity is increased in hyperglycaemic subjects. Oxygen affinity is abnormal in the diabetic erythrocyte.⁹⁷ All these factors may contribute to microvascular disturbances in DM (Figure.2.1)

2.8 Role of Anaemia in Diabetic Nephropathy

Patients with diabetic nephropathy have relatively greater degrees of anaemia than non-diabetics with similar levels of renal impairment.¹⁰¹ Erythropoietin (EPO) deficiency anaemia with renal impairment occurs early in both type 1 and type 2 DM, although the prevalence may be higher in type 1 DM.¹⁰¹ Impaired function of EPO-producing fibroblasts is associated with interstitial fibrosis¹⁰² and a defect of

"anaemia-sensing" mechanisms with autonomic neuropathy.¹⁰³ At relatively normal creatinine levels, efferent sympathetic denervation of the kidney may lead to the loss of EPO appropriate production, thus contributing to EPO deficiency.¹⁰⁴

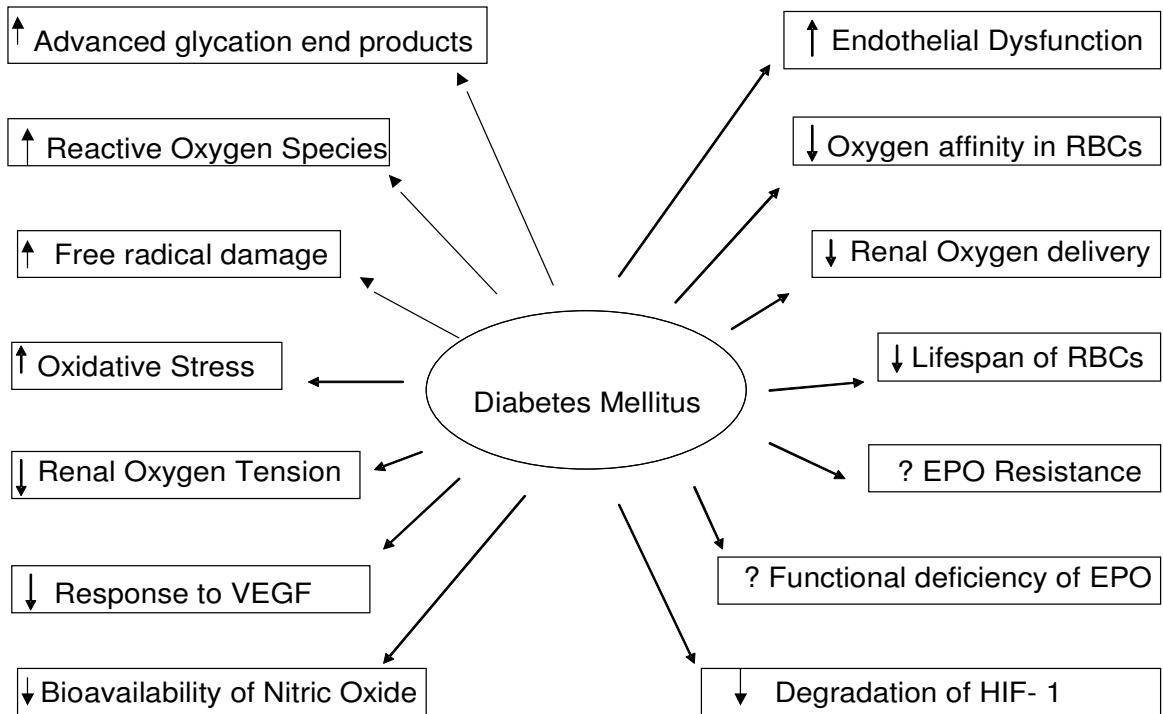


Figure 2.1 Microvascular Disturbances in Diabetes Mellitus

Patients with diabetes suffer from vascular dysfunction secondary to an impaired ability to respond to tissue ischemia. Diabetes causes defects in both the endothelial progenitor cell and peripheral tissue responses to hypoxia.¹⁰⁵ VEGF is an important survival factor for endothelial cells in hypoxic environments. High glucose concentrations regulate aspects of VEGF expression in some cell types, including proximal tubular cells. Hyperglycemia blunts the VEGF response to hypoxia as seen

in immortalized rat proximal tubular cells, an effect mediated by oxidative stress-regulated hypoxia-inducible factor (HIF)/hypoxia-responsive element (HRE) pathway.¹⁰⁶ Hence ambient glucose levels may modulate the progression of chronic kidney disease, especially diabetic nephropathy.

2.9 Early Tubular Proteinuria

Epithelial cells of the proximal tubule are major players in orchestrating events in the tubulointerstitium in early diabetic nephropathy. Proteinuria may be a marker of tubulointerstitial injury in early diabetic renal disease and result from an early dysfunction of charge-dependent tubular reabsorption, which precedes loss of the glomerular charge barrier.¹⁰⁷

In diabetic nephropathy, oxidant injury and renal tubular damage accompany and may even precede microalbuminuria.¹⁰⁸ The post glomerular capillaries may be the initial location of injury in DN.¹⁰⁹ The presence of these abnormalities in the absence of glomerular proteinuria favours the hypothesis that alterations first occur in the peritubular microcirculation, which by causing oxidant injury and tubular damage may initiate diabetic nephropathy.¹⁰⁸

2.10 Sodium Co-Transport and Glomerular Hyperfiltration

Glomerular filtration rate (GFR) is influenced by various factors including the renal nerves, hormones, the size and condition of the glomerulus and tubulo-glomerular

feedback (TGF) from the macula densa (MD) mediated by changes in sodium chloride concentrations in this segment (MD[NaCl]). No single disturbance can affect GFR without secondarily affecting the MD[NaCl] and vice versa. However, primary vascular and tubular events that have the same effect on GFR will have contrary effects on MD[NaCl]. If GFR increases due to a primary vascular effect then MD[NaCl] must also increase. If GFR increases due to a tubular effect, then MD[NaCl] must decrease.¹¹⁰

In animal models, with recent onset diabetes, increased sodium reabsorption in the proximal tubule has been shown to reduce sodium concentrations at the macula densa, which in turn elicited a tubulo-glomerular feedback-dependent increase in single nephron GFR.¹¹¹ Stimulation of tubular Na+/glucose co-transport by reducing the tubulo-glomerular feedback signal at the macula densa may contribute to glomerular hyperfiltration. This tends to compensate for the rise in fractional tubular reabsorption to partly restore the electrolyte load to the distal nephron.¹¹² Normal rats adapt to changes in dietary NaCl primarily by adjusting sodium transport downstream of the macula densa. In contrast, the presence of diabetes renders reabsorption in the proximal tubule sensitive to dietary NaCl with subsequent effects on the TGF signal. This helps to explain the paradoxical effect of dietary NaCl on GFR in early DM.¹¹²

Sodium retention occurs early in DM. Diabetics who are unable to compensate for these early changes in sodium handling may be at greater risk of developing hypertension and progressive nephropathy.¹¹³ The absolute total tubular sodium reabsorption in type 1 diabetics is increased by approximately 30-40 per cent, as is the filtered sodium load.¹¹⁴ Insulin and hyperglycemia both reduce renal sodium

excretion; the proximal tubule being the likely site of action for hyperglycemia, and the distal portion of the nephron for insulin.¹¹⁵

Glucose is reabsorbed in the early portion of the proximal tubules coupled to Na⁺ transport, utilizing a common carrier protein. An increased load of glucose will therefore be expected to induce an increase in the proximal reabsorption rate of sodium and water, at least as long as the proximal tubular reabsorption capacity for glucose is not exceeded to a degree, which might induce osmotic diuresis. This deviation from normal in proximal renal sodium and fluid handling may be relevant to the development of hypertension in long-term type 1 diabetes.¹¹⁴

It has been suggested that the primary event in diabetic glomerular hyperfiltration is due to an increase in proximal tubular sodium reabsorption and changes in tubular sodium handling most probably influence tubulo-glomerular feedback.¹¹⁶ Sodium-lithium counter-transport, which may reflect the activity of the Na-H pump,¹¹⁷ is altered in essential hypertension and may be a marker for DN.¹¹⁸

The proximal tubule accounts for most of the increased kidney mass in early diabetes.¹¹⁹ As the proximal tubule grows, it reabsorbs more, thus causing MD [NaCl] to decline incrementally. As MD [NaCl] declines, GFR increases through the TGF mechanism, while TGF resets upward through a slower macula densa mechanism.¹²⁰ The hyperglycaemic milieu, chronic tubulointerstitial hypoxia, sympathetic denervation of kidney, disturbed renin angiotensin system (RAS) and resetting of TGF, all promote glomerular hyperfiltration. Glomerular hyperfiltration results in

increased workload on the surviving tubular cells and this in turn may lead to functional tubular hypoxia through the overwork of tubular reabsorption process.

2.11 Vitamin D and Vitamin D Receptor

The proximal tubular epithelial cell is the site of hydroxylation of 25 hydroxyvitamin D to 1, 25-dihydroxyvitamin D. Reduced serum levels of 1, 25-dihydroxyvitamin D occur early in CKD.¹²¹ In a recent study of CKD patients in various stages, 13% of patients with GFR > 80 ml/min were found to have low levels (<22pg/ml) of 1, 25-dihydroxyvitamin D, whilst 12% of patients had high PTH levels (>65pm/dl).¹²² Altered vitamin D metabolism has been demonstrated in animal models of diabetic nephropathy with increased glomerular expression of vitamin D3 1alpha-hydroxylase, vitamin D binding protein and calcium binding proteins like calbindins and calcyclin, which are important in calcium metabolism.¹²³

The binding of 1, 25-dihydroxyvitamin D or calcitriol to the vitamin D nuclear receptor (VDR) activates the VDR. The VDR has been found in more than 30 tissues including the intestines, bone, kidney (glomerular podocytes, proximal and distal tubules, collecting ducts), parathyroid gland, pancreatic β -cells, monocytes, T-cells, keratinocytes and many cancer cells.¹²⁴ Earlier studies demonstrated the presence of the VDR in cultured human mesangial cells.¹²⁵ 1, 25-dihydroxyvitamin D inhibits proximal tubular epithelial cell proliferation in a dose-dependent manner. Diminished local production of 1, 25-dihydroxyvitamin D by tubular epithelial cells in CKD may facilitate interstitial fibrosis as a result of decreased inhibitory control of 1, 25-dihydroxyvitamin D on renal cell proliferation.¹²⁶

Chronic hyperglycemia is associated with cytokine (NF- κ B) activation induced renal injury, enhanced production of fibronectin and collagen IV protein, which is blocked by 1, 25-dihydroxyvitamin D.¹²³ It has been suggested that 1, 25-dihydroxyvitamin D is protective in glomerular disease, particularly in conditions associated with excess matrix production.¹²⁷ Since adequate local concentrations of active vitamin D may be required to maintain structural and functional integrity of renal parenchyma, lower vitamin D levels may promote progression of CKD. This may lead to further parenchymal damage, another feedback loop.

In vitro studies have demonstrated that the effect of 1, 25-dihydroxyvitamin D on renin regulation is independent of serum calcium and PTH levels.¹²⁸ Vitamin D serves as a down regulator of the RAS, directly and independently suppressing Renin gene expression.¹²⁹ The VDR is present in the tubular epithelial cells, and regulates many functions in these cells in addition to its role in calcium homoeostasis.

Megalin, an endocytic receptor on the apical membranes of proximal tubule, is involved in the reabsorption and metabolism of various proteins that have been filtered by glomeruli. In the early stages following kidney injury, there is down-regulation of Megalin in the renal tubular cells. Lower Megalin levels affect endocytosis, which leads to decreased 25-hydroxyvitamin D reabsorption and increased proteinuria.¹³⁰

2.12 The Hypoxic Tubular Hypothesis

Chronic hyperglycemia affects the renal interstitium in multiple ways (Figure. 2.2).

Recently, chronic hypoxia in the renal interstitium has been proposed as a common mechanism for the progression of different types of renal diseases.¹³¹ This hypothesis, which emphasizes the role of chronic oxygen deprivation or hypoxia in the tubulointerstitium as a common pathway in end-stage kidney injury, has been supported by several studies.^{132, 133} Chronic hypoxic milieu may play a dominant pathogenetic role in DN as well, not only in promoting progression but also in the initiation of the condition.

Hypoxia is a potent regulator of gene expression for a wide spectrum of molecules including growth factors, hormones, vasoactive compounds and enzymes involved in intermediary metabolism.¹³⁴ Vasoactive compounds such as Angiotensin II not only constrict efferent arterioles but also, by inducing oxidative stress, hamper the efficient utilization of oxygen in tubular cells.¹³³ Reduced peritubular capillary flow and tubular dysfunction in type 2 diabetes with normoalbuminuria has been reported.¹³⁵ Relative hypoxia in the kidney also results from increased metabolic demand in tubular cells. Furthermore, renal anaemia tends to hinder oxygen delivery. These factors may affect the kidney even before the appearance of significant pathologic changes in the vasculature and predispose to tubulointerstitial injury.

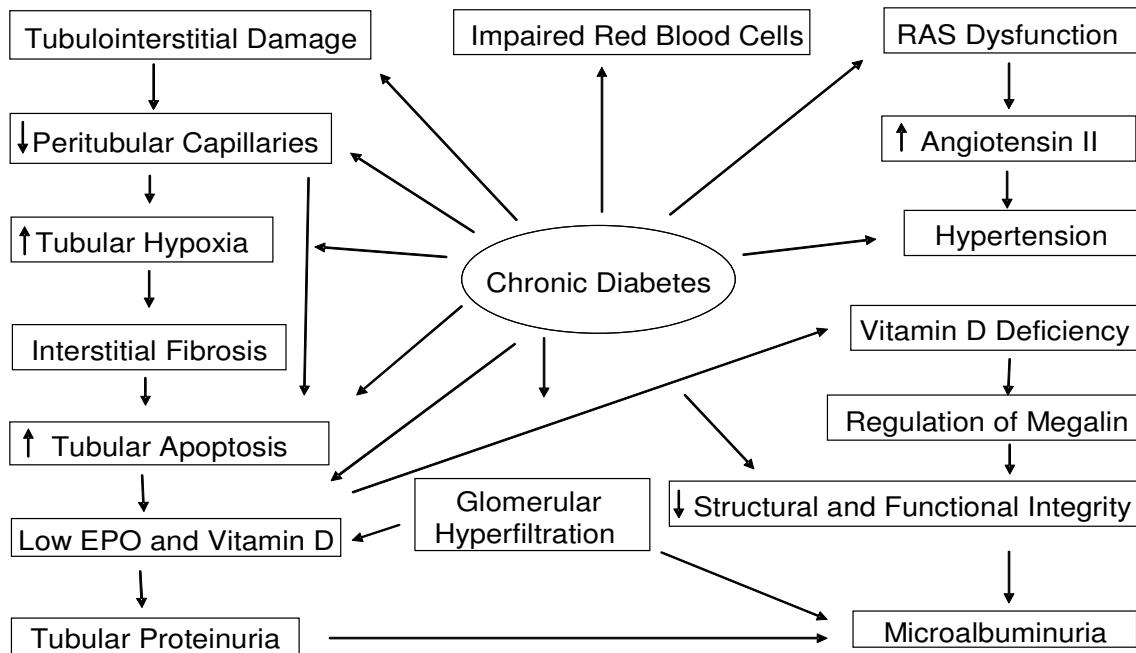


Figure 2.2 Hypoxic Tubular Hypotheses

Diabetic status, microproteinuria with dysfunction of charge-dependent tubular reabsorption prior to a loss of glomerular charge barrier, and hypoxia due to insufficient blood supply may lead to apoptosis of the tubular cells. This is not seen in the glomerular cells. This early tubular apoptosis may play a role in gradual loss of renal mass.¹³⁶ Initially, the remaining peritubular cells may have to work more in response to hypoxia, producing adequate or higher EPO levels to maintain adequate haemoglobin levels. However, continuing adverse factors like chronic hypoxic milieu, hyperglycemia, oxidant injury and endothelial dysfunction may lead to tubular fatigue, resulting in increased tubular apoptosis. This may lead to loss of EPO producing fibroblasts leading to low EPO levels.

EPO degradation by endocytosis process in the EPO receptors is the dominant mechanism of EPO removal from the body.¹³⁷ EPO, following desialation in the liver, may also be cleared by glomerular filtration;¹³⁷ especially when EPO receptors are saturated.¹³⁸ The number of functional EPO receptors may be reduced due to modulation by chronic hyperglycemia and tubular apoptosis. This together with glomerular hyperfiltration may result in increased urinary EPO loss, lowering blood levels, aggravating hypoxia, and initiating a detrimental cycle.

2.13 Erythropoietin levels in Diabetic Nephropathy

Diabetic nephropathy is associated with low EPO levels,¹³⁹ functional EPO deficiency⁷⁸ or a state of relative EPO resistance,¹⁴⁰ with reduced haemoglobin production that further aggravates hypoxia. Theoretically, glycation of endogenous EPO might occur analogously to glycation of apo-lipoproteins¹⁴¹ with possible modification of EPO receptors by glycation.¹⁴² So even though the surviving peritubular cells may mount a response to hypoxia by producing increased EPO, glycated EPO may bind ineffectively to the EPO receptors. The initially increased EPO levels may lead to down regulation of EPO receptors. This is akin to ‘starvation amidst plenty’ scenario seen in insulin resistance. With increased tubular apoptosis, the number of EPO receptors may decrease, further aggravating the hypoxic milieu.

One of the most potent causes of suboptimal response to EPO is chronic inflammation.¹⁴³ This is associated with an increased production of cytokines, such as tumour necrosis factor- α , interleukin-1, or interferon- γ ,¹⁴⁴ which may suppress erythrocyte stem cell proliferation.¹⁴⁵ Although the synthesis of erythropoietin in

response to renal anaemia appears to be reduced in diabetes beyond that seen in other renal diseases,¹⁰¹ patients with diabetes are still able to mount an appropriate response to acute hypoxia,¹⁰⁴ suggesting that the peritubular cells that produce erythropoietin are not simply lost.

The changes due to damaged tubular cells lead to deranged sodium metabolism and disturbances of the RAS system (angiotensin converting enzyme (ACE) causing constriction of the efferent arteriole) contributing to hypertension and hyperfiltration in an attempt to increase the perfusion of the kidney and meet the metabolic requirement of the tubules. Hyperfiltration and hypertension increase the load on the glomerular filter eventually leading to microalbuminuria and ultimately overt proteinuria.

This sequence of events suggests that early diabetic nephropathy is essentially a tubular disease, which is superseded by the glomerular component after the onset of hyperfiltration. Diabetic status, low concentrations of 1, 25-dihydroxyvitamin D which affects the structural and functional integrity of renal parenchyma, abnormalities of RBCs, oxidative stress, sympathetic denervation of the kidney due to autonomic neuropathy, diabetes induced tubular apoptosis, could all lead to hypoxia and ultimately reduced number of functional tubular cells. The remaining tubular cells react by producing more erythropoietin to meet the metabolic demand. With continuing hypoxia, cellular exhaustion and early tubular proteinuria, the functional tubular cells are unable to produce the required amount or there is inappropriately low production of EPO. Along with low EPO levels some patients have been found to have functional EPO deficiency leading to anaemia in established nephropathy.

2.14 Potential Therapeutic Interventions

Various treatment modalities to ameliorate hypoxia of chronic renal disease are being developed. Therapies targeting VEGF and HIF-1 are still in their infancy. In current clinical practice, two agents, which appear to be promising in the therapy of early diabetic nephropathy, are 1, 25-dihydroxyvitamin D and EPO. 1, 25-dihydroxyvitamin D is a trophic hormone and has many important functions including immunomodulation, anti-proliferation and pro-differentiation as well as down-regulation of RAS. There is a growing amount of experimental evidence that 1, 25-dihydroxyvitamin D may also be reno-protective.¹⁴⁶

EPO is a trophic hormone, which exerts its haematopoietic effects by stimulating the proliferation of early erythroid precursors and the differentiation of later precursors of the erythroid lineage¹⁴⁷. Mature endothelial cells also express EPO receptors¹⁴⁸ and EPO induces a pro-angiogenic response in cultivated mature endothelial cells, as evidenced by stimulation of endothelial cell proliferation, migration and endothelin-1 release.¹⁴⁹

Early EPO administration in predialysis patients has been shown to slow the progression of CKD, with a significant reduction in mortality and the need for renal replacement therapy.¹⁵⁰ The beneficial effect was ascribed to increased erythrocyte number, i.e. the haematopoietic effect of EPO. On the down side, an increased haematocrit level could possibly contribute to elevated blood pressure and/or vascular thrombosis.¹⁵¹ Recent large multicentre trials such as CHOIR,¹⁵² CONSORT¹⁵³ and

CREATE¹⁵⁴ have raised questions about the relationship between anaemia correction and progression of renal disease. However, this review focuses on exploring non-haemopoietic trophic properties of EPO, which may be achieved by using low doses of EPO, which may be well below the required dose to elicit haematopoietic response.

EPO has pleiotropic effects well beyond the maintenance of red blood cell mass.¹⁵⁵ It has important cytoprotective effects, including protection from ischemic injury, inhibition of apoptotic death-related pathways^{156, 157} and cisplatin induced renal injury.¹⁵⁸ EPO has been recognized to be a multifunctional cytokine that plays a key role in ischemic preconditioning in the brain and heart.¹⁵⁹ In animal models, EPO has been demonstrated to possess anti-oxidant and anti-inflammatory properties.¹⁶⁰

The non-haematopoietic pleiotropic effects of EPO have major implications.¹⁵⁵ EPO exhibits its pleiotropic effects by the activation of functional EPO receptor (EPOR) on the tissue surface.¹⁶¹ EPORs have been demonstrated throughout the kidney, including both proximal and distal tubular cells.¹⁶² Also, EPO-producing fibroblast-like interstitial cells are in direct contact with the basal aspects of proximal and distal tubular cells.¹⁶³ However, the affinity of EPOR to EPO is well below the normal plasma EPO concentration, suggesting that these EPORs respond to the circulating EPO in a paracrine fashion.¹⁶⁴ The anatomical relationship between EPO-secreting cells, the intrarenal capillary network, and tubular and other renal cells could facilitate endocrine and paracrine actions of EPO within the kidney.¹⁶²

Administration of EPO promotes impressive reno-protection in experimental ischemic and toxic acute renal failure.¹⁶⁵ In vitro, primary cultures of human proximal tubule cells have demonstrated significant degree of apoptosis in presence of hypoxia.¹⁶⁶ Co-incubation of these hypoxic proximal tubule cells with EPO inhibited apoptosis, enhanced tubular epithelial regeneration and hastened renal functional recovery. Also, in experimental models, chronic low-dose therapy with EPO has shown to confer vascular and tissue protection, lower blood pressure, preservation of renal functions, and improved survival.¹⁶⁷ The anti-apoptotic and reno-protective effects of EPO may be due to direct action of EPO on the EPO receptors in the kidney tissue. These observations in animal models can be the basis for further studies in humans.

2.15 Conclusions

The pathogenesis of diabetic nephropathy is multifactorial. Clinical strategies revolve around suppression of known 'causes' of progression by targeting hyperglycemia, high blood pressure as well as the renin–angiotensin system (RAS). Although these therapies slow the progression of CKD, the residual risk of these patients for both renal and cardiovascular end points remains high; a significant number of patients continue to have progressive deterioration of renal function. Chronic hypoxia in the renal interstitium is widely recognised as an important element along with proteinuria for the progression of chronic kidney disease.

In current clinical practice, EPO therapy is primarily indicated for treatment of anaemia in EPO deficient individuals. Current practice also confines the role of 1, 25-

dihydroxyvitamin D to its role in mineral metabolism. The trophic properties of EPO and 1, 25-dihydroxyvitamin D have not been adequately explored. The available evidence encourages us to study the relationship between early tubular proteinuria and deficiencies of these hormones, and to explore their potential role in ameliorating early hypoxic injury.

The following chapter discusses the consequence of EPO deficiency in patients with DM. Even before any apparent EPO deficiency, chronic hyperglycemia may result in generalised haematopoietic stress, which may promote early anaemia in DM. Chronic anaemia may complicate the pre-existing vasculopathy in patients with DM.

CHAPTER 3

A REVIEW OF ANAEMIA IN DIABETES MELLITUS

A REVIEW OF ANAEMIA IN DIABETES MELLITUS

3.1 Introduction

Diabetes mellitus (DM) is a major risk factor for CVD¹⁶⁸ and congestive heart failure (CHF)¹⁶⁹ with more than two-fold increased risk of cardiovascular mortality in patients with diabetes.⁴⁸ Majority of patients with diabetes, with or without end stage renal disease (ESRD), die of CVD related causes.¹⁷⁰ Improved medical care and scientific advances in treatment of patients with diabetes have helped to prolong their lifespan. Ironically, increased survival potentially predisposes a patient with diabetes to long-term vascular complications and consequent reductions in quality of life.⁴⁶ Anaemia may play an important role in the pathogenesis and progression of diabetic complications.¹⁷¹

The pathogenesis of anaemia in chronic diseases is multifactorial. Anaemia has been found to be a common association of, and an important potential contributant in, the development and progression of diabetic complications.¹⁷² Anaemia is also common in chronic kidney disease (CKD) and an important risk factor for CVD¹⁷³ and CHF.¹⁷⁴ Although, anaemia is a modifiable risk factor, its correction in patients with CKD is far from optimal, though it is known that correction of anaemia significantly improves the quality of life of patients with CKD,¹⁷⁵ and may help to arrest CKD progression.¹⁷⁶

3.2 Prevalence of Anaemia

The World Health Organisation (WHO) defines anaemia as haemoglobin levels below 13 g/dl in males and 12 g/dl in females.¹⁷⁷ Chronic anaemia may impact adversely on psychological and physical development, cognitive function and appetite, and exercise tolerance.¹⁷⁸ Common symptoms include easy fatigability, malaise, dyspnoea and palpitations. Anaemia is traditionally classified on the basis of red cell size and shape as normochromic normocytic, hypochromic microcytic and macrocytic anaemia. Normocytic normochromic anaemia has been reported in subjects with diabetes without significant renal failure.¹⁰¹

3.3 Anaemia in Diabetes

Anaemia is commonly associated with diabetes. In a recent, cross sectional study,¹⁷⁹ approximately a quarter of patients attending a diabetic clinic were found to be anaemic. Anaemia is an independent risk factor for development and progression of CKD.¹⁸⁰ It also contributes to the pathogenesis and progression of diabetic retinopathy by aggravating hypoxic environment in the retinal blood vessels.¹⁸¹ Patients with diabetes may be more vulnerable to the adverse effects of anaemia in presence of cardiovascular disease and hypoxia-induced organ damage.¹⁷⁹

The etiopathogenesis of anaemia in diabetes is multifactorial (Figure 3.1). Erythropoietin (EPO) deficiency as a result of DN is the most important cause of anaemia in patients with diabetes.¹⁰¹ However, even before any functional or organic deficiency of EPO is evident, several other factors, may contribute to the development

of a chronic hypoxic milieu, promoting erythropoietic stress and potentiating the genesis of early anaemia in diabetes. Also, low levels of testosterone, a common finding in males with type 2 diabetes, may contribute to anaemia in these patients.¹⁸²

3.4 Role of chronic inflammation

Studies of pro-inflammatory cytokines such as Interleukin –1, Tumour Necrosis Factor-A and Interferon-gamma in the development of anaemia, in chronic disease states, suggest an important role for these cytokines mediated through suppression and apoptosis of erythroid progenitor cells.¹⁸³ Diabetes is a chronic inflammatory state with increased levels of pro-inflammatory cytokines, even before the development of renal impairment.¹⁸⁴

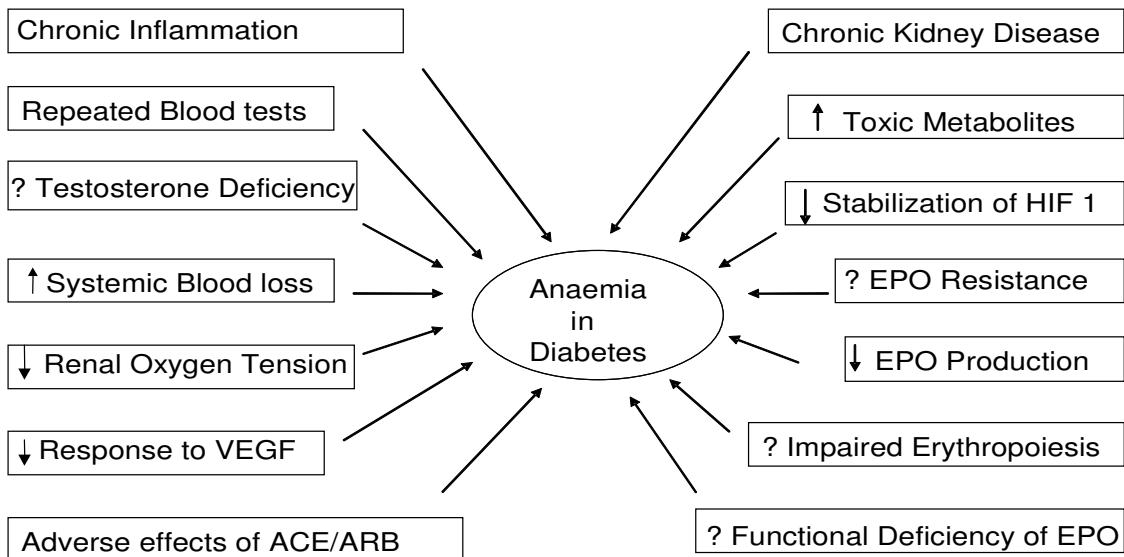


Figure 3.1 Contributing factors for Anaemia in Diabetes Mellitus

3.5 Advanced Glycation End Products and Oxidative Stress

Increased production of advanced glycation end products (AGEs) has been implicated in the genesis of microvascular complications in diabetes.¹⁸⁵ Increased accumulation of these products may promote non-enzymatic glycation of red cell-membrane glyco-proteins and hemoglobin and impaired erythrocyte deformability in diabetes.¹⁸⁶ AGEs may also increase the level of oxidative stress in diabetes by stimulating increased production of free oxygen radicals.¹⁸⁷ AGEs have been shown to play a significant role in the pathogenesis and progression of DN¹⁸⁸ and diabetic neuropathy.¹⁸⁹ Increased production of oxidative stress boosters such as reactive oxygen species (ROS) may also have a role.¹⁹⁰

ROS combines with nitrite oxide (NO) in the endothelium to form reactive oxygen intermediates such as peroxynitrite, thus reducing the total bioavailability of NO.⁸⁵ This may lead to an increase in the vascular tone and increased oxygen expenditure.

⁸⁸ A reduction in the renal oxygen tension along with impaired bioavailability of NO may increase free radical damage in the renal tubulointerstitium.

Chronic hyperglycemia may give rise to alterations in the regulation of intra-renal blood flow,⁸⁷ which along with an increased lactate concentration in the renal medulla,⁸⁸ may result in reduced oxygen delivery to this region.⁸⁶ All these perturbations promote a hypoxic milieu in the tubulointerstitium, potentially hampering the functional ability of peritubular fibroblasts, which are primarily responsible for hydroxylation of 25-hydroxyvitamin D to 1, 25-dihydroxyvitamin D and production of EPO (see chapter 2).

3.6 Haematinic Status

Diabetes may be associated with several metabolic derangements, which may contribute to anaemia. Patients with diabetes have an increased prevalence of chronic gastritis¹⁹¹ and H. Pylori infection,¹⁹² that may potentially influence absorption of nutrients from the stomach. Some patients with type 1 DM may harbour antibodies to parietal cells, which may increase their risk of developing iron deficiency anaemia and atrophic gastritis.¹⁹³ A few may also have an associated malabsorptive disorder like coeliac disease, hampering overall nutrient intake.¹⁹⁴

Inadequate iron stores may result in decreased responsiveness to EPO action on erythropoiesis.¹⁹⁵ In clinical practice, iron deficiency is the commonest cause of resistance to exogenous EPO.¹⁹⁶ The prevalence of iron deficiency has been reported to be higher in patients with diabetes than in non-diabetics.¹⁹⁷ Modulation of transferrin receptors by glycation may impair their capacity to bind iron and potentially reduce iron availability in diabetes.¹⁹⁸

3.7 Adverse effects of Medications

Patients with type 2 DM often take multiple medications for control of diabetes and other concomitant conditions. Metformin, one of the most commonly used oral anti-diabetic agents has been associated with malabsorption leading to vitamin B¹² deficiency,¹⁹⁹ potentially resulting in megaloblastic anaemia in susceptible individuals. Glitazones may exacerbate anaemia, probably due to haemodilution secondary to fluid retention.²⁰⁰ Anti hypertensive medications such as Angiotensin

converting enzyme Inhibitors (ACEI) and Angiotensin II Receptor Blockers (ARBs) may adversely influence erythropoiesis and promote anaemia.²⁰¹

3.8 Abnormalities of Erythrocytes

The life span of red blood cells (RBCs) may be decreased in DM.⁹⁷ RBCs are affected by various disturbances in the haematopoietic milieu, such as chronic hyperglycemia and hyperosmolarity, elevated internal viscosity of RBCs,²⁰² overactivity of the polyol pathway⁹⁸ and enhanced erythrocyte membrane rigidity due to AGEs.²⁰³ Several other metabolic and functional abnormalities of the RBCs have been described in experimental models of diabetes, which include elevated sorbitol levels, diminished Na/K-ATPase function⁹⁹ and modulated activity of acetyl cholinesterase.¹⁰⁰ There is an increase in the cell size, osmotic fragility and membrane viscosity of the RBCs along with reduced filterability.²⁰⁴

Oxygen affinity is altered in the diabetic erythrocyte.⁹⁷ These abnormalities may contribute to oxidative stress in the diabetic erythrocyte and may modulate the flexibility of the RBCs making them prone to trapping and sequestration in the reticulo-endothelial system.¹⁷² Diabetes has been associated with impaired red blood cell deformability, a hemorheologic perturbation, promoting microvascular complications and anaemia. A number of other studies have supported the role of RBCs in the vascular damage associated with diabetic vasculopathy.²⁰⁵ All these factors may contribute to microvascular disturbances and erythropoietic stress in diabetes mellitus. (Figure 3. 2)

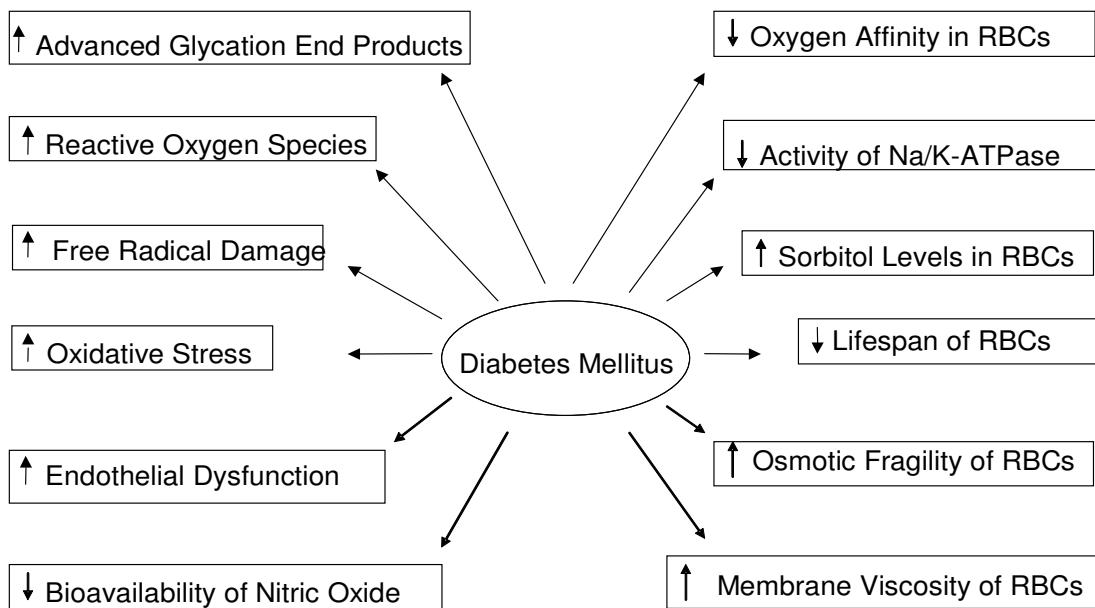


Figure 3.2 Erythropoietic Stress in Diabetes Mellitus

3.9 Role of Diabetic Neuropathy

Diabetic neuropathy is one of the earliest microvascular complications seen in patients with diabetes. Studies in experimental models of diabetes have suggested a role for splanchnic denervation, secondary to autonomic neuropathy, leading to blunted erythropoietin response to anaemia. Recently, a few small clinical studies have reported similar findings, advocating a role for autonomic neuropathy in initiating anaemia by alteration of the ‘anaemia sensing’ mechanisms.¹⁰⁴ These studies, in patients with type 1 DM, with neuropathy and normal creatinine, demonstrated low circulating levels of EPO. Erythropoietin resistance or blunted response to

endogenous EPO has been reported in diabetics with severe autonomic neuropathy as compared to those without neuropathy.²⁰⁶ At relatively normal creatinine levels, efferent sympathetic denervation of the kidney may lead to the loss of appropriate EPO production, and contribute to EPO deficiency.¹⁰⁴

3.10 Role of Diabetic Nephropathy

DN is a long-term microvascular complication of diabetes. With increased life expectancy of patients with diabetes, due to improved healthcare, DN has become the most common cause of end-stage renal disease (ESRD) and renal anaemia in developed countries.²⁰⁷ Anaemia in non-diabetic CKD is normally not apparent before the advent of moderate renal failure.²⁰⁸ Diabetic nephropathy is a major cause of renal anaemia, resulting in low circulating EPO levels, which occur earlier in the course of progressive DN, than in the course of CKD due to other causes.²⁰⁹ Patients with diabetes are more anaemic than non-diabetics with similar degrees of renal impairment.¹⁰¹

3.11 Tubular proteinuria and Ischemia

Microalbuminuria is the first detectable clinical sign of an increased risk of DN.¹⁸⁰ However, early dysfunction of renal tubules, the primary site of EPO production, has been reported in patients with diabetes before the onset of microalbuminuria.⁷⁴ Increased urinary excretion of tubular injury markers such as N-acetyl- β -D-glucosaminidase (NAG), a tubular enzyme, and Retinol Binding Protein (RBP) have

been reported in type 1²¹⁰ and type 2 DM,⁷⁴ which may imply disturbed function of the renal tubules, well before the onset of microalbuminuria.

Glucose uptake by renal tubules is independent of insulin action. This renders the tubules especially vulnerable to injury by chronic hyperglycemia.⁷⁹ Chronic hyperglycemia may result in early tubular apoptosis, reducing the number of functional tubular cells.¹³⁶ In addition, chronic hyperglycemia is associated with increased production of AGEs, and polyols, and increased stimulation of protein kinase C and angiotensin II in the renal tubules. These changes may result in vasoconstriction and tubular ischemia.⁸⁰

Peritubular capillaries (PTCs) are vital for survival and functioning of tubular cells. Chronic hyperglycemia coupled with renal vasoconstriction and the above-mentioned cellular changes, in absence of adequate NO may adversely affect the survival of PTCs.⁸¹ Inadequate availability of NO may lead to an increased vascular tone and enhanced oxygen uptake.⁸⁶ Chronic hyperglycemia may thus compromise the microcirculation in the renal interstitium.⁸⁷ It also enhances lactate concentration in the renal medulla, reducing pH, further hampering oxygen availability in the renal interstitium.⁸⁸

Tubular and interstitial cells in the juxtamedullary region and outer medulla are, even under normal conditions, in a state of relative hypoxia. This is a result of the countercurrent exchange of oxygen within the vasa recta and high oxygen consumption in the medullary thick ascending limb and the S3 segments of the proximal tubules.²¹¹ The hypoxic milieu is a stimulus for production of mediators for cellular adaptation to hypoxia such as Hypoxia Inducible Factor (HIF). HIF-1 is one of

the major mediators of cell adaptation to hypoxia. HIF-1 manifests its effect by promoting vasculogenesis, improving oxygen availability and modulation of cellular metabolism.⁹¹

In addition, HIF-1 influences pathways involved in glucose metabolism,⁹² cellular growth and apoptosis,⁹³ EPO production, vascular endothelial growth factor (VEGF) production, iron metabolism, and extracellular matrix metabolism.⁹⁴ Hyperglycemia, in a dose dependant fashion, impairs the stabilisation of HIF-1 against protease degradation in the body.²¹² HIF-1 may protect against fibrotic processes within the kidney by influencing genes promoting fibrosis. Reduced HIF-1 action on these pro-fibrotic genes due to inhibition of HIF-1 by hyperglycemia, may promote interstitial fibrosis.⁹¹ This may exacerbate local ischemia by inhibiting diffusion from the capillary to the tubule.

3.12 Erythropoietin levels in Diabetes

Erythropoietin, a glycoprotein hormone is produced by the peritubular fibroblast in the kidneys, and stimulates erythropoiesis in response to hypoxia.¹⁶³ Erythropoietin deficiency anaemia occurs early in patients with type 1 and type 2 DM, though the prevalence is greater in type 1 DM. A defect in 'anaemia sensing'¹⁰¹ or resistance to EPO action has been suggested as a probable mechanism of early onset anaemia in type 1 DM and may be related to splanchnic denervation as a result of diabetic autonomic neuropathy.¹⁰⁴

Erythropoietin is a multidimensional hormone,¹⁴⁷ its actions extending well beyond erythropoiesis.¹⁵⁵ The extra-haemopoietic properties of EPO include cytoprotection and inhibition of apoptotic death-related processes.²¹³ It also has anti-oxidant and anti-inflammatory properties.¹⁶⁰ The extra-haemopoietic properties of EPO may have major implications.²¹⁴ EPO manifests its trophic properties by stimulation of the EPO receptors (EPOR) on the tissue surface.¹⁶¹ EPO receptors have been located throughout the substance of the kidney, including on glomerular podocytes²¹⁵ and on tubular cells.²¹⁶

Inadequate EPO response in diabetes may be due to low levels,¹³⁹ functional EPO deficiency⁷⁸ and/or EPO resistance.¹⁴⁰ Also, EPO may be ineffective due to modulation of erythropoietin receptors as a result of glycation.¹⁴² Absence of adequate EPO action, may promote hypoxia as a result of low haemoglobin levels, which in turn may initially stimulate EPO production by the surviving peritubular cells. This, in the persisting hypoxic environment, might promote cellular fatigue and exacerbate apoptosis. Unabated tubular apoptosis, as a result of chronic hyperglycemia and interstitial fibrosis, would also cause a reduction in functional EPO receptors. These processes may have a cumulative effect in precipitating early anaemia in diabetes.

3.13 Correction of Anaemia

There is no clear consensus on the level of haemoglobin, which should trigger investigation into the cause of anaemia. Most clinical guidelines²¹⁷⁻²¹⁹ suggest a cut-off of 11.5 g/dl in a CKD patient. With no clinical data to support benefits of very high

or normalised haemoglobin on survival, the optimum level of target haemoglobin is still debated.²²⁰ In view of this, National institute of clinical excellence (NICE) guidelines recommend haemoglobin levels should be maintained between 10.5 and 12.5 g/dl.²¹⁷

Theoretically, correction of anaemia should be able to reverse the effects of anaemia. However, there are conflicting reports on benefits of anaemia correction. Early EPO administration in pre-dialysis patients with EPO deficiency anaemia has been reported to alter CKD progression and reduce mortality.¹⁵⁰ However, recent major clinical trials such as CHOIR (Correction of Haemoglobin and Outcomes in Renal Insufficiency),¹⁵² and CREATE (Cardiovascular Risk Reduction by Early Anaemia Treatment with Erythropoietin Beta),¹⁵⁴ which were designed to look at the beneficial aspects of correction of anaemia and the optimal haemoglobin levels, reported increased mortality with higher levels of haemoglobin and no beneficial effect on progression of renal disease. CHOIR,¹⁵² and CREATE,¹⁵⁴ studied CKD patients in general, though a significant proportion had diabetes.

Currently, the TREAT (Trial to Reduce Cardiovascular Events with Aranesp Therapy) study is being carried out to study the impact on mortality and nonfatal cardiovascular events in patients with type 2 DM of anaemia correction to high (haemoglobin 13 g/dl) and low (haemoglobin 9 g/dl) levels.²²¹ Another multicentre study, ACORD (Anaemia Correction in Diabetes),²²² is underway to study the effects of anaemia correction on cardiac structure, function, and outcomes in patients with diabetes with anaemia and early DN. The primary results from ACORD reported beneficial effects of anaemia correction in prevention of progression of left ventricular hypertrophy in patients with

diabetes.²²² On completion, TREAT and ACORD may provide us a better insight into the benefits of anaemia correction in diabetes.

3.14 Conclusion

The effects of erythropoietic stress are a multidimensional occurrence in patients with diabetes. It occurs early and plays an important role in the genesis of vasculopathy. Erythropoietic stress, resulting from chronic hyperglycemia, may play a major role in promoting hypoxic milieu, oxidative stress, hampering nutritional support, impairing the functioning of “stress busters”, contributing to sympathetic denervation and thus precipitating anaemia early in the course of the disease.

Factors promoting erythropoietic stress in diabetes include chronic hyperglycemia, increased production of AGEs, elevated levels of free radicals, increased oxidative stress, reduced NO production, decreased stabilization of HIF-1, enhanced endothelial dysfunction, and abnormal erythrocyte morphology and function. Chronic inflammation, abnormal haematinic absorption and adverse effects of drugs, may worsen hematopoietic stress.

Erythropoietic stress coupled with chronic hyperglycemia may play an important role in obliterating peritubular capillaries, hampering nutrition of the tubulointerstitium, promoting hypoxia and early tubular damage, and resulting in tubular dysfunction and/or tubular apoptosis. Surviving tubular cells may have to work ‘overtime’ to produce enough EPO to maintain adequate haemoglobin levels. However, the

increased workload, in presence of chronic hypoxia may prove counter-productive, and result in further loss of tubular cells; thus setting up a vicious cycle and leading to EPO deficiency and anaemia.

Diabetic vasculopathy and endothelial dysfunction, occurring as a result of erythropoietic stress, may modulate the vascular system, and in absence of adequate repair processes, may impair the tone and flexibility of the blood vessels. By the time a patient with diabetes develops EPO deficiency anaemia; the blood vessels may be badly damaged, with poor tone and reduced flexibility.

Correction of anaemia with EPO leads to an increased haematocrit level, however, the damaged blood vessels with reduced tensile strength, may not be able to accommodate the elevated erythrocyte numbers. EPO therapy must be used cautiously in patients with CHF and ischemic heart disease on haemodialysis since these patients are at an increased risk due to widespread vasculopathy. Aiming for very high haematocrit levels, may lead to elevated blood pressure and/or vascular thrombosis, known complications of EPO therapy.¹⁵¹

The results of TREAT and ACORD are awaited; and in absence of clear guidelines on anaemia management, it would be fairly reasonable to aim to maintain the haemoglobin levels between 10.5 and 12.5 g/dl as recommended by NICE.²¹⁷ The results of ACORD and TREAT may also provide a better understanding of the role of anaemia in the progression of vasculopathy in DM. The ACORD study, if positive, may potentially open the way for trying low dose EPO supplementation, in DM

patients with EPO deficiency, to prevent the progression of DN. Similarly, positive indications from TREAT study may brighten the prospects of use EPO in prevention of progression of CVD.

Along with the promotion of haematopoietic stress milieu resulting in anaemia, chronic hyperglycemia also promotes vascular damage by several mechanisms. The previous two chapters discussed the role of chronic hyperglycemia in promoting anaemia and microvasculopathy (DN). The following chapter discusses the role of chronic hyperglycemia in the pathogenesis of macrovascular disease and vascular calcification.

CHAPTER 4

VASCULAR CALCIFICATION IN DIABETIC MACROVASCULOPATHY

VASCULAR CALCIFICATION IN DIABETIC MACROVASCULOPATHY

4.1 Introduction

Macrovascular complications of diabetes mellitus (DM) such as cardiovascular disease (CVD) and peripheral vascular disease (PWD) are the leading cause of increased mortality²²³ and morbidity²²⁴ respectively, in these patients. The pathogenesis of macrovasculopathy in DM is multifactorial and not completely understood.²²⁵ The pathogenetic processes for macrovasculopathy are different in type 1²²⁶ and type 2 DM²²⁷ due to different aetiologies of these two conditions. The pathogenetic processes in these two categories may have a common beginning in generalised endothelial dysfunction followed by atherosclerosis and vascular calcification (VC). However, there may be an overlap of the last two stages. Macrovasculopathy in DM is associated with structural and functional modulation of the vasculature, resulting in reduced tone and flexibility. In the long run, with onset of VC, these changes contribute to the development and progression of CVD in these patients.²²⁸

The process of VC incorporates the laying down of calcium phosphate material, usually in the form of hydroxyapatite in the vessel wall. VC may be localized as manifested in an atherosclerotic plaque or it may be generalized as seen in medial arterial calcification (MAC). Usually it is a mixed picture with simultaneous presence of both types of calcification. MAC, though traditionally associated with the ageing process, is a characteristic feature of VC in DM. MAC refers to concentric calcification and stiffening of the elastic layer of the arterial wall, however, in contrast to the

eccentric intimal arterial calcification, as seen in atherosclerosis; MAC does not occlude the arterial lumen.²²⁹

MAC is also seen in advanced renal failure with abnormal mineral metabolism. However, MAC may manifest with normal serum calcium and phosphate levels in patients with DM.²³⁰ The pathogenesis of MAC is multifactorial and poorly understood. Vascular calcification is a huge area of research. This concise review will discuss the plausible role of i) endothelial cell dysfunction in genesis of vasculopathy in type 1 and type 2 DM ii) Renin Angiotensin System (RAS) and various contributory factors in the blood in the progression of vasculopathy iii) OPG/RANKL/TRAIL axis in the genesis and progression of MAC in DM.

4.2 Normal Endothelium

The endothelium is a layer of endothelial cells (EC) lining the internal lumen of the blood vessels and serves as a biological barrier between the blood and vascular smooth muscle cells (VSMC).²³¹ The physiological actions of EC include modulation of vascular tone (vasoconstriction and vasodilation), haemostasis, regulation of growth and differentiation of VSMC and modulation of inflammation in the vasculature.²³² In addition, EC also contributes to mitogenesis, angiogenesis, vascular permeability and fluid balance.²³³ EC modulates the vascular tone by regulating the release of vasodilators such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) and vasoconstrictors such as Endothelin-1, Prostaglandin H₂,²³⁴ Reactive oxygen species (ROS),²³⁵ Angiotensin II (ANG II)²³⁶ and Thromboxane A₂.²³⁷

EC plays a primary role in haemostasis by maintaining a balance between the processes of coagulation and fibrinolysis in the vascular milieu. This function is carried out by four distinct but interrelated mechanisms such as modulation of procoagulant pathways, suppression of procoagulant proteins, modulation of fibrinolysis by regulating the production of various substances such as tissue factor inhibitor, von Willebrand (vWF), fibrinogen, plasminogen activator (PA)²³⁸ and plasminogen activator inhibitors (PAI-1, PAI-2)²³⁹ and modulation of thrombus formation by regulating production of compounds such as Thromboxane A₂ (platelet proaggregator) and prostacyclin (platelet antiaggregator).²⁴⁰

Due to its close proximity, the EC is an important mediator of growth and differentiation of the VSMC. The vascular remodelling is carried out primarily by EC regulation of growth factors. Insulin like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF) prevent apoptosis of VSMC and hence are important for VSMC survival.²⁴¹ Fibroblast growth factor, ANG II and transforming growth factor-beta (TGF-β) are other potent growth factors for VSMC. ANG II and TGF-β activate both proliferative and antiproliferative pathways and hence have bifunctional effects on VSMC growth.²⁴²

The EC expresses a range of adhesion molecules on its surface to facilitate cell-to-cell interaction with circulating polymorphonuclear leukocytes. Some of these adhesion molecules are derived from the immunoglobulin superfamily - the intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1),²⁴³ while the selectins (E-, P- and L-selectin) are the other important group.²⁴⁴ The EC regulates the dissociation of leukocytes from the vascular bed by

modulating the expression of adhesion molecules²⁴⁵ and control of NO release from its surface.²⁴⁶ EC also regulates platelet adhesion by NO release.²⁴⁷

4.3 Endothelial Cell Dysfunction

The pathogenesis of endothelial cell dysfunction (ECD) is not completely understood and may involve multiple mechanisms. There is no single definition of ECD. Broadly, ECD may be defined as deviation or incapacity of the EC to regulate some or all its functions. ECD may result in an imbalance of i) vascular relaxing and contracting factors ii) procoagulant and anticoagulant mediators iii) vascular growth-inhibiting and growth-promoting substances. ECD may lead to various patho-physiological complications such as enhanced expression of adhesion molecules²⁴⁸ resulting in increased leukocyte-EC adhesions,²⁴⁹ promotion of procoagulant state as a result of increased activation of platelets and clotting factors,²⁵⁰ and finally, impaired NO release as a result of ECD may lead to defective modulation of vascular growth and remodeling in the vessel wall.²⁵¹

There is no definitive marker of ECD. However, ECD is determined on observations of surrogate markers of ECD such as estimation of flow-mediated vasodilation subsequent to transient ischemia²⁵² and evaluation of vessel resistance in small or big arteries to physiological stimuli.²⁵³ These two measurements are employed to broadly assess the NO release capacity of the EC in response to various stimuli. The other indirect estimates of ECD are assessment of i) of vascular permeability of macromolecules,²⁵⁴ levels of vasoactive (vasoconstrictors/vasodilators) factors,²⁵⁵ prothrombotic/procoagulant activity,²⁵⁶ and inflammatory markers such as adhesion

molecules (VCAM-1, ICAM-1, and E-selectin),²⁵⁷ cytokines [interleukin (IL)-1beta, IL-6, and tumor necrosis factor-alpha (TNF-alpha)]²⁵⁸ and C reactive protein (CRP).²⁵⁹

4.4 ECD in Type 1 DM

The current literature is inconclusive about the pathogenesis of ECD in type 1 DM. Although, type 1 DM patients with poor glycemic control may demonstrate features of ECD,²⁶⁰ features ECD may be absent in uncomplicated type 1 DM patients with good glycemic control.²⁶¹ However, type 1 patients may manifest some features of ECD, later in the course of the disease, before the onset of microalbuminuria (MA).²⁶² MA is considered as a marker of extensive ECD and an independent risk factor for atherosclerosis, CVD, and premature mortality due to CVD in patients with hypertension and type 1 and type 2 DM.²⁶³ However, there may be a subset of 'resistant' type 1 DM patients who may have no ECD despite having MA,²⁶⁴ giving rise to the speculation that although diabetic state may predispose to the development of ECD, it may not be sufficient to cause it.

4.5 ECD in Type 2 DM

The pathogenesis of ECD in type 2 DM is multifactorial (Figure 4.1). Type 2 DM develops in the background of an array of risk factors such as obesity, glucose intolerance, hypertension, dyslipidemia, hyperinsulinemia, insulin resistance (IR) and elevated inflammatory markers, collectively termed as metabolic syndrome. All the components of metabolic syndrome may potentially promote ECD.²⁶⁵ Individuals at

risk of type 2 DM have been demonstrated to have higher levels of biochemical markers of ECD such as ET-1, vWF, ICAM, VCAM with normoglycaemia,²⁶⁶ suggesting that ECD may well precede the development of type 2 DM.²⁶⁷

Insulin may modulate ET-1 release²⁶⁸ and also has a vasodilatory effect on the skeletal muscle vasculature by promoting synthesis/release of NO from the EC.²⁶⁹ IR is one of the major components in the pathophysiology of ECD in type 2 DM.²⁷⁰ IR may lead to functional insulin deficiency and elevated levels of lipids and glucose in the vasculature. All these factors may contribute to impaired NO production,²⁷¹ and potentially impair the vasodilatory action of EC. Whether ECD leads to IR or vice versa is still debated.²⁷²

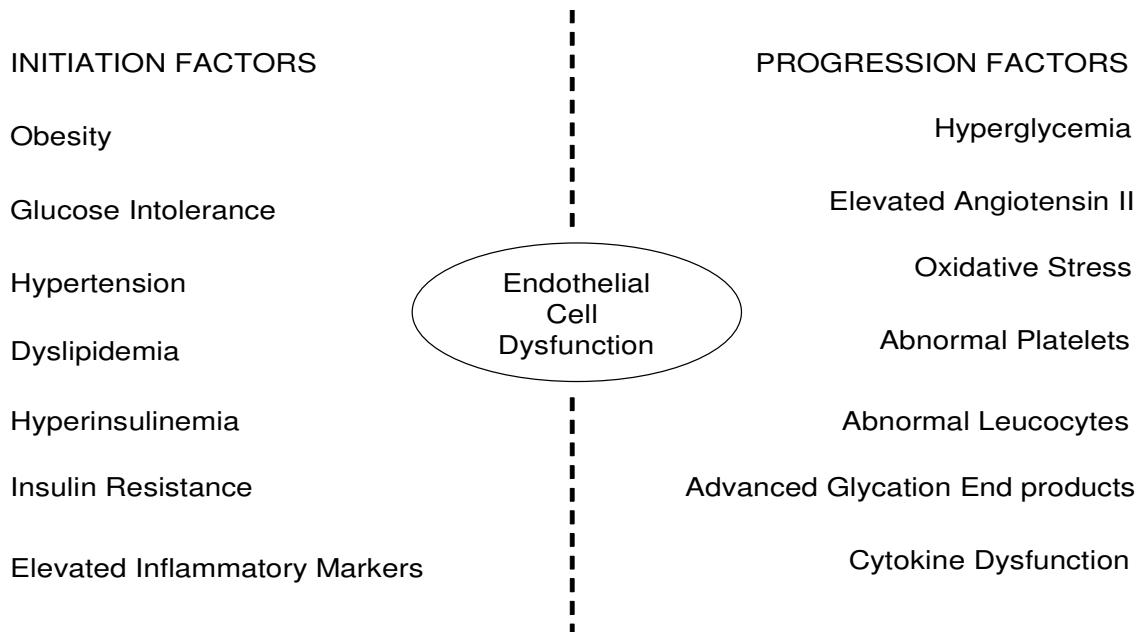


Figure 4.1 Endothelial Cell Dysfunction in Type 2 Diabetes Mellitus

4.6 Renin Angiotensin System in ECD

RAS inhibition reduces cardiovascular risk in patients with DM, suggesting an important role of RAS in diabetic macrovasculopathy.²⁷³ ANG II, the active, central metabolite of RAS, has multiple actions throughout the cardiovascular system such as promoting vasoconstriction, cardiac hypertrophy, VSMC proliferation, and generation of ROS.²⁷⁴ RAS inhibition by angiotensin converting enzyme inhibitors may reverse ECD, thus pointing towards the potential role of RAS dysfunction in the pathogenesis of ECD.²⁷⁵

4.7 Effects of Chronic Hyperglycemia

Hyperglycemia may modulate several aspects of EC through activation of various biochemical pathways (sorbitol, diacylglycerol/protein kinase C, hexosamine) of glucose metabolism.²³² The transport of glucose across EC is insulin independent, which may make the EC potentially susceptible to the adverse effects of hyperglycemia due to prolonged direct exposure. Chronic hyperglycemia may modulate several aspect of EC function such as promotion of a procoagulant state (elevated PAI-1 and reduced fibrinolytic activity),²⁷⁶ increased blood viscosity,²⁷⁷ enhanced production of basement membrane components (collagen type IV and fibronectin),²⁷⁸ increased EC and VSMC proliferation,²⁷⁹ elevated inflammatory markers (haptoglobin, C-reactive protein and alpha-1 acid glycoprotein),²⁸⁰ enhanced adhesion molecule expression²⁸¹ and apoptosis of EC.²⁸²

4.8 Role of Advanced Glycation End Products

Advanced glycation end products AGEs are a heterogenous group of end product substances derived from nonenzymatic glycation of proteins, lipids, and nucleic acids. In DM there is increased production and accumulation AGEs along with increased expression of the receptor for AGEs (RAGE) on the EC.²⁸³ AGEs can potentially promote atherosclerosis by modulation of extracellular matrix expression (type I and type IV collagen) and oxidation of low density lipid cholesterol particles.²⁸⁴ Excessive AGEs accumulation promotes ECD by increasing endothelial permeability, altered coagulant properties (down regulation of anticoagulant endothelial cofactor thrombomodulin and expression of procoagulant cofactor tissue factor)²⁸⁵ and production of Endothelin-1.²⁸⁶ AGEs also promote increased cellular proliferation and expression of fibronectin in the VSMC through activation of TGF- β .²⁸⁷

4.9 Role of Oxidative stress

Oxidative stress plays an important role in the pathogenesis and progression of vascular complications of DM.⁸³ AGEs promote oxidative stress by increased production of ROS²⁸⁸ and enhanced depletion of oxidative stress busters (glutathione and ascorbic acid).²⁸⁹ AGEs quench NO and reduce its bioavailability in the EC milieu.²⁹⁰ In addition, ROS impairs NO bioavailability by interacting with NO to form intermediate molecules such as peroxynitrite.⁸⁵ Oxidative stress promotes ECD by increasing vascular permeability and increased adhesion of leucocytes.²⁹¹

4.10 Role of Platelets

The normal function of platelets is hampered as a result of chronic hyperglycemia leading to decreased production of NO and Prostacyclin, reduced antioxidant and platelet vitamin C levels, increased aggregability and adhesiveness, enhanced platelet production, and increased production of Thromboxane A₂. Platelets in diabetes are at an increased risk of damage due to reduced membrane fluidity, altered mineral metabolism of calcium and magnesium in the platelet cell membrane and elevated fibrinogen levels.²⁹²

4.11 Role of Leucocytes

The filterability and deformability of polymorphonuclear leukocytes is reduced in diabetes.²⁹³ Also, there is an increased expression of receptors for fibronectin, a plasma protein. This leads to increased adhesiveness of monocytes to fibronectin in diabetes. In addition, monocytes have increased pro-coagulant activity.²⁹⁴ These changes may result in reduced leukocyte deformability and impaired microcirculation, resulting in aggravation of flow disturbances in the vessel wall, and potentially contributing to vasculopathy in diabetes.²⁹⁵

4.12 Role of Dyslipidemia

Abnormally elevated total cholesterol, LDL cholesterol and triglycerides and low HDL levels characterize dyslipidemia in DM. There is an increased oxidation of LDL

particles by AGE in the vasculature. Oxidized LDL particles are toxic to the EC and increased accumulation of these particles may promote ECD and eventually atherosclerosis in the vasculature by i) enhancing monocyte attachment to EC ii) contributing to Macrophage and Foam cell formation iii) and reduced NO levels by decreasing NO synthase availability.²²⁵

4.13 Role of Cytokine Activation

DM is characterized by chronic low-grade inflammation with enhanced activation of cytokines (TNF cytokine family) and growth factors (TGF- β , PDGF, epidermal growth factor and vascular endothelial growth factor).²⁹⁶ On activation, TNF- α may promote ECD by i) reducing the bioavailability of NO ii) increasing oxidative stress by generation of free radicals iii) increased expression of adhesion molecules on EC and modulation of the lipid metabolism.²⁹⁷ In addition, modulation of extended TNF family members such as Osteoprotegerin (OPG), receptor activator of nuclear factor kappa β ligand (RANKL) and TNF- related apoptosis inducing ligand (TRAIL) may play a key role in the pathogenesis of vascular calcification in the background of ECD.

4.14 Endothelial Cell Dysfunction and Calcification

ECD is an independent marker and a key early step in the pathogenesis of vascular disease and accelerated atherosclerosis in DM.²⁹⁸ In non-diabetics, ECD promotes atherosclerosis by facilitating i) thrombogenic milieu with fibrin and platelet adhesion on its surface ii) deposition of oxidized lipid particles iii) adhesion of inflammatory

macrophage and T cells, leading to atherosclerotic plaque formation in the intimal layer of the blood vessels and finally calcification of the intima, also termed as intimal vascular calcification.²⁹⁹ In patients with DM, atherosclerotic changes are seen in the intima and the media, with more pronounced medial sclerotic changes and termed as MAC or Mönckeberg's sclerosis (Figure 4.2).²²⁹

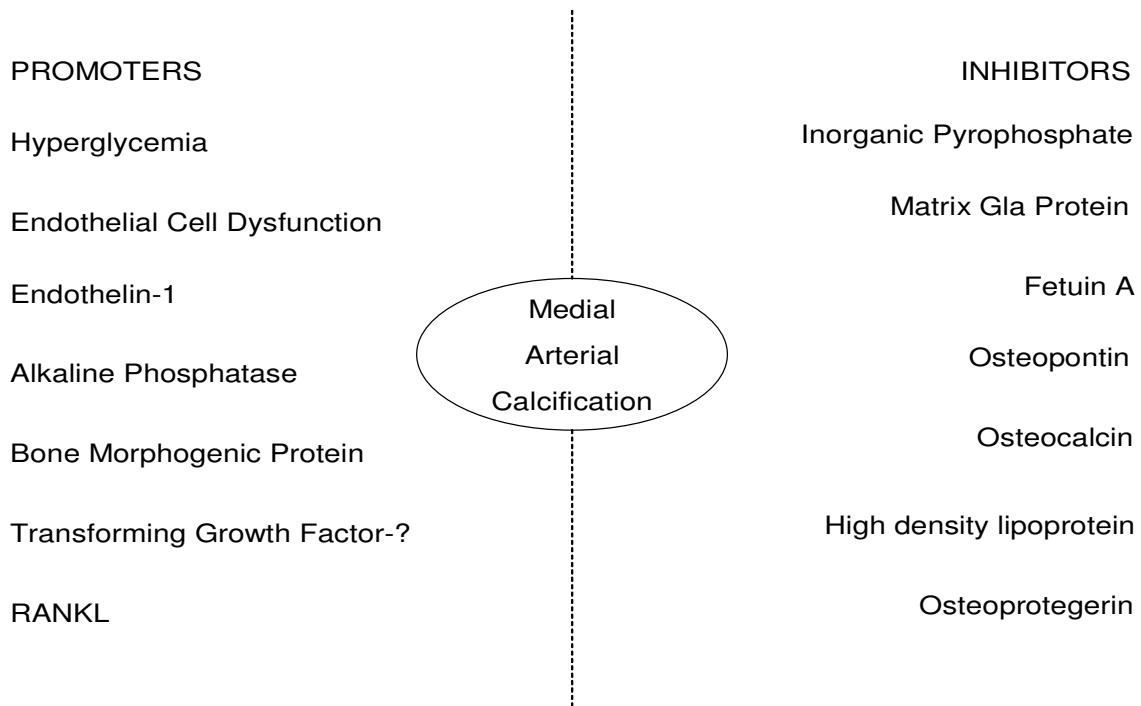


Figure 4.2 Regulators of Medial Arterial Calcification in Diabetes Mellitus

4.15 OPG/TRAIL Axis

Patients with DM have greater atheroma burden, extensive atherosclerosis, impaired compensatory remodelling, enhanced plaque progression, reduced lumen size with no difference in the external elastic layer as compared with non diabetics.³⁰⁰ MAC

leads to stiffening of the elastic layer in the arterial wall and is seen more commonly in the elderly general population and patients with DM.²²⁹ MAC is an important predictor of CVD and occurs independent of atherosclerosis in DM.³⁰¹ OPG and TRAIL have been detected in calcified regions of atherosclerosis and MS along with enhanced apoptosis, suggesting a significant role for the regulators of bone metabolism in the pathogenesis of MAC in patients with DM.³⁰²

4.16 Regulators of Calcification

Traditionally, VC has been assumed to be a process of passive relocalisation of calcium from bone to the vasculature. However, it is now perceived to be a complex interaction between inhibitors and promoters of VC. These regulators act in concert to inhibit or promote VC. In the normal vascular milieu, a diverse group of regulatory factors such as inorganic pyrophosphate (PPi), matrix Gla protein (MGP), Fetuin, osteopontin and OPG overwhelmingly inhibit the process of VC. However, in phenotypically altered vascular milieu, this defence mechanism may be weakened, making the vascular milieu susceptible to the actions of promoters of VC such as glucotoxicity, endothelin-1, alkaline phosphatase (ALP), bone morphogenetic protein (BMP)-2, BMP-4, TGF- β and RANKL.³⁰³

4.17 Pathogenesis of Calcification

Vascular EC activation as a response to inflammatory stimuli is a key step in the transformation of the EC from a quiescent barrier to a dynamic state.³⁰⁴ EC plays a

key role in bone development and remodeling by modulating formation and activity of Osteoclasts. In addition, EC orchestrates the inflammatory response and immune mediated mechanisms in various vascular diseases such as vascular calcification.³⁰⁵ The evidence for TRAIL induced apoptosis in a normal EC is contradictory. However, TRAIL may activate the process of apoptosis in the EC when its phenotype is altered.³⁰⁴ EC apoptosis as a result of prolonged ECD or other factors (Figure. 1), may compromise with the continuity of the endothelial barrier and expose the VSMC to the hazards of hyperglycemia and cytokine action.

4.18 VSMC Dysfunction and Apoptosis

The VSMCs are multi-lineage cells and have an enormous ability to undergo phenotypic transformation. A subset of VSMC also termed as calcifying vascular cells (CVC) can potentially express chondrogenic, leiomyogenic and stromogenic (osteoblast) markers on activation.³⁰³ Glucotoxicity and oxidative stress as a result of chronic hyperglycemia may play an important role in activation and transformation of CVC into osteoblast-like cells,³⁰⁶ which produces hydroxyapatite mineral for VC . TRAIL induced apoptosis of EC and VSMC cells serve as nidus or matrix for initiation of VC.³⁰²

4.19 Calcification Cascade Activation

In the background of hyperglycemia and oxidative stress, apoptosis of vascular cells (EC and VSMC) may be a trigger for TNF- α to induce the production of BMP-2 from

surviving ECs.³⁰⁷ BMP-2, a potent osteoblastic differentiation factor, in conjunction with TNF- α , galvanizes the osteo/chondrogenic cascade by activating the homeobox homolog (Msx2) and Wnt signalling pathways. These signals are expressed concentrically in the medial arterial layer through the vasa vasorum.³⁰⁸ The activation of these pathways results in mobilization of osteogenic enzymes and matrix proteins to facilitate calcium phosphate mineral deposition.³⁰⁹

4.20 Calcification of Elastic laminae

The deposition of calcium material in the vicinity of elastin fibres in the medial arterial layer adversely modulates the elastin matrix metabolism, resulting in reduced expression of major elastic fibre components such as tropoelastin, fibrillin-1, elastin-related enzyme and lysyl oxidase. In addition, it may result in reduced formation of new elastic fibres and increased vascular stiffness.³¹⁰ The process of calcium deposition starts in lipid vesicles located along and between the elastic fibres of the elastic laminae.

4.21 Initiation of Calcification

Inorganic phosphate (PPi) is a direct and powerful inhibitor of hydroxyapatite formation in vitro and PPi inhibition is a critical step in the initiation of VC.²³⁰ Elevated levels of ALP degrade and inactivate tissue PPi, and helps in mobilising calcium and phosphate ions to the site of VC initiation. The up-regulation of ALP activity is mediated by TNF- α and BMP 2.³⁰⁹ The calcification process in MAC is similar to the

intra-membranous bone formation.²⁹⁹ With the activation of the osteo/chondrogenic pathways, chondrocytes produce a layer of cartilage, which, on maturing, degenerates. The CVC differentiated osteoblasts may now supersede and generate a layer of bone matrix on the existing cartilaginous base.³¹¹ This mineralised matrix may be subsequently replaced by osteoid, and with osteoblast and osteoclast action, remodelled into mature bone, as in endochondral ossification.³¹²

4.22 Role of Calcification Regulators

The interaction of inhibitors and promoters of VC is complex and the underlying mechanisms are being unravelled. VC may be termed as failure of the inhibitory mechanism in the background of various metabolic and cytokine insults. However, the process is not straightforward and there are checks and balances with attempts at various stages, by the inhibitors, to influence the process of VC by countering the effects of promoters. PPi is a by-product of intracellular enzymatic reactions and is produced by several cells in the body including VSMC.²³⁰ It is responsible for stabilization of the VSMC and a reduction in PPi levels may render the VSMC susceptible to osteo/chondrogenic trans-differentiation.³¹³

The VSMC also produce MGP, calcium binding matrix protein, which inhibits the process of VC by blocking the production of ALP by antagonising BMP-2 and binding to matrix elastin.³⁰⁸ TGF- β facilitates VC by mineralization of extracellular matrix in the vessel wall. Fetuin, a glycoprotein released by liver, is a potent inhibitor of VC and it manifests its effects by blocking the action of profibrotic TGF-beta and BMP-2.³¹⁴ The trans-differentiated osteoblast like cells, from the CVC, expresses several bone

matrix regulatory factors such as alkaline phosphatase, collagen type I, bone sialoprotein, osteocalcin, osteonectin and osteopontin, akin to osteogenesis.³¹⁵

Osteopontin, a potent inhibitor of VC is normally not present in the non-diseased vasculature. However, it has been localized in mineralized matrix in VC, pointing towards its role of inhibitor of VC, in the advanced stages.³¹¹ The precise mechanism of actions of other suggested regulators of VC such as bone sialoprotein, osteocalcin and osteonectin is still elusive. All the above-mentioned inhibitors seem to become activated after the initiation of VC with matrix formation in the vascular apoptotic cells, however, OPG seems to be the foremost inhibitor in this sequence and it is expressed by the ECs, in response to ECD and TRAIL induced apoptosis.³⁰²

4.23 OPG/RANKL and Calcification

OPG and RANKL are key regulators of bone metabolism and vascular disease and OPG/RANKL/TRAIL axis plays an important role in the osteogenic modulation of vasculature in DM.³⁰² OPG/RANKL system is a relatively new avenue in understanding of the connection between the bone milieu and the vasculature. A huge amount of research is underway to explore the role of this system and to untangle the intricacies of the cell-to-cell talk between the bone and blood milieu. The precise metabolism and clearance of OPG/RANKL in the body is still obscure. In addition, there is little information on the biological variation of these regulators as well as their modulators such as PTH and 1, 25-dihydroxyvitamin D in the healthy population.

OPG is produced by a diverse range of tissues including ECs and VSMCs, with high levels in aortic and renal arteries. In contrast, RANKL is frequently not detectable in normal vasculature.³¹⁶ OPG has indirect anti-apoptotic and protective effects on the EC by serving as a decoy receptor for TRAIL and minimising its effect on ECs.³¹⁷ In the bone milieu, RANKL functions as a major osteoclast maturation factor, promoting osteoclast activity and bone resorption, whereas OPG undermines the action of RANKL by acting as a soluble decoy receptor for RANKL, thus diminishing its effects.

³¹⁶ RANKL promotes calcification in the vessel wall, whilst OPG protects against it.³¹⁸

High OPG levels, as seen in ECD,³¹⁹ may be a compensatory mechanism by the EC to protect against TRAIL induced apoptosis and in this regard, high OPG may be a survival factor for EC.³¹⁷ The localisation of OPG/RANKL in MAC and atherosclerotic calcification points towards their active role and association in these processes.³⁰² As mentioned above, the process of inhibition of VC is very tightly controlled in the body. Although, in the background of EC and VSMC apoptosis, the process of VC may be initiated, however, the overwhelming response of VC inhibitors at various stages of VC suggests that the vascular cells (EC and VSMC) do not concede easily in face of various metabolic and inflammatory insults.

Despite adverse metabolic milieu, in the stage of ECD, these vascular cells may respond by over expression of OPG, which serves as a survival factor for these cells by blunting the action of TRAIL, through binding of its receptor on EC.³¹⁷ In addition, the over expression of OPG by the vascular cells may continue through the process of VC and in fact, the levels may increase further, in response to need, by simultaneous production of OPG by the newly differentiated osteoblast-like cells from

the CVC. The additional OPG expression from the osteoblast-like cells may be required to counter the VC promoting action of RANKL, which may now be expressed at the site of new bone formation in the vessel wall.³¹²

High OPG levels are reported to have a positive correlation with inflammatory markers (CRP, IL-6 and fibrinogen), HbA1c and insulin resistance.³²⁰ OPG expression by the vascular cells seems to be one of the most important protective mechanisms to prevent VC and thus MAC, in the vessel wall. However, in the context of an unabated adverse environment in form of hyperglycemia, ongoing oxidative stress and an activated cytokine network may facilitate the calcification process by supporting the promoters of VC. To counter the progressive calcification, the osteoblast-like cells and the vascular cells in the vicinity of the matrix may boost OPG production. This process of inhibition and promotion may be progressive and continue till formation of bone like tissue in the vessel wall.

4.24 Conclusions

ECD is the primary event in the initiation of vasculopathy in DM. The etiopathogenesis of ECD is different in type 1 and type 2 DM. It may precede the onset of type 2 DM and some features of ECD may be seen in uncontrolled type 1 DM. The blood milieu in DM with chronic hyperglycemia, enhanced AGE production, oxidative stress, abnormal leucocytes and platelets, activation of renin-angiotensin system and enhanced inflammatory status with overactive cytokine network may all contribute to the progression of vasculopathy. The EC cells may be susceptible to cellular apoptosis as a result of chronic hyperglycemia and/or TRAIL activity.

The ECs attempt to protect themselves with increased expression of OPG to counter TRAIL action. However, in the event of overwhelming metabolic insults, the calcification inhibiting defence mechanism may be weakened and this may favour the promoters of VC, which in turn may initiate the process of VC. Vascular cell apoptosis may serve as nidus for matrix formation for VC initiation. However, this process may not be one of easily 'give way' but involve a continuous interplay of various inhibitors and promoters of VC. In addition to other factors and regulators, OPG plays a major role in modulating the response of EC and VSMC to the onslaught of VC promoters; right from the stage of ECD to MAC.

The enhanced atherosclerotic burden in DM coupled with stiffened elastic layer due to MAC may lead to reduced arterial lumen, seen in these patients. In the long run, decreased arterial lumen along with hyperglycemia, hypertension, increased arterial stiffness, impaired remodelling, oxidative stress, dyslipidemia, abnormal leucocytes and platelets may promote a hypoxic vascular milieu and promote ischemic injury in the cardiac muscle, leading to infarction and death.

An estimation of OPG levels in patients with DM may provide an insight into the status of mineral metabolism and vasculature in these patients. This may help to pick up patients who are at risk of VC and provide an opportunity for timely interventional therapy to slow and/or prevent the progression of VC in these patients. Vascular calcification is an important component of diabetic macrovasculopathy. The following chapter summarises the current understanding of various factors, which may potentially contribute to the pathogenesis and progression of micro and macrovasculopathy in patients with DM.

CHAPTER 5

AN OVERVIEW OF DIABETIC VASCULOPATHY

AN OVERVIEW OF DIABETIC VASCULOPATHY

5.1 Introduction

Diabetic Vasculopathy (DV) is a broad subject comprising microvascular complications of Diabetes Mellitus (DM) such as diabetic neuropathy, diabetic nephropathy (DN), diabetic retinopathy and macrovascular complications of DM such as cardiovascular disease (CVD), cerebrovascular disease and peripheral vascular disease (PWD). Microvascular and macrovascular complications are equally important because they are the major cause of mortality and morbidity in patients with DM.

DN is now the leading cause of end stage renal disease (ESRD) in the world. People with diabetes may form up to 40% of patients who require renal replacement therapy worldwide. The most important cause of mortality in patients with DM is CVD. Anaemia is an important modifiable risk factor for CVD and especially in patients with DM. A patient with DM is twice at risk of MI as compared to the nondiabetic population. In fact, so huge is the risk that patients with history of DM are considered to be equivalent to a nondiabetic patient with a history of MI. PWD is one of the most important causes of morbidity in patients with DM resulting in increased rates of amputation as compared to the nondiabetic general population.

The microvascular and macrovascular complications do not occur in isolation and often may co-exist. The presence of one may be indicative of the presence of the other and requires further investigation to assess the status of the other systems. The cost of DV is enormous and an increasing burden on the health care expenditure

worldwide. On top of this, the burgeoning population of people with DM coupled with limited resources for health care funding may cripple the health care system worldwide, especially so in the developing countries.

The key to this daunting problem is to devise ways to i) prevent the escalation of number of patients with DM, ii) develop early identification of patients at risk of vascular complications in diabetes, and iii) aggressively intervene to control modifiable factors such as glycemic status, diet, physical activity, cessation of smoking, blood pressure control, anaemia correction if present, and timely follow-ups and monitoring. At the base of all these interventions is to understand the pathogenesis of the various vascular complications to guide therapy and timely intervention. Hence an understanding of the mechanisms of various vascular complications in DM is of utmost importance to take a further step in the prevention of these complications.

The aim of this thesis is to contribute to this understanding. To achieve this, a series of clinical studies have been undertaken. The bases for these studies are set out below.

5.2 Normal Biological Variation

How reliable are our measurements of the potential modulators of diabetic vasculopathy? What is their normal biological variation?

I have previously alluded to the potential role of EPO, 1, 25-dihydroxyvitamin D and the OPG/RANKL system in the pathogenesis of diabetic vasculopathy. The normal biological variation of these and related modulators, such as PTH, is not known. In order to be able to interpret potential variations in the levels of these hormones across various pathological states, it is important to have an understanding of the extent to which they vary in normal subjects. This gives rise to the hypothesis that our measurements of the potential modulators of diabetic vasculopathy are sufficiently reliable to allow differences in mean values between groups to be interpreted with confidence.

5.3 Tubulointerstitial Dysfunction

Does tubulointerstitial dysfunction occur before the onset of microalbuminuria in patients with diabetes mellitus?

The etiopathogenesis of DN is complex. Although traditionally considered to be a glomerular disease, there are suggestions that the pathogenesis of DN may originate in the tubules. Recent studies have strengthened this view and support a major role for the tubules in the pathogenesis of DN. The renal tubulointerstitium may be damaged by hypoxia occurring as the result of chronic hyperglycemia. Chronic

hypoxia may result in injury and early apoptosis of tubular cells leading to reduction of total tubular and peritubular cells. This tubular injury may result in increased excretion of tubular injury markers such as NAG and RBP.

In presence of continuing hypoxic conditions, the remaining tubular cells may try to maintain their function of production and maintenance of levels of important hormones such as erythropoietin (EPO) and 1, 25-dihydroxyvitamin D by increasing production in the surviving cells. However, with decreasing number of tubular cells and continuing hypoxia, the surviving over-burdened cells may not be able to maintain the levels of EPO and 1, 25-dihydroxyvitamin D. This gives rise to the hypothesis that tubular dysfunction occurs before the onset of microalbuminuria and results in low levels of EPO and 1, 25-dihydroxyvitamin D.

5.4 Pleiotropic effects of Erythropoietin supplementation

Does erythropoietin therapy ameliorate tubular injury?

The renal peritubular cells are the primary sites of erythropoietin (EPO) synthesis. EPO is a trophic hormone with multidimensional properties. The actions EPO extend well beyond stimulation of erythropoiesis. EPO exerts its action on the target cells by modulation of the EPO receptor (EPOR) on the cells. EPOR has been identified in several tissues such as tubules, glomerular and endothelial cells. The presence of the EPOR receptors on these tissues indicates the likely sites of EPO action. This gives rise to the hypothesis that supplementation of EPO in patients, who are EPO deficient, as seen in patients with DN, may reduce tubular injury.

5.5 Vascular calcification and Osteopenia in Diabetes Mellitus

Are vascular calcification and osteopenia linked in patients with diabetes mellitus? What is their relationship to modulators of diabetic vasculopathy?

Vascular calcification is a complex process of deposition of calcium hydroxyapatite in the vasculature and has been associated with increasing age, atherosclerosis, increasing duration of diabetes and presence of chronic kidney disease. Endothelial dysfunction may lead to endothelial cell apoptosis in presence of adverse metabolic conditions. The apoptotic endothelial cell forms a nidus for vascular calcification. OPG is an important survival factor for endothelial cells. In event of adverse metabolic conditions and enhanced cytokine activation, the endothelial cells express increased amounts of OPG to thwart the process of vascular calcification.

Low bone mineral density (BMD) is now a recognized feature of type 1 DM, though the literature is inconsistent in patients with type 2. Chronic hyperglycemia may potentially modulate the bone metabolism by its effect on osteoblasts through osmotic and non-osmotic pathways. Longer duration of diabetes and poor metabolic control may have an adverse impact upon the bone metabolism as upon the prevalence of other complications.

This gives rise to three hypotheses that in type 2 DM i) the degree of vascular calcification and bone mineral density are inversely related, ii) both are related to other clinical indicators of microvascular disease such as the degree of proteinuria,

and the presence of neuropathy and iii) both are related to modulators of diabetic vasculopathy such as the OPG/RANKL axis.

5.6 Modulators of diabetic vasculopathy in chronic kidney disease

What is the relationship between modulators of diabetic vasculopathy and the degree of chronic kidney disease?

The kidney plays an important role in the metabolism of PTH and 1, 25-dihydroxyvitamin D. With enhanced tubular injury, as seen in progressive renal deterioration, there is decreased production of 1, 25-dihydroxyvitamin D, which results in reduced levels of calcium absorption from the gut. This in turn leads to increased levels of PTH secretion to act on the tubular cells to stimulate 1, 25-dihydroxyvitamin D production. However, with dwindling numbers of tubular cells, there is a progressive deterioration in the levels of 1, 25-dihydroxyvitamin D and a reactive increase in the PTH levels.

Diabetic nephropathy (DN) is a progressive condition and the modulators of OPG/RANKL such as PTH and 1, 25-dihydroxyvitamin D are altered early in patients with DN. All these modulators act in concert. The altered balance of PTH and 1, 25-dihydroxyvitamin D is progressive in DN. This may impact upon their modulatory action on OPG/RANKL giving rise to the hypothesis that the OPG/RANKL ratio is disturbed in progressive DN along with altered PTH and 1, 25-dihydroxyvitamin D levels.

5.7 Conclusions

In summary, the pathogenesis of Diabetic Vasculopathy is multifactorial and multilayered with contributions from several modulators. Microvascular and macrovascular complications may co-exist and presence of one may be indicative of the presence of the other. A review of the literature on Diabetic Vasculopathy (above) raises several questions, which have been listed below – they form the ideas that are examined in each of the 5 clinical studies that are reported in chapters 7 to 11.

- Chapter 7 explores the biological variation of modulators of diabetic vasculopathy such as OPG, RANKL, PTH and 1, 25-dihydroxyvitamin D and Erythropoietin.
- Chapter 8 examines two hypotheses i) tubular injury with higher excretion of tubular injury markers such as NAG and RBP occurs before the onset of microalbuminuria in DM and ii) low levels of erythropoietin and 1, 25-dihydroxyvitamin D occur before the onset of microalbuminuria.
- Chapter 9 examines the hypothesis that supplementation of erythropoietin in patients with erythropoietin deficient anaemia reduces tubular injury.
- Chapter 10 examines three hypotheses on the relationship of bone, blood and kidney in type 2 DM patients. i) the degree of vascular calcification and bone mineral density are inversely related, ii) both are related to other clinical

indicators of microvascular disease such as the degree of proteinuria, and the presence of neuropathy and iii) both are related to modulators of diabetic vasculopathy such as the OPG/RANKL axis.

- Chapter 11 examines the hypothesis that OPG/RANKL ratio is progressively disturbed with increasing severity of CKD.

SECTION II

CHAPTER 6

GENERAL METHODOLOGY

GENERAL METHODOLOGY

6.1 Introduction

All the reported studies in this thesis are pilot studies. A summary of the methods used is provided in each chapter; however a detailed general consideration of the methods used has been described in this chapter. All the studies described in this thesis were carried out in the clinic room of the Renal Research unit at Lister hospital; a 500 bedded District General Hospital in Stevenage, UK. The studies were carried out in joint collaboration with the department of Diabetes and Endocrinology in the East and North Herts NHS Trust. The routine laboratory measurements were carried out in the department of pathology at Lister Hospital.

6.2 Study Design and Sample size

The idea and design of each study reported in this thesis have been based on the respective hypothesis (mentioned in chapter 5). The design and plan for each study was borne out of discussions between the author and his supervisors. All the studies in the thesis are cross-sectional studies with a single point estimation of various variables in each subject, except estimation of biological variation study, in which blood samples were collected on the same day and time of the day over five weeks.

6.2.1 Biological Variation Study

Chapter 7 reports the biological variation of various potential modulators of diabetic vasculopathy. The diurnal variation of these modulators such as erythropoietin (EPO), parathyroid hormone (PTH), 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D is already known, however the normal biological variation in these parameters over a period of time is not known. A small change in levels of these modulators may bring about a significant effect on its target parameter. Until the biological variation of these modulators has been established in healthy individuals, it will be difficult to interpret the levels of such moieties found in patients with conditions such as DM and CKD. Since all the planned clinical studies were of a cross-sectional design with single point estimation, it became apparent that an understanding of the normal biological variation would be important in interpreting the levels of those modulators deployed in these studies. To carry out these assessments, the method previously described by Harris et al³²¹ was deployed.

In this method, serum samples were collected from healthy individuals, who were not known to have any current medical illness and not on any active medication. The serum sample for the present study was collected on the same day and approximately same time of the week over a period of 5 weeks. Along with median, minimum and maximum values for each parameter, the intra individual and inter individual analytical variation and coefficient of variation was calculated to assess the normal biological variation in each individual and between individuals.

6.2.2 Cross Sectional Studies

Chapters' 8-11 report cross sectional studies carried out in different forms and stages of diabetic vasculopathy. Each study is unique. The study criteria and organisation of study groups have been outlined in the respective chapters. A cross sectional design was chosen over a longitudinal one because of various issues with longitudinal studies such as comparatively longer duration of the study and high dropout rates. Cross sectional studies may not be helpful in determining causal relationships; however, it does provide a glimpse of the incident status of the biochemical environment and the various associated factors. The prospective clinical studies in this thesis required an assessment of current status of various biological systems. In this respect, cross sectional estimation of various parameters, in structured patient groups based on known clinical stages of the disease was deemed to be appropriate.

The concept and hypothesis for each study was novel and in absence of any similar previous study, the sample size for each study was agreed in consultation with a senior statistician at the University of Hertfordshire. The number of participants differed in each study. As the relationship between variables in the hypothesis had not been examined previously, there were no published statistical estimates upon which to base a power calculation. However, for a standard correlation, each study was powered at least 0.80 (alpha value of 0.05), to arrive at reasonable conclusion.

6.3 Research Protocol and Ethical Clearance

The research protocol for each study along with the patient information sheet, informed consent forms were based on the guidelines of the National Research Ethics Service guidelines and developed through active consultation with the supervisors. All the studies received ethical approval (including amendments) from the Hertfordshire Research Ethics Committee. In addition, the Research and Development department of the East and North Herts NHS Trust considered all the studies.

Each participant was given enough time to go through the patient information sheet and decide about their participation in the study with at least 24 hour gap between the patient actually receiving the information sheet and agreeing to their participation, in accordance with the good clinical practice guidelines. A written informed consent was obtained from each participant before any research related procedures were carried out. All the studies adhered to the declaration of Helsinki.

6.4 Patient Selection and Recruitment

The participants for the biological variation study were recruited from the medical staff at Lister hospital. For the cross sectional studies, the participants were recruited from different sources. Patients with diabetes were recruited from local General Practices in Hertfordshire. Patients with diabetic nephropathy and nondiabetic patients with renal disease were recruited from the renal outpatient clinic in Lister Hospital. Non-diabetic healthy controls for different studies were recruited from the hospital staff at

Lister Hospital. All the participants in the study were screened according to predefined inclusion and exclusion criteria for the respective study.

6.5 Sample Collection and Management

Part of the collected blood and urine samples collected by the author from the participants, were submitted to the laboratory for routine laboratory tests as soon as possible. The specialized assays such as urinary retinol binding protein (RBP), urinary N-acetyl- β -D-glucosaminidase (NAG), serum erythropoietin (EPO), parathyroid hormone (PTH), 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D, Osteoprotegerin (OPG), receptor activator of nuclear factor kappa- β -ligand (RANKL), N-linked telopeptide of collagen (Ntx) and Bone specific alkaline phosphatase (BAP) were performed in duplicate.

For those assays, which could not be analysed routinely, a portion of the blood and urine sample was allocated. The blood samples for specialized assays were centrifuged at 4000 rpm for 10 minutes to separate the serum from the cells. Several aliquots were made out of the serum and the remaining urine samples. These aliquots were labelled and packed in designated plastic bags and stored in -80⁰ C freezers till all the samples for that particular study was collected. The freezer had an inbuilt temperature display which was monitored on a regular basis to make sure that an average temperature of -80⁰ C was maintained.

6.6 Routine Laboratory Measurements

Full blood count measurement was performed on the ABX Pentra ® (Horiba Diagnostics, Northampton, UK) according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), gamma glutamyl transferase (GGT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin) were performed on the Olympus AU 2700 ® multi-analyzer (Olympus Diagnostics, Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access ® 2 immunoassay system (Beckman Coulter, (High Wycombe, UK).

6.7 Specialised Assays

The specialised assays for erythropoietin, NAG, RBP, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D, Osteoprotegerin and RANKL was not performed by the author. They were carried out by a senior biomedical scientist Mr. G. Sivakumar with assistance from the author. To do the specialized assays, an automated ELISA (enzyme linked immunosorbent assay) analyzer, was hired from Triturus ® Grifols, Cambridge, UK. The Triturus® is a fully automated, completely open system, bench top, fully automated enzyme immunoassay (EIA) analyzer. It is manufactured by Grifols, Spain and marketed in the UK by Grifols, UK. The system has been designed for use with ELISA and EIA assay systems. Using 96 well-plates, it can perform all steps of any microplate EIA such as sample dilution and dispensing, incubation, washing, reagent addition, absorbance reading and calculation and interpretation of

results. It is a completely open system with the ability to perform all steps of any micro-well EIA test, independent of the manufacturer of the assay.

Triturus® (Figure 6.1) has multi-test and multi-batch ability and can perform simultaneously up to 8 different tests per batch on a given sample or a group of samples. In addition, the machine can run up to 4 different batches simultaneously, with the provision of loading new work batches, while the other batches are being processed. It is a single unit connected to an external computer that coordinates operations and it also allows for a bi-directional host communication, and on-line connection for data management and reporting.



Figure 6.1 Triturus ELISA Machine

The reagents for the assays were obtained from the following manufacturers: Urinary retinol binding protein - Immunodiagnostik AG, Bensheim, Germany, N-acetyl- β -d-

glucosaminidase - PPR diagnostics Ltd. London, Erythropoietin - IBL, Hamburg, Germany, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D - Immunodiagnostic Systems Ltd, Boldon, UK. Osteoprotegerin (OPG), Receptor activator of nuclear factor kappa- β -ligand (RANKL), bone specific alkaline phosphatase (BAP) and N-linked telopeptide of collagen (Ntx), Oxford Biosystems Ltd. Oxon, UK. All analyses were performed as a single batch. These analytes were also measured in duplicate to exclude the occurrence of random analytical sample.

The specialised assays used in each chapter are as follows. Chapter 7 - EPO, PTH, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D, chapter 8 - EPO, PTH, 25 hydroxyvitamin D, 1, 25 hydroxyvitamin D, NAG and RBP, chapter 9 - PTH, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D, OPG, RANKL, BAP and Ntx, chapter 10 – OPG, RANKL and PTH, chapter 11 – NAG and RBP.

6.8 Precision

The following within-run analytical coefficients of variation ranges were obtained for the respective concentration ranges; RBP (range 0.01 – 0.94 mg/l, CV 0.6-8.3%), EPO (range 0.07 – 47.8 mIU/ml, CV 1.9-6.7%), 25-dihydroxyvitamin D (range 29.7 – 99.0 nmol/l, CV 3.5-11.2%), 1, 25-dihydroxyvitamin D (range 15.4 – 89.3 pmol/l, CV 0.4-10.2%) and NAG (range 609 – 1459 umol/h, CV 0.2 – 6.8%), OPG (range 0.81 – 6.64 pmol/l, CV 0.2-9.4%), RANKL (range 0.5 – 9.17 pmol/l, CV 0.6-10.5%) and Ntx (range 35.0 – 342 nm BCE, CV 1.4-8.5%).

6.9 Data Collection and Management

A detailed medical history was obtained from all the participants according to the protocol. The medical history included recording of full name, age, gender, race, type of diabetes and duration of diabetes (if applicable), lifestyle (history of smoking, alcohol and exercise), family history of any medical illness, duration and treatment of any medical condition, if present and finally all concomitant medication. In addition, specific medical information pertaining to that particular study was sought to corroborate with the laboratory findings. All the information was initially captured by the author and the research nurse on a case report form prepared for each study.

The laboratory reports for the routine assessments were collected as and when they arrived and stored in a secure place. The results from the specialised assays were initially collected and scrutinised on the attached computer of the ELISA Analyzer. After careful inspection and confirmation of the results by a consultant chemical pathologist, the results were downloaded and entered on a excel spreadsheet. On completion of the study, the data obtained from medical history, routine laboratory measurements and specialised assays were transferred on a large excel data spreadsheet for that particular study and stored in a memory stick with a backup copy on a personal laptop. The data was then checked for any missing values and errors and then formatted in accordance with SPSS requirements for statistical analysis.

6. 10 Statistics and Sample Size

The sample sizes for each of the studies were estimated in collaboration with one of my supervisors, Dr. David Wellsted, a senior statistician. Most of the studies undertaken were pilot studies, in areas with little available data upon which to base the power calculations.

The statistical methods employed in all the studies were based on the distribution of the data and accordingly parametric and non-parametric tests as appropriate were employed. The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA) by the author, under the guidance of supervisors. The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median values, when the distribution was not normal. Comparisons between groups were carried out using the Kruskal-Wallis and Mann-Whitney U-tests for non-normally distributed data and One Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc testing when data was normally distributed.

Differences between the groups with respect to the distribution of categorical variables were examined using the Chi-squared test. In all statistical tests a p-value of ≤ 0.05 was considered to be statistically significant. Multiple linear regression and logistic regression was employed, where necessary, to examine the ability of an independent variable to be a predictive factor for a dependent variable. Various associated factors which demonstrated significant correlations with the dependent

variable on univariate analysis, were put together as independent variables in the logistic regression model.

6.11 Data Analysis and Reporting

The data was analysed initially by the author with the above mentioned statistical methods and conclusions were drawn from the results. The whole data and the statistical analysis was critically scrutinised by the supervisors to pick up any errors in the statistical methods and the tests applied to detect the differences across and/or the groups. The write up of the findings for each study was done in the format of introduction, research methods, statistical analysis, results, discussion and conclusions by the author and critically edited by the supervisors.

The following section discusses the various prospective studies and their findings have been reported in chapters 7-11.

SECTION III

CLINICAL STUDIES IN DIABETIC VASCULOPATHY

CHAPTER 7

BIOLOGICAL VARIATION OF MEDIATORS OF DIABETIC VASCULOPATHY

BIOLOGICAL VARIATION OF MEDIATORS OF DIABETIC VASCULOPATHY

7.1 Introduction

The pathogenesis of diabetic vasculopathy (DV) is complex with contributions from several systems. The blood milieu, bone metabolism and the kidney are intricately linked with several common modulators that orchestrate events in these systems. Tubular hormones such as erythropoietin (EPO) and 1, 25-dihydroxyvitamin D are important modulators in the kidney and blood milieu. Both hormones have pleiotropic effects as well. EPO receptors have been located on vascular endothelial cells, apart from the tubules and glomerular tissue (see chapter 2), suggesting a role for EPO action on these tissues. Similarly, along with the known role in mineral metabolism, receptors for 1, 25-dihydroxyvitamin D have been identified on endothelial cells³²² suggesting their sphere of influence.

Low serum levels of EPO and 1, 25-dihydroxyvitamin D may adversely impact erythropoiesis and calcium metabolism, respectively. In addition, low levels of these hormones may impact upon their pleiotropic effects as well. It is known that EPO deficiency results in anaemia and low 1, 25-dihydroxyvitamin D may lead to disturbed mineral metabolism by reduction in serum calcium levels. However, the impact of low levels of these hormones on their pleiotropic effects is not known.

Parathyroid hormone (PTH) is one of the major regulators of mineral metabolism, which exerts its action on the bone and the kidney to maintain the homeostasis of serum calcium and phosphate levels in the blood milieu. Disturbances of mineral

metabolism may play an important role in the manifestation of DV. Along with 1, 25-dihydroxyvitamin D and EPO, PTH may potentially play an important role in the bone, blood and kidney milieu and contribute to the pathogenesis of DV.

Although there is information available on the diurnal variation, there is little information on the biological variation of these modulators, over a period of time, in the healthy population. Data on biological variation forms part of the comprehensive evaluation required for analytes that are measured in the clinical laboratory and is an essential prerequisite to the introduction of new analytes.

³²³ Biological variation data have several important clinical and laboratory applications that include: setting analytical quality specifications, evaluating the significance of changes in serial results (the reference change value or “critical difference”, assessing the utility of population-based reference intervals, and calculating the number of specimens required to estimate the homeostatic set point.³²⁴

A comprehensive biological variation database of all known analytes that is frequently updated contains more than 300 analytes and references more than 200 Publications and serves as a useful reference for many clinical laboratories.

³²⁵ Interestingly, no data are available for the biological variation of PTH, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D and EPO as opposed to many other more esoteric tests. Without information on biological variation in the healthy population, it becomes difficult to objectively assess the effect and importance of these parameters in patients with and without diabetes mellitus (DM).

Estimation of PTH, EPO, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D is not done routinely, except in certain clinical conditions, where their estimation is warranted. Since it was intended to study these parameters in the subsequent studies, it was important to have an idea of analytical variance and coefficient of variance of these assays to address the hypothesis- How reliable are our measurements of the potential modulators of diabetic vasculopathy and what is their normal biological variation? An investigation of biological variation of these parameters in the healthy population was carried out to gain a better insight and understanding and help us to interpret these values in various patient groups.

7.2 Research Design and Methods

7.2.1 Subjects

A total of 20 apparently healthy individuals with 10 men and 10 women; median age 37 years (range 19-60 years) participated in the study. All subjects provided written consent prior to the study, which had been approved by the local Research and Ethics Committee. Table 7.1 shows the characteristics and routine biochemistry investigations for this group. None of the subjects were taking any prescription medication, had any illnesses in the recent past (previous 3 months) or during the study that required at least general practitioner consultation and none had a history of current or previous renal impairment or pathology-related bone and/or mineral ion metabolism.

No abnormalities in the relevant routine biochemistry investigations were detected for any of the subjects. Venous blood was collected into 5-mL tripotassium EDTA tubes (Vacuette, Greiner Bio-One, Frickenhausen, Germany), taking care to ensure that the sample collection vessel was completely filled. Samples were collected between 08:45 and 09:30 h on the same day of the week, weekly for 5 weeks as described by Fraser and Harris.³²⁶ All the samples were collected by the author. The samples were centrifuged at 4000 rpm to separate the plasma, which was immediately stored at -80⁰C.

7.3 Laboratory Investigations

Routine biochemistry investigations were performed on an Olympus AU 2700 analyzer (Olympus, Watford, UK) according to the manufacturer's specifications. PTH analyses were performed on the Beckman Access 2 immunoassay system (Beckman Coulter, High Wycombe, UK). This is a two-site immunoenzymatic assay with chemiluminescence as the method of detection. Prior to analysis, all of the serum samples were thawed, thoroughly mixed, and then centrifuged at 2500 rpm for 5 min. The samples were analysed once before being randomized, and then reanalysed. One analyst performed all the analyses, using the same batches of reagent quality control material and calibrators.

7.4 Statistical Analysis

Biovariability data were analysed using Excel (Microsoft Corporation, Reading, UK). The analytical, within-subject (intra-individual) and between-subject (inter-individual) variances (SD_A^2 , SD_i^2 and SD_G^2) were calculated as described by Fraser and Harris.³²⁶ Using this technique, analytical variance (SD_A^2) were calculated as the difference between duplicate results for each specimen ($SD_A^2 = \Sigma d^2/2n$, where d is the difference between duplicates, and n is the number of paired results). The variance of the first set of results for each subject was used to calculate the average biological intra-individual variance (SD_i^2) by subtraction of SD_A^2 from the observed dispersion ($SD_i^2 + SD_A^2$). Subtraction of $SD_i^2 + SD_A^2$ from the overall variance of the set of first results yielded the between-subject variance (SD_G^2).

7.5 Results

The patient characteristics and baseline biochemistry has been displayed in Table 7.1. The mean age of males (39.7 ± 10.11 years) was not significantly different from females (33.9 ± 13.2 years). None of them suffered from any acute or chronic illness and were not on any medication.

Table 7.1 Patient characteristics and baseline biochemistry

Subject	Age years	Sex	Calcium mmol/l (2.2-2.65)	Creatinine umol/l (45-125)	Phosphate mmol/l (0.75-1.36)	Magnesium mmol/l (0.74-1.0)	Alk. Phos IU/L (30-115)
1	23	M	2.43	103	1.15	0.83	77
2	38	F	2.32	77	0.96	0.81	62
3	39	M	2.17	88	1.13	0.78	80
4	49	M	2.4	87	1.17	0.89	80
5	45	M	2.41	94	0.57	0.89	89
6	37	M	2.29	78	1.05	0.81	50
7	43	F	2.33	87	0.99	0.87	47
8	21	M	2.38	104	1.36	0.87	84
9	40	F	2.37	85	1.1	0.77	55
10	43	M	2.24	95	0.96	0.74	64
11	44	F	2.31	80	1.21	0.8	45
12	44	M	2.5	106	0.83	0.78	74
13	49	M	2.34	75	1.07	0.84	90
14	24	F	2.35	77	0.96	0.81	98
15	22	F	2.46	85	1.15	0.76	51
16	60	F	2.4	78	1.38	0.85	97
17	19	F	2.25	82	1.11	0.71	70
18	27	F	2.44	92	1.38	0.82	71
19	22	F	2.26	90	1.21	0.77	61
20	47	M	2.29	102	1.24	0.69	80

7.5.1 PTH levels

The median and range for PTH concentrations are shown in Figure 7.1. Of the total test variance, analytical variance contributed 0.4%, within-subject variance contributed 25.3% and between-subject variance contributed 74.3%. The analytical coefficient of variation (CV_A), within-subject coefficient of variation (CV_I) and between-subject coefficient of variation (CV_G) were 3.3%, 25.3% and 43.4%, respectively.

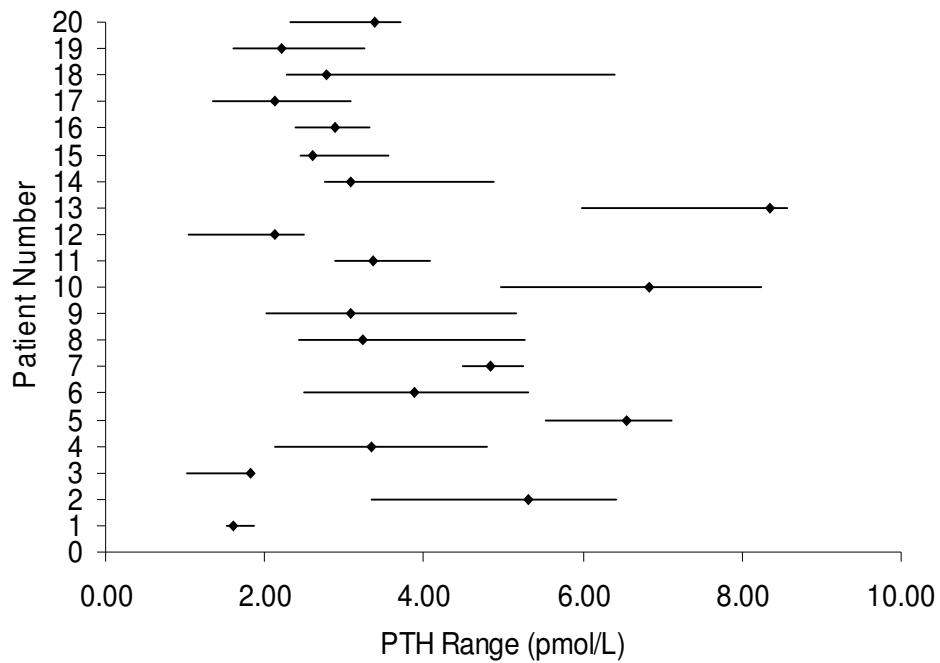


Figure 7.1 Biological variation of Parathyroid Hormone

7.5.2 25 hydroxyvitamin D levels

The median and range for 25 hydroxyvitamin D concentrations are shown in Figure 7.2. Of the total test variance, analytical variance contributed 3.1%, within-subject variance contributed 7.7% and between-subject variance contributed 89.2%. The analytical coefficient of variation (CV_A), within-subject coefficient of variation (CV_i) and between-subject coefficient of variation (CV_G) were 6.7%, 12.1% and 40.3%, respectively.

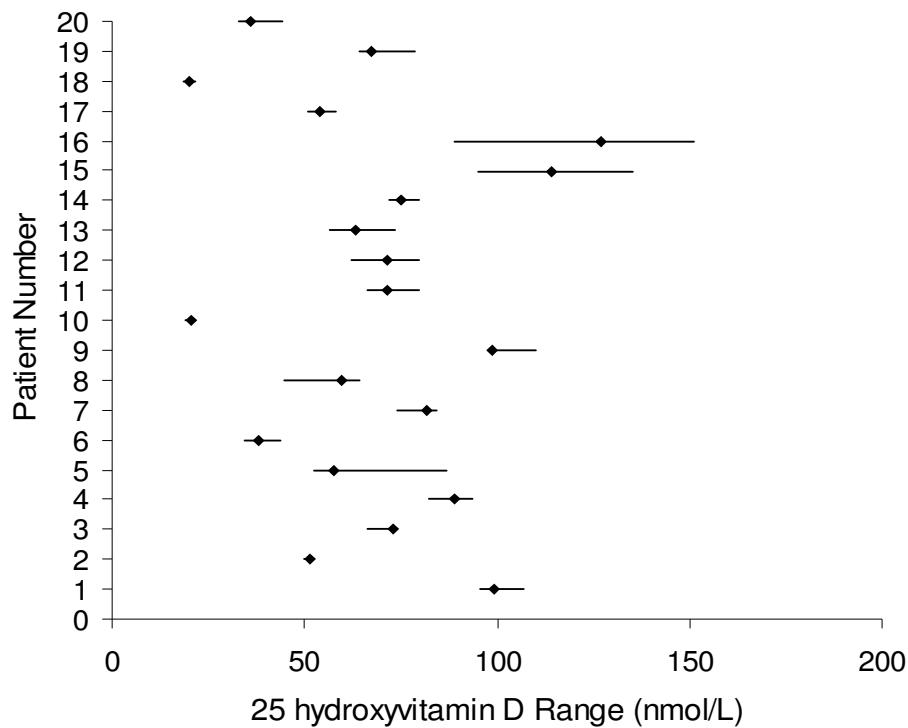


Figure 7. 2 Biological variation of 25 hydroxyvitamin D

7.5.3 1, 25-dihydroxyvitamin D levels

The median and range for 1, 25-dihydroxyvitamin D concentrations are shown in Figure 7.3. Of the total test variance, analytical variance contributed 1.9%, within-subject variance contributed 6.2% and between-subject variance contributed 91.9%. The analytical coefficient of variation (CV_A), within-subject coefficient of variation (CV_i) and between-subject coefficient of variation (CV_G) were 5.7%, 10.3% and 39.7%, respectively.

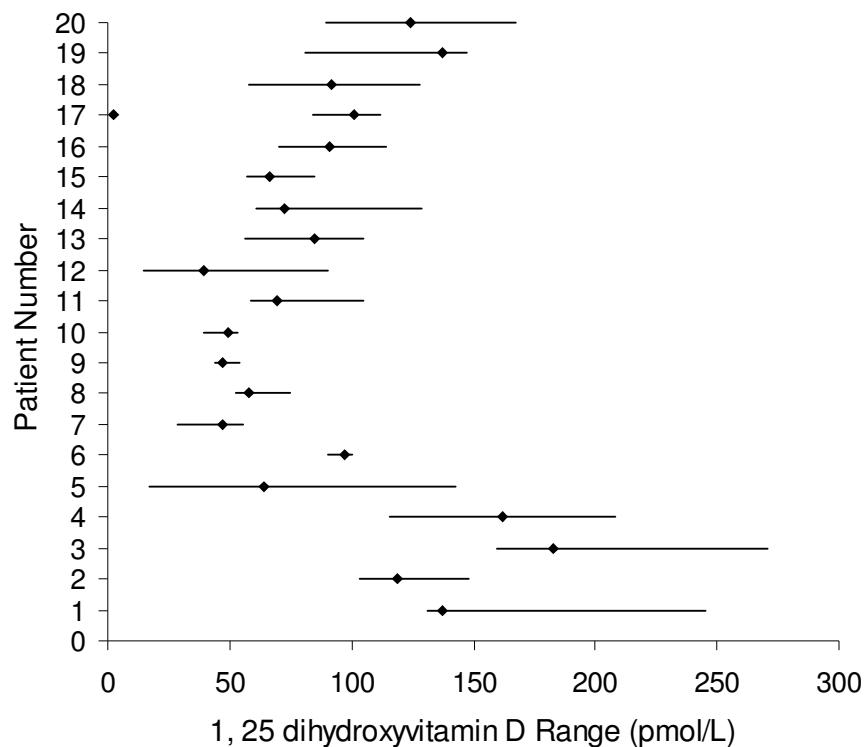


Figure 7.3 Biological variation of 1, 25-dihydroxyvitamin D

7.5.4 Erythropoietin levels

The median and range for EPO concentrations are shown in Figure 7.4. Of the total test variance, analytical variance contributed 2.3%, within-subject variance contributed 5.7% and between-subject variance contributed 92%. The analytical coefficient of variation (CV_A), within-subject coefficient of variation (CV_i) and between-subject coefficient of variation (CV_G) were 5.7%, 8.9% and 35.7%, respectively.

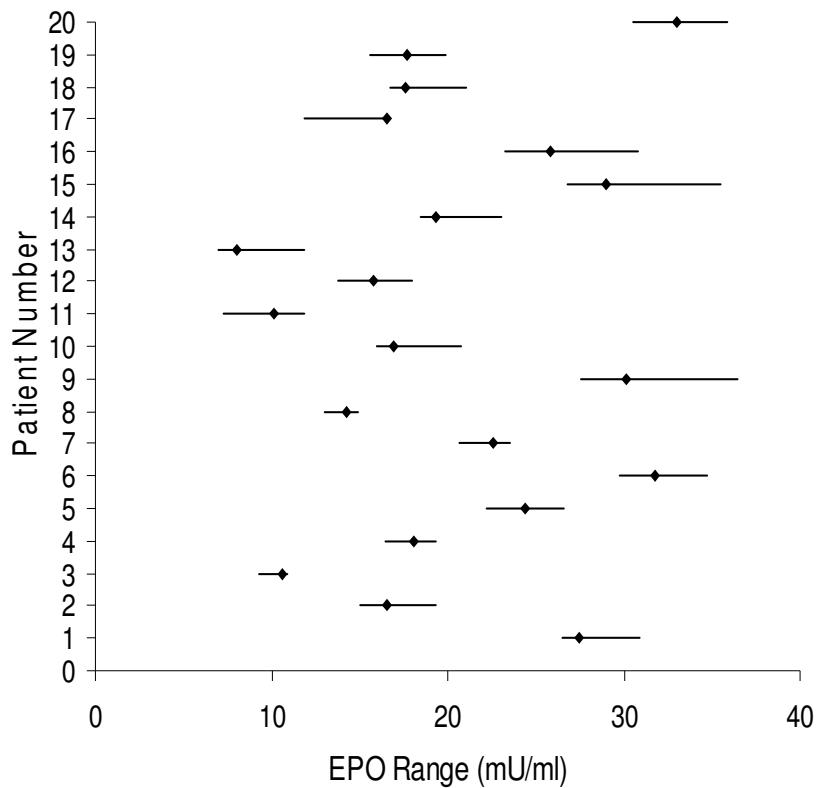


Figure 7. 4 Biological variation of Erythropoietin

Table 7. 2 Biological Variation of Modulators of Diabetic Vasculopathy

Variables	V _A	V _i	V _G	CV _A	CV _i	CV _G	Analytical Imprecision	CV _i /CV _G
PTH	0.4	25.3	74.3	3.3	25.3	43.4	optimum	0.58
25 OH D	3.1	7.7	89.2	6.7	12.1	40.3	minimal	0.30
1,25 (OH)₂D	1.9	6.2	91.2	5.7	10.3	39.7	minimal	0.26
EPO	2.3	5.7	92.0	5.7	8.9	35.7	minimal	0.25

Legend

PTH = Parathyroid hormone, 25 OH D = 25 hydroxyvitamin D, 1, 25 (OH)₂D = 1, 25-dihydroxyvitamin D, EPO = Erythropoietin, V_A = Analytical variance, V_i = Within subject variation, V_G = Between subject variation, CV_A = Analytical coefficient of variation, CV_i = Within subject coefficient of variation, CV_G = Between subject coefficient of variation.

7.6 Discussion

Analytical quality specifications may be based on components of biological variation. Desirable performance for analytical imprecision is given by CV_A < 0.5CV_i, optimal performance as CV_A < 0.25CV_i, and minimum performance as CV_A < 0.75CV_i. It can be seen by these criteria, that the analytical imprecision of the PTH assay was optimal whereas for other assays such as 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D and EPO, it was minimum.

Reference intervals are usually based on the mean and SD of a population sample, but these reference intervals become more useful for making decisions when CV_i is greater than CV_G. When CV_i/CV_G (Index of Individuality)³²¹ is low and particularly

when it is less than 0.6, the dispersion of values for any individual spans only a small part of the reference interval. Reference values in this situation are of limited use in deciding for instance whether a significant change has occurred. In contrast, when CV_i/CV_G is high, and particularly when it is greater than 1.4, then the range of values for an individual spans the greater part of the entire distribution of the reference interval.

In this setting reference intervals will be of significant benefit in interpreting individual results. For the analytes studied above (Table 7.2) – PTH, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D and EPO, the index of individuality was low. In the following studies we have therefore utilised control groups or comparison groups rather than reference ranges, to help to interpret measured values of these moieties.

7.7 Conclusions

There is a wide within-subject and between-subject biological variation in various modulators of bone metabolism and EPO in healthy individuals. These ‘normal’ variations may be altered in patients with DM and potentially contribute to the process of DV. In the current study, the analytical variance and coefficient of variance of PTH, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D and EPO assays were established. The wide variation in healthy individuals suggests a need for a control group to be incorporated in future studies in different patient groups, to establish a reference range and that any data on patients with chronic diseases such as CKD and DM should be treated cautiously. Whether, the so-called

differences from the baseline in various parameters is due to the disease process or part of the biological variation phenomenon may not always be clear.

The following chapter examines the status of the above-mentioned modulators of DV in type 1 and type 2 DM patients before the onset of microalbuminuria, as compared to non-diabetic controls.

CHAPTER 8

TUBULAR DYSFUNCTION IN EARLY DIABETIC NEPHROPATHY

TUBULAR DYSFUNCTION IN EARLY DIABETIC NEPHROPATHY

8.1 Introduction

Diabetic nephropathy (DN) is a major microvascular complication of diabetes, and is now the leading cause of end stage renal failure (ESRD) worldwide.³²⁷ Classically, DN has been described as a glomerular disease, with five different stages, starting with hyperfiltration, and progressing through a silent or incipient phase, through microalbuminuric and macroalbuminuric stages to a final stage of ESRD.⁵⁹ In contrast to this traditional understanding, histological studies have suggested a major pathogenetic role for the tubulointerstitium.^{68, 69} Altered tubular function, suggesting early tubulointerstitial injury has been reported in normoalbuminuric patients with Type 2 DM.¹³⁵

N-acetyl- β -D-glucosaminidase (NAG) and Retinol-binding protein (RBP) are sensitive markers of renal tubular injury.³²⁸ Early tubular dysfunction may be seen in diabetes subjects, before the onset of microalbuminuria. Increased urinary excretion of NAG has been reported in patients with type 1 DM without microalbuminuria.³²⁹ Normoalbuminuric type 2 DM patients have been reported to have increased excretion of RBP.³³⁰ These observations of tubular dysfunction in absence of microalbuminuria suggest early involvement of renal tubulo-interstitium in the genesis of DN.

The renal tubulointerstitium is the primary site of hydroxylation of 25-hydroxyvitamin D to 1, 25-dihydroxyvitamin D.³³¹ Also, EPO synthesis is primarily carried out in the

tubulointerstitial area by the peritubular fibroblasts.³³² Anaemia, due to low levels of EPO, develops early in patients with DN.¹⁰¹ Low EPO levels in these patients may result from damage to the EPO-producing fibroblasts.³³² Abnormalities in mineral metabolism have also been described in DM patients, early in the course of chronic kidney disease (CKD) and have been associated with reduced serum levels of 1, 25-dihydroxyvitamin D.¹²²

To address the question - Does tubulointerstitial dysfunction occur before the onset of microalbuminuria in patients with diabetes mellitus? We investigated the relationship between markers of tubular dysfunction (NAG and RBP) and tubulointerstitial hormone (EPO and 1, 25-dihydroxyvitamin D) synthesis in nondiabetic healthy controls and patients with type 1 and type 2 diabetes without persistent microalbuminuria.

8.2 Research Design and Methods

8.2.1 Subjects

Patients with type 1 and type 2 DM were recruited from local General Practices in East and North Hertfordshire, according to predefined inclusion and exclusion criteria. Inclusion criteria were age >18 years, normal estimated creatinine clearance (>90 ml/min) by Cockcroft-Gault formula,³³³ no evidence of microalbuminuria on screening, no evidence of documented peripheral neuropathy or retinopathy at screening. Exclusion criteria were history of renal disease, malignancy, current pregnancy,

current immunosuppressive therapy and regular use of non-steroidal anti-inflammatory drugs within the previous month.

Non-diabetic controls were mainly recruited from hospital staff at Lister Hospital. Controls were age and sex-matched with type 1 DM and sex matched with type 2 DM subjects. It was not possible to age match controls and type 1 DM with type 2 DM subjects. Neither the presence of hypertension nor the use of antihypertensive medications was a bar to recruitment either in the control or diabetic groups. The study was approved by the Hertfordshire research ethics committee. After screening the medical records, suitable patients were approached with the patient information sheet and explained about the study. The prospective study subjects, who agreed to participate, gave written informed consent and there were no drop-outs post consent. Forty-one non-diabetics, 40 Type 1 DM and 40 Type 2 DM, met the criteria (Total 121 subjects). The study was carried out in the summer months (July and August).

8.2.2 Data collection

A detailed medical history was recorded including, age, gender, race, type of diabetes, duration of diabetes, treatment for diabetes with insulin or oral hypoglycemics, history of hypertension, duration of hypertension, type of antihypertensive therapy including angiotensin converting enzyme inhibitors or angiotensin receptor blockers and finally all other concomitant medication. All patients with diabetes had no prior documentation of retinopathy or peripheral neuropathy in the GP surgery, at the time of screening.

8.3 Laboratory Investigations

A single overnight fasting blood sample was collected to measure baseline blood chemistry including fasting blood glucose (FBG), HbA_{1c}, full blood count, blood urea, serum ferritin, B₁₂ and folate levels, serum creatinine, serum albumin, C reactive protein, serum bilirubin, serum alanine transferase (ALT), serum alkaline phosphatase, serum parathyroid hormone (PTH), serum calcium, serum phosphate, and serum magnesium. Samples were also taken for serum erythropoietin, 25-hydroxyvitamin D and 1, 25-dihydroxyvitamin D estimation. A spot urine sample was collected to assess levels of urinary albumin excretion, urine phosphate levels, urinary creatinine, urinary magnesium, fractional excretion of magnesium, NAG and RBP excretion.

8.3.1 Laboratory Methodology

Full blood count measurement was performed on the ABX Pentra ® (Horiba Diagnostics, Northampton, UK) according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), gamma glutamyl transferase (GGT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin) were performed on the Olympus AU 2700 ® multi-analyzer (Olympus Diagnostics, Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access ® 2 immunoassay system (Beckman Coulter, (High Wycombe, UK)). Urine retinol binding protein (RBP), serum EPO, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D were

performed in duplicate on the automated ELISA (enzyme linked immunosorbent assay) analyzer, Triturus® (Grifols, Cambridge, UK).

The reagents were obtained from the following manufacturers: RBP (Immunodiagnostik AG, Bensheim, Germany), EPO (IBL, Hamburg, Germany), 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D (Immunodiagnostic Systems Ltd, Boldon, UK). Urine NAG was also measured on the Triturus® (Grifols, Cambridge, UK) employing colorimetric detection at 505 nm, using reagents supplied by PPR Diagnostics Ltd (London, UK).

8.4 Sample Size and Statistical Analysis

The primary objective of the study was to determine whether EPO level is inversely correlated with NAG and RBP levels in diabetic patients. As this relationship was not examined before, there were no published statistical estimates upon which to base a power calculation. However for a standard correlation, given an alpha value of 0.05, and study power of 0.90 a sample size of 40 patients would enable a correlation with effect size 0.45 to be reliably detected (a moderate effect size).

Secondary analyses would examine interrelations between diabetic (type 1 and 2) and non-diabetic patients, and the interrelation between the other outlined parameters using a multivariate analysis for all 120 patients. A total sample size of 120 patients would enable a multivariate analysis (e.g. ANCOVA, regression) using a reasonable number of independent variables, and would enable interactions between groups (diabetic and non-diabetic patients) to be examined.

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA). The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median values, when the distribution was not normal. Thus routine biochemical measurements have been reported as means, whereas, median values has been reported for EPO, 1, 25-dihydroxyvitamin D, NAG and RBP. Comparisons between groups were carried out using the Kruskal-Wallis and Mann-Whitney U-tests for non-normally distributed data and One Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc testing when data was normally distributed. Differences between the groups with respect to the distribution of categorical variables were examined using the Chi-squared test. P-values ≤ 0.05 were considered to be statistically significant.

8.5 Results

8.5.1 Demographics and clinical factors (Table 8.1)

There was no difference in the age of type 1 DM patients and controls. However, patients with type 2 DM were significantly older than other groups ($p < 0.001$ in both cases). There were also racial differences between the groups, the proportion of non-whites being lower in patients with Type 1 DM than in those with Type 2 disease ($p = 0.057$) and in controls ($p = 0.002$). There was no difference in the gender ratio across the groups. More patients with type 2 than with type 1 DM gave a history of ever smoking ($p = 0.042$), the difference with controls was not significant. The proportion of

current smokers did not differ significantly across the groups. Patients with type 2 DM were significantly heavier than those with type 1 disease ($p = 0.001$) and controls ($p < 0.001$). Systolic blood pressure was significantly higher in patients with type 2 DM (137 mm of Hg: $p = 0.002$) as compared to controls (124 mm of Hg). A greater proportion of patients with type 2 DM took ACEI or ARB than type 1 ($p = 0.041$) or controls ($p = 0.011$).

Table 8.1 Patient Characteristics and Demographics in Tubular study

Variables	Groups			Comparison of Groups		
	Controls (C)	Type 1 DM (T1)	Type 2 DM (T2)	C v T1 p-value	C v T2 p-value	T 1 v T 2 p-value
Number	41	40	40	NS	NS	NS
Age (yrs)	43.1 ± 9.5	42.4 ± 11.6	56.3 ± 9.0	NS	<0.001	<0.001
Gender (M:F)	20/21	20/20	21/19	NS	NS	NS
White/non-white	29/12	40/1	33/7	0.002	NS	0.057
DM Duration (yrs)	NA	19.9 ± 10.7	6.6 ± 3.7	<0.001	<0.001	<0.001
SBP (mmHg)	124 ± 15	132 ± 18	138 ± 20	NS	0.004	NS
DBP (mmHg)	85 ± 10	84 ± 10	90 ± 9	NS	NS	NS
BMI	26.4 ± 4.3	27.0 ± 4.3	33.4 ± 6.2	NS	<0.001	<0.001
Ever-Smoked (%)	39.0 (14.6)	32.5 (17.5)	58.5 (19.5)	NS	NS	0.042
ACEI/ARB (%)	7.3	30.0	56.1	0.011	<0.001	0.041

Legend

Demographic and clinical characteristics in control subjects and patients with type 1 and type 2 Diabetes. The p value represents the statistical significance of changes as determined by ANOVA test, BMI = Body Mass Index, ACEI = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor blocker, DM = diabetes mellitus, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, NS = non significant.

8.5.2 Baseline Biochemistry and Haematology (Table 8.2)

Patients with type 2 DM had significantly lower HbA_{1c} (7.6 ± 1.5 vs. $8.3 \pm 1.4\%$: $p = 0.022$) as compared to type 1 DM. There was no difference in renal function between the groups. Serum creatinine and eGFR by the MDRD-4³³⁴ formula did not differ significantly across the groups, though creatinine clearance calculated by the Cockcroft Gault method³³³ was slightly higher in type 2 DM when compared with controls ($p = 0.018$). Between the three groups, haemoglobin, serum calcium, phosphate and parathyroid hormone levels did not differ significantly. Serum magnesium levels were lower in type 1 DM (0.83 mmol/l: $p = 0.009$) and type 2 DM (0.82 mmol/l: $p < 0.001$) as compared to controls (0.88 mmol/l).

Serum ferritin and vitamin B₁₂ levels did not differ across the groups, but serum folate levels in patients with type 1 DM were significantly higher than in controls ($p = 0.004$). Serum alkaline phosphatase levels were higher in patients with type 1 DM than in those with type 2 DM ($p = 0.007$) and controls ($p = 0.001$), though ALT levels did not differ. In addition, serum albumin was lower in type 2 DM as compared to controls.

8.5.3 Serum Erythropoietin Levels

Median serum EPO levels were lower in type 1 (2.57 mIU/ml: $p < 0.001$) and type 2 DM (5.69 mIU/ml: $p = 0.044$) compared to controls (8.76 mIU/ml), (Figure 8.1), though there were no significant differences in haemoglobin levels between the groups. (Table 8.2) EPO levels did not correlate with age, sex and fasting blood glucose and

HbA1c, in any of the groups. There were no significant differences in EPO levels in patients on ACE/ARB therapy and those not on this treatment.

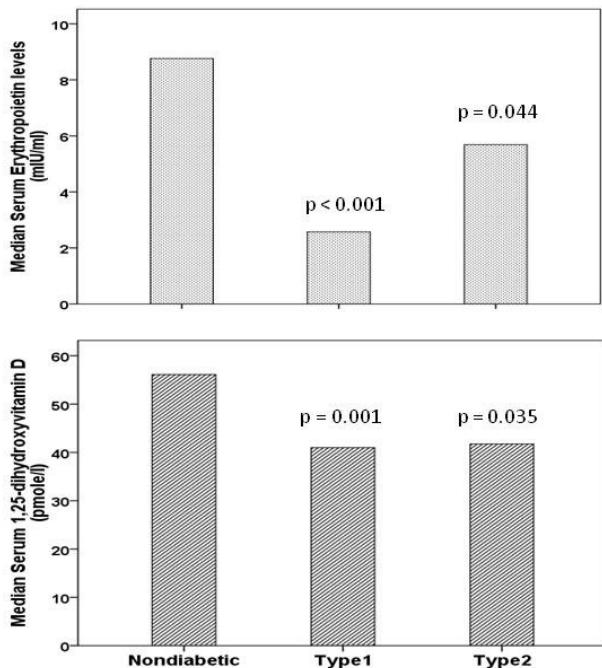


Figure 8.1 Erythropoietin and 1, 25-dihydroxyvitamin D levels

Legend

Median serum Erythropoietin and 1, 25-dihydroxyvitamin D levels in patients with type 1 and type 2 diabetes and in non-diabetic controls. The p values quoted reflect significance of differences from controls.

8.5.4 Serum Vitamin D levels

Median serum 25-hydroxyvitamin D levels in patients with type 2 DM (63.8 nmol/l) were lower than those in controls (69.1 nmol/l: $p = 0.034$). Median levels in patients with type 1 DM (65.5 nmol/l) did not differ from control values. Median 1, 25-dihydroxyvitamin D levels were lower in type 1 (41.0 pmol/l: $p = 0.001$) and type 2 DM (41.8 pmol/l: $p = 0.035$) compared to controls (56.1 pmol/l), (Figure 8.1) though the groups did not differ with respect to calcium, phosphate and PTH levels (Table 8.2). There was no effect of age, sex, race, smoking, BMI, ACEI and ARB therapy on 25-hydroxyvitamin D and 1, 25-dihydroxyvitamin D levels.

8.5.5 Tubular dysfunction markers (Table 8.2)

Median RBP excretion was significantly higher in patients with type 2 DM (0.35 mg/l: $p = 0.013$) as compared to controls (0.23 mg/l). In type 1 DM (0.27 mg/l) patients, the median RBP excretion, although higher than in controls, was not significantly different. Median RBP: creatinine ratio was significantly higher in patients with type 2 DM than in controls (0.027 vs. 0.018 mg/l/mmol: $p = 0.023$). Median NAG excretion was higher in type 1 (1079 umol/h: $p = 0.048$) as compared to controls (1030 umol/h). NAG: creatinine ratio did not differ significantly across the groups.

In all subjects, NAG: creatinine ratio correlated significantly with RBP: creatinine ratio ($r = 0.676$, $p < 0.001$), and with fractional excretion of magnesium ($r = 0.179$, $p = 0.05$). In the diabetes group, NAG: creatinine ratio correlated significantly only with RBP: creatinine ratio ($r = 0.676$, $p < 0.001$). Correlations were similar in the DM

groups singly and as a whole. There were no significant correlations between urinary NAG, NAG: creatinine ratio, RBP or RBP: creatinine ratio and EPO levels or 1, 25-dihydroxyvitamin D in the group as a whole or within individual subgroups.

In the whole group, urinary RBP: creatinine ratio correlated significantly with age ($r = 0.195$, $p < 0.032$), and urinary RBP: creatinine ratio was higher in smokers (0.027 v 0.018: $p = 0.021$), but sex, race, BMI and the use of ACEI or ARB therapy had no effect on this ratio. Neither age nor smoking, were significant determinants of urinary RBP: creatinine ratios in multivariate analysis. Sex, race, smoking, BMI and being on ACEI or ARB therapy had no effect on urinary RBP and NAG levels or on urinary NAG: creatinine ratios.

8.5.6 Urinary albumin levels

Although patients with diabetes were selected on the basis of having no microalbuminuria on screening (defined as albumin creatinine ratio (ACR) < 2.5 in males and < 3.5 mg/mmol in females on two or more consecutive tests, within a period of one to three months),³³⁵ when urinary ACR estimates were repeated as part of the study protocol, 13 subjects were found to have high ACR levels - 4 controls, 5 patients with type 1 DM, and 4 patients with type 2 DM. Mean ACR levels were minimal and did not differ between the groups (Non-diabetics 0.10 ± 0.30 , type 1 DM , 0.11 ± 0.33 , type 2 DM 0.10 ± 0.30 mg/mmol: $p = NS$). There was no correlation between urinary RBP, NAG, RBP: creatinine ratio, NAG: creatinine ratio and ACR levels. The exclusion of those patients with high ACR from the analysis, did not significantly impact upon the overall result.

Table 8.2 Biochemical and Haematological Parameters in Tubular study

Variables	Groups			Comparison of Groups		
	Controls (C)	Type 1 DM (T1)	Type 2 DM (T2)	C v T1 p-value	C v T 2 p-value	T 1 v T 2 p-value
Serum Parameters						
Glucose (mmol/l)	4.9 ± 0.6	8.6 ± 3.9	7.8 ± 2.7	<0.001	<0.001	NS
HbA1c (%)	5.5 ± 0.4	8.3 ± 1.4	7.6 ± 1.5	<0.001	<0.001	0.022
Creatinine (umol/l)	84 ± 10	82 ± 11	79 ± 14	NS	NS	NS
eGFR (MDRD 4)	80 ± 12	84 ± 10	84 ± 14	NS	NS	NS
Creat clearance (C-G)	100 ± 24	106 ± 18	115 ± 31	NS	0.018	NS
Urea (mmol/l)	4.9 ± 1.3	5.2 ± 1.3	5.3 ± 1.3	NS	NS	NS
Haemoglobin (g/dl)	14.1 ± 1.3	14.2 ± 1.2	14.0 ± 1.3	NS	NS	NS
CRP (mg/l)	1.8 ± 2.6	2.8 ± 4.5	3.2 ± 4.4	NS	NS	NS
Calcium (mmol/l)	2.29 ± 0.08	2.29 ± 0.07	2.31 ± 0.07	NS	NS	NS
Phosphate (mmol/l)	1.05 ± 0.16	1.1 ± 0.18	1.08 ± 0.15	NS	NS	NS
PTH (pmol/l)	4.4 ± 2.5	4.1 ± 1.8	4.0 ± 1.8	NS	NS	NS
Magnesium (mmol/l)	0.88 ± 0.05	0.84 ± 0.06	0.82 ± 0.08	0.009	<0.001	NS
Alkaline Phos (IU/l)	74.6 ± 23.9	94.2 ± 25.7	77.9 ± 20.7	0.001	NS	0.007
ALT (IU/l)	26.0 ± 12.3	33.7 ± 23.8	34.5 ± 15.6	NS	NS	NS
Albumin (g/l)	45.4 ± 2.5	44.6 ± 2.6	43.9 ± 2.7	NS	0.033	NS
Urinary Parameters						
Median NAG (umol/h)	1030 (670)	1079 (756)	989 (780)	0.048	NS	NS
Median NAG: creat. ratio	67.9 (848)	83.1 (373)	93.5 (411)	NS	NS	NS
Median RBP (mg/l)	0.23 (0.73)	0.27 (0.85)	0.35 (0.93)	NS	0.013	NS
Median RBP: creat. ratio	0.02 (0.41)	0.02 (0.15)	0.03 (0.25)	NS	0.023	NS

Legend

Baseline biochemical and haematological parameters in control subjects and patients with type 1 and type 2 Diabetes. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (range). The p value represents the statistical significance of changes as determined by ANOVA test. ALT = alanine transaminase, Alkaline phos = Alkaline phosphatase, CRP = C - reactive protein, PTH = parathyroid hormone. eGFR (ml/min/1.73m²) = estimated glomerular filtration rate calculated using MDRD 4 formula. Creat Clearance (ml/min) = Creatinine Clearance estimated by Cockcroft-Gault method (C-G). NAG = N-acetyl-β-D-glucosaminidase, RBP = Retinol binding protein, NAG: creat. ratio = N-acetyl-β-D-glucosaminidase: creatinine ratio, RBP: creatinine ratio = Retinol binding protein: creatinine ratio.

8.6 Discussion

The major findings in this study are that both type 1 and type 2 DM patients have low EPO and 1, 25-dihydroxyvitamin D levels with normal estimated creatinine clearance and prior to the onset of persistent microalbuminuria. These subjects with diabetes also had evidence of early tubular dysfunction as reflected by higher excretion of NAG in Type 1 DM and RBP in Type 2 DM patients.

Though serum EPO levels were lower in type 1 and type 2 DM patients compared to controls, there were no significant differences in hemoglobin levels between the groups. Low EPO levels with normal haemoglobin have been reported in normoalbuminuric^{139, 336} and microalbuminuric Type 2 DM patients.³³⁷ However, the previous studies in normoalbuminuric Type 2 DM were limited by non-characterization of patient groups in terms of GFR and albuminuria¹³⁹ and/or absence of a control group,³³⁶ and lack of data on haematinic levels.^{139, 336} Also, the previous studies did not include patients with Type 1 DM.

Erythropoietin stimulates erythropoiesis and has other pleiotropic properties. Erythropoietin levels were not associated with low haemoglobin levels perhaps because the levels in the diabetes groups remained above the threshold required to maintain normal hemoglobin. It may be, though, that these low levels of EPO could potentially influence its pleiotropic properties in some individuals. In agreement with previous observations,³³⁶ Erythropoietin levels did not differ significantly in patients who were on ACE-I/ARB therapy compared to those who were not.

Low 25-hydroxyvitamin D levels have been reported and implicated in the pathogenesis of type 1³³⁸ and type 2 DM³³⁹. This is the first study to report low levels of 1, 25-dihydroxyvitamin D in type 1 and type 2 DM before the onset of persistent microalbuminuria. It is noteworthy that these changes occurred in the absence of changes in calcium, phosphate, or parathyroid hormone levels, though magnesium levels were lower in both DM groups. Magnesium deficiency has been associated with low serum levels of 1, 25-dihydroxyvitamin D and impaired secretion of parathyroid hormone.³⁴⁰

The observations of higher excretion of tubular injury markers are in agreement with previous studies which have shown increased excretion of NAG in type 1 DM³²⁹ and RBP in type 2 DM.³³⁰ The differences in RBP and NAG excretion in type 1 and type 2 DM may reflect different pathogenetic factors operating in these conditions, and merit further study. As discussed above, the reductions in EPO and 1, 25-dihydroxyvitamin D levels were more marked in patients with type 1 DM, whilst the evidence for tubular dysfunction was stronger in type 2 patients. This dissociation also merits further attention.

Microalbuminuria has been considered to be the first significant marker of DN. Up to 30% of patients with Type 2 DM may have microalbuminuria or proteinuria at diagnosis.³⁴¹ Early microalbuminuria may result from both glomerular and proximal tubular dysfunction.⁷⁶ In DM patients with normal renal function, increased urinary excretion of NAG and RBP may indicate proximal tubular injury and potentially help in identifying patients at high risk of developing DN.³²⁹

Due to a chronic hypoxic milieu as a result of sustained hyperglycemia, the renal tubule may be damaged prior to the glomerulus in early DN.³⁴² Early loss of tubular and peritubular cells could impair the synthesis of 1, 25-dihydroxyvitamin D and EPO which, together with dysfunction of their receptors caused by the diabetic state, and other mechanisms, such as early removal of EPO from circulation as a result of abnormal glycosylation,¹³⁹ might diminish the local trophic effects of these hormones. This could potentially result in further compromise to the functional and structural integrity of the renal parenchyma, and contribute to the gradual decline of renal function. The findings of the current study are compatible with this hypothesis.

Although the excretion of NAG and RBP was elevated in patients with diabetes and the serum levels of EPO and 1, 25-dihydroxyvitamin D were low, there were no significant correlations between the markers of tubular injury and those reflecting diminished functional capacity. This lack of correlation may be accounted for by 1) the different cellular locations of the hormone function (EPO synthesis and 1 α hydroxylation of 25-hydroxyvitamin D) and the markers of tubular injury 2) a dissociation between the mechanisms of tubular and peritubular injury 3) lack of sensitivity of urinary NAG and RBP excretion as markers of tubular damage.

The present study has a number of limitations. These include small patient numbers and incomplete matching with respect to age and duration of both the types of diabetes. In addition, although the diabetes groups were selected on the basis of having been screened negative for microalbuminuria, when, during the course of the study, microalbuminuria was retested on the basis of ACR in the spot urine sample, similar numbers in all groups (4-5 patients, 10-12.5%) were found to be in

microalbuminuric range. The diagnosis of microalbuminuria requires ACR levels exceeding threshold values on 2 or more consecutive occasions, ideally within one to three months.³⁴³

The finding of 10 -12.5% of patients in the microalbuminuric range, based on a single ACR value, may reflect a natural variability in urinary albumin excretion these patients or may indicate evolving microalbuminuria. Interestingly, the prevalence of microalbuminuria, based on a single ACR sample, in the control group was also 10%. This was lower compared with a previous study, which reported the prevalence of microalbuminuria in non-diabetic general population of up to 13%.³⁴⁴ NAG and RBP may not be the gold standard markers of tubular injury. However, NAG and RBP are most widely assessed tubular injury markers. It may be though that further studies in this area will need to employ a range of markers of tubular function, and include timed collections of urine to improve sensitivity.

8.7 Conclusions

Diminished functional ability of tubulointerstitium in DM patients with normal levels of serum creatinine and estimated GFR, in the absence of persistent microalbuminuria was observed, as reflected by reduced levels of EPO and 1, 25-dihydroxyvitamin D, in presence of significantly higher excretion of NAG and RBP. EPO and 1, 25-dihydroxyvitamin D levels in this setting may be useful markers for the diagnosis and monitoring of early DN. These findings are also compatible with the hypothesis that in the evolution of DN, significant tubulointerstitial damage may precede clinically

evident glomerular injury. Further studies with larger patient groups are required to confirm and extend these findings.

The following chapter examines the effect of EPO supplementation on tubular injury markers in diabetic and non-diabetic patients in different stages of chronic kidney disease.

CHAPTER 9

ERYTHROPOIETIN THERAPY AND TUBULAR INJURY MARKERS

ERYTHROPOIETIN THERAPY AND TUBULAR INJURY MARKERS

9.1 Introduction

Anaemia, as a result of erythropoietin (EPO) deficiency, usually develops early in the course of diabetic nephropathy (DN).¹⁰¹ The associated factors that may contribute to early anaemia in DN include shortened red cell survival, decreased erythropoietin production, blood loss because of defective platelet function, and impaired erythropoiesis secondary to inhibitors or toxic metabolites.³⁴⁵ Patients with diabetes mellitus (DM) have been reported to have significantly low serum EPO levels as compared to nondiabetic patients with chronic kidney disease (CKD).¹³⁹

EPO synthesis is primarily carried out in the tubulointerstitial area by the peritubular fibroblasts.³³² EPO is a trophic hormone, which exerts its haematopoietic effects by stimulating the proliferation of early erythroid precursors and the differentiation of later precursors of the erythroid lineage.¹⁴⁷ EPO has pleiotropic effects well beyond the maintenance of red blood cell mass.¹⁵⁵ It has important cytoprotective effects, including protection from ischemic injury, inhibition of apoptotic death-related pathways.^{156, 157} EPO has been recognized to be a multifunctional cytokine that plays a key role in ischemic pre-conditioning in the brain and heart.¹⁵⁹ In animal models, EPO has been demonstrated to possess anti-oxidant and anti-inflammatory properties.¹⁶⁰

EPO exhibits its pleiotropic effects by the activation of functional EPO receptor (EPOR) on the tissue surface.¹⁶¹ EPORs have been demonstrated throughout the

kidney, including both proximal and distal tubular cells¹⁶² Also, EPO-producing fibroblast-like interstitial cells are in direct contact with the basal aspects of proximal and distal tubular cells.¹⁶³ However, the affinity of EPOR to EPO is well below the normal plasma EPO concentration, suggesting that these EPORs respond to the circulating EPO in a paracrine fashion.¹⁶⁴

Chronic hypoxia in the tubulointerstitium may be a trigger in the initiation of early tubular damage and nephropathy in DM. This tubular damage is evident in form of increased shedding of tubular injury markers such as N-acetyl-β-D-glucosaminidase (NAG) and Retinol-binding protein (RBP) in normoalbuminuric type 1³²⁹ and type 2 DM,³³⁰ respectively. The early proteinuria seen in DN is partly tubular in origin;⁷⁶ and reduction of early proteinuria may arrest the progression from micro- to macroalbuminuria in DN.³⁴⁶

Early EPO administration in predialysis patients has shown to slow the progression of CKD, with a significant reduction of the risk of initiating renal replacement or death.¹⁵⁰ Whether the renoprotective effects of EPO are due to correction of anaemia and/or due to pleiotropic properties, are not clear. The beneficial effect of recombinant human EPO in the treatment of anaemia in chronic renal failure has been established.³⁴⁷ In current clinical practice, the use of EPO is restricted to correct anaemia in patients with EPO deficiency anaemia; however, given the extent of presence of EPO receptors throughout the kidney, the use of EPO to target these tissues remains unexplored. The aim of this study was to examine the hypothesis that supplementation with erythropoietin ameliorates tubular injury.

9.2 Research Design and Methods

9.2.1 Patient recruitment

All the patients (DM and non-diabetics) were recruited from the renal clinic at Lister Hospital, Stevenage, UK. The patients were screened according to predefined inclusion and exclusion criteria. Inclusion criteria were age >18 years, DM or non-diabetic with history of CKD and clinical diagnosis of EPO deficiency anaemia. The exclusion criteria were immunosuppressive therapy, currently pregnancy, hormone replacement therapy and malignancy.

The study was approved by the Hertfordshire research ethics committee. After screening the medical records, suitable patients were approached with the patient information sheet and explained about the study. The prospective study subjects, who agreed to participate, gave written informed consent and there were no drop-outs post consent. The patients were divided into the DM and non-diabetic groups. There were 10 patients in the non-diabetic group and 9 patients in the DM group. In total there were 19 patients.

9.2.2 Data collection

A detailed medical history was recorded including, age, gender, type of diabetes, weight, height, blood pressure, recording of medical history including, duration of diabetes, treatment for diabetes, history of hypertension, duration of hypertension,

history of erectile dysfunction, type of antihypertensive therapy, duration of CKD in non-diabetics, dose of EPO at initiation and finally all other concomitant medications.

9.3 Laboratory Investigations

A single overnight fasting blood sample was collected to measure baseline blood chemistry including fasting blood glucose (FBG), HbA_{1c}, full blood count, blood urea, serum ferritin, B₁₂ and folate levels, serum creatinine, serum albumin, C reactive protein, serum bilirubin, serum alanine transferase (ALT), serum alkaline phosphatase, serum parathyroid hormone (PTH), serum calcium, serum phosphate, and serum magnesium. A spot urine sample was collected to assess levels of urinary albumin excretion, urinary phosphate, urinary creatinine, and urinary NAG and RBP excretion. The blood and urine samples were collected at EPO initiation and repeated after 3 months of EPO therapy to look at the difference in the various parameters.

9.3.1 Laboratory Methodology

Full blood count measurement was performed on the ABX Pentra ® (Horiba Diagnostics, Northampton, UK), according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin) were performed on the Olympus AU 2700 ® multi-analyzer (Olympus Diagnostics, Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access ® 2 immunoassay system (Beckman Coulter,

(High Wycombe, UK). RBP was performed in duplicate on the automated ELISA (enzyme linked immunosorbent assay) analyzer, Triturus® (Grifols, Cambridge, UK). Urine NAG was measured on the Triturus® (Grifols, Cambridge, UK) employing colorimetric detection at 505 nm, using reagents supplied by PPR Diagnostics Ltd (London, UK). All analyses were performed as a single batch. The within-run analytical coefficients of variation obtained for the respective assays have been reported in the general methodology chapter. These analytes were also measured in duplicate to exclude the occurrence of random analytical error.

9.4 Sample Size and Statistical Analysis

The primary aim of the study is to determine the extent to which NAG and RBP change in patients treated for EPO deficient anemia. Changes in these parameters from before to after treatment will be considered initially using an appropriate paired comparison (e.g. t-tests). Additional analyses will take into account other clinical and demographic factors using appropriate multivariate methods (e.g. ANCOVA or regression).

There is no currently published data examining changes in NAG and RBP after EPO supplementation, so a formal power model cannot be considered. However previous studies of changes in NAG given treatment for hypertension show that the changes in NAG are highly correlated (>0.7) and are large in comparison to the standard error of change ($D'>1$). A sample size of 20 patients will enable changes in NAG and RBP to be detected with power in excess of 90%. Given a 2 sided test, $\alpha=0.05$, and $n=20$, a

effect size of 0.77 can be detected with power of 90%, and an effect size of 0.66 with power of 80%.

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA). The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median values, when the distribution was not normal. Paired t test was employed to study the significant differences in various parameters before and after EPO therapy. P-values ≤ 0.05 were considered to be statistically significant.

9.5 Results

9.5.1 Demographics and clinical factors

The mean age of the whole group was 62.4 ± 13 . There was no difference in the age between the DM and non-diabetics. The proportion of females was 42% across the whole group. There was no difference in the systolic blood pressure; however, the diastolic blood pressure was significantly higher post EPO therapy (87 vs. 80 mm of hg: $p = 0.011$).

9.5.2 Pre and Post EPO therapy biochemistry (Table 9-1)

There were no differences in any parameter between diabetic and non-diabetic patients. The glycemic control in the diabetes group was not significantly different ($HbA1c - 7.24$ vs. 7.44% : $p = NS$), in the pre and post therapy period. Compared to

baseline, there was a significant rise in serum haemoglobin levels (11.9 vs. 9.9 g/dl: p < 0.001) post EPO therapy in the whole group. Serum bilirubin was significantly higher (7.9 vs. 6.7: p = 0.027) as compared to baseline. There was a significant reduction in ALT (24 vs. 19 IU/l: p = 0.001) post treatment. There was a marked increase in urinary phosphate excretion (13 vs. 8.4 p = 0.004). In addition, there was a significant rise in the serum phosphate levels 1.38 vs. 1.29: p = 0.049). There was no difference in urinary proteinuria and urinary creatinine. In addition, there were no differences in serum levels of PTH, folate, ferritin, HbA1c, transferrin, CRP, creatinine, urea, corrected calcium, magnesium, alkaline phosphatase and albumin.

9.5.3 NAG and RBP levels (Table 9.1)

The mean NAG levels was significantly higher post EPO therapy (924 vs. 848 umol/l: p = 0.002). However, there was no significant difference in the NAG: creatinine ratio in the pre and post therapy levels (181.8 vs. 157.8: p = NS). The RBP levels did not differ significantly in the pre and post EPO therapy period (2 vs. 1.6 mg/l: p = NS). Similarly, there were no significant differences in the pre and post therapy RBP: creatinine ratio (0.34 vs. 0.36: p = NS). In the baseline assessments, NAG correlated significantly with ACR ($r = 0.674$: p = 0.016) and serum urea ($r = 0.611$: p = 0.005). RBP correlated with serum folate ($r = 0.486$: p = 0.035) and BMI ($r = 0.529$: p = 0.020). In the post treatment assessment, NAG correlated significantly only with age ($r = 0.471$: p = 0.042) whereas, RBP correlated with only with HbA1c ($r = 0.537$: p = 0.047).

Table 9.1 Patient Characteristics and Biochemistry: Pre and Post EPO Therapy

Variables	Groups		
	Before Treatment	After Treatment	BT v AT p-value
Number	19	19	NS
Systolic BP (mmHg)	150 ± 23	154 ± 27	NS
Diastolic BP (mmHg)	80 ± 14	87 ± 16	0.011
BMI	28 ± 7.3	29 ± 7.5	NS
eGFR MDRD (ml/min)	17.5	16.8	NS
NAG (umol/h)	848 ± 78	924 ± 53	0.002
NAG:Creatinine Ratio	181.8 ± 99	157.8 ± 103	NS
RBP (mg/l)	1.6 ± 0.9	2 ± 1.04	NS
RBP:Creatinine Ratio	0.34 ± 0.22	0.36 ± 0.32	NS
Creatinine	316	331	NS
Urea	18.5	20	NS
ACR	22.5	23.6	NS
Haemoglobin (g/dl)	9.9	11.9	<0.001
Vitamin B 12	305	307	NS
Folate	483	568	NS
Urine phosphate	8.4	13.3	0.004
PTH (pmol/l)	27.8	23.9	NS
Calcium (mmol/l)	2.3	2.3	NS
Phosphate	1.29	1.38	0.49
Magnesium	0.91	0.93	NS
Alk Phos (IU/l)	112	118	NS
ALT (IU/l)	24.2	18.6	0.001
Albumin (g/l)	38.5	39	NS
Bilirubin	6.7	7.9	0.027

Legend

Clinical characteristics and biochemistry in chronic kidney disease patients, with and without diabetes. The p value represents the statistical significance of changes as determined by paired t test. BP = Blood pressure, BMI = Body mass index, eGFR (MDRD) = estimated glomerular filtration rate by Modification of Diet in Renal Disease Study Equation, NS = non significant. NAG = N-acetyl-β-D-glucosaminidase, RBP = Retinol binding protein, ACR = Albumin creatinine ratio, PTH = Parathyroid hormone, Co Calcium = Corrected Calcium. Alk. Phos = Alkaline Phosphatase, ALT = Alanine Transaminase.

9.6 Discussion

The principal finding in this study is that EPO therapy does not have any effect on the rate of excretion of NAG and RBP in CKD patients with advanced renal disease. In addition, we did not find any significant difference in pre and post EPO therapy excretion of NAG and RBP, in the diabetic and non-diabetic patients. As expected, treatment of anemic CKD patients with EPO therapy resulted in a significant rise in haemoglobin. In addition, there was a significant rise in proteinuria with progression of the disease.

In current clinical practice, a multi-pronged approach is applied in the treatment of patients with CKD. This incorporates identification and treatment of suspected risk factor/s and therapy for underlying associated diseases such as hypertension, dyslipidemia and/or diabetes, dietary intervention, correction of anaemia, therapy for disturbed mineral metabolism (hyperphosphatemia and hyperparathyroidism) and cessation of adverse personal habits such as smoking.

Anaemia is an early feature in patients with CKD, more so in those with DM.¹⁰¹ EPO therapy is the mainstay of treatment for EPO deficiency anaemia in patients with CKD. Several small experimental studies^{156, 157, 159, 160} have suggested pleiotropic properties of EPO apart from its known main function in stimulating erythropoiesis. These observations are further bolstered by the fact that EPORs have been demonstrated all over the kidney, including the tubular cells.¹⁶² The presence of EPORs on tissue other than those connected with erythropoiesis suggests the extension of EPO action on these tissues.

The above observations provided a valid ground for studying the effect of EPO supplementation on renal tubules, which are damaged in progressive CKD. NAG and RBP are known markers of tubulointerstitial injury and are widely employed to assess the damage to the renal tubules in renal diseases.³⁴⁸ Higher excretion of NAG and RBP reflects tubulointerstitial damage in patients with^{329, 330} or without diabetes.³⁴⁸

Atubular glomeruli with loss of renal tubule are a feature of progressive renal disease in non diabetics³⁴⁹ and type 1³⁵⁰ and type 2 DM.³⁵¹ The patients examined in our study were predominantly drawn from advanced renal disease (stage 4 and 5 CKD), which is characterised by a significant loss in the amount of functional nephrons. It is obvious that in absence of adequate EPORs, due to a significant drop in functional tubules, there was a limited scope for EPO action on the remaining tubules. This may be the primary reason for non-demonstration of any effect of EPO supplementation on the level of tubular injury markers in this study.

Progressive CKD is associated with a significant increase in inflammatory markers.³⁵² It is widely known that presence of inflammation blunts the responsiveness of EPO therapy in Anaemia.³⁵³ This could be another reason for lack of any effect of EPO therapy on EPORs on the remaining tubules. However, data from recent experimental studies^{354, 355} looks promising and suggestive of beneficial effects of EPO therapy in early/moderate renal disease as opposed to advanced renal disease, wherein there may not be adequate number of functional nephrons for pleiotropic action of EPO.

9.7 Conclusions

EPO therapy was significantly beneficial in correction of anaemia in the present study group. However, EPO supplementation did not show any of its suggestive pleiotropic actions, with no effect on NAG and RBP levels post therapy. In addition, we did not find any difference in the diabetic and non-diabetic groups. Primarily, this may be attributed to non-availability of adequate functional nephrons as a result of advanced renal disease. However, further support is derived from recent experimental studies suggesting the beneficial action of EPO in reno-protection. In this context, the effect of EPO supplementation on tubular injury should be studied in patients with early and moderate CKD with substantial number of functioning nephrons.

Micro and macrovascular complications of DM often co-exist together and the presence of one complication may be suggestive of presence of the other. The following chapter assesses the presence of vascular calcification and bone health in patients with type 2 DM patients with varying degrees of proteinuria and examines their relationship with the potential modulators of DV.

CHAPTER 10

VASCULAR CALCIFICATION AND BONE MINERAL DENSITY IN DIABETES

VASCULAR CALCIFICATION AND BONE MINERAL DENSITY IN DIABETES

10.1 Introduction

Vascular calcification (VC) is a strong independent predictor of cardiovascular mortality,³⁵⁶ the most common cause of death in patients with diabetes mellitus (DM).³⁵⁷ VC is widespread in patients with DM, coronary artery disease and peripheral vascular disease.³⁵⁸ VC is a complex process of bio-mineralization resembling osteogenesis and is enhanced in people with DM.³⁵⁹ Hyperglycemia induced apoptosis of vascular cells (endothelial and vascular smooth muscle cells)³⁶⁰ may provide a nidus for the initiation of the process of VC.³⁰² The process of initiation of VC is tightly regulated by an interaction of inhibitors and promoters of VC. In DM, hyperglycemia is a significant predictor of peripheral vascular calcification.³⁶¹ The manifestation of VC may be viewed as a failure of the inhibitory mechanism on the background of various metabolic and cytokine insults in DM.

Albuminuria is a strong predicting factor of VC and enhanced albuminuria has been associated with VC independently of peripheral neuropathy in DM.³⁶² Several studies have reported association between VC and autonomic denervation in the intima media of the small muscular arteries of the foot; with increased calcification following sympathectomy.^{363, 364} The severity of VC has been reported to have a significant correlation with the duration of DM.³⁶⁵

The burden of diabetic foot disease is enormous, with patients requiring regular input to prevent or treat foot ulceration, infection, vascular insufficiency and amputation.

The overall risk for foot amputation in patients with DM is 15 times greater than in non-diabetics.²²⁴ Peripheral vascular disease (PWD) is a leading cause of morbidity in DM,²²⁴ but the underlying mechanisms of VC in PWD are not clear. Multiple predictors and pathways have been proposed but major gaps still exist in our understanding.

The impact of DM and its complications on general bone health is not clearly established; although peripheral bone disease has been linked to both neuropathy and nephropathy. Type 1 DM patients with severe neuropathy have significantly less cortical bone mass in the hands and feet than non-diabetics.³⁶⁶ In type 2 diabetics, there are conflicting reports of reduced, normal or higher BMD as compared to controls by various groups.³⁶⁷ Patients with VC often have low bone mineral density (BMD). Whether osteoporosis and PWD are interrelated and linked by a common etiopathogenesis is ambiguous.³⁶⁸ It is not clear whether the osteopenia noted in neuropathic patients is causally related to neuropathy. Poor glycemic control is associated with reduced bone mass and improvement of glycaemic control has shown to have protective effects on bone in type 2 DM.³⁶⁹

Evidence supports the assumption that the Osteoprotegerin/Receptor activator of nuclear factor kappa B ligand (OPG/RANKL) complex cytokine network may be expressed, regulated, and function in vascular physiology and pathology to regulate vascular smooth muscle cell osteogenesis and calcification.³¹² RANKL is one of the major osteoclast maturation factors, whereas OPG functions as a soluble decoy receptor for RANKL and inhibits its effects.³¹⁶ Therefore at the bone level, OPG promotes bone formation, whereas RANKL promotes bone resorption.³¹⁸ In the

vessel wall, RANKL promotes calcification and OPG has a protective role against calcification.³¹⁸

The relationship between bone health, mineral metabolism, neurovascular function and OPG/RANKL system in the diabetic foot has not been examined before. Previous studies in this area have been carried out mostly in DM patients with advanced vascular complications such as subjects at the end stage of diabetic-renal-foot disease. The aim of this study is to examine three hypotheses in type 2 DM patients, i) the degree of vascular calcification and bone mineral density are inversely related, ii) both are related to other clinical indicators of microvascular disease such as the degree of proteinuria, and the presence of neuropathy and iii) both are related to modulators of diabetic vasculopathy such as the OPG/RANKL axis.

10.2 Research Design and Methods

10.2.1 Patient recruitment

The type 2 DM patients with varying degrees of proteinuria were recruited from local GP surgeries, diabetic and renal clinic in East and North Herts NHS Trust hospitals in Hertfordshire, UK. The patients were screened according to predefined inclusion and exclusion criteria. Inclusion criteria were age >18 years, type 2 DM, proteinuria less than 3 grams/day. The exclusion criteria were type 1 DM, history of current or recent urinary tract infection, history of chronic non-diabetic renal disease, serum creatinine in excess of 125 umol/L, active foot ulceration, immunosuppressive therapy, current pregnancy, hormone replacement therapy and malignancy.

The study was approved by the Hertfordshire research ethics committee. After screening the medical records, suitable patients were approached with the patient information sheet and explained about the study. Prospective subjects, who agreed to participate, gave written informed consent. There were no drop-outs post consent. Microalbuminuria was defined as albumin creatinine ratio (ACR) < 2.5 in males and < 3.5 mg/mmol in females on two or more consecutive tests, within a period of one to three months.³⁴³ Patients with no microalbuminuria were included in the normoalbuminuric group and patients with high ACR, were tested for urine dipstick protein and if found positive, were included in the proteinuric group. The patients were age-matched across the groups. After screening there were 65 patients who were examined.

10.2.2 Data collection

A detailed medical history was recorded including, age, gender, race, type of diabetes, duration of diabetes, treatment for diabetes with insulin or oral hypoglycemics, weight, height, history of foot ulcers, history of erectile dysfunction, postural hypotension, history of hypertension, duration of hypertension, type of antihypertensive therapy and recording of all concomitant medications.

10.2.3 Peripheral neuropathy

Peripheral neuropathy was assessed by two modalities and patients with abnormal sensation on any of the methods were labelled as having peripheral neuropathy.

The first method employed using a 10-gram Semmes-Weinstein monofilament examination. At the start of the assessment with this modality, the patient was given a reference sensation by application of the stimulus to the palm and then asked the nature of the sensation perceived if it was correct the patient was asked to perceive the sensation, with eyes closed, sequentially at different sites such as the plantar surface of the first toe and the 1st, 3rd and 5th metatarsal heads in each foot. In total the patient was asked to perceive the sensations at 8 sites. It was repeated four times on both feet in an arrhythmic manner and correct responses were scored on a scale of 0 to 8. A patient with a score below 8 was labeled as having peripheral neuropathy.

The second method entailed using the neurothesiometer to assess vibration perception thresholds to detect peripheral neuropathy as described before. In this procedure again, the patient was given a reference vibration sensation by application of the neurothesiometer knob to the palm and then asked the nature of the sensation perceived. The subject was asked to close the eyes and thereafter a neurothesiometer knob was placed on the plantar surface of the big toe. The patient was asked to prompt as soon as the vibration was perceived. The vibration mode was switched on and was controlled by a regulator. The voltage at which the patient perceived and prompted was labeled as the score for that particular foot and then repeated in the other feet. A vibration perception threshold from 0-14 was considered as normal and any score beyond that was considered as abnormal.

10.2.4 Radiology

Each patient underwent a Multidetector helical CT scan. The Scans were performed on a Siemens sensation 16 Multidetector scanner. The Images were obtained with the patient in the supine position. No intravenous contrast was used. A standardised section of the common and superficial femoral artery was imaged in 3mm slices for a length of 7.2cm from the superior margin of the hip joints. A similar section of the posterior tibial and dorsalis pedis arteries was imaged in 3mm slices for a length of 7.2cm from the level of the tibial dome with the Feet plantar flexed to lie flat on the scanning table. Any additional slices were excluded from evaluation. Where quantification was not possible due to contact between the calcified vessels and adjacent bone, a note was made regarding the same. The slices were not overlapped and each slice scored individually by the investigator using the calcification software in the Siemens Leonardo workstation as described by Agatston et al.³⁷⁰ Calcification was considered to be present if an area $\geq 1 \text{ mm}^2$ displayed a density >130 Hounsfield units (HU).

Plain radiographs of both feet were also performed in AP position. The presence of linear or curvilinear calcification in the expected anatomical location of dorsalis pedis and posterior tibial arteries were scored as 1 and 0 for presence and absence of calcification, respectively as suggested in a previous study.³⁷¹ Patchy isolated calcifications were excluded. Analysis of both radiographs and CT scans were performed by single experienced radiologist blinded to patient's clinical details.

10.2.5 Doppler assessment

Peripheral blood flow and assessment of plaque and patency were carried out using Doppler –ultrasonography (G.E Logiq 7 ultrasound machine and 10 MHz linear array probe). The procedure was carried out and reported by a trained vascular sonologist, blinded to the patient's medical history. Longitudinal and transverse B-mode (black and white mode) imaging of i) a 5cm segment of the arteries of the groin from the distal CFA, PFA and SFA ii) PTA at the level of the medial malleolus iii) DPA just below the level of the medial malleolus, on the anterior surface of the foot. Any plaques noted were measured in 3 dimensions and categorised as either soft, mixed (both soft and calcified) and calcified in nature. Colour and spectral Doppler imaging was also performed and peak systolic velocities (PSV) and pulsatility index (PI) recorded in the CFA, proximal PFA, proximal SFA, distal PTA at the level of the medial malleolus and DPA on the anterior portion of the foot.

10.2.6 DEXA Scan

To assess the bone mineral density, a dual energy absorptiometry (DEXA) scan was carried out of the spine, hip and heel by quantitative digital radiography (QDR) application by a senior radiographer. QDR employs emitting alternating high, 140kVp, and low, 100kVp, X-rays. For our procedure, third generation QDR densitometers (Hologic's bone densitometry systems), which employs multiple detectors and a dual energy X-ray fan-beam, was used. The radiation dose is approximately 1/100th of conventional X-ray dose. The results were reported as total BMD, Z- score (measure

of difference between the patient's BMD and that of healthy people of the same age and ethnicity) and T-score (measure of the difference between the patient's BMD and that of a young adult population of the same sex and ethnicity).

10.3 Laboratory Investigations

A single overnight fasting blood sample was collected to measure baseline blood chemistry including fasting blood glucose (FBG), HbA_{1c}, full blood count, blood urea, serum ferritin, B₁₂ and folate levels, serum creatinine, serum albumin, C reactive protein, serum bilirubin, serum alanine transferase (ALT), serum alkaline phosphatase, serum parathyroid hormone (PTH), serum calcium, serum phosphate, and serum magnesium. Samples were also taken for serum OPG, RANKL, bone specific alkaline phosphatase (BAP), 25-hydroxyvitamin D and 1, 25-dihydroxyvitamin D estimation. A spot urine sample was collected to assess levels of urinary albumin excretion, urine phosphate, creatinine, magnesium, Cross-linked N-telopeptide of type I collagen (Ntx) excretion. .

10.3.1 Laboratory Methodology

Full blood count measurement was performed on the ABX Pentra ® (Horiba Diagnostics, Northampton, UK) according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin) were performed on the Olympus AU 2700 ® multi-analyzer (Olympus Diagnostics,

Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access ® 2 immunoassay system (Beckman Coulter, (High Wycombe, UK).

Serum OPG, RANKL, BAP, 25 hydroxyvitamin D, 1, 25-dihydroxyvitamin D and urinary Ntx were performed in duplicate on the automated ELISA (enzyme linked immunosorbent assay) analyzer, Triturus ® (Grifols, Cambridge, UK). The reagents were obtained from the following manufacturers: OPG, RANKL, BAP, and Ntx (Oxford biosystems, Oxford, UK), 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D (Immunodiagnostic Systems Ltd, Boldon, UK). All analyses were performed as a single batch. The following within-run analytical coefficients of variation ranges were obtained for the respective concentration ranges These analytes were also measured in duplicate to exclude the occurrence of random analytical;

10.4 Sample Size and Statistical Analysis

Patients with type 2 diabetes were split into three groups of 20 patients: normoalbuminuria, microalbuminuria and overt proteinuria. Univariate analysis comparing levels of OPG and RANK-L with VC severity score and BMD T score would be conducted using correlations for each group. Since the data is likely to be skewed Spearman's rank correlation coefficient should be calculated. Sample sizes of 20 will allow for detection of correlation coefficients larger than .53 with 80% power and a one sided alpha level of .05

Multivariate analysis, using linear regression models, would be used to investigate the predictive ability of OPG/RANK-L with BMD and VC with the inclusion interactions between OPG/RANK-L and group indicator variables to allow for the examination of between group effects. A sample size of 20 in each group will allow for the significant detection of a change in the variance explained at around 19% with 80% power ($\alpha = .05$).

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA). The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median values, when the distribution was not normal. Comparisons between groups were carried out using the Kruskal-Wallis and Mann-Whitney U-tests for non-normally distributed data and Students t-test and One Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc testing as appropriate when data was normally distributed. Differences between the groups with respect to the distribution of categorical variables were examined using the Chi-squared test. Multivariate analysis was carried out using multiple linear regression and logistic regression as appropriate. P-values ≤ 0.05 were considered to be statistically significant.

10.5 Results

10.5.1 Demographics and clinical factors

The mean age of subjects was 62 ± 11 years. The majority (80%) of study subjects were Caucasian, the remainder were Asian or Afro-Caribbean. Females constituted almost one third (32%).

Table 10.1 Vascular Calcification: Demographics and Biochemistry

Variables	Groups			Comparison of Groups		
	NA	MA	P	NA v MA p-value	NA v P p-value	MA v P p-value
Age (years)	60 ± 12	64 ± 12	63 ± 11	NS	NS	NS
Gender (M:F)	14:11	16:4	14:6	NS	NS	NS
DM Duration (yrs)	6.6 ± 4.8	11.5 ± 7.7	13.3 ± 9.7	NS	0.011	NS
Ever Smoked (%)	48	60	75	NS	NS	NS
SBP (mmHg)	141 ± 18	139 ± 22	159 ± 25	NS	0.031	0.018
DBP (mmHg)	81 ± 10	75 ± 11	78 ± 12	NS	NS	NS
Weight (kg)	91.6 ± 16.6	95.1 ± 27.8	100.4 ± 29.7	NS	NS	NS
BMI	32.4 ± 4.9	32.9 ± 7.1	31.1 ± 10.4	NS	NS	NS
Neuropathy (%)	60	65	75	NS	NS	NS
Glucose (mmol/l)	8.7 ± 2.9	8.5 ± 3.1	10.4 ± 4	NS	NS	NS
HbA1c (%)	7.7 ± 1.5	7.8 ± 1.1	8.8 ± 2.3	NS	NS	NS
Creatinine (umol/l)	90 ± 14	97 ± 13	97 ± 16	NS	NS	NS
eGFR (MDRD 4)	72 ± 14	82 ± 19	69 ± 15	NS	NS	0.038
Creat clear.(C-G)	96 ± 33	91 ± 30	97 ± 42	NS	NS	NS
Urea (mmol/l)	5.6 ± 1.4	6.8 ± 2.5	6.6 ± 1.6	NS	NS	NS
Haemoglobin (g/dl)	14.7 ± 2.4	14.2 ± 1.9	13.3 ± 2.3	NS	NS	NS
CRP (mg/l)	1.0 (1,13)	1.0 (1,11)	1.0 (1,15)	NS	NS	NS
Calcium(mmol/l)	2.35 ± 0.1	2.34 ± 0.11	2.42 ± 0.07	NS	NS	NS
Phosphate (mmol/l)	1.07 ± 0.17	1.05 ± 0.16	1.08 ± 0.15	NS	NS	NS
Hb g/dl	14.7 ± 2.4	4.2 ± 1.9	13.3 ± 2.3	NS	NS	NS
Bilirubin	10.7±5.8	11.1±5.6	9.1±3.9	NS	NS	NS
Albumin g/l	44.9 ± 3	45.5 ± 2.7	42.6 ± 3.2	NS	0.039	0.009
BAP (IU/l)	19 ± 4.9	24.1 ± 8.2	22.5 ± 8.4	NS	NS	NS
Ntx (nm BCE)	305 ± 207	502 ± 359	409 ± 370	NS	NS	NS
OPG (pmol/l)	4.6 ± 1.3	4.6 ± 1.2	5.4± 1.5	NS	NS	NS
RANKL (pmol/l)	0.1 (0.03, 0.94)	0.1 (0.02, 0.32)	0.04 (0.02, 0.04)	NS	0.008	0.044
OPG/RANKL Ratio	54 ± 38	71 ± 70	135 ± 93	NS	0.001	0.013
25 OH D	52 ± 25	60 ± 35	67 ± 33	NS	NS	NS
1, 25 (OH) ₂ D	43.4 ± 15	43.9 ± 12	37.2 ± 11	NS	NS	NS
PTH (pmol/l)	4.5 ± 2.7	4.5 ± 3.2	4.3 ± 2.7	NS	NS	NS
Urinary ACR	0.6 (0, 19.4)	6.8 (2.7,26.8)	84.7(30,500)	<0.001	<0.001	<0.001

Legend

Baseline clinical and biochemical parameters in the three groups. Normally distributed parameters represented as mean \pm standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by ANOVA test, NA = Normoalbuminuria, MA = Microalbuminuria, P = Proteinuria, CRP = C - reactive protein, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, eGFR (ml/min/1.73m²) = estimated glomerular filtration rate calculated using MDRD 4 formula. Creat Clear. (ml/min) = Creatinine Clearance estimated by Cockcroft-Gault method (C-G), Hb – Haemoglobin, Ntx- N linked telopeptide of collagen, OPG = Osteoprotegerin, RANKL = Receptor activator of Nuclear factor kappa B ligand, 25 OH D = 25 hydroxyvitamin D, 1, 25 (OH)₂ D = 1, 25-dihydroxyvitamin D, PTH = Parathyroid hormone, ACR = urinary albumin to creatinine ratio.

10.5.2 Proteinuria and biochemical parameters (*Table 10.1*)

Based on ACR, the proportion of patients with normoalbuminuria (NA), microalbuminuria (MA) and proteinuria (P) was 38%, 31% and 31% respectively. There was no difference between the proteinuria groups with respect to age, sex distribution, ethnic mix, smoking history, or clinical evidence of peripheral neuropathy. Glycemic control as reflected by HbA_{1c} was similar across the groups. Serum creatinine and creatinine clearance calculated by the Cockcroft Gault method ³³³ did not differ significantly, though the mean eGFR by the MDRD-4 ³³⁴ formula was significantly lower in the P group as compared to MA group (p = 0.038).

Haemoglobin, serum calcium, phosphate and parathyroid hormone levels were similar but serum albumin was significantly lower in the P group (42.6 g/L) than the NA (44.9 g/L: p = 0.039) and MA (45.5 g/L: p = 0.009) groups. Serum ferritin, folate and vitamin

B_{12} levels did not differ across the groups, nor did serum luteinizing hormone, follicle stimulating hormone, Oestradiol, progesterone and testosterone. Mean 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D levels did not differ significantly across the three groups (Table 10.1), nor did mean BAP and Ntx levels (Table 10.1).

Mean OPG levels did not differ significantly across the NA, MA and P groups (Table 10.1). However it was significantly higher in the group with established proteinuria (5.4 vs. 4.6 pmol/l: $p = 0.027$) (Table 10.2) as compared to the non-proteinuric groups (Normoalbuminuric plus microalbuminuric). Median RANKL levels were significantly lower in the proteinuric group (0.04 pmol/l) as compared to normoalbuminuric (0.1 pmol/l: $p = 0.008$) and microalbuminuric groups (0.1 pmol: $p = 0.044$). The OPG/RANKL ratio was significantly higher in the proteinuric group compared to NA (135 vs. 54: $p = 0.001$) and MA (135 vs. 71: $p = 0.013$) groups (Table 10.1).

Table 10.2 Proteinuria versus No-proteinuria

Variable	No-Proteinuria	Proteinuria	p-value
OPG	4.6±1.2	5.4±1.5	0.027
OPG/RANKL	61.7±54	135.5±93	0.003
Albumin	45.1±2.8	42.6±3.2	0.002
Systolic BP	140±19	159±25	0.002

Legend

Baseline clinical and biochemical parameters in the proteinuria versus no-proteinuria groups. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by student t test, Systolic BP = Systolic blood pressure, OPG = Osteoprotegerin, RANKL = Receptor activator of Nuclear factor kappa B ligand.

10.5.3 Proteinuria: Calcification, BMD and Doppler assessments

The median maximal Agatston calcification scores in the femoral artery were 20, 185.5 and 310.5 in the normoalbuminuric, microalbuminuric and proteinuric groups respectively ($p=0.048$). The score in the proteinuric group was significantly higher than that in the normoalbuminuric group (Table 10.3). The proportion of patients with calcification in the foot on CT was 44%, 45% and 40% in the NA, MA and P groups respectively ($p=NS$). There were only minimal differences between proteinuric groups with respect to bone density and Doppler characteristics (Table 10.3). There were no significant differences in lumbar spine BMD, neck of femur BMD, heel BMD, across the NA, MA and P groups (Table 10.3). The proportion of patients with peripheral neuropathy was not significantly different across the three groups.

Table 10.3 Radiology, Ultrasonographic and BMD assessments

Variables	Groups			Comparison of Groups		
	NA	MA	P	NA v MA p-value	NA v P p-value	MA v P p-value
Max Fem Cal (AS)	20 (0, 5999)	185.5 (0, 3073)	310 (0, 5486)	NS	0.013	NS
Max Foot Cal (AS)	0 (0, 700)	0 (0, 700)	0 (0, 700)	NS	NS	NS
Foot X ray Cal (%)	36	30	45	NS	NS	NS
Max CFVI	88 ± 23	90 ± 21	94 ± 31	NS	NS	NS
Max CFPI	9.5 (2.1, 139)	8.8 (4.5, 46)	11.4 (6, 155)	NS	NS	NS
Max PFVI	64.2 ± 30	65 ± 15	75 ± 28	NS	NS	NS
Max PFPI	10.7 ± 8.2	13.1 ± 13.1	13.1 ± 8.9	NS	NS	NS
Max SFVI	87.1 ± 24.2	89.2 ± 17.8	97 ± 20	NS	NS	NS
Max SFPI	12 (2, 60)	138 (5, 34)	13.5 (6, 203)	NS	NS	NS
Max PTVI	71.4 ± 30.1	78 ± 21	81 ± 20	NS	NS	NS
Max PTPI	16.1 ± 13	19 ± 14	17 ± 7	NS	NS	NS
Max DPVI	58 ± 24	58 ± 26	59 ± 30	NS	NS	NS
Max DPPI	14.5 (2, 73)	15.6 (4, 54)	14.3 (5, 52)	0.049	NS	NS
Spine BMD	1.06 ± 0.15	1.1 ± 0.23	1.1 ± 0.17	NS	NS	NS
Spine T Score	-0.6 (-2, 4.2)	-0.25 (-2.4, 4.8)	0.45 (-2, 3)	NS	0.045	NS
NOF BMD	0.86 ± 0.14	0.85 ± 0.15	0.84 ± 0.14	NS	NS	NS
Heel BMD	0.48 (0.34, 0.61)	0.51 (0.2, 0.74)	0.48 (0.24, 435)	NS	NS	NS
Heel T Score	-0.6 (-2.3, 1.3)	-0.5 (-4.3, 2.5)	-0.55 (-3.7, 1.7)	NS	NS	NS

Legend

Radiology, ultrasonographic and bone mineral density assessments in the three groups. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by ANOVA test, Max Fem Cal = Maximum Femoral calcification in either left or right femoral artery, AS = Agatston Score, Max Foot Cal = Maximum Foot calcification in either left or right foot, CFVI = Common Femoral Velocity Index, CFPI = Common Femoral Pulsatility Index, PFVI = Profunda Femoral Velocity Index, PFPI = Profunda Femoral Pulsatility Index, SFVI = Superficial Femoral Velocity Index, SFPI = Superficial Femoral Pulsatility Index, PTVI = Posterior Tibial Velocity Index, PTPI = Posterior Tibial Pulsatility Index, DPVI = Dorsalis Pedis Velocity Index, DPPI = Dorsalis Pedis Pulsatility Index, BMD = Bone Mineral Density, NOF = Neck of femur.

10.5.4 Peripheral Neuropathy (Table 10.4)

Compared to those without, patients with peripheral neuropathy had significantly lower mean serum levels of 1, 25-dihydroxyvitamin D (38.3 vs. 48.1 pmol/l: $p = 0.004$) and higher femoral blood flow velocity (73 vs. 58: $p = 0.025$) and pulsatility (14 vs. 9: $p = 0.010$) index; reflecting comparatively higher blood flow. The mean age (65.7 vs. 55.4: $p < 0.001$) and duration of diabetes (11.6 vs. 7.3 yrs: $p = 0.037$) was significantly higher in the neuropathy group.

The median Agatston calcification score was significantly higher in the femoral artery (247 vs. 0: $p = 0.001$) and foot (7.6 vs. 0: $p < 0.001$), in those with peripheral neuropathy. Those with peripheral neuropathy were more likely to have evidence of calcification by CT in the femoral (88 vs. 45%: $p < 0.001$) and foot (58 vs. 14%: $p = 0.001$), and by foot X ray (51 vs. 9%: $p = 0.001$) (Table 10.4). In addition, the median lumbar spine BMD (1.14 vs. 1.02: $p = 0.013$) and heel BMD (0.51 vs. 0.46: $p = 0.046$), was significantly higher in the peripheral neuropathy group, reflecting higher bone mineral density as compared to those without neuropathy.

Table 10.4 Neuropathy versus No-neuropathy

Variable	Normal	Neuropathy	p-value
Number	22/65	43/65	
Age (yr)	55.4±12.4	65.7±9.3	0.002
Weight (kg)	101±25	84±19	0.006
Duration (yr)	7.3±7.6	11.6±7.8	0.037
Creatinine (umol/l)	88±16	97±13	0.011
HDL (mmol)	1.4±0.4	1.2±0.3	0.048
1,25 (OH)₂D	125.1±30.2	99.7±33.4	0.004

Legend

Baseline clinical and biochemical parameters in the neuropathy versus no-neuropathy groups. Normally distributed parameters represented as mean \pm standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by student t test. HDL = High density lipoprotein, I, 25(OH)₂D = 1, 25-dihydroxyvitamin D.

10.5.5 OPG/RANKL relationships

Subjects with higher than median levels of OPG (Table 10.5) were significantly different in terms of age (67.5 vs. 57.1 yrs: p < 0.001), eGFR (66.7 vs. 81.2: p < 0.001), serum creatinine (99 vs. 90 umol/l: p = 0.015), ACR (15.9 vs. 3.5: p = 0.038), serum calcium (2.38 vs. 2.33 mmol/l: p = 0.043), systolic blood pressure (157 vs. 135 mm of Hg: p < 0.001), lumbar spine BMD (1.14 vs. 1.05: p = 0.056), maximum femoral calcification (474 vs. 16: p < 0.001), and longer duration of DM (12.6 vs. 7.7), compared to those with lower than median levels.

Table 10.5 Associations with Osteoprotegerin levels

Variable	< Median	> Median	p-value
Age (yrs)	57.1 \pm 10.2	67.5 \pm 10.4	<0.001
E-GFR (ml/min)	81.2 \pm 15.3	66.7 \pm 14.5	<0.001
ACR	3.5	15.9	0.038
Creatinine (umol/l)	90 \pm 14	99 \pm 14	0.015
Calcium (mmol/l)	2.33 \pm 0.06	2.38 \pm 0.12	0.043
Systolic BP (mm of Hg)	135 \pm 17	157 \pm 22	<0.001
Spine BMD	1.05 \pm 0.16	1.14 \pm 0.19	0.056
Fem Cal (AS)	16	474	<0.001
Duration (yrs)	7.7 \pm 5.8	12.6 \pm 9.1	0.014

Legend

Baseline clinical and biochemical parameters in the higher versus lower the median Osteoprotegerin groups. Normally distributed parameters represented as mean \pm standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by student t test. E-GFR (ml/min/1.73m²) = estimated glomerular filtration rate calculated using MDRD-4 formula. ACR = Urinary albumin to creatinine ratio, Systolic BP = Systolic blood pressure, Fem cal = Femoral calcification, AS = Agatston score, BMD = Bone mineral density.

OPG correlated significantly with age ($r = 0.576$: $p < 0.001$), corrected calcium ($r = 0.252$: $p = 0.043$), Cockcroft-Gault creatinine clearance ($r = 0.345$: $p = 0.005$), eGFR ($r = 0.382$: $p = 0.002$), duration of diabetes ($r = 0.322$: $p = 0.009$), maximal femoral calcification ($r = 0.551$: $p < 0.001$), maximal foot calcification ($r = 0.251$: $p = 0.043$), systolic blood pressure ($r = 0.529$: $p < 0.001$), blood flow velocity in the common femoral artery ($r = 0.254$: $p = 0.041$), profunda femoral artery ($r = 0.480$: $p < 0.001$), superficial femoral artery ($r = 0.470$: $p < 0.001$) and pulsatility index of the dorsalis pedis artery ($r = -0.338$: $p = 0.006$).

Subjects with higher than median levels of RANKL had significantly lower 25 hydroxyvitamin D (50.4 vs. 67.2 nmol/l: $p = 0.025$) and ACR (3.3 vs. 22.3: $p = 0.044$), and higher haemoglobin levels (14.7 vs. 13.5 g/dl) as compared to those below the median levels. In the whole group, RANKL correlated significantly only with ACR ($r = 0.366$: $p = 0.003$).

Those with higher than median values of OPG/RANKL ratio had significantly higher 25 hydroxyvitamin D levels (66.5 vs. 50.1 nmol/l: $p = 0.022$) and ACR (22.3 vs. 3.0: p

= 0.01). OPG/RANKL ratio correlated significantly with ACR ($r = 0.418$: $p = 0.001$), haemoglobin ($r = 0.248$: $p = 0.047$), systolic blood pressure ($r = 0.322$: $p = 0.009$), and maximal femoral calcification ($r = 0.249$: $p = 0.047$).

10.5.6 Vitamin D relationships and other associations

Those with higher than median 25 hydroxyvitamin D levels had significantly higher OPG/RANKL ratio (97 vs. 44: $p = 0.009$) and posterior tibial velocity (84.6 vs. 70.4: $p = 0.045$) compared to those below the median levels. Patients with higher than median levels of 1, 25-dihydroxyvitamin D had significantly higher systolic blood pressure (150 vs. 140 mm of Hg: $p = 0.047$) and posterior tibial velocity (83.7 vs. 67.7: $p = 0.017$) as compared to those with below median levels.

In the whole group, 25 hydroxyvitamin D correlated significantly with maximal femoral calcification ($r = 0.295$: $p = 0.018$). 1, 25-dihydroxyvitamin D levels did not correlate significantly with any measured parameter. BAP correlated significantly with Ntx ($r = 0.467$: $p < 0.001$), maximal foot calcification ($r = 0.249$: $p = 0.045$) and hip BMD ($r = 0.267$: $p = 0.038$). In addition to BAP, Ntx correlated significantly with hip BMD ($r = 0.399$: $p = 0.001$), and heel BMD ($r = 0.273$: $p = 0.028$).

10.5.7 Vascular Doppler Studies (Table 10.3)

There were no significant differences in the velocity, pulsatility and resistance index of common femoral, profunda femoral, superficial femoral, posterior tibial and dorsalis

pedis arteries across the groups, whether characterised by presence and absence of proteinuria, calcification or osteopenia.

10.5.8 Detection of calcification

Seventy-five percent of subjects had evidence of femoral artery calcification on CT compared with 45% with foot calcification. Seventy-seven percent of subjects had calcification at one or both sites by this method. Ninety three percent of those with CT foot calcification also had CT femoral calcification, whilst only in 59% of those without CT evidence of foot calcification, was femoral calcification present ($p = 0.003$). There was also a degree of agreement between the presence of common femoral calcification detected by CT and by ultrasound. Fifty-eight percent of patients had calcification in one or both femoral arteries on ultrasound, compared with 75% by CT. Ninety-two percent of subjects with calcification detected by US also had calcification on CT, whilst only 52% of those with a negative US also had a negative CT ($p <0.001$).

Assuming CT estimates to be the “gold standard”, relying on US for detection of femoral calcification is subject to a 7% false positive rate but a 48% false negative rate. Ultrasound was poor in detecting calcification in the vessels in the foot. Thirty-seven percent of patients had evidence of calcification on plain X-ray of the foot. There was a higher degree of correspondence with the findings on foot CT, 72.4% of patients with positive foot X-ray having a positive CT, whilst 91.7% of those with a negative foot X-ray had a negative CT. Assuming the CT estimates to be the “gold standard”, then relying on a foot X-ray to detect calcification is subject to an 8.3% false positive rate and a 27.6% false negative rate.

10.5.9 Associations of Calcification

There were significant correlations between femoral calcification severity on CT and age ($r = 0.682$, $p < 0.001$), duration of diabetes ($r = 0.325$, $p = 0.009$), OPG levels ($r = 0.558$: $p < 0.001$), 25-OH D ($r = 0.295$: $p = 0.017$), eGFR-MDRD ($r = -0.336$: $p = 0.006$), serum creatinine ($r = 0.291$, $p = 0.019$), ACR ($r = 0.266$: $p = 0.033$), maximal foot calcification ($r = 0.512$, $p < 0.001$), lumbar spine BMD ($r = 0.261$: $p = 0.042$) systolic blood pressure ($r = 0.285$, $p = 0.021$), and diastolic blood pressure ($r = -0.248$: $p = 0.046$). CT calcification in the foot was also correlated with age ($r = 0.420$: $p < 0.001$), duration of diabetes ($r = 0.263$: $p = 0.045$), bone alkaline phosphatase ($r = -0.249$: $p = 0.045$), OPG ($r = 0.251$, $p = 0.043$), serum creatinine ($r = 0.289$: $p = 0.020$), lumbar spine BMD ($r = 0.324$: $p = 0.011$) and diastolic blood pressure ($r = -0.418$: $p = 0.001$).

The associations with calcification detected by CT in the foot, in the femoral artery and at either site are shown in Table 10.6. Greater age, longer duration of DM, worse renal function, the presence of peripheral neuropathy, and higher OPG levels were consistently associated with the presence of calcification. There were less consistent associations with bone mineral density at the lumbar spine [which tended to be higher in those with evidence of calcification], and with BMD at the neck of femur [which tended to be lower in those with calcification]. Other associations were with lower triglyceride levels (foot), and higher 25 hydroxyvitamin D levels (foot). The associations with systolic and diastolic blood pressure were likely to be consequences of higher levels of vessel calcification.

Table 10.6 Associations of Vascular Calcification

	FOOT CALCIFICATION			FEMORAL CALCIFICATION			ANY CALCIFICATION		
	Yes	No	P-value	Yes	No	P-value	Yes	No	P-value
Age (years)	68 ± 9	57±11	<0.001	66 ± 9	51 ± 11	<0.001	66 ± 9	51 ± 11	<0.001
Duration (years)	-	-	-	11.2±8.3	6.9±5.7	0.024	11.4±8.2	5.9±4.9	0.003
Creatinine (umol/l)	99 ±11	90 ± 15	0.010	-	-	-	96±14	87±15	0.031
E-GFR MDRD (ml/min/1.73m²)	69±15	78±16	0.037	71 ± 16	84 ± 15	0.003	71 ± 16	85 ± 15	0.004
Systolic BP (mmHg)	-	-	-	150±23	136±21	0.033	-	-	-
Diastolic_BP (mmHg)	74±10	83±10	0.001	-	-	-	-	-	-
Neuropathy (%)	90	47	<0.001	79	29	<0.001	80	20	<0.001
Triglycerides (mmol)	1.8±0.8	2.8±2.2	0.027	-	-	-	-	-	-
25 OH D (pmol/l)	67±34	51±22	0.026	-	-	-	-	-	-
OPG (pmol/l)	5.2±1.3	4.6±1.3	0.056	5.2±1.2	4.0±1.3	0.002	5.1±1.2	4.0±1.3	0.005
Spine BMD	1.16±0.21	1.06±0.15	0.015	-	-	-	1.12±0.2	1.02±0.12	0.067
Neck of Femur BMD	-	-	-	0.83±0.14	0.94±0.13	0.013	0.83±0.14	0.92±0.13	0.052

Legend

Associations of vascular calcification. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by student t test. E-GFR (ml/min/1.73m²) = estimated glomerular filtration rate calculated using MDRD 4 formula. BP = Blood pressure, 25 OH D = 25 hydroxyvitamin D, OPG = Osteoprotegerin, BMD = Bone mineral density.

Multivariate modelling

Factors significant in univariate analysis were included in a number of logistic regression models. Factors included in the model predicting the presence of CT calcification in the foot were, age, duration of diabetes, eGFR, bone alkaline phosphatase levels, OPG levels, 25 hydroxyvitamin D levels, triglyceride levels, the presence of neuropathy, and lumbar spine BMD. Factors were added to the model in order of degree of statistical significance in univariate analysis. They were retained as a component of the multivariate model provided they improved its predictive power. When all factors had been added, a backwards LR model was performed (Table 10.7) which identified age, neuropathy and 25 hydroxyvitamin D levels as independent predictors of foot calcification. The model successfully predicted the presence or absence of foot calcification in 77% of cases.

Table 10.7 Regression Model for Vascular Calcification in the Foot

Variables		R square = 0.515							
		B	S.E.	Wald	df	Sig.	Exp(B)	95% CI for B	
								Lower	Upper
Step 1	Age	.057	.041	1.912	1	.167	1.059	.976	1.148
	Neuropathy	2.462	.999	6.077	1	.014	11.729	1.656	83.060
	Spine BMD	2.578	2.319	1.236	1	.266	13.176	.140	1240.44
	25 OH D	.027	.014	3.663	1	.056	1.028	.999	1.057
	Triglycerides	-.508	.374	1.850	1	.174	.602	.289	1.251
	E_GFR_MDRD	-.026	.026	.957	1	.328	.975	.926	1.026
	Constant	-7.203	4.263	2.855	1	.091	.001		
Step 2	Age	.076	.038	3.981	1	.046	1.079	1.001	1.163
	Neuropathy	2.482	.993	6.242	1	.012	11.963	1.707	83.836
	Spine BMD	2.007	2.208	.826	1	.364	7.440	.098	564.199
	25 OH D	.028	.015	3.789	1	.052	1.029	1.000	1.059
	Triglycerides	-.398	.339	1.374	1	.241	.672	.346	1.306
	Constant	-9.917	3.531	7.889	1	.005	.000		
Step 3	Age	.086	.038	5.172	1	.023	1.089	1.012	1.173
	Neuropathy	2.645	.972	7.400	1	.007	14.079	2.094	94.652
	25 OH D	.027	.014	3.485	1	.062	1.027	.999	1.056
	Triglycerides	-.389	.331	1.385	1	.239	.677	.354	1.296
	Constant	-8.366	2.972	7.925	1	.005	.000		
Step 4	Age	.095	.038	6.419	1	.011	1.100	1.022	1.184
	Neuropathy	2.462	.976	6.370	1	.012	11.732	1.734	79.403
	25 OH D	.031	.014	4.836	1	.028	1.031	1.003	1.060
	Constant	-9.921	2.789	12.655	1	.000	.000		

Factors included in the model predicting the presence of CT calcification in the femoral artery were, age, duration of diabetes, eGFR, OPG levels, 25 hydroxyvitamin D levels, urinary Albumin: creatinine ratio, the presence of neuropathy, lumbar spine BMD and

neck of femur BMD. Factors were added to the model in order of degree of significance on univariate analysis, and retained if they improved its predictive power. When all factors had been added, a backwards LR model was performed (Figure 10.8) which identified age, neuropathy and neck of femur BMD as independent predictors of foot calcification. The model successfully predicted the presence or absence of foot calcification in 90.2% of cases.

Table 10.8 Regression Model for Vascular Calcification in the Femoral Artery

R square = 0.633		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I for B	
	Variables							Lower	Upper
Step 1	Age	.100	.050	4.113	1	.043	1.106	1.003	1.218
	Neuropathy	2.495	1.023	5.942	1	.015	12.116	1.630	90.032
	E_GFR_MDRD	-.037	.032	1.337	1	.247	.964	.906	1.026
	Neck of Femur BMD	-6.601	3.949	2.794	1	.095	.001	.000	3.124
Step 2	Constant	2.666	5.786	.212	1	.645	14.376		
	Age	.111	.048	5.417	1	.020	1.118	1.018	1.228
	Neuropathy	2.567	1.029	6.220	1	.013	13.031	1.733	97.998
	Neck of Femur BMD	-6.601	3.822	2.983	1	.084	.001	.000	2.434
	Constant	-.971	4.559	.045	1	.831	.379		

Factors included in the model predicting the presence of CT calcification at either site were, age, duration of diabetes, eGFR, OPG levels, 25 hydroxyvitamin D levels, presence of neuropathy, lumbar spine BMD and neck of femur BMD. Factors were added to the model in order of degree of significance on univariate analysis, and retained if they improved its predictive power. When all factors had been added, a backwards LR model was performed (Figure 10.9) which identified age, neuropathy as

the only independent predictors of calcification at either site. The model successfully predicted the presence or absence of calcification in 86.9% of cases.

Table 10.9 Regression Model for Vascular Calcification at either Site

R square = 0.613		Variables	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I for B	
									Lower	Upper
Step 1	Age	.098	.054	3.346	1	.067	1.103	.993	1.226	
	Neuropathy	2.871	1.088	6.960	1	.008	17.659	2.092	149.055	
	E_GFR_MDRD	-.040	.032	1.545	1	.214	.961	.902	1.023	
	Neck of Femur BMD	-4.777	4.176	1.309	1	.253	.008	.000	30.193	
Step 2	Constant	1.538	6.271	.060	1	.806	4.656			
	Age	.129	.050	6.579	1	.010	1.138	1.031	1.256	
	Neuropathy	2.544	.972	6.848	1	.009	12.727	1.894	85.536	
	E_GFR_MDRD	-.041	.031	1.743	1	.187	.960	.903	1.020	
Step 3	Constant	-4.194	3.715	1.274	1	.259	.015			
	Age	.136	.048	7.878	1	.005	1.146	1.042	1.260	
	Neuropathy	2.452	.932	6.925	1	.008	11.611	1.870	72.108	
	Constant	-7.821	2.810	7.749	1	.005	.000			

On exclusion of age as a factor, and inclusion of BMD at various sites, the model which was generated (Table 10.10) successfully predicted the presence or absence of calcification at any site in 86.9% of cases. In this model, bone mineral density at the lumbar spine and neck of femur were independent predictors of calcification along with peripheral neuropathy.

Table 10.10 Regression Model for Vascular Calcification at either Site (excluding age)

R square = 0.654		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I for B	
Variables								Lower	Upper
Step 1	Neuropathy	3.442	1.168	8.680	1	.003	31.247	3.165	308.466
	Duration	.090	.097	.877	1	.349	1.095	.906	1.323
	OPG	.387	.480	.650	1	.420	1.472	.575	3.770
	Neck of Femur BMD	-14.079	5.596	6.330	1	.012	.000	.000	.045
	Spine BMD	6.924	4.654	2.214	1	.137	1016.815	.111	930308
	Constant	2.012	4.175	.232	1	.630	7.475		
Step 2	Neuropathy	3.408	1.148	8.821	1	.003	30.218	3.187	286.517
	Duration	.114	.094	1.493	1	.222	1.121	.933	1.347
	Neck of Femur BMD	-15.781	5.538	8.122	1	.004	.000	.000	.007
	Spine BMD	8.688	4.367	3.958	1	.047	5933.218	1.138	3.094E7
	Constant	3.261	3.846	.719	1	.397	26.075		
	Step 3	Neuropathy	3.472	1.094	10.069	1	.002	32.205	3.772
	Neck of Femur BMD	-14.635	4.956	8.719	1	.003	.000	.000	.007
	Spine BMD	9.698	4.352	4.966	1	.026	16278.895	3.216	8.240E7
	Constant	2.083	3.639	.327	1	.567	8.025		

10.5.9 Bone Mineral Density

Table 10.11 shows the associations with osteopenia (defined as T score < -1) at the lumbar spine, neck of femur and heel and at any of the three sites. Subjects with osteopenia of the lumbar spine tended to have lower body weight, peripheral neuropathy, and higher HDL cholesterol levels. Subject with osteopenia of the neck of femur also tended to be lighter, to have less renal function (only by Cockcroft-Gault creatinine clearance estimation), to have lower levels of bicarbonate, and to have better controlled diabetes as judged by lower levels of HbA1c. Subjects with osteopenia at the heel also tended to be lighter, and were also older, had higher levels of OPG and 25

hydroxyvitamin D, worse renal function, and higher HDL cholesterol levels. Patients with osteopenia at any site were heavier, had higher levels of Ntx, and 25 hydroxyvitamin D, less renal function, better control of DM, and higher HDL levels.

Lumbar spine BMD correlated with age ($r = 0.256$: $p = 0.047$), weight ($r = 0.317$: $p = 0.013$), duration of DM ($r = 0.302$, $p = 0.018$), OPG levels ($r = 0.229$: $p = 0.076$), HDL levels ($r = -0.315$: $p = 0.013$), CT femoral calcification ($r = 0.261$: $p = 0.042$), and CT foot calcification ($r = 0.344$: $p = 0.007$). Neck of femur BMD correlated with age ($r = -0.265$: $p = 0.039$), weight ($r = 0.427$: $p = 0.001$), Cockcroft-Gault creatinine clearance ($r = 0.463$: $p < 0.001$), PTH ($r = -0.263$: $p = 0.040$), and HDL cholesterol ($r = -0.329$: $p = 0.010$).

Heel BMD correlated with age ($r = -0.263$: $p = 0.036$), weight ($r = 0.516$: $p < 0.001$), renal function (Cockcroft-Gault creatinine clearance [$r = 0.518$: $p < 0.001$] and MDRD eGFR [$r = 0.279$: $p = 0.025$]), indices of bone resorption (PTH [$r = -0.333$: $p = 0.007$], Ntx [$r = -0.302$: $p = 0.015$], bone alkaline phosphatase [$r = -0.277$: $p = 0.027$]), OPG levels (-0.230: $p = 0.068$), 25 OH D levels ($r = -0.254$: $p = 0.043$), and lipid fractions (total cholesterol levels [$r = -0.349$: $p = 0.005$] and HDL cholesterol levels [$r = -0.396$: $p = 0.001$]). Heel BMD was significantly higher in males than in females (0.53 vs. 0.42: $p < 0.001$).

Table 10.11 Associations of Osteopenia at various Sites

Variables	Spine Osteopenia		p-value	Neck of Femur Osteopenia		p-value	Heel Osteopenia		p-value	Osteopenia any site		p-value
	Yes	No		Yes	No		Yes	No		Yes	No	
Number	14	51		19	46		26	39		37	28	-
Age (years)	-	-	-	-	-	-	66±11	60±11	0.043	-	-	-
Sex (m:f ratio)	-	-	-	-	-	-	-	-	-	1.3	4.6	0.001
OPG (pmol/l)	-	-	-	-	-	-	5.3±1.5	4.6±1.1	0.026	-	-	-
Ntx (nm BCE)	-	-	-	-	-	-	-	-	-	466±332	307±282	0.046
25 OH D ng/ml	-	-	-	-	-	-	72.2±33.5	48.8±21.2	0.003	65.2±32.2	48.9±21.1	0.016
Weight (kg)	82.0±16.5	97.7±22.9	0.020	82.7±15.6	100.6±25.8	0.001	82.5±17.6	103.9±25.1	<0.001	84.3±17.5	110±25	0.001
Neuropathy (%)	43	73	0.056	-	-	-	-	-	-	-	-	-
CrCl_{CG} (ml/min)	-	-	-	78.8±22.1	101.6±36.8	0.015	81.3±31.5	104.0±33.9	0.008	84.1±28.7	109±36	0.003
Bicarbonate	-	-	-	24.5±2.7	26.0±2.7	0.051	-	-	-	-	-	-
HbA1c (%)	-	-	-	7.4±1.6	8.4±1.8	0.040	-	-	-	7.6±1.4	8.8±1.9	0.007
HDL (mmol/l)	1.45±0.33	1.24±0.33	0.044	-	-	-	1.45±0.36	1.18±0.28	0.001	1.38±0.35	1.16±0.27	0.007
Tchol:HDL	2.7±0.6	3.7±1.2	<0.001	-	-	-	--	-	-	-	-	-

Legend

Associations of osteopenia at various sites. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by student t test. OPG = Osteoprotegerin, Ntx = N -linked telopeptide of type 1 collagen, 25 OH D = 25 hydroxyvitamin D, CrCl-CG = Creatinine clearance by Cockcroft-Gault method, HDL = High density lipoprotein, T chol = Total Cholesterol.

Multivariate models

Factors significantly associated with bone mineral density were included in multiple regression analysis to determine the predictors of bone mineral density. The model for the lumbar spine is shown in table 10.12. At this site significant predictors of bone mineral density were age, weight, peripheral neuropathy, and HDL cholesterol levels.

Table 10.12 Predictors of BMD at Lumbar Spine

R square = 0.231 Variables					t	Sig.	95% CI for B	
		B	S.E	Beta			Lower	Upper
Step 1	(Constant)	.752	.188		4.007	.000	.376	1.129
	Age	.004	.002	.238	1.531	.132	-.001	.008
	Weight	.002	.001	.264	2.105	.040	.000	.005
	Neuropathy	.000	.055	.000	-.007	.995	-.110	.109
	OPG	.016	.020	.122	.804	.425	-.024	.057
	Duration	.003	.003	.136	1.014	.315	-.003	.009
	HDL	-.163	.069	-.300	-2.354	.022	-.301	-.024
Step 2	(Constant)	.752	.181		4.158	.000	.390	1.115
	Age	.004	.002	.238	1.772	.082	.000	.008
	Weight	.002	.001	.264	2.243	.029	.000	.005
	OPG	.016	.020	.122	.816	.418	-.024	.056
	Duration	.003	.003	.136	1.034	.306	-.003	.009
	HDL	-.163	.066	-.300	-2.465	.017	-.295	-.030
Step 3	(Constant)	.757	.180		4.199	.000	.396	1.118
	Age	.004	.002	.287	2.406	.019	.001	.008
	Weight	.002	.001	.265	2.255	.028	.000	.005
	Duration	.004	.003	.182	1.548	.127	-.001	.010
	HDL	-.150	.064	-.276	-2.347	.023	-.278	-.022
Step 4	(Constant)	.743	.182		4.077	.000	.378	1.108
	Age	.005	.002	.342	2.966	.004	.002	.009
	Weight	.003	.001	.283	2.396	.020	.000	.005
	HDL	-.159	.064	-.293	-2.468	.017	-.288	-.030
a. Dependent Variable: L_Spine_BMD								

If age was omitted from the model OPG became a significant predictor though R square was reduced to 0.213 (Table 10.13).

Table 10.13 Predictors of BMD (excluding age) at Lumbar Spine

R square = 0.213					t	Sig.	95% C I for B	
		Variables	B	S.E			Lower	Upper
Step 1	(Constant)	.908	.160		5.692	.000	.589	1.228
	Weight	.002	.001	.216	1.756	.085	.000	.004
	Neuropathy	.041	.048	.109	.849	.400	-.056	.138
	OPG	.030	.018	.224	1.616	.112	-.007	.067
	Duration	.003	.003	.128	.948	.347	-.003	.009
	HDL	-.146	.069	-.270	-2.117	.039	-.285	-.008
Step 2	(Constant)	.915	.159		5.756	.000	.597	1.233
	Weight	.002	.001	.241	2.019	.048	.000	.004
	OPG	.032	.018	.243	1.778	.081	-.004	.069
	Duration	.003	.003	.145	1.089	.281	-.003	.009
	HDL	-.159	.067	-.294	-2.375	.021	-.294	-.025
Step 3	(Constant)	.907	.159		5.704	.000	.589	1.226
	Weight	.002	.001	.252	2.115	.039	.000	.004
	OPG	.042	.016	.318	2.708	.009	.011	.074
	HDL	-.173	.066	-.318	-2.610	.012	-.305	-.040

a. Dependent Variable: L_Spine_BMD

The model for the neck of femur is shown in table 10.14. The predictors of BMD at this site were weight, PTH levels and HDL levels.

Table 10.14 Predictors of BMD at Neck of Femur

R square = 0.241 Variables					t	Sig.	95.0% CI for B	
		B	S.E	Beta			Lower	Upper
Step 1	(Constant)	.599	.261		2.294	.026	.075	1.122
	Age	.000	.002	-.057	-.307	.760	-.005	.004
	Weight	.002	.002	.221	.917	.363	-.002	.005
	E_GFR_C-G	.001	.001	.130	.481	.633	-.002	.003
	PTH	-.007	.006	-.140	-1.144	.258	-.019	.005
	HDL	-.088	.053	-.204	-1.657	.103	-.195	.019
	Bicarbonate	.007	.007	.125	1.074	.288	-.006	.020
Step 2	(Constant)	.549	.202		2.714	.009	.143	.954
	Weight	.001	.001	.176	.930	.357	-.001	.004
	E_GFR_C-G	.001	.001	.192	1.093	.279	.000	.002
	PTH	-.007	.006	-.138	-1.140	.259	-.019	.005
	HDL	-.088	.053	-.205	-1.679	.099	-.194	.017
	Bicarbonate	.007	.007	.125	1.086	.282	-.006	.020
	HbA1c	.009	.011	.110	.801	.426	-.013	.031
Step 3	(Constant)	.566	.200		2.823	.007	.164	.967
	Weight	.002	.001	.248	1.493	.141	.000	.004
	E_GFR_C-G	.001	.001	.179	1.024	.310	.000	.002
	PTH	-.007	.006	-.143	-1.187	.241	-.019	.005
	HDL	-.084	.052	-.194	-1.605	.114	-.188	.021
	Bicarbonate	.007	.007	.131	1.138	.260	-.006	.020
Step 4	(Constant)	.573	.200		2.863	.006	.172	.975
	Weight	.003	.001	.368	3.134	.003	.001	.004
	PTH	-.009	.006	-.178	-1.531	.131	-.021	.003
	HDL	-.093	.051	-.216	-1.806	.076	-.196	.010
	Bicarbonate	.008	.007	.135	1.173	.246	-.005	.021
Step 5	(Constant)	.764	.118		6.471	.000	.527	1.000
	Weight	.003	.001	.367	3.111	.003	.001	.004
	PTH	-.010	.006	-.197	-1.713	.092	-.021	.002
	HDL	-.086	.051	-.200	-1.682	.098	-.189	.016

a. Dependent Variable: Neck of Femur_BMD

The model for the heel is shown in table 10.15. Factors which were significant in univariate analysis but omitted from model were Cockcroft Gault creatinine clearance

(significantly correlated with MDRD eGFR), Ntx (significantly correlated with PTH), and total cholesterol (significantly correlated with HDL cholesterol). The predictors of BMD at this site were age, weight, sex, bone alkaline phosphatase levels, 25 OH D levels.

Table 10.15 Predictors of Heel BMD

Variables				t	Sig.	95.0% CI for B	
	B	S.E	Beta			Lower	Upper
Step 1 (Constant)	.731	.263		2.773	.008	.202	1.259
Weight	.001	.000	.344	3.617	.001	.001	.002
PTH	-.005	.004	-.130	-1.171	.247	-.013	.003
25 OH D	.000	.000	-.264	-2.813	.007	-.002	.000
OPG	-.003	.008	-.046	-.416	.679	-.020	.013
Calcium	-.024	.105	-.023	-.227	.821	-.235	.187
E_GFR_MDRD	9.149E-5	.001	.015	.136	.892	-.001	.001
BAP	-.002	.002	-.131	-1.282	.206	-.005	.001
HDL	-.026	.030	-.085	-.871	.388	-.085	.033
Sex	-.084	.023	-.387	-3.669	.001	-.130	-.038
Age	.000	.001	-.085	-.771	.444	-.003	.001
Steps 2-5							
Step 6 (Constant)	.678	.076		8.875	.000	.525	.831
Weight	.002	.000	.358	4.034	.000	.001	.002
25 OH D	.000	.000	-.256	-2.871	.006	-.002	.000
BAP	-.003	.001	-.185	-2.099	.040	-.005	.000
Sex	-.100	.019	-.462	-5.194	.000	-.138	-.061
Age	-.001	.001	-.151	-1.715	.092	-.003	.000

a. Dependent Variable: L_Heel_BMD

Omitting age from the model (Table 10.16), PTH replaced bone alkaline phosphatase in the model with no significant change in R square (= 0.533) Omitting sex from the model

did not bring any other factors into significance but significantly reduces the R square value.

Table 10.16 Predictors of Heel BMD (excluding age)

Variables				t	Sig.	95% CI for B	
	B	S.E.	Beta			Lower	Upper
Step 1 (Constant)	.674	.252		2.674	.010	.169	1.179
Weight	.001	.000	.352	3.739	.000	.001	.002
PTH	-.005	.004	-.143	-1.308	.196	-.013	.003
25 OH D	.000	.000	-.273	-2.932	.005	-.002	.000
OPG	-.006	.007	-.084	-.858	.395	-.021	.009
Calcium	-.017	.104	-.016	-.164	.871	-.226	.192
E_GFR_MDRD	.000	.001	.032	.307	.760	-.001	.002
BAP	-.002	.002	-.140	-1.384	.172	-.005	.001
HDL	-.027	.029	-.088	-.909	.367	-.086	.032
Sex	-.080	.022	-.371	-3.601	.001	-.125	-.036
Steps 2-5							
Step 6 (Constant)	.552	.057		9.699	.000	.438	.666
Weight	.002	.000	.386	4.314	.000	.001	.002
PTH	-.008	.003	-.233	-2.457	.017	-.015	-.002
25 OH D	-.001	.000	-.296	-3.290	.002	-.002	.000
Sex	-.087	.021	-.401	-4.191	.000	-.128	-.045

Table 10.17 shows a logistic regression model (R square = 0.588) for the predictors of osteopenia at any site, obtained by entering the factors shown to be associated with osteopenia in univariate analysis. The significant predictors were age, sex, weight, NTX levels, and Cockcroft-Gault creatinine clearance

Table 10.17 Predictors of Osteopenia at any Site

R square = 0.588		B	S.E.	Wald	df	95% CI for B	Exp(B)
	Variables						
Step 1	Age	.146	.063	5.401	1	.020	1.157
	Sex(1)	2.115	.978	4.677	1	.031	8.289
	Weight	-.155	.053	8.608	1	.003	.856
	Ntx	.004	.002	4.911	1	.027	1.004
	HDL	2.306	1.730	1.775	1	.183	10.031
	25 OH D	.012	.016	.584	1	.445	1.012
	E_GFR(CG)	.084	.032	6.759	1	.009	1.088
	HbA1c	-.298	.293	1.032	1	.310	.742
	Constant	-4.917	5.282	.867	1	.352	.007
Steps 2 &3							
Step 4	Age	.154	.058	7.129	1	.008	1.166
	Sex(1)	1.987	.966	4.232	1	.040	7.292
	Weight	-.168	.051	10.795	1	.001	.845
	Ntx	.004	.002	6.801	1	.009	1.004
	E_GFR(CG)	.078	.030	6.522	1	.011	1.081
	Constant	-2.575	3.186	.653	1	.419	.076

a. Variable(s) entered on step 1: Age, Sex, Weight, Ntx, HDL, 25 OH D, E_GFR(CG), HbA1c.

Hence in multivariate analysis the significant predictors of calcification were age and the presence of peripheral neuropathy, other potential factors being 25 OH D levels and BMD at the lumbar spine and neck of femur. The significant predictors of osteopenia were age, weight, sex, and indices of bone turnover (PTH, Ntx, bone alkaline phosphatase – the most significant depending on the particular model used). Other potential factors were renal function (particularly Cockcroft-Gault creatinine clearance), 25 OH D levels, HDL levels and OPG levels.

Apart from age, there was minimal overlap of predictors of calcification and osteopenia. However, at the heel, there were indications that the greater the degree of bone thinning, the greater the degree of calcification (Table 10.18: $p = 0.029$). The prevalence of frank osteoporosis at sites other than the heel was minimal.

**Table 10.18 Relationship between Heel Osteopenia and Foot Calcification
(Likelihood ratio = 0.029)**

				Total
	Normal	Osteopenia	Osteoporosis	
CT foot calcification No	24	12	0	36
	15	10	4	29
Total	39	22	4	65

10.6 Discussion

This is the first study to report the status of vasculopathy in relation to peripheral vascular calcification, bone mineral density, limb blood flow and biochemical markers of vascular and bone metabolism in type 2 diabetes patients with early microvascular complications (nephropathy and neuropathy). Among the principal findings are i) there is extensive peripheral vascular calcification in patients with normal eGFR, serum calcium, phosphate, PTH levels, and before the onset of microalbuminuria ii) the severity of calcification increases with the degree of albuminuria iii) patients with peripheral neuropathy have significantly more vascular calcification as compared to those without iv) there is high prevalence of osteopenia in this group of patients v) serum osteoprotegerin levels are higher in those with calcification and in those with proteinuria.

The presence of peripheral vascular calcification in DM patients without microvascular complications, as assessed by x ray of the feet, has been reported before.^{363, 365} However, there are very few reports on structured assessments of the diabetic foot involving the use of CT scanning.^{372, 373} Previous studies in this area employed cruder methodologies^{363, 365} or where CT scanning was deployed, the study groups were unstructured³⁷³ or consisted of patients with advanced chronic kidney disease.³⁷² Vascular calcification was associated with increasing age, duration of DM, serum creatinine, OPG levels and falling GFR. Femoral calcification had significant associations with cardiovascular risk factors (age, diabetes duration, and blood pressure), renal parameters (serum creatinine and MDRD eGFR), mineral metabolism (25 hydroxyvitamin D and OPG) and bone (lumbar spine BMD). Foot calcification had similar correlations, except that in place of OPG, foot calcification had significant correlation with BAP.

A significant proportion of patients in the current study had bone mineral density in the osteopenic range (T score < -1). The high prevalence of osteopenia in type 2 DM patients found in our patient group is in agreement with previous studies in similar patient groups.^{374, 375} Lumbar spine BMD correlated with age, weight, duration of DM, OPG levels, HDL levels and CT femoral calcification. Neck of femur BMD correlated with age, weight, Cockcroft-Gault creatinine clearance, PTH levels, and HDL cholesterol levels. Age, weight, peripheral neuropathy and HDL cholesterol were independent predictors of spine BMD, whereas, weight, PTH and HDL cholesterol levels were independent predictors of neck of femur BMD. Apart from being an independent predictor of BMD, weight had a protective effect on osteopenia. This is in agreement with a previously published report.³⁷⁶

Heel BMD had a significant correlation with age, weight, renal function (Cockcroft-Gault creatinine clearance and MDRD eGFR, indices of bone resorption (PTH, Ntx, bone alkaline phosphatase, OPG levels, 25 hydroxyvitamin D levels, and lipid fractions (total cholesterol levels and HDL cholesterol levels). Heel BMD was significantly higher in males than in females. The independent predictors of heel BMD were age, weight, sex, BAP and 25 hydroxyvitamin D levels. In overall analysis for independent predictors for osteopenia at any of the three sites, the predictors were age, sex, weight, Ntx levels, and Cockcroft-Gault creatinine clearance. Weight had an overwhelming protective effect on BMD and those with osteopenia at any site weighed significantly less than those without.

It has been speculated that there may be a causal link between vascular calcification and bone mineral density.³⁷⁷ We hypothesized that there is an inverse relationship between vascular calcification and BMD, and found only sparse support for this from this study. We found that neck of femur and lumbar spine BMD were weak predictors of femoral calcification. However, neck of femur BMD had an inverse relationship, whereas, lumbar spine BMD had a direct relationship with vascular calcification at either site. This dissociation may be an artefact due to interference of aortic calcification in the assessment of lumbar spine BMD. Alternatively it may reflect different mechanisms of regulation of BMD at these sites. This phenomenon needs further investigation. In the foot, there were indications that the greater the degree of bone thinning, the greater the degree of calcification (Table 10.18).

A second hypothesis was that both vascular calcification and osteopenia are related to clinical indicators of microvascular disease, particularly the degree of proteinuria and

the presence of neuropathy. It has been established that peripheral neuropathy is associated with vascular calcification.³⁷⁷ We found that age, peripheral neuropathy and 25 hydroxyvitamin D were independent predictors of calcification in the foot whereas, the predictors of femoral calcification were age, peripheral neuropathy and neck of femur BMD. Age and peripheral neuropathy were independent predictors of VC irrespective of the site. Those with peripheral neuropathy had a significantly higher Agatston score of femoral and foot calcification than those without, and a significantly higher proportion of patients with peripheral neuropathy had evidence of calcification on CT and foot x ray. Those with peripheral neuropathy had significantly lower 1, 25-dihydroxyvitamin D levels along with significantly higher lumbar spine and heel BMD. However, in multivariate analysis peripheral neuropathy was a strong independent predictor of vascular calcification but not of BMD or osteopenia. This dissociation warrants further investigation.

In addition, whilst peripheral neuropathy was a strong independent predictor of calcification, the association of proteinuria with calcification was weak. There was a weak correlation of albumin: creatinine ratio with femoral but not foot calcification. The median Agatston scores for femoral calcification, but not foot calcification was significantly higher in a stepwise fashion in the normoalbuminuric, microalbuminuric and proteinuric groups. However, proteinuria was not a significant predictor of calcification in logistic regression model. In addition, albumin: creatinine ratio did not correlate with BMD at any site. The dissociation of the effects of these different microvascular complications (proteinuria and peripheral neuropathy) also warrants further study.

The final component of the hypotheses driving this study was that both vascular calcification and osteopenia are related to modulators of diabetic vasculopathy such as the OPG/RANKL axis. The study findings suggest further dissociations relating to the impact of such potential modulators as 1, 25-dihydroxyvitamin D, 25 hydroxyvitamin D, PTH and OPG on VC and osteopenia. While, 1, 25-dihydroxyvitamin D did not have any association with VC or osteopenia, 25 hydroxyvitamin D was a significant predictor of foot calcification and heel BMD but not femoral calcification or BMD at other sites. In agreement with a previous report,³⁷⁸ there was no correlation between OPG and RANKL levels with BMD at the spine, hip and heel. If age was excluded as a factor as a factor in multivariate modelling, OPG emerged as an independent predictor of BMD at lumbar spine and PTH as an independent predictor of BMD at heel. The bone formation marker, BAP, was an independent predictor of heel BMD whereas, the bone resorption marker, Ntx, was a significant predictor of osteopenia at any site. Surprisingly, HDL cholesterol was a significant predictor of BMD at the spine and neck of femur. It is difficult to explain this association and further investigation is required.

The process of vascular aging is accelerated in patients with DM³⁷⁹ due to several vascular risk factors associated with chronic hyperglycemia such as dyslipidemia, hypertension, defects in fibrinolysis, chronic low-grade inflammation and others.³⁸⁰ In addition, peripheral neuropathy leading to sympathetic denervation of the smooth muscle of the tunica media has been reported as an important predictor of MAC in DM.³⁶³ Age and neuropathy were independent predictors of VC in the current study, supporting a role for these factors in the pathogenesis of VC^{379, 380, 363}. Osteoprotegerin has been reported to have a strong correlation with age and vascular

disease in diabetes.³⁸¹ The predictive effect of age on VC in the current study may well be mediated through OPG. However, this too needs further investigation.

Deterioration in glomerular filtration rate (GFR) results in abnormal mineral metabolism, which in turns favours the pathogenesis of VC in patients with renal impairment.²³⁰ In this study, despite normal serum creatinine, patients with proteinuria had significantly lower eGFR (MDRD) and significantly higher femoral calcification compared to those without proteinuria. However; there were no significant differences in the level of foot calcification. This suggests that although deterioration in renal function may contribute to deranged mineral metabolism in advanced stages, its contribution to early VC, as seen in the current cohort, remains limited.

Subjects with proteinuria had significantly higher levels of OPG and OPG/RANKL ratio, suggesting increased activity of this system in the proteinuric compared to the non-proteinuric group. The mean OPG levels increased with proteinuria and were significantly higher in the proteinuric group. This is in agreement with a recent report of higher OPG levels with increasing albumin excretion in type 2 DM.³⁸² The median RANKL levels reduced significantly with increasing proteinuria and were significantly different across the groups. This had an effect on the OPG/RANKL ratio, which increased significantly with proteinuria.

OPG has been detected in calcified regions of blood vessels, suggesting a significant role for the regulators of bone metabolism in the pathogenesis of VC in patients with DM.³⁰² OPG/RANKL ratio is a better indicator of the activity of OPG/RANKL axis in the bone and vascular milieu.³¹² OPG/RANKL ratio was significantly higher in the

proteinuric group as compared to the microalbuminuric and the normoalbuminuric groups and it increased with the severity of proteinuria. The fact that those with proteinuria had significantly higher OPG/RANKL ratio coupled with significantly higher femoral calcification and absence of any correlation with foot calcification, suggests a complex association of OPG/RANKL axis with VC, which needs further investigation.

The endocrine hormones (LH, FSH, Oestradiol, progesterone and testosterone) did not have any significant association with OPG and BMD. This is contrary to a previous study, which reported Oestradiol and OPG to be significant determinants of bone turnover and BMD in postmenopausal woman.³⁸³ This could be due to differences in the groups studied. This study compared patients with and without diabetes, whilst previous studies focussed on postmenopausal women.

The present study had several limitations. First and foremost, it is a cross sectional study, and therefore no causal relationship can be established. In addition, the number of participants was small. Finally, the design did not include an age and sex matched healthy control group.

10.7 Conclusions

The present study demonstrates presence of a weak inverse relationship between VC and osteopenia in patients with type 2 diabetes patients with normal serum creatinine and mineral metabolism. Although the degree of calcification increased with the severity of proteinuria, only age and peripheral neuropathy were significant

independent predictors of vascular calcification. Excluding age from multivariate models uncovered lumbar spine and neck of femur BMD as independent predictors of foot calcification and OPG and PTH as independent predictors of spine and heel BMD respectively. Weight and urinary Ntx were also independent predictors of osteopenia overall.

Though estimating OPG/RANKL ratio in clinical setting may provide an insight into the mineral metabolism of patients with DM, it does not appear to be a reliable marker of VC or osteopenia in these patients. Further studies with bigger numbers are warranted to confirm these findings and help untangling the complexities the pathogenesis of vascular calcification in DM. Prevention may then become a possibility.

The following study examines the status of OPG/RANKL axis in DM patients with progressive kidney disease and those on haemodialysis as compared to non-diabetic CKD patients.

CHAPTER 11

OPG/RANKL AXIS THROUGH THE SPECTRUM OF DIABETIC NEPHROPATHY

OPG/RANKL AXIS THROUGH THE SPECTRUM OF DIABETIC NEPHROPATHY

11.1 Introduction

Diabetic nephropathy (DN) is an important complication of diabetes mellitus (DM) that may lead to end stage renal disease (ESRD). It is now the most common cause of ESRD in patients requiring renal replacement therapy (RRT) in the UK.³⁸⁴ Type 2 DM patients with DN manifest higher mortality and poor quality of life, not only when they require renal replacement therapy, but also in the predialysis stage.³⁸⁵ ESRD results in extensive loss of functioning nephrons. A common clinical syndrome, characterised by hypertension, proteinuria and a progressive decline in renal function is in place. In this stage, a patient with DM, usually suffers from a multitude of medical problems; caused by diabetic microvascular and macrovascular complications.

The development of DN often accompanies other vascular complications of DM; in particular the co-existence of peripheral and autonomic neuropathy and peripheral vascular disease (PWD) often closely parallels nephropathy. Thus, the development of a host of contributory factors alongside DN, are implicated in progressive foot problems in patients with advancing DN. The prevalence of PWD is two times higher in DM patients as compared to the general population.³⁸⁶ PWD is an independent predictor of enhanced risk of death due to cardiovascular disease (CVD).³⁸⁷ Almost 50% of patients presenting with symptoms of PWD may have underlying CVD with extensively compromised coronary circulation as evident by coronary angiography studies in these patients.³⁸⁷

The etiopathogenesis of vascular calcification in DN is multifactorial and not well understood. In patients with chronic kidney disease (CKD), several studies have reported associations of both traditional and uremic-specific risk factors such as hyperphosphatemia, hypocalcaemia and/or increased calcium intake, chronic inflammation, low serum Fetuin-A levels, dyslipidemia, elevated homocysteine and dialysis duration, with calcification. However, these associations do not successfully prove cause-effect of the associations of these factors.³⁸⁸

Diabetic bone disease is associated with low bone turnover as a result of impaired parathyroid hormone (PTH) secretion or reduced sensitivity of osteoblasts to PTH.³⁸⁹ Adynamic bone disease is common in patients with DM and may manifest in the predialysis stage, during the course of DN.³⁹⁰ Earlier bone loss in the course of DN is suggested by low bone mineral density (BMD) and bone loss is a progressive feature in patients with DN.³⁹¹ PTH acts as a uremic toxin and it may contribute to altered bone metabolism resulting in renal osteodystrophy, non-skeletal abnormalities, including generalised vascular calcification, abnormalities in cardiovascular structure and function, immune dysfunction and renal anaemia.³⁹²

The diabetic renal foot is at multiple risks in terms of chronic hyperglycemia, peripheral neuropathy, progressive endothelial dysfunction, hypoxia, deranged mineral metabolism, vascular calcification, bone loss and progressive kidney disease. All these factors work simultaneously and there is no unifying hypothesis to correlate all of them. The osteoprotegerin (OPG)/ receptor activator of nuclear factor- κ -B ligand (RANKL) system may be a possible link between bone and vascular disease.³¹² In the bone milieu, OPG promotes bone formation, whereas RANKL promotes bone

resorption.³¹⁸ In the vasculature, RANKL promotes calcification and OPG has a protective role against calcification.³¹⁸ PTH and 1, 25-dihydroxyvitamin D are important modulators of OPG/RANKL axis.³⁹³

CVD and cardiovascular mortality have been associated with elevated OPG levels³⁸¹ and the higher OPG levels has been suggested be a compensatory mechanism to counteract osteoporosis or vascular calcification. The relationship of OPG/RANKL axis with other modulators of bone metabolism, with progressive kidney disease is not known. The aim of this study was to examine the relationship between modulators of diabetic vasculopathy and the degree of chronic kidney disease, throughout the spectrum of DN as compared to non-diabetic patients with CKD.

11.2 Research Design and Methods

11.2.1 Patient recruitment

All the patients (DM and non-diabetics) were recruited from diabetic and renal clinic in East and North Herts NHS Trust hospitals in Hertfordshire, UK. The patients were screened according to predefined inclusion and exclusion criteria. Inclusion criteria were age >18 years, DM or non-diabetic with history of CKD, estimated glomerular filtration rate (eGFR) by MDRD-4 formula³³⁴ less than 60 ml/min.. The exclusion criteria were immunosuppressive therapy, currently pregnant, hormone replacement therapy and malignancy.

The study was approved by the Hertfordshire research ethics committee. After screening the medical records, suitable patients were approached with the patient information sheet and explained about the study. The prospective study subjects, who agreed to participate, gave written informed consent and there were no drop-outs post consent. Based on eGFR, the DM and non-diabetics patient groups were divided into Group 1 (Moderate or stage 3 CKD), Group 2 (Advanced or stage 4 and 5 CKD) and Group 3 (on haemodialysis). There were 20 patients each (10 each of DM and non-diabetic) in group 1 and Group 2. The haemodialysis group consisted of 30 patients (15 each of DM and non-diabetics). In the haemodialysis group, only those patients, who had undergone at least 3 months of dialysis, were considered.

11.2.2 Data collection

A detailed medical history was recorded including, age, gender, type of diabetes, weight, height, blood pressure, recording of medical history including, duration of diabetes, treatment for diabetes, history of hypertension, duration of hypertension, history of erectile dysfunction, type of antihypertensive therapy, duration of CKD in non-diabetics, and finally all other concomitant medication.

11.3 Laboratory Investigations

A single overnight fasting blood sample was collected to measure baseline blood chemistry including fasting blood glucose (FBG), full blood count, blood urea, serum creatinine, serum albumin, C reactive protein, serum bilirubin serum alanine

transferase (ALT), serum alkaline phosphatase, serum parathyroid hormone (PTH), serum calcium, serum phosphate, and serum magnesium. Samples were also taken for serum OPG, RANKL and bone specific alkaline phosphatase (BAP) estimation. In the predialysis groups, a spot urine sample was collected to assess levels of urinary albumin excretion, urine phosphate levels and urinary creatinine excretion. .

11.3.1 Laboratory Methodology

Full blood count measurement was performed on the ABX Pentra ® (Horiba Diagnostics, Northampton, UK) according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin were performed on the Olympus AU 2700 ® multi-analyzer (Olympus Diagnostics, Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access ® 2 immunoassay system (Beckman Coulter, High Wycombe, UK). The reagents for OPG, RANKL, BAP, and Ntx were obtained from Oxford biosystems, Oxford, UK. All analyses were performed as a single batch. The analytical coefficients of variation ranges are mentioned in general methodology. These analytes were also measured in duplicate to exclude the occurrence of random analytical.

11.4 Sample Size and Statistical Analysis

The primary objective of this pilot study is to determine the relationship of OPG/RANKL with markers of bone metabolism in diabetics and nondiabetics in the predialysis and haemodialysis phase. As this relationship has not been examined before, there are no published statistical estimates upon which to base a power calculation. The findings of this pilot study will be useful in evaluating the need and feasibility of future research projects exploring the role of OPG/RANKL in bone metabolism in diabetics.

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA). The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median values, when the distribution was not normal. Thus routine biochemical measurements and OPG have been reported as means, whereas, median values have been reported for PTH, RANKL, OPG/RANKL ratio and CRP.

Comparisons between groups were carried out using the Kruskal-Wallis and Mann-Whitney U-tests for non-normally distributed data and One Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc testing when data was normally distributed. Differences between the groups with respect to the distribution of categorical variables were examined using the Chi-squared test. P-values ≤ 0.05 were considered to be statistically significant.

11.5 Results

11.5.1 Demographics and clinical factors (Table 11.1)

There was no difference in the age across the moderate CKD (M), Advanced CKD (A) and Haemodialysis (HD) groups. All the participants were white. The proportion of females across the groups did not differ significantly and was 29%, 42% and 31% in the M, A and HD groups respectively. There was no difference in the systolic blood pressure; however, the diastolic blood pressure was significantly lower in the HD group (73 mm of Hg) compared to M (84 mm of Hg: $p = 0.028$) and A (95 mm of Hg: $p < 0.001$) groups. The groups did not differ as regards to known duration of diabetes. The M group had significantly higher BMI as compared to A (33.4 vs. 26.5: $p < 0.001$) and HD (33.4 vs. 27.1: $p < 0.001$) groups. The BMI did not differ significantly between the A and HD groups. In the HD group there were no significant differences in dialysis vintage between the diabetic and non-diabetic (34 vs. 38 months: $p = \text{NS}$) patients.

Table 11.1 Patient Characteristics of OPG/RANKL in CKD study

Variables	Groups			Comparison of Groups		
	Moderate CKD (M)	Advanced CKD (A)	Haemodialysis (HD)	M v A	M v HD	A v HD
Number	21	19	32	NS	NS	NS
Age (yrs)	64 ± 9.8	66 ± 17	64 ± 14	NS	NS	NS
Gender (M:F)	15/6	11/8	22/10	NS	NS	NS
DM Duration (yrs)	16 ± 8.6	20 ± 10.7	15.6 ± 10	NS	NS	NS
SBP (mmHg)	145 ± 13	143 ± 43	134 ± 17	NS	NS	NS
DBP (mmHg)	85 ± 12	95 ± 23	73 ± 11	NS	0.028	<0.001
BMI	33.4 ± 6.2	26.5 ± 3.4	27.1 ± 5.8	<0.001	<0.001	NS

Legend

Demographic and clinical characteristics in diabetics and non-diabetics with chronic kidney disease (CKD). Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by ANOVA test, M = Moderate CKD, A = Advanced CKD and HD = Haemodialysis, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, BMI = Body mass index, NS = non-significant.

11.5.2 Baseline biochemistry and haematology (Table 11.2)

The mean glucose levels were similar across the three groups. In the diabetic group, those on HD had significantly better HbA1c (6.8 vs. 9.0: p = 0.005) as compared to the M group but not with A group (9.0 vs. 8.0: p= NS). In the whole group, HbA1c had significant correlation only with age ($r = -0.423$: p = 0.011). Serum creatinine was significantly higher in the HD group (746 mmol/l) as compared to the M (150 mmol/l: p < 0.001) and the A (288 mmol/l: p = 0.024). Serum urea was significantly lower in the M group (10.3 mmol/l) as compared to A (20.3 mmol/l: p < 0.001) and HD (20.7 mmol/l: p < 0.001) groups. Haemoglobin levels were significantly higher in the M

group (12.7 g/dl) compared to A (11.2 g/dl: $p = 0.050$) and HD (11.1 g/dl: $p = 0.010$) groups. The corrected calcium was significantly higher in the HD group (2.43 mmol/l) as compared to the M group (2.38 mmol/l: $p = 0.026$). Serum albumin was significantly lower in the HD group (37.6 g/L) compared to M (41.1 g/L: $p = 0.002$) and A (41.7 g/L: $p = 0.006$) groups. The HD group had significantly higher inflammation as reflected by higher median CRP (14 vs. 7: $p = 0.033$) as compared to the predialysis groups. Serum ferritin, folate and vitamin B₁₂ levels did not differ across the groups.

11.5.3 OPG and RANKL levels (Table 11.2)

The mean OPG levels in the HD group were significantly higher than the M (9.8 vs. 5.4 pmol/l: $p < 0.001$) and A (9.8 vs. 5.8 pmol/l: $p < 0.001$) groups. There were no significant differences in the median RANKL levels across the three groups. Overall, the OPG/RANKL ratio was significantly different across the groups ($p = 0.038$). The median OPG/RANKL ratio was higher in the HD group as compared to the A (109 vs. 45: $p = 0.016$) and M (109 vs. 61: $p = 0.066$) groups. In addition, OPG/RANKL ratio was significantly higher in the HD (109 vs. 53: $p = 0.014$) group as compared to the predialysis groups (M and A) taken together.

OPG levels were significantly higher in diabetic patients compared to non-diabetics in the M (6.1 vs. 4.5 pmol/l: $p = 0.032$) and A (6.7 vs. 4.6 pmol/l: $p = 0.032$) groups (Figure 11.1). The HD group demonstrated lower OPG levels in diabetics (8.6 vs. 10.9 pmol/l: $p = 0.079$), however it was not statistically significant. There was no difference in the RANKL and OPG/RANKL ratio across the three groups and also between diabetics and non-diabetics in the M, A and HD groups. In the whole group, OPG had

a significant correlation with PTH ($r = 0.439$: $p < 0.001$), haemoglobin ($r = -0.272$: $p = 0.023$), corrected calcium ($r = 0.289$: $p = 0.015$), diastolic blood pressure ($r = -0.391$: $p = 0.001$) and BMI ($r = -0.265$: $p = 0.027$). Median RANKL levels correlated significantly only with age ($r = -0.292$: $p = 0.014$). OPG/RANKL ratio did not correlate with any parameters in the study.

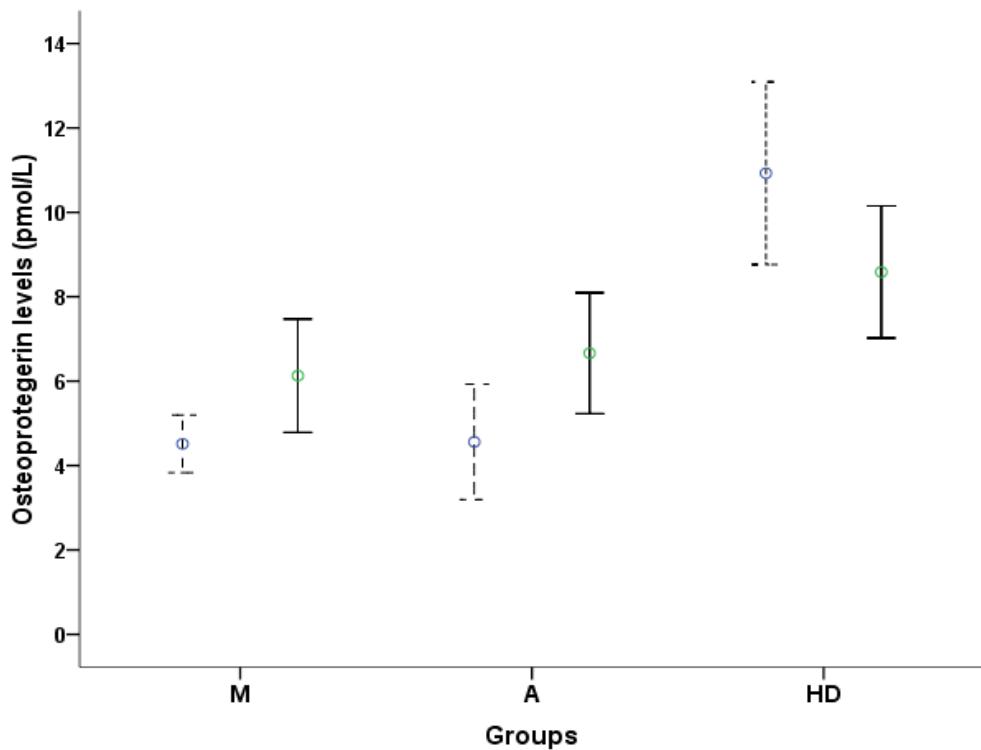


Figure 11.1 Osteoprotegerin levels in Diabetic and Non-diabetic CKD patients

Legend

CKD = Chronic kidney disease, M = Moderate CKD patients, A = Advanced CKD patients, HD = Haemodialysis, dotted lines represent non-diabetics, straight line represents diabetics.

11.5.4 Parathyroid hormone levels (Table 11.2)

The median PTH levels were significantly higher in the HD group compared to the M (19.1 vs. 3.2 pmol/l: $p < 0.001$) and A (19.1 vs. 5.5 pmol/: $p < 0.001$) groups. Serum PTH levels correlated significantly with OPG ($r = 0.439$: $p < 0.001$), haemoglobin ($r = -0.445$: $p < 0.001$), eGFR ($r = -0.322$: $p = 0.043$), diastolic blood pressure ($r = -0.375$: $p = 0.001$) and BMI ($r = -0.314$: $p = 0.008$).

11.5.5 Bone alkaline phosphatase levels (Table 11.2)

The mean BAP levels were significantly higher in the A group compared to the HD (29.4 vs. 24.0 IU/l: $p = 0.023$), but not in the M (29.4 vs. 26.2 IU/l: $p = \text{NS}$). In addition, BAP levels were significantly higher in the diabetic patients (27.5 vs. 20.9 IU/l: $p = 0.004$) as compared to non-diabetics in the HD group, but not in the other groups. BAP correlated significantly with albumin ($r = 0.363$: $p = 0.002$), calcium ($r = -0.340$: $p = 0.004$), cholesterol/HDL ratio ($r = 0.338$: $p = 0.005$), CRP ($r = -0.249$: $p = 0.038$) and diastolic blood pressure ($r = 0.292$: $p = 0.014$). In the diabetes groups BAP levels correlated significantly only with calcium ($r = -0.395$: $p = 0.017$).

Table 11.2 Biochemistry of OPG/RANKL study

Variables	Groups			Comparison of Groups		
	M	A	HD	M v A	M v HD	A v HD
				p-value	p-value	p-value
Glucose (mmol/l)	7.4 ± 4.5	8.1 ± 5.9	7.7 ± 4.3	NS	NS	NS
Creatinine (umol/l)	150 ± 29	288 ± 79	746 ± 229	0.024	<0.001	<0.001
eGFR (MDRD 4)	42 ± 18	20 ± 5	NA	<0.001	NA	NA
Urea (mmol/l)	10.3 ± 2.4	20.3 ± 6	20.7 ± 6	<0.001	<0.001	NS
Haemoglobin (g/dl)	12.7 ± 2.4	11.2 ± 2	11.1 ± 1.1	0.050	0.010	NS
CRP (mg/l)	5.0 (5,41)	5.0 (5,20)	5.5 (5,78)	NS	NS	0.027
Calcium(mmol/l)	2.38 ± 0.08	2.32 ± 0.09	2.43 ± 0.18	NS	NS	0.026
Phosphate (mmol/l)	1.11 ± 0.24	1.34 ± 0.25	1.6 ± 0.46	NS	<0.001	0.018
Bilirubin (umol/l)	7.6 ± 2.4	6.4 ± 1.1	6.4 ± 3.4	NS	NS	NS
Albumin (g/l)	41.1 ± 4.7	41.7 ± 4.7	37.6 ± 2.9	NS	0.006	0.002
BAP (IU/l)	26.2 ± 6.4	29.4 ± 7.3	24 ± 6.5	NS	NS	0.023
PTH (pmol/l)	4.4 (1.2, 18.3)	5.5 (1.2, 18.3)	19.1 (1.0, 43.7)	NS	<0.001	<0.001
OPG (pmol/l)	5.4 ± 1.76	5.78 ± 2.17	9.8 ± 3.67	NS	<0.001	<0.001
RANKL (pmol/l)	0.05 (0, 0.47)	0 (0, 0.42)	0.02 (0, 0.37)	NS	NS	NS
OPG/RANKL Ratio	61 (8, 550)	45 (14, 180)	109 (23,1450)	NS	NS	0.016

Legend

Baseline biochemical and haematological parameters in the three groups. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). The p value represents the statistical significance of changes as determined by ANOVA test. M = Moderate Chronic kidney disease, A = Advance chronic kidney disease, H = Haemodialysis, CRP = C - reactive protein, Co Calcium = corrected calcium, eGFR (ml/min/1.73m²) = estimated glomerular filtration rate calculated using MDRD 4 formula. BAP = Bone specific alkaline phosphatase, PTH = parathyroid hormone, OPG = Osteoprotegerin, RANKL = Receptor activator of Nuclear factor kappa B ligand.

11.6 Discussion

This prospective study examined the levels of markers of modulators of bone and vascular metabolism in non-diabetic patients with CKD and those with diabetic nephropathy in the predialysis and haemodialysis patients. The principal findings in this study are i) significant elevation in OPG levels with falling glomerular filtration rate ii) increased OPG/RANKL ratio with decline in renal function and iii) Compared to non-diabetics, OPG levels were significantly higher in diabetic patients in the predialysis phase, however levels were lower in the haemodialysis phase, but not significantly.

Chronic kidney disease is characterized by progressive deterioration of renal function, abnormalities of mineral and bone metabolism, and vascular complications such as vascular calcification (VC). ³⁹⁴ Although traditionally regarded as a passive process of vascular mineralization as a result of abnormalities of calcium and phosphorus metabolism, recent in-vitro studies demonstrating expression of bone matrix proteins by vascular smooth muscle cells in the vascular lumen, suggests the contrary.³⁹⁵ Vascular calcification is associated with cardiovascular mortality ³⁹⁶ and its frequency increases with progressive loss of kidney function.³⁹⁷

Several studies have suggested an association between vascular calcification and osteoporosis. ^{302, 398} The suggestion of association between these two pathological processes is based on observations of expression of osteoblast like cells in the vascular lumen.³⁰² An imbalance of common mediators of vascular and bone metabolism such as OPG/RANKL axis has been suggested to play a major role in the precipitation of vascular calcification.³¹⁶

Extensive VC with elevated levels of OPG has been reported in uncomplicated type 2 diabetes patients with DN,³⁹⁹ non-diabetic predialysis patients with CKD⁴⁰⁰ and haemodialysis patients.⁴⁰¹ However, there is dearth of literature on the association of biochemical markers of VC with progressive renal impairment in a single cohort. To my knowledge, this is the first study to report the OPG/RANKL activity in structured stages, throughout the spectrum of CKD and haemodialysis in patients with and without diabetes.

The OPG levels in the present study increased stepwise with deterioration in renal function. The haemodialysis patients had significantly higher OPG levels as compared to the moderate and advanced CKD patients. This is in agreement with previous studies, which have demonstrated elevated levels in various stages of CKD.^{399, 401, 402} OPG levels were significantly higher in the diabetic patients in the predialysis groups as compared to non-diabetics, however, OPG levels were lower in the diabetic HD patients.

OPG is secreted by the endothelial cells as a protective factor against the processes of VC in the blood milieu and vasculature. The low OPG levels in the diabetic HD group may be due to loss of endothelial cells or increased endothelial cell dysfunction as a result of chronic hyperglycaemia in a severely uremic milieu. This endothelial exhaustion needs further investigation. RANKL levels decreased serially with falling renal function in diabetics and non-diabetics. Several studies have suggested an increased risk of VC in patients with elevated levels of OPG^{381, 403} and reduced levels of RANKL.⁴⁰⁴

In view of the abnormalities of the OPG and RANKL levels, it is plausible that the present cohort may have associated VC and abnormal bone, which was not assessed in this study. As compared to the predialysis groups, the patients on haemodialysis had significantly higher OPG levels. OPG is associated with mortality in haemodialysis patients,⁴⁰¹ however, the interpretation of OPG levels in this group may be complex because not much is known about the clearance of OPG by haemodialysis and a greater accumulation of OPG in this group may result in reduced level of circulating RANKL in this population.⁴⁰¹ The disparity in OPG/RANKL levels in this group may contribute to the increased prevalence of VC in these patients.^{381, 405}

Median PTH levels were significantly higher in the haemodialysis group as compared to the moderate and advanced CKD groups. It has been reported that chronically elevated PTH promotes RANKL and suppresses OPG gene expression and increases the OPG/RANKL ratio.⁴⁰⁶ However, intermittent increase in serum PTH levels has an anabolic effect on bone, and intermittent PTH treatment prevents VC.⁴⁰⁷ In the present study PTH had a strong correlation with OPG and diastolic blood pressure, a surrogate measure of arterial stiffness, suggesting the close association of elevated PTH with modulators of mineral metabolism such as OPG and arterial stiffness.

The HD group had significantly higher calcium and phosphate levels as compared to the predialysis groups. An association of osteoporosis and VC has been seen in the elderly population in the absence of abnormal mineral metabolism.⁴⁰⁸ However, in patients with CKD, the presence of VC and abnormal bone metabolism is usually associated with abnormal levels of serum phosphate and calcium and derangement of endocrine and humoral pathways, including PTH and 1, 25-dihydroxyvitamin D.

Parathyroid hormone and 1, 25-dihydroxyvitamin D are important modulators of OPG/RANKL system³⁹³ and abnormalities of these hormones occurs early in the course of CKD, than previously thought.¹²² The early abnormalities of these hormones may orchestrate the events in the vascular milieu and the bone by the virtue of their influence on the mineral metabolism through the OPG/RANKL axis. In patients with end stage renal disease, an inverse association has been reported between bone activity and VC, independent of calcium load, age and other confounding factors suggesting a direct cell-to-cell interaction between the bone and the vessel, which may play an important role in the pathogenesis of VC in these patients.⁴⁰⁹

OPG and RANKL are common mediators of the bone and vascular milieu and may be the important interlocutor between these two systems. OPG has antagonist action against RANKL. In animal model experiments, it has been shown that OPG knockout mice develop severe calcification and suggesting that OPG may have a protective role against calcium deposition in the vessels.⁴⁰¹ Uraemia in CKD promotes the expression of calcification inhibitors such as OPG and matrix GLA protein.⁴¹⁰ The compensatory elevation in OPG levels may be enhanced in the presence of inflammation⁴⁰¹ and this could be one of the reasons of higher OPG in the haemodialysis group, which had a significantly higher CRP levels as compared to the predialysis groups.

It has been reported that amongst various modulators of mineral metabolism such as calcium, phosphate, PTH and OPG/RANKL axis, OPG levels were found to be the strongest predictor of all-cause mortality in haemodialysis patients, especially so in the presence of high inflammation.⁴⁰¹ Given the calcification burden and bone

abnormalities in patients with CKD, determination of OPG levels in this patient group may provide an early insight into the bone and vascular health of these patients.

The present study has few limitations, which need to be mentioned. First and foremost, this is a cross-sectional study and hence no causal relationships can be established. Secondly, there was no assessment of the extent of vascular calcification and bone mineral density in these patients. And finally, the OPG levels measured in this study was total OPG and it is difficult to comment upon the free and inactive forms of OPG. Nevertheless, this study demonstrates the presence of disturbed OPG/RANKL axis along with other parameters of mineral metabolism such as PTH, Calcium and phosphate in patients with different stages of CKD.

11.7 Conclusions

Prevalence of extensive vascular calcification and disturbances of bone metabolism is known in patients with CKD. Abnormalities of mineral metabolism occur early in the course of CKD and may provide the substrate for early VC and bone abnormalities in these patients. OPG/RANKL axis is an important mediator of changes occurring in the vasculature and the bone due to its modulatory action on the mineral metabolism. This axis is disturbed quite early in the course of CKD and OPG levels rise as a compensatory mechanism. This may help to prevent VC in patients with CKD.

OPG levels are higher in patients with DM in the pre-dialysis phase but lower in dialysis patients. This may reflect a failure of these protective mechanisms in the diabetic, severely uremic environment. Interestingly plasma OPG levels have been

associated with increased all-cause mortality in HD patients, though differences in diabetic and non-diabetic groups were not investigated. Assessment of OPG levels in patients with CKD may help to identify patients with disturbed mineral metabolism, who may be at risk of VC and abnormal bone health.

SECTION IV
CHAPTER 12
GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

12.1 Introduction

Diabetes Mellitus (DM) is now a recognized pandemic and treatment costs of DM and its complications are a major burden on healthcare systems throughout the world. Diabetic vasculopathy (DV) is the most important consequence of chronic hyperglycemia, in patients with DM. Clinically; DV is usually described into two main pathological entities as microvascular complications and macrovascular complications. In a patient with diabetes mellitus (DM), chronic hyperglycemia orchestrates a series of abnormalities leading to impaired function of virtually every organ in the body, as a result of, if not completely due to, vascular damage or vasculopathy (Chapter 1). Macrovasculopathy such as cardiovascular disease CVD and peripheral vascular disease (PWD) are the leading cause of mortality and morbidity, respectively in diabetes. On the other hand microvascular complications such as diabetic nephropathy (DN), diabetic retinopathy and diabetic neuropathy are common associations of macrovasculopathy and contribute to the co-morbid disease burden in DM.

This thesis discusses some facets of the pathogenesis of DV, which seem to precipitate from a complex interaction between the blood milieu, the vasculature, bone and mineral metabolism and kidney-related factors. Initially I carried out an extensive literature search to understand the current concepts of pathogenesis of macrovascular and microvascular diseases in DM and to identify gaps in literature. This process lead to the proposal of a number of hypotheses relating to various

facets of DV. Once this procedure was completed, a series of prospective studies were planned and executed to examine these hypotheses. The findings from the prospective studies were analysed and any new information obtained from the clinical studies was integrated into the current knowledge base and the significance of these findings were discussed. The information obtained from these studies has raised new questions, upon which future work might be based.

The results of the literature search have been presented in chapters 2 to 4. These original reviews suggest that the pathogenesis of microvascular and macrovascular complications of DV may be mediated by several biochemical modulators, which are common players in the blood milieu, the vasculature, bone and mineral metabolism and the kidney. An abnormality in one organ system or a modulator may be suggestive of abnormal metabolism of the other. Some of the potential modulators of DV are, erythropoietin (EPO), parathyroid hormone, 25 hydroxyvitamin D and 1, 25-dihydroxyvitamin D.

In addition to these modulators, there are some relatively new mediators, which seem to play an important role in the bone and vascular metabolism. These mediators such as osteoprotegerin (OPG) and receptor activator of nuclear factor kappa- β -ligand (RANKL) are important links in the cross talk between the bone and the blood vessel. The various hypothesis generated (chapter 5) were examined in the subsequent prospective studies.

12.2 Biological variation of potential modulators

The literature search suggested an important role of modulators (mentioned above), in the pathogenesis of DV. However, it soon dawned that there was no data in the literature on the normal biological variation of these modulators in the healthy population. In the absence of this information, it would be difficult to interpret the levels of these markers in the subsequent study cohort, which included patients with DM and chronic kidney disease (CKD). To define the normal biological variation of these modulators, a group of healthy volunteers were studied and serum samples were collected over a few weeks.

The findings from this study (chapter 7) revealed a wide biological variation of these modulators in the healthy population. In addition, the analytical variance and coefficient of variance of assays for these modulators were established. The wide variation in healthy individuals suggested the need for control groups to be incorporated in future studies to provide the context to allow interpretation of the results in different patient groups.

12.3 Tubular injury and dysfunction in early Diabetic nephropathy

The pathogenesis of DN a microvascular complication of DM is multifactorial, with many factors contributing to the initiation and progression of DN. Traditionally, DN is considered to be a glomerular disease; however, evidence from recent studies support a tubular origin (chapter 2). To examine the hypothesis, whether tubulointerstitial dysfunction occurs before the onset of microalbuminuria, a

measurement of the above mentioned parameters was carried out along with markers of tubulointerstitial injury such as N-acetyl- β -D-glucosaminidase (NAG) and Retinol-binding protein (RBP), in patients with type 1 and type 2 DM without microalbuminuria and in non-diabetic controls (chapter 8).

The study revealed that in comparison to non-diabetic healthy controls, a higher excretion of tubular injury markers such as NAG and RBP is present in both diabetes groups. In addition, low levels of the tubulointerstitial hormones, EPO and 1, 25-dihydroxyvitamin D, were demonstrated in the diabetes groups, compared to controls. There was no correlation between the level of tubular injury markers and the tubulointerstitial hormones, which could be possibly due to different the cellular locations of these two moieties. These abnormalities of tubular function, occurring before the onset of microalbuminuria, provide a strong indication of early tubular dysfunction and support the hypothesis of tubular origin for DM.

12.4 Erythropoietin therapy and tubular injury markers

Low levels of EPO were found, along with higher excretion of tubular injury markers, in patients with DM with normal haemoglobin. To examine the hypothesis that EPO therapy ameliorates tubular injury, a prospective study looking at the effects of EPO supplementation on tubular injury markers was carried out. It was considered that, given the current state of knowledge in this area, a trial of EPO supplementation in subjects with early DN and normal haemoglobin levels was premature. Instead a cohort of patients diabetic and non-diabetic, with stage 4 and 5 CKD was identified, who were about to have EPO therapy initiated to treat their anaemia (chapter 9).

The levels of tubular injury markers were assessed before and after three months of EPO therapy. We were unable to demonstrate an effect of EPO therapy on the markers of tubular injury in spite of a beneficial haematological response. This may be due to the advanced stage of CKD in these patients, which is characterised by a significant loss of functional nephrons and presence of atubular glomeruli. In absence of adequate tubular number, theoretically, there was limited scope for EPO action. However, there have been encouraging reports from recent experimental studies, which suggest that EPO therapy may confer reno-protection in patients with early and moderate CKD.

12.5 Vascular calcification and bone mineral density in diabetes

The pathogenesis of Macrovascular disease such as CVD and PVD in DM is complex with contribution from several associated factors. Vascular calcification (VC) is an integral part of macrovasculopathy. Traditionally, VC was considered to be associated with ageing, advanced renal disease and long duration of DM. Based on the literature review (chapter 4), it was proposed that the genesis of VC may be intricately entwined with endothelial cell dysfunction. To examine, whether VC and bone mineral density (BMD) was linked in patients with diabetes mellitus and their relationship to modulators of DV, an assessment of VC and BMD was undertaken in age and sex matched patients with type 2 DM who had well-preserved renal and varying degrees of proteinuria (chapter 10).

The assessment of VC was by CT scan and BMD by a DEXA scan. The modulators of DV were measured including serum OPG and RANKL. The study demonstrated a

high prevalence of VC and osteopenia in normoalbuminuric type 2 DM patients with normal serum creatinine. There was a weak inverse relationship between VC and osteopenia in these patients. The proteinuric patients had worse VC but not osteopenia and there were weak relationships between OPG levels with both, VC and osteopenia, masked by age in multivariate analysis. Age and peripheral neuropathy were independent predictors of VC, whereas, weight was the most significant predictor of BMD in these patients.

12.6 OPG/RANKL axis in Chronic Kidney Disease

The vasculature, blood milieu, bone and mineral metabolism, and the kidney are intricately linked with multiple common mediators and modulators working towards maintaining homeostasis in these systems. VC can occur on the background of normal mineral metabolism (chapter 10) and yet its severity is increased in presence of abnormal mineral metabolism. OPG/RANKL axis is a major modulator of this common pool of mineral metabolism in the bone and blood vessel. Against this background, the relationship between modulators of DV, including OPG and RANKL, and the degree of CKD was examined, in patients with and without DM.

It was found that abnormalities of OPG and RANKL occur before the onset of microalbuminuria (chapter 10) and progress with deterioration of renal function (chapter 11). In addition, there was a discrepancy in OPG levels in DM patients as compared to non-diabetics. In the predialysis phase, DM patients had significantly higher OPG levels, suggesting increased secretion of OPG by the endothelial cells as a protective mechanism against VC. However, DM patients on haemodialysis had

lower levels of OPG as compared to non-diabetics, suggesting a reduced response from endothelial cells, either due to extensive loss of endothelial cells or cellular exhaustion in the surviving endothelial cells. This phenomenon needs further investigation.

Along with abnormalities of PTH, phosphate and calcium levels, there was a significant rise in OPG/RANKL activity with falling renal function. The haemodialysis group had significantly higher OPG/RANKL ratio as compared to the predialysis groups. The study result supports the hypothesis of a major role of OPG/RANKL axis in the modulation of mineral metabolism in patients with CKD, with or without DM. The close relationship of OPG/RANKL activity with VC earlier and disturbed metabolism in the present study suggests a role for OPG estimation as a surrogate marker of VC in clinical practice. However, this needs further critical examination in bigger patient groups.

12.7 Future directions

The observations from the above-mentioned prospective studies have increased our understanding of the relationship of blood, bone and kidney in patients with Diabetes Mellitus (DM). Diabetic Vasculopathy (DV) is a huge subject and it is not practically possible to address all the issues concerned with the pathogenesis, progression, treatment and prevention of this condition. The results from these studies have led to further questions-which remain to be answered and may be the basis of future work in this field.

The focus of future work should be primarily aimed at prevention of complication studies in patients with DM before the onset of microalbuminuria. This is especially relevant in the Indian context, which has a burgeoning population of people with diabetes and little resources to deal with the diabetes epidemic. Given the genetic predisposition of the Indian population towards diabetes, there are several issues, which need to be addressed. However, based on the findings from the above-mentioned studies, the ones that need further work in near future are

- To study the relationship of a wide range of tubular injury markers such as NAG, RBP, Vimentin, Kidney injury Molecule (KIM-1) with EPO and 1, 25-dihydroxyvitamin D in a bigger groups of DM patients before the onset of microalbuminuria.
- To confirm the findings of low EPO and 1, 25-dihydroxyvitamin D in a large group of DM patients and the effects of supplementation of these two hormones on the progression of tubular injury.
- To study the effects of low dose ACEI (angiotensin converting enzyme inhibitors) and ARB (angiotensin receptor blockers) on patients with higher than normal excretion of tubular injury markers, in prevention of progression of tubular enzymuria to microalbuminuria.
- To study the protective potential of OPG in vascular calcification and the differences in this role between patients with progressive renal disease with and without DM.
- To study the role of peripheral neuropathy in the pathogenesis of VC.
- To study the effects of intensive versus conventional long-term glycemic control on OPG levels and severity of VC.

12.8 Conclusions

The interaction between the blood milieu, bone metabolism and the kidneys in the pathogenesis and progression of DV is complex and not well defined. The underlying factor is chronic hyperglycemia, which, in a concerted manner, precipitates several abnormalities, which in turn unleash a cascade of reactions, resulting in progressive vessel and ultimately organ damage in DM. The pathogenesis of DV is complex and the process begins earlier than previously thought. Microvascular and macrovascular disease do not occur in isolation. The presence of one is strongly suggestive of the presence of the other. Good glycemic control along with tight blood pressure control is of paramount importance in patients with DM and has been proven to retard the progression and reduce the risk of death due to macro and microvascular complications.⁴¹¹

In current medical practice, microalbuminuria is considered as the first clinical indicator of diabetic nephropathy. It is a strong marker of cardiovascular disease and is considered to be a state of generalised endothelial dysfunction. Unfortunately, by the time a patient has clinical microalbuminuria, there is significant loss of functional nephrons. ACEI and ARB therapy is known to reduce microalbuminuria and may retard the progression. However, however the disease may remain progressive and it is known that a significant proportion of patients with DM die of CVD, much before end stage renal failure. These observations are indirect suggestions that the prevention therapy in DM patients, especially those with type 2 DM, must begin in the pre-microalbuminuric stage.

The results from the tubular study with low EPO and 1, 25-dihydroxyvitamin D and higher excretion of tubular injury markers such as NAG and RBP are strongly suggestive of early tubular impairment before the onset of microalbuminuria. Similarly a significant proportion of type 2 DM with normoalbuminuria demonstrated early vascular calcification and osteopenia. These observations suggest that microalbuminuria may occur later in the course of vascular damage in DM, than previously thought, and there is an urgent need to devise and develop clinical markers of vascular damage in DM before the onset of microalbuminuria.

Diabetic vasculopathy is a progressive phenomenon with contributions from multiple metabolic derangements related to chronic hyperglycemia. The gap between the diagnosis of DM and the onset microalbuminuria can be regarded as a ‘window of opportunity’ for clinicians and researchers to contain the damage and prevent the progression of diabetic vasculopathy. Further work is required to untangle these complexities and to define the contribution of factors such as the adverse blood milieu, the vasculature, abnormal bone and mineral metabolism, and early tubulointerstitial damage.

These pilot studies are invaluable in terms of testing a hypothesis and to understand a trend. However, these studies have a number of limitations, which need to be considered. Due to small patient numbers and cross sectional design, the studies need to be carried out in bigger population to confirm the findings. In addition, long-term, follow-up studies are required to understand the progress of the various disease processes and the evaluation of the potential modulators of DV.

The findings from the studies reported here may help in the formulation of new hypotheses, which might contribute to future work in this area. Until definitive therapies aimed at prevention and treatments of DV are available, research proven, combined interventions such as appropriate diet and exercise, healthy lifestyle with cessation of smoking and alcohol, tight glycemic control, optimum blood pressure, correction of dyslipidemia and anaemia remain the mainstay for prevention of DV in these patients.

SECTION V

REFERENCES

Reference List

- (1) Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998 July;15(7):539-553.
- (2) King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 1998 September 1;21(9):1414-1431.
- (3) Sicree R SJZP. Diabetes and impaired glucose tolerance.: Diabetes Atlas, International Diabetes Federation; 2006 15-103.
- (4) Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes: Indian scenario. *Indian J Med Res* 2007 March;125(3):217-230.
- (5) Unnikrishnan R, Rema M, Pradeepa R, Deepa M, Shanthirani CS, Deepa R, Mohan V. Prevalence and Risk Factors of Diabetic Nephropathy in an Urban South Indian Population. *Diabetes Care* 2007 August;30(8):2019-2024.
- (6) Patlak M. New weapons to combat an ancient disease: treating diabetes. *FASEB J* 2002 December;16(14):1853.
- (7) Minkowski O. Historical development of the theory of pancreatic diabetes by Oscar Minkowski, 1929: introduction and translation by Rachmiel Levine. *Diabetes* 1989 January;38(1):1-6.
- (8) Banting FG et al. Pancreatic extracts in the treatment of diabetes mellitus: preliminary report. 1922. *CMAJ* 1991 November 15;(145)(10):1281-1286.
- (9) Himsworth H. *Diabetes mellitus: its differentiation into insulin-sensitive and insulin-insensitive types*. *The Lancet* 1936;227(5864).
- (10) WHO Expert Committee on Diabetes Mellitus: second report. *World Health Organ Tech Rep Ser* 1980;646:1-80.
- (11) *Diabetes Mellitus: Report of a WHO Study Group*. Geneva: WHO, WHO, (1985).
- (12) Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2004 January 1;27(90001):S5-10.
- (13) Foulis AK, McGill M, Farquharson MA. Insulitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes, and interferon-gamma containing cells. *J Pathol* 1991 October;165(2):97-103.

- (14) Gillespie KM. Type 1 diabetes: pathogenesis and prevention. *Canadian Medical Association Journal* 2006 July 18;175(2):165-170.
- (15) Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet* 2000 March 11;355(9207):873-876.
- (16) Devendra D EG. Immunologic endocrine disorders. *J Allergy Clin Immunol* 2003;(111(2 Suppl)):S624-636.
- (17) Hyoti H. Enterovirus infections and type 1 diabetes. *Annals Of Medicine* 2002;34(3):138.
- (18) Honeyman MC, Coulson BS, Stone NL, Gellert SA, Goldwater PN, Steele CE, Couper JJ, Tait BD, Colman PG, Harrison LC. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* 2000 August 1;49(8):1319-1324.
- (19) Ginsberg-Fellner F, Witt ME, Fedun B, Taub F, Dobersen MJ, McEvoy RC, Cooper LZ, Notkins AL, Rubinstein P. Diabetes mellitus and autoimmunity in patients with the congenital rubella syndrome. *Rev Infect Dis* 1985 March;7 Suppl 1:S170-S176.
- (20) Baekkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S. Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis. Unambiguous identification of 64K target antigen. *Diabetes* 1989 September 1;38(9):1133-1141.
- (21) Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proceedings of the National Academy of Sciences of the United States of America* 1996 June 25;93(13):6367-6370.
- (22) Palmer JP. Insulin autoantibodies: their role in the pathogenesis of IDDM. *Diabetes Metab Rev* 1987 October;3(4):1005-1015.
- (23) Vandewalle CL, Decraene T, Schuit FC, De L, I, Pipeleers DG, Goris FK. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. The Belgian Diabetes Registry. *Diabetologia* 1993 November;36(11):1155-1162.
- (24) Goris FK, Goubert P, Semakula C, Vandewalle CL, De SJ, Scheen A, Christie MR, Pipeleers DG. IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry. *Diabetologia* 1997 January;40(1):95-99.

- (25) Decochez K, Tits J, Coolens JL, Van Gaal L, Krzentowski G, Winnock F, Anckaert E, Weets I, Pipeleers DG, Gorus FK. High frequency of persisting or increasing islet-specific autoantibody levels after diagnosis of type 1 diabetes presenting before 40 years of age. *The Belgian Diabetes Registry*. *Diabetes Care* 2000 June 1;23(6):838-844.
- (26) Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA. Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 1994 April;11(3):299-303.
- (27) Humphrey AR, McCarty DJ, Mackay IR, Rowley MJ, Dwyer T, Zimmet P. Autoantibodies to glutamic acid decarboxylase and phenotypic features associated with early insulin treatment in individuals with adult-onset diabetes mellitus. *Diabet Med* 1998 February;15(2):113-119.
- (28) Betterle C, Zanette F, Pedini B, Presotto F, Rapp LB, Monciotti CM, Rigon F. Clinical and subclinical organ-specific autoimmune manifestations in type 1 (insulin-dependent) diabetic patients and their first-degree relatives. *Diabetologia* 1984 June;26(6):431-436.
- (29) Mooy JM, Grootenhuis PA, de Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care* 1995 September 1;18(9):1270-1273.
- (30) Knowler WC, Saad MF, Pettitt DJ, Nelson RG, Bennett PH. Determinants of diabetes mellitus in the Pima Indians. *Diabetes Care* 1993 January 1;16(1):216-227.
- (31) Shai I, Jiang R, Manson JE, Stampfer MJ, Willett WC, Colditz GA, Hu FB. Ethnicity, Obesity, and Risk of Type 2 Diabetes in Women: A 20-year follow-up study. *Diabetes Care* 2006 July 1;29(7):1585-1590.
- (32) Li H, Isomaa B, Taskinen MR, Groop L, Tuomi T. Consequences of a family history of type 1 and type 2 diabetes on the phenotype of patients with type 2 diabetes. *Diabetes Care* 2000 May 1;23(5):589-594.
- (33) Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS. Prevalence of Obesity, Diabetes, and Obesity-Related Health Risk Factors, 2001. *JAMA* 2003 January 1;289(1):76-79.
- (34) Helmrich SP, Ragland DR, Leung RW, Paffenbarger RS. Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus. *N Engl J Med* 1991 July 18;325(3):147-152.
- (35) England LJ, Levine RJ, Qian C, Soule LM, Schisterman EF, Yu KF, Catalano PM. Glucose Tolerance and Risk of Gestational Diabetes Mellitus in Nulliparous Women Who Smoke during Pregnancy. *Am J Epidemiol* 2004 December 15;160(12):1205-1213.

- (36) Das UN. Risk of type 2 diabetes mellitus in those with hypertension. *Eur Heart J* 2008 February 26;ehn037.
- (37) Willi C, Bodenmann P, Ghali WA, Faris PD, Cornuz J. Active Smoking and the Risk of Type 2 Diabetes: A Systematic Review and Meta-analysis. *JAMA* 2007 December 12;298(22):2654-2664.
- (38) Haffner SM. Management of dyslipidemia in adults with diabetes. *Diabetes Care* 1998 January 1;21(1):160-178.
- (39) Choi B C K SF. Risk factors for diabetes mellitus by age and sex: results of the National Population Health Survey. *Diabetologia* 2001 October;44(10):1221-1231.
- (40) Campbell PJ, Carlson MG. Impact of obesity on insulin action in NIDDM. *Diabetes* 1993 March 1;42(3):405-410.
- (41) Mahler RJ, Adler ML. Type 2 Diabetes Mellitus: Update on Diagnosis, Pathophysiology, and Treatment. *J Clin Endocrinol Metab* 1999 April 1;84(4):1165-1171.
- (42) Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988 December 1;37(12):1595-1607.
- (43) Charles MA EETNCJWJRGGJBB. The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study. *Diabetologia* 1997 September;40(9):1101-1106.
- (44) Beck-Nielsen H, Vaag A, Damsbo P, Handberg A, Nielsen OH, Henriksen JE, Thye-Ronn P. Insulin resistance in skeletal muscles in patients with NIDDM. *Diabetes Care* 1992 March 1;15(3):418-429.
- (45) Kahn SE, Montgomery B, Howell W, Ligueros-Saylan M, Hsu CH, Devineni D, McLeod JF, Horowitz A, Foley JE. Importance of Early Phase Insulin Secretion to Intravenous Glucose Tolerance in Subjects with Type 2 Diabetes Mellitus. *J Clin Endocrinol Metab* 2001 December 1;86(12):5824-5829.
- (46) Jonsson B. Revealing the cost of Type II diabetes in Europe. *Diabetologia* 2002 July;45(7):S5-12.
- (47) WHO publishes definitive atlas on global heart disease and stroke epidemic. *Indian J Med Sci* 2004 September;58(9):405-406.
- (48) Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998 July 23;339(4):229-234.
- (49) Pyorala K, Pedersen TR, Kjekshus J, Faergeman O, Olsson AG, Thorsteinsson G. Cholesterol lowering with simvastatin improves

- prognosis of diabetic patients with coronary heart disease. A subgroup analysis of the Scandinavian Simvastatin Survival Study (4S). *Diabetes Care* 1997 April;20(4):614-620.
- (50) Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators. *Lancet* 2000 January 22;355(9200):253-259.
- (51) Anderson KM, Wilson PW, Odell PM, Kannel WB. An updated coronary risk profile. A statement for health professionals. *Circulation* 1991 January;83(1):356-362.
- (52) Assmann G, Cullen P, Jossa F, Lewis B, Mancini M. Coronary heart disease: reducing the risk: the scientific background to primary and secondary prevention of coronary heart disease. A worldwide view. International Task force for the Prevention of Coronary Heart disease. *Arterioscler Thromb Vasc Biol* 1999 August;19(8):1819-1824.
- (53) Grover SA, Paquet S, Levinton C, Coupal L, Zowall H. Estimating the benefits of modifying risk factors of cardiovascular disease: a comparison of primary vs secondary prevention. *Arch Intern Med* 1998 March 23;158(6):655-662.
- (54) Stevens RJ, Kothari V, Adler AI, Stratton IM. The UKPDS risk engine: a model for the risk of coronary heart disease in Type II diabetes (UKPDS 56). *Clin Sci (Lond)* 2001 December;101(6):671-679.
- (55) Lehto S, Ronnemaa T, Pyorala K, Laakso M. Poor glycemic control predicts coronary heart disease events in patients with type 1 diabetes without nephropathy. *Arterioscler Thromb Vasc Biol* 1999 April;19(4):1014-1019.
- (56) Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. *Diabetes* 1999 May;48(5):937-942.
- (57) Marshall SM, Flyvbjerg A. Prevention and early detection of vascular complications of diabetes. *BMJ* 2006 September 2;333(7566):475-480.
- (58) Phillips AO, Steadman R. Diabetic nephropathy: The central role of renal proximal tubular cells in tubulointerstitial injury. *Histology and Histopathology* 2002;17(1):247-252.
- (59) Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. *Diabetes* 1983 May;32 Suppl 2:64-78.
- (60) Hasslacher C, Ritz E, Wahl P, Michael C. Similar Risks of Nephropathy in Patients with Type-I Or Type-II Diabetes-Mellitus. *Nephrol Dial Transplant* 1989;4(10):859-863.

- (61) Wolf G, Reinking R, Zahner G, Stahl RA, Shankland SJ. Erk 1,2 phosphorylates p27(Kip1): Functional evidence for a role in high glucose-induced hypertrophy of mesangial cells. *Diabetologia* 2003 August;46(8):1090-1099.
- (62) Kim Y, Kleppel MM, Butkowski R, Mauer SM, Wieslander J, Michael AF. Differential expression of basement membrane collagen chains in diabetic nephropathy. *Am J Pathol* 1991 February;138(2):413-420.
- (63) Brito PL, Fioretto P, Drummond K, Kim Y, Steffes MW, Basgen JM, Sisson-Ross S, Mauer M. Proximal tubular basement membrane width in insulin-dependent diabetes mellitus. *Kidney Int* 1998 March;53(3):754-761.
- (64) Katz A, Caramori ML, Sisson-Ross S, Groppoli T, Basgen JM, Mauer M. An increase in the cell component of the cortical interstitium antedates interstitial fibrosis in type 1 diabetic patients. *Kidney Int* 2002 June;61(6):2058-2066.
- (65) Alsaad KO, Herzenberg AM. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update. *J Clin Pathol* 2007 January;60(1):18-26.
- (66) Harris RD, Steffes MW, Bilous RW, Sutherland DE, Mauer SM. Global glomerular sclerosis and glomerular arteriolar hyalinosis in insulin dependent diabetes. *Kidney Int* 1991 July;40(1):107-114.
- (67) Dalla VM, Saller A, Bortoloso E, Mauer M, Fioretto P. Structural involvement in type 1 and type 2 diabetic nephropathy. *Diabetes Metab* 2000 July;26 Suppl 4:8-14.
- (68) Mauer SM, Steffes MW, Ellis EN, Sutherland DER, Brown DM, Goetz FC. Structural-Functional Relationships in Diabetic Nephropathy. *Journal of Clinical Investigation* 1984;74(4):1143-1155.
- (69) Gilbert RE, Cooper ME. The tubulointerstitium in progressive diabetic kidney disease: More than an aftermath of glomerular injury? *Kidney International* 1999;56(5):1627-1637.
- (70) Gekle M. Renal Proximal Tubular Albumin Reabsorption: Daily Prevention of Albuminuria. *News Physiol Sci* 1998 February;13:5-11.
- (71) Bourdeau JE, Carone FA. Protein handling by the renal tubule. *Nephron* 1974;13(1):22-34.
- (72) Abbate M, Remuzzi G. Novel mechanism(s) implicated in tubular albumin reabsorption and handling. *Am J Kidney Dis* 2001 July;38(1):196-204.
- (73) Burton C, Harris KP. The role of proteinuria in the progression of chronic renal failure. *Am J Kidney Dis* 1996 June;27(6):765-775.

- (74) Basturk T, Altuntas Y, Kurklu A, Aydin L, Eren N, Unsal A. Urinary N-acetyl B glucosaminidase as an earlier marker of diabetic nephropathy and influence of low-dose perindopril/indapamide combination. *Renal Failure* 2006;28(2):125-128.
- (75) Topping MD, Forster HW, Dolman C, Luczynska CM, Bernard AM. Measurement of urinary retinol-binding protein by enzyme-linked immunosorbent assay, and its application to detection of tubular proteinuria. *Clin Chem* 1986 October;32(10):1863-1866.
- (76) Gibb DM, Tomlinson PA, Dalton NR, Turner C, Shah V, Barratt TM. Renal tubular proteinuria and microalbuminuria in diabetic patients. *Arch Dis Child* 1989 January;64(1):129-134.
- (77) Ikenaga H, Suzuki H, Ishii N, Itoh H, Saruta T. Enzymuria in Non-Insulin-Dependent Diabetic-Patients - Signs of Tubular Cell Dysfunction. *Clinical Science* 1993;84(4):469-475.
- (78) Thomas MC, Tsalamandris C, MacIsaac R, Jerums G. Functional erythropoietin deficiency in patients with Type 2 diabetes and anaemia. *Diabetic Medicine* 2006;23(5):502-509.
- (79) Jones SC, Saunders HJ, Pollock CA. High glucose increases growth and collagen synthesis in cultured human tubulointerstitial cells. *Diabetic Medicine* 1999;16(11):932-938.
- (80) Phillips AO, Topley N, Steadman R, Morrisey K, Williams JD. Induction of TGF-beta 1 synthesis in D-glucose primed human proximal tubular cells by IL-1 beta and TNF alpha. *Kidney International* 1996;50(5):1546-1554.
- (81) Choi YJ, Chakraborty S, Nguyen V, Nguyen C, Kim BK, Shim SI, Suki WN, Truong LD. Peritubular capillary loss is associated with chronic tubulointerstitial injury in human kidney: Altered expression of vascular endothelial growth factor. *Human Pathology* 2000;31(12):1491-1497.
- (82) Kang DH, Kanellis J, Hugo C, Truong L, Anderson S, Kerjaschki D, Schreiner GF, Johnson RJ. Role of the microvascular endothelium in progressive renal disease. *J Am Soc Nephrol* 2002;13(3).
- (83) Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991 April 1;40(4):405-412.
- (84) Palm F, Cederberg J, Hansell P, Liss P, Carlsson PO. Reactive oxygen species cause diabetes-induced decrease in renal oxygen tension. *Diabetologia* 2003;46(8):1153-1160.
- (85) Buerk DG. Can we model nitric oxide biotransport? A survey of mathematical models for a simple diatomic molecule with surprisingly complex biological activities. *Annual Review of Biomedical Engineering* 2001;3:109-143.

- (86) Palm F, Buerk DG, Carlsson PO, Hansell P, Liss P. Reduced nitric oxide concentration in the renal cortex of streptozotocin-induced diabetic rats - Effects on renal oxygenation and microcirculation. *Diabetes* 2005;54(11):3282-3287.
- (87) Komers R, Anderson S. Paradoxes of nitric oxide in the diabetic kidney. *American Journal of Physiology-Renal Physiology* 2003;284(6):F1121-F1137.
- (88) Palm F, Hansell P, Ronquist G, Waldenstrom A, Liss P, Carlsson PO. Polyol-pathway-dependent disturbances in renal medullary metabolism in experimental insulin-deficient diabetes mellitus in rats. *Diabetologia* 2004;47(7):1223-1231.
- (89) Palm F. Intrarenal oxygen in diabetes and a possible link to diabetic nephropathy. *Clinical and Experimental Pharmacology and Physiology* 2006;33(10):997-1001.
- (90) Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S. HIF activation by pH-dependent nucleolar sequestration of VHL. *Nature Cell Biology* 2004;6(7):642-647.
- (91) Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am J Physiol Renal Physiol* 2004 December 1;287(6):F1223-F1232.
- (92) Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 alpha. *Genes & Development* 1998;12(2):149-162.
- (93) Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E. Role of HIF-1 alpha or in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 1998;394(6692):485-490.
- (94) Maxwell P. HIF-1: An oxygen response system with special relevance to the kidney. *J Am Soc Nephrol* 2003;14(11):2712-2722.
- (95) Norman JT, Clark IM, Garcia PL. Hypoxia promotes fibrogenesis in human renal fibroblasts. *Kidney International* 2000;58(6):2351-2366.
- (96) Zhong Z, Arteel GE, Connor HD, Yin M, Frankenberg MV, Stachlewitz RF, Raleigh JA, Mason RP, Thurman RG. Cyclosporin A increases hypoxia and free radical production in rat kidneys: prevention by dietary glycine. *American Journal of Physiology-Renal Physiology* 1998;44(4):F595-F604.
- (97) Jones RL, Peterson CM. Hematologic Alterations in Diabetes-Mellitus. *American Journal of Medicine* 1981;70(2):339-352.

- (98) Robey C, Dasmahapatra A, Cohen MP, Suarez S. Sorbinil partially prevents decreased erythrocyte deformability in experimental diabetes mellitus. *Diabetes* 1987 September 1;36(9):1010-1013.
- (99) Kowluru R, Bitensky MW, Kowluru A, Dembo M, Keaton PA, Buican T. Reversible Sodium-Pump Defect and Swelling in the Diabetic Rat Erythrocyte - Effects on Filterability and Implications for Microangiopathy. *Proceedings of the National Academy of Sciences of the United States of America* 1989;86(9):3327-3331.
- (100) Testa I, Rabini RA, Fumelli P, Bertoli E, Mazzanti L. Abnormal Membrane Fluidity and Acetylcholinesterase Activity in Erythrocytes from Insulin-Dependent Diabetic-Patients. *Journal of Clinical Endocrinology and Metabolism* 1988;67(6):1129-1133.
- (101) Bosman DR, Winkler AS, Marsden JT, Macdougall IC, Watkins PJ. Anemia with erythropoietin deficiency occurs early in diabetic nephropathy. *Diabetes Care* 2001;24(3):495-499.
- (102) Fioretto P, Mauer M, Brocco E, Velussi M, Frigato F, Muollo B, Sambataro M, Abaterusso C, Baggio B, Crepaldi G, Nosadini R. Patterns of renal injury in NIDDM patients with microalbuminuria. *Diabetologia* 1996;39(12):1569-1576.
- (103) Winkler AS, Marsden J, Chaudhuri KR, Hambley H, Watkins PJ. Erythropoietin depletion and anaemia in diabetes mellitus. *Diabetic Medicine* 1999;16(10):813-819.
- (104) Bosman DR, Osborne CA, Marsden JT, Macdougall IC, Gardner WN, Watkins PJ. Erythropoietin response to hypoxia in patients with diabetic autonomic neuropathy and non-diabetic chronic renal failure. *Diabetic Medicine* 2002;19(1):65-69.
- (105) Capla JM, Grogan RH, Callaghan MJ, Galiano RD, Tepper OM, Ceradini DJ, Gurtner GC. Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. *Plastic and Reconstructive Surgery* 2007;119(1):59-70.
- (106) Katavetin P, Miyata T, Inagi R, Tanaka T, Sassa R, Ingelfinger JR, Fujita T, Nangaku M. High glucose blunts vascular endothelial growth factor response to hypoxia via the oxidative stress-regulated hypoxia-inducible factor/hypoxia-responsible element pathway. *J Am Soc Nephrol* 2006;17(5):1405-1413.
- (107) Kunika K, Yamaoka T, Itakura M. Damage of charge-dependent renal tubular reabsorption causes diabetic microproteinuria. *Diabetes Research and Clinical Practice* 1997;36(1):1-9.
- (108) Yaqoob M, McClelland P, Patrick AW, Stevenson A, Mason H, White MC, Bell GM. Evidence of Oxidant Injury and Tubular Damage in Early Diabetic Nephropathy. *Quarterly Journal of Medicine* 1994;87(10):601-607.

- (109) Yaqoob M, Patrick AW, McClelland P, Stevenson A, Mason H, White MC, Bell GM. Relationship Between Markers of Endothelial Dysfunction, Oxidant Injury and Tubular Damage in Patients with Insulin-Dependent Diabetes-Mellitus. *Clinical Science* 1993;85(5):557-562.
- (110) Thomson SC, Vallon V, Blantz RC. Kidney function in early diabetes: the tubular hypothesis of glomerular filtration. *Am J Physiol Renal Physiol* 2004 January 1;286(1):F8-15.
- (111) Evangelista C, Rizzo M, Cantone A, Corbo G, Di DL, Trocino C, Zacchia M, Capasso G. [Glomerulo-tubular balance in diabetes mellitus: molecular evidence and clinical consequences]. *G Ital Nefrol* 2006 January;23 Suppl 34:S16-S20.
- (112) Vallon V, Blantz RC, Thomson S. Glomerular hyperfiltration and the salt paradox in early type 1 diabetes mellitus: A tubulo-centric view. *J Am Soc Nephrol* 2003;14(2):530-537.
- (113) Patel KP. Volume reflex in diabetes. *Cardiovascular Research* 1997;34(1):81-90.
- (114) Ditzel J, Lervang HH, Brochnermortensen J. Renal Sodium-Metabolism in Relation to Hypertension in Diabetes. *Diabetes & Metabolism* 1989;15(5BIS):292-295.
- (115) Quinones-Galvan A, Ferrannini E. Renal effects of insulin in man. *Journal of Nephrology* 1997;10(4):188-191.
- (116) Vervoort G, Veldman B, Berden JHM, Smits P, Wetzels JFM. Glomerular hyperfiltration in type 1 diabetes mellitus results from primary changes in proximal tubular sodium handling without changes in volume expansion. *European Journal of Clinical Investigation* 2005;35(5):330-336.
- (117) Mahnensmith RL, Aronson PS. Interrelationships Among Quinidine, Amiloride, and Lithium As Inhibitors of the Renal Na⁺-H⁺ Exchanger. *J Biol Chem* 1985;260(23):2586-2592.
- (118) Krolewski AS, Canessa M, Warram JH, Laffel LMB, Christlieb AR, Knowler WC, Rand LI. Predisposition to Hypertension and Susceptibility to Renal-Disease in Insulin-Dependent Diabetes-Mellitus. *New England Journal of Medicine* 1988;318(3):140-145.
- (119) Pedersen SB, Flyvbjerg A, Richelsen B. Inhibition of Renal Ornithine Decarboxylase Activity Prevents Kidney Hypertrophy in Experimental Diabetes. *American Journal of Physiology* 1993;264(2):C453-C456.
- (120) Thomson SC, Vallon V, Blantz RC. Kidney function in early diabetes: the tubular hypothesis of glomerular filtration. *Am J Physiol Renal Physiol* 2004 January 1;286(1):F8-15.

- (121) Tian J, Liu YH, Williams LA, de Zeeuw D. Potential role of active vitamin D in retarding the progression of chronic kidney disease. *Nephrol Dial Transplant* 2007;22(2):321-328.
- (122) Levin A, Bakris GL, Molitch M, Smulders M, Tian J, Williams LA, Andress DL. Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: Results of the study to evaluate early kidney disease. *Kidney International* 2007;71(1):31-38.
- (123) Wang Y, Zhou J, Minto AW, Hack BK, Alexander JJ, Haas M, Li YC, Heilig CW, Quigg RJ. Altered vitamin D metabolism in type II diabetic mouse glomeruli may provide protection from diabetic nephropathy. *Kidney International* 2006;70(5):882-891.
- (124) Holick MF. Resurrection of vitamin D deficiency and rickets. *J Clin Invest* 2006 August;116(8):2062-2072.
- (125) Weinreich T, Merke J, Schonemark M, Reichel H, Diebold M, Hansch GM, Ritz E. Actions of 1,25-Dihydroxyvitamin D₃ on Human Mesangial Cells. *American Journal of Kidney Diseases* 1991;18(3):359-366.
- (126) Weinreich T, Muller A, Wuthrich RP, Booy C, Binswanger U. 1,25-dihydroxyvitamin D-3 and the synthetic: Vitamin D analogue, KH 1060, modulate the growth of mouse proximal tubular cells. *Kidney & Blood Pressure Research* 1996;19(6):325-331.
- (127) Makibayashi K, Tatematsu M, Hirata M, Fukushima N, Kusano K, Ohashi S, Abe H, Kuze K, Fukatsu A, Kita T, Doi T. A vitamin D analog ameliorates glomerular injury on rat glomerulonephritis. *American Journal of Pathology* 2001;158(5):1733-1741.
- (128) Li YC, Kong J, Wei MJ, Chen ZF, Liu SQ, Cao LP. 1,25-Dihydroxyvitamin D-3 is a negative endocrine regulator of the renin-angiotensin system. *Journal of Clinical Investigation* 2002;110(2):229-238.
- (129) Xiang W, Kong J, Chen S, Cao LP, Qiao G, Zheng W, Liu W, Li X, Gardner DG, Li YC. Cardiac hypertrophy in vitamin D receptor knockout mice: role of the systemic and cardiac renin-angiotensin systems. *Am J Physiol Endocrinol Metab* 2005 January 1;288(1):E125-E132.
- (130) Gekle M. Renal tubule albumin transport. *Annual Review of Physiology* 2005;67:573-594.
- (131) Fine LG, Orphanides C, Norman JT. Progressive renal disease: the chronic hypoxia hypothesis. *Kidney Int Suppl* 1998 April;65:S74-S78.
- (132) Fine LG, Bandyopadhyay D, Norman JT. Is there a common mechanism for the progression of different types of renal diseases other than proteinuria? Towards the unifying theme of chronic hypoxia. *Kidney Int* 2000 April;57(S75):S22-S26.

- (133) Nangaku M. Hypoxia and tubulointerstitial injury: A final common pathway to end-stage renal failure. *Nephron Experimental Nephrology* 2004;98(1):E8-E12.
- (134) Shih SC, Claffey KP. Hypoxia-mediated regulation of gene expression in mammalian cells. *International Journal of Experimental Pathology* 1998;79(6):347-357.
- (135) Futrakul N, Vongthavarawat V, Sirisalipotch S, Chairatanarat T, Futrakul P, Suwanwalaikorn S. Tubular dysfunction and hemodynamic alteration in normoalbuminuric type 2 diabetes. *Clinical Hemorheology and Microcirculation* 2005;32(1):59-65.
- (136) Allen DA, Harwood SM, Varagunam M, Raftery MJ, Yaqoob MM. High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *Faseb Journal* 2003;17(3):908-+.
- (137) Gross AW, Lodish HF. Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). *J Biol Chem* 2006;281(4):2024-2032.
- (138) Chapel SH, Veng-Pedersen P, Schmidt RL, Widness JA. Receptor-based model accounts for phlebotomy-induced changes in erythropoietin pharmacokinetics. *Experimental Hematology* 2001;29(4):425-431.
- (139) Symeonidis A, Kouraklis-Symeonidis A, Psiroyiannis A, Leotsinidis M, Kyriazopoulou V, Vassilakos P, Vagenakis A, Zoumbos N. Inappropriately low erythropoietin response for the degree of anemia in patients with noninsulin-dependent diabetes mellitus. *Annals of Hematology* 2006;85(2):79-85.
- (140) Craig KJ, Williams JD, Riley SG, Smith H, Owens DR, Worthing D, Cavill I, Phillips AO. Anemia and diabetes in the absence of nephropathy. *Diabetes Care* 2005;28(5):1118-1123.
- (141) Laffont I, Shubaev VV, Briand O, Lestavel S, Barbier A, Taniguchi N, Fruchart JC, Clavey V, Siest G. Early-glycation of apolipoprotein E: effect on its binding to LDL receptor, scavenger receptor A and heparan sulfates. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* 2002;1583(1):99-107.
- (142) Dikow R, Schwenger V, Schomig M, Ritz E. How should we manage anaemia in patients with diabetes? *Nephrol Dial Transplant* 2002 January 1;17(suppl_1):67-72.
- (143) Kaysen GA. The microinflammatory state in uremia: Causes and potential consequences. *J Am Soc Nephrol* 2001;12(7):1549-1557.
- (144) Goicoechea M, Martin J, De Sequera P, Quiroga JA, Ortiz A, Carreno V, Caramelo C. Role of cytokines in the response to erythropoietin in hemodialysis patients. *Kidney International* 1998;54(4):1337-1343.

- (145) Zanjani ED, Mcglave PB, Davies SF, Banisadre M, Kaplan ME, Sarosi GA. Invitro Suppression of Erythropoiesis by Bone-Marrow Adherent Cells from Some Patients with Fungal Infection. *British Journal of Haematology* 1982;50(3):479-490.
- (146) Zhang Z, Sun L, Wang Y, Ning G, Minto AW, Kong J, Quigg RJ, Li YC. Renoprotective role of the vitamin D receptor in diabetic nephropathy. *Kidney International* 2008;73(2):163-171.
- (147) Krantz SB. Erythropoietin. *Blood* 1991;77(3):419-434.
- (148) Anagnostou A, Liu ZY, Steiner M, Chin K, Lee ES, Kessimian N, Noguchi CT. Erythropoietin Receptor Messenger-Rna Expression in Human Endothelial-Cells. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91(9):3974-3978.
- (149) Vogel V, Kramer HJ, Backer A, MeyerLehnert H, Jelkmann W, Fandrey J. Effects of erythropoietin on endothelin-1 synthesis and the cellular calcium messenger system in vascular endothelial cells. *American Journal of Hypertension* 1997;10(3):289-296.
- (150) Gouva C, Nikolopoulos P, Ioannidis JPA, Siamopoulos KC. Treating anemia early in renal failure patients slows the decline of renal function: A randomized controlled trial. *Kidney International* 2004;66(2):753-760.
- (151) Besarab A, Bolton WK, Browne JK, Egrie JC, Nissensohn AR, Okamoto DM, Schwab SJ, Goodkin DA. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *New England Journal of Medicine* 1998;339(9):584-590.
- (152) Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, Reddan D. Correction of anemia with epoetin alfa in chronic kidney disease. *New England Journal of Medicine* 2006;355(20):2085-2098.
- (153) Begg C, Cho M, Eastwood S, Horton R, Moher D, Olkin I, Pitkin R, Rennie D, Schulz KF, Simel D, Stroup DF. Improving the quality of reporting of randomized controlled trials - The CONSORT statement. *Jama-Journal of the American Medical Association* 1996;276(8):637-639.
- (154) Drueke TB, Locatelli F, Clyne N, Eckardt K, Macdougall IC, Tsakiris D, Burger H, Scherhag A. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *New England Journal of Medicine* 2006;355(20):2071-2084.
- (155) Lappin TR, Maxwell AP, Johnston PG. EPO's alter ego: Erythropoietin has multiple actions. *Stem Cells* 2002;20(6):485-492.
- (156) Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, Salio M, Cerami A, Brines M. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial

remodeling. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100(8):4802-4806.

- (157) Fliser D, Bahlmann FH, Haller H. EPO: renoprotection beyond anemia correction. *Pediatric Nephrology* 2006;21(12):1785-1789.
- (158) Lee SH, Li C, Lim SW, Ahn KO, Choi BS, Kim YS, Moon IS, Kim J, Bang BK, Yang CW. Attenuation of interstitial inflammation and fibrosis by recombinant human erythropoietin in chronic cyclosporine nephropathy. *Am J Nephrol* 2005 January;25(1):64-76.
- (159) Sharples EJ, Yaqoob MM. Erythropoietin in experimental acute renal failure. *Nephron Experimental Nephrology* 2006;104(3):E83-E88.
- (160) Mitra A, Bansal S, Wang W, Falk S, Zolty E, Schrier RW. Erythropoietin ameliorates renal dysfunction during endotoxaemia. *Nephrol Dial Transplant* 2007;22(8):2349-2353.
- (161) Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF. Structure, Function, and Activation of the Erythropoietin Receptor. *Blood* 1993;81(9):2223-2236.
- (162) Westenfelder C, Biddle DL, Baranowski RL. Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney International* 1999;55(3):808-820.
- (163) Bachmann S, Lehr M, Eckardt KU. Colocalization of Erythropoietin Messenger-Rna and Ecto-5'-Nucleotidase Immunoreactivity in Peritubular Cells of Rat Renal-Cortex Indicates That Fibroblasts Produce Erythropoietin. *Journal of Histochemistry & Cytochemistry* 1993;41(3):335-341.
- (164) Brines M, Cerami A. Discovering erythropoietin's extra-hematopoietic functions: Biology and clinical promise. *Kidney International* 2006;70(2):246-250.
- (165) Johnson DW, Pat B, Vesey DA, Guan Z, Endre Z, Gobe GC. Delayed administration of darbepoetin or erythropoietin protects against ischemic acute renal injury and failure. *Kidney International* 2006;69(10):1806-1813.
- (166) Vesey DA, Cheung C, Pat B, Endre Z, Gobe G, Johnson DW. Erythropoietin protects against ischaemic acute renal injury. *Nephrol Dial Transplant* 2004;19(2):348-355.
- (167) Bahlmann FH, Song R, Boehm SM, Mengel M, von Wasielewski R, Lindschau C, Kirsch T, de Groot K, Laudeley R, Niemczyk E, Guler F, Menne J, Haller H, Fliser D. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. *Circulation* 2004;110(8):1006-1012.

- (168) Diabetes Mellitus: A Major Risk Factor for Cardiovascular Disease : A Joint Editorial Statement by the American Diabetes Association; the National Heart, Lung, and Blood Institute; the Juvenile Diabetes Foundation International; the National Institute of Diabetes and Digestive and Kidney Diseases; and the American Heart Association. *Circulation* 1999 September 7;100(10):1132-1133.
- (169) Gottdiener JS, Arnold AM, Aurigemma GP, Polak JF, Tracy RP, Kitzman DW, Gardin JM, Rutledge JE, Boineau RC. Predictors of congestive heart failure in the elderly: the cardiovascular health study. *J Am Coll Cardiol* 2000 May 1;35(6):1628-1637.
- (170) Foley RN, Culleton BF, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. Cardiac disease in diabetic end-stage renal disease. *Diabetologia* 1997 November;40(11):1307-1312.
- (171) Thomas MC, Cooper ME, Rossing K, Parving HH. Anaemia in diabetes: Is there a rationale to TREAT? *Diabetologia* 2006 June;49(6):1151-1157.
- (172) Lameire N. The anaemia of silent diabetic nephropathy-prevalence, physiopathology, and management. *Acta Clin Belg* 2003 May;58(3):159-168.
- (173) Vlagopoulos PT, Tighiouart H, Weiner DE, Griffith J, Pettitt D, Salem DN, Levey AS, Sarnak MJ. Anemia as a Risk Factor for Cardiovascular Disease and All-Cause Mortality in Diabetes: The Impact of Chronic Kidney Disease. *J Am Soc Nephrol* 2005 November 1;16(11):3403-3410.
- (174) Silverberg DS, Wexler D, Blum M, Tchelbiner JZ, Sheps D, Keren G, Schwartz D, Baruch R, Yachnin T, Shaked M, Schwartz I, Steinbruch S, Iaina A. The effect of correction of anaemia in diabetics and non-diabetics with severe resistant congestive heart failure and chronic renal failure by subcutaneous erythropoietin and intravenous iron. *Nephrol Dial Transplant* 2003 January;18(1):141-146.
- (175) Kausz AT, Obrador GT, Pereira BJ. Anemia management in patients with chronic renal insufficiency. *Am J Kidney Dis* 2000 December;36(6 Suppl 3):S39-S51.
- (176) Jungers P, Choukroun G, Oualim Z, Robino C, Nguyen AT, Man NK. Beneficial influence of recombinant human erythropoietin therapy on the rate of progression of chronic renal failure in predialysis patients. *Nephrol Dial Transplant* 2001 February;16(2):307-312.
- (177) Guralnik JM, Ershler WB, Schrier SL, Picozzi VJ. Anemia in the Elderly: A Public Health Crisis in Hematology. *Hematology* 2005 January 1;2005(1):528-532.
- (178) Lundin AP. Quality of life: subjective and objective improvements with recombinant human erythropoietin therapy. *Semin Nephrol* 1989 March;9(1 Suppl 1):22-29.

- (179) Thomas MC, MacIsaac RJ, Tsalamandris C, Power D, Jerums G. Unrecognized anemia in patients with diabetes: a cross-sectional survey. *Diabetes Care* 2003 April;26(4):1164-1169.
- (180) Keane WF, Brenner BM, de ZD, Grunfeld JP, McGill J, Mitch WE, Ribeiro AB, Shahinfar S, Simpson RL, Snapinn SM, Toto R. The risk of developing end-stage renal disease in patients with type 2 diabetes and nephropathy: the RENAAL study. *Kidney Int* 2003 April;63(4):1499-1507.
- (181) Sinclair SH. Macular retinal capillary hemodynamics in diabetic patients. *Ophthalmology* 1991 October;98(10):1580-1586.
- (182) Grossmann M, Panagiotopolous S, Sharpe K, MacIsaac RJ, Clarke S, Zajac JD, Jerums G, Thomas MC. Low testosterone and anaemia in men with type 2 diabetes. *Clin Endocrinol (Oxf)* 2008 August 13.
- (183) Means RT, Jr., Krantz SB. Progress in understanding the pathogenesis of the anemia of chronic disease. *Blood* 1992 October 1;80(7):1639-1647.
- (184) Macdougall IC, Cooper AC. Erythropoietin resistance: the role of inflammation and pro-inflammatory cytokines. *Nephrol Dial Transplant* 2002;17 Suppl 11:39-43.
- (185) Kirstein M, Brett J, Radoff S, Ogawa S, Stern D, Vlassara H. Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. *Proc Natl Acad Sci U S A* 1990 November;87(22):9010-9014.
- (186) Miller JA, Gravallese E, Bunn HF. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. *J Clin Invest* 1980 April;65(4):896-901.
- (187) Hangaishi M, Taguchi J, Miyata T, Ikari Y, Togo M, Hashimoto Y, Watanabe T, Kimura S, Kurokawa K, Ohno M. Increased aggregation of human platelets produced by advanced glycation end products in vitro. *Biochem Biophys Res Commun* 1998 July 20;248(2):285-292.
- (188) Bucala R, Vlassara H. Advanced glycosylation end products in diabetic renal and vascular disease. *Am J Kidney Dis* 1995 December;26(6):875-888.
- (189) Boel E, Selmer J, Flodgaard HJ, Jensen T. Diabetic late complications: will aldose reductase inhibitors or inhibitors of advanced glycosylation endproduct formation hold promise? *J Diabetes Complications* 1995 April;9(2):104-129.
- (190) Schnackenberg CG. Physiological and pathophysiological roles of oxygen radicals in the renal microvasculature. *Am J Physiol Regul Integr Comp Physiol* 2002 February;282(2):R335-R342.

- (191) Saltzman MB, McCallum RW. Diabetes and the stomach. *Yale J Biol Med* 1983 May;56(3):179-187.
- (192) Marollo M, Latella G, Melideo D, Storelli E, Iannarelli R, Stornelli P, Valenti M, Caprilli R. Increased prevalence of Helicobacter pylori in patients with diabetes mellitus. *Dig Liver Dis* 2001 January;33(1):21-29.
- (193) De Block CE, Van Campenhout CM, De L, I, Keenoy BM, Martin M, Van H, V, Van Gaal LF. Soluble transferrin receptor level: a new marker of iron deficiency anemia, a common manifestation of gastric autoimmunity in type 1 diabetes. *Diabetes Care* 2000 September;23(9):1384-1388.
- (194) Treem WR. Emerging concepts in celiac disease. *Curr Opin Pediatr* 2004 October;16(5):552-559.
- (195) Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005 March 10;352(10):1011-1023.
- (196) Horl WH. Non-erythropoietin-based anaemia management in chronic kidney disease. *Nephrol Dial Transplant* 2002;17 Suppl 11:35-38.
- (197) Thomas MC, MacIsaac RJ, Tsalamandris C, Jerums G. Elevated iron indices in patients with diabetes. *Diabet Med* 2004 July;21(7):798-802.
- (198) Fujimoto S, Kawakami N, Ohara A. Nonenzymatic glycation of transferrin: decrease of iron-binding capacity and increase of oxygen radical production. *Biol Pharm Bull* 1995 March;18(3):396-400.
- (199) Ting RZ, Szeto CC, Chan MH, Ma KK, Chow KM. Risk factors of vitamin B(12) deficiency in patients receiving metformin. *Arch Intern Med* 2006 October 9;166(18):1975-1979.
- (200) Wagstaff AJ, Goa KL. Rosiglitazone: a review of its use in the management of type 2 diabetes mellitus. *Drugs* 2002;62(12):1805-1837.
- (201) Robles NR, Angulo E, Grois J, Barquero A. Comparative effects of fosinopril and irbesartan on hematopoiesis in essential hypertensives. *Ren Fail* 2004 July;26(4):399-404.
- (202) McMillan DE, Utterback NG, La PJ. Reduced erythrocyte deformability in diabetes. *Diabetes* 1978 September;27(9):895-901.
- (203) Maeda N, Kon K, Imaizumi K, Sekiya M, Shiga T. Alteration of rheological properties of human erythrocytes by crosslinking of membrane proteins. *Biochim Biophys Acta* 1983 October 26;735(1):104-112.
- (204) Spannhake EW, Fisher JW. Partial inhibition of hypoxia-induced erythropoietin production by cholinergic blockade in the dog (39866). *Proc Soc Exp Biol Med* 1977 October;156(1):19-23.
- (205) Wautier JL, Wautier MP, Schmidt AM, Anderson GM, Hori O, Zoukourian C, Capron L, Chappey O, Yan SD, Brett J, . Advanced glycation end

products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc Natl Acad Sci U S A* 1994 August 2;91(16):7742-7746.

- (206) Cotroneo P, Maria RB, Todaro L, Pitocco D, Manto A, Ruotolo V, Storti S, Damiani P, Caputo S, Ghirlanda G. Blunted erythropoietin response to anemia in patients with Type 1 diabetes. *Diabetes Metab Res Rev* 2000 May;16(3):172-176.
- (207) Ritz E, Rychlik I, Locatelli F, Halimi S. End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions. *Am J Kidney Dis* 1999 November;34(5):795-808.
- (208) Chandra M, Clemons GK, McVicar MI. Relation of serum erythropoietin levels to renal excretory function: evidence for lowered set point for erythropoietin production in chronic renal failure. *J Pediatr* 1988 December;113(6):1015-1021.
- (209) Radtke HW, Claussner A, Erbes PM, Scheuermann EH, Schoeppe W, Koch KM. Serum erythropoietin concentration in chronic renal failure: relationship to degree of anemia and excretory renal function. *Blood* 1979 October;54(4):877-884.
- (210) Catalano C, Winocour PH, Gillespie S, Gibb I, Alberti KG. Effect of posture and acute glycaemic control on the excretion of retinol-binding protein in normoalbuminuric insulin-dependent diabetic patients. *Clin Sci (Lond)* 1993 April;84(4):461-467.
- (211) Epstein FH, Agmon Y, Brezis M. Physiology of renal hypoxia. *Ann N Y Acad Sci* 1994 April 15;718:72-81.
- (212) Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. *Diabetes* 2004 December;53(12):3226-3232.
- (213) Chong ZZ, Kang JQ, Maiese K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* 2002 December 3;106(23):2973-2979.
- (214) Sharples EJ, Patel N, Brown P, Stewart K, Mota-Philippe H, Sheaff M, Kieswich J, Allen D, Harwood S, Raftery M, Thiemerann C, Yaqoob MM. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J Am Soc Nephrol* 2004 August;15(8):2115-2124.
- (215) Echigoya MH, Obikane K, Nakashima T, Sasaki S. Glomerular localization of erythropoietin receptor mRNA and protein in neonatal and mature mouse kidney. *Nephron Exp Nephrol* 2005;100(1):e21-e29.

- (216) Westenfelder C, Biddle DL, Baranowski RL. Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney International* 1999;55(3):808-820.
- (217) Crowe E, Halpin D, Stevens P. Early identification and management of chronic kidney disease: summary of NICE guidance. *BMJ* 2008;337:a1530.
- (218) Jacobs C, Horl WH, Macdougall IC, Valderrabano F, Parrondo I, Segner A, Abraham IL. European best practice guidelines 5: target haemoglobin. *Nephrol Dial Transplant* 2000;15 Suppl 4:15-19.
- (219) Collins AJ, Brenner RM, Ofman JJ, Chi EM, Stuccio-White N, Krishnan M, Solid C, Ofsthun NJ, Lazarus JM. Epoetin alfa use in patients with ESRD: an analysis of recent US prescribing patterns and hemoglobin outcomes. *Am J Kidney Dis* 2005 September;46(3):481-488.
- (220) Joss N, Patel R, Paterson K, Simpson K, Perry C, Stirling C. Anaemia is common and predicts mortality in diabetic nephropathy. *QJM* 2007 October;100(10):641-647.
- (221) Mix TC, Brenner RM, Cooper ME, de ZD, Ivanovich P, Levey AS, McGill JB, McMurray JJ, Parfrey PS, Parving HH, Pereira BJ, Remuzzi G, Singh AK, Solomon SD, Stehman-Breen C, Toto RD, Pfeffer MA. Rationale--Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT): evolving the management of cardiovascular risk in patients with chronic kidney disease. *Am Heart J* 2005 March;149(3):408-413.
- (222) Ritz E, Laville M, Bilous RW, O'Donoghue D, Scherhag A, Burger U, de AF. Target level for hemoglobin correction in patients with diabetes and CKD: primary results of the Anemia Correction in Diabetes (ACORD) Study. *Am J Kidney Dis* 2007 February;49(2):194-207.
- (223) Morrish NJ, Wang SL, Stevens LK, Fuller JH, Keen H. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. *Diabetologia* 2001 September;44 Suppl 2:S14-S21.
- (224) Most RS, Sinnock P. The epidemiology of lower extremity amputations in diabetic individuals. *Diabetes Care* 1983 January 1;6(1):87-91.
- (225) Rahman S, Rahman T, Ismail AA, Rashid AR. Diabetes-associated macrovasculopathy: pathophysiology and pathogenesis. *Diabetes Obes Metab* 2007 November;9(6):767-780.
- (226) Soedamah-Muthu SS, Chaturvedi N, Toeller M, Ferriss B, Rebaldi P, Michel G, Manes C, Fuller JH. Risk Factors for Coronary Heart Disease in Type 1 Diabetic Patients in Europe: The EURODIAB Prospective Complications Study. *Diabetes Care* 2004 February 1;27(2):530-537.
- (227) Stern MP. Diabetes and cardiovascular disease. The "common soil" hypothesis. *Diabetes* 1995 April;44(4):369-374.

- (228) Nicolaides E, Jones CJ. Review: Type 2 diabetes -- implications for macrovascular mechanics and disease. *The British Journal of Diabetes & Vascular Disease* 2002 January 1;2(1):9-12.
- (229) Lehto S, Niskanen L, Suhonen M, Ronnemaa T, Laakso M. Medial Artery Calcification: A Neglected Harbinger of Cardiovascular Complications in Non-Insulin-Dependent Diabetes Mellitus. *Arterioscler Thromb Vasc Biol* 1996 August 1;16(8):978-983.
- (230) Lomashvili KA, Khawandi W, O'Neill WC. Reduced Plasma Pyrophosphate Levels in Hemodialysis Patients. *J Am Soc Nephrol* 2005 August 1;16(8):2495-2500.
- (231) Calles-Escandon J, Cipolla M. Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev* 2001 February;22(1):36-52.
- (232) Schalkwijk CG, Stehouwer CD. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clin Sci (Lond)* 2005 August;109(2):143-159.
- (233) Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 2004 August;15(8):1983-1992.
- (234) Fleming I, Busse R. NO: the primary EDRF. *J Mol Cell Cardiol* 1999 January;31(1):5-14.
- (235) Schiffrin EL. A critical review of the role of endothelial factors in the pathogenesis of hypertension. *J Cardiovasc Pharmacol* 2001 November;38 Suppl 2:S3-S6.
- (236) Toop MJ, Dallinger KJ, Jennings PE, Barnett AH. Angiotensin-converting enzyme (ACE): relationship to insulin-dependent diabetes and microangiopathy. *Diabet Med* 1986 September;3(5):455-457.
- (237) Ashton AW, Ware JA. Thromboxane A₂ receptor signaling inhibits vascular endothelial growth factor-induced endothelial cell differentiation and migration. *Circ Res* 2004 August 20;95(4):372-379.
- (238) Davies MG, Hagen PO. The vascular endothelium. A new horizon. *Ann Surg* 1993 November;218(5):593-609.
- (239) Van H V, Sprengers ED, Kooistra T. Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. *Thromb Haemost* 1987 April 7;57(2):148-153.
- (240) Bull HA, Rustin MH, Dowd PM. Release of prostacyclin from cultured human endothelial cells following prolonged exposure to histamine. *Br J Dermatol* 1989 June;120(6):757-765.

- (241) Bennett MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin Invest* 1995 May;95(5):2266-2274.
- (242) Gibbons GH, Pratt RE, Dzau VJ. Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor-beta 1 expression determines growth response to angiotensin II. *J Clin Invest* 1992 August;90(2):456-461.
- (243) Bevilacqua MP. Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol* 1993;11:767-804.
- (244) Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest* 1993 February;91(2):379-387.
- (245) Cartwright JE, Whitley GS, Johnstone AP. The expression and release of adhesion molecules by human endothelial cell lines and their consequent binding of lymphocytes. *Exp Cell Res* 1995 April;217(2):329-335.
- (246) Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 1991 June 1;88(11):4651-4655.
- (247) Radomski MW, Palmer RM, Moncada S. The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun* 1987 November 13;148(3):1482-1489.
- (248) Li H, Forstermann U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol* 2000 February;190(3):244-254.
- (249) Pieper GM. Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. *Diabetologia* 1999 February;42(2):204-213.
- (250) Ding H, Triggle CR. Endothelial cell dysfunction and the vascular complications associated with type 2 diabetes: assessing the health of the endothelium. *Vasc Health Risk Manag* 2005;1(1):55-71.
- (251) Rudic RD, Sessa WC. Nitric oxide in endothelial dysfunction and vascular remodeling: clinical correlates and experimental links. *Am J Hum Genet* 1999 March;64(3):673-677.
- (252) Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002 January 16;39(2):257-265.
- (253) Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in

- response to insulin. *Am J Physiol Endocrinol Metab* 2003 July;285(1):E123-E129.
- (254) Vervoort G, Lutterman JA, Smits P, Berden JH, Wetzels JF. Transcapillary escape rate of albumin is increased and related to haemodynamic changes in normo-albuminuric type 1 diabetic patients. *J Hypertens* 1999 December;17(12 Pt 2):1911-1916.
- (255) Vapaatalo H, Mervaala E. Clinically important factors influencing endothelial function. *Med Sci Monit* 2001 September;7(5):1075-1085.
- (256) Kario K, Matsuo T, Kobayashi H, Matsuo M, Sakata T, Miyata T. Activation of tissue factor-induced coagulation and endothelial cell dysfunction in non-insulin-dependent diabetic patients with microalbuminuria. *Arterioscler Thromb Vasc Biol* 1995 August;15(8):1114-1120.
- (257) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., Boerwinkle E. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 1997 December 16;96(12):4219-4225.
- (258) Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 2003 March;52(3):812-817.
- (259) Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001 July 18;286(3):327-334.
- (260) Shachor-Meyouhas Y, Pillar G, Shehadeh N. Uncontrolled type 1 diabetes mellitus and endothelial dysfunction in adolescents. *Isr Med Assoc J* 2007 September;9(9):637-640.
- (261) Vervoort G, Wetzels JF, Lutterman JA, van Doorn LG, Berden JH, Smits P. Elevated skeletal muscle blood flow in noncomplicated type 1 diabetes mellitus: role of nitric oxide and sympathetic tone. *Hypertension* 1999 November;34(5):1080-1085.
- (262) Stehouwer CD, Fischer HR, van Kuijk AW, Polak BC, Donker AJ. Endothelial dysfunction precedes development of microalbuminuria in IDDM. *Diabetes* 1995 May;44(5):561-564.
- (263) Naidoo DP. The link between microalbuminuria, endothelial dysfunction and cardiovascular disease in diabetes. *Cardiovasc J S Afr* 2002 July;13(4):194-199.
- (264) Stehouwer CD, Yudkin JS, Fioretto P, Nosadini R. How heterogeneous is microalbuminuria in diabetes mellitus? The case for 'benign' and

'malignant' microalbuminuria. *Nephrol Dial Transplant* 1998 November;13(11):2751-2754.

- (265) Yamagishi S, Nakamura K, Jinnouchi Y, Takenaka K, Imaizumi T. Molecular mechanisms for vascular injury in the metabolic syndrome. *Drugs Exp Clin Res* 2005;31(3):123-129.
- (266) Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, King GL, Logerfo FW, Horton ES, Veves A. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. *Diabetes* 1999 September;48(9):1856-1862.
- (267) Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 2004 April 28;291(16):1978-1986.
- (268) Wolpert HA, Steen SN, Istfan NW, Simonson DC. Insulin modulates circulating endothelin-1 levels in humans. *Metabolism* 1993 August;42(8):1027-1030.
- (269) Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* 1994 September;94(3):1172-1179.
- (270) Cersosimo E, DeFronzo RA. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. *Diabetes Metab Res Rev* 2006 November;22(6):423-436.
- (271) Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. *Diabetologia* 2002 May;45(5):623-634.
- (272) Pinkney JH, Stehouwer CD, Coppock SW, Yudkin JS. Endothelial dysfunction: cause of the insulin resistance syndrome. *Diabetes* 1997 September;46 Suppl 2:S9-13.
- (273) Mann JF, Gerstein HC, Pogue J, Bosch J, Yusuf S. Renal insufficiency as a predictor of cardiovascular outcomes and the impact of ramipril: the HOPE randomized trial. *Ann Intern Med* 2001 April 17;134(8):629-636.
- (274) Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000 March;52(1):11-34.
- (275) O'Driscoll G, Green D, Maiorana A, Stanton K, Colreavy F, Taylor R. Improvement in endothelial function by angiotensin-converting enzyme inhibition in non-insulin-dependent diabetes mellitus. *J Am Coll Cardiol* 1999 May;33(6):1506-1511.
- (276) Maiello M, Boeri D, Podesta F, Cagliero E, Vichi M, Odetti P, Adezati L, Lorenzi M. Increased expression of tissue plasminogen activator and its

inhibitor and reduced fibrinolytic potential of human endothelial cells cultured in elevated glucose. *Diabetes* 1992 August;41(8):1009-1015.

- (277) McMillan DE. Disturbance of serum viscosity in diabetes mellitus. *J Clin Invest* 1974 April;53(4):1071-1079.
- (278) Cagliero E, Maiello M, Boeri D, Roy S, Lorenzi M. Increased expression of basement membrane components in human endothelial cells cultured in high glucose. *J Clin Invest* 1988 August;82(2):735-738.
- (279) Graier WF, Grubenthal I, Dittrich P, Wascher TC, Kostner GM. Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. *Eur J Pharmacol* 1995 December 27;294(1):221-229.
- (280) McMillan DE. Increased levels of acute-phase serum proteins in diabetes. *Metabolism* 1989 November;38(11):1042-1046.
- (281) Baumgartner-Parzer SM, Wagner L, Pettermann M, Gessl A, Waldhausl W. Modulation by high glucose of adhesion molecule expression in cultured endothelial cells. *Diabetologia* 1995 November;38(11):1367-1370.
- (282) Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessl A, Waldhausl W. High-glucose--triggered apoptosis in cultured endothelial cells. *Diabetes* 1995 November;44(11):1323-1327.
- (283) Ritthaler U, Deng Y, Zhang Y, Greten J, Abel M, Sido B, Allenberg J, Otto G, Roth H, Bierhaus A, . Expression of receptors for advanced glycation end products in peripheral occlusive vascular disease. *Am J Pathol* 1995 March;146(3):688-694.
- (284) Basta G, Schmidt AM, De CR. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res* 2004 September 1;63(4):582-592.
- (285) Esposito C, Gerlach H, Brett J, Stern D, Vlassara H. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *J Exp Med* 1989 October 1;170(4):1387-1407.
- (286) Quehenberger P, Bierhaus A, Fasching P, Muellner C, Klevesath M, Hong M, Stier G, Sattler M, Schleicher E, Speiser W, Nawroth PP. Endothelin 1 transcription is controlled by nuclear factor-kappaB in AGE-stimulated cultured endothelial cells. *Diabetes* 2000 September 1;49(9):1561-1570.
- (287) Sakata N, Meng J, Takebayashi S. Effects of advanced glycation end products on the proliferation and fibronectin production of smooth muscle cells. *J Atheroscler Thromb* 2000;7(3):169-176.

- (288) Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994 April 1;269(13):9889-9897.
- (289) Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Berentshtain E, Tritschler H, Muller M, Wahl P, Ziegler R, Nawroth PP. Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. *Diabetes* 1997 September;46(9):1481-1490.
- (290) Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 1991 February;87(2):432-438.
- (291) Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 2001 April;280(4):C719-C741.
- (292) Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. *Diabetes Care* 2003 July;26(7):2181-2188.
- (293) Vermes I, Steinmetz ET, Zeyen LJ, van d, V. Rheological properties of white blood cells are changed in diabetic patients with microvascular complications. *Diabetologia* 1987 June;30(6):434-436.
- (294) Jude B, Watel A, Fontaine O, Fontaine P, Cosson A. Distinctive features of procoagulant response of monocytes from diabetic patients. *Haemostasis* 1989;19(2):65-73.
- (295) Miyamoto K, Ogura Y, Kenmochi S, Honda Y. Role of leukocytes in diabetic microcirculatory disturbances. *Microvasc Res* 1997 July;54(1):43-48.
- (296) Cooper ME, Bonnet F, Oldfield M, Jandeleit-Dahm K. Mechanisms of diabetic vasculopathy: an overview. *Am J Hypertens* 2001 May;14(5 Pt 1):475-486.
- (297) Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, Dellasperger KC, Zhang C. Role of TNF-alpha in vascular dysfunction. *Clin Sci (Lond)* 2009 February;116(3):219-230.
- (298) Stehouwer CD, Lambert J, Donker AJ, van H, V. Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Cardiovasc Res* 1997 April;34(1):55-68.
- (299) Vattikuti R, Towler DA. Osteogenic regulation of vascular calcification: an early perspective. *Am J Physiol Endocrinol Metab* 2004 May;286(5):E686-E696.
- (300) Nicholls SJ, Tuzcu EM, Kalidindi S, Wolski K, Moon KW, Sipahi I, Schoenhagen P, Nissen SE. Effect of diabetes on progression of coronary atherosclerosis and arterial remodeling: a pooled analysis of 5

intravascular ultrasound trials. *J Am Coll Cardiol* 2008 July 22;52(4):255-262.

- (301) Shanahan CM, Cary NRB, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. Medial Localization of Mineralization-Regulating Proteins in Association With Monckeberg's Sclerosis : Evidence for Smooth Muscle Cell-Mediated Vascular Calcification. *Circulation* 1999 November 23;100(21):2168-2176.
- (302) Schoppet M, Al-Fakhri N, Franke FE, Katz N, Barth PJ, Maisch B, Preissner KT, Hofbauer LC. Localization of osteoprotegerin, tumor necrosis factor-related apoptosis-inducing ligand, and receptor activator of nuclear factor-kappaB ligand in Monckeberg's sclerosis and atherosclerosis. *J Clin Endocrinol Metab* 2004 August;89(8):4104-4112.
- (303) Guzman RJ. Clinical, cellular, and molecular aspects of arterial calcification. *J Vasc Surg* 2007 June;45 Suppl A:A57-A63.
- (304) Chen PL, Easton A. Apoptotic phenotype alters the capacity of tumor necrosis factor-related apoptosis-inducing ligand to induce human vascular endothelial activation. *J Vasc Res* 2008;45(2):111-122.
- (305) Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. *J Biol Chem* 2001 June 8;276(23):20659-20672.
- (306) Hayden MR, Tyagi SC, Kolb L, Sowers JR, Khanna R. Vascular ossification-calcification in metabolic syndrome, type 2 diabetes mellitus, chronic kidney disease, and calciphylaxis-calcific uremic arteriolopathy: the emerging role of sodium thiosulfate. *Cardiovasc Diabetol* 2005;4(1):4.
- (307) Csiszar A, Smith KE, Koller A, Kaley G, Edwards JG, Ungvari Z. Regulation of Bone Morphogenetic Protein-2 Expression in Endothelial Cells: Role of Nuclear Factor-{kappa}B Activation by Tumor Necrosis Factor-{alpha}, H₂O₂, and High Intravascular Pressure. *Circulation* 2005 May 10;111(18):2364-2372.
- (308) Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lessons learned from the aorta. *Arterioscler Thromb Vasc Biol* 2006 July;26(7):1423-1430.
- (309) Al-Aly Z, Shao JS, Lai CF, Huang E, Cai J, Behrmann A, Cheng SL, Towler DA. Aortic Msx2-Wnt Calcification Cascade Is Regulated by TNF-{alpha} Dependent Signals in Diabetic Ldlr / Mice. *Arterioscler Thromb Vasc Biol* 2007 December 1;27(12):2589-2596.
- (310) Sugitani H, Wachi H, Mecham RP, Seyama Y. Accelerated calcification represses the expression of elastic fiber components and lysyl oxidase in cultured bovine aortic smooth muscle cells. *J Atheroscler Thromb* 2002;9(6):292-298.

- (311) Trion A, van der LA. Vascular smooth muscle cells and calcification in atherosclerosis. *Am Heart J* 2004 May;147(5):808-814.
- (312) Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res* 2004 November 26;95(11):1046-1057.
- (313) Johnson K, Polewski M, van ED, Terkeltaub R. Chondrogenesis mediated by PPi depletion promotes spontaneous aortic calcification in NPP1^{-/-} mice. *Arterioscler Thromb Vasc Biol* 2005 April;25(4):686-691.
- (314) Demetriou M, Binkert C, Sukhu B, Tenenbaum HC, Dennis JW. Fetuin/alpha 2-HS Glycoprotein Is a Transforming Growth Factor-beta Type II Receptor Mimic and Cytokine Antagonist. *J Biol Chem* 1996 May 31;271(22):12755-12761.
- (315) Watson KE, Bostrom K, Ravindranath R, Lam T, Norton B, Demer LL. TGF-beta 1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. *J Clin Invest* 1994 May;93(5):2106-2113.
- (316) Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. *Arterioscler Thromb Vasc Biol* 2002 April 1;22(4):549-553.
- (317) Malyankar UM, Scatena M, Suchland KL, Yun TJ, Clark EA, Giachelli CM. Osteoprotegerin is an alpha vbeta 3-induced, NF-kappa B-dependent survival factor for endothelial cells. *J Biol Chem* 2000 July 14;275(28):20959-20962.
- (318) Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997 April 18;89(2):309-319.
- (319) Shin JY, Shin YG, Chung CH. Elevated serum osteoprotegerin levels are associated with vascular endothelial dysfunction in type 2 diabetes. *Diabetes Care* 2006 July;29(7):1664-1666.
- (320) Shin JY, Shin YG, Chung CH. Elevated serum osteoprotegerin levels are associated with vascular endothelial dysfunction in type 2 diabetes. *Diabetes Care* 2006 July;29(7):1664-1666.
- (321) Harris EK. Effects of intra- and interindividual variation on the appropriate use of normal ranges. *Clin Chem* 1974 December;20(12):1535-1542.
- (322) Chung I, Han G, Seshadri M, Gillard BM, Yu Wd, Foster BA, Trump DL, Johnson CS. Role of Vitamin D Receptor in the Antiproliferative Effects of Calcitriol in Tumor-Derived Endothelial Cells and Tumor Angiogenesis In vivo. *Cancer Res* 2009 February 1;69(3):967-975.

- (323) Fraser CG. Data on biological variation: essential prerequisites for introducing new procedures? *Clin Chem* 1994 September;40(9):1671-1673.
- (324) Fraser CG, Petersen PH. Analytical performance characteristics should be judged against objective quality specifications. *Clin Chem* 1999 March;45(3):321-323.
- (325) Ricós et al. Biological variation database, and quality specifications for imprecision, bias and total error . The 2008 update. 2009.
- (326) Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci* 1989;27(5):409-437.
- (327) Marshall SM. Recent advances in diabetic nephropathy. *Clin Med* 2004 May;4(3):277-282.
- (328) Dillon SC, Taylor GM, Shah V. Diagnostic value of urinary retinol-binding protein in childhood nephrotic syndrome. *Pediatr Nephrol* 1998 October;12(8):643-647.
- (329) Salem MA, el-Habashy SA, Saeid OM, el-Tawil MM, Tawfik PH. Urinary excretion of n-acetyl-beta-D-glucosaminidase and retinol binding protein as alternative indicators of nephropathy in patients with type 1 diabetes mellitus. *Pediatr Diabetes* 2002 March;3(1):37-41.
- (330) Holm J, Hemmingsen L, Nielsen NV. Low-molecular-mass proteinuria as a marker of proximal renal tubular dysfunction in normo- and microalbuminuric non-insulin-dependent diabetic subjects. *Clin Chem* 1993 March;39(3):517-519.
- (331) Brunette MG, Chan M, Ferriere C, Roberts KD. Site of 1,25(OH)₂ vitamin D₃ synthesis in the kidney. *Nature* 1978 November 16;276(5685):287-289.
- (332) Lacombe C, Da Silva JL, Bruneval P, Fournier JG, Wendling F, Casadevall N, Camilleri JP, Bariety J, Varet B, Tambourin P. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. *J Clin Invest* 1988 February;81(2):620-623.
- (333) Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976;16(1):31-41.
- (334) Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999 March 16;130(6):461-470.
- (335) Burden R, Tomson C. Identification, management and referral of adults with chronic kidney disease: concise guidelines. *Clin Med* 2005 November;5(6):635-642.

- (336) Mojiminiyi OA, Abdella NA, Zaki MY, El Gebely SA, Mohamedi HM, Aldhahi WA. Prevalence and associations of low plasma erythropoietin in patients with Type 2 diabetes mellitus. *Diabet Med* 2006 August;23(8):839-844.
- (337) Inomata S, Itoh M, Imai H, Sato T. Serum levels of erythropoietin as a novel marker reflecting the severity of diabetic nephropathy. *Nephron* 1997;75(4):426-430.
- (338) Littorin B, Blom P, Scholin A, Arnqvist HJ, Blohme G, Bolinder J, Ekbom-Schnell A, Eriksson JW, Gudbjornsdottir S, Nystrom L, Ostman J, Sundkvist G. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). *Diabetologia* 2006 December;49(12):2847-2852.
- (339) Isaia G, Giorgino R, Adami S. High prevalence of hypovitaminosis D in female type 2 diabetic population. *Diabetes Care* 2001 August;24(8):1496.
- (340) Fatemi S, Ryzen E, Flores J, Endres DB, Rude RK. Effect of experimental human magnesium depletion on parathyroid hormone secretion and 1,25-dihydroxyvitamin D metabolism. *J Clin Endocrinol Metab* 1991 November;73(5):1067-1072.
- (341) Tarchini R, Bottini E, Botti P, Marseglia CD, Talassi E, Baraldi O, Lambertini D, Gaetti L, Bellomi A. [Type 2 diabetic nephropathy: clinical course and prevention proposals 2004]. *G Ital Nefrol* 2005 January;22 Suppl 31:S15-S19.
- (342) Singh DK, Winocour P, Farrington K. Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy. *Nat Clin Pract Nephrol* 2008 April;4(4):216-226.
- (343) Burden R, Tomson C. Identification, management and referral of adults with chronic kidney disease: concise guidelines. *Clin Med* 2005 November;5(6):635-642.
- (344) Haffner SM, Stern MP, Gruber MK, Hazuda HP, Mitchell BD, Patterson JK. Microalbuminuria. Potential marker for increased cardiovascular risk factors in nondiabetic subjects? *Arteriosclerosis* 1990 September;10(5):727-731.
- (345) Eschbach JW, Adamson JW. Modern aspects of the pathophysiology of renal anemia. *Contrib Nephrol* 1988;66:63-70.
- (346) Viberti G, Mogensen CE, Groop LC, Pauls JF. Effect of captopril on progression to clinical proteinuria in patients with insulin-dependent diabetes mellitus and microalbuminuria. European Microalbuminuria Captopril Study Group. *JAMA* 1994 January 26;271(4):275-279.

- (347) Erslev AJ, Besarab A. Erythropoietin in the pathogenesis and treatment of the anemia of chronic renal failure. *Kidney Int* 1997 March;51(3):622-630.
- (348) Xu X, Fang Y, Ji J, Jiang S, Xing D, Fei S, Ding X. Clinical value of urinary kidney biomarkers for estimation of renal impairment in elderly Chinese with essential hypertension. *J Clin Lab Anal* 2008;22(1):86-90.
- (349) Chevalier RL, Forbes MS. Generation and evolution of atubular glomeruli in the progression of renal disorders. *J Am Soc Nephrol* 2008 February;19(2):197-206.
- (350) Najafian B, Crosson JT, Kim Y, Mauer M. Glomerulotubular junction abnormalities are associated with proteinuria in type 1 diabetes. *J Am Soc Nephrol* 2006 April;17(4 Suppl 2):S53-S60.
- (351) White KE, Marshall SM, Bilous RW. Prevalence of atubular glomeruli in type 2 diabetic patients with nephropathy. *Nephrol Dial Transplant* 2008 November;23(11):3539-3545.
- (352) Stenvinkel P. Inflammation in end-stage renal failure: could it be treated? *Nephrol Dial Transplant* 2002;17 Suppl 8:33-38.
- (353) van de WA. Anemia in critically ill patients. *Eur J Intern Med* 2004 December;15(8):481-486.
- (354) Schiffer M, Park JK, Tossidou I, Bartels J, Shushakova N, Menne J, Fliser D. Erythropoietin prevents diabetes-induced podocyte damage. *Kidney Blood Press Res* 2008;31(6):411-415.
- (355) Logar CM, Brinkkoetter PT, Krofft RD, Pippin JW, Shankland SJ. Darbepoetin alfa protects podocytes from apoptosis in vitro and in vivo. *Kidney Int* 2007 August;72(4):489-498.
- (356) Niskanen L, Siitonen O, Suhonen M, Uusitupa MI. Medial artery calcification predicts cardiovascular mortality in patients with NIDDM. *Diabetes Care* 1994 November;17(11):1252-1256.
- (357) Morrish NJ, Wang SL, Stevens LK, Fuller JH, Keen H. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. *Diabetologia* 2001 September;44 Suppl 2:S14-S21.
- (358) Demer LL, Tintut Y. Pitting Phosphate Transport Inhibitors Against Vascular Calcification. *Circ Res* 2006 April 14;98(7):857-859.
- (359) Abedin M, Tintut Y, Demer LL. Vascular Calcification: Mechanisms and Clinical Ramifications. *Arterioscler Thromb Vasc Biol* 2004 July 1;24(7):1161-1170.
- (360) Recchioni R, Marcheselli F, Moroni F, Pieri C. Apoptosis in human aortic endothelial cells induced by hyperglycemic condition involves

mitochondrial depolarization and is prevented by N-acetyl-L-cysteine. *Metabolism* 2002 November;51(11):1384-1388.

- (361) Ishimura E, Okuno S, Kitatani K, Kim M, Shoji T, Nakatani T, Inaba M, Nishizawa Y. Different risk factors for peripheral vascular calcification between diabetic and non-diabetic haemodialysis patients--importance of glycaemic control. *Diabetologia* 2002 October;45(10):1446-1448.
- (362) Psyrogiannis A, Kyriazopoulou V, Vagenakis AG, Vagenakis AG. Medial Arterial Calcification Is Frequently Found in Patients with Microalbuminuria. *Angiology* 1999 December 1;50(12):971-975.
- (363) Edmonds ME, Morrison N, Laws JW, Watkins PJ. Medial arterial calcification and diabetic neuropathy. *Br Med J (Clin Res Ed)* 1982 March 27;284(6320):928-930.
- (364) Goebel FD, Fuessl HS. Monckeberg's sclerosis after sympathetic denervation in diabetic and non-diabetic subjects. *Diabetologia* 1983 May;24(5):347-350.
- (365) Young MJ, Adams JE, Anderson GF, Boulton AJ, Cavanagh PR. Medial arterial calcification in the feet of diabetic patients and matched non-diabetic control subjects. *Diabetologia* 1993 July;36(7):615-621.
- (366) Cundy TF, Edmonds ME, Watkins PJ. Osteopenia and metatarsal fractures in diabetic neuropathy. *Diabet Med* 1985 November;2(6):461-464.
- (367) Leidig-Bruckner G, Ziegler R. Diabetes mellitus a risk for osteoporosis? *Exp Clin Endocrinol Diabetes* 2001;109 Suppl 2:S493-S514.
- (368) Pennisi P, Signorelli SS, Riccobene S, Celotta G, Di PL, La MT, Fiore CE. Low bone density and abnormal bone turnover in patients with atherosclerosis of peripheral vessels. *Osteoporos Int* 2004 May;15(5):389-395.
- (369) Okazaki R, Totsuka Y, Hamano K, Ajima M, Miura M, Hirota Y, Hata K, Fukumoto S, Matsumoto T. Metabolic improvement of poorly controlled noninsulin-dependent diabetes mellitus decreases bone turnover. *J Clin Endocrinol Metab* 1997 September;82(9):2915-2920.
- (370) Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M, Jr., Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990 March 15;15(4):827-832.
- (371) Adragao T, Pires A, Lucas C, Birne R, Magalhaes L, Goncalves M, Negrao AP. A simple vascular calcification score predicts cardiovascular risk in haemodialysis patients. *Nephrol Dial Transplant* 2004 June;19(6):1480-1488.
- (372) Porter CJ, Stavroulopoulos A, Roe SD, Pointon K, Cassidy MJD. Detection of coronary and peripheral artery calcification in patients with

chronic kidney disease stages 3 and 4, with and without diabetes. *Nephrol Dial Transplant* 2007 November 1;22(11):3208-3213.

- (373) Costacou T, Huskey ND, Edmundowicz D, Stolk R, Orchard TJ. Lower-extremity arterial calcification as a correlate of coronary artery calcification. *Metabolism* 2006 December;55(12):1689-1696.
- (374) Ishida H, Seino Y, Matsukura S, Ikeda M, Yawata M, Yamashita G, Ishizuka S, Imura H. Diabetic osteopenia and circulating levels of vitamin D metabolites in type 2 (noninsulin-dependent) diabetes. *Metabolism* 1985 September;34(9):797-801.
- (375) Isaia G, Bodrato L, Carlevatto V, Mussetta M, Salamano G, Molinatti GM. Osteoporosis in type II diabetes. *Acta Diabetol Lat* 1987 October;24(4):305-310.
- (376) Hannan MT, Felson DT, wson-Hughes B, Tucker KL, Cupples LA, Wilson PW, Kiel DP. Risk factors for longitudinal bone loss in elderly men and women: the Framingham Osteoporosis Study. *J Bone Miner Res* 2000 April;15(4):710-720.
- (377) Jeffcoate W. Vascular calcification and osteolysis in diabetic neuropathy-is RANK-L the missing link? *Diabetologia* 2004 September;47(9):1488-1492.
- (378) Browner WS, Lui LY, Cummings SR. Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. *J Clin Endocrinol Metab* 2001 February;86(2):631-637.
- (379) Nilsson PM. Early vascular aging (EVA): consequences and prevention. *Vasc Health Risk Manag* 2008;4(3):547-552.
- (380) Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* 1993 February;16(2):434-444.
- (381) Schoppet M, Sattler AM, Schaefer JR, Herzum M, Maisch B, Hofbauer LC. Increased Osteoprotegerin Serum Levels in Men with Coronary Artery Disease. *J Clin Endocrinol Metab* 2003 March 1;88(3):1024-1028.
- (382) Xiang GD, Pu JH, Zhao LS, Sun HL, Hou J, Yue L. Association between plasma osteoprotegerin concentrations and urinary albumin excretion in Type 2 diabetes. *Diabet Med* 2009 April;26(4):397-403.
- (383) Rogers A, Saleh G, Hannon RA, Greenfield D, Eastell R. Circulating estradiol and osteoprotegerin as determinants of bone turnover and bone density in postmenopausal women. *J Clin Endocrinol Metab* 2002 October;87(10):4470-4475.

- (384) Roderick P, Armitage A. Renal services for people with diabetes in the UK. *Diabet Med* 2002 July;19 Suppl 4:56-60.
- (385) Biesenbach G, Zazgornik J. High mortality and poor quality of life during predialysis period in type II diabetic patients with diabetic nephropathy. *Ren Fail* 1994;16(2):263-272.
- (386) Gregg EW, Sorlie P, Paulose-Ram R, Gu Q, Eberhardt MS, Wolz M, Burt V, Curtin L, Engelgau M, Geiss L. Prevalence of Lower-Extremity Disease in the U.S. Adult Population >=40 Years of Age With and Without Diabetes: 1999-2000 National Health and Nutrition Examination Survey. *Diabetes Care* 2004 July 1;27(7):1591-1597.
- (387) Tierney S, Fennessy F, Hayes DB. ABC of arterial and vascular disease: Secondary prevention of peripheral vascular disease. *BMJ* 2000 May 6;320(7244):1262-1265.
- (388) Moe SM, Chen NX. Pathophysiology of Vascular Calcification in Chronic Kidney Disease. *Circ Res* 2004 September 17;95(6):560-567.
- (389) Inaba M, Nagasue K, Okuno S, Ueda M, Kumeda Y, Imanishi Y, Shoji T, Ishimura E, Ohta T, Nakatani T, Kim M, Nishizawa Y. Impaired secretion of parathyroid hormone, but not refractoriness of osteoblast, is a major mechanism of low bone turnover in hemodialyzed patients with diabetes mellitus. *Am J Kidney Dis* 2002 June;39(6):1261-1269.
- (390) Spasovski GB, Bervoets AR, Behets GJ, Ivanovski N, Sikole A, Dams G, Couttenye MM, De Broe ME, D'Haese PC. Spectrum of renal bone disease in end-stage renal failure patients not yet on dialysis. *Nephrol Dial Transplant* 2003 June;18(6):1159-1166.
- (391) Rigalleau V, Lasseur C, Raffaitin C, Perlemoine C, Barthe N, Chauveau P, Aparicio M, Combe C, Gin H. Bone loss in diabetic patients with chronic kidney disease. *Diabet Med* 2007 January;24(1):91-93.
- (392) Horl WH. The clinical consequences of secondary hyperparathyroidism: focus on clinical outcomes. *Nephrol Dial Transplant* 2004 August;19 Suppl 5:V2-V8.
- (393) Khosla S. Minireview: the OPG/RANKL/RANK system. *Endocrinology* 2001 December;142(12):5050-5055.
- (394) Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K, Ott S, Sprague S, Lameire N, Eknoyan G. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 2006 June;69(11):1945-1953.
- (395) Tintut Y, Parhami F, Bostrom K, Jackson SM, Demer LL. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. *J Biol Chem* 1998 March 27;273(13):7547-7553.

- (396) London GM, Guerin AP, Marchais SJ, Metivier F, Pannier B, Adda H. Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. *Nephrol Dial Transplant* 2003 September 1;18(9):1731-1740.
- (397) Fox CS, Larson MG, Keyes MJ, Levy D, Clouse ME, Culleton B, O'Donnell CJ. Kidney function is inversely associated with coronary artery calcification in men and women free of cardiovascular disease: the Framingham Heart Study. *Kidney Int* 2004 November;66(5):2017-2021.
- (398) Hofbauer LC, Brueck CC, Shanahan CM, Schoppe M, Dobnig H. Vascular calcification and osteoporosis--from clinical observation towards molecular understanding. *Osteoporos Int* 2007 March;18(3):251-259.
- (399) Anand DV, Lahiri A, Lim E, Hopkins D, Corder R. The relationship between plasma osteoprotegerin levels and coronary artery calcification in uncomplicated type 2 diabetic subjects. *J Am Coll Cardiol* 2006 May 2;47(9):1850-1857.
- (400) Toussaint ND, Lau KK, Strauss BJ, Polkinghorne KR, Kerr PG. Associations between vascular calcification, arterial stiffness and bone mineral density in chronic kidney disease. *Nephrol Dial Transplant* 2008 February;23(2):586-593.
- (401) Morena M, Terrier N, Jausset I, Leray-Moragues H, Chalabi L, Rivory JP, Maurice F, Delcourt C, Cristol JP, Canaud B, Dupuy AM. Plasma Osteoprotegerin Is Associated with Mortality in Hemodialysis Patients. *J Am Soc Nephrol* 2006 January 1;17(1):262-270.
- (402) Toussaint ND, Lau KK, Strauss BJ, Polkinghorne KR, Kerr PG. Associations between vascular calcification, arterial stiffness and bone mineral density in chronic kidney disease. *Nephrol Dial Transplant* 2008 February;23(2):586-593.
- (403) Kiechl S, Schett G, Wenning G, Redlich K, Oberholzer M, Mayr A, Santer P, Smolen J, Poewe W, Willeit J. Osteoprotegerin Is a Risk Factor for Progressive Atherosclerosis and Cardiovascular Disease. *Circulation* 2004 May 11;109(18):2175-2180.
- (404) Schoppe M, Schaefer JR, Hofbauer LC. Low serum levels of soluble RANK ligand are associated with the presence of coronary artery disease in men. *Circulation* 2003 March 25;107(11):e76.
- (405) Schoppe M, Schaefer JR, Hofbauer LC. Low serum levels of soluble RANK ligand are associated with the presence of coronary artery disease in men. *Circulation* 2003 March 25;107(11):e76.
- (406) Huang JC, Sakata T, Pfleger LL, Bencsik M, Halloran BP, Bikle DD, Nissenson RA. PTH differentially regulates expression of RANKL and OPG. *J Bone Miner Res* 2004 February;19(2):235-244.

- (407) Shao JS, Cheng SL, Charlton-Kachigian N, Loewy AP, Towler DA. Teriparatide (human parathyroid hormone (1-34)) inhibits osteogenic vascular calcification in diabetic low density lipoprotein receptor-deficient mice. *J Biol Chem* 2003 December 12;278(50):50195-50202.
- (408) Rubin MR, Silverberg SJ. Vascular Calcification and Osteoporosis--The Nature of the Nexus. *J Clin Endocrinol Metab* 2004 September 1;89(9):4243-4245.
- (409) London GM, Marty C, Marchais SJ, Guerin AP, Metivier F, de Vernejoul MC. Arterial calcifications and bone histomorphometry in end-stage renal disease. *J Am Soc Nephrol* 2004 July;15(7):1943-1951.
- (410) Moe SM, Reslerova M, Ketteler M, O'Neill K, Duan D, Koczman J, Westenfeld R, Jähnen-Dechent W, Chen NX. Role of calcification inhibitors in the pathogenesis of vascular calcification in chronic kidney disease (CKD). *Kidney Int* 2005 June;67(6):2295-2304.
- (411) UK Prospective Diabetes Study Group. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. *BMJ* 1998 September 12;317(7160):703-713.

SECTION VI

PUBLISHED PAPERS

Papers and Presentations arising from this work

Published Papers

- Singh DK, Winocour P, Farrington K. Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy. *Nat Clin Pract Nephrol.* 2008 Apr; 4(4):216-26.
- Viljoen A, Singh DK, Twomey PJ, Farrington K. Analytical quality goals for parathyroid hormone based on biological variation. *Clin Chem Lab Med.* 2008; 46 (10):1438-42.
- Singh DK, Winocour P, Farrington K. Erythropoietic stress and anemia in diabetes mellitus. *Nat Rev Endocrinol.* 2009 Apr; 5 (4):204-10.
- Singh DK, Winocour P, Summerhayes B, Viljoen A, Sivakumar G, Farrington K. Are low erythropoietin and 1, 25-dihydroxyvitamin D levels indicative of tubulo-interstitial dysfunction in diabetes without persistent microalbuminuria? *Diabetes Res Clin Pract.* 2009 Sep; 85 (3):258-64.

Accepted Papers

- Singh DK, Winocour P, Summerhayes B, Viljoen A, Sivakumar G, Farrington K. Low Osteoprotegerin levels in type 1 Diabetes Mellitus
- Singh DK, Winocour P, Farrington K. Endothelial Cell Dysfunction, Medial Arterial Calcification and Osteoprotegerin In Diabetes Mellitus

SECTION VII

APPENDIX

APPENDIX

- 1. Copy of Good Clinical Practice Certificate**
- 2. Copies of Ethics Committee Approval**
- 3. Patient Consent Forms**