



Stevens, Kathryn K. (2014) *Phosphate as a cardiovascular risk factor: effects on vascular and endothelial function*. PhD thesis.

<http://theses.gla.ac.uk/5301/>

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten:Theses
<http://theses.gla.ac.uk/>
theses@gla.ac.uk

**Phosphate as a cardiovascular risk factor: effects on vascular
and endothelial function**

Kathryn K. Stevens

BSc. (Med.Sci.) Hons, MB.Ch.B., MRCP (UK)

**Submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy (Ph.D.)**

School of Medicine

College of Medical, Veterinary and Life Sciences

University of Glasgow

LIST OF TABLES	9
LIST OF FIGURES	10
ACKNOWLEDGEMENT	14
AUTHOR'S DECLARATION	15
DEFINITIONS/ABBREVIATIONS	16
SUMMARY	20
1 CHAPTER ONE – INTRODUCTION	23
1.1 General Introduction	24
1.2 Chronic kidney disease	25
1.2.1 Staging of chronic kidney disease	25
1.2.2 Cardiovascular disease in chronic kidney disease	25
1.2.3 Cardiovascular risk factors in chronic kidney disease	27
1.2.4 Progression of chronic kidney disease	27
1.2.5 Proteinuria	29
1.2.6 Renin-angiotensin-aldosterone system blockade	29
1.2.7 Phosphate and progression of chronic kidney disease	30
1.3 The vasculature in chronic kidney disease	31
1.3.1 Arterial stiffness, pulse wave velocity and the augmentation index	31
1.3.2 Arterial stiffness in chronic kidney disease	32
1.4 The endothelium in health and chronic kidney disease	34
1.4.1 The nitric oxide pathway and endothelial dysfunction	35
1.4.2 Reactive oxygen species and oxidative stress	36
1.4.3 Endothelial dysfunction in chronic kidney disease	38
1.4.4 Flow mediated dilatation	39
1.5 Phosphate	41
1.5.1 Phosphorus or phosphate	41
1.5.2 Phosphate homeostasis in health	42
1.5.2.1 Phosphate and diet.....	42
1.5.2.2 Phosphate transport in the intestine.....	43
1.5.2.3 Phosphate transport in the kidney	45
1.5.2.4 Phosphate transport in other tissues and organs.....	45
1.5.2.5 Hormonal regulators of phosphate homeostasis	46
1.5.2.6 Parathyroid hormone	46
1.5.2.7 Vitamin D.....	47
1.5.2.8 Fibroblast growth factor 23	49

1.5.2.9	Klotho.....	50
1.5.2.10	Emerging phosphatonins	52
1.5.2.11	Phosphate homeostasis summary	52
1.5.3	Dysregulation of phosphate in chronic kidney disease	54
1.5.3.1	The intact nephron hypothesis	54
1.5.3.2	The extent of the problem	54
1.5.3.3	The current ‘trade off’ hypothesis.....	56
1.5.3.4	FGF-23 in CKD.....	56
1.5.3.5	Klotho in CKD	58
1.5.3.6	PTH and Vitamin D in CKD	59
1.5.3.7	Summary	60
1.6	Guidelines for phosphate control in chronic kidney disease	60
1.6.1	UK Renal Association Guideline	62
1.7	Phosphate and cardiovascular risk	62
1.7.1	Mortality, CV risk and phosphate: in chronic kidney disease and in health	62
1.7.2	Phosphate and left ventricular hypertrophy	65
1.7.3	Phosphate and vascular calcification	65
1.7.4	Phosphate and endothelial function	67
1.7.5	Mortality, cardiovascular risk and phosphate regulation	69
1.8	Clinical consequences of altered phosphate homeostasis	71
1.8.1	Chronic kidney disease mineral bone disorder	71
1.8.2	Symptoms and signs attributable to elevated phosphate	72
1.8.3	Progression of CKD: phosphate and proteinuria	73
1.9	Management of CKD-MBD	73
1.9.1	Diet	74
1.9.2	Phosphate binders	75
1.9.2.1	Calcium containing phosphate binders	76
1.9.2.2	Non-calcium containing phosphate binders	76
1.9.2.3	Conclusion.....	77
1.9.3	Dialysis	78
1.9.4	New treatments	79
1.9.5	Potential therapeutic targets	80
1.10	Work leading up to this thesis	81
1.10.1	Methods	81
1.10.2	Results	81
1.10.3	Discussion	81
1.11	Aims of this thesis	85
1.12	Hypothesis	85

2	CHAPTER TWO – MATERIALS AND METHODS	87
2.1	Introduction	88
2.2	General laboratory practice	88
2.3	Vessel studies	89
2.3.1	Animals	89
2.3.2	Selection of human subjects	90
2.3.3	Wire myography and dissection of vessels	90
2.3.3.1	Rat mesenteric vessels.....	95
2.3.3.2	Human subcutaneous vessels	95
2.3.4	Mounting of vessels on the wire myograph	95
2.3.5	Normalisation and experimental procedure - rat mesenteric vessels	97
2.3.6	Standardisation and experimental procedure – human subcutaneous vessels	97
2.3.7	Statistical analysis	98
2.3.8	cGMP ELISA	98
2.3.9	Preparation of rat vessel samples for cGMP ELISA	99
2.3.10	Superoxide measurement in whole blood	99
2.4	Cell culture	100
2.4.1	Trypsinization and cell storage	100
2.4.2	Cell based ELISA for E-selectin and VCAM	101
2.4.3	Cell staining and imaging	103
2.4.4	MTT Proliferation assay	103
2.4.5	Griess reaction	104
2.4.6	Preparation of cell lysates	105
2.4.7	Quantification of protein concentration	105
2.4.8	Polyacrylamide gel electrophoresis and immunoblotting	106
2.4.9	VEGF ELISA	106
2.4.10	Epi-fluorescence and FURA -2-AM	107
2.4.11	EPR superoxide measure from cell supernatant	111
2.5	Molecular biology	111
2.5.1	RNA extraction and complementary DNA synthesis	111
2.5.2	Polymerase chain reaction: primer design	112
2.5.3	Polymerase chain reaction	112
2.5.3.1	Agarose gel electrophoresis of DNA	112
2.5.3.2	TaqMan® real time polymerase chain reaction.....	113
2.5.3.3	TaqMan® RT-PCR protocol.....	115
2.5.4	Microarray	115
2.5.4.1	Cell culture, RNA extraction, quantification and validation.....	115
2.5.4.2	cDNA synthesis and double strand cDNA purification	115
2.5.4.3	<i>In vitro</i> transcription for cRNA synthesis, purification and validation..	116
2.5.4.4	Microarray Hybridisation & Data Collection	117
2.5.4.5	Gene Expression Data Analysis	118

2.6	Clinical Studies	119
2.6.1	Study 1: FGF-23 and left ventricular mass in chronic kidney disease	119
2.6.2	Study 2: Effects of sustained phosphate loading on endothelial function	120
2.6.2.1	Inclusion Criteria.....	121
2.6.2.2	Exclusion Criteria.....	121
2.6.2.3	Study Design	121
2.6.2.4	Randomisation, power calculation and statistical analysis	122
2.6.2.5	Blood pressure measurement	123
2.6.2.6	Blood sampling	123
2.6.2.7	Measures of vascular stiffness - the SphygmoCor® Vx system.....	126
2.6.2.8	Acquisition and analysis of pulse wave velocity	126
2.6.2.9	Acquisition and analysis of the augmentation index.....	128
2.6.2.10	Acquisition and analysis of brachial artery flow mediated dilatation	128
2.6.2.11	Blood and urine analysis - routine biochemistry and haematology ...	131
2.6.2.12	Vitamin D analysis	131
2.6.2.13	FGF-23 analysis	131
2.6.2.14	Cyclic GMP.....	131
3	CHAPTER THREE – FGF-23: A TALE OF TWO STUDIES. RELATIONSHIP WITH LEFT VENTRICULAR MASS IN CKD AND PHOSPHATE, FGF-23 AND CELL ADHESION MOLECULE FORMATION <i>IN VITRO</i>.	132
3.1	Introduction	133
3.2	Materials and Methods	134
3.3	Results	134
3.4	Discussion	139
3.5	Conclusion	144
4	CHAPTER FOUR – EFFECTS OF PHOSPHATE ON THE FUNCTION OF RAT RESISTANCE VESSELS AND ON RAT VASCULAR SMOOTH MUSCLE CELLS	145
4.1	Introduction	146
4.2	Materials and Methods	149
4.2.1	Cyclic GMP measurement, VEGF ELISA and Griess Reaction	150
4.2.2	Immunoblotting; eNOS and PKG	150
4.3	Results	150
4.3.1	Sample size and baseline demographics	150

4.3.2	Contraction with PEP in the presence and absence of LNAME	150
4.3.3	Endothelium dependent vasodilatation – carbachol	152
4.3.4	Endothelium independent vasodilatation – SNP	152
4.3.5	Effect of incubation with ascorbic acid.	158
4.3.6	Effect of incubation with zaprinast (PDE5I)	162
4.3.7	cGMP Measurements	167
4.3.8	Immunoblotting for eNOS and PKG	167
4.3.9	VEGF ELISA	173
4.3.10	NO production: Griess reaction	173
4.3.11	Summary of results	176
4.4	Discussion	177
4.5	Conclusion	181
5	CHAPTER FIVE – PHOSPHATE; EFFECTS ON THE FUNCTION OF HUMAN RESISTANCE VESSELS AND ON THE NITRIC OXIDE PATHWAY IN HUMAN CELL LINES	182
5.1	Introduction	183
5.2	Materials and Methods	184
5.2.1	Vessel studies	184
5.2.2	Superoxide, FGF-23 and vitamin D measurement	185
5.2.3	Imunoblotting, Griess reaction and epi-fluorescence	185
5.3	Results	187
5.3.1	Sample size and baseline demographics	187
5.3.2	Contraction and relaxation response in vessels from living kidney donors	188
5.3.3	Contraction and relaxation response in vessels from patients with CKD	193
5.3.4	Superoxide measurement	197
5.3.5	Immunoblotting for eNOS and nitrotyrosine	197
5.3.6	Griess reaction in HUVECs	197
5.3.7	Intra-cellular calcium measurements	197
5.3.8	Summary of results	205
5.4	Discussion	206
5.5	Conclusion	209
6	CHAPTER SIX – STUDIES OF THE EFFECT OF PHOSPHATE ON THE GROWTH AND PROLIFERATION OF CULTURED CELLS.	210
6.1	Introduction	211
6.2	Materials and Methods	212
6.2.1	Staining and imaging cells	212
6.2.2	MTT proliferation assay	212
6.2.3	Microarray and PCR	212
6.3	Results	212

6.3.1	Cell staining and imaging	212
6.3.2	Qualitative PCR	212
6.3.3	MTT proliferation assays	215
6.3.3.1	Endothelial cells	215
6.3.3.2	Human Cardiac Myocytes	215
6.3.3.3	Rat VSMCs	215
6.3.4	Gene expression studies	218
6.3.4.1	Microarray	218
6.3.4.2	Microarray Data Quality Control and Beadstudio analysis	218
6.3.4.3	MetaCore™ Analysis	218
6.3.4.4	Confirmation of differential expression of candidate genes	225
6.3.4.5	Metacore™ network process analysis	225
6.3.5	Summary of Results	226
6.4	Discussion	230
6.4.1	Direct effects of phosphate on cell growth	230
6.4.2	FGF-23, Klotho and cell growth and proliferation.	231
7	CHAPTER SEVEN – SUSTAINED PHOSPHATE LOADING AND ENDOTHELIAL FUNCTION: A CROSS OVER STUDY	234
7.1	Introduction	235
7.2	Materials and Methods	236
7.3	Results	237
7.3.1	Sample size and demographic data	237
7.3.2	Demographic data following each intervention	237
7.3.3	Determinants of vascular stiffness, endothelial function	240
7.3.4	Flow mediated dilatation	240
7.3.5	Urinary cGMP	247
7.3.6	Side effects of lanthanum and phosphate	247
7.3.7	Summary of results	247
7.4	Discussion	248
7.4.1	Phosphate homeostasis and total body phosphate level	248
7.4.2	Associations with phosphate and FGF-23	249
7.4.3	Vascular stiffness	250
7.4.4	Endothelial dysfunction	251
7.4.5	Strengths and limitations	253
7.4.6	Conclusion	253
7.5	Further work	254
8	CHAPTER EIGHT – GENERAL DISCUSSION	255
8.1	Summary of findings	256

8.2	Strengths and limitations of these studies	257
8.3	Future work	261
8.4	Conclusion	263
9	APPENDIX	265
9.1	Publications, awards and presentations	265
9.2	Supplementary Material	267

List of Tables

Table 1-1: Comparison of the five year survival in a patient aged between 60-64 years with different diagnoses.	26
Table 1-2: Associations between serum phosphate and outcome in different populations.	64
Table 1-3: Characteristics of the study population.	83
Table 2-1: Composition of normal and high phosphate concentration solutions.	94
Table 2-2: Final electrolyte concentration of standard and high phosphate PSS.	94
Table 2-3: Details of cell line and media suppliers and the passage range at which cell lines were used in experimental work.	102
Table 2-4: The concentration of electrolytes in each type of media used; endothelial cell (EC), smooth muscle cell (SMC) and cardiac myocyte (CM).	102
Table 2-5: Immunochemicals used in experimental work.	108
Table 2-6: Oligonucleotide gene targets and characteristics.	114
Table 3-1: Comparison of the baseline characteristics between the patients with CKD and control patients with essential hypertension (EH).	135
Table 3-2: Comparison between patients with CKD with and without LVH.	140
Table 3-3: Multivariate analysis showing significant predictors of LVH in CKD.	140
Table 4-1: Comparison of numbers of vessels studied for each drug and the corresponding size of the vessels.	151
Table 5-1: Demographics of the study population.	189
Table 5-2: Comparison of numbers of vessels studied for each patient group.	189
Table 7-1: Baseline demographics.	238
Table 7-2: Participant demographics at baseline, following lanthanum and phosphate.	238
Table 7-3: Significant correlations between PWV, AIx@75bpm, post cuff FMD, serum phosphate and serum FGF-23.	241
Table 7-4: The raw data used to calculate the ICC coefficient (top).	242
Table 7-5: Multiple regression model with post cuff FMD as the outcome measure.	245

List of Figures

Figure 1-1: Traditional and non-traditional risk factors for CVD in CKD.	28
Figure 1-2: Graphical representation of pulse wave velocity and derived functions.	33
Figure 1-3: The nitric oxide pathway.	37
Figure 1-4: Schematic representation of FMD.	40
Figure 1-5: Phosphate balance within the body.	44
Figure 1-6: Effects of parathyroid hormone release on bone, kidney and intestine.	48
Figure 1-7: Phosphate homeostasis in health.	53
Figure 1-8: Dysregulation of phosphate in CKD.	55
Figure 1-9: The original and updated 'trade off' hypotheses.	57
Figure 1-10: Unwelcome consequences of hyperphosphataemia.	70
Figure 1-11: Transplant recipient survival stratified by serum phosphate at one year post transplant and censored for transplant failure.	84
Figure 1-12: Summary of thesis - the interaction between phosphate and FGF-23 and effects on the vasculature and the endothelium and the pertinent chapters.	86
Figure 2-1: Dissected rat mesentery pinned out onto a petri dish.	92
Figure 2-2: A typical four chamber wire myograph (top) with the micrometer indicated by the black arrow and oxygen supply by the red arrow.	93
Figure 2-3: A four chamber wire myograph system (top) and a cartoon of vessel mounted on two wires, attached to the two jaws of a wire myograph (bottom).	96
Figure 2-4: A fluorescence recording from ECs using the Clampfit analysis package.	110
Figure 2-5: Study Protocol.	124
Figure 2-6: Labels affixed to the outer bag (top) containing study medication and the inner box (bottom) of the medication.	125
Figure 2-7: Example of the SphygmoCor® Vx system PWV recording.	127
Figure 2-8: FMD set-up with an example recording of the brachial artery (bottom).	130
Figure 3-1: Median FGF-23 concentration in the presence and absence of CKD and LVH.	136
Figure 3-2: Scatter plot showing the relationship between FGF-23 concentration and LVMI in patients with CKD.	137
Figure 3-3: Scatter plot showing the relationship between FGF-23 concentration and renal function by the MDRD4 equation in all patients.	138
Figure 3-4: E-selectin production in HUVECs cultured in standard (0.5mM) and high (3mM) phosphate concentration media.	141

Figure 3-5: VCAM production in HUVECs cultured in standard (0.5mM) and high (3mM) phosphate concentration media.	142
Figure 4-1: The site of action of the study drugs on the NO pathway.	148
Figure 4-2: Contraction to PEP in vessels incubated for 16 hours in normal or high phosphate PSS.	153
Figure 4-3: Contraction to PEP in the presence and absence of L-NAME in vessels incubated for 16 hours in normal phosphate PSS.	154
Figure 4-4: Contraction to PEP in the presence and absence of L-NAME in vessels incubated for 16 hours in high phosphate PSS.	155
Figure 4-5: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	156
Figure 4-6: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	157
Figure 4-7: Contraction to PEP in the presence of ascorbic acid (AA) in vessels incubated for 16 hours in normal or high phosphate PSS.	159
Figure 4-8: Vasodilatation to increasing concentrations of carbachol, in the presence of ascorbic acid (AA), expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	160
Figure 4-9: Vasodilatation to carbachol in the presence and absence of ascorbic acid (AA) in vessels incubated in high phosphate PSS, expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	161
Figure 4-10: Vasodilatation to increasing concentrations of carbachol, in the presence of PDE5I expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	163
Figure 4-11: Vasodilatation to increasing concentrations of SNP, in the presence of the PDE5I, zaprinast, expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.	165
Figure 4-12: Comparison of cGMP concentration (pM/ μ g protein) in vessels incubated for 24 hours in normal or high phosphate concentration PSS.	168
Figure 4-13: Phospho eNOS expression in rat VSMCs (in triplicate).	169
Figure 4-14: Phospho eNOS expression in rat VSMCs (in duplicate).	170
Figure 4-15: PKG expression in rat VSMCs (in triplicate).	171
Figure 4-16: Expression of PKG in rat VSMCs (in triplicate).	172
Figure 4-17: VEGF concentration in supernatant of cells cultured in 0.5mM and 3mM phosphate in standard and hypoxic ($-O_2$) conditions.	174
Figure 4-18: Relative NO production in rat VSMCs cultured in high (3mM) and standard (0.5mM) phosphate concentration medium +/- L-NAME (LN) and L-arginine (LA).	175

Figure 5-1: Confocal microscopy pictures of human artery stained with QAPB dye (green), CGP12177 (red) and syto62 (blue).	186
Figure 5-2: Contraction to PEP in vessels from living kidney donors.	190
Figure 5-3: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M (living kidney donors).	191
Figure 5-4: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M (living kidney donors).	192
Figure 5-5: Contraction to PEP in vessels from patients with CKD.	194
Figure 5-6: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M (CKD).	195
Figure 5-7: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M (CKD).	196
Figure 5-8: The rate of superoxide production (expressed as superoxide production/ng of protein/minute) in patients with and without CKD.	198
Figure 5-9: Superoxide production from HUVECs.	199
Figure 5-10: Total eNOS expression in HUVECs.	200
Figure 5-11: Phospho eNOS expression in HUVECs.	201
Figure 5-12: Nitrotyrosine expression in HUVECs. The antibody detects nitrotyrosine adducts on proteins.	202
Figure 5-13: Relative NO concentration in HUVECs.	203
Figure 5-14: Intracellular calcium content of HUVECs (top) and rat VSMCs (bottom) cultured in normal (0.5mM) and high (3mM) phosphate concentration medium.	204
Figure 6-1: Rat smooth muscle cell cultured in A, normal phosphate (0.5mM) and B, high phosphate (3mM) concentration media.	213
Figure 6-2: Human cardiac myocytes, cultured in A, normal phosphate (0.5mM) and B, high phosphate (3mM) concentration media.	213
Figure 6-3: Qualitative PCR showing expression of Klotho (KL), eNOS, the FGFR (FGF1) and NaPi2b in HUVECs. Control bands run with water and not DNA are also shown (suffix b).	214
Figure 6-4: Cell proliferation in HUVECs +/- L-NAME (top) and +/-FGF-23 and Klotho (bottom).	216
Figure 6-5: Cell proliferation in cardiac myocytes +/-L-NAME.	217
Figure 6-6: Agilent Bioanalyzer 2100 Assessment of Total RNA Quality.	219
Figure 6-7: Agilent Bioanalyzer 2100 Assessment of cRNA Quality.	220
Figure 6-8: MetaCore™ pathway analysis of BeadStudio Microarray data.	221
Figure 6-9: The Role of APC in cell cycle regulation.	222

Figure 6-10: Chromosome condensation in prometaphase.	223
Figure 6-11: Legend for Figures 6-9 and 6-10.	224
Figure 6-12: BeadStudio candidate gene mRNA expression relative to GAPDH.	227
Figure 6-13: Candidate gene mRNA expression relative to GAPDH.	228
Figure 6-14: An example of a network process identifying possible relationships between objects of interest.	229
Figure 7-1: Levels of serum phosphate (A) and FGF-23 (B) and fractional excretion of urinary phosphate (C) at baseline (green) and after lanthanum (blue) and phosphate (red) in the study population. Values are median and IQR. * denotes a significant change ($p < 0.05$) compared with baseline values.	239
Figure 7-2: Change in post cuff FMD (top) and post GTN vessel dilatation (bottom).	243
Figure 7-3: The relationship between FeP (FrExp), FGF-23 and post cuff FMD.	246
Figure 8-1: Effects of phosphate leading to ED, based on the findings in this thesis.	262
Figure 8-2: Possible mechanism of action of phosphate resulting in endothelial dysfunction and a pro-inflammatory state based on the main findings in this thesis.	264

Acknowledgement

I am indebted to my supervisor, Professor Alan Jardine not only for the opportunity to undertake this research but for his unwavering support and guidance throughout the last four years. I am incredibly grateful for his patience, dedication and vision. I have been fortunate to have an excellent co-supervisor in Dr Christian Delles. He has an uncanny ability to remain positive at all times which has been very much appreciated. Thank you to The British Heart Foundation who facilitated this work by supporting it financially with a Junior Clinical Fellowship.

I am grateful to the renal research group: Dr Emily McQuarrie, Dr Rajan Patel, Dr Patrick Mark and Dr Alison Taylor who provided support, fun and diet coke in equal measures. I would like to thank the scientists who accepted a medic into their midst and patiently helped with the laboratory techniques: Dr Billy Sands, Dr Dianne Hillyard, Dr Martin McBride, Dr John McLure, Ms Lesley Anderson, Dr Lorraine Work, Dr Robert McDonald, Ms Wendy Crawford, Dr Ruth Mackenzie, Dr Tony Workman and Dr Carlene Hamilton. I am indebted to Professor Godfrey Smith and Dr Sarah Kettlewell for their patience teaching me the importance of calcium; Mrs Elisabeth Beattie for her help during the early myography days; Dr Laura Denby for her wisdom; and The Loughrey-Elliotts who know why!

The support and kindness of each of the consultants within the Renal Unit has been overwhelming. I would like to particularly acknowledge the transplant surgeons (Mr Marc Clancy, Mr Enric Murio, Mr David Kingsmore, Mr Vlad Shumeyko, Miss Laura Buist and Mr John Asher) for their help with vessel collection. Dr Ellon McGregor deserves special mention for being incredibly accommodating as do Dr Colin Geddes, Dr Mun Woo and the team at Inverclyde for their invaluable help with recruitment and for providing coffee and cake on numerous occasions.

I could not have achieved this without the unfailing belief and support of my wonderful parents (with special thanks to my dad for his proof reading skills), sister, Martin and friends who have endured the highs and lows! Finally I am exceptionally grateful to the healthy volunteers and the patients of The Glasgow Renal Unit for giving so willingly of their time to participate in this research. Without them, it would not have been possible.

Author's declaration

The work presented in this thesis was that of the author and her supervisors, Professor Alan Jardine and Dr Christian Delles. All experimental work was carried out by the author, unless otherwise stated.

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not previously been submitted for a higher degree.

Kathryn K. Stevens

September 2013

Definitions/Abbreviations

24(OH)ase	24 hydroxylase
25-OH-D	25 hydroxyvitamin D (inactive form of vitamin D)
1-25-(OH) ₂ -D	1,25 hydroxyvitamin D (active form of vitamin D)
25-OH-D 1- α -hydroxylase	25 hydroxy vitamin D 1- α -hydroxylase
ACE(i)	Angiotensin converting enzyme (inhibitors)
ADHR	Autosomal dominant hypophosphataemic rickets
ADORA2A	Adenosine receptor 2A
AIx	Augmentation index
ANOVA	Analysis of variance
ARB	Angiotensin II receptor blocker
AUC	Area under the curve
BMI	Body mass index
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease mineral bone disorder
CP	3-carboxyl-proxyl radical
CPH	1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine
CREB 1	cAMP responsive element binding protein 1
CRP	C reactive protein
Ct	Cycle threshold
CT	Computerised tomography (scan)
CV	Cardiovascular
CVD	Cardiovascular disease
DCOR	Dialysis clinical outcomes revisited
DCT	Distal convoluted tubule
DF	Deferoxamine
DM	Diabetes mellitus
DMSO	Dimethyl Sulphoxide
DOPPS	Dialysis outcomes and practice patterns study
DTEC	Diethyldithiocarbamic acid
DTT	Dithiothreitol
EC	Endothelial cell

ED	Endothelial dysfunction
EDTA	Ethylenediaminetetraacetic acid
(e)GFR	(Estimated) glomerular filtration rate
EGF (-R)	Epidermal growth factor (-receptor)
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal related protein kinase (a MAPK)
EPR	Electroparamagnetic spin resonance
ESRD	End stage renal disease
FCS	Foetal calf serum
FDR	False discovery rate
FeP	Fractional excretion of urinary phosphate
FGF-23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
FISP	Fast imaging with steady state precession
FMD	Flow mediated dilatation
FRP-4	Frizzled related protein 4
FURA-2-AM	FURA-2-acetoxymethyl ester
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLM	General linear model
GTN	Glyceryl tri-nitrate
H&E	Haematoxylin and eosin
HD	Haemodialysis
HDL	High density lipoprotein
HEPES	Hydroxyethyl piperazineethanesulphonic acid
HRP	Horseradish peroxidase
HSP 70	Heat shock protein 70
HUVECs	Human umbilical vein endothelial cells
ICC	Intraclass correlation coefficient
IGF-1	Insulin like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IQR	Inter-quartile range
IVT	<i>In vitro</i> transcription
Kd	Dissociation constant
KDOQI	Kidney disease outcomes quality initiative

KDIGO	Kidney Disease Improving Global Outcomes
KPSS	High potassium concentration physiological saline solution
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDL	Low density lipoprotein
LDS	Lithium dodecyl sulphate
L-NAME	N-nitro-L-arginine methyl ester
LVEF	Left ventricular ejection fraction
LVH	Left ventricular hypertrophy
LVM(I)	Left ventricular mass (index)
MAPK	Mitogen activated protein kinase
MDRD 4	Modification of diet in renal disease (with 4 variables)
MEPE	Matrix extracellular protein
(C)MRI	(Cardiac) Magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaPi2a	Type 2a sodium phosphate co-transporter
NaPi2b	Type 2b sodium phosphate co-transporter
NaPi2c	Type 2c sodium phosphate co-transporter
NF-κB	Nuclear factor kappa-light-chain-enhancer of B cells
NHANES	National health and nutrition examination survey
NHERF-1	Sodium-proton exchanger regulatory factor 1
NICE	National Institute for Health and Care Excellence
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NOSIP	Nitric oxide synthase interacting protein
NSAID	Non-steroidal anti-inflammatory drug
p53	Protein 53/tumour protein 53
p-Akt	Phospho Akt
PBS	Phosphate buffered saline
(RT-)PCR	(Real time) Polymerase chain reaction
PCT	Proximal convoluted tubule
PD	Peritoneal dialysis
PDE5I	Phosphodiesterase 5 inhibitor (zaprinast)
PEP	Phenylephrine
PKC	Protein kinase C

PKG	Protein kinase G
PLC γ	Phosphoinositide phospholipase C γ
PMSF	Phenylmethanesulphonyl fluoride
PSS	Physiological saline solution
PTH	Parathyroid hormone
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RA	The UK Renal Association
RAAS	Renin-angiotensin-aldosterone system
Redox	Reduction-oxidation reactions
REIN	Ramipril in non-diabetic renal failure
RIN	RNA integrity number
RIPA	Radio-immunoprecipitation assay buffer
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RRT	Renal replacement therapy
SD	Standard deviation
SEEK	Study to evaluate early kidney disease
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SP1	Specificity protein 1
TAE	Tris-acetate electrophoresis
TBS(T)	Tris buffered saline(with tween)
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRPV5	Transient receptor potential vallinoid 5
UPCR	Urinary protein to creatinine ratio
VC	Vascular calcification
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WKY	Wistar-Kyoto rat strains
Wnt	Wingless/integration-1

Summary

Chronic kidney disease (CKD) is prevalent affecting 8.5% of the population in the UK and it is associated with premature cardiovascular disease (CVD) and death. The association between CKD and accelerated CVD arises as a consequence of traditional and non-traditional cardiovascular (CV) risk factors, including serum phosphate. Currently, there is no therapeutic intervention which has been shown to effectively reverse the increased CV risk in CKD. Phosphate metabolism is disordered in CKD particularly in the advanced stages (CKD 4 and 5). Even at the upper limit of the normal reference range, serum phosphate has been shown to be associated with CV mortality and morbidity including left ventricular hypertrophy, vascular calcification (VC) and endothelial dysfunction (ED). These associations also extend to populations without CKD. Serum phosphate is an appealing CV risk factor because it can be modified by dietary and pharmacological therapies. However, there has been no study linking lowered phosphate with improved outcomes and whilst several small mechanistic studies have suggested a role of phosphate in VC, oxidative stress and more recently ED, the precise mechanism of action of phosphate as a CV risk factor remains elusive. Our lack of understanding of the mechanism of action of phosphate makes it difficult to ascertain how to best manage phosphate.

The hypothesis of this thesis is that long term exposure to elevated phosphate is associated with endothelial and vascular dysfunction and it is this which contributes to the elevated CV risk seen in CKD. Endothelial and vascular dysfunction will be evident in individual cell lines and in blood vessels as well as in humans, who have been exposed to sustained oral phosphate. This hypothesis has been explored in a translational manner in three ways:

1. The function of resistance vessels from rats and from humans, with and without CKD, has been studied. Experiments were performed looking at endothelium dependent and independent relaxation of vessels exposed to either normal or high phosphate concentration medium. The anti-oxidant, ascorbic acid and zaprinast, a phosphodiesterase 5 inhibitor were studied in the rat vessels to assess if these additions altered the vessels' relaxation response.
2. Cells were cultured in normal and high phosphate concentration medium from the outset to mimic the chronicity of the uraemic environment. The nitric oxide (NO)

pathway was studied considering eNOS expression, VEGF and cGMP production, intracellular calcium concentration and NO production. Proliferation and cell growth pathways have also been studied.

3. A cross-over clinical trial was performed in 19 healthy volunteers, without CKD. Volunteers attended for three visits. Prior to each visit, they fasted for 12 hours and performed a 24 hour urine collection. Bloods were drawn and endothelial function was measured with flow mediated dilatation and vascular stiffness with pulse wave velocity and analysis. There was a baseline visit and then two further visits. Prior to visit two, volunteers were randomised to take phosphate supplementation or phosphate binding medication for two weeks, followed by a wash out period and then volunteers took the other tablet for two weeks, before attending for a final visit.

In rat vessels, there was impaired endothelium dependent and independent relaxation in vessels exposed to high phosphate concentration medium. Vessels in high phosphate produced less basal NO and less cGMP. The impaired relaxation could be ameliorated with the addition of a phosphodiesterase 5 inhibitor. This suggests reversibility of the detrimental effects of phosphate. In human vessels from patients without CKD, there was similarly attenuated endothelium dependent and independent relaxation. In vessels from patients with CKD, there was impaired endothelium dependent relaxation but independent relaxation was preserved. The CKD vessels exposed to normal phosphate medium relaxed to the same degree as their counterparts from patients without CKD, again suggesting that the effects of phosphate may be reversible. These effects are independent of intracellular calcium concentration which was found to be similar in cells cultured in normal or high phosphate medium. There was evidence of disruption to the NO pathway with reduced eNOS expression in human and rat endothelial cells and reduced protein kinase G expression in vascular smooth muscle cells. NO measured by the Griess reaction was lower in cells cultured in high phosphate medium. NO has an inhibitory effect on growth and cells cultured in high phosphate proliferated more (measured with the MTT assay) and were bigger than cells cultured in normal phosphate medium. Gene expression studies showed alterations in growth genes and cell cycle regulators.

ED was demonstrated in healthy volunteers exposed to sustained oral phosphate loading. This was independent of serum phosphate level which was unchanged. Urinary phosphate and fibroblast growth factor 23 level independently predicted ED and suggest that whilst

the normal homeostatic mechanisms maintain serum phosphate within the normal reference range, total body phosphate was elevated and urinary phosphate excretion was a surrogate for this. These relationships are novel and have not been demonstrated previously.

It has previously been difficult to separate the effects of phosphate from other effects of the uraemic environment, including acidosis. The studies in this thesis have achieved this and the results provide strong evidence of an association between phosphate and ED. There is also evidence that these effects may be reversible. In contrast to conventional thinking that the effects of phosphate, like VC, are largely irreversible, these studies suggest that there may be dynamic effects of phosphate. This may be explained by alterations in intracellular phosphate. These findings have important implications for patients with CKD because they provide a sound explanation for the increased CV risk seen with phosphate and advocate further study of phosphate lowering (and outcome) as a therapeutic strategy to reverse this elevated CV risk.

1 Chapter One – Introduction

1.1 General Introduction

This thesis will explore the mechanism of action of phosphate as a cardiovascular (CV) risk factor in a translational manner. Specifically, the effects of phosphate on the vasculature and the endothelium will be addressed in cell lines, in blood vessels and in humans both in the context of chronic kidney disease (CKD) and in the healthy population.

In this introduction, CKD and CV risk will be discussed in sections 1.2 and 1.7.

Endothelial dysfunction (ED) and vascular calcification (VC) seen in CKD are discussed in sections 1.3, 1.4 and 1.7. Phosphate homeostasis in health and dysregulation in CKD is described in sections 1.5, 1.6 and 1.8 with current management strategies for elevated phosphate discussed in section 1.9. Section 1.10 details work leading up to this thesis, section 1.11 the aims of this thesis and section 1.12 the hypotheses.

The association between CKD and accelerated CV ageing arises as a consequence of both traditional and non-traditional CV risk factors. Phosphate metabolism is disordered in CKD, probably at all stages but especially in advanced CKD (stages 4 and 5) (1;2). In observational studies of CKD, elevated phosphate has been linked to VC, left ventricular hypertrophy (LVH), arrhythmogenic death and ED (3-6). This increased CV risk seen with phosphate also extends to populations without CKD (7;8).

Superficially, hyperphosphataemia is an appealing risk factor for cardiovascular disease (CVD) in CKD because it can be modified through diet and currently available pharmacological agents; phosphate binders. Yet, no firm evidence links lower serum phosphate levels with improved CV or patient outcomes (9;10). Furthermore, despite several mechanistic studies establishing that hyperphosphatemia plays a role in pathological processes including VC, ED and oxidative stress, the precise role and independent mechanism of action of phosphate as a CV risk factor remains elusive (4;6;11). It is very difficult to determine how best to manage elevated phosphate, whilst our understanding of the mechanism of action of phosphate as a CV risk factor remains limited.

1.2 Chronic kidney disease

1.2.1 Staging of chronic kidney disease

CKD is classified according to the measured or estimated glomerular filtration rate (eGFR). There are five stages: stage one indicates normal excretory function and stage five either severe impairment of renal function or the need for renal replacement therapy (RRT). The suffix p denotes the presence of significant proteinuria of more than 1g/day (12).

1.2.2 Cardiovascular disease in chronic kidney disease

CKD is increasingly prevalent, affecting 8.5% of the population in the U.K. and 10.9% of the population in the US (13;14). The burden of CVD in these patients is substantially higher than that of the general population. Moreover, patients with CKD are significantly more likely to die of CVD than to progress to end stage renal disease (ESRD) (15-17). Even with adjustment for age, the presence of diabetes mellitus (DM), racial and sex differences, CV mortality in end stage CKD is 10-30 times that of the general population such that a 25-year-old receiving haemodialysis (HD) maintenance has a mortality which is roughly equivalent to that of an 80-year-old in the general population (14;17). Dialysis is expensive costing approximately £35,000 per patient annually (18). Renal transplantation is currently the only definitive treatment available and offers a survival advantage over maintenance dialysis (19;20). Yet CVD mortality rates of at least twice that of the general population persist and the overall prevalence of CVD continues to be approximately four times higher (14;21). Furthermore there is a shortage of deceased donor kidneys and renal transplantation is not a viable option for everyone (22).

Without transplantation, the five year survival for a patient aged around 60 years on dialysis is 46% which is equivalent to five year survival from ovarian cancer and significantly worse than five year survival from breast, colon or bladder cancer. Notably if a cancer sufferer survives, their chance of survival at ten years increases whereas in dialysis patients, it continues to reduce leading to a trend survival of less than 15% (Table 1-1) (23-25). Thus, CKD represents a significant, worldwide public health concern and socio-economic burden.

Diagnosis	Approximate 5 year patient (aged 60-64) survival (%)	Incidence Per 100,000 UK population aged 60-64 years
CKD (on dialysis)	46	15
Renal Transplant	75	62 (prevalence)
Ovarian Cancer	42	49
Colon Cancer	60	132
Lung Cancer	11	136
Skin Cancer (melanoma)	85	39
Breast Cancer	90	360

Table 1-1: Comparison of the five year survival in a patient aged between 60-64 years with different diagnoses.

Adapted from Hutchison et al and based on statistics from Cancer Research UK and The UK Renal Registry (23-26).

1.2.3 Cardiovascular risk factors in chronic kidney disease

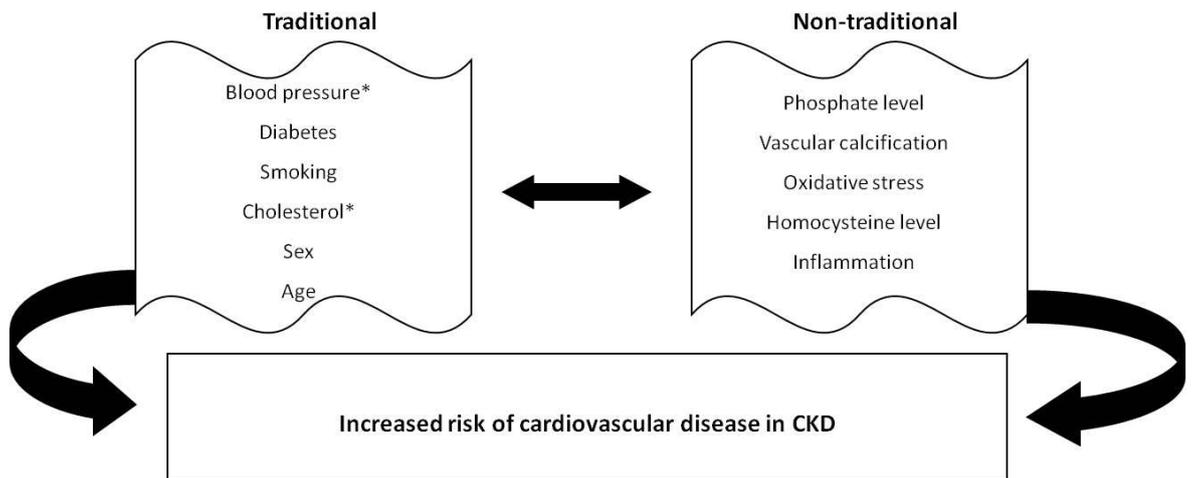
The disproportionate CV risk seen in CKD is not adequately explained by the traditional atherosclerotic risk factors such as hypertension and DM, and the pattern of CVD differs with an excess of sudden death and death due to chronic heart failure rather than myocardial infarction (27;28). Some traditional risk factors including body mass index (BMI) and hypertension exhibit ‘reverse epidemiology’ in HD patients whereby low BMI and hypotension are risk factors for CVD compared to obesity and hypertension in the general population (29;30).

In an attempt to explain the gap in risk between the CKD and the general population, ‘non-traditional’ risk factors which may be unique to the CKD population have been described (2;14). In reality, excess CV risk probably reflects a combination of traditional CV risk factors, including hypertension and ‘non-traditional’ risk factors including serum phosphate level (Figure 1-1), ED and oxidative stress (31).

Serum phosphate level, even within the normal range, has been shown to be an independent risk factor for CVD in the CKD population, including recipients of a renal transplant, and in healthy volunteers (2;8;32;33). It is of particular relevance in CKD because the normal homeostatic mechanisms are disrupted leading to disequilibrium with clinically significant consequences (3;4). Serum phosphate is inversely proportional to eGFR, although there is individual variation (34). The mechanisms of action of phosphate as a CV risk factor are not well understood and may relate to direct effects on VC (dependent upon the calcium phosphate product), effects on endothelial function and indirect effects mediated via regulators of phosphate homeostasis including fibroblast growth factor 23 (FGF-23) (6;35;36).

1.2.4 Progression of chronic kidney disease

Several risk factors have been demonstrated to increase the likelihood of progressive loss of renal function including poor control of hypertension and low eGFR at presentation (37;38). However, the best independent predictor of outcome in CKD is proteinuria (39). This is true in patients with both primary renal disease and diabetic renal disease (40;41).



**Exhibit U shaped curve with mortality and a degree of reverse epidemiology so both high and low blood pressure and high and low cholesterol increase an individual's risk.*

Figure 1-1: Traditional and non-traditional risk factors for CVD in CKD.

Proteinuria is also a sensitive predictor of chronic renal transplant dysfunction and of long-term graft survival (42-44) and also predicts all-cause and CV mortality in the general, renal transplant and CKD populations (45-48).

1.2.5 Proteinuria

Proteinuria indicates renal damage and reflects the increased permeability of the glomerulus as a consequence of renal disease and tubular losses (49). Evidence also points to a directly toxic effect of proteinuria causing stimulation of pro-fibrotic, inflammatory chemokines including interleukin (IL) 8 and transforming growth factor (TGF) β markers in the proximal convoluted tubule (PCT) and proximal tubule cell apoptosis (50). The extent of interstitial fibrosis (and associated tubular depletion), determined by renal biopsy, is the best histological correlate of risk of progressive renal decline (51).

The increased CV risk seen with proteinuria is multi-factorial. In part it relates to its association with a more rapid decline in renal function and thus more rapid progression to the increased risk associated with low eGFR. Other postulated mechanisms include inflammation, hypertension and ED (52). Proteinuria might not only represent renal damage but increased endothelial permeability throughout the body. In 2003, Paisley et al demonstrated macro-vascular ED in individuals with proteinuria in addition to a low grade inflammatory response which was associated with impaired micro-vascular endothelial function (53). Stroes et al showed a similar impairment in macrovascular endothelial function and postulated that this might relate to defective nitric oxide (NO) release (54). Associations have been demonstrated between proteinuria and alterations in the NO and vascular endothelial growth factor (VEGF) pathways (52). No study demonstrates a causative link and whilst proteinuria may be directly toxic to the vasculature, it may simply be a marker reflecting widespread ED.

1.2.6 Renin-angiotensin-aldosterone system blockade

The benefit of therapeutic strategies which reduce proteinuria to slow the progression of renal decline has been clearly established (55-58). The renin-angiotensin-aldosterone system (RAAS) plays a well recognised role in the pathogenesis of CV and renal disease with activation resulting in oxidative stress, a pro-inflammatory environment and fibrosis

(59). Proteinuria may be a surrogate marker for the damage inflicted by activation of the RAAS. In the last decade, inhibition of RAAS with angiotensin converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARB) has become the mainstay of the management of the common manifestations and complications of CKD: CVD, hypertension and proteinuria. The reno-protective effects of ACEi and ARB in CKD appear to extend beyond their blood pressure lowering capabilities (56). However, despite RAAS blockade, a proportion of patients will develop end stage renal disease and no study has demonstrated that these agents reduce CV risk or CVD in this population.

One reason for the incomplete benefits of ACEi/ARB may be that neither agent blocks the RAAS completely and aldosterone may be independently regulated. The detrimental effects of aldosterone include ED and fibrosis (59). Blockade of the mineralocorticoid receptor may confer additional benefits in terms of reno-protection particularly when taken in combination with ACEi or ARB (60). Similarly, the direct renin inhibitor, aliskeren, blocks RAAS at an earlier point and may confer additional benefits (61). There is a paucity of trials looking at either renal or patient outcome with these drugs and any such study would require a large cohort and an extensive follow up period.

1.2.7 Phosphate and progression of chronic kidney disease

Interest in phosphate waned in the 1980s and 1990s with research focussing on proteinuria, hyperparathyroidism and VC in CKD progression and related mortality. However with the discovery of new factors involved in bone mineral metabolism in CKD, interest in phosphate has revived.

Thirty years ago, Ibels et al demonstrated dietary phosphate restriction in a remnant-kidney rat model prevented histological change, reduced renal functional decline, ameliorated proteinuria and reduced mortality (62). A second study in the 5/6 nephrectomised rat confirmed that those animals fed a high phosphate diet were more likely to develop progressive renal impairment and myocardial hypertrophy (63). In the 1980s, a clinical study demonstrated that a phosphate and nitrogen restricted diet had a renoprotective effect (64). In 2006 and 2007, three further observational clinical studies highlighted an association between progression of renal disease and higher serum phosphate levels (65-67). A further observational study in 2011 confirmed that elevated serum phosphate increased the likelihood of progression of renal disease independently of eGFR (68).

Observation does not confirm causality nor does it give insight into the mechanism by which higher phosphate levels might accelerate renal decline.

1.3 The vasculature in chronic kidney disease

The vasculature becomes progressively dysfunctional in CKD with evidence of increased stiffness and ED ubiquitously present from the large vessels down to the very small resistance vessels.

1.3.1 Arterial stiffness, pulse wave velocity and the augmentation index

In health, the arterial vessels are both elastic and compliant allowing forward and retrograde pressure waves to propel blood from and towards the heart. Their compliance affords the ability to smooth the transition of the pressure wave throughout the arterial tree ensuring a constant flow of blood to the tissues. Proximal arteries (eg ascending aorta) are comprised of a higher proportion of elastin and collagen and thus are more elastic than their distal counterparts (eg femoral artery). Compliance of an artery is the product of the vessel's distensibility and the volume of blood contained within the vessel. In less distensible and therefore stiffer arteries, blood flow dynamics are affected; forward and retrograde pressure waves travel more rapidly through a stiffer vessel (69). Pulse wave velocity (PWV) therefore acts as a surrogate for arterial stiffness. It is derived from the distance between two recording sites in the line of pulse travel (typically the carotid and femoral arteries) and the time delay between the corresponding points of the pressure wave. PWV measurement is non-invasive, straightforward and reproducible and it is acknowledged to be the gold standard measure of arterial stiffness (70).

PWV is higher in distal compared with proximal arteries. As a pulse wave travels through the distal vessels, its amplitude will increase such that in a fit, healthy, young individual with a normal heart rate, brachial pulse pressure may be up to 50% higher than that of the aorta (71). Consequently, brachial pressure cannot be considered to be the same as aortic pressure. The augmentation index (AIx) measured by pulse wave analysis (PWA) is another reflection of arterial stiffness. In a stiffer artery, the PWV increases such that the pulse wave will arrive 'early' in systole (71). As a result, the forward pressure wave is

enhanced, systolic blood pressure rises and diastolic blood pressure is proportionately lower. The amount by which the reflected wave augments the forward pressure wave is the AIx. This is summarised in Figure 1-2. Any factor which tips the balance between production and degradation of elastin and collagen towards reduced quantities of the proteins can contribute to vascular stiffness; ageing, DM, inflammation, hypertension, CKD, hormonal and dietary factors (72). The more compliant proximal arteries are those predominantly affected by stiffening. As a consequence of arterial stiffness, arterial pressure rises, cardiac performance and coronary perfusion are compromised. Not unexpectedly, increased arterial stiffness is linked to ageing, CV morbidity and mortality, all cause mortality and dementia (72-74). Measurements of PWV and AIx are predictive of CV events and mortality in the general population and in CKD (75-78). In CKD, both also correlate with progression of renal disease (79;80).

1.3.2 Arterial stiffness in chronic kidney disease

Arterial stiffness increases as renal function declines. This is true even if changes in eGFR are minor and within the normal range (81;82) and may reflect the association of uraemia with factors which are known to contribute to arterial stiffness including inflammation, ED, oxidative stress, malnutrition, DM, insulin resistance and hypertension (35;72).

However there are other factors, prevalent in CKD, which contribute to arterial stiffness - notably VC, the hallmark of which is calcium and phosphate deposition as hydroxyapatite (83). In the CKD population compared with the non-CKD population, extensive VC is often present. In those with advanced CKD this is classically calcification of the arterial media - Mönckeberg sclerosis - which occurs independently of atheromatous disease of the intima (4;84). Intimal calcification, in the context of atheroma, may also develop but medial calcification is significantly more common (4;85).

Explanations for the increased prevalence of VC seen in CKD include disordered bone mineral metabolism and the recognition of a complex and active osteochondrogenic differentiation process whereby the vascular smooth muscle cell (VSMC) of the media undergoes phenotypic transformation into an osteoblast like cell (86;87). There is increasing evidence that phosphate plays a key role in the development of VC and this will be discussed more fully in section 1.7.3.

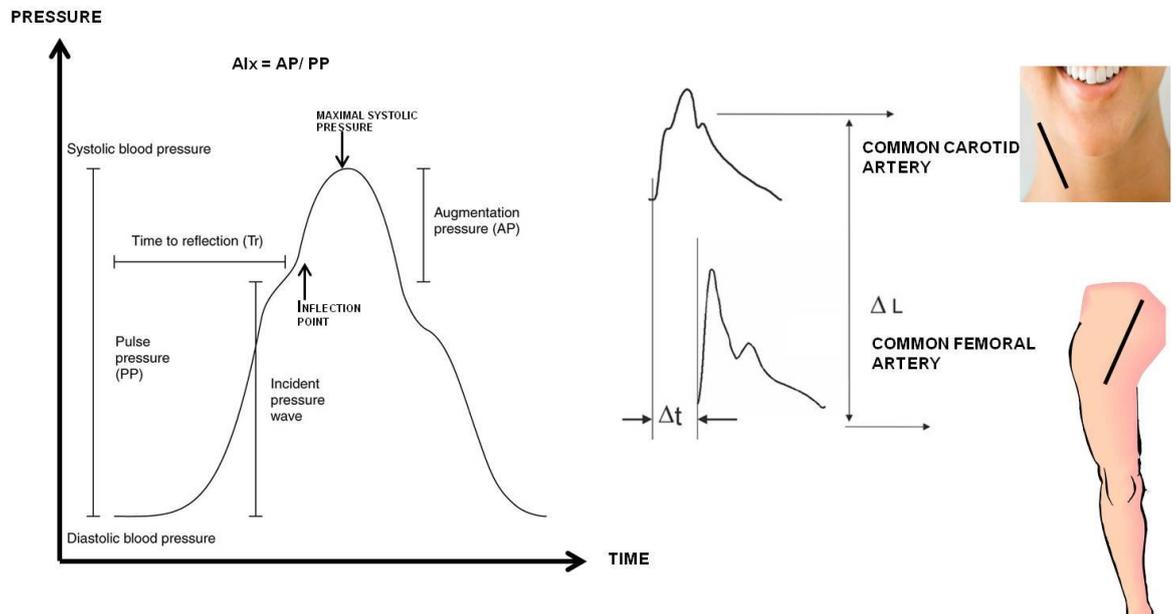


Figure 1-2: Graphical representation of pulse wave velocity and derived functions.

The augmentation pressure (AP) is the pressure difference between the maximal systolic peak and the inflection point. Aix is the augmentation index and is calculated by dividing AP by the pulse pressure (PP). To ascertain the pulse wave velocity: Δt is the difference in time between the upstroke in the common carotid artery and the upstroke in the common femoral artery, after the R wave and ΔL is the distance the pulse wave has to travel. Adapted from Laurent et al and Janner et al (71;88).

1.4 The endothelium in health and chronic kidney disease

The endothelium is a mono-layer of squamous cells which lines the inner surface of every blood vessel. It serves as a pivotal connection between the vascular wall and the blood circulating through the vessel lumen and plays a central role both in health and disease (89). In health, the endothelium is central to the maintenance of vascular tone and provides a non-thrombotic, smooth surface to line blood vessels and provide structural integrity. Additionally, directly or indirectly, the endothelium produces an abundance of mediator molecules involved in coagulation, VSMC growth and proliferation and inflammation (89;90).

Endothelial cells (ECs) 'sense' and respond to mechanical signals created by blood flow (pressure, stretch and shear stress), as well as humoral eg adrenaline, noradrenaline, aldosterone, acetylcholine, angiotensin II and endothelin-I and chemical (glucose, reactive oxygen species (ROS) and homocysteine) factors (90-93). Detection of these signals, alone or in combination triggers ECs to release mediators, including NO, which modulate processes involving the blood vessel wall, including adhesion of leucocytes, release of pro-thrombotic and antithrombotic factors and release of vasoactive substances (91).

When the endothelium is damaged, ED develops and this refers to a pro-thrombotic and pro-inflammatory environment in which there is increased vascular reactivity with impaired endothelium dependent vasodilation, leucocyte adhesion and platelet aggregation (90;94). This can affect any area of the vasculature, and is the hallmark of all major vascular causes of mortality and morbidity: CKD, atherosclerosis, hypertension, pulmonary hypertension and DM (89;95-98).

ED has been shown to be an independent predictor of CV events in patients with CV risk factors or pre-existing CVD (99;100). In healthy volunteers without DM or pre-existing CVD, it is independently predictive of progression of carotid arterial disease and of future CV events (101;102).

ED may be partly reversed by statin therapy and with ACEi and ARBs, independently of any blood pressure lowering effect (103;104). Antioxidants may also be effective although the evidence is mixed with one study showing an improvement in endothelial function with treatment with a combination of vitamins C and E and α -lipoic acid in elderly but not in

younger patients (105). In women with polycystic ovarian syndrome, metformin and pioglitazone improve endothelial function (106).

1.4.1 The nitric oxide pathway and endothelial dysfunction

The major modulatory molecule released by ECs is NO. The NO pathway is summarised in Figure 1-3.

NO is produced under basal conditions as well as in response to the mechanical, humoral and chemical stress as described above (107). Basal NO production is reduced in conditions including coronary artery disease, where ED is present (108). NO is a powerful relaxing factor which is synthesised from the oxidation of L-arginine by nitric oxide synthase (NOS). There are three isoforms of this enzyme: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). The form predominantly present in endothelial cells is eNOS. eNOS has two phosphorylation sites: Ser1177 which activates eNOS and Thr495 which inhibits eNOS (90;109). eNOS produces NO continuously in the picomolar range. iNOS is present in VSMC and is not responsible for basal NO production; it needs to be 'switched on' by cytokines, mechanical stress or endotoxins; production of NO is delayed and levels produced are in the nanomolar range (110).

NO is a paracrine mediator, diffusing from ECs to adjacent VSMCs where it triggers a signalling cascade mediated by activation of guanylate cyclase and the generation of cyclic guanosine monophosphate (cGMP). This promotes the stimulation of protein kinase G (PKG) and results in the activation of several transcription factors; the end result is inhibition of calcium mediated vasoconstriction (90;111).

In addition to its role as a potent vasodilator, NO inhibits platelet aggregation, leucocyte adhesion (107) and VSMC proliferation (possibly by inhibition of the enzyme, ornithine decarboxylase which is essential for cell growth) (112). VEGF is produced by a number of different cells and promotes vasodilation. It acts on ECs to increase endothelial permeability and stimulates phosphorylation of eNOS thus increasing NO levels (113). Levels of VEGF have been shown to be reduced in the setting of ED (114).

When ED is present, there is reduced NO release. This promotes a pro-inflammatory state and encourages leucocyte adhesion and platelet aggregation with increased expression of the cell adhesion molecules including soluble E-selectin and vascular cell adhesion

molecule (VCAM) (115). Elevated levels of soluble E-selectin have been shown to be independently predictive of mortality in patients with pre-existing CVD (116). In the pro-inflammatory state of ED, the adenosine receptor, ADORA2A may be protective: it is activated which enhances production of the intracellular signalling molecule, cyclic adenosine monophosphate (cAMP) and reduces cell adhesion molecule expression (117).

Thus, NO has a central role in the relationship between ED and coagulation which explains the importance of ED in CVD specifically occlusive coronary disease and stroke.

1.4.2 Reactive oxygen species and oxidative stress

Reduction-oxidation reactions (redox) occur *in vivo* to convert oxygen to water.

Intermediates, termed ROS are produced as part of the reduction of oxygen. Free radicals are species with an unpaired electron. ROS include both free radicals (superoxide (O_2^-), peroxynitrite) and peroxides (hydrogen peroxide) (118).

In health, ROS are products of normal cellular metabolism and the concentration of ROS is dependent upon the balance between production and removal. Removal occurs via ROS scavengers including traditional antioxidants like vitamins C and E as well as enzymes and proteins (118). An anti-oxidant is a substance which inhibits oxidation of oxidizable substrates by direct competition (119). ROS stimulate expression of 'defence genes' eg NADPH oxidases and activate transcription factors to control endothelial function and maintain vascular tone (90;94;120). Oxidative stress occurs when there is impaired redox signalling and a pro-oxidant environment. In this circumstance, ROS induce cellular injury and play a pathological role in inflammation, hypertrophy, proliferation, angiogenesis and fibrosis, all of which contributes to ED.

NO is highly reactive with free radicals and this is of benefit; it has been shown to scavenge peroxyl radicals and to inhibit lipid peroxidation. (121). However, a by-product of the reaction between NO and O_2^- is peroxynitrite, which is itself a ROS. The superoxide dismutase (SOD) enzymes are thought to balance this reaction by controlling levels of O_2^- (121).

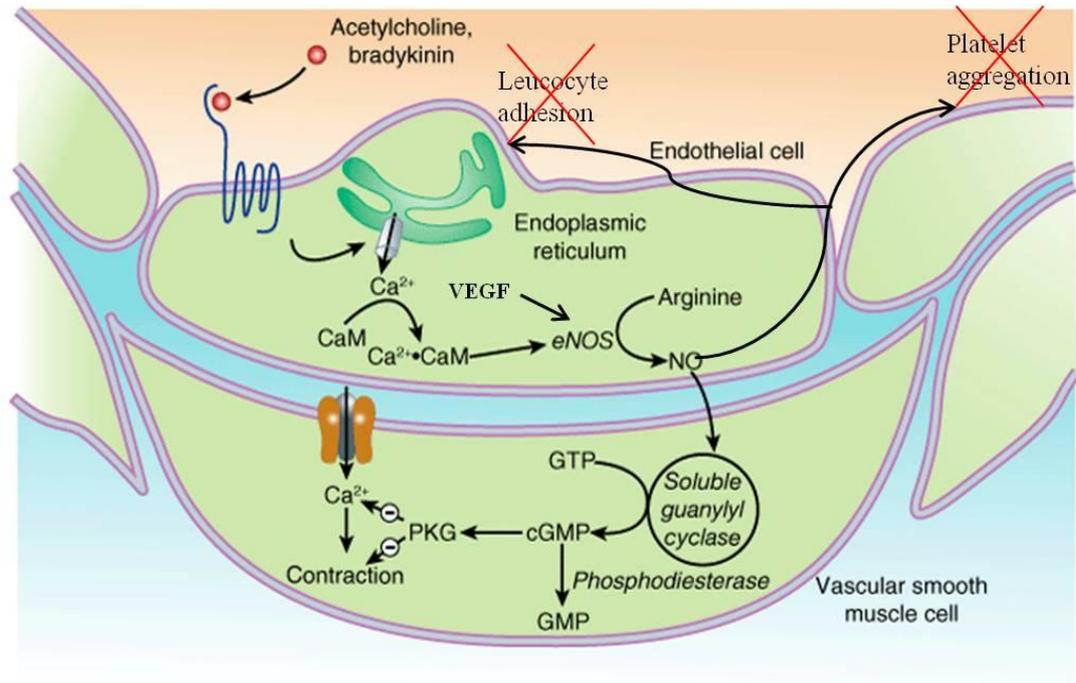


Figure 1-3: The nitric oxide pathway.

Upon stimulation of an EC, the calcium regulatory protein, calmodulin (CaM) binds eNOS resulting in the production of NO. Additional stimulation of NO production comes via the vascular endothelial growth factor (VEGF) pathway. NO diffuses into the VSMC where it stimulates the production of cGMP from guanosine-5'-triphosphate (GTP) via activation of the enzyme, guanylyl cyclase. cGMP activates protein kinase G (PKG) which has a negative effect on calcium and causes subsequent relaxation of the muscle cell and vasodilatation of the blood vessel as a whole. cGMP is broken down to the guanosine monophosphate (GMP) by a group of enzymes, the phosphodiesterases. NO also prevents platelets and leucocytes from sticking to the blood vessel wall. Adapted from Katzung et al (122).

1.4.3 Endothelial dysfunction in chronic kidney disease

ED is present in CKD and the degree worsens with severity of renal disease. Low GFR, is linked to clinical measures of endothelial function and increased oxidative stress (123-125). Many of the clinical studies in this area have used forearm plethysmography or flow mediated dilatation (FMD), measured in the brachial artery, to assess endothelial function. The differences seen demonstrate that in CKD, ED is widespread and not confined to the renal vascular bed. Thus ED is very likely to play a role in the increased premature CV risk seen in the CKD population which is unexplained by traditional CV risk factors.

The mechanism of ED seen in CKD is not well understood, and much of the work in this area has been in animal models. ED may be either a cause or a consequence of CKD. In reality it is likely that there is an initial insult leading to endothelial damage which then self-propagates the problem with imbalances in redox as described in section 1.4.2.

Alterations in eNOS and O_2^- expression have been demonstrated as well as evidence of increased lipid peroxidation products, abnormalities in EC growth and impaired relaxation in human resistance vessels from patients with CKD (6;95;124;125).

Renal EC dysfunction plays a central role in the pathogenesis of disorders including vasculitis, glomerulonephritis, DM and haemolytic uraemic syndrome (126;127).

Proteinuria (sections 1.2.5 and 1.2.6), when present, is likely to be a marker of ED, specifically glomerular EC dysfunction as a marker of generalised ED (52;53) and may explain the link between ED and progression of CKD (126).

ED is an early marker of CVD, facilitating the development of atherosclerosis and hypertension. An improved understanding of the mechanism of ED in CKD may allow for targeted therapies to reduce CV risk; the contribution of phosphate to ED is one possibility.

1.4.4 Flow mediated dilatation

In vivo, FMD is the best validated, non-invasive technique for measurement of endothelial function and correlates closely with invasive measurements at coronary angiography following acetylcholine injection (128). A schematic representation of FMD is presented in the cartoon in Figure 1-4.

Using high resolution B mode doppler ultrasound, the brachial artery can be imaged to quantify changes in artery diameter secondary to increased blood flow (Figure 2-7, Chapter 2). This can provide an indicator of vasomotor function. A blood pressure cuff is inflated on the forearm to occlude the arterial inflow for five minutes. This causes ischaemia and dilation of downstream blood vessels as a result of auto-regulation. When the cuff is deflated there is a brief period of increased blood flow through the artery, reactive hyperaemia, which in the healthy endothelium stimulates NO release from the endothelium with consequent vasodilation of the brachial artery – FMD (130). This measure has been validated in a number of populations including healthy volunteers and those with CKD (123;131).

Endothelium independent vasodilation can also be measured with this technique. Glyceryl tri-nitrate (GTN) is a NO donor which causes dilation of the brachial artery independently of the endothelium. It allows determination of the maximum possible vasodilation response (seen approximately 3-4 minutes following administration of GTN) and provides an assessment of VSMC function (129).

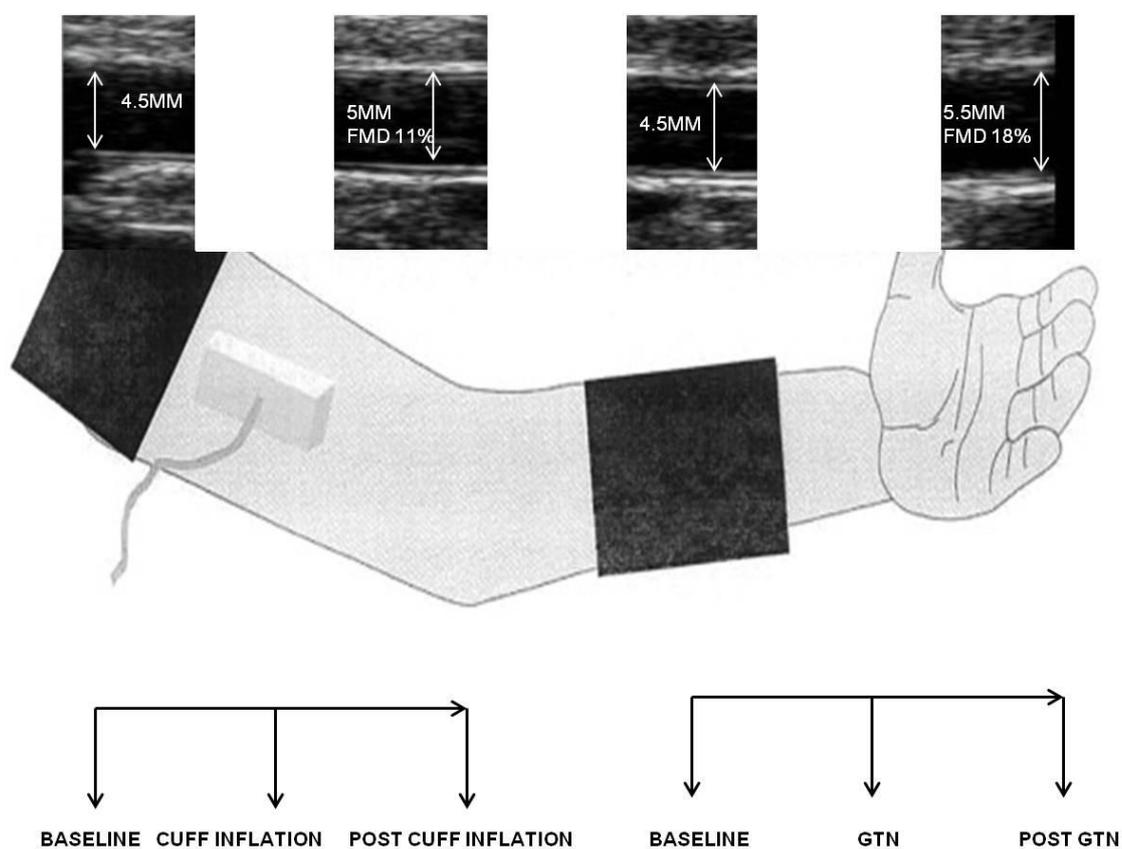


Figure 1-4: Schematic representation of FMD.

Baseline brachial artery diameter is measured pre and post inflation of a blood pressure cuff and the % difference is calculated as a measure of endothelial function. When the endothelium is intact the vessel dilates when the cuff is released and blood flow is increased: 'reactive hyperaemia'. The same measurements can be undertaken after administration of GTN spray to assess endothelium independent function. Adapted from Corretti et al (129).

1.5 Phosphate

1.5.1 Phosphorus or phosphate

Phosphorus, described as the ‘Devil’s element’ because of its use in explosives, poisons and nerve agents, was first discovered in 1669 by a German chemist, Hennig Brand. Brand was trying to produce ‘The Philosopher’s Stone’ by evaporating urine when he isolated a white substance which glowed in the dark - phosphorus. In ancient Greece, phosphorus was the name for the planet Venus and has its origins in the Greek words for ‘light’ and ‘carry’ thus phosphorus translates roughly as light carrier (132).

Phosphorous is a non-metal element which is part of the nitrogen group in the periodic table (atomic number 15). It is extremely reactive and does not exist as a free element, but rather in an oxidised state as phosphate (PO_4^{3-}), a component of many minerals (133). Throughout this thesis, the term phosphate will be used.

Phosphate is a tetrahedral anion and is found within the human body in several forms. Approximately 80% of total body phosphate is stored in the teeth and bones as solid minerals, calcium phosphate, hydroxyapatite (and fluoroapatite, which is harder and created when hydroxyl groups are replaced by fluoride ions). 14% is found intracellularly and 1% in the serum (134).

Serum phosphate acts as an important buffer system existing in four forms; the two main forms are hydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate (H_2PO_4^-). Briefly, the reaction scheme for this buffer system is: $\text{H}^+ + \text{HPO}_4^{2-} \rightleftharpoons \text{H}_2\text{PO}_4^-$

At a neutral pH of 7.4, the pK value of H_2PO_4^- is 6.8 and $\text{HPO}_4^{2-}:\text{H}_2\text{PO}_4^-$ exist in a ratio of 4:1. In strongly acidic conditions, a third form, H_3PO_4 (phosphoric acid) predominates. In a strongly alkaline environment, the fourth form, the phosphate ion is the dominant form (PO_4^{3-}). Remaining serum phosphate (about 14%) is found bound to protein. Phosphate forms chains and rings with phosphoanhydride bonds (P-O-P bonds). Both adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are polyphosphates (135;136).

Although serum phosphate is only a small percentage of total body phosphate, it can easily be measured and provides some insight into total phosphate stores (134). The concentration of phosphate is generally expressed as millimoles/litre (mmol/L or mM); in

the USA, it is often expressed as milligrams/decilitre (mg/dL). mM can be converted to mg/dL by dividing by 0.323 such that 1mg/dL is equivalent to 0.323mM (134). The reference range for serum phosphate measurements in our laboratory (Gartnavel General Hospital) is 0.7-1.4mM (2.2-4.3mg/dL) in an adult.

1.5.2 Phosphate homeostasis in health

Despite the accolade of the sixth most abundant element present in the human body and playing a crucial role in several biological processes and functions including genetic coding, energy metabolism, oxygen carrying by haemoglobin, and intracellular signal transduction, the precise details of phosphate homeostasis are poorly defined (137).

To maintain phosphate at physiological levels, there has to be a tightly regulated sensing and signalling mechanism. In phosphate homeostasis two types of cell sensing have been described: metabolic sensing which is how human cells sense inorganic phosphate to regulate cell metabolism and endocrine sensing which describes how extracellular phosphate is regulated via feedback systems (138). Mechanisms for phosphate sensing in yeast and bacteria have been well described leading to alterations in protein phosphorylation and transcription (139;140). In humans and other mammals, identifying the phosphate sensor(s) has proved elusive. The four main organs involved in phosphate regulation: kidney, bone, parathyroid gland and intestine all 'talk' to each other and there is now a sizeable body of evidence describing how this occurs and this in turn provides further evidence that a phosphate sensor must exist in humans although we have yet to find the sensor or its effector mechanisms.

1.5.2.1 Phosphate and diet

Phosphate is present in many foods, most importantly dairy products, meat, whole grains, fizzy juice and food additives. The phosphate content of selected foods is incorporated into Figure 1-5 (141). In the Western world, average daily phosphate consumption approximates 1000-1500mg. However, the modern diet is high in processed foods which often contain food additives including mono-calcium phosphate and sodium phosphate (9;141). High consumption of processed food contributes up to 1000mg of additional phosphate per day (142). Currently, the amount of phosphate does not need to be quantified on product labels and thus the actual phosphate intake and the estimated phosphate intake might differ significantly (143). In health, bone contains an exchangeable pool of phosphate and

behaves as a reservoir (Figure 1-5). There is constant exchange of phosphate to and from this pool between bone and the serum; in combination with dietary absorption and renal resorption, this maintains serum phosphate levels within the normal range (144).

1.5.2.2 Phosphate transport in the intestine

The majority of phosphate from the diet is absorbed in the small intestine via two processes: a passive diffusional process and an active process via sodium dependent phosphate co-transporters. Passive absorption relies upon electrochemical gradients across the intestinal epithelial cells with phosphate diffusing through the junctions between adherent neighbouring cells. This process is dependent upon phosphate load in the intestine from ingested food (145).

The type II sodium dependent phosphate co-transporters are responsible for active absorption of phosphate from the small intestine. There are three type II transporters: NaPi2a, NaPi2b and NaPi2c, encoded for by the SLC34A1, SLC34A2 and SLC34A3 genes respectively(146). NaPi2a and NaPi2c are largely responsible for renal transport of phosphate and are discussed in section 1.5.2.3.

NaPi2b is principally expressed in the brush border membrane of the small intestine and is responsible for the active absorption of phosphate here (147). Expression of this transporter is primarily regulated by vitamin D although FGF-23 is also thought to play a key role (145;147). Other factors including oestrogen, glucocorticoids and thyroid hormones may also be involved, and a study in a knock out mouse model demonstrated that phosphate itself may directly stimulate expression of the receptor independently of vitamin D (145;148). In this model, serum phosphate levels were unchanged which is likely to be explained by the observed increased expression of NaPi2a in the kidney with consequent increased renal reabsorption of phosphate. In humans with genetic mutations and resultant inactivation of NaPi2b, serum phosphate levels are unaltered which is at odds with NaPi2b playing an essential role in serum phosphate regulation (149).

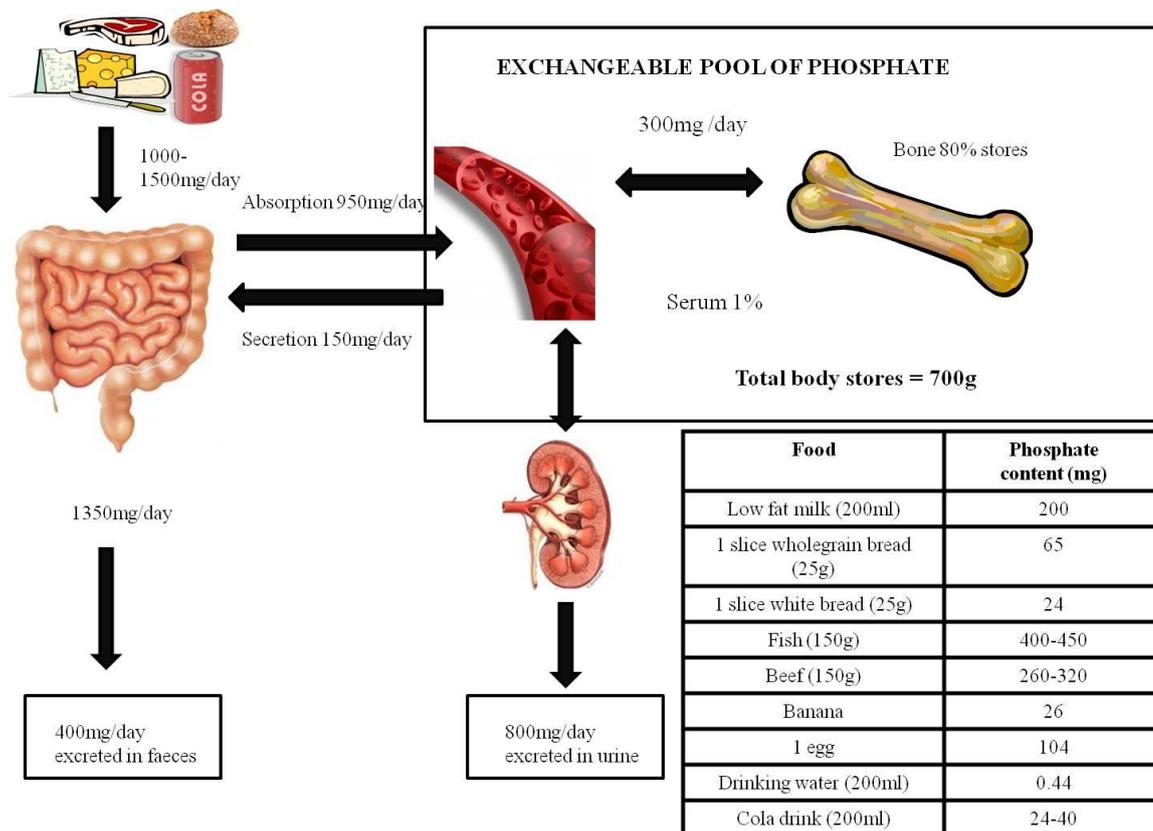


Figure 1-5: Phosphate balance within the body.

Phosphate is consumed within the diet and excreted mainly via the kidneys but also via the intestine. The bone acts as a reservoir and constantly exchanges phosphate with the serum. Phosphate content of commonly consumed foods is also shown.

1.5.2.3 Phosphate transport in the kidney

Phosphate is filtered in the glomerulus and approximately 70% of filtered phosphate is reabsorbed in the PCT in the kidney (150). NaPi2a and NaPi2c are expressed in the PCT and facilitate phosphate transport across the apical brush border epithelial membrane of the PCT cells with efflux at the basolateral membrane (147). Parathyroid hormone (PTH) is the major regulator of transporter expression (discussed in detail in section 1.5.2.6). High phosphate concentration in the glomerular filtrate also directly stimulates release of these transporters from brush border vesicles to reduce phosphate reabsorption (144).

NaPi2a appears to play a more prominent role in phosphate reabsorption than NaPi2c and might be responsible for up to 70% of phosphate reabsorption (147;150). Several differences between the transporters have been identified. NaPi2a transports three sodium ions with phosphate whereas NaPi2c carries only two (146). PTH reduces expression of NaPi2a and therefore reduces renal phosphate reabsorption. In a knock out mouse

(NaPi2a^{-/-}), proven to express NaPi2c, PTH does not have this effect and thus PTH does not seem to be able to alter renal phosphate reabsorption by altering the expression of NaPi2c (150).

In animal studies, NaPi2c seems to be more abundantly expressed in the neonatal period and expression reduces thereafter (151). Humans with hereditary hypophosphataemic rickets have profound hypophosphataemia, rickets and short stature. In this condition, loss of function mutations in the SLC34A3 (encodes NaPi2c) have been sequenced (152). It is likely therefore that, at least in humans, NaPi2c plays a more important role than initially appreciated in renal phosphate transport.

1.5.2.4 Phosphate transport in other tissues and organs

PiT1 and PiT2 are type 3 phosphate transporters encoded for by SLC20A1 and SLC20A2. Both transport phosphate with a high affinity and are widely expressed throughout the body (145;150). Current thinking assumes that these transporters are ‘housekeepers’ responsible for intracellular transport of phosphate to provide cells with their basic phosphate requirements. These transporters have not been as well studied as the type II phosphate transporters and there is little cellular data to support the ‘housekeeper’ theory. Recent evidence suggests that the importance of PiT1 and 2 may have been overlooked. In

an animal model, PiT1 has been shown to have a pivotal role in liver development (153). In human cell lines depleted of PiT1, cell proliferation is markedly reduced and cells are more sensitive to the pro-apoptotic effects of tumour necrosis factor (TNF) α which implies a role in cell growth and possibly in cancer pathogenesis (154;155). These observed effects are independent of phosphate transport function.

1.5.2.5 Hormonal regulators of phosphate homeostasis

Maintaining phosphate balance centres upon regulation of phosphate excretion by the kidney. This is controlled by three major regulatory factors; PTH, vitamin D and FGF-23. Homeostasis depends upon co-ordination between these factors and not upon on any one factor individually.

1.5.2.6 Parathyroid hormone

PTH is produced by the parathyroid glands and its main role relates to calcium homeostasis (150). In health it is produced primarily in response to reduced serum calcium but also to increased serum phosphate levels. PTH exerts its effects on bone and the kidney via the type 1 PTH receptor with several consequences: increased urinary phosphate excretion, reduced urinary calcium excretion, increased production of vitamin D and increased calcium release from bone (36;147;156). This serves to maintain serum calcium and phosphate at physiological levels. The role of PTH in phosphate homeostasis is summarised in Figure 1-6.

PTH binds to the type 1 PTH receptor in the PCT cells and reduces the expression of NaPi2a. This occurs via one of three pathways: protein kinase C (PKC) signalling, the cAMP dependent phospholipase C pathway and the mitogen activated protein kinase (MAPK) pathway. Correct targeting of PTH and NaPi2a seems to require the presence of an additional factor: sodium-proton exchanger regulatory factor 1 (NHERF1) which interacts with the PTH receptor and phospholipase C (146;147;150). Thus PTH reduces renal phosphate transport and increases urinary phosphate excretion.

PCT expression of 25-hydroxyvitamin D 1- α -hydroxylase is increased by PTH resulting in the conversion of inactive vitamin D, 25-hydroxyvitamin D (25-OH-D) to active vitamin D, 1,25-dihydroxyvitamin D₃ 1-25-(OH)₂-D. Vitamin D acts on the renal distal convoluted tubule (DCT) to increase calcium reabsorption although its major effect is on the intestine where it increases both calcium and phosphate absorption (36). The

contrasting effects of vitamin D and PTH on the kidney and intestine serve to balance serum phosphate levels whilst maintaining calcium homeostasis. PTH also increases bone resorption by osteoclasts thereby stimulating calcium release from bone (157). Lastly PTH may increase the expression and production of FGF-23, which is discussed in section 1.5.2.8 (36).

1.5.2.7 Vitamin D

Vitamin D circulates in an inactive form, 25-OH-D which is filtered by the kidneys and reabsorbed in the PCT (158). The active form, 1-25-(OH)₂-D, is secreted in the kidney following conversion of 25-OH-D by 25-OH-D 1- α -hydroxylase and it mediates its effects via the vitamin D receptor (159). Elevated serum phosphate and lowered serum calcium levels enhance the activity of 25-OH-D 1- α -hydroxylase. PTH stimulates transcription of 25-OH-D 1- α -hydroxylase (147). Active vitamin D increases calcium and phosphate absorption in the small intestine. FGF-23 reduces expression of 25-OH-D 1- α -hydroxylase thereby reducing 1-25-(OH)₂-D production (158).

1-25-(OH)₂-D has a negative regulatory effect on PTH and on itself by reducing expression of 25-hydroxyvitamin D 1- α -hydroxylase and increasing expression of 24-hydroxylase (24-OH-ase) which converts active vitamin D into an inactive form. 1-25-(OH)₂-D increases production of FGF-23 from bone and increases gene expression of Klotho, the FGF-23 co-factor. This promotes additional negative feedback on 1-25-(OH)₂-D production (158;159).

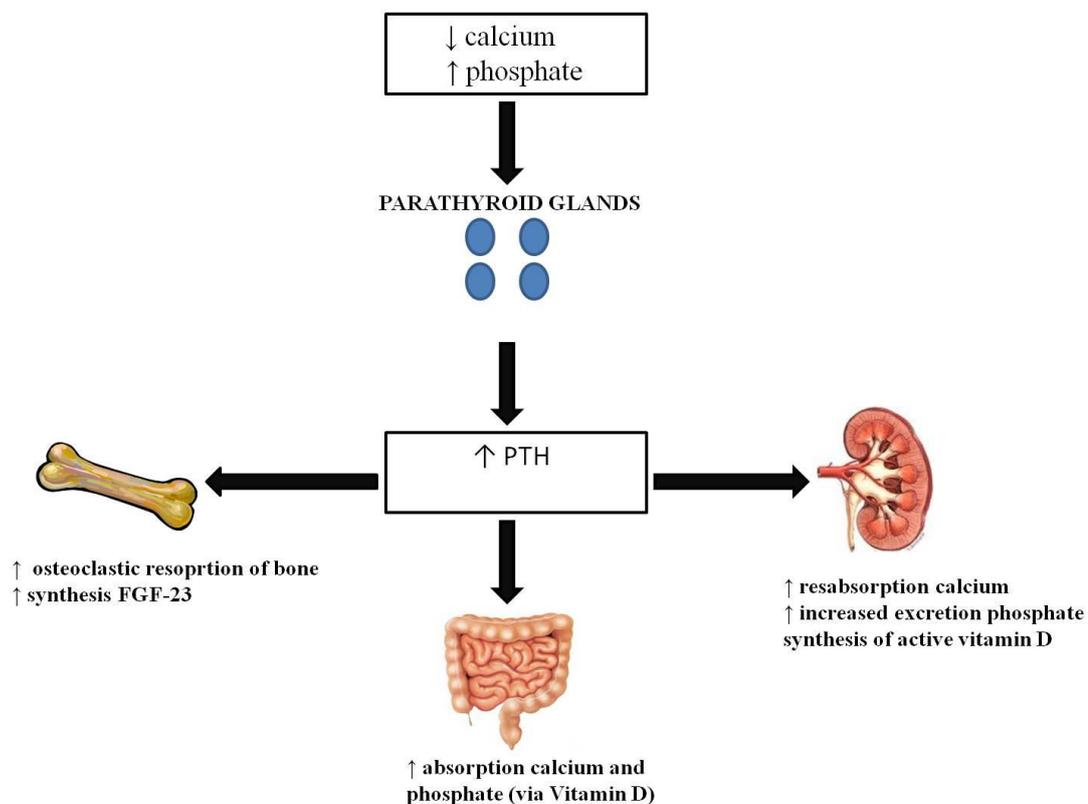


Figure 1-6: Effects of parathyroid hormone release on bone, kidney and intestine.

Hypocalcaemia and hyperphosphataemia stimulate release of parathyroid hormone which acts on bone to increase bone resorption and stimulate FGF-23; on kidney to reabsorb calcium, synthesise vitamin D and excrete phosphate; and on the intestine to increase calcium and phosphate absorption (mediated via vitamin D)

1.5.2.8 Fibroblast growth factor 23

FGF-23 is a phosphaturic hormone which was discovered in 2000 following genetic studies in patients suffering from autosomal dominant hypophosphataemic rickets (ADHR) (160). ADHR is one of a handful of genetic conditions, including tumour induced osteomalacia, characterised by hypophosphataemia, hyperphosphaturia and disrupted bone mineralisation. An additional circulating factor was suggested to have a role in these conditions to explain the surprising finding that serum phosphate concentrations are altered without observing any significant changes in serum calcium level, PTH or vitamin D (161). Subsequent genomic work led to the discovery of FGF-23. In ADHR, there is a genetic defect in PHEX, a metalloprotease with a role in FGF-23 breakdown. The consequence is elevated serum levels of FGF-23 (162).

FGF-23 is a 251 amino acid protein with a molecular weight of 26kDa which is produced in bone by osteocytes and osteoblasts in response to increases in serum phosphate and 1-25-(OH)₂-D levels (36;150;163). It is encoded for by the FGF-23 gene located on chromosome 12 (147). There are seven FGF families; FGF-23 is a member of the FGF-19 sub-family (163). FGF-23 shares a common structure, an 'FGF-like sequence' at amino acid residues 25-180, with other FGFs but it has a unique 72 amino acid sequence extension (residues 179-251) at the carboxyl C terminal tail (164). Full length FGF-23 is the biologically active form but the C terminal is essential for its biological effects (147;164). Only full length FGF-23 causes a reduction in serum phosphate levels (165). However there is evidence in rats that the C terminal of FGF-23 retains significant phosphaturic activity separate from the 'FGF-like' main amino acid sequence (166). Inactivation of FGF-23 occurs by cleavage of the FGF-like domain from the C terminal tail of the intact protein (147). The enzyme responsible has not been identified.

FGF-23 acts mainly to alter renal tubular handling of phosphate by reducing the expression of NaPi2a and NaPi2c and thus reducing phosphate re-absorption and increasing urinary phosphate excretion. This action is mediated in the kidney via the interaction of FGF-23 with Klotho, its co-factor and the FGF receptor (36). The molecular mechanism underlying the phosphaturic effects of FGF-23 remain elusive but there is evidence that FGF-23 activates ERK 1 / 2 and serum-glucocorticoid-regulated kinase-1 resulting in phosphorylation of NHERF-1 leading to downregulation of NaPi2a (167).

Although 1-25-(OH)₂-D levels increases the mRNA levels encoding FGF-23, FGF-23 itself is a counter-regulatory hormone for 1-25-(OH)₂-D – it reduces expression of Cyp27b1 which encodes 25-OH-D 1- α -hydroxylase and upregulates Cyp24 which encodes 24 (OH)ase (168). FGF-23 reduces 1-25-(OH)₂-D production and inactivates available 1-25-(OH)₂-D. In patients with normal renal function, if there is excess FGF-23 in the serum, hypophosphataemia occurs; this relates to the effects of FGF-23 on the renal sodium phosphate co-transporters and 1-25-(OH)₂-D with consequent renal phosphate wasting.

FGF-23 binds to and activates FGF receptors (FGFRs) 1c, 3c and 4 (168). Most FGF molecules use heparin sulphate as a cofactor to facilitate binding to the FGFRs. However, FGF-23 and other members of the FGF-19 sub-family possess a disulfide bond, not found in other FGFs, and this confers a conformational change in the core structure such that FGF-23 has a low affinity for heparin (163;169). However when an FGFR forms a complex with Klotho, the FGFR becomes specific for FGF-23 (168).

1.5.2.9 Klotho

Klotho is an anti-ageing, trans-membrane protein, named after one of the daughters of the Greek god, Zeus. Klotho spins the thread of life whilst her sisters determine life's length and end (163;170). Accelerated ageing and widespread atherosclerosis is observed in Klotho deficient mice and mice who over-express Klotho experience an extended lifespan and reduced levels of oxidative stress (171-173). The mice also develop hyperphosphataemia (168).

FGF-23 null mice develop hyperphosphataemia and display a similar accelerated ageing phenotype to Klotho deficient mice but with the addition of elevated vitamin D levels and resultant metastatic calcification and abnormal bone mineralization (174). In both knock-out models, a low phosphate diet slows the ageing process and in the FGF-23 null mouse corrects hyperphosphataemia and VC although elevated vitamin D and calcium levels persist (169;175). This supports the notion of a shared signalling pathway. Klotho makes the FGFR specific for FGF-23 and the biological effects of FGF-23 are mediated in a Klotho dependent fashion; the Klotho/FGFR complex binds FGF-23 with much higher affinity than the receptor or Klotho alone (176). The FGFR is expressed throughout the body, yet Klotho is expressed at high levels only in the DCT of the kidney, the parathyroid gland, the sino-atrial node of the heart, the choroid plexus, the testis, the ovary and the pituitary gland (168;177). There is also evidence of expression in the PCT of the kidney

but to a far lesser extent (167). This limited expression of Klotho has not yet been explained but it supports the notion of a targeted, organ specific effect of FGF-23. However, in the kidney the disparity between the expression of Klotho and the site of action of FGF-23 is not easily explained.

There are two hypotheses: FGF-23, via Klotho, acts on the DCT and induces a paracrine signal (perhaps secreted Klotho) which acts at the PCT to downregulate expression of NaPi2a and NaPi2c permitting the phosphaturic effects of FGF-23 or FGF-23 acts directly on the PCT and the role of Klotho in the DCT is unclear. In a mouse study by Farrow et al, following injection of FGF-23, the FGF-23 signalling pathway was activated only in the DCT (178). This supports the former hypothesis.

Furthermore there is a secreted form of Klotho which arises from the extracellular portion of the protein. This is cleaved by membrane proteases and secreted in blood, urine and cerebrospinal fluid (169). Secreted Klotho is phosphaturic, independently of FGF-23, and can directly inhibit NaPi2a and NaPi2c (179). Additionally it has the ability to act upon the DCT calcium channel, TRPV5, promoting calcium reabsorption and reducing calciuria (180).

There is increasing evidence that Klotho plays a part in maintaining endothelial function; reducing vascular inflammation and attenuating oxidative stress. In human ECs, Klotho has been shown to up-regulate ACE activity via the cAMP pathway, and in mice Klotho increases endothelial derived NO production (181-183). Thus Klotho may have some involvement in the regulation of vascular tone mediated via the interplay between NO and the RAAS. Angiotensin II negatively regulates Klotho expression via an angiotensin 1 or 2 receptor dependent pathway (184;185). Inhibition of insulin like signalling is a recognised as a mechanism for conserving life span and in VSMCs, Klotho inhibits the insulin/IGF-1 signalling cascade and reduces oxidative stress induced apoptosis (170;182). Removal of ROS is facilitated by Klotho which induces manganese superoxide dismutase expression thus reducing oxidative stress (170). Furthermore, the ED displayed in Klotho deficient mice can be attenuated by adenovirus mediated Klotho gene delivery (183). Cell adhesion molecules including VCAM and E-selectin are up-regulated when the vascular endothelium is activated (186). In human umbilical vein ECs (HUVECs), Klotho reduces TNF α induced VCAM expression, possibly acting via NF- κ B. TNF α induced suppression of eNOS phosphorylation is also attenuated by Klotho (177).

1.5.2.10 Emerging phosphatonins

Kumar defined a phosphatonin as a substance which fulfils four criteria: it inhibits renal phosphate tubular reabsorption; it inhibits vitamin D; it is produced by tumours in tumour induced osteomalacia; and it is elevated in the serum of these patients (187).

Current literature suggests that FGF-23 is the major phosphatonin. However there are others whose importance may not yet be recognised. They have not been well studied but include frizzled-related protein 4 (FRP4), matrix extracellular protein (MEPE) and stanniocalcin. FRP4 inhibits renal phosphate transport, mediated by effects on the Wnt/ β -catenin pathway within kidney and bone (161). MEPE induces renal phosphate wasting and is over-expressed in tumour induced osteomalacia. It is unclear if effects on renal phosphate wasting are independent or the result of an interaction with FGF-23 (150). Stanniocalcin is a calcium regulating hormone thought to exert paracrine effects affecting intestinal absorption and renal transport of phosphate (137).

1.5.2.11 Phosphate homeostasis summary

Current understanding of phosphate homeostasis is summarised in Figure 1-7. The maintenance of serum phosphate within a tightly controlled range is the result of a complex interplay between PTH, vitamin D, FGF-23, Klotho and a multi-organ system: the bone-parathyroid-kidney axis. Our understanding of these factors, their interaction and their precise regulation is incomplete but it is continuing to evolve. In CKD, the homeostatic mechanisms described are disrupted leading to hyperphosphataemia. The emergence of serum phosphate as a CV risk factor has prompted increased study of this dysregulation and has helped to enhance our understanding of phosphate balance.

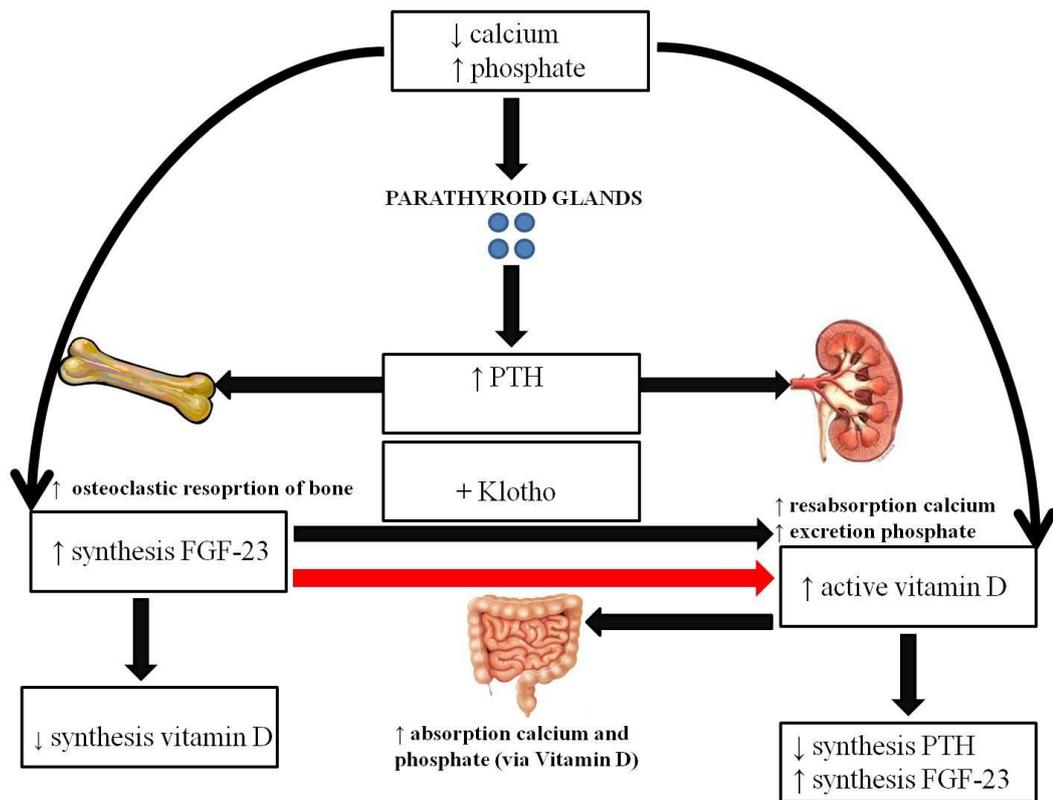


Figure 1-7: Phosphate homeostasis in health.

Bold black arrows indicate a stimulatory effect and the bold red arrow, an inhibitory effect.

1.5.3 Dysregulation of phosphate in chronic kidney disease

Patients with advanced CKD experience disarray of the systems described in section 1.5.2 which are designed to regulate phosphate balance. This leads to a clinical syndrome characterised by hyperphosphataemia, elevated FGF-23, reduced vitamin D, reduced Klotho and hyperparathyroidism and with widespread detrimental effects on the bone, the heart, the vasculature and the endothelium. Furthermore, hyperphosphataemia itself is linked independently with progression of CKD and thus not only contributes directly to CV risk, it also perpetuates it by promoting renal decline.

1.5.3.1 The intact nephron hypothesis

In 1960, Bricker described the concept of the intact nephron hypothesis whereby in CKD as the number of functioning nephrons reduces, the workload of the remaining nephrons, which are believed to be functionally intact, must increase so that any individual nephron must perform a greater fraction of renal excretion (188;189). In the case of phosphate, individual nephrons must excrete an increased amount of phosphate to maintain serum phosphate balance. In CKD, serum phosphate levels are initially preserved within the physiological range via the regulators described in sections 1.5.2.5 to 1.5.2.10 and it is not until advanced CKD that homeostasis is no longer possible and hyperphosphataemia inevitable (Figure 1-8) (190).

1.5.3.2 The extent of the problem

There are limited data looking at the prevalence of phosphate dysregulation in patients with CKD and not on dialysis. Kestenbaum et al published observational data from a cohort of approximately 3500 patients and reported serum phosphate levels to be maintained within the reference range until eGFR fell below 30ml/minute; serum phosphate then began to rise above the upper limit of normal (2). This happens insidiously but it has not been determined exactly at which point in CKD phosphate levels start to rise within the normal reference range. There is, however, some evidence to support a gradual rise from mild CKD (1). Between 40 and 70% of patients with ESRD on dialysis have a serum phosphate elevated above the reference range (191-193). An Indian study found that 65% of patients with CKD stage 4 had a phosphate higher than the reference range (191). In German live kidney donors evaluated 11±7 years from nephrectomy, Gossman et al found creatinine rose significantly from the time of donation, serum phosphate to be 0.9±0.19mmol/L, PTH to be elevated in 19% of donors and 30% to have reduced tubular

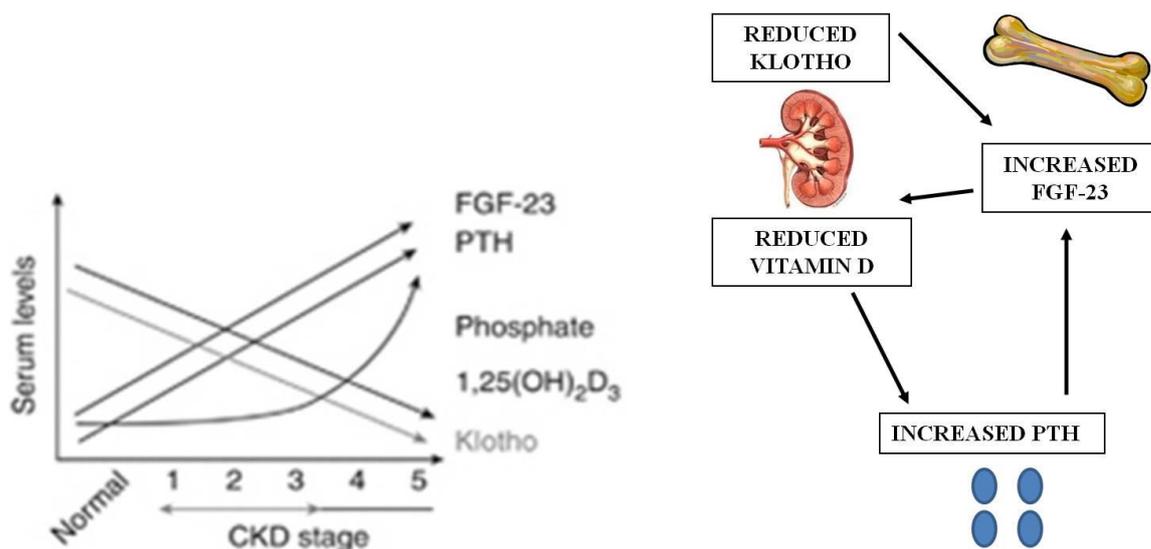


Figure 1-8: Dysregulation of phosphate in CKD.

Initially phosphate levels are maintained within the normal range but as nephrons are lost and CKD advances, the normal homeostatic mechanisms are overwhelmed and there is hyperparathyroidism, elevated FGF-23 and hyperphosphataemia whilst vitamin D and Klotho levels fall. Adapted from Kuro (169).

reabsorption of phosphate. They did not compare phosphate values at the time of donation and at the time of evaluation (194).

1.5.3.3 The current ‘trade off’ hypothesis

This was described by Gutierrez in 2010: in order to maintain serum phosphate levels by increasing phosphate excretion per nephron there has to be a ‘trade off’ between normal serum phosphate and alterations in circulating levels of key regulatory factors (195). Prior to the discovery of FGF-23, the standard thinking was that in CKD reduced nephron mass led to a reduction in phosphate excretion and thereby an increase in serum phosphate causing a reduction in vitamin D and calcium. All three of these factors then stimulate PTH release and this was believed to be the key hormone responsible for phosphate homeostasis. We now know that there is much more to the phosphate story and the current updated ‘trade off’ hypothesis acknowledges the role of FGF-23 in maintaining serum phosphate levels and a second consequent ‘trade off’ between elevated FGF-23 and lowered vitamin D levels (Figure 1-9).

1.5.3.4 FGF-23 in CKD

FGF-23 levels are elevated from early in CKD; initially this maintains phosphate within the physiological range (36;195). Increased FGF-23 secretion from bone is the first compensatory step for the reduced number of functioning nephrons in the kidney and by enhancing renal excretion and reducing intestinal absorption of phosphate (directly and indirectly via reduced vitamin D) allows serum phosphate levels to be maintained. Lowered vitamin D levels are a very important consequence of elevated FGF-23. FGF-23 levels rise before phosphate and before PTH leading to widespread conclusion that FGF-23 may be a useful biomarker to detect disorder in phosphate homeostasis early in CKD (196). By advanced CKD, FGF-23 levels can be more than 1000 fold above the normal range (197;198). FGF-23 remains biologically active even at these supra-physiological levels (199). It has been postulated that FGF-23 at such high levels may have effects in the absence of Klotho. The phenotype of mice without Klotho and FGF-23 is no different to that of those lacking only Klotho (despite very high FGF-23 levels) which does not support the notion of FGF-23 effects independent of Klotho (200). However, there is an association between FGF-23 and LVH (section 1.7.5) and Klotho is not expressed in myocardial cells. Faul et al demonstrated evidence of a Klotho independent pathway (acting via the PLC γ pathway) leading to LVH in mice.

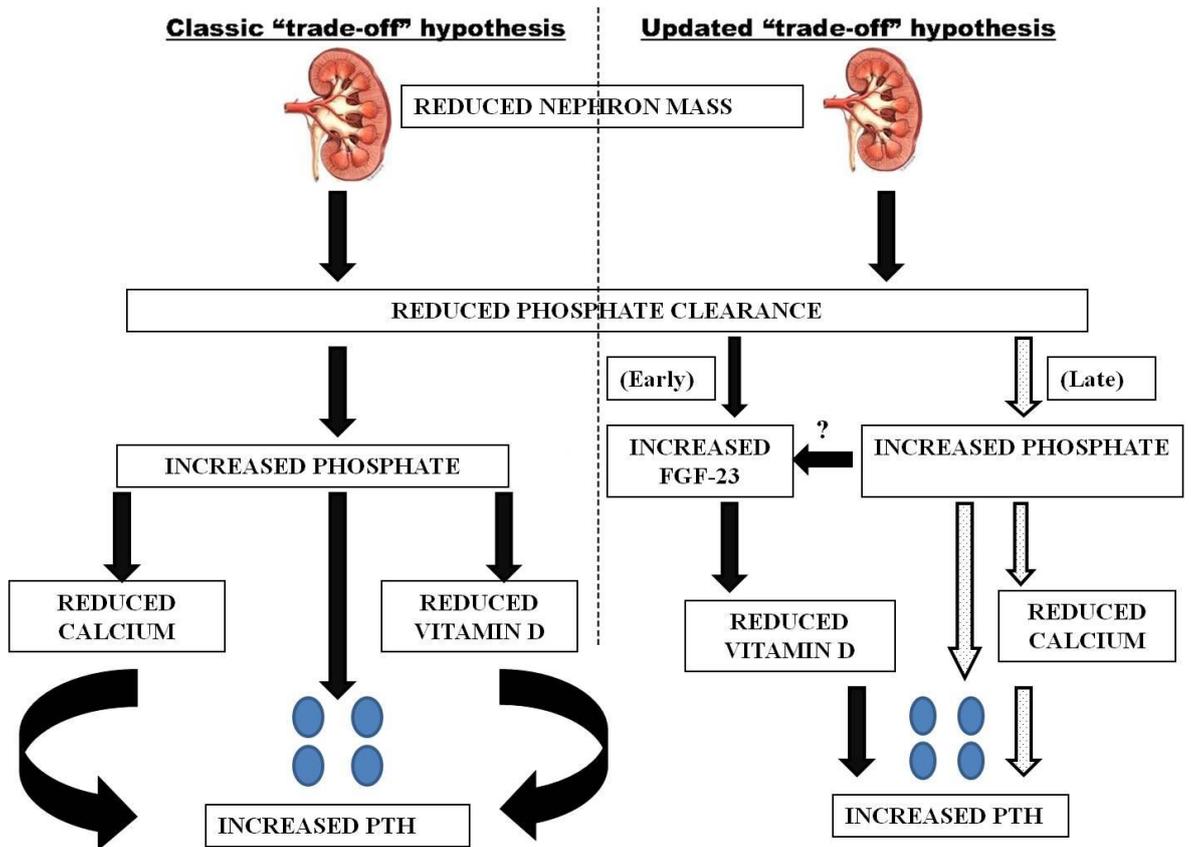


Figure 1-9: The original and updated 'trade off' hypotheses.

The updated version acknowledges the previously unrecognized role of FGF-23. Adapted from Gutierrez et al (195).

This does support the hypothesis that FGF-23 may be able to exert effects on atypical target organs, including the myocardium (201). Elevated FGF-23 can down-regulate Klotho expression in mice which may contribute to target organ resistance to FGF-23 in CKD and consequently encourage further FGF-23 release to perpetuate this cycle (202). In another animal study, FGF-23 (when injected) was seen to up-regulate Klotho expression (203). The explanation for the difference may be that short term exposure to and the long term effects of elevated FGF-23 are different.

1.5.3.5 Klotho in CKD

Klotho has been reported to be reduced in CKD (169). Koh et al, in 2001, demonstrated that in patients on HD, there is a reduced number of functioning nephrons and in the remaining nephrons, membrane bound Klotho expression (protein and mRNA) is reduced to approximately 5% of the level seen in healthy control specimens (204). In hyperplastic parathyroid glands from patients with CKD including recipients of a renal transplant, expression of Klotho is reduced along with expression of FGFR1. The expression of both correlates with renal function and declines as renal function declines (205). The study populations in both studies were small but there is support for the notion of reduced Klotho expression in CKD from animal studies (169;206). Klotho expression is difficult to measure in humans when it requires a tissue sample. However, Yamazaki et al have developed a commercially available ELISA kit which allows measurement of secreted Klotho in blood and urine (207). Use is not yet widespread but evidence is now becoming available that, like membrane bound Klotho, secreted Klotho is also reduced in CKD. There is evidence of reduced secreted Klotho in the urine of those with CKD compared to urine from controls without CKD. This reduction is evident from CKD stage 1 and in a similar fashion to FGF-23 has led to speculation that Klotho is one of the earliest biomarkers for disordered phosphate metabolism (208). In dialysis patients, serum Klotho is lowered compared with control serum and is positively correlated with serum phosphate levels and negatively correlated with serum calcium levels (209). In contrast, Seiler et al looked at patients with CKD stages 2-4 and found no association between secreted plasma Klotho and declining renal function or with any parameter of calcium-phosphate metabolism. There was only a weak association with age (210). This study did not look at Klotho as a continuous variable but rather in tertiles, it did not look at urinary Klotho and by excluding patients with CKD 1, there is the possibility that a very early reduction in Klotho was missed and, of course, in dialysis patients the story may be different.

In rats with acute kidney injury induced by ischaemia reperfusion injury, expression of membrane Klotho and levels of secreted Klotho (blood and urine) are reduced. The administration of recombinant Klotho after induction of acute kidney injury, increased Klotho levels and minimised the extent of kidney damage (211). Similarly, in a mouse model, angiotensin II infusion down-regulated Klotho and induced kidney damage; gene transfer of Klotho in an adenovirus vector ameliorated much of this damage (212).

Thus, whilst there is some evidence of Klotho deficiency in CKD, studies present conflicting findings. We do not yet have a full understanding of this and further studies should help to clarify the relationship between membrane bound Klotho, secreted Klotho and CKD. Membrane bound Klotho may decline progressively in CKD with progressive urinary decline of secreted Klotho. Plasma secreted Klotho may decline in early CKD and then may remain fairly constant until the later stages of CKD or it may decline progressively as FGF-23 rises progressively. The rise in FGF-23 may in part be the result of end organ resistance secondary to a decline in Klotho, its co-factor. However, in a mouse model of renal disease, the reduction in Klotho arises prior to the rise in FGF-23, raising the possibility that Klotho and not FGF-23 is the earliest biomarker for CKD and a potentially causative factor in the accompanying phosphate dysregulation as CKD advances (169).

1.5.3.6 PTH and Vitamin D in CKD

Prior to the interest in phosphate as a CV risk factor, PTH and vitamin D were believed to be the primary regulators in phosphate homeostasis as explained by the classical ‘trade off’ hypothesis (Figure 1-10) (195). Reduced nephron mass leads to phosphate retention and a deficiency of 1α -hydroxylase which in turn leads to a deficiency of active vitamin D, $1,25-(\text{OH})_2\text{-D}_3$. Both these factors stimulate production of PTH. The discovery of FGF-23 and other molecules changed this thinking. However, the reduction in vitamin D levels and the elevation of parathyroid hormone seen in CKD undoubtedly remains an integral part of the phosphate story.

FGF-23 reduces vitamin D synthesis; low vitamin D levels stimulate PTH release and secondary hyperparathyroidism develops. In early CKD, when phosphate levels are normal, reduced vitamin D levels and elevated PTH levels are observed (213). Under normal circumstances vitamin D should suppress PTH but the reduced levels of vitamin D, perpetuated by the raised levels of FGF-23 prevent this. When parathyroid cells are

exposed to FGF-23 *in vitro*, there is suppressed PTH expression and secretion (203). PTH is elevated in CKD, and reduced Klotho expression in the parathyroid gland in CKD may explain why FGF-23 does not suppress PTH release in CKD. In addition to the possible effects of FGF-23 on Klotho expression, Vitamin D up-regulates Klotho expression and therefore reduced vitamin D levels in CKD also attenuate Klotho expression (214). Removing the parathyroid gland in a rat model of uraemia reduces FGF-23 levels. This raises the possibility that PTH itself may stimulate FGF-23 production (215).

We still do not fully understand the various complex interactions between Phosphate, FGF-23, Klotho, vitamin D and PTH either in health or in CKD. In CKD, it is evident that the relationships become disordered and that the extent of this increases with eGFR decline. In CKD stages 4-5, increased levels of FGF-23 are no longer able to compensate for dietary phosphate intake with consequent hyperphosphataemia, vitamin D deficiency and hyperparathyroidism and parathyroid hyperplasia.

1.5.3.7 Summary

Klotho plays an important role in ageing and expression levels naturally decline with age (216). CKD can be considered a condition of accelerated ageing with life expectancy markedly reduced, most commonly secondary to premature CVD (16). The phenotype seen in humans with CKD including early CVD, infertility and VC is very similar to the phenotype of the Klotho deficient mouse (173). CKD accelerated ageing is associated with a syndrome of hyperphosphataemia, hyperparathyroidism and hyper-FGF-23-aemia; this may be a cause or a consequence of altered Klotho expression. It may be adaptive or it may be maladaptive. Regardless this syndrome, arising from disordered phosphate balance, leads to widespread detrimental effects throughout the body and is associated with a significantly shortened life expectancy and progression of CKD.

1.6 Guidelines for phosphate control in chronic kidney disease

In CKD, phosphate can be lowered by modification of dietary intake or with readily available pharmacological therapies which bind phosphate in the gut (phosphate binders) before it can be absorbed (section 1.9). Additionally in dialysis patients, the dialysis

process itself facilitates phosphate removal; this can be improved by optimising the quality and quantity of dialysis received. Thus, guidelines have been produced to target phosphate levels in CKD.

The Kidney Disease Improving Global Outcomes (KDIGO) work group produce international guidelines for various aspects of management of CKD including phosphate control (217). KDIGO focus the guideline on minimising risk, based on most up-to-date evidence. Some countries, including the UK and the USA produce their own guidelines, taking into account the recommendations from KDIGO. The rationale for the difference in the UK relates to ensuring that the guideline allows *'targeting ideal clinical practice targets for biochemical parameters.'* (218)

The UK guideline is produced by The Renal Association (RA) and the USA guideline by The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) (218;219). Traditionally guidelines present recommendations and grade these according to the strength of evidence as per the GRADE approach (220). The strength of recommendation is graded according to the strength of the evidence A (strong), B (moderate) C (low) D (very low). Grade A is randomised controlled trial evidence whereas grade D is often simply opinion.

All guidelines suggest that in patients with CKD stage 3-5 (not on RRT) serum phosphate should be maintained within the normal range (graded C). In dialysis patients the recommendation is to lower elevated serum phosphate levels toward the normal range (graded C). The guidelines for target phosphate level in CKD are arbitrary and are not evidence based. There is no recommendation in any guideline pertaining to phosphate control, a target serum phosphate level or how to achieve a target serum phosphate which is graded above level C.

One large retrospective study in HD patients showed an association between attaining a phosphate within the normal range and a reduction in all cause mortality of between 5 and 33% depending upon the number of phosphate results within target (221). The study was retrospective and was not able to look at adjusted mortality.

There is no evidence that lowering phosphate to a specific target translates to an improvement in clinical outcomes and there is no evidence to point to a phosphate level at which the detrimental effects seen with phosphate are attenuated.

1.6.1 UK Renal Association Guideline

In the UK, the RA advise that, in CKD stages 3-5 (not on RRT), serum phosphate levels should be maintained at 0.9-1.5mM. In patients on dialysis, pre-dialysis phosphate levels should be maintained at 1.1-1.7mM. Both of these recommendations are graded at C. In March 2013, The National Institute for Health and Care Excellence (NICE) issued its first guideline for the management of hyperphosphataemia in CKD (222). The aim of the guideline was to standardise management across the UK, and they suggest that a calcium based phosphate binder be offered as first line management in conjunction with dietetic input. They acknowledge that the evidence for this is poor but taking account of what evidence there is and on the basis of a thorough cost analysis, this was the most robust recommendation they could make.

1.7 Phosphate and cardiovascular risk

In 1998, Block et al demonstrated a link between aberrant phosphate homeostasis and adverse clinical outcomes. This association remained present after adjustment for other variables including co-morbidity (192). Prior to this, hyperphosphataemia was a recognised problem in CKD but focus centred on its causative role in hyperparathyroidism and consequent metastatic VC rather than on any directly toxic effects of phosphate itself. This was the first study to demonstrate an independent effect of phosphate on mortality in patients on dialysis and led to renewed interest in the effects of phosphate and indirectly to a better understanding of phosphate homeostasis in health and CKD. Naturally with better understanding, more questions have arisen which remain unanswered.

1.7.1 Mortality, CV risk and phosphate: in chronic kidney disease and in health

In 1997, Narang et al studied a population of patients undergoing elective coronary angiography. They found a strongly positive correlation between serum phosphate levels and severity of occlusive coronary disease. On the basis of their data they concluded that *“A moderate change in the serum level of...phosphorus confers a risk similar to that associated with smoking, as estimated by the odds ratios.”*(223). Smoking, and the risks associated with it, is a highly publicised public health concern. This study put a serum

phosphate level, elevated within the normal range, in the same risk category as smoking, a major killer in the Western world. Many observational studies since 1997 have demonstrated that Narang et al did not overestimate the relevance of serum phosphate as a risk factor for CVD. Fifteen years on, however, a causative link underlying the observed association between serum phosphate and outcomes remains elusive. Higher serum phosphate levels even within the 'normal' range are linked with a significant increase in all cause mortality, CV mortality and CV risk in the CKD population, in the general 'healthy' population and in recipients of a renal transplant. The risk is present regardless of pre-existing CVD and is maintained following adjustment for standard CV risk factors (Table 1-2) (7;8;32;67;224-227). Increased risk remains present when adjustments are made for GFR and thus it is not an anomaly attributable to reduced renal function.

In 2009, a systematic review identified seventeen studies, published between 1990 and 2007, which assessed the association between hyperphosphataemia and death in dialysis patients (228). Sixteen of these studies concluded that hyperphosphataemia was a risk factor for death in patients on dialysis. Stevens et al demonstrate an increase in risk of 56% for each 1mM rise in serum phosphate (229). This holds true in the pre-dialysis population with an increase in risk of death of 62% (67). Six studies identified by the same review considered the effect of serum phosphate on CV mortality in dialysis patients; all six reported an increase in risk of up to 13% per 0.32mM rise in serum phosphate (228). The increase in CV mortality was evident in patients using peritoneal dialysis (PD) or HD as their mode of RRT (230).

The Western diet is high in phosphate rich foods and in preservatives but effects of phosphate certainly in the non CKD population might be unrelated to dietary consumption. The NHANES III study with just over 15,500 participants (1,500 of whom had CKD) found only a very weak association with phosphate and dietary intake and an inverse relationship between higher serum phosphate levels and Framingham coronary heart disease risk scores (based upon traditional risk factors for CVD including smoking and hypertension) (231). This supports an independent effect of phosphate on CV risk rather than an effect mediated through either dietary phosphate intake or an already unfavourable CV risk profile. It remains unclear if these results can be extrapolated to the CKD population.

Author , year	Population	n	Outcome measure	Adjusted Risk (95%) CI	Median Follow Up
Naves-Diaz, 2010	HD	16173	Mortality CV mortality CV risk (CV related Hospitalisation)	Comparison with Pi 1.6-1.8mM For Pi>2.4mM HR 2.24 (1.5-3.34) HR 2.51 (1.34-4.72) For Pi>2.3mM HR 3.96 (1.17-13.38)	1.35 years
Voormolen, 2007	Advanced CKD (GFR<20mls/min/1.73m ²)	448	Mortality	For each 0.32mM increase in Pi RR 1.62 (1.02-2.59)	11 months
Chue, 2012	CKD stages 2-4 without CVD	208	LVMI (on MRI scan)	57g/m ² v 61g/m ² Pi quartile 1 and quartile 4.	n/a
Connolly, 2009	Transplant recipients	379	Mortality	Comparison with Pi <0.92mM 0.92-1.12 HR 2.2 (1.1-4.4) >1.12 HR 3.5 (1.8-6.8)	6.5 years
Tonelli, 2005	Post myocardial infarction (no CKD)	4127	Mortality MI New heart failure	For each 0.32mM increase in Pi HR 1.27 (1.02-1.58) HR 1.24 (1.00-1.53) HR 1.22 (0.95-1.57)	5 years
Abramowitz, 2010	Medical outpatients	10,743	CVD Hospitalisation Mortality	Per SD increase in Pi (0.2mM) HR 1.06 (1.01-1.12) HR 1.08 (1.01-1.15)	6.8 years
Dhingra, 2007	Healthy adults, mean age 44 years	3368	Composite CVD outcome	For each 0.32mM increase in Pi HR1.31 (1.05-1.63)	16 years
Foley, 2009	Healthy adults, mean age 25 years	3015	Coronary artery calcification score (15 years later)	Each 0.16mM increase in Pi from baseline OR 1.17 (1.01-1.34)	15 years

Table 1-2: Associations between serum phosphate and outcome in different populations.

A selection of eight clinical studies demonstrating an association between serum phosphate level and mortality, CVD or VC in patients with and without pre-existing CVD and with and without CKD (7;8;32;67;224-227).

The mechanism by which phosphate confers this increased CV risk is not understood: association does not always imply causality and thus a plausible mechanism of action for phosphate as a CV risk factor is crucial.

1.7.2 Phosphate and left ventricular hypertrophy

LVH is a common manifestation of CVD in CKD with a prevalence of 40% in pre-dialysis patients increasing to 75% for those on dialysis (232). Several studies have shown an association between serum phosphate level and left ventricular mass (LVM) which is independent of blood pressure (225;233). No causative relationship has been demonstrated but Ayus et al showed that improving phosphate control in HD patients by 12% over a 12 month period resulted in a reduction in LVH, which was independent of other confounders (233). In individuals without CKD, higher dietary phosphate intake has been shown to correlate with greater risk of LVH in women but not men, without CKD (234).

The increased CV risk seen with phosphate levels even within the normal range may be in part explained by the association with LVM. However an appreciation of the mechanisms by which phosphate might contribute to the risk of LVH is essential. Phosphate may be directly toxic to the myocardium or it may be that it triggers its effect via disruption of the usual regulators of phosphate homeostasis: FGF-23, PTH or vitamin D. That LVH is seen in early CKD, before significant alterations in serum phosphate are evident, lends plausibility to this hypothesis.

1.7.3 Phosphate and vascular calcification

Patients with CKD have diffuse arterial calcification; this begins before the onset of end stage renal disease, may be unrelated to the degree of renal impairment and is present at least at moderate level in up to 70% of patients with CKD stages 3-5 (235). More than 60% of dialysis patients have significant VC (236). Medial arterial calcification is a strong prognostic marker of CV mortality in dialysis patients (237). In patients with CKD stage 3-5, progression of coronary artery calcification, measured by total calcium scoring on CT scan, was more prominent than in control subjects without CKD (238). Serum phosphate level was correlated with coronary artery calcification. In another study of patients with mild CKD (mean eGFR 51ml/min) after adjustment for confounders, for each 1mg/dL (0.323mmol/L) increase in serum phosphate within the normal range, there was a 21% greater prevalence of coronary artery calcification and a 62% increase in mitral valve

calcification (239). This relationship holds within the general population, where Foley et al showed that a higher phosphate, within the normal range, led to a >50% increase in risk of coronary artery calcification 15 years later (8). Demonstration that phosphate at the high end of the normal range is associated with risk of VC lends support to the concept that phosphate load and abnormal regulation of phosphate are important mediators of ectopic VC. Further support for this comes from Russo et al (240). In 90 patients with CKD stage 3-5 not on dialysis, a comparison was made between three interventions: low phosphate diet alone or in combination with one of two phosphate binders, sevelamer or calcium carbonate. (Phosphate binders are discussed in section 1.9.2). Total calcium score was the outcome measure as a marker of VC. The groups on the low phosphate diet alone or in combination with calcium carbonate had significant progression in VC over the course of the study (one year). In contrast, in the sevelamer group total calcium score did not change significantly. Serum phosphate levels were unchanged from baseline in all three groups but urinary phosphate reduced in the groups taking calcium carbonate or sevelamer (240). This suggests that lower phosphate absorption might reduce progression of VC. Additionally, perhaps other measures of phosphate load or excretion should be made; serum phosphate is probably not a reflection of total body phosphate.

In animal and cell models, a significant number of studies have demonstrated that VC is an active, regulated process much like the formation of bone (5). Under normal circumstances, a balance is maintained between pro and anti calcification factors such that VC does not occur. In pathological conditions, such as CKD, ectopic calcification occurs because this normal homeostasis is upset (241). A number of different risk factors, including elevated phosphate level can tip this balance in favour of ectopic VC (242). Reynolds et al showed that calcification can be induced in human VSMCs maintained in culture with medium containing high calcium or high phosphate concentration; treatment with medium containing both high calcium and phosphate concentration had a synergistic effect (243). However, they showed that in VSMCs exposed to elevated phosphate and calcium, apoptosis was induced and cells released vesicles with high concentrations of both phosphate and calcium. Usually, factors including fetuin A and matrix GLA protein inhibit matrix mineralisation, but in CKD, these factors are reduced creating a pro-calcification environment (244). Concomitantly, VSMCs undergo phenotypic change and essentially become bone cells with osteochondrogenic differentiation (245). Li et al have shown that blocking phosphate uptake into cells (via the type 3 sodium dependent phosphate co-transporter, Pit1) *in vitro* can ameliorate phosphate induced VC (246). Similarly, Mathew et al, demonstrated that controlling hyperphosphataemia in an animal

model of CKD reduced VC (247). There is a lack of evidence that strategies to reduce serum phosphate prevent or reverse VC or impact upon hard clinical endpoints like patient survival.

1.7.4 Phosphate and endothelial function

In the past, most clinical and laboratory studies have focussed on VC as an explanation for the observed increase in CV risk with hyperphosphataemia. In many clinical studies, VC is used as the surrogate outcome measure. However, there is growing evidence that phosphate has a direct detrimental effect on endothelial function. Much of the evidence comes from isolated cell and animal studies, the detailed molecular mechanism remains elusive and whether phosphate is a direct or indirect endothelial toxin is not clear. Furthermore, cells are usually treated with phosphate for a short period (24 hours or less) testing acute, rather than long term exposure to elevated phosphate. Patients with CKD are exposed to higher levels of phosphate for a prolonged period and thus long term effects of phosphate *in vitro* and *in vivo* are of more clinical relevance.

Several studies have shown effects of phosphate on the expression of eNOS, NO and ROS production in cell culture (6;248-250). Peng et al showed that elevated phosphate in culture media (3mmol/L) reduced cell number and caused apoptosis, associated with reduced eNOS phosphorylation at residues Ser-1177 and Ser-615 and reduced NO. Intracellular calcium levels were also lower and they found evidence of alterations in phospho-Akt and NF- κ B-p65 signalling (249). Shuto et al, found elevated phosphate increased phosphorylation of eNOS at Thr⁴⁹⁷ (inactivates eNOS) and increased ROS production in a bovine cell line (6;248). This effect was ameliorated by phosphonoformic acid, a specific inhibitor of sodium dependent phosphate transporters (6). They also found that PKC was increased, and postulated that phosphate may mediate its effects via PKC but unlike Peng et al, they did not demonstrate a change in intracellular calcium concentration.

Several studies suggest an effect of phosphate on cell growth and cell death with elevated phosphate causing increased apoptosis (249;250), the cause of which is not clear, although may involve cell cycle regulators. Of interest, similar effects have been seen with low phosphate (0.5mmol/L) with increased expression of p53 and cyclin dependent kinase 2 (CDK2), a cell cycle regulator known to induce apoptosis (249). Di Marco et al, found that apoptosis and increased ROS production was present in ECs at phosphate concentrations of 2.5mmol/L and above, concluding that high phosphate caused

mitochondrial dysfunction and that increased ROS production was the trigger for apoptosis (250).

In studies of isolated blood vessels *in vitro*, rat aortic rings incubated for one hour in a high phosphate concentration medium (2.4mmol/L) showed impaired endothelium dependent vasodilatation to acetylcholine (6). This effect on endothelium dependent vasodilatation has also been shown in resistance vessels from rats with induced renal impairment and fed a high phosphate diet (2 fold increase in phosphate content compared with normal rat chow) over several weeks (251). In the same study, the use of L-NAME also suggested that there were lower levels of basal NO than in control animals and levels of serum phosphate, brain natriuretic peptide (BNP) and PTH were higher than in control animals (251). BNP level is known to be an independent predictor of endothelial function in man (252).

Morris et al, showed that resistance vessels from patients with CKD stage 5 showed impaired endothelium dependent relaxation when compared with healthy controls with preserved endothelium independent relaxation (95). Whilst there are many studies evaluating ED in renal disease, there is paucity of studies looking at phosphate and its effects specifically the effects of long term exposure to elevated phosphate.

In healthy subjects, acute phosphate loading with a meal containing 1200mg of phosphate significantly reduced FMD two hours post ingestion when compared with a meal containing 400mg of phosphate (6).

There are no outcome data in clinical trials demonstrating a beneficial effect of lowering phosphate, although we have the tools to do so (section 1.9). In a rat model of adenine induced CKD, rats fed a low phosphate diet (0.2% compared with 1% in standard rat chow) for 16 days exhibited significant improvement in markers of bone biochemistry (phosphate, calcium, PTH, vitamin D and FGF-23). Phosphorylation of eNOS (Ser-1177) was significantly increased and the impaired endothelial dependent vasodilatation seen in aortic rings with animals fed standard rat chow was ameliorated in those fed chow containing low phosphate (253).

Part of the difficulty with clinical studies of phosphate lowering is the endpoints tend to be surrogate outcomes, for example VC, rather than mortality or CV events. Additionally many of the studies compare different phosphate lowering agents with each other rather

than against a placebo or no intervention. No single study has demonstrated that phosphate lowering reduces mortality.

1.7.5 Mortality, cardiovascular risk and phosphate regulation

Abnormal regulation of phosphate is likely to be as detrimental to CV risk as hyperphosphataemia itself. The consequences of hyperphosphataemia and resultant abnormalities in phosphate regulation are summarised in Figure 1-10.

In the general population, and in CKD, vitamin D deficiency is linked with ED, vascular stiffness and increased mortality (254-256). In animal models, vitamin D deficiency has been shown to induce myocardial hypertrophy and myocyte proliferation and reduce myocardial contractility (257;258). Treatment with vitamin D inhibits both myocyte proliferation and hypertrophy (259;260). In HD patients, treatment with vitamin D (calcitriol) for 15 weeks, led to a marked reduction in LVM (261) although evidence of long term reduction in CVD or mortality is lacking.

The dialysis outcomes and practice patterns study (DOPPS) an observational study in >25,000 patients showed that patients with phosphate levels >7mg/dl (2.3mM) or PTH levels >600pg/ml were at increased risk of death and if both were present the risk was highest (262). Additionally higher PTH levels predict worse renal outcomes (263). In animal models, elevated PTH has been linked with myocardial hypertrophy and fibrosis (264). In dialysis patients, higher levels of PTH predict increased rate of all cause and CV hospitalisation (11).

There has been much focus on FGF-23 and its co-receptor Klotho. FGF-23 levels inversely correlate with renal function (197). Gutierrez et al demonstrated an independent association with elevated FGF-23 and mortality in patients commencing dialysis (198). There is evidence that this relationship is true across the spectrum of CKD and that elevated FGF-23 is a predictor of ESRD (265). Additionally, higher FGF-23 levels are associated with increased arterial stiffness in CKD and with ED in the healthy population (266). In dialysis patients, FGF-23 levels correlate with carotid artery atherosclerosis and with LVM (267;268). Increased LVM is also seen with higher FGF-23 levels in the CKD population (236). Faul et al showed in a cohort of >3000 patients with CKD, FGF-23 level was an independent predictor of LVH (201). In >1200 patients with largely preserved renal function, dietary phosphate intake, PTH level and standard CV risk factors including

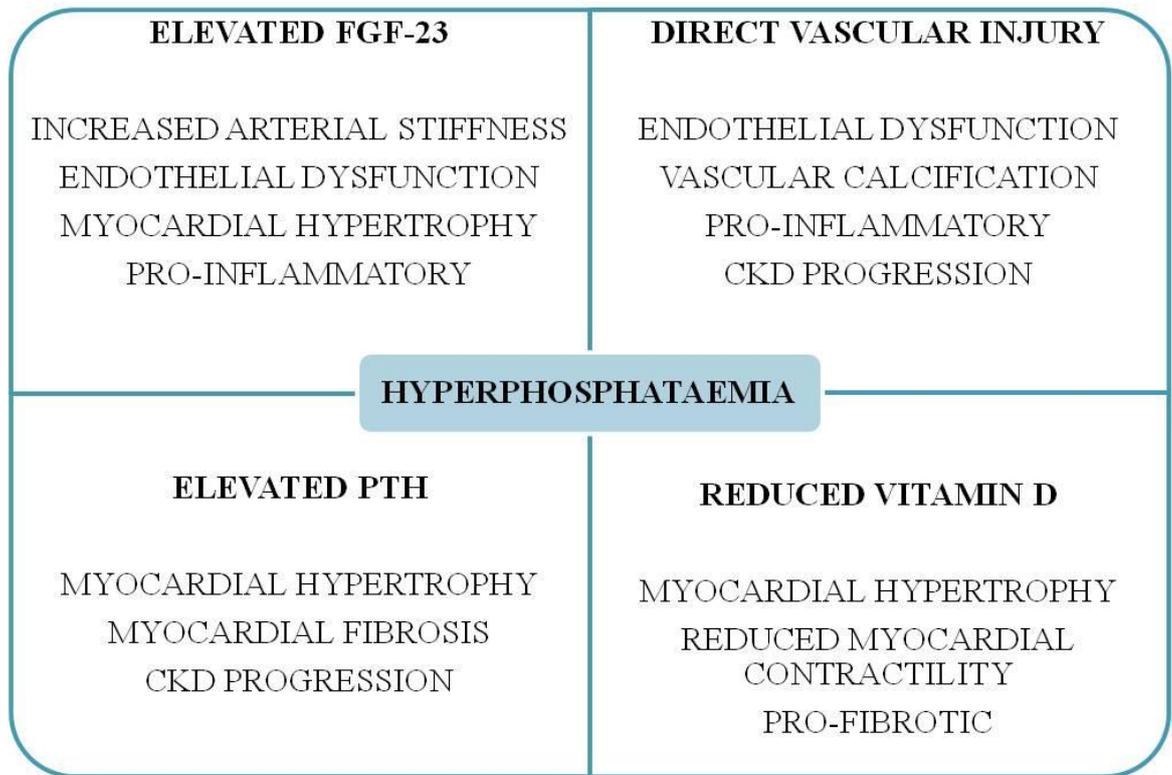


Figure 1-10: Unwelcome consequences of hyperphosphataemia.

Hyperphosphataemia results in abnormal regulation of phosphate in CKD. The consequences of this include elevated FGF-23, elevated PTH and reduced vitamin D. Each of these four factors impacts upon CV risk and has detrimental effects on the vasculature and the heart.

triglyceride level, smoking status and hypertension were all independently predictive of higher FGF-23 level (269).

At a molecular level, FGF-23 is believed to need Klotho, its co-receptor to mediate its effects (36). The observational studies described above do not determine whether FGF-23 is directly toxic or if it is simply a biomarker for myocardial damage and hypertrophy. Faul et al demonstrated that not only is FGF-23 directly toxic to the myocardium causing LVH in an animal model but that it does this via a Klotho independent pathway (the calcineurin-NFA signalling pathway) (201). They went on to show that treating animals with established CKD with an FGF receptor blocker (PD173074) could attenuate LVH whilst making no alteration to blood pressure; supporting a blood pressure independent effect of FGF-23 on LVH. FGF-23 and its receptor may therefore be promising therapeutic targets in early CKD before the detrimental effects are observed.

1.8 Clinical consequences of altered phosphate homeostasis

1.8.1 Chronic kidney disease mineral bone disorder

A new clinical syndrome was defined by KDIGO in 2006 in recognition of the strong association between phosphate and diseases of the bone-vascular axis: chronic kidney disease mineral bone disorder (CKD-MBD) (270). Previously the term renal osteodystrophy was used to explain the histological bone changes and the abnormalities in phosphate and calcium metabolism seen in CKD (271). However, CKD-MBD encompasses the systemic nature of the problems seen in bone and soft tissue including the clinical features and is defined by an abnormality of any of the following (or a combination): serum calcium, phosphate, PTH, or vitamin D levels; alterations in bone turnover, mineralization, volume, linear growth, or strength; and calcification in the vasculature or soft-tissue. This has raised the profile of phosphate and highlighted its pivotal role in what is now known to be a multisystem disorder. The creation of CKD-MBD as a separate disease entity has further emphasised the importance of establishing the ideal target for phosphate lowering, whether phosphate lowering translates to improvement in clinically relevant outcomes (reduced hospitalisation, improved mortality and CV risk

rather than surrogate outcomes like VC) and focussed attention on establishing a mechanism to explain the observed effect of phosphate on CV risk and mortality.

1.8.2 Symptoms and signs attributable to elevated phosphate

Hyperphosphataemia is similar to hypertension in that the symptoms tend to be those of the consequences rather than directly attributable to hyperphosphataemia itself. The lack of visible symptoms, early on, is likely to make patients less compliant with treatment, particularly if that treatment has side effects. Education may improve adherence, however it is difficult to educate and to justify intervention when there is not a target phosphate level. Our understanding of the mechanism is limited and evidence of a beneficial effect with treatment is lacking. The consequences of hyperphosphataemia experienced by the patient include calciphylaxis, bone disease, VC and CVD.

Calciphylaxis has a prevalence of around 4% in HD patients and is a debilitating skin condition caused by calcification in the small arteries with consequent necrosis and panniculitis (272). It is difficult to treat and increases the mortality of HD patients by eightfold usually secondary to sepsis (272;273). The development of calciphylaxis is linked to serum phosphate levels and treatment options are limited and often futile, usually centring around improved control of PTH and phosphate (273).

There is a spectrum of bone disease which occurs in CKD-MBD with altered bone turnover rate and altered mineralisation (271). Clinical features tend to develop when abnormalities in phosphate and calcium metabolism have been present for some time. At which point, clinical features may include symptomatic hypocalcaemia, fractures, bone and joint pain and muscle weakness (271;274). In paediatric patients, there may be consequent abnormalities of growth (271)

VC occurs via the processes outlined in section 1.7.3 and is initially asymptomatic.

Ultimately, the patient can experience a number of problems as a consequence of VC.

Transplant is the best method we have currently of lowering CV risk in CKD (19).

However, with significant VC it can become technically impossible to perform a transplant operation. When the vessels are heavily calcified, vascular access can become problematic and impossible in extreme cases leaving no method of undertaking HD (275). If other forms of RRT are not an option, a patient will die. Calcified valvular heart disease is more common in CKD and associated with elevated phosphate and increased all cause mortality

(276;277). Arrhythmias and sudden cardiac death are more common, and VC within the cardiac conducting system may contribute (278-280).

ED is not currently included in the manifestations of CKD-MBD. This may relate to the multi-factorial nature of ED in CKD, the small current evidence base for the link between abnormalities and ED and to the lack of routine measurement in clinical practice. However the 'symptoms' of ED experienced by the patient include CVD and its consequences including death.

1.8.3 Progression of CKD: phosphate and proteinuria

Higher serum phosphate levels and proteinuria have been shown to promote progression of CKD. Blockers of the RAAS system are a key strategy to reduce proteinuria and slow progression of CKD. Zoccali et al showed in a post hoc analysis of 331 patients from the REIN study population that not only is phosphate an independent risk factor for progression of CKD (1mg/dl (0.323mM), (increase in serum phosphate translated to an 84% excess risk for progression to ESRD, requiring RRT) but a high phosphate load may limit the renoprotective effect of ACEi (ramipril) (281). The relationship between phosphate and risk of progression of renal disease was present even with phosphate levels within the normal range (albeit at the upper limit). Whilst these findings require confirmation in further study populations, they support the need to establish exactly how phosphate acts to increase CV risk, mortality and risk of progression of renal disease. Optimising our understanding of the mechanism of action of phosphate as a risk factor would allow for targeted and more effective interventions in an attempt to improve outcomes. This is necessary not least because serum phosphate appears to weaken one of the only strategies we currently have to slow progression of proteinuric CKD. It is also plausible to hypothesise that in the presence of a higher serum phosphate, the protective effects of ACEi and ARB on the CV system seen in non-CKD populations might be ameliorated (282).

1.9 Management of CKD-MBD

Phosphate increases CV risk and is associated with ED and VC. Thus there is a drive, which is perhaps misplaced, to lower phosphate in CKD. There is no evidence to support

that lowering phosphate translates to improved outcomes and furthermore there is no evidence that any of our currently available phosphate lowering methods confer any benefit to patients. The two currently available management strategies are dietary modification to reduce the phosphate load and phosphate binders to reduce phosphate bioavailability within the body. Mostly, these methods are poorly tolerated and perhaps, if we better understood the mechanism of action of phosphate as a CV risk factor, the opportunity to develop novel, more effective strategies for reducing phosphate would become available. Ultimately this might translate to improved clinical outcomes. Dietician input and phosphate binders are expensive and yet using these resources patients consistently fail to achieve the targets we have set. Although the current targets set for phosphate lowering are opinion rather than evidence based, in the 1,814 participants of the SEEK study only 15% had a serum phosphate below or at the target set by the KDOQI guidelines (190). Our focus should be on better understanding the *in vivo* effects of hyperphosphataemia, the consequences of lowering phosphate and then using this information to tailor patient management. This may include the development of novel phosphate lowering strategies.

1.9.1 Diet

Phosphate is present in many common foods (section 1.5.2.1). By altering cooking methods, reducing quantities of or eliminating certain foods it is possible to lower the dietary phosphate content. However, using diet as a management strategy for hyperphosphataemia poses several problems: there is no clear consensus on when dietary advice should be instigated, access to dietetic services varies between centres, and even the most motivated patients find it difficult to drastically change their diet and to maintain compliance with these changes in the long term.

Phosphate containing foods are often protein rich and thus there is a theoretical risk of malnutrition in patients on a phosphate restricted diet (283). This is over and above the malnutrition often present with uraemia in advanced CKD (284). In a review article, Sigrist et al provide a standard phosphate containing diet and a low phosphate alternative; both contain similar quantities of protein (283). This demonstrates it is possible to limit dietary phosphate content without compromising protein intake but it requires thought, effort, motivation and planning from patients. Even in the most motivated patient, this can prove challenging particularly if there are other dietary restrictions in place. This includes patients with DM or coeliac disease or those following a low potassium diet which often

runs in parallel with a phosphate restricted diet. The advice and food choices can be conflicting and difficult to follow as well as socially isolating and could lead to a reduced quality of life for some - limiting social engagements where often food is a focus. In HD patients, up to 75% struggle to adhere to dietary guidelines (including low phosphate dietary advice) (285).

Additionally, patients with CKD are more likely to be depressed than the general population, and in the CKD population, those who are malnourished are most at risk of depression (286). In patients with CKD, as in many chronic conditions, there is evidence that improving nutritional status generally improves quality of life (284). However, nutrition, depression, quality of life and phosphate restriction are closely linked and present an impossible situation in patients with advanced CKD. Restricting already limited food choices may worsen nutritional status, exacerbate depression and worsen quality of life. Worrying about non-adherence and non-compliance may contribute additional pressures. Thus in the management of hyperphosphataemia, dietary phosphate restriction is not the Holy Grail and alternative strategies for management would be useful and may prove superior.

1.9.2 Phosphate binders

Phosphate binders are drugs which bind phosphate in the intestine to prevent its absorption and thus lower serum phosphate levels when CKD prevents normal phosphate metabolism and excretion. They were first introduced in the 1970s and initially were used to prevent or slow the complications of secondary hyperparathyroidism (287). Currently, following the recognition of the importance of phosphate as a CV risk factor, these drugs are used in conjunction with dietary measures (and dialysis in patients with ESRD) to lower phosphate to a specific therapeutic target with the aim of reducing CV risk. This is despite no study showing a benefit of lowering serum phosphate to a specific level. These drugs are prescribed to over 90% of patients with CKD at a cost of approximately \$750 annually (9). Whilst there is evidence that phosphate binders effectively lower serum phosphate in CKD, in the 40 years since they were first introduced there has been no randomised controlled trial which proves benefit of phosphate lowering in terms of meaningful patient outcomes like mortality or CVD (288).

Broadly speaking there are two categories of phosphate binder: calcium and non-calcium containing. The most effective would be one which lowers phosphate with few side effects and the minimum number of tablets.

1.9.2.1 Calcium containing phosphate binders

The two main binders in this category are called calcium carbonate and calcium acetate. Up to 50% of patients may develop hypercalcaemia with this class of drug (288). The binding ability of calcium carbonate is pH dependent and can be reduced by concomitant use of proton pump inhibitors. Calcium acetate has superior binding abilities and theoretically could be used in smaller doses, reducing calcium content. However it causes a higher proportion of gastrointestinal side effects and is less well tolerated (23).

1.9.2.2 Non-calcium containing phosphate binders

Aluminium was the first binder in this category. It was effective at lowering phosphate but is no longer widely used because of an extensive side effect profile as a result of systemic aluminium toxicity (289).

Sevelamer hydrochloride is an anion exchange resin (9). It was the first non-calcium and non-aluminium containing binder to become available (290). Studies have shown that it lowers serum phosphate levels but perhaps less effectively than calcium containing binders (288). Much of the trial evidence in this area is conflicting but sevelamer seems to reduce VC when compared to calcium containing phosphate binders and may have positive effects on low density lipoprotein (LDL) cholesterol levels, inflammation and endothelial dysfunction (291-294). In terms of mortality, the DCOR trial with over 2000 participants (on HD) showed no difference in mortality when compared with calcium containing phosphate binders except in a subgroup analysis of patients aged over 65 years where a survival benefit was observed (295). There was a significant reduction in all cause hospitalisations in the sevelamer treated group. However, almost half of the original study population did not complete the study. In CKD patients, not on dialysis, a pilot study showed a possible mortality benefit with sevelamer compared with calcium carbonate (296). Oliveria et al showed that in CKD stages 3 and 4, whilst both calcium containing binders and sevelamer reduce serum PTH and urinary phosphate levels, only sevelamer reduced FGF-23 levels significantly (297).

The problems with sevelamer include optimal phosphate binding occurring at a pH higher than that of the intestinal tract, large pill size, a high pill burden and possible interference with the absorption of fat soluble vitamins including vitamin D (290;298). Additionally there is some evidence that sevelamer may aggravate acidosis (it lowers bicarbonate levels possible because of its hydrochloride content) and hyperkalaemia in dialysis patients (299).

The only placebo controlled blinded randomised trial in this area compared sevelamer to placebo over a 40 week period in patients with mild CKD (stage 3) and found no improvement in LVM, left ventricular systolic or diastolic function nor in arterial stiffness (300). They did not measure endothelial function and compliance was poor.

Lanthanum carbonate (this will be referred to as lanthanum throughout this thesis) is the newest non-calcium containing phosphate binder. Advantages compared to sevelamer include a smaller pill burden and reduced risk of acidosis and hyperkalaemia, optimal phosphate binding at a broad pH range (thus it is effective in both the stomach and small intestine) and no effect on the absorption of fat soluble vitamins (290). Lanthanum may be better at attaining serum phosphate targets and at a reduced cost (301;302). Lanthanum is a heavy metal with the potential for accumulation in a similar fashion to aluminium. However, it is mainly excreted by the liver and has a low bioavailability (303). It may be absorbed; bone biopsy specimens from patients treated with lanthanum for approximately five years show rising levels of lanthanum over time (9;304). Despite this, there is no evidence that it causes direct bone toxicity and unlike aluminium it does not seem to cross the blood brain barrier (303). Lanthanum is manufactured in different tablet strengths unlike any other phosphate binder and so helps with pill burden (23). In CKD stages 4 and 5, lanthanum effectively lowers FGF-23 levels (305).

Magnesium has been studied as a potential phosphate binder and may have therapeutic potential (306;307). However, there is sparse data on safety and efficacy and more work is needed with larger patient numbers (9).

1.9.2.3 Conclusion

There is good evidence that all currently available phosphate binders work: they all lower serum phosphate. However, despite many studies, none has been shown to be superior in terms of meaningful patient outcomes. Furthermore a study by Isakova et al in 10,000 HD patients showed that those prescribed a phosphate binder had improved survival compared

with those not prescribed a binder. This survival benefit was seen independently of serum phosphate levels and regardless of which phosphate binder was prescribed (308). If the effect is not related to serum phosphate is that because serum phosphate is not a true marker of total body phosphate and a different surrogate e.g. urinary phosphate would have been a better measure of true body phosphate levels? Or is it that the phosphate binder has an effect on something else e.g. FGF-23 and it is alterations in that which may confer a benefit?

The pill burden in patients with CKD, particularly those on dialysis is high and higher than that of many other chronic diseases. A recent study by Chiu et al found that in 233 dialysis patients, the average total daily pill burden was 19 with 25% taking in excess of 25 pills per day (309). Phosphate binders account for 50% of the pill burden. A higher pill burden correlated independently with poorer quality of life, and increasing the number of phosphate binder pills did not seem to improve hyperphosphataemia and in fact came at the expense of poorer quality of life. 40 – 60% of dialysis patients admit to non-adherence with phosphate binding medication (309;310).

Furthermore according to registry data, almost 50% of patients on dialysis fail to achieve targets for phosphate control (with the caveat that optimal phosphate levels are not known) (311). When we consider the cost of phosphate binders, the poor compliance and the lack of evidence for improved patient outcomes surely for now we should concentrate on determining why phosphate increases CV risk?

1.9.3 Dialysis

The dialysis process removes phosphate. This works best with long, slow HD sessions (eight hours) as is the case with nocturnal dialysis which removes up to 40% more phosphate than conventional dialysis (four hour session) (312). There is diurnal variation in serum phosphate levels in healthy patients and this variation is maintained in CKD with phosphate levels at their highest in the evening (313). This might contribute to the success of nocturnal dialysis in reducing phosphate. Nocturnal, home HD is the only dialysis modality which has been shown to normalise serum phosphate levels (314). Most patients are however not on nocturnal nor on home but on conventional in-hospital HD as dictated by resource, patient preference and co-morbid factors. For PD, depending upon an individual's ability to transport different solutes, continuous dialysis with manual exchanges may more effectively remove phosphate than automated PD performed over-

night (315). Neither HD nor PD alone lower phosphate sufficiently to meet the set targets; this is particularly true in patients with residual renal function (316). Thus, patients are prescribed dietary advice and phosphate binders in conjunction with dialysis.

1.9.4 New treatments

The problems with current methods of lowering phosphate as outlined above have led to the study of alternative management strategies. Wang et al showed that oral activated charcoal effectively reduces serum phosphate in HD patients. 90% of patients achieved a serum phosphate below 5.5mg/dL(1.78mM) with the average reduction in serum phosphate being 2.6mg/dL (0.83mM) over the 24 week treatment period (317). However this was a small pilot study and not a randomised control trial; the safety and efficacy of activated charcoal would need to be studied in a larger population and over a longer time period.

Vitamin B₃ or niacin has been shown to improve lipid profiles and slow the progression of atherosclerosis in patients, without CKD, with CV risk factors (318;319). It exerts its effects by inhibiting adipocyte lipolysis and raising high density lipoprotein (HDL) cholesterol levels (320-322). In animal studies, niacin metabolites have also been shown to have effects on the type 2 sodium dependent phosphate co-transporters in the intestine (323;324). The problem with niacin is that it stimulates prostaglandin release and thus one of the major side effects is facial flushing which has the potential to markedly reduce patient tolerance (318). Nicotinamide is a circulating form of niacin which does not induce prostaglandin release to the same extent and thus has fewer side effects (325).

Nicotinamide does not act through the nicotinic acid receptor, activation of which is thought to be essential for observed effects on HDL cholesterol levels (326). It does, however exert effects on the intestinal phosphate transporters as well as in PCT cells where it has been shown to inhibit phosphate uptake (327).

The observed effects of niacin and nicotinamide on phosphate transporters have sparked interest in the potential phosphate lowering effects of these drugs. In HD patients, Muller et al studied an oral modified release preparation of niacin and Takahashi et al studied oral nicotinamide in a similar population (325;326). Both studies showed that the drugs lowered serum phosphate levels. Niacin was not well tolerated with 35% of patients not completing the study secondary to side effects and a further 29% experiencing flushing or diarrhoea but not to the extent that they discontinued the drug. Although serum phosphate was lowered, this was not significant and the mean phosphate level following treatment

was 5.8mg/dL (1.87mM). With nicotinamide, serum phosphate was significantly lowered to a mean of 5.4mg/dL (1.74mM) and only 9% of patients experienced side effects. Both drugs raised HDL cholesterol levels. Neither drug has been studied in meaningful numbers in patients with CKD not yet on dialysis. To limit the side effects of niacin, Muller et al concomitantly administered a non-steroidal anti-inflammatory drug (NSAID) to inhibit prostaglandin release. NSAIDs have a detrimental effect on kidney function and thus whilst safe in patients on dialysis, they can accelerate renal decline in those who do not yet have ESRD and are thus not a viable option in these patients. Other small studies have confirmed a modest phosphate lowering effect with both drugs but the side effect profile and tolerability seems to be variable (328;329). Both niacin and nicotinamide can be taken once daily and do not need to be taken with meals (9). Neither drug is currently a serious contender in the management of hyperphosphataemia without further large multi-centre studies including outcome and pharmacokinetic studies in CKD.

Patients with CKD secrete more phosphate in the saliva than the general population and this correlates with renal function and serum phosphate levels (330). Chitosan, a natural polymer, has a good binding capacity for phosphate. In 2007, Savica et al showed that if chitosan gum was chewed in between meals and sevelamer was taken with meals, a lower phosphate could be achieved in dialysis patients previously established on sevelamer regularly (331). No adverse effects were reported. In a separate study, the same investigators found that the chitosan containing chewing gum significantly reduced CRP levels in HD patients and that this reduction correlated with serum phosphate reduction (332). This supports a pro-inflammatory effect of phosphate which might be modifiable if serum phosphate is lowered. This is pilot data only but it does suggest that salivary phosphate might be a therapeutic target.

No information is available as to whether any of the above therapies impact upon VC, endothelial function, mortality, rate of hospitalisation or bone histology.

1.9.5 Potential therapeutic targets

In order to establish novel therapeutic targets, the exact mechanism of action of phosphate as a CV risk factor needs to be established and an improvement in patient outcome has to be demonstrated with lowered phosphate. Rather than binding phosphate in the intestine, one possibility might be to block the uptake of phosphate into cells. There may be more of a role for FGF-23 and the use of medication to alter the level.

1.10 Work leading up to this thesis

Although, renal transplantation improves quality of life and offers a survival advantage over maintenance dialysis (19;20), the prevalence of CVD still remains 3-4 times that of the general population (21). An association has been demonstrated between elevated serum phosphate and increased mortality post renal transplantation (32) and I, therefore, sought to ascertain if this relationship was evident in our local renal transplant population.

1.10.1 Methods

The electronic patient record was used to identify patients who received a first deceased donor renal transplant between January 1999 and December 2008. Patient demographics, laboratory parameters, cause and date of death and of transplant failure were recorded at one year post transplantation. A history of smoking, DM or CVD was recorded. The primary endpoint was death with a functioning transplant.

1.10.2 Results

377 transplant recipients were followed up for a median of 1798 days (337-3919 days). 62% (n=235) of patients were male, 10% (n=38) were diabetic and 18.8% (n=71) had a history of vascular disease at the time of transplantation. Characteristics of the patients are shown in Table 1.3. Forty-four patients had died at the time of analysis: 11 from a CV cause. Survivors and non-survivors were compared; survivors were younger, less likely to be diabetic, had a higher eGFR and a lower phosphate at one year post transplantation. Figure 1-11 shows Kaplan-Meier analysis for transplant recipient survival stratified by serum phosphate at one year post transplantation. A serum phosphate in the range of 0.9 to 1.1mM afforded the best survival outcome; serum phosphate which was greater than 1.1mM was associated with all cause mortality (p=0.006). When adjusting for history of DM, history of smoking, age at the time of transplantation and eGFR, serum phosphate at one year post transplantation remained a significant predictor of all cause mortality (HR 3.36 (1.26-8.97), p=0.016).

1.10.3 Discussion

There is an independent association between serum phosphate at one year post renal transplantation and death from all causes. Optimal serum phosphate appears to be between 0.9mM and 1.1mM; levels above or below confer increased risk of death. This

observation, for the first time, of a possible J shaped relationship between serum phosphate and all cause mortality, suggests that addressing the impact of phosphate is more complicated than the use of phosphate binders and phosphate supplementation. A history of CVD was not significantly associated with mortality. In our centre, patients undergo CV screening prior to being listed for transplantation; high risk patients are selected out which may explain this finding (333).

This study is a single centre study with small numbers and its retrospective nature means there is incomplete data on important CV risk factors. However, the association of phosphate with all cause mortality concurs with other data of CV risk and ED associated with increased phosphate (6;32). Understanding the mechanism is a crucial step in allowing us to identify phosphate targets and management strategies to lower CV risk in 'at risk' populations, particularly those with CKD.

The prevalent view is that elevated serum phosphate increases CV risk by promoting VC. An alternative possibility is that phosphate directly causes ED. Phosphate may act alone or in conjunction with its regulators, eg FGF-23. These possibilities are explored in detail, throughout this thesis, by examining vascular function *in vitro* and *in vivo*, gene expression, and quantitative and functional changes in signal transduction pathways.

	All patients n=377*	Deceased n=44	Survivors n=333	P value
Age (years)**	45.8 ± 13.2	50.6 ± 13.9	45.2 ± 13	0.018
Male sex	62% (n=235)	61% (n=27)	62% (n=208)	0.89
CVD**	19% (n=71)	25% (n=11)	18% (n=60)	0.18
Diabetic**	10% (n=38)	23% (n=10)	8% (n=28)	0.007
Smoker**	17% (n=63)	32% (n=14)	15% (n=49)	0.008
eGFR (ml/min)	48.5 ± 19.4	43.8 ± 21.2	49.1 ± 16.6	0.047
Systolic BP(mmHg)	140.9 ± 18.8	141.4 ± 25.4	146.8 ± 17.8	0.84
Diastolic BP(mmHg)	78.4 ± 10.2	76.1 ± 10.6	78.8 ± 10.2	0.1
Phosphate at 1 year (mM)	0.96 ± 0.24	1.04 ± 0.26	0.95 ± 0.23	0.031
Calcium (mM)	2.4 ± 0.2	2.4 ± 0.2	2.46 ± 0.2	0.07
Albumin (mM)	41.5 ± 3.8	40.2 ± 4.7	41.7 ± 3.6	0.016
Haemoglobin (g/dl)	13.1 ± 1.8	12.6 ± 2.0	13.2 ± 1.8	0.041
Phosphate at 5 years (mM)*	1.02 ± .32	1.31 ± 0.37	1 ± 0.31	0.013
Transplant failure	43	13	31	0.002

Table 1-3: Characteristics of the study population.

*p values refer to comparison between survivors and deceased patients. * n=190 at 5 years and **at time of transplantation.*

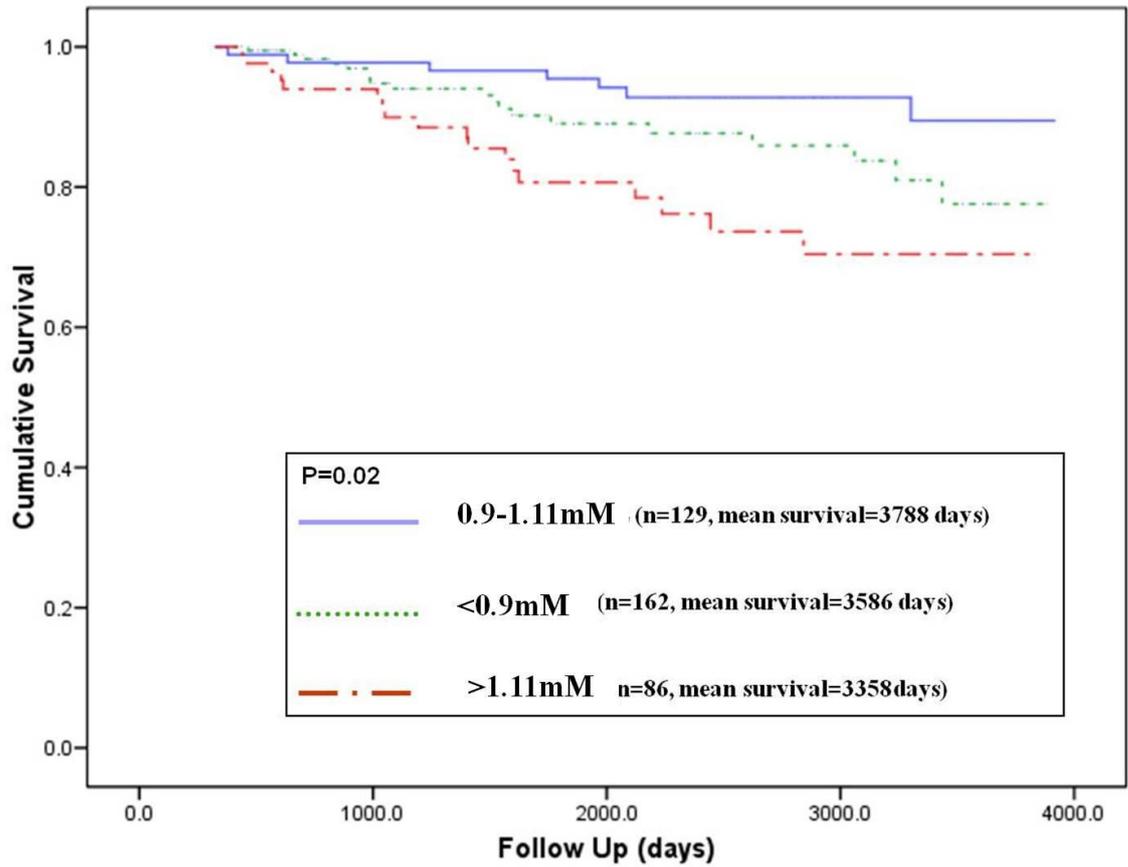


Figure 1-11: Transplant recipient survival stratified by serum phosphate at one year post transplant and censored for transplant failure.

1.11 Aims of this thesis

The aims of this thesis are to determine the effects of phosphate *in vitro* and *in vivo*. The specific questions that I have answered are:

- Whether phosphate and FGF-23 level correlate with LVM in patients with CKD and whether this may be explained by an effect on cell adhesion molecules?
- Does elevated phosphate concentration affect the function of resistance vessels from healthy rats and from humans with and without CKD?
- What are the effects of culturing different cell lines, long term, in high phosphate concentration medium particularly whether long term exposure to high phosphate causes endothelial dysfunction and altered growth patterns?
- Whether intracellular calcium concentrations are altered when cells are cultured in high phosphate medium?
- In healthy volunteers, what are the effects of phosphate supplementation and phosphate binding on endothelial function, vascular stiffness, urinary phosphate excretion, urinary cGMP levels and serum phosphate and FGF-23 levels?

1.12 Hypothesis

The hypothesis of this thesis is that long term exposure to elevated phosphate is associated with direct endothelial and vascular dysfunction and it is this which contributes to the elevated CV risk seen in those with CKD. Endothelial and vascular dysfunction will be evident in individual cell lines, in blood vessels and in humans exposed to prolonged oral phosphate loading. Figure 1-12 summarises which aspects of this hypothesis are covered in which chapters.



Figure 1-12: Summary of thesis - the interaction between phosphate and FGF-23 and effects on the vasculature and the endothelium and the pertinent chapters.

2 Chapter Two – Materials and Methods

2.1 Introduction

This chapter contains a comprehensive guide to the methods used in the work contained within this thesis. The effects of phosphate were studied in a translational manner and the findings described in the individual results chapters involved the use of cell lines (chapters three to six), resistance vessels from rats (chapter four), resistance vessels from humans (chapter five) and a cross over trial in human volunteers (chapter seven).

2.2 General laboratory practice

A laboratory coat and latex free gloves were worn during all experimental work. The highest available grade of laboratory reagents and equipment was used. When handling hazardous materials, laboratory glasses and/or a fume hood were utilised in accordance with The Control of Substances Hazardous to Health Regulations (COSHH).

Gilson pipettes (Gilson Medical Instruments) were used to dispense volumes between 0.1 µl and 1,000 µl whilst a Gilson battery powered pipetting aid with sterile disposable pipettes (Corning Inc., NY, USA) allowed dispensing of larger volumes between 1 ml and 25 ml. When appropriate, a multi-channel pipette (Eppendorf) was used. Pipettes were calibrated annually.

Centrifugation of samples at 4-20 °C was performed in either a Sigma 1-15K microfuge (volumes up to 2 ml) or a Beckman GS-6KR centrifuge (volumes up to 50 ml). Vortexing was undertaken with a FSA Laboratory Supplies WhirliMixer. A plate shaker, 'Belly dancer' was used where indicated (Denville Scientific, Inc.). A Mettler Toledo digital pH meter was used to determine the pH of solutions. This was calibrated regularly with solutions of pH 4.0, 7.0 and 10.0 prepared from buffer tablets (Sigma-Aldrich Ltd, Poole, UK). To weigh reagents, use was made of either an Ohaus portable advanced balance (sensitive to 0.01 g) or a Mettler HK160 balance (sensitive to 0.0001 g).

For protein quantification, a Beckman DU640B spectrophotometer was utilized. An automated microplate washer (LT-3500, Labtech) was used where indicated. A hot block was used to heat substances up to 70°C (Dri-Block DB-2A Techna) and a Heidolph Polymax 1040 plate shaker was used to mix solutions when appropriate.

In the preparation of aqueous solutions, distilled water was used unless otherwise stated and a Jenway 1000 hotplate/stirrer aided dissolving and mixing.

Sterile, disposable plastic ware from Corning Inc., NY, USA was utilised including: 0.5ml, 1.5ml and 2ml microcentrifuge tubes (Greiner Bio-One); 15ml and 50ml centrifuge tubes; 5ml and 20ml universal containers (Sterilin); assorted sterile, disposable pipette tips; 100mm x 20mm cell culture dishes; Six well, 12 well, 24 well and 96 well cell culture plates; and 25cm², 75cm² and 150cm² culture flasks with vented caps.

Glassware was cleaned in Decon 75 detergent (Decon Laboratories Ltd.), rinsed in distilled water and finally dried in a 37°C cabinet. If laboratory equipment required to be sterilised this was undertaken in an autoclave (Priorclave Tactrol 2). All cell culture took place under sterile conditions in a laminar flow hood. Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich Ltd, Poole, UK.

2.3 Vessel studies

Two separate series of studies were undertaken looking at the function of animal and human vessels *in vitro*. The vessels used were: rat mesenteric resistance vessels from 12 week old male Wistar-Kyoto rats (WKY) and human resistance vessels from subcutaneous abdominal adipose tissue from patients with and without CKD. The West of Scotland Research Ethics Committee 4 approved the human studies.

2.3.1 Animals

Work with experimental animals was in accordance with the Animals Scientific Procedures Act 1986. Pre 9/11/2011 this was under the project licence held by Professor A.F. Dominiczak (60/3618) and post 9/11/2012 under the project licence held by Dr Delyth Graham (60/4286). Inbred colonies of WKYs were maintained “in-house” by brother-sister mating. Microsatellite screening was used to confirm homozygosity of all loci within a random group from the strain.

WKYs were sacrificed (schedule one) and the mesentery removed and placed into a chilled normal phosphate concentration physiological saline solution (PSS). The constituents of PSS are defined in Table 2-1. This was undertaken by trained staff. Prior to sacrifice, all animals were housed under controlled environmental conditions: temperature maintained

at 21°C with 12 hour light/dark cycles. Rats were fed standard rat chow (rat and mouse No.1 maintenance diet, Special Diet Services) and water was provided ad libitum. The mesentery was stored in the fridge for up to one hour before it was pinned on a petri dish containing fresh PSS as illustrated in Figure 2-1.

2.3.2 Selection of human subjects

Patients without CKD undergoing nephrectomy for living kidney donation and patients with CKD undergoing live donor renal transplant were identified and provided with a participant information sheet (section 9.2, Appendix).

Informed consent was obtained (section 9.2, Appendix). Blood samples were collected the day prior to the operation and in the case of patients with CKD, prior to dialysis where possible. Samples were sent for full blood count and routine biochemistry including bone biochemistry, urea and electrolytes, CRP and PTH. EDTA plasma was stored for FGF-23 analysis and serum was stored for vitamin D measurement.

At the time of the operation, and prior to the use of either diathermy/harmonic scalpel, a piece of skin approximately 5mm wide and 70mm long was dissected with adherent subcutaneous abdominal fat. The sample was immediately placed into chilled PSS and transported to the laboratory.

2.3.3 Wire myography and dissection of vessels

Blood flow to tissues is controlled by resistance vessels including all small arteries with an internal diameter of less than 500µm. Arterioles have an internal diameter of less than 80µm. Wire myography allows the study of the function of all pre-arteriolar arteries: tiny resistance vessels with an internal diameter of 100-500µm as defined by Mulvany (334).

In 1972, Bevan and Osher described the first wire myograph which allowed segments of arterial vessels with internal diameters as small as 100µm to be mounted on two wires fixed under tension at both ends (335). The isometric response of a vessel to an agonist could then be recorded. Prior to this, detailed study of the properties of vascular smooth muscle was confined to ring preparations of large vessels using the traditional organ bath method. Technical difficulties prevented direct study of smaller vessels and thus information about their mechanical and pharmacological properties could only be

extrapolated from these large vessel studies or gleaned from perfusion experiments or histological examination (336).

In the mid-70s, Mulvany and Halpern honed the technique further. Vessel segments of approximately 2mm in length are mounted on fine wires (40 μ m) with both wire ends fixed under tension onto two stainless steel jaws. One jaw is attached to an isometric force transducer and the other to a micrometer, allowing the vessel wall tension to be measured at a pre-determined circumference (Figure 2-2) (337;338). This method differs from others where only one end of the wire is fixed, necessitating manipulation of the vessel on the wire with dissecting equipment with almost inevitable consequent damage to the vessel (339). Additionally, when only one end of the wire is fixed, for contractions to be isometric, the wire has to be thicker (100-200 μ m) precluding the use of smaller vessels.

Mulvany and Halpern performed their original experiments using rat resistance vessels from the mesentery. Human omental vessels were first examined with wire myography in 1981 (337;338;340). Human vessels are taken from subcutaneous fat usually from buttock biopsies or at the time of a surgical procedure or from the myometrium in pregnancy (95;108;340;341). In the rat, mesenteric vessels are commonly studied (338).

Once vessels are mounted, the contractile response can be studied with the addition of phenylephrine (PEP) or noradrenaline and acetylcholine and its mimetics (e.g. carbachol) can be administered to trigger NO production in the endothelium. NO donors including sodium nitroprusside (SNP) allow the study of endothelial independent vasodilation by donating NO to VSMCs, bypassing the need for a functioning endothelium. N-nitro-L-arginine methyl ester (L-NAME) is an inhibitor of NO synthesis and thus can be used in the study of NO and its effects, particularly to study basal NO production.

All vessels were dissected, on a petri dish with 5mm thick layer of sylgaard to hold fixing pins and fresh PSS, under a dissection microscope (Zeiss). Micro dissection instruments were used (World precision instruments). Dissection was performed at room temperature without oxygenating PSS. Following dissection, vessels were stored in standard PSS before being transferred to universal containers containing either normal (1.18mM) or high (2.5mM) phosphate concentration PSS. The composition of these solutions is shown in Tables 2-1 and 2-2. The solutions were stored at 4°C for 16 hours prior to use in experiments. For the human vessels, EDTA was also added to both the normal and high concentration PSS at a concentration of 0.023mM.

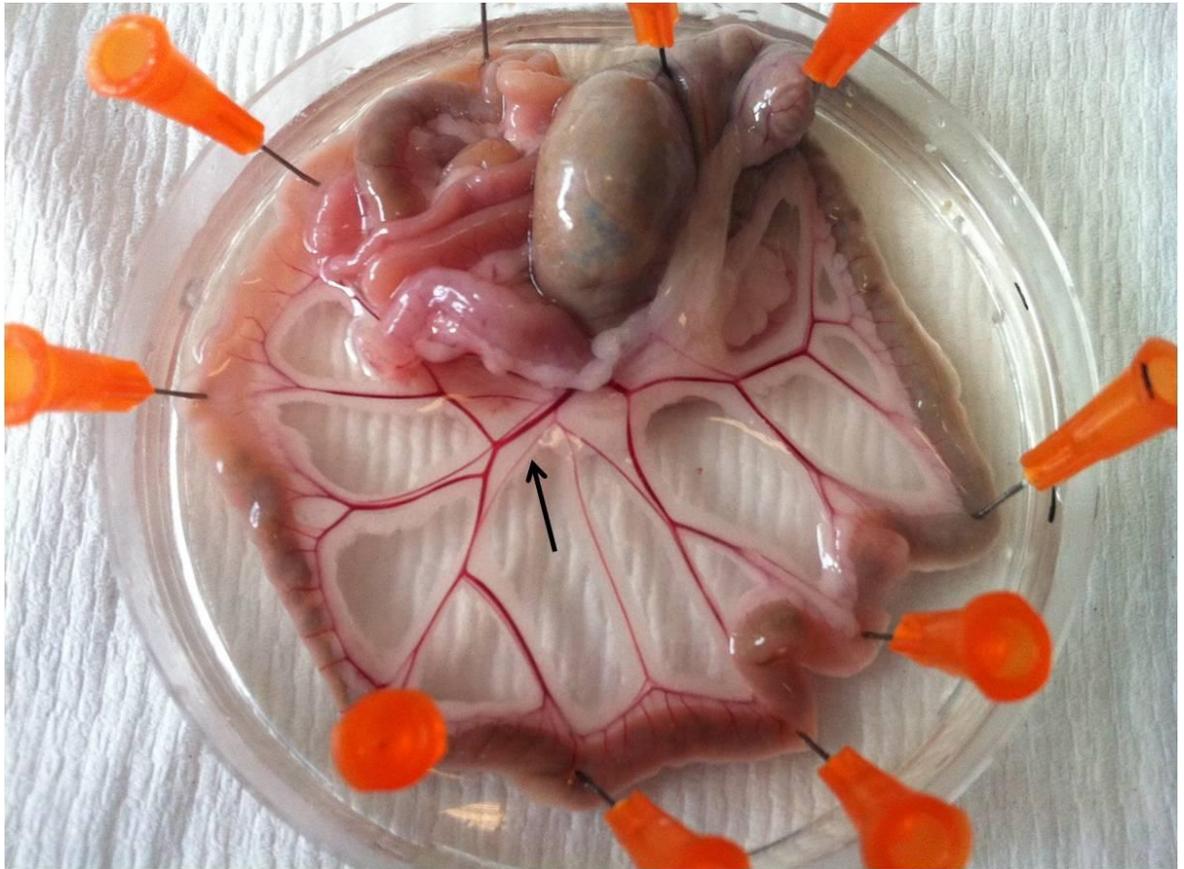


Figure 2-1: Dissected rat mesentery pinned out onto a petri dish.

The resistance vessels (example indicated by the black arrow) are easily identified under a dissecting microscope; they have a thicker wall and are structurally different to the veins.

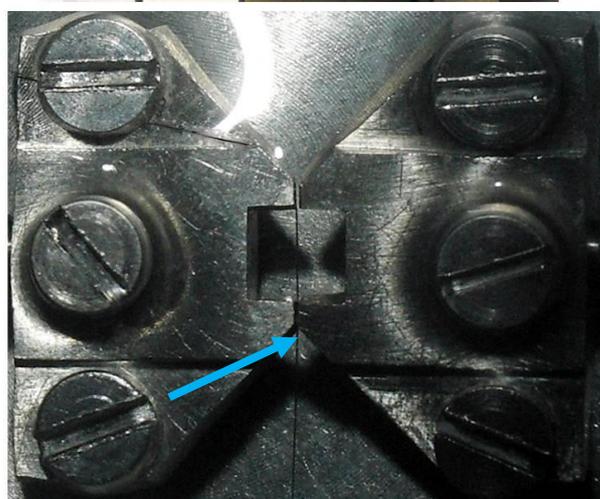
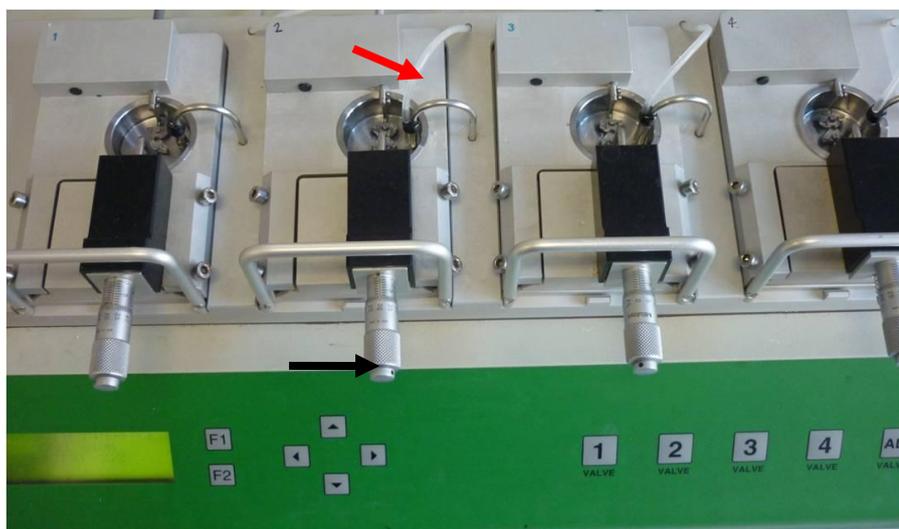


Figure 2-2: A typical four chamber wire myograph (top) with the micrometer indicated by the black arrow and oxygen supply by the red arrow.

Inside each of the four chambers are stainless steel jaws (middle) between which two wires can be fixed to hold a vessel in place. One wire is indicated by the blue arrow (bottom).

Substance	Standard PSS (mM)	High phosphate PSS (mM)
NaCl	118.4	118.4
KCl	4.7	3.3
MgSO ₄ .H ₂ O	1.2	1.2
NaHCO ₃	24.9	24.9
KH ₂ PO ₄	1.18	2.5
Glucose	11.1	11.1
CaCl ₂	2.5	2.5

Table 2-1: Composition of normal and high phosphate concentration solutions.

The solutions were aerated with a 5% CO₂/95% O₂ mixture and had a pH of 7.4

Electrolyte	Standard PSS (mM)	High phosphate PSS (mM)
Sodium	143.3	143.3
Potassium	5.9	5.8
Magnesium	1.2	1.2
Phosphate	1.18	2.5
Calcium	2.5	2.5
Glucose	11.1	11.1
Chloride	120.9	120.9

Table 2-2: Final electrolyte concentration of standard and high phosphate PSS.

2.3.3.1 Rat mesenteric vessels

Third order resistance vessels were identified according to the technique described by Mulvany and dissected under a dissecting microscope with all adherent fat removed (336).

2.3.3.2 Human subcutaneous vessels

Resistance vessels were usually identified running from the skin. Dissected vessels along with a small amount of attached fat were transferred to a second petri dish also containing PSS and pinned at either end. The surrounding fat was carefully removed taking care not to damage the vessel. The vessels were then transferred to a universal containing PSS and stored in the fridge for between three and four hours. At time zero, the vessels were transferred to two further universals containing normal (1.18mM) or high (2.5mM) phosphate concentration PSS.

2.3.4 Mounting of vessels on the wire myograph

All myography experiments were performed on a four chamber wire myograph (multimyograph model 610, Danish Myo Technology). This is illustrated in Figure 2-3. Experiments were conducted after storage of the vessels at 4°C in normal (1.18mM) or high phosphate (2.5mM) concentration PSS for 16 hours. Each chamber of the myograph was filled with PSS; two chambers contained normal phosphate and two high phosphate concentration PSS. Stainless steel wires, forty microns in diameter, were cut into lengths of approximately 2.5cm. One wire was attached by one of the two available fixing screws onto one jaw of the myograph thus leaving a free straight segment of wire onto which a vessel lumen could be threaded. Under the dissecting microscope, the vessels were, using micro-forceps, fed onto the wire attached to the myograph. The free end of the wire was then attached to the second fixing screw on the myograph jaw. A second wire was then carefully threaded, through the vessel lumen, under the first wire. The jaws were closed clamping both wires. The second wire was secured to each fixing screw on the opposite jaw of the myograph and the jaws were carefully opened putting a small amount of tension on the vessel. This allowed the wires to be levelled in a horizontal plane. Any part of the vessel protruding beyond the jaws was cut away taking care not to damage the remaining vessel. Using a micrometer eyepiece attached to the dissecting microscope, the length of the vessel segment was measured and recorded. The wires were then just touching and the micrometer reading recorded at that point. Figure 2-3 incorporates a cartoon of a vessel mounted on a myograph.

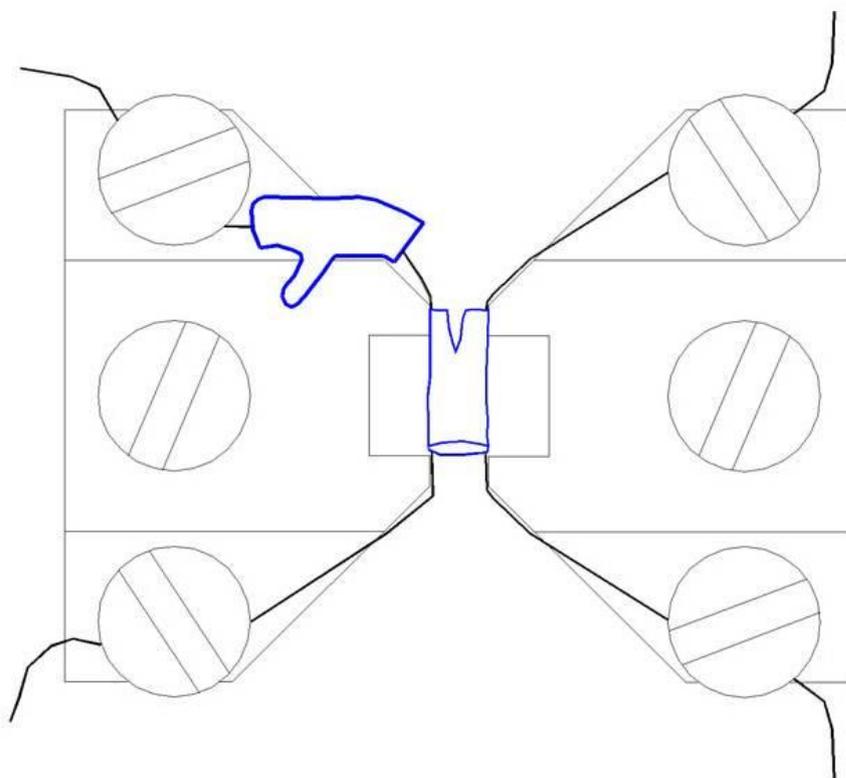
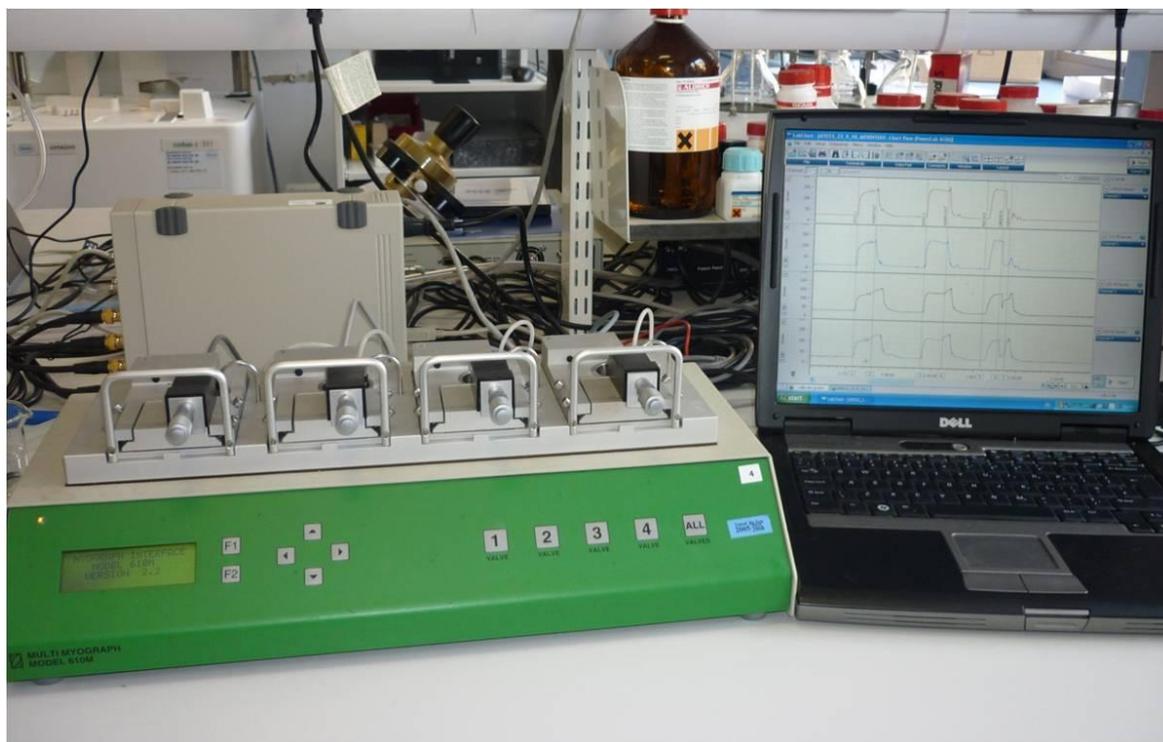


Figure 2-3: A four chamber wire myograph system (top) and a cartoon of vessel mounted on two wires, attached to the two jaws of a wire myograph (bottom).

One jaw is attached to a micropositioner allowing the vessel to be put under tension and the other jaw attaches to a force transducer which is connected to a computer. Adapted from Mulvany et al (336).

2.3.5 Normalisation and experimental procedure - rat mesenteric vessels

Mulvany's original work described a process by which to standardise third order rat resistance vessels on a myograph (338). This is necessary for three reasons: the active response of a vessel is dependent upon the degree of stretch which it is under, the sensitivity of a vessel to pharmacological agents is dependent upon the degree of stretch which it is under and the size of an elastic vessel is influenced by trans-mural pressure and this requires to be defined. Normalisation establishes the internal circumference that a vessel would have when relaxed under a trans-mural pressure of 100mmHg (L_{100}). This is undertaken by gradually distending the vessel using the micrometer and recording the micrometer and force readings. The Laplace equation is then used to calculate the L_{100} . The internal circumference is then set to 90% of the L_{100} (342)

Vessels were mounted as described and then following normalisation subjected to a 'waking up' protocol where KPSS (PSS with potassium chloride substituted for sodium chloride on an equimolar basis) was added to the bath and the vessels contracted. This was repeated. The vessels were then contracted with phenylephrine (PEP) and relaxed with carbachol. Vessels which failed to contract to KPSS or PEP or failed to relax >50% to carbachol were discarded. The vessels were then left for 30 minutes prior to concentration response curves being calculated. The specific protocols are discussed in more detail in the relevant results chapters; four (rat vessels) and five (human vessels). Briefly, vessels were contracted with PEP in the presence and absence of N-nitro-L-arginine methyl ester (L-NAME). Following contraction with PEP, they were relaxed with carbachol or with sodium nitroprusside (SNP). Vessels were also contracted and relaxed in the presence of ascorbic acid or zaprinast, a phosphodiesterase-5-inhibitor (PDE5I) (Tocris bioscience, Bristol, UK).

2.3.6 Standardisation and experimental procedure – human subcutaneous vessels

Mulvany's process of normalisation refers specifically to third order rat resistance vessels and there is debate as to the applicability to other types of vessel (343). Therefore, for the human resistance vessels, active and passive tension curves were constructed as described by Van den Akker (343). The force on the vessels was standardised at the point where the constructed active and passive tension curves crossed: 0.8g of tension. Approximately 0.2g of tension was added to the vessels every 15 minutes to reach 0.8g. The vessel diameter

was measured manually by taking the micrometer reading when the wires were just touching and subtracting this from the reading when the wires were touching the vessel wall and adding 80 microns (wires 40microns). The vessel length was measured also. The vessels were 'woken' with KPSS. They were then contracted with PEP to achieve what was considered to be a maximal response. After wash out the vessels were contracted with PEP to 60% of the maximum response and then relaxed with carbachol. Following a wash out period, the vessels were then contracted again with PEP and relaxed with SNP. Only vessels which did not contract to KPSS were discarded.

2.3.7 Statistical analysis

For each vessel, a concentration response curve was calculated for each drug added to the bath. Area under the curve (AUC) was then calculated in Microsoft Excel using the formula: $AUC = (X1/2)+X2+X3+X4+X5+X6+X7+(X8/2)$. X_n is the % relaxation or contraction at each point on the concentration curve. A Student's T test or one-way analysis of variance (ANOVA) (if comparison of more than two groups) was then used to compare the AUC values. Maximum contraction and vasodilatation responses were compared directly with a Student's T test. EC_{50} values were also calculated. This is the concentration where 50% of the maximal drug effect is observed. The median L_{100} (rat vessels only) and the median vessel lengths were compared with a Mann Whitney U test. Statistical analysis was performed in SPSS v 19 (IBM, UK) and results are expressed as mean \pm standard error of the mean (SEM).

2.3.8 cGMP ELISA

This was measured according to the manufacturer's instructions using an ELISA kit (Cell Signaling technology, distributed by New England Biolabs Ltd, Hertfordshire, UK). A standard curve was plotted using the samples provided and plotted in Microsoft Excel on a logarithmic scale with a logarithmic trendline and the appropriate equation ($y=a*\ln(x) + b$). Each measure was made in duplicate and an average absorbance taken, the average background absorbance was subtracted from this to allow cGMP calculation (nM). Results were then expressed as cGMP pM/ μ g protein. Analysis was by Mann Whitney U test in GraphPad Prism, CA, USA .

2.3.9 Preparation of rat vessel samples for cGMP ELISA

Rat mesenteric resistance vessels were incubated in 1.18mM or 2.5mM PSS for 24 hours and then snap frozen using a combination of dry ice and methanol and stored at -80°C until use. The vessels were thawed at 4°C, sonicated and the protein concentration quantified using the Bradford's assay (section 2.4.7). The solution (180µl) was washed with a 10% (v/v) excess of 1:1 tri-n-octylamine and 1,2,2-tri chlorofluoroethane (198µl) vortex mixed and centrifuged (14,000rpm at 4°C for three minutes). The top layer (150µl) was removed and washed a second time as above. The top layer was removed (120µl) and stored at -80°C until use.

2.3.10 Superoxide measurement in whole blood

This was undertaken by Ms Elaine Friel (Technician, Institute of Cardiovascular and Medical Sciences). Whole blood samples were collected from patients with and without CKD. The samples were placed on ice immediately after collection. Electroparamagnetic spin resonance (EPR) spectroscopy allows measurement of chemical species which have one or more unpaired electron, like O_2^- . The Bruker e-scan EPR machine was calibrated using a reduced form of the spin probe, CP (3-carboxyl-proxyl radical) at concentrations of 1µM, 5µM and 10µM. Each was read ten times and the mean, standard deviation (SD) and coefficient of variation were calculated from these results. A HEPES buffer containing 100mM DTT (dithiothreitol) and 20% (v/v) Tween-20 was used to keep the pH of the solutions constant at 7.4. Deferoxamine (DF) and diethyldithiocarbamic acid (DTEC) were used as chelators at final concentrations of 25µM and 5µM respectively. CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine) was then used as a spin probe, labelling the superoxide present in the sample (final concentration 1mM). Everything was kept on ice throughout. Blood was added to HEPES buffer and chelators and finally CPH was added immediately before processing. The solution was drawn up by a 15µl capillary tube and sealed with 'cristoseal' sealant. After ten minutes on the EPR machine, the sample was removed and disposed of. Each blood sample was processed three times. The results were analysed in Microsoft excel using a linear equation and plotted in GraphPad Prism.

2.4 Cell culture

Four cell lines were utilised: HUVECs, rat thoracic aortic ECs, rat myoblasts and human cardiac myocytes. All cultures were incubated at 37°C in 5% CO₂. For all cell types, two types of media appropriate to the cell line were used. Media was either standard phosphate concentration media (0.5mM) or custom formulated with a phosphate concentration of 3mM from PromoCell, Heidelberg, Germany. Table 2-3 details the cell line supplier, the media supplier, the appropriate catalogue number and the passage range at which each cell type was used in experimental work. All media were supplemented with 10U/ml penicillin and 10µg/ml streptomycin (Gibco by Life technologies Ltd., UK). In order to check the electrolyte composition of the media, the biochemistry department at Gartnavel General Hospital analysed sodium, potassium, phosphate, magnesium and calcium concentrations in each of the medium utilised (Table 2-4).

On arrival, cells were seeded into flasks and grown in standard phosphate concentration medium until they reached 90% confluence. At the first passage in our laboratory, they were divided into normal phosphate (0.5mM) and high phosphate (3mM) concentration cells and grown in the appropriate phosphate concentration media from that point until they were discarded. HUVECs were used between the second and fifth passages, rat ECs were used between the fourth and sixth passages and cardiac myocytes between the fourth and sixth passages. The rat myoblasts used, A10s, are an immortalized cell line with many properties of VSMCs and thus throughout this thesis will be referred to as VSMCs.

2.4.1 Trypsinization and cell storage

At 90% confluence, all cell lines were trypsinized and transferred to new flasks or onto plates at a density appropriate to the plate size. Media was removed from flasks and 10mls of phosphate buffered saline (PBS) (Gibco by Life technologies Ltd., UK) was added to wash away trypsin neutralisers. Trypsin-EDTA (Gibco by Life technologies Ltd., UK) was added and the flask was incubated at 37°C for five to ten minutes (cardiac myocytes were incubated at room temperature as per manufacturer's instructions). Immediately after detachment of cells, media was added to neutralise the trypsin, and the solution was transferred to a universal container and centrifuged at 1500rpm for five mins at 20°C. The cell pellet was re-suspended in media. 10µl of this solution was put onto a haemocytometer to allow the cells to be counted. Cells were left to adhere at least overnight before any experimental work was undertaken. Unless otherwise stated, the cell densities used were:

- Six well plate and glass bottom dish: 1×10^4 /well or dish.
- Twelve well plate: 1×10^5 /well.
- Ninety-six well plate and four chamber glass slides: 1.8×10^3 /well or chamber.
- Passage into new culture flasks: a 1 in 10 dilution.

For cell storage, cells were trypsinised and then re-suspended in standard growth medium for those cells, i.e. 0.5mmol/L or 3mmol/L phosphate concentration, containing 10% (v/v) DMSO and 20% (v/v) FCS. One ml of this cell suspension was frozen containing 1×10^6 /ml cells and frozen in an isopropanol containing cryopreservation container ('Mr Frosty') at a rate of $-1^\circ\text{C}/\text{minute}$ to -80°C before being transferred to liquid nitrogen after 24 hours.

2.4.2 Cell based ELISA for E-selectin and VCAM

This was performed in HUVECs cultured in 0.5mM phosphate and 3mM phosphate. The cells were plated onto a 96 well plate and left for 24 hours before being treated with FGF-23, at a final concentration of $3.5 \times 10^{-7}\text{M}$ (ProSpec, Tany TechnoGene Ltd, Israel) and Klotho, $2 \times 10^{-10}\text{M}$ (R&D Systems, Abingdon, UK) either alone or in combination; controls were untreated. After a four hour incubation with FGF-23 and Klotho, some wells were also treated with $\text{IL}1\beta$, and all plates left for a further six hours. The wells were washed with PBS and then the cells were fixed with 4% (w/v) paraformaldehyde and incubated at 4°C overnight. The wells were washed with a PBS (purchased from Sigma Aldrich) and 0.1% (w/v) bovine serum albumin (BSA) solution for one hour to block non specific protein binding. For E-selectin, mouse anti-human antibody (AbD Serotec, Oxford, UK) at a 1/2000 dilution was added to each well. For VCAM sheep anti-human antibody (R&D Systems, Oxford, UK) at a 1/2000 dilution was added to each well. After a one hour incubation, the wells were washed and the secondary antibody added (horseradish peroxidase (HRP) rabbit anti-mouse for E-Selectin and rabbit anti-goat for VCAM). This was washed off after incubation for one hour and developer was added. The wells were read at 630nm absorbance on a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Stripping buffer was added to the wells which were then probed for mouse anti-human GAPDH (R&D Systems, Abingdon, UK).

Product	Supplier	Catalogue number	Cell passage
Myocyte growth medium	Promocell, Heidelberg, Germany	C-22070, C-97124*, C-39275**	
SMC growth medium 2	Promocell, Heidelberg, Germany	C-22062, C-97109*, C-39267**	
EC basal medium	Promocell, Heidelberg, Germany	C-22215, C-97084*, C-39215**	
HUVECs	Promocell, Heidelberg, Germany	C-12203	2-6
Rat Aortic ECs	Cell applications Inc., San Diego, CA.	06090770	4-6
Human cardiac myocytes	Promocell, Heidelberg, Germany	C-12811	3-6
A10 (rat myoblasts)	ATCC, Rockville, MD	CRL-1476	Immortalized

Table 2-3: Details of cell line and media suppliers and the passage range at which cell lines were used in experimental work.

**Indicates custom made media formulation with 3mmol/L phosphate concentration and ** indicates the supplement mix added to the media.*

Medium	Na mM	K mM	Cl mM	Phos mM	Ca mM	Mg mM
Standard EC	130	4.4	122	0.58	1.66	9.12
Customised EC	131	4.2	119	2.87	1.47	9.37
Standard SMC	122	4.3	105	0.62	1.62	0.94
Customised SMC	128	4.3	106	3.04	1.63	0.91
Standard CM	121	4.2	105	0.63	1.62	0.97
Customised CM	126	4.3	105	2.86	1.6	0.93

Table 2-4: The concentration of electrolytes in each type of media used; endothelial cell (EC), smooth muscle cell (SMC) and cardiac myocyte (CM).

Standard media phosphate concentration for all cell lines is listed by Promocell as 0.5mM. The custom formulation media was to have a phosphate concentration of 3mM.

Cell based ELISA for E-Selectin and VCAM was performed in three separate experiments with each group represented in triplicate in each experiment, and results were normalized to GAPDH. For cell based ELISA comparisons, the different groups were compared using a two way ANOVA and *post-hoc* analysis was carried out with Tukey's test.

2.4.3 Cell staining and imaging

All cell lines were plated onto four chamber glass slides (Nunc Lab-Tek Chamber Slide). The following day they were stained with haematoxylin (0.1% w/v) and eosin (0.2% v/v) (H&E). Initially, the cells were washed twice with PBS and then they were fixed with paraformaldehyde (4% (w/v) dissolved in a 5% (w/v) sucrose solution) and incubated for ten minutes. The cells were washed again with PBS and then the slide was placed into haematoxylin for two minutes. After washing for five minutes with tap water, the slide was transferred to eosin for a further minute and then dipped into tap water to remove the excess for ten seconds. Slides were then taken to the fume hood where they were left for five minutes each in 70% (v/v) ethanol, 90% (v/v) ethanol and 100% (v/v) ethanol. The slides were then placed in histoclear for ten minutes. A cover slip was placed onto the slide and the cells were then imaged and photographed with an Olympus BX40 microscope and a Micropublisher 3.3 RTV camera (Q Imaging, UK) using Image Pro Software (Media Cybernetics, UK).

2.4.4 MTT Proliferation assay

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazolium salt is reduced to blue/purple formazan in the mitochondria of living cells via mitochondrial dehydrogenase. NADH is thought to be responsible for the majority of MTT reduction and MTT reduction is associated with the cytoplasm, the plasma membrane and other non-mitochondrial membranes in addition to the mitochondria. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. The formazan product is insoluble and the crystals therefore need to be solubilised (344). Absorbance can then be measured at a wavelength of 590nm with a reference filter of 620nm on a microplate reader. An increased cell number results in an increased amount of formazan and therefore increased absorbance (345).

MTT was used at a concentration of 5mg/ml, filtered through a 0.2µm filter and stored at -20°C, protected from light. A series of experiments using the MTT assay was performed in HUVECs, rat ECs, cardiac myocytes and rat VSMCs. Cells were incubated in twelve well plates and assayed for mitochondrial dehydrogenase activity by MTT. For the basic proliferation assay, cells were plated onto twelve well plates on day one. On days two and three the media was carefully changed. On day four, 20µl MTT/100µl media (final concentration 1mg/ml) was added to HUVECs, rat ECs and VSMCs. 10µl MTT/100µl media (final concentration 0.5mg/ml) was added to cardiac myocytes. The cells were then incubated at 37°C for three and a half hours (HUVECs and rat ECs), two hours (VSMCs) and one hour (cardiac myocytes). The media/MTT solution was carefully removed and 150 µl of MTT solvent was added (1:1 ethanol/DMSO mixture). The plate was wrapped in aluminium foil and agitated on an orbital plate shaker for fifteen minutes. The plate was then read on a microplate reader. In each plate there were control wells which contained media (both 0.5mM and 3mM as appropriate) and no cells. The basic proliferation assay was performed a minimum of three times for each cell line. Within each plate, four wells contained cells cultured in 0.5mM phosphate concentration and four wells, cells cultured in 3mM phosphate concentration. The remaining four wells were control wells.

The proliferation assays were repeated with the cells exposed to different chemicals: L-NAME (1×10^{-4} M), IL1β (5ng/ml), FGF-23 (3.5×10^{-7} M) and Klotho (2×10^{-6} M). The cells were plated as described above and then on day two, one of these substances, with the exception of IL1β, was added when the media was changed. This was repeated on day three. IL1β was only added to the cells six hours before the addition of MTT. Comparisons were made using Graph Pad Prism (San Diego, CA) and the Student's T test, one way ANOVA or Kruskal-Wallis test with post hoc (Dunn's multiple comparison test) analysis as appropriate.

2.4.5 Griess reaction

Measurement of NO is difficult because it has short half life and reacts rapidly with other molecules including oxygen radicals (346). Nitrate and nitrite are the main NO metabolites. The Griess reaction detects nitrites; when Griess reagent is added to a sample containing nitrite, a red-pink colour develops. Griess reagent contains sulphanilic acid which forms a diazonium salt with nitrites and an azo dye (N-alpha-naphthyl-ethylenediamine) which results in the red-pink colour. On day one, cells were plated onto a 96 well plate. After 24 hours fresh media was added to each well. Simultaneously cells

were treated with L-NAME ($1 \times 10^{-4} \text{M}$) or L-Arginine ($1 \times 10^{-5} \text{M}$); controls were left untreated. L-NAME was used as a NOS inhibitor and thus a reduction in NO would be expected. L-arginine was added to increase the available substrate for eNOS with an anticipated subsequent increase in NO production. The media was replaced daily with fresh L-NAME or L-arginine as appropriate until day four when a Griess reaction was performed. Sodium nitrite was used to generate a standard curve in each plate at a concentration of 0-100 μM . 50 μl of Griess was added to each well, the plate was then wrapped in tinfoil and replaced in the incubator for 30 minutes before being read at an absorbance of 540nm on a microplate reader. This was performed in duplicate and a minimum of six replicates were used for each condition.

2.4.6 Preparation of cell lysates

Supernatant was removed from cells and either discarded or stored in Eppendorf tubes at -80°C . On ice, cells were washed three times in ice cold PBS, before 70 μL of radio-immunoprecipitation assay buffer (RIPA) was added to individual wells. RIPA was made in house (150mM NaCl, 1mM EDTA, 1% (v/v) TX100, 0.5% (w/v) deoxycholic acid and 50mM Tris pH7.4; concentrations are final concentrations) and stored at 4°C . Prior to use, RIPA was supplemented with the protease inhibitors benzamidine and soya bean trypsin inhibitor, sodium orthovanadate and PMSF (phenylmethanesulphonyl fluoride). A pipette tip was used to scrape the adherent cells and the plate were placed in the freezer (-20°C) for 20 minutes. Lysates were collected after thawing and stored at -80°C until use. Repeated freeze-thaw was avoided.

2.4.7 Quantification of protein concentration

The Bradford assay was used to quantify protein concentration; it is a colorimetric protein assay. Under acidic conditions, the red form of Coomassie brilliant blue G-250 dye is converted to a blue form to bind to the protein being assayed. The blue form of the dye is stabilised when it binds to the protein and the protein concentration can then be estimated by measuring absorbance at 595nm. The increase of absorbance at 595nm is proportional to the amount of bound dye, and therefore to the concentration of protein present in the sample. BSA standards were used to construct a standard curve at a concentration of 0-0.225 μM . 0.5ml of Bradford reagent (BioRad Laboratories, Germany) was added to a cuvette containing 5 μL of sample. The samples were read using a spectrophotometer

(Beckman DU 640D) at an absorbance of 595nm. The standards and samples were routinely measured in duplicate and an average taken.

2.4.8 Polyacrylamide gel electrophoresis and immunoblotting

Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system (Life technologies, Paisley, UK formerly Invitrogen). Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS (lithium dodecyl sulphate at a pH of 8.4) sample buffer (Life technologies, Paisley, UK) by heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing (unless otherwise stated). Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TRIS buffered saline (TBS) (20mM Tris-HCl pH 7.4, 135mM sodium chloride sterilized by autoclave) containing 0.1% (v/v) Tween 20 (TBST). The nitrocellulose membranes were then blocked in either 5% (w/v) non-fat powdered milk (Marvel) or 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with primary antibodies at the optimal concentration in either milk or BSA overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with the appropriate horseradish peroxidase conjugated secondary antibody for two hours in milk or 5% (w/v) BSA at room temperature. Blots were then washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The antibodies used are detailed in Table 2-5.

2.4.9 VEGF ELISA

VEGF was measured on cell supernatants. The cells were cultured in six well plates and the supernatant from each well of one six well plate was combined and then concentrated and used to represent one replicate for each phosphate concentration. Three replicates were used for each condition and stored at -80°C. After a single thaw, the supernatant was concentrated using appropriately sized Amicon® ultra centrifugal filters (Merck Millipore, Abingdon, UK). A solid phase ELISA kit purchased from R&D systems was used and the ELISA was performed and analysed in accordance with the manufacturer's instructions. A

standard curve is generated by plotting absorbance versus VEGF concentration for each standard. The concentration of VEGF in each sample is subsequently read from this curve. VEGF is known to be induced when cells are exposed to hypoxic conditions (347). Cells exposed to hypoxic conditions were therefore used as a positive control.

Cells were put into an air tight chamber for 24 hours to induce hypoxia. The reliable air tight seal and the fast gas exchange properties of the chamber, allow for the rapid and economical creation of a non-fluctuating hypoxic environment. Once sealed, this is a closed air tight system. The gas is a mixture of 5% CO₂ and 95% N₂. The cells are placed into the chamber in six well plates in the hood. The gas is bubbled through for four minutes then the chamber is sealed and put into the incubator. The lids are left off the plates (there is O₂ in media). After 12 hours, fresh gas was bubbled through to ensure hypoxia was maintained. After 24 hours, the cells were removed and the supernatant and lysates obtained and stored.

2.4.10 Epi-fluorescence and FURA -2-AM

Intracellular calcium concentration was measured in HUVECs, rat VSMCs and rat ECs with epifluorescence using a dual-wavelength spectrophotometric method and the fluorescent dye FURA-2-AM (FURA-2-acetoxymethyl ester).

FURA-2-AM is a membrane permeable derivative of the ratiometric calcium indicator, FURA-2. FURA-2-AM crosses into a cell through the membrane and the acetoxymethyl groups are cleaved by cellular esterases; this process regenerates FURA-2, the pentacarboxylate calcium indicator. Cells imaged under fluorescence can be measured at two wavelengths, 340nm and 380nm. Intracellular calcium concentration can then be calculated based on 340/380 ratios. Using the ratio automatically cancels out variables which may lead to artefact when imaging intracellular calcium concentration; including local differences in FURA-2 concentration and variations in cell thickness (348). The intracellular calcium value (R) which corresponds to a particular 340/380 ratio is calculated with this formula: $((R-R_{\min})/(R_{\max}-R))*1.2 \times 10^{-6} \text{M}$

Protein Target (Site recognised)	Manufacturer	Catalogue Number	Species	Concentration
p eNOS (Ser1177)	Cell Signaling Technology	#9571	Rabbit	1/1000
Total eNOS	Cell Signaling Technology	#9586	Rabbit	1/1000
p PKC (β II Ser660)	Cell Signaling Technology	#9371	Rabbit	1/1000
Total PKC	Cell Signaling Technology	#9368	Rabbit	1/1000
p ERK p44/42 MAPkinase (Thr202/Tyr204)	Cell Signaling Technology	#9101	Rabbit	1/1000
Total ERK	Cell Signaling Technology	#9102	Rabbit	1/1000
Nitrotyrosine	R&D Systems	MAB3248	Mouse	1/1000
PKG	Enzo Life Sciences	ADI-KAP-PK002	Rabbit	1/1000
GAPDH	Abcam	9484	Rabbit	1/2000
Anti-mouse HRP	Amersham	NA9310	Sheep	1/2000
Anti-rabbit HRP	Amersham	NA9340	Donkey	1/2000

Table 2-5: Immunochemicals used in experimental work.

Includes manufacturer information, the host species and the concentration at which the antibody was utilised. p denotes phospho-.

$1.2 \times 10^{-6} \text{M}$ is the dissociation constant of FURA-2-AM. The minimum ratio (R_{\min}) is the fluorescence obtained using a zero calcium solution (10mM EGTA). The maximum calcium ratio (R_{\max}) is the fluorescence ratio obtained when the cell is lysed, thus releasing all of the intracellular FURA-2.

Cells were plated in glass bottomed plates and allowed to adhere overnight. The following day, cytosolic loading of FURA-2-AM (Life technologies, Paisley UK, formerly Invitrogen) was achieved by incubating the cells with FURA-2-AM (10 μM endothelial cells and 5 μM SMCs) at 37°C for 40 minutes (ECs) or 15 minutes (VSMCs). The cells were then washed with 1ml of media and incubated at 37°C for a further 15 minutes. Plates were mounted on an inverted microscope and isolated cells were imaged. Using an oscilloscope and the Clampex data acquisition software (pCLAMP, Molecular Devices LLC, USA), baseline fluorescence was recorded. 2 μM calcium ionophore (non-fluorescing form, A23187) was added to prove that a calcium sensitive signal could be generated. On addition of calcium ionophore, the 340/380 fluorescence ratio rises and plateaus. Following this the cell lyser, saponin was added (10 μM) to trigger cell lysis. A recording was made until the 380 nm wavelength began to fall. The Clampfit programme (Molecular Devices LLC, USA) was used to analyse the data and to determine the baseline fluorescence and the R_{\max} values. An example recording is shown in Figure 2-4.

To calculate the R_{\min} of the cells and the dissociation constant (K_d) of FURA-2-AM, a three point calibration curve was constructed using HUVECs. Cells were plated in three dishes as described above and then incubated with 10 μM FURA-2-AM for 40 minutes at 37°C. The cells were washed and incubated for a further 5 minutes. 2 μM calcium ionophore was added and the plates were left at 37°C for a further 2 minutes. A zero calcium HEPES (100mM KCl, 1mM MgCl_2 , 25mM HEPES, 10mM NaCl) solution was used to wash the cells and then a 10mM EGTA HEPES solution was added to one plate, a 5mM/5mM calcium EGTA/HEPES solution was added to the 2nd and to the 3rd a 10mM calcium EGTA solution was added.

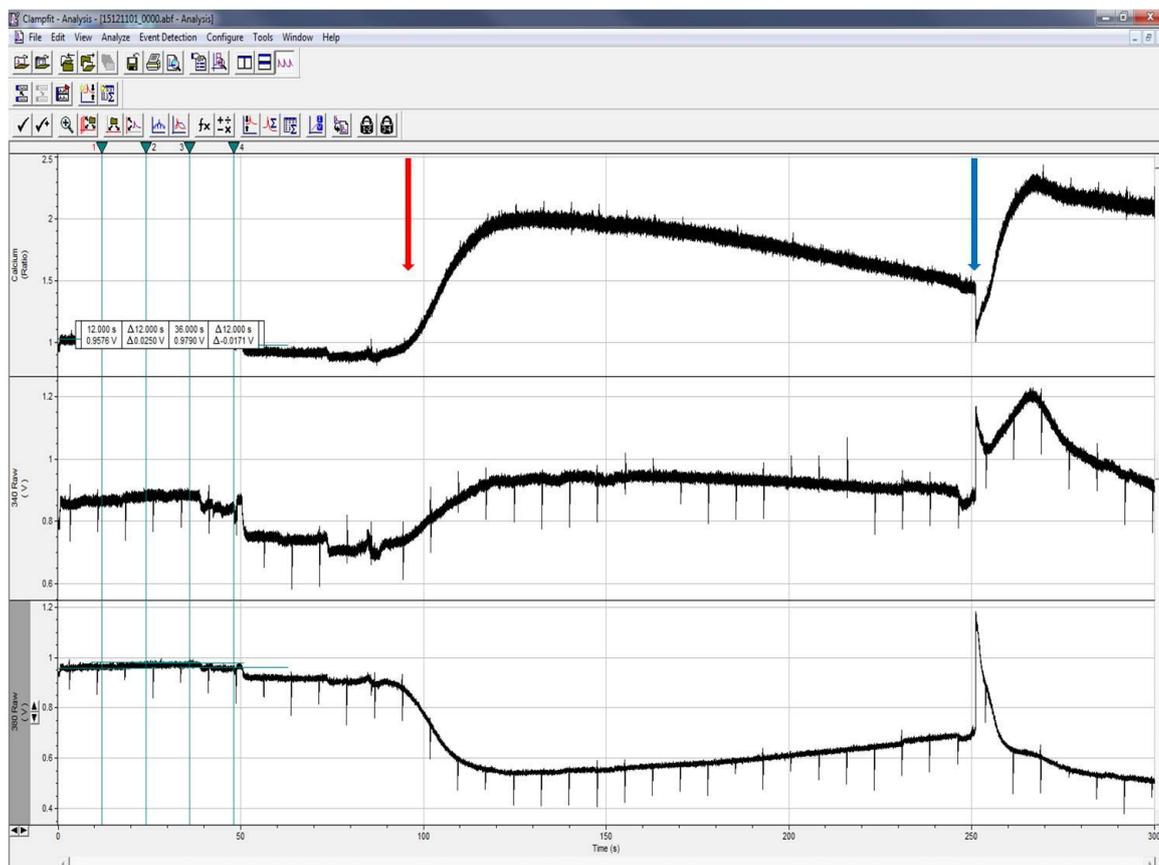


Figure 2-4: A fluorescence recording from ECs using the Clampfit analysis package.

The red arrow indicates the addition of calcium ionophore and the blue, saponin. The top row shows the 340/380 fluorescence ratio, the middle row the 340nm raw data and the bottom the 380nm raw data.

The plates were incubated for 10 minutes at 37°C. The fluorescence ratio was measured on the oscilloscope. The free calcium concentration of each of the three added solutions was calculated using REACT software (10nM, 37.5µM and 61.7µM respectively). The free calcium concentration was then plotted on a log scale on the X axis and the fluorescence ratio (R_{\min}) recorded for each solution was plotted on the Y axis. A sigmoidal curve was drawn. The 50% value is equivalent to the K_d of FURA-2-AM and the R_{\min} value for the cell line is equivalent to the fluorescence ratio seen with the 10mM EGTA solution.

2.4.11 EPR superoxide measure from cell supernatant

This was undertaken in HUVECs using a similar method to that described in section 2.3.10. The cells were cultured and plated onto six well plates. Upon reaching confluence, the cells were washed twice with PBS and then incubated with a Krebs-HEPES buffer (118.4mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4 \cdot H_2O$, 11.1mM glucose, 2.5mM $CaCl_2$, 25mM HEPES) and the CPH spin probe for one hour at 37°C. The CPH spin probe and Krebs-HEPES buffer were also incubated in a well without cells for the same time period. The supernatant was then removed and immediately put onto ice before being run through the EPR machine in triplicate. The CPH without exposure to cells was used to assess the natural oxidation of the spin probe. The protein content of each well was quantified using the Bradford's assay. For analysis, Microsoft excel was used. The figures obtained from the spin probe alone were subtracted from those attained with cells present and then normalised to the protein content of each well. Thus the final value is superoxide production/ng of protein/minute.

2.5 Molecular biology

2.5.1 RNA extraction and complementary DNA synthesis

Cytoplasmic RNA was extracted from trypsinised cells according to the manufacturer's instructions with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined by measuring absorbance at 260nm and purity estimated by checking the 260nm:280nm absorbance ratio using the ThermoScientific NanoDrop® ND-100 Spectrophotometer (Ratio >1.6). RNA was stored at -80°C until use and repeated freeze thaw was avoided.

cDNA was synthesised using the Invitrogen M-MLV reverse transcriptase kit. A mix comprising 1µg of RNA, 1µl hexamers (0.5µg/µl), 1µl 10 mM dNTP mix and sterile water to a final volume of 12µl was heated to 65°C for 5 minutes and then chilled on ice. 4µl of 5x First-Strand buffer, 2µl 0.1M DTT and 1µl RNaseOUT (recombinant ribonuclease inhibitor) were added to the mix and samples were incubated for 2 minutes at 37°C before adding 1µl of reverse transcriptase (M-MLV RT (200U/µl)). Thereafter, samples were incubated first at 37°C for 50 minutes and then at 70°C for 15 minutes. Each sample was also amplified without reverse transcriptase to assess for DNA contamination. Samples were subsequently diluted 1:5 with distilled water to a final volume of 100µl and stored at -20°C.

2.5.2 Polymerase chain reaction: primer design

The Roche universal probe library was used to design intron spanning primers. (<https://www.roche-applied-science.com/xis/rtpcr/upl/index.jsp?id=UP030000>). Primers were subsequently ordered from Integrated DNA Technologies (Belgium). Table 2-6 lists the primers and their characteristics.

2.5.3 Polymerase chain reaction

The polymerase chain reaction (PCR) was performed using a PTC-100 programmable thermal controller with the Invitrogen Superscript III™ first strand system for RT PCR. A 1µl of template (0.2ng), 0.25µl, 100nM forward and reverse primers, 0.5mls of 10mM dNTPs, 0.25µl Taq polymerase (5U/µl), 5µl 10x buffer (200mM Tris-HCl (pH 8.4), 500mM KCl) and PCR-grade water were combined to give a 25µl reaction volume. Samples were denatured at 94°C for 30 seconds then primers annealed at 55°C for 45 seconds and DNA extended at 68°C for 1 minute. This thermal cycling profile was used for 35 cycles.

2.5.3.1 Agarose gel electrophoresis of DNA

The DNA samples were mixed with blue loading buffer (Promega) and 15µl of sample/loading buffer mix was loaded onto the agarose gel. The DNA samples were run at 100V for 1 hour with tris-acetate electrophoresis (TAE) buffer (0.04M Tris-acetate, 0.001M EDTA). The 2% Agarose gels were made using 0.8g agarose for molecular biology, 40ml of TAE buffer and 4µl ethidium bromide. An ultraviolet transilluminator

was used to visualise bands and images were recorded on a BioRad ChemiDoc XRS digital imaging system.

2.5.3.2 TaqMan® real time polymerase chain reaction

Following reverse transcription, TaqMan® real time PCR (RT-PCR) was used to ascertain the relative quantity of the mRNA transcripts. The Roche Universal Probe Library was used in a probe-based detection scheme to allow time sensitive quantification rather than endpoint detection of the PCR template: 'real time'. The fluorescent reporter signal is detected and quantified at the end of every PCR cycle and this signal increases proportionally to the amount of PCR product present in the reaction. A fixed fluorescence threshold is set above baseline called the threshold cycle (Ct) and this is the cycle number at which the fluorescence emission exceeds the threshold and is exponential. The Taqman probe is an oligonucleotide which is shorter than primers and has a fluorescent dye at the 5' base and a quenching dye on the 3' base. When the probe is intact, no fluorescence is emitted. The probes are designed to anneal to a region of the PCR product. When polymerase replicates a template on which a Taqman probe is bound, the 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye and the reporter emits fluorescence – this increases with each cycle proportional to the rate of cleavage. The accumulation of product is detected by monitoring the increase in fluorescence compared with a passive reference dye e.g. ROX. An internal control gene which remains constant in proportion to the total RNA is also amplified e.g. GAPDH. The mRNA target can be normalized to this control gene to allow for differences in the total RNA added to each reaction. Relative quantification of gene expression is determined using the comparative Ct method, where the difference between the Ct values of two PCRs for the same initial template amount varies. This is normalised to a baseline reference sample for comparison to be made ($\Delta\Delta\text{Ct}$ method).

Target gene	ENSEMBL (ENSG00000..)	Fwd primer	Rev primer	Ann. Temp	Probe No.	Amp. Size (nt)
PiT1 (SLC20A1)	144136	ggccttcccatcagtaaac	aagagacgccagtaacacgc	55	36	92
PiT2 (SLC20A2)	168575	goggaatgacgtgagtaa	ttaccccgctgtttgtaa	60	5	72
AURKA	087586	gcagatttgggtggtcagt	tcatcatgcatccgacctt	60	79	107
AURKB	178999	attgctgactcggctggt	gtccagggtccacacat	60	69	69
CYCLIN A	145386	ggtactgaagtcgggaacc	gtgacatgcatcatttacagg	60	84	105
CYCLIN B	134507	ttcgccctgagcctatttg	gcacatccagatgttccatt	60	34	64
E-selectin	007908	accagcccagggtgaatg	ggftggacaaggctgtgc	60	86	89
iNOS	007171	gctcaaatctcggcagaatc	gccatcctcacaggagagtt	55	42	79
eNOS	164867	cacatggccttgactgaa	cagagccctggccttttc	60	83	82
NaPi2A	131183	catcatcgtcagcatggtct	atgatgggatggcagag	59	67	60
NOSIP	142546	aaggagtcggctatcgtgag	gcccaggttgacatcat	59	76	88
FGFR1	077782	ggagtatctggcctccaaga	tcacattgtcctctgtacca	60	71	77
KLOTHO	133116	tccaatggaatcgatgacg	ccatccagtatgtggcttt	60	73	104

Table 2-6: Oligonucleotide gene targets and characteristics.

ENSEMBL gene identification number, oligonucleotide sequences (forward and reverse primers), optimal annealing temperature (Ann. temp), paired Universal Probe number (Probe No.) and amplicon size (Amp. Size).

2.5.3.3 TaqMan® RT-PCR protocol

384 well PCR plates were utilized with each sample run in triplicate. 10µL was added to each well (2µL of 0.2ng of template, 0.1µL of 100nM probe, 0.4µL of 100nM forward and reverse primers, 2.1µL of PCR-grade water, 5µL QPCR mix containing 120nM ROX). The control gene was GAPDH. Control wells were run with non reverse transcribed template and mastermix without template. The plate was loaded onto a TaqMan® RT-PCR cycler where the block was heated to 50°C for two minutes then samples heated to 95°C for 10 minutes followed by 40 cycles of denaturing at 95°C for 15 seconds and one minute annealing / extension at 60°C. To assess specificity of amplified product and to avoid spurious results from primer-dimer formation and amplification, dissociation curves showing product melting points were assessed.

2.5.4 Microarray

Microarray technology allows exploration of the expression levels of multiple genes simultaneously and thus provides insight into disease processes, regulatory pathways and gene function through comparison of mRNA transcript levels in cells or tissues.

Biotinylated, amplified complementary RNA (cRNA), for hybridisation with Illumina Sentrix® arrays, was generated using the Illumina® TotalPrep RNA Amplification Kit according to the manufacturer's protocol.

2.5.4.1 Cell culture, RNA extraction, quantification and validation

HUVECs were cultured in standard or high phosphate concentration medium on 96 well plates. Total RNA was extracted, DNase treated and quantified using the NanoDrop® ND-100 Spectrophotometer. Total RNA sample integrity was verified by electrophoresis of 500 ng on an Agilent Bioanalyzer 2100.

2.5.4.2 cDNA synthesis and double strand cDNA purification

First strand cDNA was synthesised by reverse transcription. Nuclease free H₂O was added to 500 ng of total RNA to a final volume of 11 µl. 9 µl of reverse transcription master mix was then added. The master mix was prepared at room temperature and contained 1 µl T7 Oligo(dT) primer, 2 µl 10 X first strand buffer, 4 µl dNTP mix, 1 µl RNase inhibitor and 1 µl ArrayScript for each single 20 µl reaction. After thorough mixing the samples were

incubated at 42°C for two hours. Subsequently, second strand cDNA was synthesised immediately by adding 80 µl second strand master mix to each sample; prepared on ice and containing 63 µl nuclease-free H₂O, 10 µl 10 X second strand buffer, 4 µl dNTP mix, 2 µl DNA polymerase and 1 µl RNase H for a single 100 µl reaction. After mixing thoroughly and flicking the tube 3-4 times, tubes were centrifuged briefly to collect the reaction in the bottom of the tube. Samples were then incubated for two hours at 16°C in a thermal cycler and following this, cDNA purification was carried out immediately. cDNA was purified to remove RNA, primers, enzymes and salts which would inhibit *in vitro* transcription. 250 µl cDNA binding buffer was added to each sample and the mixture mixed thoroughly with pipetting and flicking the tube prior to being applied to the centre of a cDNA filter cartridge seated in a wash tube. Cartridges were centrifuged for a minute at 10,000x g, the flow-through was discarded and the cartridges replaced in wash tubes. 500 µl wash buffer was added to each cDNA filter cartridge and cartridges centrifuged at 10,000x g for a minute. Flow-through was discarded and the cartridges centrifuged for a further minute to remove all traces of wash buffer. The cDNA filter cartridges were then transferred to cDNA elution tubes and 10µl of pre-heated (50-55°C) nuclease-free H₂O added to the centre of the filters. The samples were incubated at room temperature for two minutes and then centrifuged at 10,000x g for 90 seconds. 9µl preheated nuclease-free H₂O was added to the filters and samples before a final centrifugation at 10,000 x g for two minutes to elute the double-stranded cDNA in an approximate volume of 17.5 µl. *In vitro* transcription (IVT) to synthesise cRNA was carried out immediately.

2.5.4.3 *In vitro* transcription for cRNA synthesis, purification and validation

To generate biotinylated RNA from the cDNA template firstly, 7.5µl IVT master mix was added to each sample. Master mix was prepared at room temperature with 2.5 µl T7 10 X reaction buffer, 2.5 µl T7 enzyme mix and 2.5 µl biotin-NTP mix for each single 25 µl reaction). Samples were thoroughly mixed and subsequently centrifuged briefly to collect the reaction mixture at the bottom of the tube. Samples were then incubated at 37°C for between four and 14 hours before the reaction was stopped by adding 75µl of nuclease-free H₂O. Purification of cRNA was carried out immediately. Each sample of cRNA was purified to remove unincorporated NTPs, salts, enzymes and inorganic phosphate by the addition of 350µl cRNA binding buffer followed by 250µl 100% ethanol. Samples were mixed, added to the centre of a cRNA filter cartridge placed in a cRNA collection tube and centrifuged for one minute at 10,000 x g. The flow-through was discarded and the cartridges replaced in new collection tubes. 650µl wash buffer was added to each cartridge

and samples were centrifuged for one minute at 10,000 x g. Flow-through was once again discarded and the samples centrifuged for a further minute to ensure complete removal of wash buffer. Cartridges were transferred to fresh cRNA collection tubes and 100µl of pre-heated, nuclease-free H₂O was added. The samples were left at room temperature for two minutes and then centrifuged for 90 seconds at 10,000 x g to elute the purified cRNA. Purified cRNA was quantified using the NanoDrop® ND-100 Spectrophotometer and quality assessed via electrophoresis of 500ng on an Agilent Bioanalyzer 2100.

2.5.4.4 Microarray Hybridisation & Data Collection

The Illumina® Whole-Genome Expression Direct Hybridization Assay was used to prepare cRNA for hybridisation with The Illumina® HumanWG-6 v3.0 Expression Beadchips. The Illumina® Beadchips contain 48,804 probes which target >25,000 annotated genes. RNase-free H₂O was added to 1.5µg of cRNA to a final volume of 10µl. Samples were mixed and then to re-suspend cRNA, incubated at room temperature for 10 minutes. The hybridization reagents, GEX-HYB and GEX-HCB were incubated at 58°C for 10 minutes to dissolve any salts which may have precipitated in storage. 20µl GEX-HYB was added to each cRNA sample and 200µl GEX-HCB was added to each hybridisation chamber and the beadchips inserted into the chambers. cRNA samples were preheated at 65°C for five minutes then vortexed and centrifuged. Samples were cooled to room temperature before 30µl was added to each array. The samples were randomised on the chips and technical duplicates were included. The hybridization chambers were incubated for overnight in a 58°C oven rotating at 60 rpm.

The following day, the beadchips were removed from the chambers and submerged in 250 ml of the wash solution, E1BC (3mls E1BC buffer in 1L RNase-free H₂O). 1 x high-temp wash buffer (50ml of 10 x stock in 450ml RNase-free H₂O) was preheated to 55°C and the beadchips were then incubated in this buffer for 10 minutes. Following this, the beadchips were transferred, at room temperature, to fresh E1BC wash solution and placed on an orbital shaker at maximum speed for 5 minutes. The beadchips were transferred to a 250ml 100% (v/v) ethanol solution and again placed on the orbital shaker for 10 minutes. A two minute wash in E1BC wash solution was performed before each chip was blocked in 4ml Block E1 buffer for 10 minutes at medium rocking speed and room temperature. Beadchips were then placed in 2ml Block E1 buffer with streptavidin-Cy3 (2µl 1 mg/ml stock per chip) and covered to protect from light before rocking at medium speed for 10 minutes. Beadchips were washed in 250ml E1BC wash solution and then placed on the

shaker for five minutes. The chips were dried by centrifugation at room temperature for 4 minutes at 275 x g. Finally the beadchips were scanned and the images extracted using specific protocols on an Illumina® BeadArray Reader. Illumina® Gene Expression Beadchips have internal control features to monitor both sample-independent and sample-dependent data quality. Control data results were visualised with BeadStudio software.

2.5.4.5 Gene Expression Data Analysis

Initial analysis of microarray data was performed using Illumina® BeadStudio software. Quantile normalisation of the data was carried out without background subtraction. Each probe is represented by multiple beads on an Illumina® Gene Expression Beadchip. Normalised values for each bead representing a given probe were averaged to obtain the estimate of expression for that probe. Unpaired two sample T tests were then performed on these averaged values using the Illumina® custom error model. Differentially expressed probes were identified using a difference score cut-off of +/- equating to a p value ≤ 0.05 . Differentially expressed probes with a fold increase or decrease of ≥ 2 and a false discovery rate (FDR) of ≤ 0.05 were uploaded to ThomsonReuters MetaCore™ software and analysed to identify pathways of interest containing the most significantly differentially expressed genes. Pathways analysis identified the pathways from the MetaCore™ library of pathways that were most significantly associated with the data set. Genes from the data set which met the p value cut-off of 5% and were associated with a canonical pathway in the MetaCore™ database were considered for the analysis. The significance of the association between the data set and the pathway was measured in two ways. Firstly, a ratio of the number of genes with differentially expressed probes from the data set that mapped to the pathway divided by the total number of genes with probes in the data set that mapped to the pathway was displayed. Secondly, in addition to this ratio, Fisher's exact test was used to calculate a p value determining the probability that the association between the significant genes in the data set and the canonical pathway could be explained by chance alone, the FDR. TaqMan® RT-PCR as described in section 2.5.3.2 was utilised to confirm differential expression observed in microarray experiments. The same total RNAs isolated for microarray experiments were used as templates for the synthesis of cDNA. Applied Biosystems Custom TaqMan® Gene expression assays or SYBR® Green based detection assays (Invitrogen) were used for RT-PCR.

2.6 Clinical Studies

2.6.1 Study 1: *FGF-23 and left ventricular mass in chronic kidney disease*

The lead investigator for this study was Dr Emily McQuarrie (formerly clinical research fellow, University of Glasgow) who kindly allowed me to make use of her data and her stored samples to measure FGF-23 levels and study any association with LVM.

The Renal Unit at the Western Infirmary, Glasgow provides renal services to 2.8 million patients in the West of Scotland. Adult patients attending renal clinics in The West of Scotland were recruited with a diagnosis of either diabetic nephropathy or biopsy-proven IgA nephropathy and CKD stages 3–4. Patients with essential hypertension in the absence of CKD were recruited from the Glasgow blood pressure clinic. Patients who were receiving immunosuppression, had evidence of active infection or who were unable to undergo cardiac magnetic resonance imaging (CMRI) scanning were excluded. Written informed consent was obtained from all patients and the study was approved by the local ethics committee.

Patients attended the Glasgow Clinical Research Facility. Demographic data including height, weight and waist circumference were recorded. The lowest of three blood pressure measurements was recorded. Investigations included measures of renal function, bone biochemistry (PTH, vitamin D, calcium and phosphate levels), haemoglobin and CRP). Proteinuria was estimated from a spot protein:creatinine ratio (UPCR), and 24-h proteinuria quantification. All prescribed medications were continued throughout the study. Visits were undertaken by Dr Emily McQuarrie who also performed cMRI on each patient. The cMRI protocol has previously been described (349;350). Briefly, non-contrast cMRI was performed to determine left ventricular mass index (LVMI) using 8mm thick short-axis cine slices from a 1.5-Tesla Siemens (Erlangen) CMRI scanner, and a fast imaging with a steady-state precision (FISP) sequence. LVM was analysed by a blinded observer from short-axis cine loops using manual tracing of epicardial and endocardial end-systolic and end-diastolic contours. End-systolic volume, end-diastolic volume and LVM were calculated using commercial software (Argus; Siemens). Values were adjusted for body surface area (Mosteller formula, $BSA (m^2) = \sqrt{((weight (kg) \times height$

(cm))/3600)), and LVH was defined as LVMI $> 84.1 \text{ g/m}^2$ for men and $>76.4 \text{ g/m}^2$ for women; LV systolic dysfunction was defined as LV ejection fraction $<55\%$ (351).

Statistical analysis was carried out using the IBM SPSS statistics package version 18.0 (IL, USA). Normality of the data was determined using Kolmogorov-Smirnoff analysis with normally distributed data expressed as mean \pm SD and non-normally distributed data as median (interquartile range (IQR)). Parametric correlations were calculated using Pearson's correlation, non-parametric using Spearman's. Variables were log transformed to obtain a normal distribution as required. Comparisons were made using Student's T test, Mann Whitney U test and one way analysis of variance as appropriate. Multivariate analysis was performed using binary logistic regression to determine independent predictors of LVH.

2.6.2 Study 2: Effects of sustained phosphate loading on endothelial function

In subjects without CKD, phosphate levels at the higher end of the normal range and acute phosphate loading (with a phosphate rich meal) can result in ED which corrects once loading has ceased (6). This study looked at the effects of manipulating physiological phosphate levels, using two different drugs and over a longer time course, on endothelial and blood vessel function in human subjects without CKD. The two drugs utilised to manipulate phosphate levels were phosphate (Sandoz UK, subsidiary of Novartis), a phosphate supplement and lanthanum (Shire Pharmaceuticals, UK), a phosphate binding medication. One 500mg phosphate tablet contains 16.1mM of phosphate which is roughly equivalent to the quantity of phosphate found in one can of fizzy juice (352).

Hyperphosphatemia in the short term is not known to cause any adverse long term effects. Long term effects of prolonged hyperphosphataemia include VC and increased CV risk; these are seen in people who have been exposed to elevated phosphate over a significant period of time (35;192). It was anticipated that phosphate levels would be lowest with the binding medication, highest with the phosphate supplement and in the middle at the baseline visit, visit one.

This study was approved by The West of Scotland Research Ethics Committee two. All participants read a copy of the participant information sheet (section 9.2, Appendix) and were given the opportunity to ask questions and discuss the study further before informed

consent was obtained (section 9.2, Appendix). With the participant's agreement, a letter was sent to inform the General Practitioner of their participation (section 9.2, Appendix).

2.6.2.1 Inclusion Criteria

Adult patients were recruited via email and word of mouth from University and National Health Service Staff (section 9.2, Appendix).

- Patients/Volunteers without CKD (eGFR>60mls/min)
- Individuals with capacity
- Age \geq 18 years

2.6.2.2 Exclusion Criteria

- Patients on any form of RRT (HD, PD or renal transplant)
- eGFR<60mls/min
- History of peripheral vascular, cerebrovascular or coronary artery disease (symptoms, positive exercise tolerance test, CT/MRI brain findings, angiography findings)
- Individuals without capacity
- Age<18 years
- Pregnant or lactating women
- Known drug abuse

2.6.2.3 Study Design

This was a single blind, cross-over study with participants acting as their own control. Each participant attended The Glasgow Clinical Research Facility (fasted) at 9am for three visits. Prior to each visit, for the preceding 24 hours, participants performed a 24 hour urine collection; following bladder emptying, first thing in the morning, all urine was

collected during the 24 hour period finishing on the morning of the visit. From this, three aliquots of 20ml of urine were stored at -80°C for urinary FGF-23 and cGMP analysis and the remainder was sent for routine laboratory measurement of urinary protein and electrolytes including urinary phosphate and calcium level.

At visit one, a history was taken to ascertain current health, medications, smoking history and history of CVD. At all visits, simple anthropometric measurements of height, weight and waist circumference were recorded along with blood pressure and an ECG. Endothelial function was measured using FMD and vascular stiffness using SphygmoCor® Vx system (Atcor Medical, Sydney, Australia). Blood samples were collected for routine analysis, storage for future analysis and extraction of white blood cells. At visit one, patients were randomised to receive either one phosphate tablet (500mg) or 1000mg of lanthanum (phosphate binder) three times daily for two weeks. The participant knew which tablet they were taking but the investigator did not. After consultation with the Medicines and Healthcare Products Regulatory Agency, neither phosphate nor lanthanum was being used as an investigative medicinal product simply as a means of altering serum phosphate concentration *in vivo*. After two weeks, patients attended for visit two. After a further two week wash out period, patients received the other drug for two weeks, before attending for the third and final visit. Figure 2-5 illustrates the study visit protocol.

2.6.2.4 Randomisation, power calculation and statistical analysis

Dr Patrick Mark (Senior Clinical Lecturer, University of Glasgow) generated a randomisation list in Microsoft Excel. This was given to the Pharmacy Preparation Unit at the Western Infirmary who put the drugs into an inner box and an outer bag. The inner box was labelled with details of the drug and the outer bag with participant identifiers but no drug identifiers. (Figure 2-6). The Western Infirmary pharmacy dispensed the drugs.

Statistical advice with regards to sample size and power calculations was taken from an independent statistician, Professor Stephen Senn (formerly of The School of Mathematics & Statistics, University of Glasgow). We anticipated that FMD measures would differ between the highest and lowest phosphate measures. For a clinically relevant difference of 3% in FMD, and a within-patient standard deviation of three (which corresponds to a standard deviation for the difference between repeated measures of about 4.25) approximately 18 patients would be needed to have 80% power for a 5% significance level (two sided). The aim was to recruit between 18 and 24 patients, to be randomised in equal

numbers, per sequence. This would provide 90% power should all patients complete but allowed some margin in the event of drop-out. In fact 20 patients were recruited and randomised in equal numbers, 10 per sequence. Data were assessed for normality and log transformed where appropriate. Correlations were carried out using either Pearson's or Spearman's Rho. An ANOVA with *post hoc* analysis using Tukey's honestly significant difference test was employed. Univariate and multivariate linear regression models were constructed with outcome (change in FMD from baseline) fitted as a function of treatment, patient and period but in such a way that the relevant contrasts could be specified. The issue of multiple testing was addressed in the protocol using the global F-test followed (if significant) by three pairwise comparisons. One third of the images were selected at random and re-analysed to test reproducibility and intra-observer variability. Intra-class correlation coefficient was computed using a two factor mixed effects model (type: consistency) for baseline diameter, post cuff diameter and FMD.

Results for urinary phosphate are presented as the fractional excretion of phosphate calculated as follows:

$$(\text{Urinary phosphate} * \text{Serum creatinine}) / (\text{Urinary creatinine} * \text{Serum phosphate}) \%$$

By taking into account urinary creatinine, this is more likely to be an accurate reflection of true urinary phosphate excretion.

2.6.2.5 Blood pressure measurement

After 15 minutes of supine rest, blood pressure was measured twice in each arm using the Omron MX2. An average of three readings was obtained and used in further analysis.

2.6.2.6 Blood sampling

After 30 minutes of supine rest, 50ml of blood was taken for routine biochemistry (urea and electrolytes, bicarbonate, bone profile, liver function tests, CRP, urate, glucose, lipids and PTH) and haematology (full blood count). Serum samples were obtained for vitamin D analysis and EDTA plasma samples for FGF-23 analysis. These were stored at -80°C until analysis. Additional plasma samples were obtained for extraction of white blood cells and an EDTA sample was obtained for genotyping.

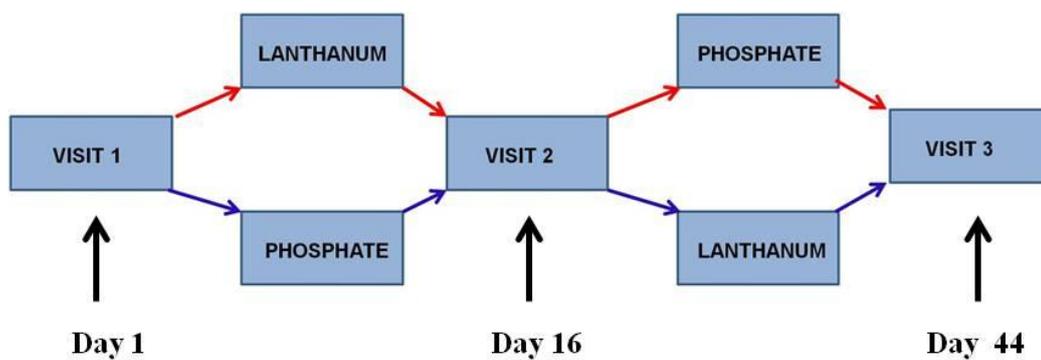


Figure 2-5: Study Protocol.

Volunteers attended for a baseline visit followed by two further visits. Prior to visit two, volunteers took either lanthanum carbonate or phosphate supplements and after a wash out period took the other drug before attending for the final visit. Patients were randomised at visit one to follow arm one (red arrows) or arm two (purple arrows).

VERSION 2

PEPBV STUDY
 Chief Investigator- Prof Alan Jardine Renal Unit Western Infirmary Glasgow
 R & D No. GN10RE236

**3 x 20 EFFERVESCENT TABLETS PHOSPHATE SANDOZ
 OR
 2 x 21 CHEWABLE TABLETS LANTHANUM 1000mg**

STORAGE INSTRUCTIONS: STORE AT ROOM TEMPERATURE BELOW 25°C
 FOR CLINICAL TRIAL USE ONLY
 Batch
 Expires 00/00/00

Patient Name..... Date of Dispensing.....
 Patient No Treatment No

MS/24712 PHARMACY DEPARTMENT WESTERN INFIRMARY GLASGOW POM

KEEP OUT OF THE REACH AND SIGHT OF CHILDREN

MAURICE
13/06/11

VERSION 2

PEPBV STUDY
 20 EFFERVESCENT TABLETS PHOSPHATE SANDOZ
 Chief Investigator- Prof Alan Jardine Renal Unit Western Infirmary Glasgow
 R & D No. GN10RE236

**Take ONE tablet dissolved in water three times a day
 for FOURTEEN DAYS.**

Dissolve or mix with water before taking.
 Take with or just after food, or a meal

THIS IS ONE OF THREE CONTAINERS OF THE SAME PRESCRIPTION.
 PLEASE USE UP THE CONTENTS OF ONE BEFORE STARTING ANOTHER.

STORAGE INSTRUCTIONS: STORE AT ROOM TEMPERATURE BELOW 25°C
 FOR CLINICAL TRIAL USE ONLY
 Batch
 Expires 00/00/00

Patient Name..... Date of Dispensing.....
 Patient No Treatment No

MS/24712 PHARMACY DEPARTMENT WESTERN INFIRMARY GLASGOW POM

KEEP OUT OF THE REACH AND SIGHT OF CHILDREN

MAURICE
13/06/11

Figure 2-6: Labels affixed to the outer bag (top) containing study medication and the inner box (bottom) of the medication.

2.6.2.7 Measures of vascular stiffness - the SphygmoCor® Vx system

This is a non-invasive system which uses the gold-standard carotid-femoral PWV and the AIx. SphygmoCor® Vx is based upon the process of applanation tonometry whereby the artery is compressed against a rigid structure (usually bone) and the tonometer detects alterations in pressure as each pulse wave is generated. The system utilises a Millar tonometer which records and feeds these waves back to an attached computer for analysis. All measurements were undertaken following an ECG, to confirm sinus rhythm, and blood pressure recording. In certain settings, PWV cannot be performed, for example non-sinus rhythm. Measurements were standardised according to recommendations published by Van Bortel et al (69).

Participants were assessed in the morning. There is diurnal variation in arterial vessel diameter and vessels are usually bigger in the evening. Participants were semi-recumbent for at least 10 minutes prior to any measurements. All usual medications were continued and because eating reduces systemic vascular resistance, participants were fasted for 12 hours before measurements. Participants also abstained from caffeine for at least 12 hours beforehand. There were no smokers amongst the study population; smokers should refrain from smoking for a minimum of three hours pre measurement because smoking increases sympathetic tone and arterial stiffness. Finally room temperature was kept at a consistent 21-23°C to standardise peripheral vasoconstriction.

2.6.2.8 Acquisition and analysis of pulse wave velocity

Three ECG electrodes were attached to the participant's chest and the recording inspected to ensure that the R waves were upright and to confirm that the rhythm was sinus. The right carotid and right femoral arteries were identified and the distances between each artery and the suprasternal notch were measured in millimeters. The distal distance is that of the sternum to the femoral artery (via the umbilicus) and the proximal that of the sternum to the carotid. These values are entered into the SphygmoCor® Vx which calculates overall length by subtracting the proximal distance from the distal.

Measurements were performed by one of four supervised, trained research nurses or by the researcher. Pulse wave velocity was recorded in metres/second and an average of three readings was used in analysis. An example carotid-femoral pulse wave velocity recording is shown in Figure 2-7.



Figure 2-7: Example of the SphygmoCor® Vx system PWV recording.

This illustrates, in the top row, the femoral (distal) and on the bottom carotid (proximal) pulse wave velocity traces. The calculated pulse wave velocity, based on these readings, is circled in red.

Recordings were taken at the right carotid and femoral arteries with the patient supine. Three readings, with a standard deviation of less than 10% of the mean, were obtained for each subject. The SphygmoCor® Vx measures the time between ventricular systole (represented by the R wave of the ECG) and the foot of the pressure wave at each site; carotid and femoral. The transit time is the time of travel of the foot of the wave over a pre determined distance. To ascertain the transit time, the time between the R wave and the proximal upstroke (carotid) is subtracted from the time between the R wave and the distal upstroke (femoral).

2.6.2.9 Acquisition and analysis of the augmentation index

With the right wrist in dorsiflexion, the augmentation index was measured at the right radial artery. On acquisition of an acceptable waveform, recordings were undertaken for 15 seconds. For the recording to be accepted, the operator index had to be greater than 80%. The operator index is a composite measure of individual quality indices: pulse height variation <5%, average pulse height >100 units, diastolic variation <5% and shape variation <5%. The SphygmoCor® Vx system then uses Fourier transformation to convert the radial waveform to a central (aortic) waveform. It is from this derived central waveform that the arterial pressure waveform is analysed. The aortic AIx is calculated by dividing the augmentation pressure by the pulse pressure. The AIx is automatically corrected to a heart rate of 75 beats per minute; the calculated aortic augmentation index (AIx@75). This provides a standardized measure for comparison.

Three acceptable recordings were taken for each participant and an average of these was taken. Analysis was performed using the average AIx@75.

2.6.2.10 Acquisition and analysis of brachial artery flow mediated dilatation

Measurements were standardized according to guidelines published by Thijssen et al in *The American Journal of Physiology: Heart and circulatory physiology* (130). Influences on FMD include recent ingestion of caffeine and alcohol, dietary intake, smoking and recent exercise. Participants avoided these for at least 12 hours prior to a visit.

Acute activation of the sympathetic nervous system and temperature can affect FMD thus to minimize these effects, participants lay semi-recumbent for 10 minutes prior to any measurements and the room was quiet and darkened with the temperature kept at a

consistent 21-23°C. All recordings and analyses were performed by the same experienced investigator (the researcher) who was blinded as to which drug (phosphate or lanthanum) the participant was taking. Three ECG electrodes were attached to the participant's chest to allow ECG recording to take place throughout the study. The right arm was extended and supported on a level table (Figure 2-8). Using an 8.0MHz linear array transducer (Acuson 8L5 system, Sequoia 512, Siemens) the brachial artery was identified and scanned longitudinally approximately 10 cm proximal to the antecubital fossa. A blood pressure cuff was placed on the forearm, just below the antecubital fossa and the ultrasound probe was then fixed over the artery using an adjustable steel arm. Baseline measurements of the artery diameter were recorded at the end of diastole (images were triggered on the R wave). Vessel diameter was assessed with ultrasonic calipers measuring the distance between the anterior and posterior interface between the media and adventitia. Within the brachial artery, clear vascular boundaries were identified to allow imaging of the double lines of Pignoli (353) and this in turn allows a more precise diameter measurement (≤ 0.05 mm) to be calculated by automated edge-detection software (Vascular Research Tools package version 5, Medical Imaging Applications LLC, USA) (354). Baseline recording was for two minutes. The blood pressure cuff on the forearm was then inflated for five minutes to 250mmHg. Upon release of the cuff, recording was re-commenced for five minutes; to record reactive hyperaemia. After a 10 minute rest, a 2nd baseline reading was recorded for two minutes. Two puffs of GTN spray were then administered and 60 seconds later a final recording was commenced for five minutes.

Each study was recorded and analysed with automated edge detection software from Medical Imaging Applications LLC. This type of software is known to reduce intra-observer variability, improve reproducibility and the validity of FMD measures (354). FMD was expressed as the percentage change in the arterial diameter pre and post cuff occlusion, relative to the baseline diameter. For endothelium independent measures following GTN, the change in arterial diameter was expressed as the percentage change in the arterial diameter pre and post GTN spray, relative to the 2nd recorded baseline diameter. An example recording is shown in Figure 2-8.

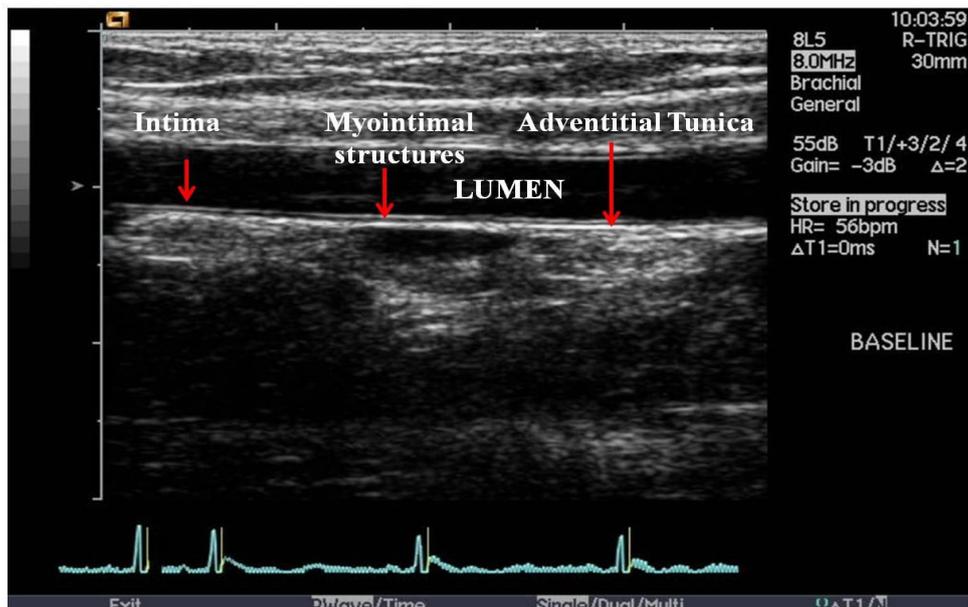
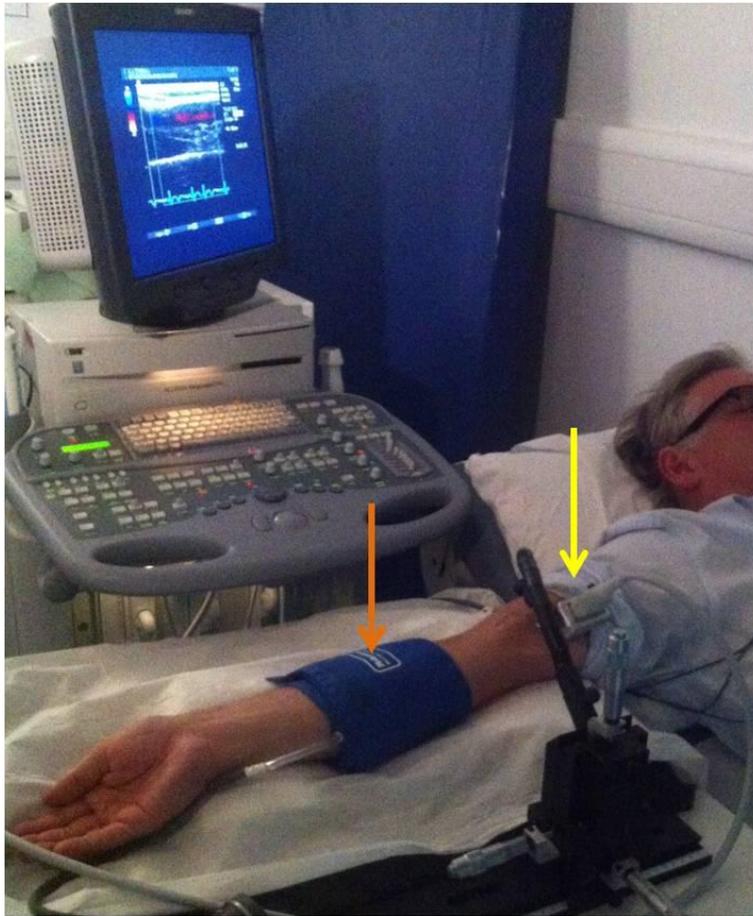


Figure 2-8: FMD set-up with an example recording of the brachial artery (bottom).

The yellow arrow indicates the ultrasound probe and the orange the blood pressure cuff. The brachial artery recording shows the different layers of the vessel wall. The intimal layer is used to calculate the brachial artery diameter.

2.6.2.11 Blood and urine analysis - routine biochemistry and haematology

Samples were sent to biochemistry for analysis of urea and electrolytes, bicarbonate, bone profile, liver function tests, CRP, urate, lipids and PTH (Diasorin Liason). Full blood count was measured by haematology. Immediately prior to each visit, a 24 hour urine collection was collected in a bottle without preservative. Urine was analysed in the routine laboratory for electrolytes using flame photometry.

2.6.2.12 Vitamin D analysis

This was undertaken at Glasgow Royal infirmary by Dr Susan Knox. 25-hydroxyvitamin D2 and D3 concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters UPLC with the ACQUITY TQD. The current assay Coefficient of variation is D3 mean 14nM CV 8.9%, mean 52.7nM CV 7.4%; D2 mean 14nM CV 9.8%, mean 47.7nM CV 8.8%.

2.6.2.13 FGF-23 analysis

After a single thaw, FGF-23 was measured on EDTA plasma samples by ELISA (Immunotopics, Inc. San Clemente, CA). The ELISA was performed in accordance with the manufacturer's instructions. The plate was read on a Wallac Victor2 1420 multilabel counter. A standard curve was generated by plotting absorbance versus FGF-23 concentration for each standard. The concentration of FGF-23 in each sample was subsequently read from this curve. Urinary FGF-23 was measured with the same ELISA kit. 10mls of urine from the 24 hour collections was used and the urine was concentrated using Amicon® ultra centrifugal filters (Merck Millipore, Abingdon, UK). The final quantity of FGF-23 in the urine was adjusted for the 24 hour urine volume and creatinine.

2.6.2.14 Cyclic GMP

Urinary cGMP was measured by ELISA (R&D systems Europe Ltd. Abingdon, UK) on samples from a 24 hour urine collections. Samples were diluted 10 fold using the calibrator dilutant (RD5-5) supplied with the kit. Recommended study procedure was followed using 96 well plates, with results read on the Wallac Victor2 1420 multilabel counter. Sample concentrations were calculated from standard curves. Results were expressed as pM/ml.

**3 Chapter three – FGF-23: a tale of two studies.
Relationship with left ventricular mass in CKD
and phosphate, FGF-23 and cell adhesion
molecule formation *in vitro*.**

3.1 Introduction

In this chapter the association between phosphate, FGF-23, ED and LVH is considered.

The excess CV risk seen in CKD is secondary to a combination of traditional and ‘non-traditional’ CV risk factors (2). The abnormalities arising as a result of disordered bone mineral metabolism in CKD; hyperphosphataemia, hyperparathyroidism and hypovitaminosis D are examples of ‘non-traditional’ CV risk factors and each has been linked independently with increased CV mortality (3-5;163). The mechanisms are poorly described but there is evidence that all can induce VC and ED (6;163).

FGF-23 (sections 1.5.2 and 1.5.3) is now recognised as an essential player in serum phosphate regulation. FGF-23 potently lowers serum phosphate levels by inducing renal phosphate wasting and is a sensitive biomarker of abnormal renal phosphate handling. FGF-23 rises earlier, and to a greater extent, than serum phosphate in progressive CKD (197). Levels are elevated, often more than 1000 fold, in patients with ESRD and in advanced CKD, hyperphosphataemia develops despite increased levels of FGF-23. This reduced responsiveness to FGF-23 may reflect the reduction in the number of intact nephrons, and the resulting reduced expression of Klotho on DCT cells (355).

Elevated FGF-23 is predictive of adverse outcome including premature mortality, CKD progression, vascular dysfunction and LVH (163;236;267;268). It is unclear whether FGF-23 exerts directly toxic effects or whether it is simply a biomarker for these disease states. The cell adhesion molecules, soluble E-selectin and soluble VCAM are markers of ED associated with accelerated CVD in CKD (186;356). CMRI scanning is acknowledged as the gold standard for LVM measurement (357). The relationship between FGF-23 and cell adhesion molecules and FGF-23 and LVM, measured by CMRI has not been studied previously. The aims of this study were, therefore:

- To assess the relationship between FGF-23 and LVH using CMRI in patients with CKD stages 3 and 4.
- To assess the effects of phosphate, FGF-23 and Klotho on the expression of E-selectin and VCAM in HUVECs.

3.2 Materials and Methods

For the clinical study, these are discussed fully in section 2.6.1. Two patient groups were recruited: those with a diagnosis of either diabetic nephropathy or biopsy-proven IgA nephropathy and CKD stages 3–4 and those with essential hypertension in the absence of CKD. LVH was defined as LVMI $> 84.1 \text{ g/m}^2$ for men and $>76.4 \text{ g/m}^2$ for women; LV systolic dysfunction was defined as LV ejection fraction (LVEF) $<55\%$ (351). Cell culture and cell based ELISA are described in section 2.4.2.

3.3 Results

Forty-eight patients with CKD were included in the analysis. 62% (n=36) had a diagnosis of diabetic nephropathy, and 18% had IgA nephropathy. 50% (n=24) had LVH. 27 ‘control’ patients with essential hypertension but without CKD were also included in the analysis; 56% (n=15) of them had LVH. Comparison between the patients with and without CKD is shown in Table 3-1. There was a similar age distribution (60.0 ± 12 vs 55.5 ± 9.5 years) and a similar proportion of males (75% vs 74%). LVMI was also similar between the two groups ($81.2 (70.2-97.6)$ vs $86 (71.6-99.6) \text{ g/m}^2$). Patients with CKD had significantly higher phosphate, PTH and FGF-23 concentration ($237.9 (109.6-393.3)$ vs $12.5 (1.5-35.9) \text{ RU/ml}$; $p < 0.001$).

Figure 3-1 shows stratification of patients by median FGF-23 concentration into four groups according to the presence or absence of LVH and CKD. Those with both CKD and LVH had the highest FGF-23 concentration followed by those with CKD without LVH ($283.4 (166.4-414.8)$ vs $118.4 (61.5-295.0) \text{ RU/ml}$; $p < 0.001$). A scatter plot of FGF-23 concentration and LVMI in patients with CKD is shown in Figure 3-2 demonstrating significant positive correlation between the two ($r^2=0.064$; $p=0.005$). Figure 3-3 shows the negative correlation with FGF-23 concentration and renal function ($r^2=0.072$; $p=0.004$). There was also a positive correlation with phosphate ($r^2=0.073$, $p=0.006$) (data not shown).

	CKD (n=48)	EH (n=27)	p value
Age (years)	60±12	55.5±9.5	0.072
Male sex	75% (n=36)	74% (n=20)	NS
DM nephropathy	62% (n=30)	NA	
eGFR (ml/min/1.73m ²)	30.2±11	94.4±11.1	<0.001
LVM index	81.2 (70.2-97.6)	86 (71.6-99.6)	NS
Systolic BP (mmHg)	148.8±22.9	152.3±19.9	NS
Diastolic BP (mmHg)	81.8±12	94.4±11.1	<0.001
Phosphate (mM)	1.2±0.2	0.99±0.22	<0.001
Vitamin D (ng/ml)	13(5-20)	20.5 (14.0-26.8)	0.004
FGF-23 (RU/ml)	237.9 (109.6-393.3)	12.5 (1.5-35.9)	<0.001
PTH (pg/ml)	14.5 (6.7-21.6)	5.7 (4.8-7.3)	<0.001
Adjusted calcium (mM)	2.38±0.1	2.4±0.1	NS
UPCR (mg/mM)	82.5 (19.8-218)	NA	

Table 3-1: Comparison of the baseline characteristics between the patients with CKD and control patients with essential hypertension (EH).

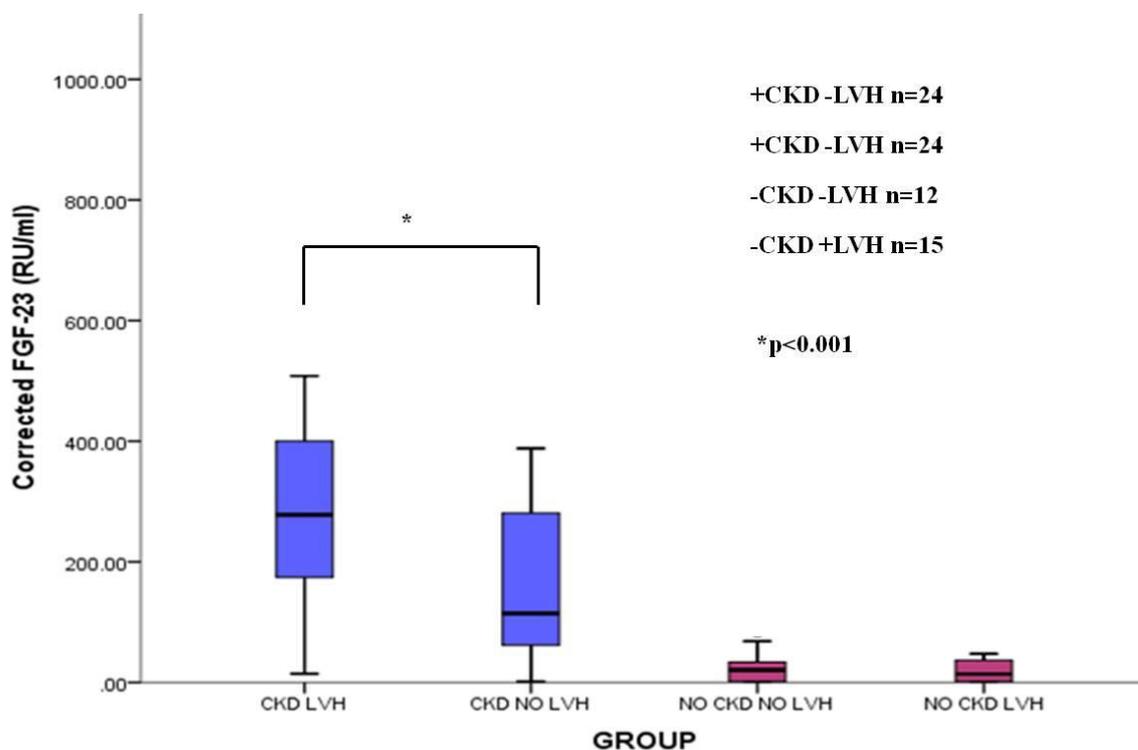


Figure 3-1: Median FGF-23 concentration in the presence and absence of CKD and LVH.

Patients were stratified according to the presence or absence of CKD and then further stratified according to the presence or absence of LVH (defined as $LVMl > 84.1 \text{g/m}^2$ for men and 76.4g/m^2 for women), measured by cMRI using a 1.5-Tesla Siemens (Erlangen) scanner. FGF-23 concentration was measured on EDTA plasma samples, after a single thaw, by ELISA (Immunotopics, Inc. San Clemente, CA). Patients with CKD had higher concentrations of FGF-23 than those without CKD and if LVH was also present, these patients had the highest levels of FGF-23. Values represent median and IQR.

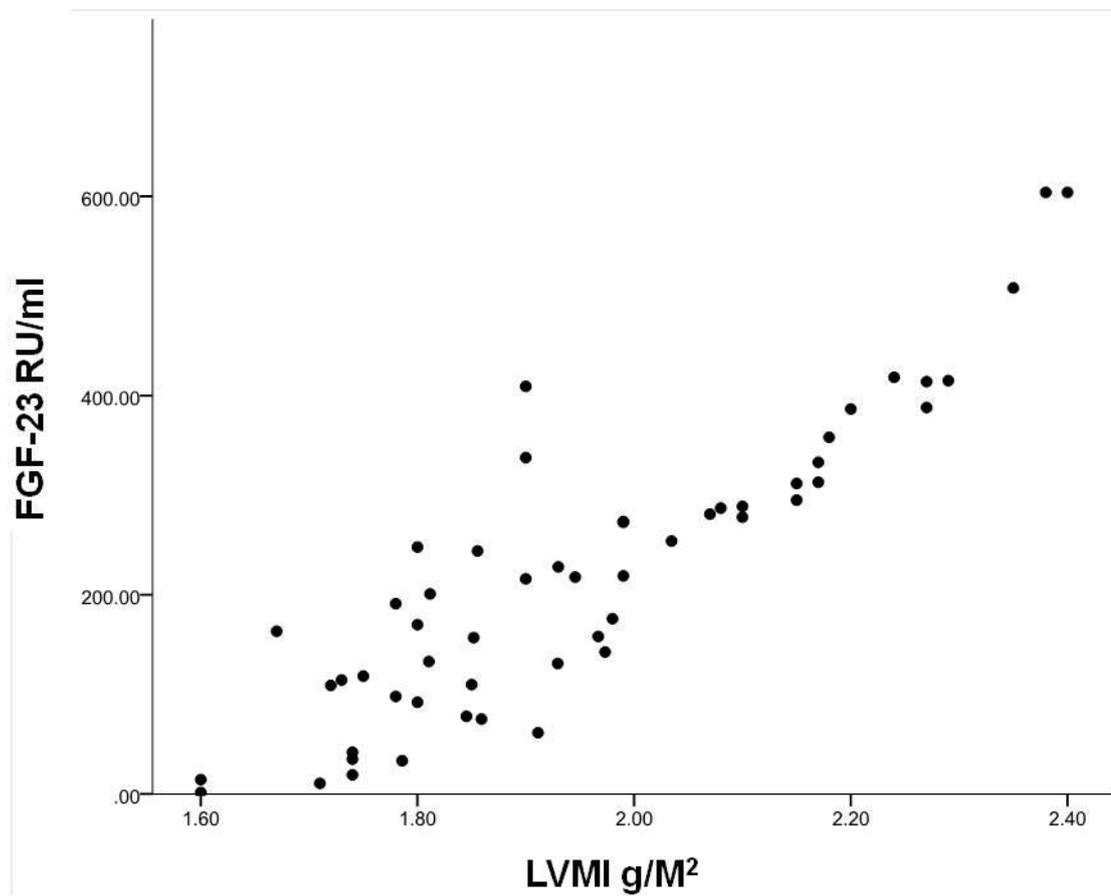
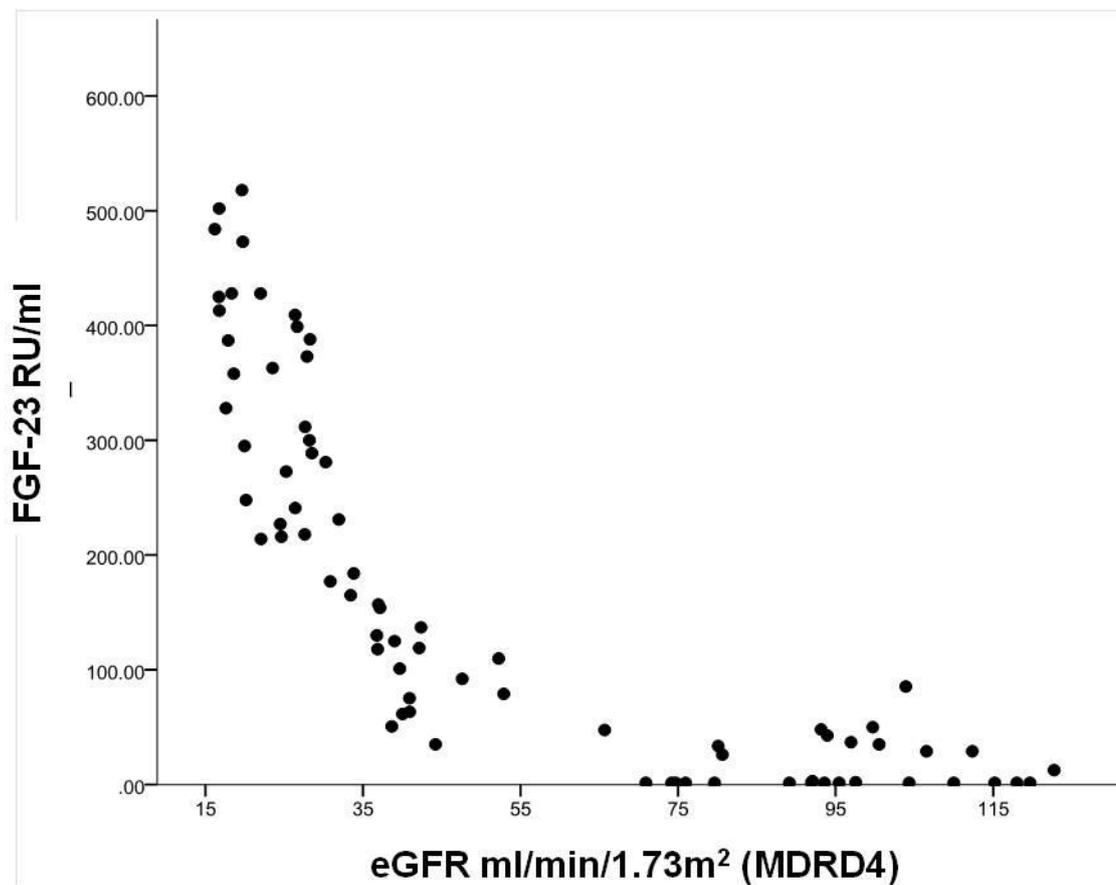


Figure 3-2: Scatter plot showing the relationship between FGF-23 concentration and LVMI in patients with CKD.



Further comparison between the patients with CKD +/- LVH is shown in Table 3-2. eGFR was significantly lower in those with LVH (27.5 ± 11.7 vs 34.3 ± 11.3 mls/min/ 1.73m^2 ; $p=0.044$), systolic blood pressure and UPCR were significantly higher (158.8 ± 19.7 vs 139.2 ± 22.2 mmHg; $p=0.002$ and 82.5 (19.8-218) vs 67.4 (12.5-196) mg/ml; $p=0.004$). Multivariate analysis including creatinine, FGF-23, systolic blood pressure and UPCR revealed all, except creatinine, to be independent predictors of LVH (Table 3-3). The r^2 value was 0.56. Creatinine was used rather than eGFR because LVH takes into account body mass. I tried the model with eGFR and this was not a significant determinant of LVH.

In the second part of our study we looked at the effects of altered phosphate concentration in the presence and absence of FGF-23, Klotho and IL1 β on the production of E-selectin and VCAM. Compared with HUVECs stimulated only with IL1 β , E-selectin production is significantly elevated in HUVECs cultured in high phosphate media (3mM) in the presence of FGF-23 and IL1 β or Klotho and IL1 β ($p=0.005$ and 0.04 respectively). This effect is attenuated in HUVECs exposed to both FGF-23 and Klotho with IL1 β (Figure 3-4). Production of VCAM was also increased in HUVECs cultured in high phosphate medium (3mM) in the presence of FGF-23 and IL1 β or Klotho and IL1 β ($p=0.008$ and 0.02). Again the effect was attenuated in HUVECs exposed to both FGF-23 and Klotho with IL1 β (Figure 3-5). There was no significant difference seen between HUVECs which were not stimulated with IL1 β (data not shown).

3.4 Discussion

In this study, we look at the associations of FGF-23 and vascular structure in CKD, with additional *in vitro* studies on the underlying mechanism. It is the first study to assess the relationship between FGF-23 and LVH measured with cardiac MRI. We confirm the findings of other studies which have used less sensitive measures of LVH; FGF-23 level is an independent predictor of LVH in patients with CKD 3 and 4 secondary to diabetic or IgA nephropathy.

Patients without CKD had much lower levels of FGF-23 regardless of the presence or absence of LVH (13.8 (1.5-38) vs 20.7 (1.5-42.2) RU/ml; $p=0.43$). Left ventricular wall thickness increases as the heart adapts to chronic pressure overload with resultant LVH (358). LVH is found in up to 25% of essential hypertensives without end

	LVH (n=24)	No LVH (n=24)	p value
Age (years)	60.4±11.6	58.8±12.8	NS
Male sex	75% (n=18)	71% (n=17)	NS
DM nephropathy	67% (n=16)	58% (n=14)	NS
eGFR (ml/min/1.73m ²)	27.5±11.7	34.3±11.3	0.044
LVM index	87.9 (84.3-95.1)	79.6(73.2-83.8)	0.045
Systolic BP (mmHg)	158.8±19.7	139.2±22.2	0.002
Phosphate (mM)	1.2±0.19	1.16±0.22	NS
Vitamin D (ng/ml)	13 (5-20)	14 (4-22)	NS
FGF-23 (RU/ml)	283.4 (166.4-414.8)	118.4(61.5-295)	0.008
PTH (pg/ml)	14.5 (6.7-21.6)	13.1 (5.9-19.6)	NS
Adjusted calcium (mM)	2.34±0.06	2.4±0.07	NS
UPCR (mg/mM)	82.5 (19.8-218.0)	67.4(12.5-196)	0.004

Table 3-2: Comparison between patients with CKD with and without LVH.

	Exp β	Confidence Interval		p value
		Lower	Upper	
Systolic BP (mmHg)	1.08	1.03	1.14	0.003
FGF-23 (RU/ml)	4.9	1.2	20.3	0.027
UPCR (mg/mM)	1.87	1.54	2.23	0.005

Table 3-3: Multivariate analysis showing significant predictors of LVH in CKD.

*R*² value for the model was 0.56.

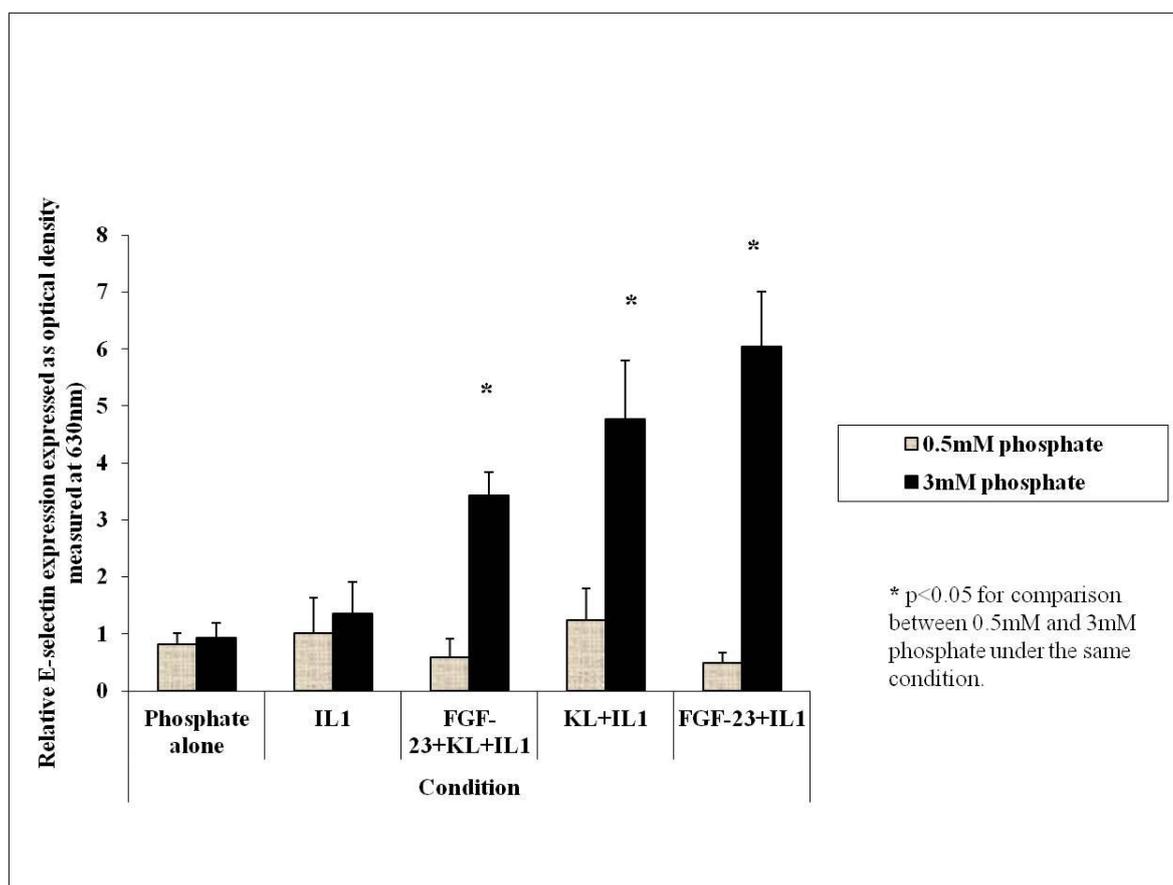


Figure 3-4: E-selectin production in HUVECs cultured in standard (0.5mM) and high (3mM) phosphate concentration media.

HUVECs cultured in either 0.5mM or 3mM phosphate concentration media were seeded onto 96 well plates at a density of 1.8×10^3 cells per well and left for 24 hours before being treated with FGF-23 (final concentration $3.5 \times 10^{-7} M$) and Klotho (KL; final concentration $2 \times 10^{-10} M$) either alone or in combination; controls were untreated. After a four hour incubation, some wells were also treated with IL1 β (IL1), and all plates left for a further six hours. The wells were washed with PBS and then the cells were fixed with 4% (w/v) paraformaldehyde. Wells were washed with PBS and 0.1% (w/v) BSA and then probed for E-selectin, mouse anti-human antibody (1/2000 dilution). After a one hour incubation, the wells were washed and HRP coupled rabbit anti-mouse antibody added. This was washed off and developer added. The wells were read at 630nm absorbance on a microplate reader. The figure shows the results of 3 separate experiments, with each condition represented in triplicate in each experiment. Values are mean \pm SEM.

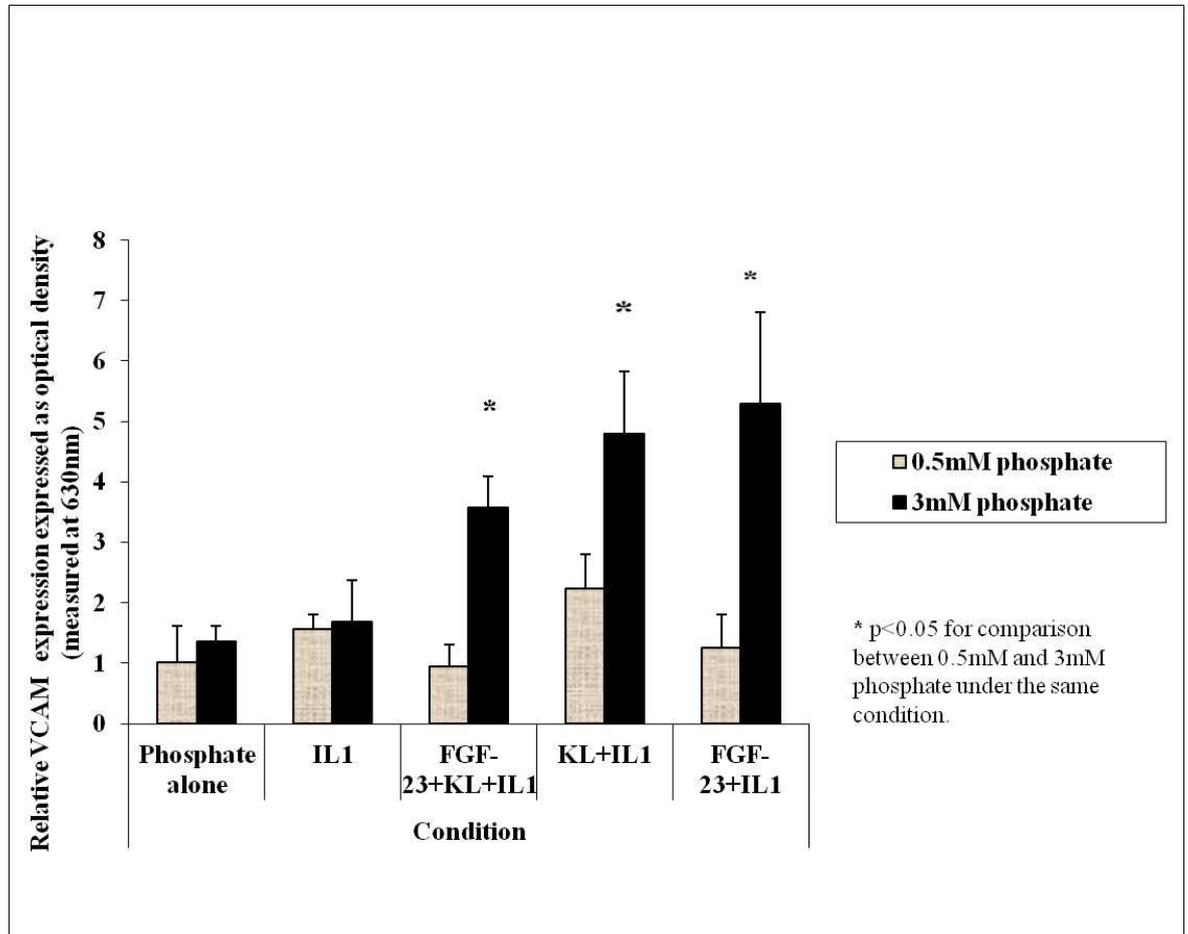


Figure 3-5: VCAM production in HUVECs cultured in standard (0.5mM) and high (3mM) phosphate concentration media.

HUVECs cultured in either 0.5mM or 3mM phosphate concentration media were seeded onto 96 well plates at a density of 1.8×10^3 cells per well and left for 24 hours before being treated with FGF-23 (final concentration $3.5 \times 10^{-7} M$) and Klotho (KL; final concentration $2 \times 10^{-10} M$) either alone or in combination; controls were untreated. After a four hour incubation, some wells were also treated with IL1 β (IL1), and all plates left for a further six hours. The wells were washed with PBS and then the cells were fixed with 4% (w/v) paraformaldehyde. Wells were washed with PBS and 0.1% (w/v) BSA and then probed for VCAM, sheep anti-human antibody (1/2000 dilution). After a one hour incubation, the wells were washed and HRP coupled rabbit anti-goat antibody added. This was washed off and developer added. The wells were read at 630nm absorbance on a microplate reader. The figure shows the results of 3 separate experiments, with each condition represented in triplicate in each experiment. Values are mean \pm SEM.

organ damage elsewhere, and up to 60% in those with end-organ damage in other territories (359). The low levels of FGF-23 seen in our essential hypertension group with LVH suggest that FGF-23 is neither a biomarker for LVH nor does it contribute significantly to the development of LVH in patients without CKD.

LVH is present in up to 75% of patients starting dialysis. Bregman et al studied factors affecting LVH in patients with CKD 3 and 4. They demonstrated that it affects approximately 50% of patients with this level of CKD and is more common with more advanced CKD (360). Both systolic and diastolic blood pressure are described in the literature as independent predictors of LVH in patients with and without CKD (361;362). There is an increased prevalence of LVH in patients with CKD and it may develop after shorter exposure to hypertension and possibly at lower values. In animal models, LVH develops in CKD even without significant hypertension (363). This supports the argument that additional factors contribute to the development of LVH. There are many other factors that may be associated with LVH in CKD including age, anaemia, proteinuria and FGF-23 level (163;349;359;364). In our studies of patients with stage 5 CKD, blood pressure is the major determinant of LV mass. However calcium-phosphate product is an additional contributor to LVH in keeping with the hypothesis that CKD mineral bone disease dysregulation is implicated in the pathogenesis of LVH in CKD (350). LVH is a major predictor of cardiovascular mortality in advanced CKD and potentially a risk factor for progression to dialysis (365). Regression of LVH therefore represents a possible strategy for improving cardiovascular outcomes in CKD patients.

In the current study, I show that FGF-23 level is an independent predictor of LVH in CKD and show the levels to be significantly higher than in those with CKD who do not have LVH. Since patients with CKD develop LVH at lower blood pressure levels than patients without CKD, some other factor present in CKD is likely to be involved. My data support the notion that FGF-23 may be responsible.

To support a pathophysiological role for FGF-23, it is necessary to show that FGF-23 either has direct effects on the growth or matrix production of cardiac myocytes (or other vascular cells) or that it has the potential to increase blood pressure. I examined the effect of FGF-23 on endothelial cells and show that in the hyperphosphataemic environment, FGF-23 stimulates the production of the cell adhesion molecules, E-selectin and VCAM. Higher levels of E-selectin and VCAM, indicate activation of the vascular endothelium and are present in patients with essential hypertension, who have ED (186). In the presence of

Klotho, this effect is attenuated consistent with the established role of Klotho as an anti-ageing protein (366). Thus, FGF-23 may cause activation and dysfunction of the vascular endothelium, and contribute to hypertension and LVH in patients with CKD. This is a small pilot study and my cell based ELISA findings require confirmation with Western blot. Additionally, gene expression of E-selectin and VCAM requires evaluation in cells treated with phosphate, FGF-23 and Klotho.

It remains to be shown whether there are similar direct effects on cardiac myocytes, but our findings support the hypothesis that FGF-23 is more than a biomarker of abnormal renal phosphate handling and may be a therapeutic target as phosphate binding medication reduces FGF-23 as well as phosphate levels (367).

3.5 Conclusion

In this chapter, the results of a small, single-centre, pilot study are presented showing that FGF-23 is associated with the development of LVH in CKD, as measured by the ‘gold standard’ method of CMRI. Preliminary evidence of a direct effect of FGF-23, in a high phosphate environment, *in vitro* is also provided: this may contribute to observed ED and LVH in CKD. I do not prove causality with elevated FGF-23 and LVH. FGF-23 might be a realistic therapeutic target and lowering levels of FGF-23 might offer benefit in terms of improved clinical outcomes but until we understand the mechanism of disordered bone mineral metabolism and ED and LVH we cannot progress. Further work is necessary to ascertain the specific mechanisms of ED in the high phosphate environment and in chapters four, five and six, I explore this *in vitro* with cell lines and resistance vessels.

4 Chapter Four – Effects of phosphate on the function of rat resistance vessels and on rat vascular smooth muscle cells

4.1 Introduction

In work leading up to this thesis, I demonstrated an association between mortality and phosphate in renal transplant recipients (section 1.10); a similar association has been shown in other populations. In chapter three, I demonstrated an association between FGF-23 and LVH (a marker of increased CV risk) and showed that in HUVECs, stimulated with FGF-23, cultured in a high phosphate environment, production of the cell adhesion molecules, E-selectin and VCAM is significantly increased. These cell adhesion molecules are markers of ED and suggest that in combination with a hyperphosphataemic environment, FGF-23 has a definite role to play in the development of ED, and thus CV risk and CVD.

ED occurs in CKD although independent effects on its relationship to the pathogenesis of renal disease are unclear and the mechanism is poorly understood. However, evidence suggests a role for the NO pathway, both in laboratory and clinical studies of CKD (6;123;124). Phosphate also has a detrimental effect on endothelial function both *in vitro* and *in vivo* in CKD (6;249;250;368). The effects of phosphate are not limited to CKD but also occur in the healthy population, supporting the concept that phosphate may have detrimental effects on CV risk even in the face of a normal serum phosphate level (6).

ED and elevated serum phosphate level are both risk factors for CVD in CKD and in the general population but their relationship to each other has not been explored in detail. It is likely that CKD causes an environment which initiates endothelial damage and dysfunction, leading to a self-perpetuating cycle, oxidative stress, an imbalance in redox and sustained ED. Whether the ED seen in CKD is reversible is not known; or whether there may be reversible phosphate-dependent effects on ED and the associated CV risk.

In vivo, resistance vessels regulate blood flow to the tissues: experimental data has shown that the function of resistance blood vessels is impaired in CKD with ED and impaired relaxation (95). However, no studies in human vessels have separated the effects of phosphate exposure and other abnormalities of the uraemic environment (eg hyperkalaemia or acidosis). In a rat model of CKD, where impaired vasodilation of aortic rings was seen in animals fed a high phosphate diet; this was ameliorated in animals fed a low phosphate diet (253). Rat aortic rings incubated in high phosphate for one hour exhibit evidence of ED (6). In humans, the effect of phosphate on endothelial function and CV risk is likely to relate to long term exposure and thus effects of short term exposure to a high phosphate

environment may have limited relevance. However, there is a paucity of studies examining either the direct effects of phosphate, separately from the other abnormalities seen in the uraemic environment, or the effects of longer exposure to a high phosphate environment in humans or experimental animals.

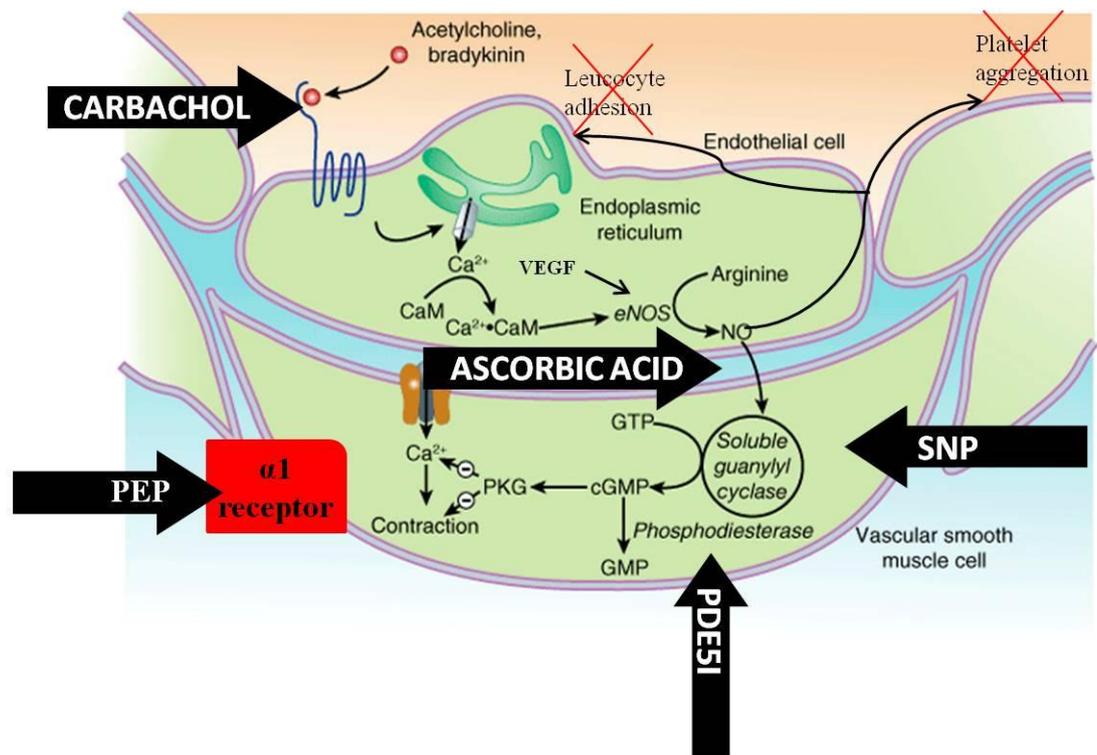
The aim of the present series of studies was to assess the effects of exposure to high phosphate alone on the function of resistance vessels from rats without renal disease by examining the:

- Contractile response to PEP
- Vasodilator response to carbachol: endothelium dependent vasodilatation
- Vasodilator response to SNP: endothelium independent vasodilatation
- Levels of basal NO produced using the NOS inhibitor, L-NAME
- Contractile response to PEP and vasodilator responses to carbachol and SNP in the presence of ascorbic acid, an anti-oxidant.
- Contractile response to PEP and vasodilator responses to carbachol and SNP in the presence of a PDE5I (zaprinast).
- Levels of cGMP present in the vessels, incubated in normal and high phosphate concentration PSS

Figure 4-1 illustrates where each of these drugs affects the NO pathway.

Two further aims addressed using rat VSMCs cultured in normal and high phosphate concentration were:

- To measure expression of eNOS and PKG.
- To measure NO production by the Griess reaction
- To measure VEGF production.



Drug	Final concentration (M)	Incubation period (minutes)
Phenylephrine	1×10^{-8} to 3×10^{-5}	n/a
Carbachol	1×10^{-8} to 3×10^{-5}	n/a
Sodium nitroprusside	1×10^{-8} to 3×10^{-5}	n/a
L-NAME	1×10^{-4}	30
PDE5I	1×10^{-6}	60
Ascorbic acid	1×10^{-5}	60

Figure 4-1: The site of action of the study drugs on the NO pathway.

Carbachol is an acetylcholine mimetic and thus causes relaxation by direct stimulation of NO production. L-NAME is a NOS inhibitor and so blocks production of NO allowing a measure of basal NO to be made. SNP is a NO donor and so causes endothelium independent vasodilatation by blocking the need for an intact endothelium to produce NO. PDE5I is a phosphodiesterase 5 inhibitor (zaprinast) which prevents break down of cGMP and thus enhances relaxation. PEP acts on $\alpha 1$ adrenoceptors on VSMCs to increase calcium and thus stimulate contraction. Adapted from Katzung et al (122). The table illustrates the concentration range for each drug, which the vessels were exposed to in the myography chamber.

4.2 Materials and Methods

Rats were sacrificed and vessels were then dissected and incubated for 16 hours in either normal (1.18mM) or high phosphate concentration (2.5mM) PSS and then mounted on a four chamber wire myograph as described in section 2.3. Following normalisation, the vessels were subjected to one of four protocols:

- Contraction with PEP followed by vasodilatation with increasing concentrations of carbachol; pre and post incubation with L-NAME for 30 minutes.
- Contraction with PEP followed by vasodilatation relaxation with increasing concentrations of SNP.
- Contraction with PEP followed by vasodilatation with carbachol, followed by a 30 minute wash out period to allow the re-establishment of a baseline and then repeat contraction with PEP followed by vasodilatation with SNP in the presence of PDE5I (incubation for one hour)
- Contraction with PEP followed by vasodilatation with carbachol followed by a 30 minute wash out period to allow the re-establishment of a baseline and repeat contraction followed by vasodilatation with SNP in the presence of ascorbic acid (incubation for one hour).

Increasing concentrations of PEP, carbachol and SNP were added in a step wise fashion to induce contraction or vasodilatation and to allow construction of cumulative concentration-response curves. A fixed concentration of L-NAME, ascorbic acid or PDE5I was added. PEP was used to elicit contraction in all protocols. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. For vasodilatation with carbachol and SNP, vessels were exposed to each concentration for two minutes or until a plateau had been reached. If a complete concentration-response curve was not possible, the vessel was discarded. The concentration range used for each drug is illustrated in the table incorporated into Figure 4-1. Blood samples were also taken from rats at the time of sacrifice and stored at -80°C. Renal function, calcium and phosphate level was measured in the routine biochemistry laboratory.

4.2.1 Cyclic GMP measurement, VEGF ELISA and Griess Reaction

Some vessels were snap frozen after 24 hours of incubation and then defrosted for cGMP using an ELISA kit as described in sections 2.3.8 and 2.3.9. VEGF ELISA is described in section 2.4.9 and the Griess reaction in section 2.4.5.

4.2.2 Immunoblotting; eNOS and PKG

Rat VSMCs were cultured from the outset in either standard (0.5mM) or high (3mM) phosphate concentration as described in section 2.4. This was intended to mimic chronic exposure to a high phosphate environment. Immunoblotting was performed as described in section 2.4.8 using antibodies for total and phospho eNOS (Ser 1177) and PKG. GAPDH was used as the housekeeping protein. Additionally, cells cultured in normal phosphate medium were exposed to high phosphate medium for 48 hours and cells cultured in high phosphate medium were exposed to normal phosphate medium. This was designed to test whether effects of high phosphate exposure might be reversible. The experiments were repeated a minimum of three times.

4.3 Results

4.3.1 Sample size and baseline demographics

The number of vessels studied for each drug and the corresponding size of the vessels (L_{100}) is detailed in Table 4-1. Blood samples from six rats were analysed in the biochemistry laboratory for creatinine, phosphate and calcium. Phosphate and calcium values were at the high end of the normal range for human samples (1.39 ± 0.22 mM and 2.56 ± 0.12 mM) and creatinine measures were low as would be expected in a small animal with normal renal function; 41.8 ± 1.5 μ M.

4.3.2 Contraction with PEP in the presence and absence of LNAME

Contraction was not significantly different between the vessels incubated in normal or high phosphate concentration PSS (Figure 4-2).

	Incubated in standard PSS	Incubated in high phosphate PSS	p value
Phenylephrine			
Number	12	10	
L100	366.5 (292.7-422.3)	401.6 (226.3-525.4)	NS
Carbachol			
Number	12	10	
L100	366.5 (292.7-422.3)	401.6 (226.3-525.4)	NS
L-Name			
Number	12	10	
L100	366.5 (292.7-422.3)	401.6 (226.3-525.4)	NS
SNP			
Number	11	9	
L100	391.9 (311.2-504.3)	(396.9 (267.5-525.3)	NS
Carbachol and ascorbic acid			
Number	10	8	
L100	363.2 (301-425.6)	(372.8 (298.4-408.3)	NS
SNP and ascorbic acid			
Number	10	9	
L100	363.2 (301-425.6)	(371.3 (296.5-408.3)	NS
Carbachol and PDE5I			
Number	10	13	
L100	387.2 (324.2-412.1)	(384.1 (341-411.6)	NS
SNP and PDE5I			
Number	10	13	
L100	387.2 (324.2-412.1)	(384.1 (341-411.6)	NS

Table 4-1: Comparison of numbers of vessels studied for each drug and the corresponding size of the vessels.

Number is the number of vessels for which a complete concentration response curve was obtained.

L₁₀₀ is the normalised internal diameter of the vessels with comparison by Mann-Whitney U test.

The potency of PEP was similar in both groups with no significant difference in the EC_{50} between vessels incubated in normal compared with high phosphate concentration PSS ($3.6 \pm 1.9 \times 10^{-6}$ M and $5.3 \pm 2.8 \times 10^{-6}$ M). With the addition of L-NAME, in the vessels incubated in normal phosphate PSS, the PEP concentration-response curve shifted to the left with a significant difference in the AUC. This difference was not seen in the vessels incubated in high phosphate PSS; L-NAME produced no discernible difference in contraction to PEP in these vessels. These results are illustrated in Figures 4-3 and 4-4.

4.3.3 Endothelium dependent vasodilatation – carbachol

The results for the endothelium dependent vasodilator, carbachol are shown in Figure 4-5. Following constriction with PEP, the vessels incubated in high phosphate PSS vasodilated significantly less well to carbachol than those incubated in normal phosphate PSS. Maximum vasodilatation obtained in the vessels incubated in high phosphate was $64\% \pm 9\%$ compared with $95\% \pm 1\%$ ($p < 0.001$) in the vessels incubated in normal phosphate.

4.3.4 Endothelium independent vasodilatation – SNP

The results for the NO donor and endothelium independent vasodilator, SNP are shown in Figure 4-6. Following constriction with PEP, the vessels incubated in high phosphate PSS vasodilated significantly less well to SNP than those incubated in normal phosphate PSS. Maximum vasodilatation obtained in the vessels incubated in high phosphate was $45 \pm 22\%$ compared with $73\% \pm 26$ ($p = 0.02$) in the vessels incubated in normal phosphate PSS.

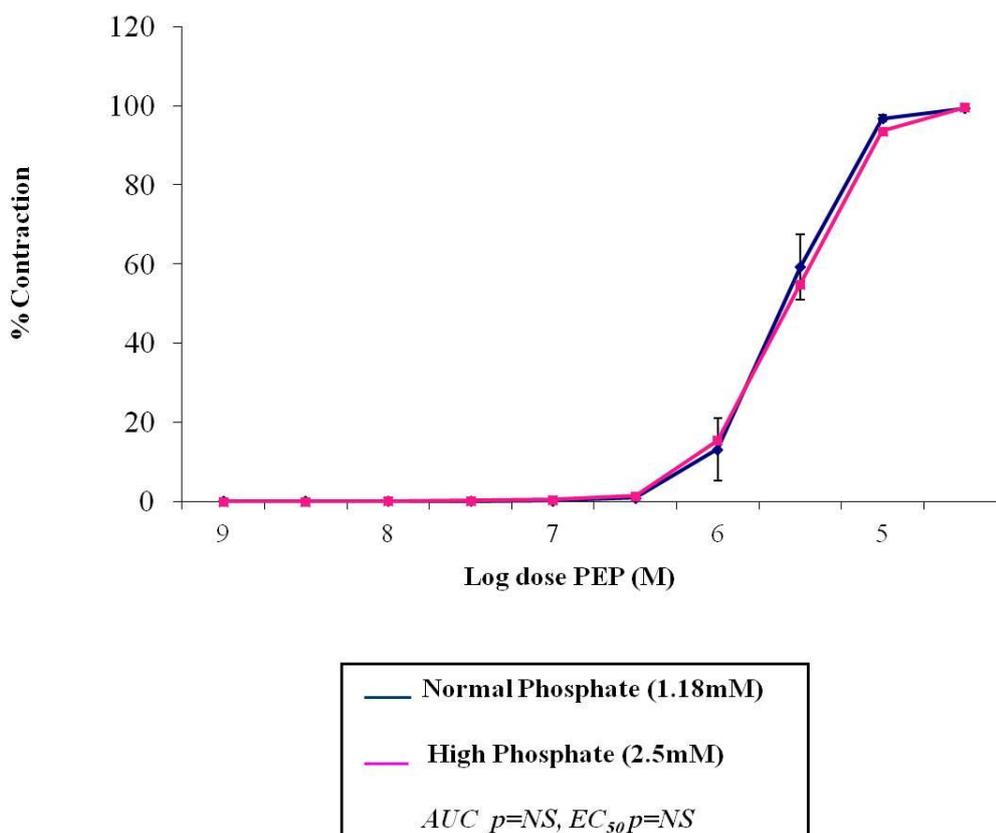


Figure 4-2: Contraction to PEP in vessels incubated for 16 hours in normal or high phosphate PSS.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=12; high phosphate, n=10) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1×10^{-8} to 1×10^{-5} M) to induce contraction and to allow construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Response expressed as mean \pm SEM and comparison made between AUC and the EC_{50} of both groups using the Student's T test.

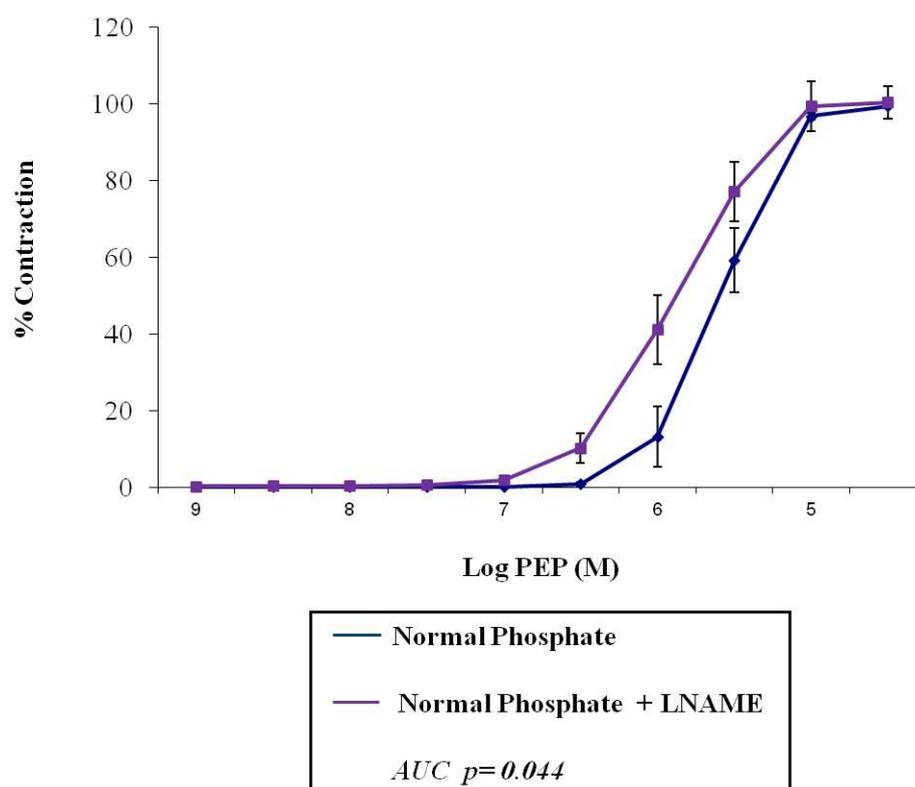


Figure 4-3: Contraction to PEP in the presence and absence of L-NAME in vessels incubated for 16 hours in normal phosphate PSS.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) phosphate concentration PSS for 16 hours. Vessels (n=12) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1×10^{-8} to 1×10^{-5} M) to induce contraction and to allow construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Following a wash out, the vessels were left for 30 minutes before L-NAME was added (1×10^{-4} M) and the vessels were then left for a further 30 minutes before repeating the addition of PEP at increasing concentrations. L-NAME is a NOS inhibitor and thus the concentration-response curve for PEP should shift significantly to the left because basal NO production is reduced in the presence of L-NAME. Response expressed as mean \pm SEM and comparison made between AUC of both groups with the Student's T test.

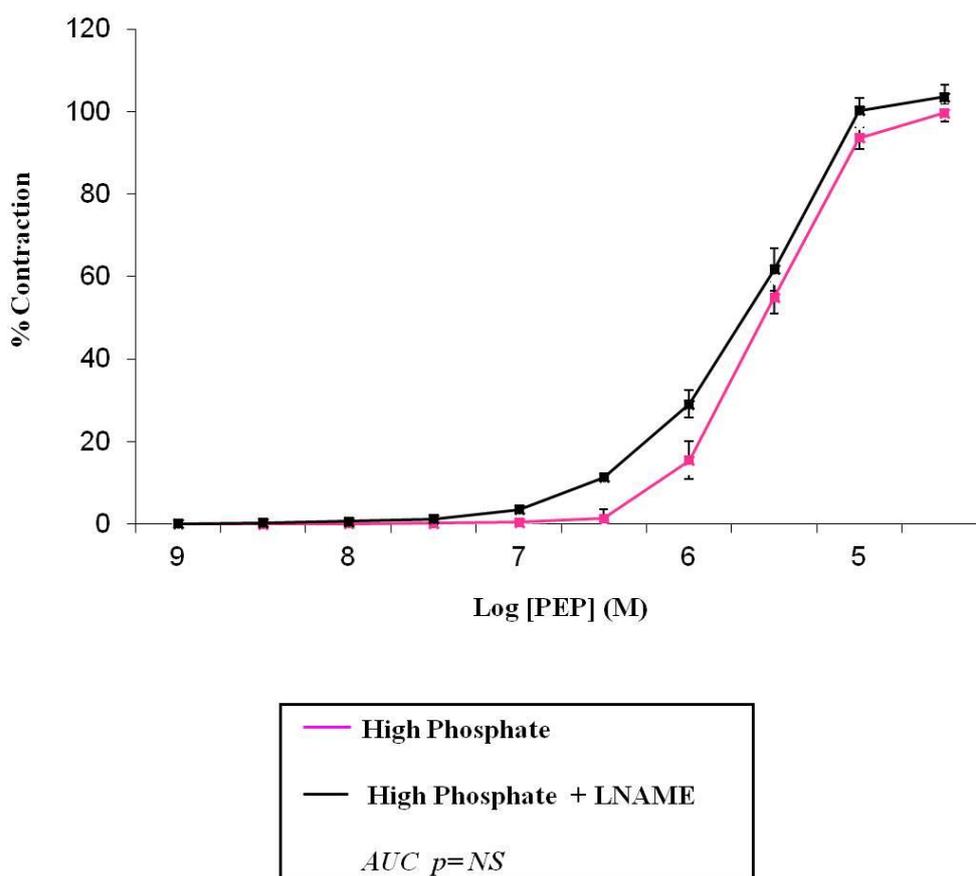


Figure 4-4: Contraction to PEP in the presence and absence of L-NAME in vessels incubated for 16 hours in high phosphate PSS.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in high (2.5mM) phosphate concentration PSS for 16 hours. Vessels ($n=10$) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1×10^{-8} to $1 \times 10^{-5}M$) to induce contraction and to allow construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Following a wash out, the vessels were left for 30 minutes before L-NAME was added ($1 \times 10^{-4}M$) and the vessels were then left for a further 30 minutes before repeating the addition of PEP at increasing concentrations. L-NAME is a NOS inhibitor and thus the concentration-response curve for PEP should shift significantly to the left because basal NO production is reduced in the presence of L-NAME. The concentration-response curve for PEP does not shift significantly to the left suggesting lower basal NO production. Response expressed as mean \pm SEM and comparison made between AUC of both groups with the Student's T test.

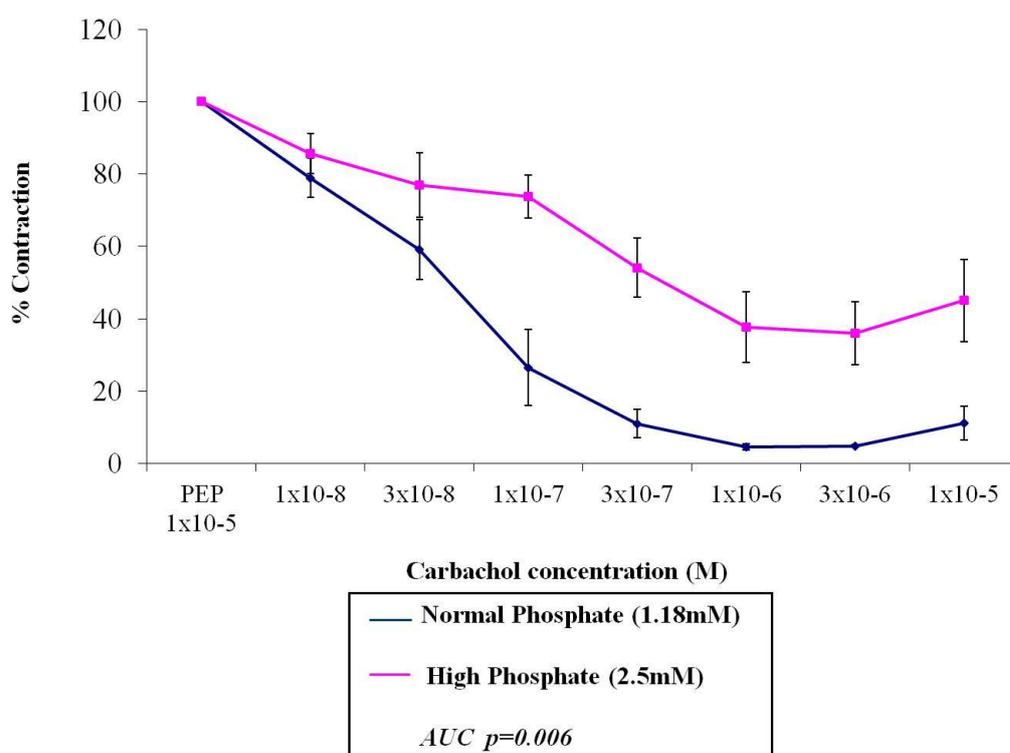


Figure 4-5: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=12; high phosphate, n=10) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁸ to 1x10⁻⁵M) to induce contraction. Carbachol was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups using the Student's T test.

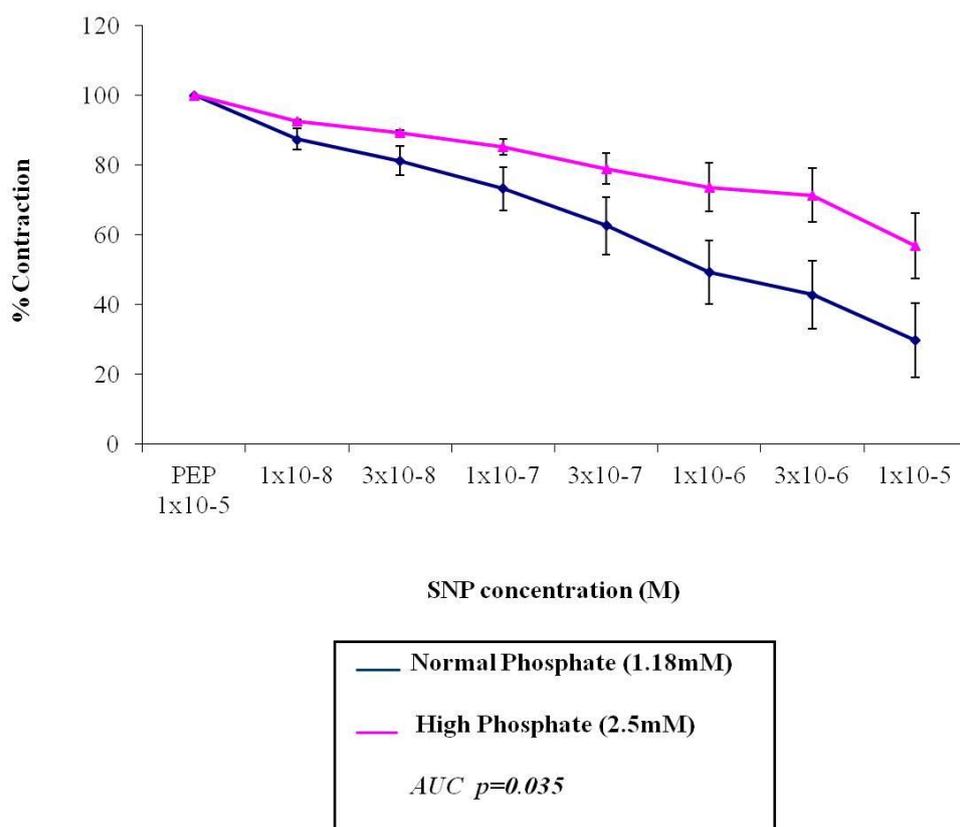


Figure 4-6: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=11; high phosphate, n=9) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁸ to 1x10⁻⁵M) to induce contraction. SNP was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and SNP for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

4.3.5 Effect of incubation with ascorbic acid.

The results of incubation with the anti-oxidant, ascorbic acid for one hour prior to contraction with PEP and then vasodilatation with carbachol and SNP are shown in Figures 4-7 to 4-9. Contraction with PEP remained similar between the vessel groups (Figure 4-7).

Endothelium dependent vasodilatation with carbachol, in the presence of ascorbic acid, is illustrated in Figure 4-8. In the vessels incubated in high phosphate PSS, vasodilatation with carbachol initially appeared to improve with the addition of ascorbic acid but this improvement was not sustained across the dose range and the maximum vasodilatation was $70\pm 3\%$ compared with $97\pm 5\%$ in the vessels incubated in normal phosphate PSS ($p=0.007$).

There was no significant difference in vasodilatation to carbachol between the normal phosphate concentration vessels incubated with and without ascorbic acid (maximum vasodilatation $97\pm 5\%$ vs $96\pm 4\%$). The same was true of the vessels incubated in high phosphate PSS with and without ascorbic acid (maximum vasodilatation $70\pm 3\%$ vs $74\pm 8\%$) (Figure 4-9).

The addition of ascorbic acid, to the vessels incubated in high phosphate PSS, improved endothelium independent maximum vasodilatation with SNP; maximum vasodilatation was $63.1\pm 5.9\%$ in the presence of ascorbic acid compared with $56.1\pm 12.2\%$ when no ascorbic acid was present ($p=0.011$). There was no significant difference in AUC comparing the vessels incubated in high phosphate PSS in the presence and absence of ascorbic acid (data not shown).

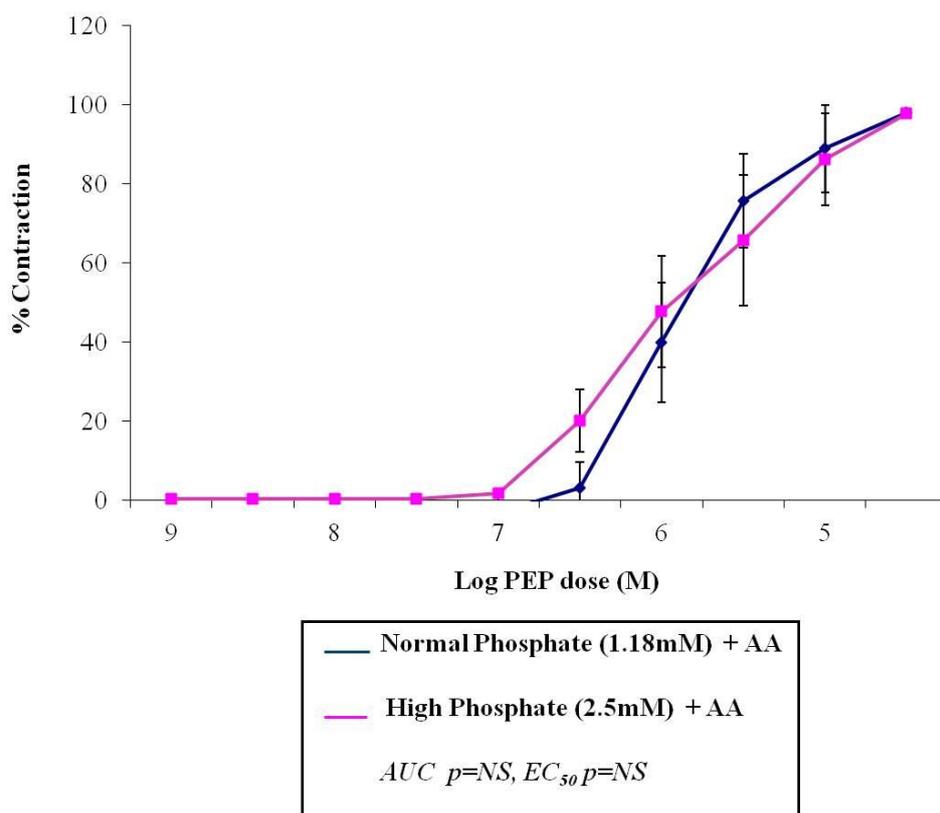


Figure 4-7: Contraction to PEP in the presence of ascorbic acid (AA) in vessels incubated for 16 hours in normal or high phosphate PSS.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=10; high phosphate, n=8) were then mounted on a wire myograph and following normalisation, the vessels were incubated with ascorbic acid ($1 \times 10^{-5} M$) for one hour. PEP was then added in increasing concentrations in a stepwise fashion (1×10^{-8} to $1 \times 10^{-5} M$) to induce contraction and to allow the construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Response is expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

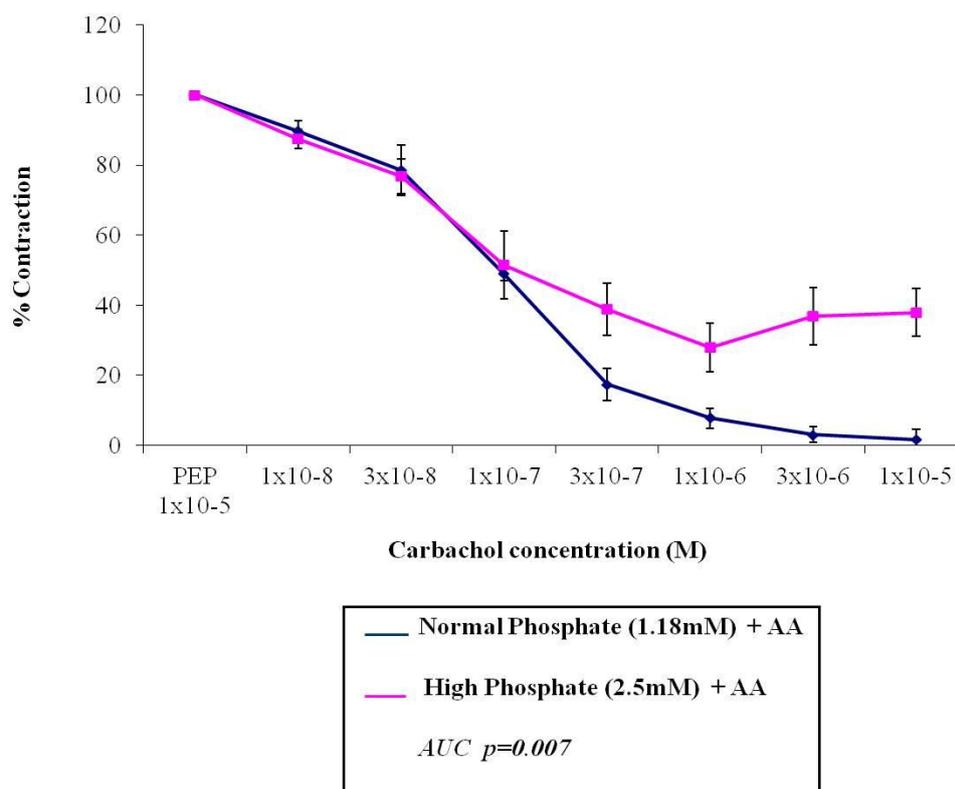


Figure 4-8: Vasodilatation to increasing concentrations of carbachol, in the presence of ascorbic acid (AA), expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=10; high phosphate, n=8) were then mounted on a wire myograph and following normalisation, the vessels were incubated with ascorbic acid (1x10⁻⁵M) for one hour. PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁸ to 1x10⁻⁵M) to induce contraction. Carbachol was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response is expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

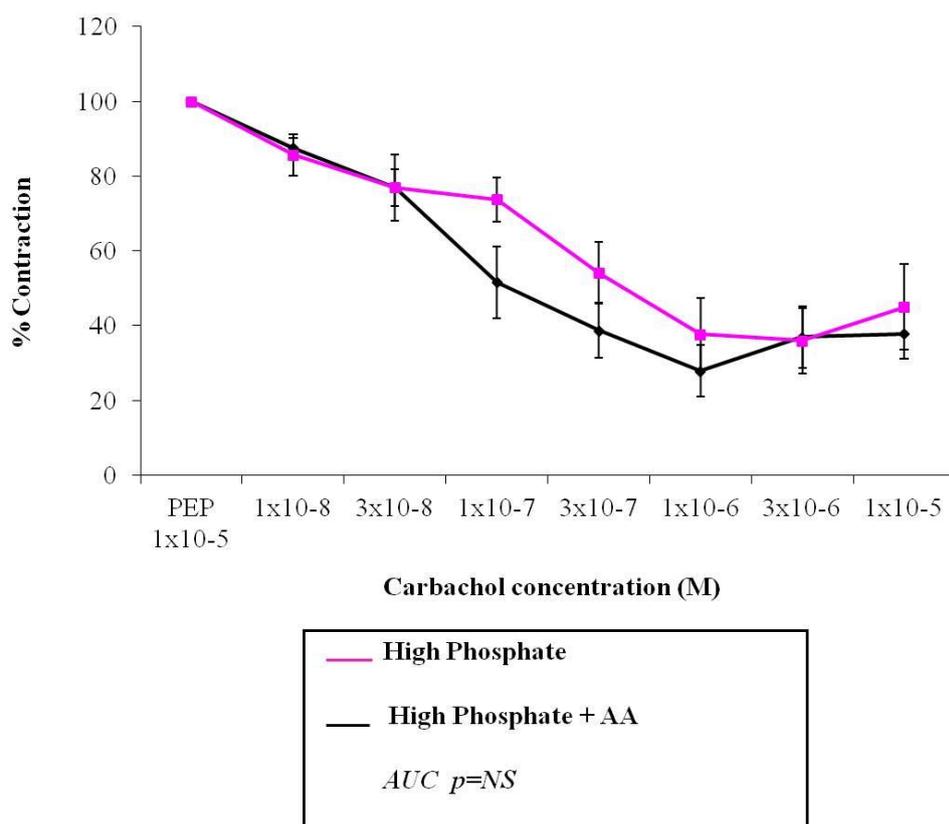


Figure 4-9: Vasodilatation to carbachol in the presence and absence of ascorbic acid (AA) in vessels incubated in high phosphate PSS, expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (n=10) were then mounted on a wire myograph and following normalisation, PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁸ to 1x10⁻⁵M) to induce contraction. Carbachol was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. This was repeated in a second group of vessels (n=8) in the presence of ascorbic acid with which the vessels were incubated for one hour following normalisation at a concentration of 1x10⁻⁵M. Response is expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

4.3.6 Effect of incubation with zaprinast (PDE5I)

Incubation with PDE5I had no significant effect on contraction to PEP in vessels incubated in normal compared with high phosphate PSS. However, incubation with PDE5I ameliorated the impaired vasodilatation seen in vessels incubated in high phosphate PSS to both the endothelium dependent vasodilator, carbachol and the endothelium independent vasodilator, SNP (Figures 4-10 and 4-11). In the presence of zaprinast, the maximum vasodilatation in the vessels incubated in normal phosphate PSS to carbachol was $86\pm 6\%$ and $93\pm 6\%$ in the vessels incubated in high phosphate (p=NS). With SNP, the maximum vasodilatation seen in the vessels incubated in normal phosphate PSS was $70\pm 7\%$ compared with $73\pm 7\%$ in the vessels incubated in high phosphate PSS (p=NS).

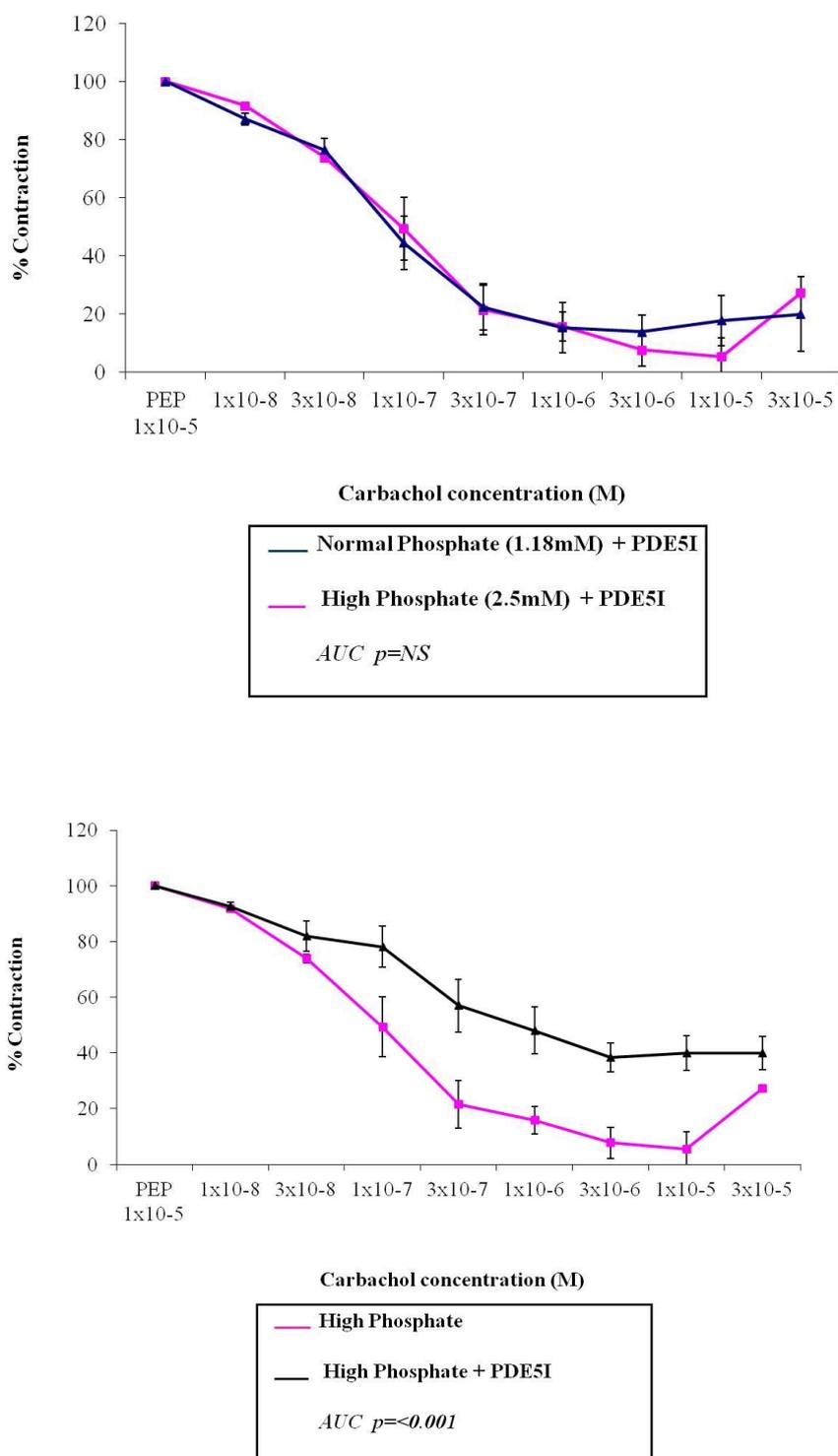


Figure 4-10: Vasodilatation to increasing concentrations of carbachol, in the presence of PDE5I expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=10; high phosphate, n=13) were mounted on a wire myograph and following normalisation, the vessels were incubated with PDE5I (1×10^{-6} M) for one hour. PEP was added in increasing concentrations (1×10^{-8}

to $1 \times 10^{-5} M$). Carbachol was then added in increasing concentrations (1×10^{-8} to $3.5 \times 10^{-5} M$) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. The bottom figure shows a comparison between vessels incubated in high phosphate with ($n=13$) and without ($n=10$) exposure to PDE5I. Response is expressed as mean \pm SEM and comparison made using AUC and the Student's T test.

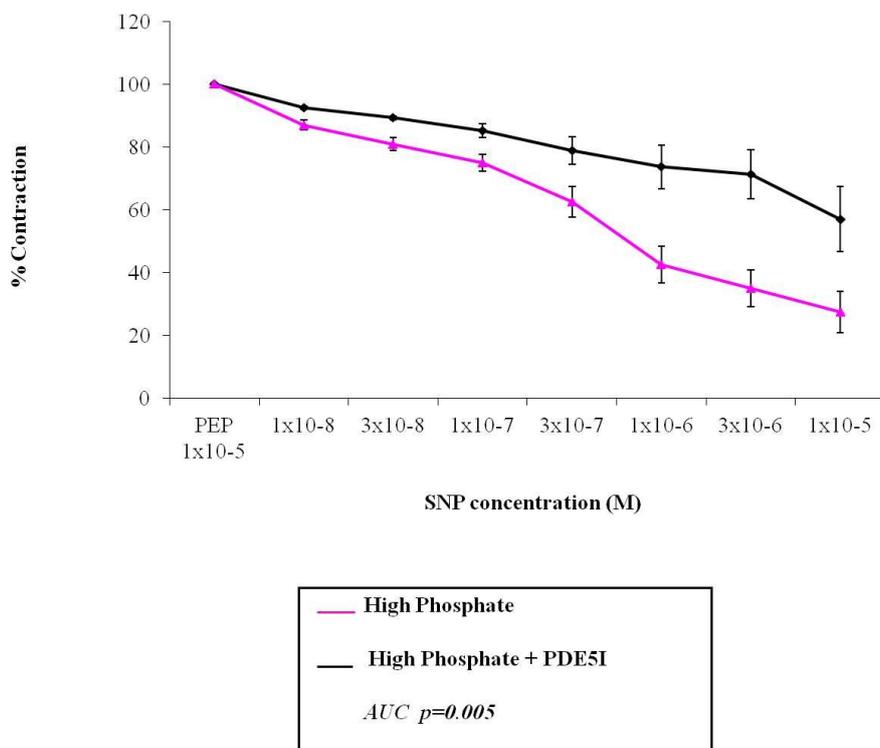
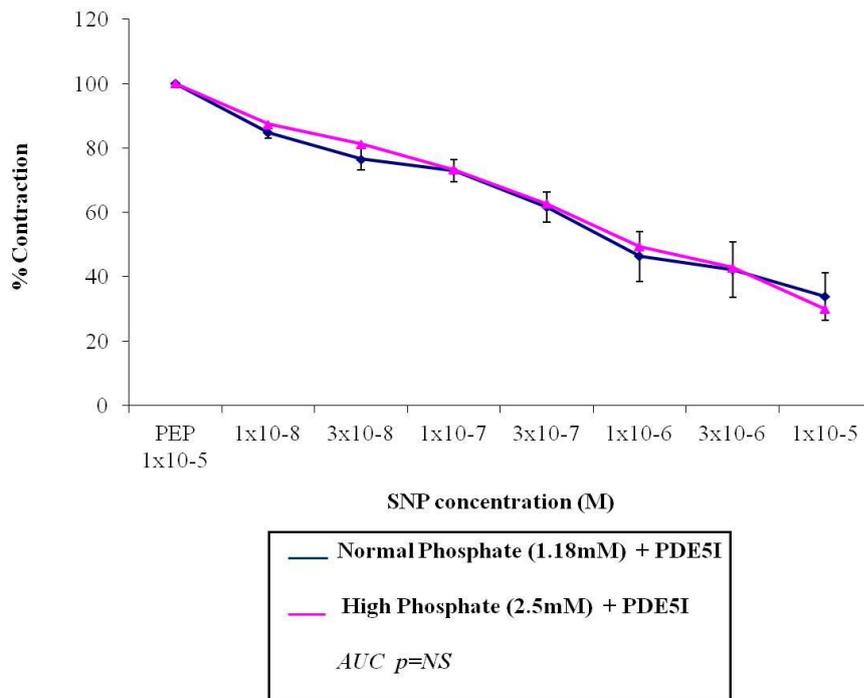


Figure 4-11: Vasodilatation to increasing concentrations of SNP, in the presence of the PDE5I, zaprinast, expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M.

Mesenteric resistance vessels from 12 week old WKY rats were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=10; high phosphate, n=13) were mounted on a wire myograph and following normalisation, the vessels were

incubated with PDE5I ($1 \times 10^{-6} M$) for one hour. PEP was added in increasing concentrations (1×10^{-8} to $3.5 \times 10^{-5} M$). SNP was then added in increasing concentrations (1×10^{-8} to $1 \times 10^{-5} M$) to induce relaxation. Vessels were exposed to each concentration of PEP and SNP for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. The bottom figure shows a comparison between vessels incubated in high phosphate with ($n=13$) and without ($n=9$) exposure to PDE5I. Response is expressed as mean \pm SEM and comparison made using AUC and the Student's T test.

4.3.7 cGMP Measurements

cGMP concentration was measured in vessels from 11 rats. There were 11 vessels incubated in each of normal and high phosphate concentration PSS. Figure 4-12 shows that there was significantly more cGMP/ μ g protein in the vessels incubated in normal phosphate concentration PSS compared with the vessels incubated in high phosphate PSS (715.6 (418-1541) vs 330.6 (284.1-500.1)pM/ μ g; $p=0.0026$).

4.3.8 Immunoblotting for eNOS and PKG

Phospho and total eNOS expression was measured in cell lysates from rat VSMCs cultured in normal and high phosphate concentration medium. There was no discernible difference in either phospho or total eNOS expression (normalised to GAPDH) between cells cultured in normal or high phosphate (Figure 4-13).

When cells cultured in normal phosphate were exposed to high phosphate conditions for 48 hours, eNOS expression increased significantly and in cells cultured in high phosphate conditions, exposed to normal phosphate, eNOS expression reduced significantly (Figure 4-14).

In cells cultured in 3mM phosphate, PKG expression was significantly reduced compared with cells cultured in 0.5mM phosphate (Figure 4-15). When cells cultured in 3mM phosphate were exposed to 0.5mM phosphate for 48 hours, expression of PKG increased (Figure 4-16).

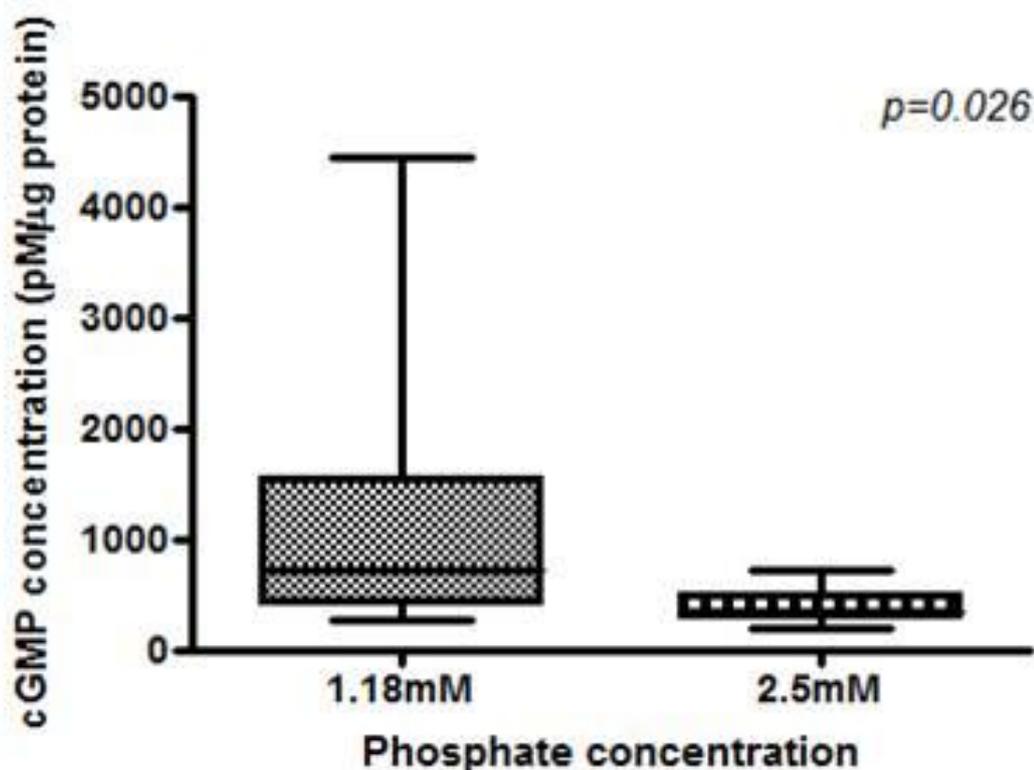


Figure 4-12: Comparison of cGMP concentration (pM/μg protein) in vessels incubated for 24 hours in normal or high phosphate concentration PSS.

Rat mesenteric resistance vessels were incubated in 1.18mM (n=11) or 2.5mM (n=11) PSS for 24 hours and then snap frozen using a combination of dry ice and methanol and stored at -80°C until use. The vessels were thawed at 4°C, sonicated and the protein concentration per μl quantified using the Bradford's assay. The solution (180μl) was washed with a 10% (v/v) excess of 1:1 tri-n-octylamine and 1,2,2-tri chlorofluoroethane (198μl) vortex mixed and centrifuged (14,000rpm at 4°C for three minutes). The top layer (150μl) was removed and washed a second time as above. The top layer was removed (120μl) and stored at -80°C until use. This was measured according to the manufacturer's instructions using an ELISA kit (Cell Signaling technology, distributed by New England Biolabs Ltd, Hertfordshire, UK). A standard curve was plotted using the samples provided and plotted in Microsoft Excel on a logarithmic scale with a logarithmic trendline and the appropriate equation ($y=a*\ln(x) + b$). Each measure was made in duplicate and an average absorbance taken, the average background absorbance was subtracted from this to allow cGMP calculation (nM). Results were then expressed as cGMP pM/μg protein. Analysis was by Mann Whitney U test.

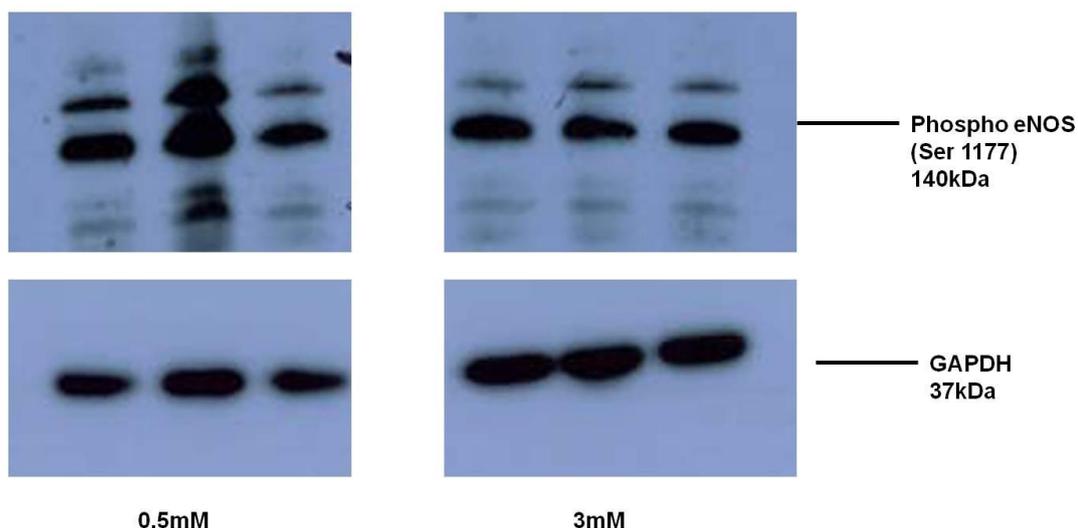


Figure 4-13: Phospho eNOS expression in rat VSMCs (in triplicate).

VSMCs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells were cultured until 90% confluence and then cells were placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a phospho eNOS, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top left panel shows the cells cultured in 0.5mM phosphate concentration medium and the top right, those cultured in 3mM phosphate medium. The bottom panels show GAPDH. The panels were all taken from a single gel and each band represents one well from three separate six well plates.

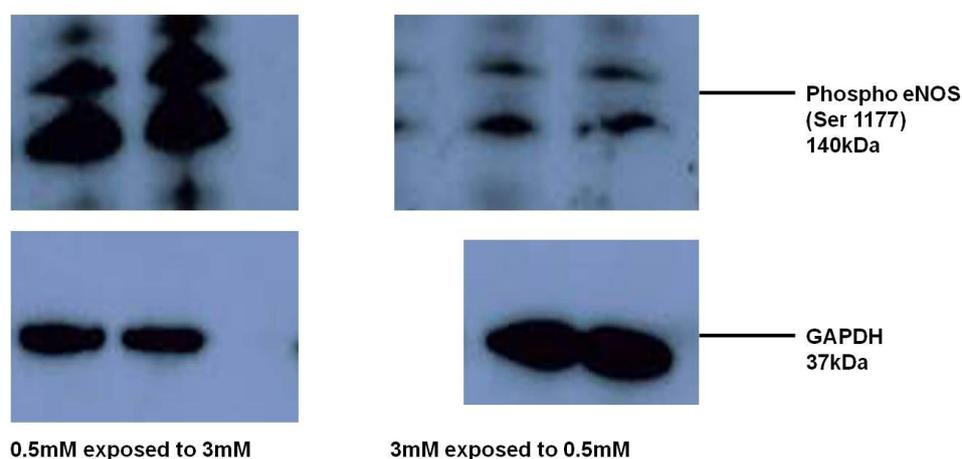


Figure 4-14: Phospho eNOS expression in rat VSMCs (in duplicate).

VSMCs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells utilised had been cultured in the appropriate medium for two weeks in total before being plated on the six well plates. After 48 hours, the media was changed such that cells cultured in 0.5mM media were exposed to 3mM media and cells cultured in 3mM media were exposed to 0.5mM media. After 48 hours, the cells reached 90% confluence and were then placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a phospho eNOS, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top left panel shows the cells cultured in 0.5mM phosphate concentration medium initially and then exposed to 3mM medium and the top right, those cultured in 3mM phosphate medium initially and then exposed to 0.5mM medium. The bottom panels show GAPDH. The panels were all taken from a single gel and each band represents one well from two separate six well plates.

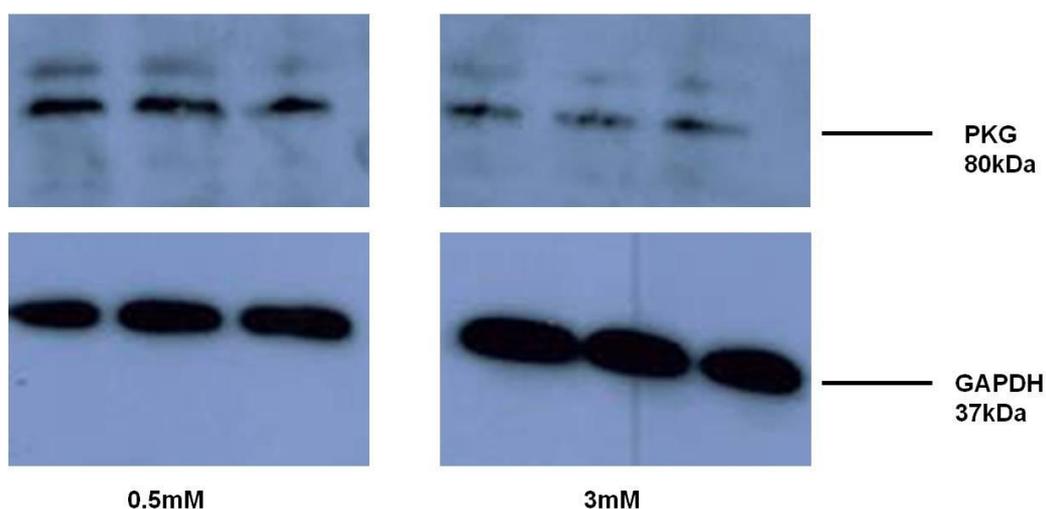


Figure 4-15: PKG expression in rat VSMCs (in triplicate).

VSMCs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells were cultured until 90% confluence and then cells were placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a PKG, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top left panel shows the cells cultured in 0.5mM phosphate concentration medium and the top right, those cultured in 3mM phosphate medium. The bottom panels show GAPDH. The panels were all taken from a single gel and each band represents one well from three separate six well plates.

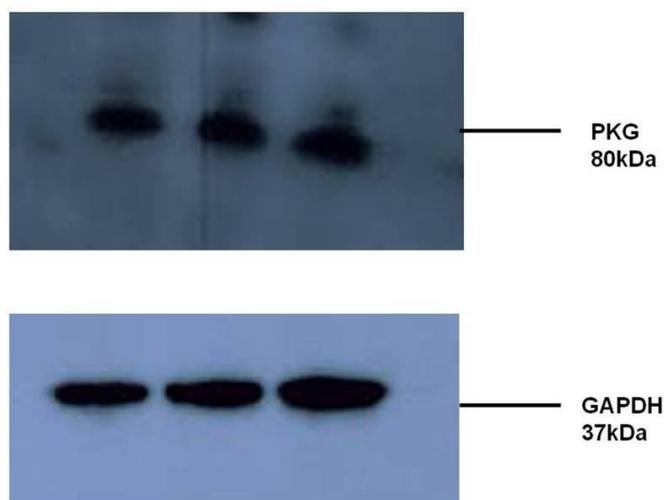


Figure 4-16: Expression of PKG in rat VSMCs (in triplicate).

VSMCs were cultured on six well plates at a cell density of 1×10^4 cells/well in 3mM phosphate concentration medium. After 48 hours, the media was changed and the cells were exposed to 0.5mM media. After 48 hours, the cells reached 90% confluence and were then placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a PKG, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top panel shows the bands for PKG and the bottom panel shows GAPDH. The panels were all taken from a single gel and each band represents one well from two separate six well plates.

4.3.9 VEGF ELISA

There was no difference in VEGF production in cells cultured in high phosphate medium. In cells stressed by exposure to hypoxic conditions for 24 hours, VEGF production increased significantly in cells cultured in standard phosphate concentration medium (0.5mM) and decreased significantly in cells cultured in high phosphate medium (3mM) when compared with cells cultured in 0.5mM medium in standard conditions (Figure 4-17).

4.3.10 NO production: Griess reaction

There was a clear albeit non significant trend to a reduction in NO in the cells cultured in high phosphate. Treatment with L-NAME significantly reduced the quantity of NO further and with L-arginine there was a non-significant trend towards an increase in NO production. In the cells cultured in normal phosphate concentration medium, there was a reduction in NO production with the addition of L-NAME. Culturing with L-arginine did not alter the level of NO (Figure 4-18).

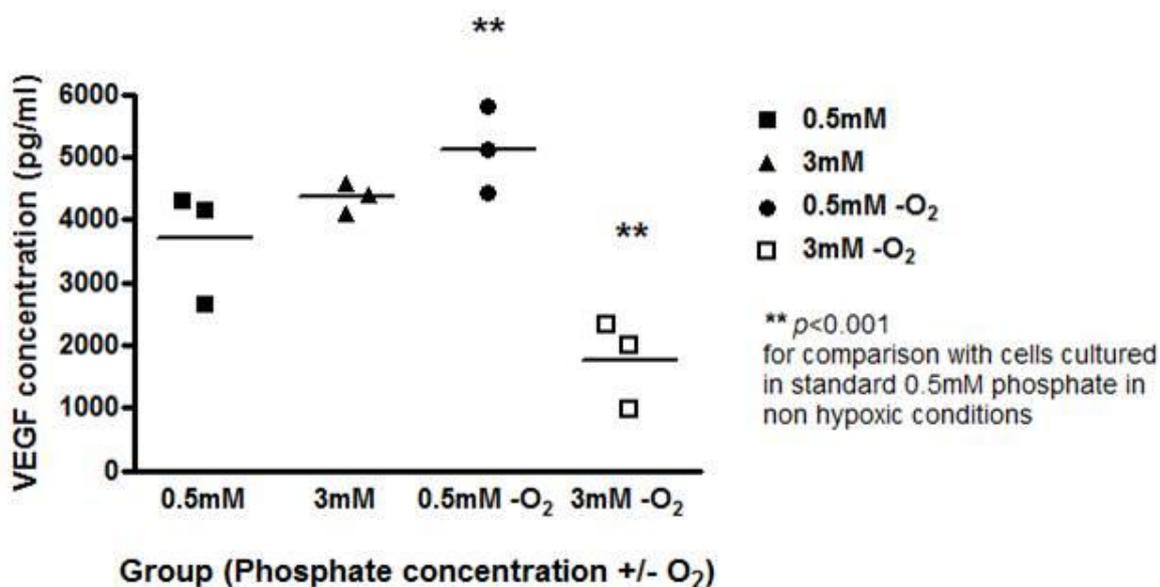


Figure 4-17: VEGF concentration in supernatant of cells cultured in 0.5mM and 3mM phosphate in standard and hypoxic (-O₂) conditions.

VEGF was measured on VSMC supernatants. The cells were cultured in six well plates at a density of 1×10^4 cells /well. The supernatant from each well of one six well plate was combined and then concentrated and used to represent one replicate for each phosphate concentration. Three replicates were used for each condition and stored at -80°C . After a single thaw, the supernatant was concentrated using appropriately sized Amicon® ultra centrifugal filters. A solid phase ELISA kit purchased from R&D systems was used and the ELISA was performed and analysed in accordance with the manufacturer's instructions. VEGF is known to be induced when cells are exposed to hypoxic condition. Cells exposed to hypoxic conditions were therefore used as a positive control. Cells were put into an air tight chamber for 24 hours to induce hypoxia. The reliable air tight seal and the fast gas exchange properties of the chamber, allow for the rapid and economical creation of a non-fluctuating hypoxic environment. Once sealed, this is a closed air tight system. The gas is a mixture of 5% CO₂ and 95% N₂. The cells are placed into the chamber in six well plates in the hood. The gas is bubbled through for four minutes then the chamber is sealed and put into the incubator. The lids are left off the plates (there is O₂ in media). After 12 hours, fresh gas was bubbled through to ensure hypoxia was maintained. After 24 hours, the cells were removed and the supernatant obtained, combined (as above) and stored. Values are mean \pm SEM.

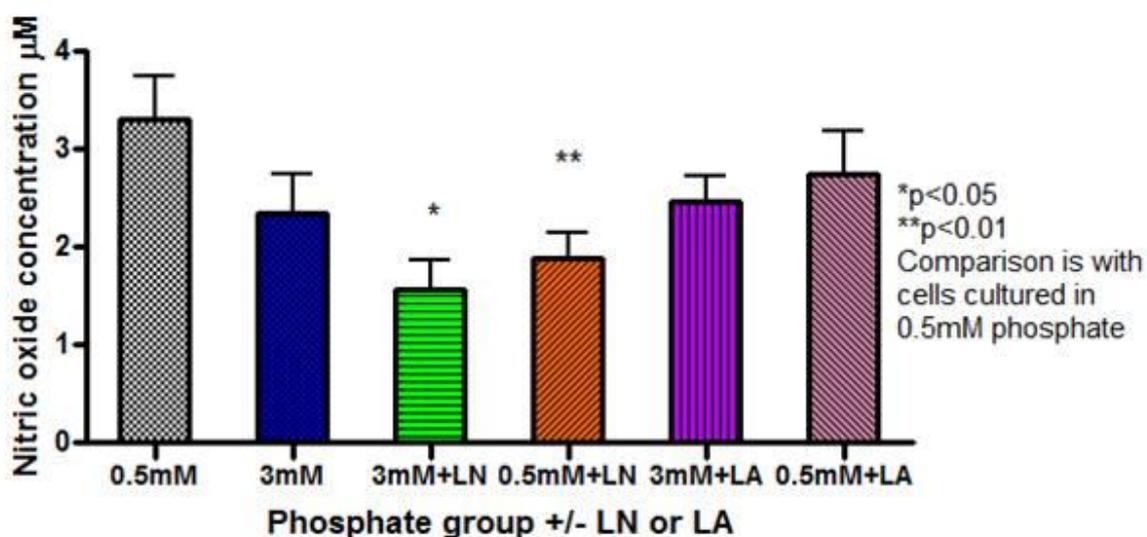


Figure 4-18: Relative NO production in rat VSMCs cultured in high (3mM) and standard (0.5mM) phosphate concentration medium +/- L-NAME (LN) and L-arginine (LA).

On day one, VSMCs were plated onto a 96 well plate at a cell density of 1×10^3 cells/well. After 24 hours fresh media was added to each well. Simultaneously cells were treated with L-NAME ($1 \times 10^{-4} M$) or L-Arginine ($1 \times 10^{-5} M$); controls were left untreated. L-NAME was used as a NOS inhibitor and thus a reduction in NO would be expected. L-arginine was added to increase the available substrate for eNOS with an anticipated subsequent increase in NO production. The media was replaced daily with fresh L-NAME or L-arginine as appropriate until day four when a Griess reaction was performed. Sodium nitrite was used to generate a standard curve in each plate at a concentration of 0-100µM. 50µl of Griess was added to each well, the plate was then wrapped in tinfoil and replaced in the incubator for 30 minutes before being read at an absorbance of 540nm on a microplate reader. This was performed in duplicate and a minimum of six replicates were used for each condition. Values are mean±SEM

4.3.11 Summary of results

In rat resistance vessels incubated in high phosphate concentration PSS (2.5mM) for 16 hours, using wire myography to assess vessel function there is evidence of:

- Reduced basal NO production
- Impaired endothelium dependent vasodilatation to carbachol
- Impaired endothelium independent vasodilatation to SNP
- Improved initial but not overall endothelium dependent vasodilatation in the presence of the anti-oxidant, ascorbic acid.
- Significant improvement in maximum endothelium independent vasodilatation in the presence of ascorbic acid
- Significant improvement in both endothelial dependent and independent vasodilatation in the presence of the PDE5I, zaprinast.
- Significantly lower concentration of cGMP.

In rat VSMCs cultured in standard phosphate medium (0.5mM) and high phosphate medium (3mM):

- There is no difference in expression of total or phospho eNOS (Ser 1177) in the chronic high phosphate environment but acute exposure (48 hours) to 3mM phosphate results in significantly increased expression of total and phospho eNOS in cells cultured in normal phosphate concentration medium.
- Acute exposure (48 hours) to 0.5mM phosphate results in significantly reduced expression of total and phospho eNOS in cells cultured in high phosphate concentration medium.

- There is reduced expression of PKG in cells cultured in high phosphate medium and PKG expression increases in cells cultured in 3mM phosphate medium upon exposure to normal phosphate medium (0.5mM) for 48 hours.
- VEGF concentration is comparable in cells cultured in normal and high phosphate concentration medium but upon exposure to stress (hypoxia), cells cultured in normal phosphate produce significantly higher quantities of VEGF and cells cultured in high phosphate produce significantly less VEGF.
- A non-significant trend towards reduced NO production estimated by the Griess reaction in cells cultured in high phosphate compared with normal phosphate.

4.4 Discussion

These *ex vivo* studies have demonstrated for the first time that exposing resistance vessels, from healthy rats, to elevated phosphate for a sustained period of time, causes impaired endothelium dependent and independent vasodilatation. This suggests a direct effect of phosphate on the endothelium and on the VSMC and separates an effect of phosphate from other effects of uraemia. Thus, the endothelium in the high phosphate environment is less able to produce NO, upon stimulation. Similarly, the VSMC is less able to respond to NO possibly as a consequence of altered cGMP levels or guanylate cyclase expression or activity. The contractile response was preserved in the high phosphate environment in our study. However, in the presence of L-NAME, there was a significant increase in contraction in vessels incubated in normal phosphate concentration PSS which was not seen in the vessels incubated in high concentration PSS. This suggests that the vessels exposed to high phosphate produce less basal NO.

My aim was to separate the effects of phosphate exposure from other effects of uraemia and this is the first study to do this in resistance vessels. My findings are in keeping with those of the only other study which has looked at the direct effect of phosphate on vasodilatation (6). This demonstrated impaired endothelium dependent relaxation in rat aortic rings, not resistance vessels, exposed to a high phosphate environment for a much shorter time period (one hour). They did not study endothelium independent vasodilatation.

In models of CKD, Wang et al performed studies using resistance vessels from rats with mild CKD and also found evidence that there was reduced basal NO production when compared with control animals (369). Our results suggest this observation may be directly related to phosphate rather than other aspects of the uraemic milieu. Interestingly our results conflict with those of Thuraisingham et al who demonstrated no difference in either endothelium dependent or independent relaxation in resistance vessels from uraemic rats compared with control animals and the spontaneously hypertensive rat (370). However, this study had a number of limitations including using a smaller number of vessels and sacrificing the animals under anaesthesia which itself may affect the ability of the vessels to vasodilate and may have clouded the observed findings. Furthermore they added a single and not an increasing concentration of SNP and all experiments were performed in the presence of the cyclo-oxygenase inhibitor, indomethacin which attenuates the production of prostaglandins. It may be that some of my findings, particularly in relation to the observed impaired endothelial independent vasodilatation, could be explained by alterations in the productions of prostaglandins. I did not, however, perform studies with indomethacin.

Morris et al demonstrated impaired endothelial vasodilatation in human resistance vessels from patients with and without CKD and although endothelium independent vasodilatation was not significantly different in this study there was a trend towards impaired relaxation with SNP in uraemic vessels (95). Interestingly, they do not provide information about serum phosphate level in the uraemic patients compared with controls although it is likely to have been significantly higher. The observed effects may be the result of exposure to higher phosphate and further studies in human vessels from patients with and without CKD exposed to different phosphate concentrations would address this.

There are a number of possible mechanistic explanations for our results; it may be that exposure to elevated phosphate leads to one or a combination of:

- Down-regulation of eNOS, production of ROS and less bioavailable NO
- Altered expression or activity of guanylate cyclase
- Altered production of cGMP
- Alterations in intra-cellular calcium

- Alterations in cell growth as a consequence of reduced levels of NO and cGMP leading to hypertrophy and dysfunction of both ECs and VSMCs.

To explore the mechanistic explanation for my observed effects, I went on to look at the response of the vessels in the presence of an antioxidant, ascorbic acid which may redress an imbalance in redox and in the presence of a PDE5I which should improve relaxation by reducing cGMP breakdown.

I found that ascorbic acid was able to significantly improve maximal endothelial independent relaxation in the vessels incubated in high phosphate. Endothelial dependent vasodilatation was improved at low but not high dose carbachol. This suggests that oxidative stress might contribute to the mechanism by which phosphate results in impaired endothelial dependent and independent vasodilatation. Ascorbic acid is an electron donor and it can interact with free radicals to form a less reactive ascorbyl radical (371). Phosphate might trigger the production of ROS which results in EC and VSMC damage. In clinical studies, ascorbic acid appears to increase NO synthesis and can improve the impaired endothelial function seen in the setting of hyperglycaemia (372;373). Although I did not see a significant improvement in endothelium dependent vasodilatation, this might be explained by too short an incubation time or too low a dose of ascorbic acid. Another possibility is that NO levels are so reduced (as a result of reduced bioavailability or reduced expression on eNOS or both) that an antioxidant alone is not enough to reverse the effects.

The addition of PDE5I ameliorated the impaired endothelium dependent and independent vasodilatation seen in the vessels incubated in high phosphate PSS. Zaprinast, the PDE5I utilised in our studies has been shown to increase NO levels and cGMP levels and to have anti-proliferative effects (374). It has not previously been studied in the context of CKD but in other disease processes, for example pulmonary hypertension, where ED is prominent studies have shown beneficial effects mediated by improvements in NO and cGMP levels (375). Long term phosphate might trigger structural changes in the vessel wall in addition to shorter term effects on cGMP and NO level. To explore this further, I measured cGMP levels in the vessels and found that those incubated in high phosphate concentration PSS had significantly lower concentrations of cGMP.

I studied expression of eNOS in rat VSMCs and found there to be no difference in expression in cells cultured in high compared with normal phosphate concentration

medium. This is not unexpected because the majority of eNOS is expressed in the EC rather than the VSMC. eNOS expression must therefore be further studied in the EC.

The increase in eNOS expression seen in cells cultured in 0.5mM phosphate and exposed acutely for 48 hours to 3mM phosphate does however suggest that there is an effect of phosphate acutely and perhaps a degree of adaption in the VSMC chronically exposed to the high phosphate environment. Perhaps acute exposure to high phosphate conditions triggers oxidative stress resulting in increased eNOS expression and thus NO production to counter balance the detrimental effects of phosphate. There may be alterations in signalling pathways including phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (p13/Akt) or MAPK. Chronic exposure to high phosphate does not influence eNOS expression and thus perhaps longer term VSMCs have the capacity to adapt to the higher phosphate environment. Interestingly, exposing cells cultured in 3mM phosphate to 0.5mM phosphate for 48 hours results in significantly reduced eNOS expression. The explanation for this is unclear but might relate at least in part to the cells adapting to the high phosphate environment such that introduction of normal phosphate concentration medium results in stress to the cells and thus increased free radical production but an inability to react appropriately and thus reduced NO production. It does suggest that any changes induced by high phosphate can be modified although these modifications may not be positive.

Similarly VEGF production is unaltered in cells cultured in 3mM compared with 0.5mM medium. However, exposing the cells to hypoxia should result in an increase in VEGF. This does not occur in cells cultured in 3mmol/L phosphate which suggests that the high phosphate environment results in an abnormal 'stress' response in these cells. This is likely to have atypical and abnormal effects on many signal transduction pathways leading to abnormal activation and inhibition of many factors including possibly eNOS, cGMP, guanylate cyclase activity and expression and PKG.

I examined NO production in the VSMCs using the Griess reaction; there was a non-significant trend towards lower NO in the cells cultured in high phosphate. However, the Griess reaction cannot accurately detect small changes and NO is very difficult to measure directly. Moreover, these experiments require to be repeated in the EC which is the major source of NO. The trend towards reduced NO even in the VSMC does lend some weight to my hypothesis that elevated phosphate causes disruption to the NO pathway thus leading to ED. In reality it is likely that phosphate interrupts multiple pathways, mediators and factors which leads to impaired vasodilatation and ED.

4.5 Conclusion

Exposure to elevated phosphate concentration PSS results in impaired endothelium dependent and independent relaxation in isolated rat vessels. These deleterious effects can be improved following incubation with an antioxidant and particularly with a PDE5I. This suggests a degree of reversibility at least in the early stages of phosphate exposure.

I have attempted to study the effects of longer term exposure to elevated phosphate than in previous studies. In the context of myography experiments, incubation for longer than 16 hours is not possible because the vessels do not survive for more than 24 hours *ex vivo*.

To further tease out the underlying mechanism of action of phosphate as a cardiovascular risk factor, in chapter five I describe studies in human resistance vessels and ECs. These studies were designed to assess the effects of the high phosphate environment in vessels from patients with and without CKD, the rate of superoxide production in whole blood from patients with and without CKD and to study eNOS and nitrotyrosine expression in HUVECs. Additionally, I compare intracellular calcium levels in cells cultured in normal and high phosphate concentration medium.

5 Chapter Five – Phosphate; effects on the function of human resistance vessels and on the nitric oxide pathway in human cell lines

5.1 Introduction

In chapter four of this thesis, a series of studies were presented to assess the effects of exposure to high phosphate on the function of resistance vessels from healthy rats and on rat VSMCs. The major finding was that exposure to elevated phosphate concentration leads to impaired endothelium dependent and independent relaxation of the vessels, mediated, in part, by the NO pathway. The cell studies confirmed differences in aspects of the NO pathway between VSMCs cultured in high and normal phosphate. There was not, however, a significant difference in eNOS expression or in NO production in VSMCs cultured in high phosphate, which might relate to the relatively low eNOS expression in this cell line compared with ECs. Whilst these studies add to the existing literature and offer insight into the possible mechanism of phosphate as a CV risk factor, there remain unanswered questions regarding exactly how phosphate might increase CV risk.

The results presented in chapter four cannot necessarily be extrapolated to humans. Thus it is necessary to study human resistance vessels to ascertain if phosphate has similar effects. *In vivo*, the resistance vessels play a key role in regulating blood flow to tissues; dysfunction is associated with increased CV risk. The effect of phosphate in vessels from healthy patients has not been examined and whilst Morris et al studied resistance vessels from patients with CKD and demonstrated impaired relaxation, they did not look separately at the effects of phosphate (95). Teasing out the mechanism of action of phosphate requires that phosphate exposure be considered separately from other abnormalities of the uraemic environment. In 5/6 nephrectomised rats, fed a high calcium, high phosphate or standard diet, Koobi et al showed that resistance vessels from uraemic animals demonstrated impaired endothelium dependent relaxation. When fed a high phosphate diet the impaired relaxation was worsened further compared to controls; the group did not look at the effect on vasodilatation of a high phosphate diet alone (251). Interestingly, they observed an improvement in vasodilatation with the uraemic animals fed a high calcium diet.

The impaired endothelium dependent and independent relaxation observed in the rat vessels is consistent with disruption of the NO pathway and reduction in available NO. Reduced eNOS expression has previously been demonstrated in bovine ECs and in two human EC lines (EAhy926 cells and GM-7373), exposed to high phosphate, phosphate also induces ROS production (6;250). In the context of hyperphosphataemia the NO pathway has not been extensively studied; beyond the observation of altered eNOS

expression. Thus, disruption of the NO pathway seen upon exposure to elevated phosphate concentration needs to be explored in human vessels and human EC lines. The possibility remains that any observed effects of phosphate on endothelium dependent and independent relaxation relate to changes in intracellular calcium concentration and the impact of phosphate on calcium levels requires further investigation.

The aims of the studies presented in this chapter, therefore, were to study resistance vessels from patients without CKD and from patients with advanced CKD (stage 5); to assess the effects of exposure to elevated phosphate on human cell lines specifically looking at the NO pathway. Additionally I quantified intracellular calcium content in both rat and human cell lines exposed to altered concentrations of phosphate. The specific experiments involved were:

- Contractile response of vessels to PEP
- Vasodilatation response to both carbachol and SNP
- Measurement of O_2^- in whole blood from patients with and without CKD
- Expression of eNOS and nitrotyrosine in HUVECs cultured in standard (0.5mM) and high (3mM) phosphate concentration
- Measurement of NO in HUVECs
- Intracellular calcium content of human and rat ECs and rat VSMCs cultured in normal and high phosphate media.

5.2 Materials and Methods

5.2.1 Vessel studies

Patients with and without CKD, undergoing nephrectomy for living kidney donation and patients with CKD 5 undergoing live donor renal transplant were recruited (section 2.3.2). The wire myography technique is described in section 2.3. Vessels were dissected and

stored for 16 hours in either normal (1.18mM) or high phosphate (2.5mM) concentration PSS. The composition of these solutions is presented in Tables 2-1 and 2-2. EDTA was also added at a final concentration of 0.023mM. Vessels were contracted with PEP and relaxed with increasing concentrations of carbachol. This was repeated with PEP and SNP. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. For vasodilatation with carbachol and SNP, vessels were exposed to each concentration for two minutes or until a plateau had been reached. The concentration range used for each drug was: PEP 1×10^{-9} to 3×10^{-5} M; carbachol 1×10^{-8} to 3×10^{-5} M; SNP 1×10^{-8} to 3×10^{-5} M.

Vessels were not available from all recruited subjects due to either absence of adipose tissue or suitable vessels. The use of human vessels is more difficult and success was lower than in rat vessels which is in keeping with the literature (376). There is also debate as to the applicability of normalisation to vessels other than third order rat resistance vessels and so I did not use this in the human vessels (343).

Additionally, I experimented with different drugs working on different receptors. Dr Craig Daly (Life Sciences Research Fellow, University of Glasgow) stained the vessels with syto62, a nuclear stain; QAPB dye, fluorescent prazosin, a selective $\alpha 1$ antagonist; and CGP12177, a $\beta 1$ and $\beta 2$ antagonist and partial agonist and used confocal microscopy to determine which receptors were present in human vessels to help guide the final choice of drug. The vessels expressed $\alpha 1$ adrenoceptors on both the smooth muscle and the endothelium (Figure 5-1).

5.2.2 Superoxide, FGF-23 and vitamin D measurement

O_2^- was measured by EPR in HUVEC cell supernatant (section 2.4.11) and in whole blood (section 2.3.10). Vitamin D and FGF-23 measurements were performed on stored samples as described in sections 2.6.2.12 and 2.6.2.13.

5.2.3 Immunoblotting, Griess reaction and epi-fluorescence

HUVECs were cultured from the outset in either 0.5mM or 3mM phosphate concentration (section 2.4). This was intended to mimic chronic exposure to a high phosphate environment. Immunoblotting was performed on cell lysates using antibodies for total and phospho-eNOS (Ser 1177) and nitrotyrosine (section 2.4.8).

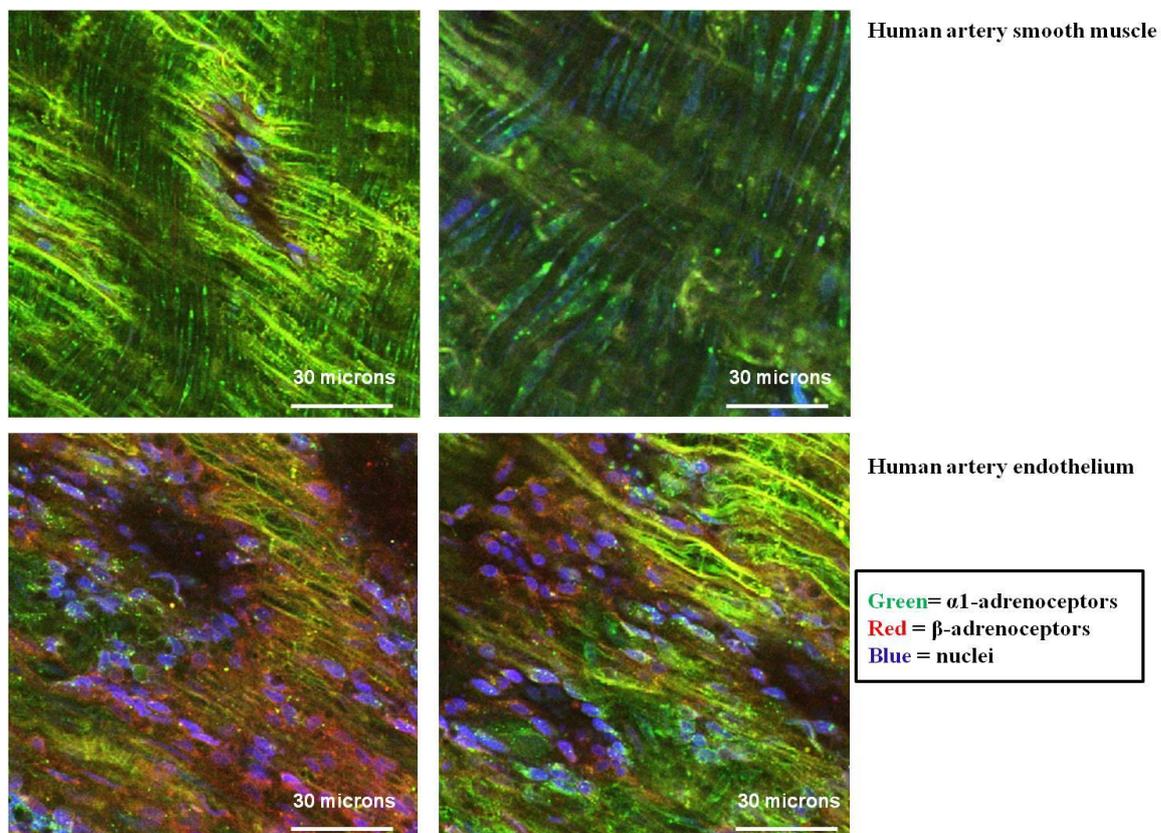


Figure 5-1: Confocal microscopy pictures of human artery stained with QAPB dye (green), CGP12177 (red) and syto62 (blue).

Dr Craig Daly (Life Sciences Research Fellow, University of Glasgow) stained two human resistance vessels, from subcutaneous abdominal fat, from three live donors with syto62, a nuclear stain; QAPB dye, fluorescent prazosin, a selective α 1 antagonist; and CGP12177, a β 1 and β 2 antagonist and partial agonist and used confocal microscopy to determine which receptors were present in human vessels. The vessels expressed α 1 adrenoceptors on both the smooth muscle and the endothelium. The top two panels are human artery smooth muscle and α 1 adrenoceptors are clearly present but there are no β adrenoceptors. This is in contrast to the endothelium which is seen in the bottom two panels. Here there is highly autofluorescent internal lamina (yellow) and many β adrenoceptors with some α 1 adrenoceptors. The images are representative images from one vessel.

GAPDH was used as the housekeeping protein. A minimum of three experiments were performed. Griess reaction to estimate NO was performed as detailed in section 2.4.5, in duplicate on five replicates.

Intracellular calcium was measured by epifluorescence with FURA-2AM (section 2.4.10). This was performed in three cell lines; HUVECs, rat aortic ECs and rat VSMCs. For each cell line, the intracellular calcium content was measured in a minimum of three cells on three separate occasions. The mean intracellular calcium concentration was calculated for each concentration of phosphate and values were compared with a Student's T test.

5.3 Results

5.3.1 Sample size and baseline demographics

The baseline demographics of the patients whose vessels were used in the myography experiments are detailed in Table 5-1. None of the patients had a history of DM or CVD. Comparison is between living kidney donors, without CKD, and patients with CKD. As would be expected, patients with CKD had a lower eGFR, higher phosphate and higher FGF-23. 89% (n=8) of the patients with CKD were taking a phosphate binder; of them 33% (n=3) were taking a calcium containing phosphate binder and 67% (n=6) a non-calcium containing phosphate binder. 89% (n=8) were taking at least one anti-hypertensive.

The number of vessels studied for each drug and the corresponding length and diameter of the vessels is shown in Table 5-2. There was no significant difference in the size of the vessels obtained from patients with or without CKD nor between vessels which were incubated in high or normal phosphate concentration PSS.

5.3.2 Contraction and relaxation response in vessels from living kidney donors

Contraction with PEP did not differ significantly between the vessels incubated in normal or high phosphate concentration PSS (Figure 5-2). The potency of PEP was similar with no significant difference in the EC_{50} ($3.3 \pm 2.9 \times 10^{-6} M$ and $2.8 \pm 2.9 \times 10^{-6} M$). Maximal contraction with PEP at a concentration of $3 \times 10^{-5} M$ was significantly higher in the vessels incubated in normal compared with high phosphate PSS ($1.76 \pm 0.71 g$ and $1.16 g \pm 0.66 g$; $p=0.05$).

Relaxation response to the endothelium dependent vasodilator, carbachol is illustrated in Figure 5-3. The vessels incubated in high phosphate PSS vasodilated significantly less well than those incubated in normal phosphate PSS (AUC, $p=0.009$). Maximum vasodilatation obtained in the vessels incubated in high phosphate was lower than in the vessels incubated in normal phosphate ($42.9 \pm 12\%$ vs $79.4 \pm 8.2\%$; $p=0.003$). The results for the endothelium independent vasodilator, SNP are shown in Figure 5-4. The vessels incubated in high phosphate PSS vasodilated significantly less well than those incubated in normal phosphate PSS (AUC $p=0.04$). Maximum vasodilatation obtained in the vessels incubated in high phosphate was lower than in the vessels incubated in normal phosphate ($59.7 \pm 13.8\%$ compared with 99.8 ± 20.2 ; $p=0.02$).

Parameter	Myography studies			Superoxide study		
	LD	CKD	p value	LD	CKD	p value
Age (years)	48.8±10.2	44.6±14.5	NS	47.1±7.8	51.1±10.5	NS
Male sex (%)	64	44		44	78	
Systolic blood pressure (mmHg)	134±16	134±24	NS	131±15	139±15	NS
BMI	29.5±3.6	25.4±3.4	0.02	29.7±3.0	28.1±4.2	NS
Dialysis (%)	0	44		0	56	
eGFR (mls/min/1.73m ²)	95.8±21	8.0±3	<0.001	105.1±9.5	7.8±2.8	<0.001
Phosphate (mmol/L)	1.1±0.1	1.7±0.6	0.003	1.0±0.2	1.6±0.5	0.002
Adj. calcium (mmol/L)	2.4±0.1	2.5±0.1	NS	2.4±0.1	2.4±0.2	NS
FGF-23 (RU/ml)	45.8 (40.5-51.7)	1644.0 (1135.7-1750.4)	<0.001	50.37 (49.1-51.3)	1727.1 (1717.7-1737.6)	<0.001
On any medication (%)	18	100		11	100	
Calcium containing phosphate binder	0	33		0	22	
Non-calcium containing phosphate binder	0	67		0	44	
Alfacalcidol (%)	0	78		0	78	
Cinacalcet (%)	0	0		0	22	
Anti hypertensive (%)	18	89		0	100	

Table 5-1: Demographics of the study population.

Demographics for the patients whose vessels were used are shown in columns two to four (LD n=14 and CKD n=14) and for the patients whose blood samples were used in the O₂⁻ study (n=9) are shown in the last three columns.

	CKD		Live Donor		p value
	Normal	High	Normal	High	
Length	4.76 ±0.36	4.52±0.49	4.59 ±0.35	4.51±0.44	ns
Diameter	494.3 (339-700)	460.9 (340-792)	534.2 (286-879)	438.4 (297-684)	ns
Phenylephrine	12	9	13	12	
Carbachol	12	9	11	12	
SNP	12	9	13	12	

Table 5-2: Comparison of numbers of vessels studied for each patient group.

The number of vessels in which a complete concentration response curve was obtained for each drug and the corresponding size of the vessels is presented. Comparison was made with a one-way ANOVA.

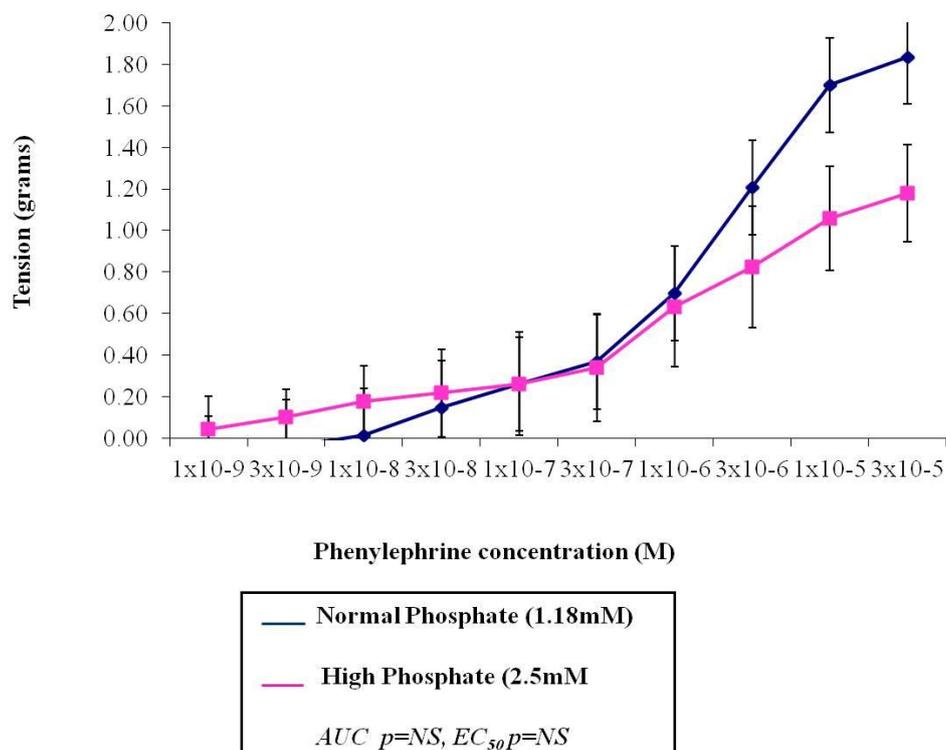


Figure 5-2: Contraction to PEP in vessels from living kidney donors.

Subcutaneous resistance vessels from abdominal fat from living kidney donors, collected at the time of donation, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=13; high phosphate, n=12) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was then added in increasing concentrations in a stepwise fashion (1×10^{-9} to 3.5×10^{-5} M) to induce contraction and to allow the construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Response is expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

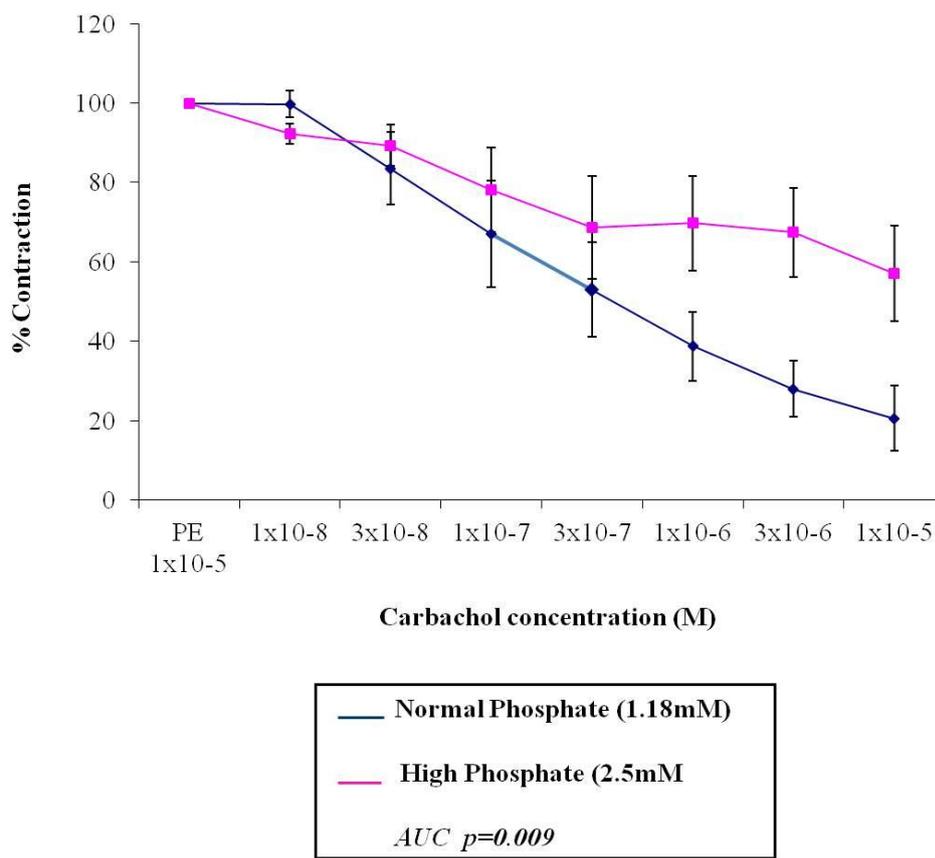


Figure 5-3: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M (living kidney donors).

Subcutaneous resistance vessels from abdominal fat from living kidney donors, collected at the time of donation, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=13; high phosphate, n=12) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁹ to 1x10⁻⁵M) to induce contraction. Carbachol was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

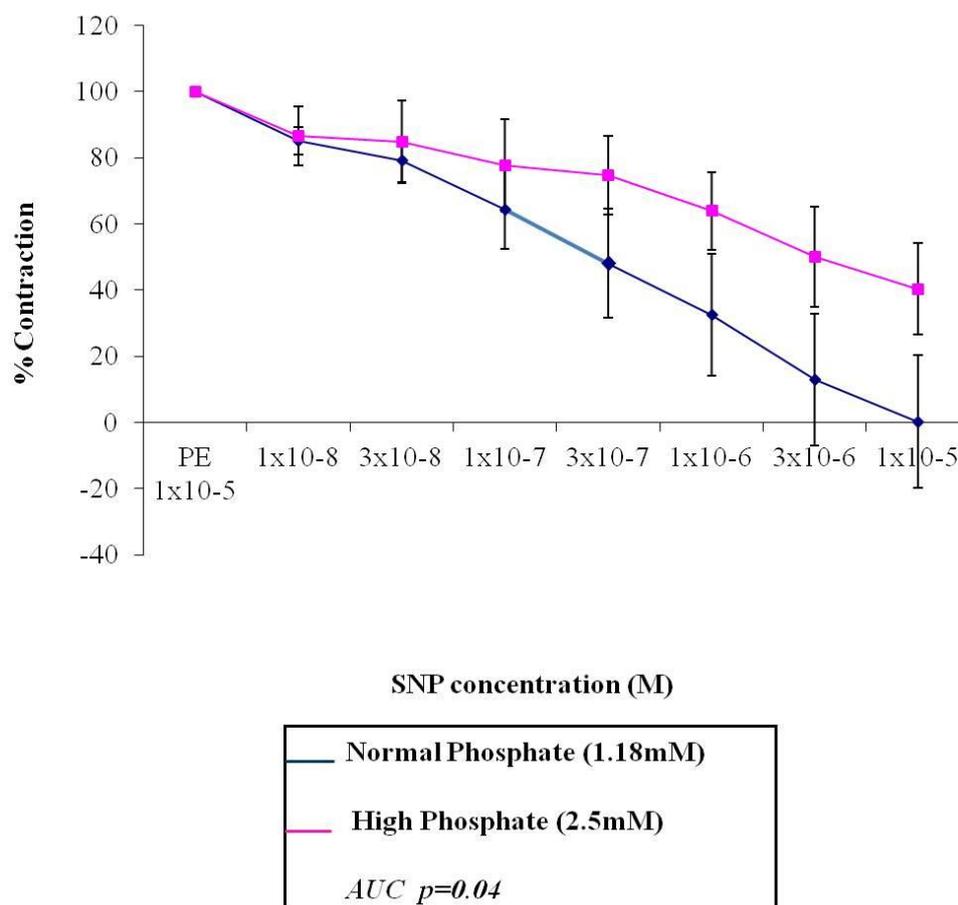


Figure 5-4: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1×10^{-5} M (living kidney donors).

Subcutaneous resistance vessels from abdominal fat from living kidney donors, collected at the time of donation, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, $n=13$; high phosphate, $n=12$) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was added in increasing concentrations in a stepwise fashion (1×10^{-9} to 1×10^{-5} M) to induce contraction. SNP was then added in increasing concentrations (1×10^{-8} to 3.5×10^{-5} M) to induce relaxation. Vessels were exposed to each concentration of PEP and SNP for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's *T* test.

5.3.3 Contraction and relaxation response in vessels from patients with CKD

Contraction with PEP was not significantly different between the vessels incubated in normal or high phosphate concentration PSS (Figure 5-5). The potency of PEP was similar in both groups with no significant difference in the EC_{50} ($1.77 \times 10^{-6} \pm 1.17 \times 10^{-6}$ M vs $1.08 \times 10^{-6} \pm 1.12 \times 10^{-6}$ M) or in maximal contraction (1.62 ± 0.69 g vs 1.37 ± 0.73 g). When compared with vessels from live kidney donors, there was no difference in EC_{50} , the maximal contraction with PEP or in AUC.

Relaxation response to carbachol is illustrated in Figure 5-6. The vessels incubated in high phosphate PSS vasodilated significantly less well to carbachol than those incubated in normal phosphate PSS (AUC, $p=0.008$). Maximum vasodilatation obtained in the vessels incubated in high phosphate was lower than in the vessels incubated in normal phosphate ($25.3 \pm 11.1\%$ vs $75.7 \pm 13.6\%$; $p < 0.001$). When compared with vessels from live kidney donors, there was not a significant difference in either AUC or maximal relaxation between the two groups of vessels incubated in normal phosphate PSS. Compared with the vessels from patients with CKD, the vessels from live donors incubated in high PSS relaxed significantly better to carbachol (AUC $p=0.03$)

The results for vasodilatation to SNP are shown in Figure 5-7. Following constriction with PEP, there was not a significant difference in relaxation to SNP between the vessels incubated in high or normal phosphate PSS. Maximum relaxation was not significantly different. When compared with the vessels from live kidney donors, the vessels from the patients with CKD relax less well to SNP both in term of AUC and maximal relaxation but this does not achieve statistical significance.

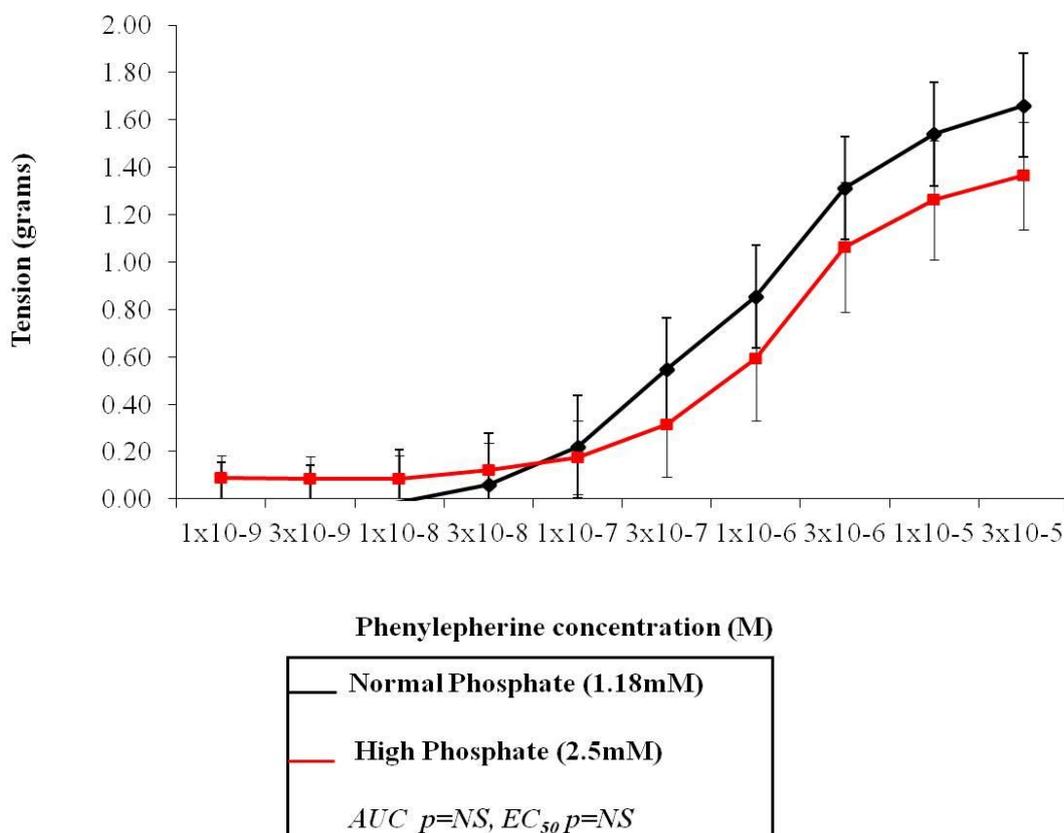


Figure 5-5: Contraction to PEP in vessels from patients with CKD.

Subcutaneous resistance vessels from abdominal fat from patients with CKD, collected at the time of live kidney transplant, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=12; high phosphate, n=9) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was then added in increasing concentrations in a stepwise fashion (1×10^{-9} to 3.5×10^{-5} M) to induce contraction and to allow the construction of cumulative concentration-response curves. Vessels were exposed to each concentration of PEP for three minutes or until a peak response at that concentration was reached. Response is expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

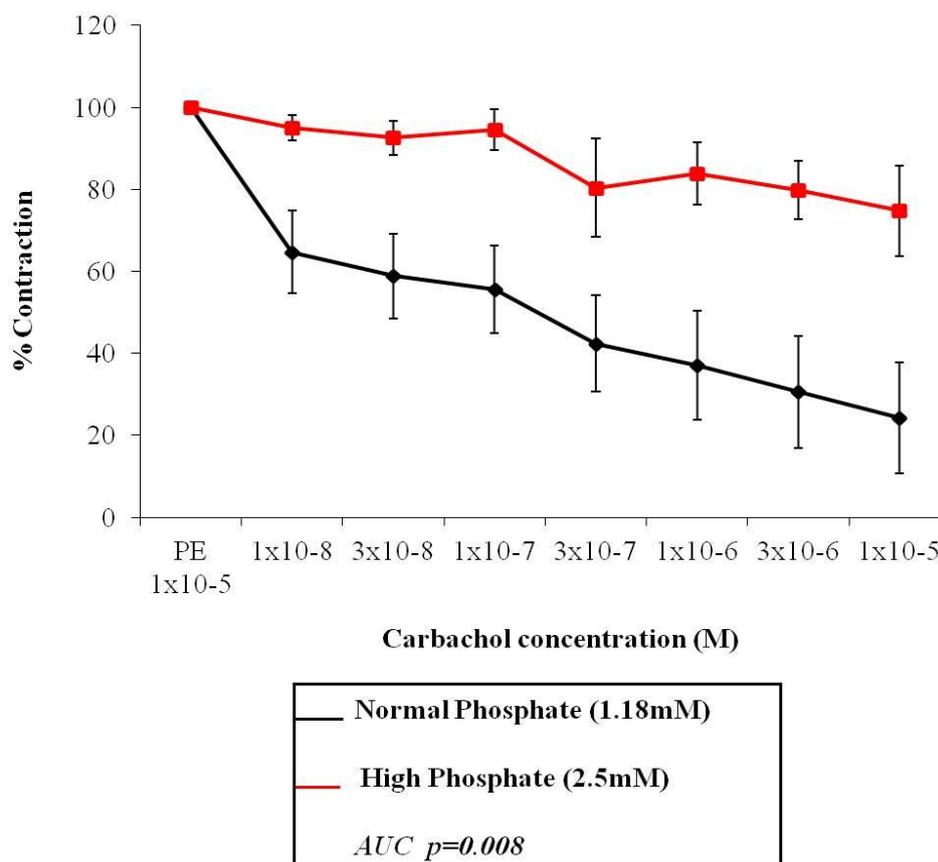


Figure 5-6: Vasodilatation to increasing concentrations of carbachol expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M (CKD).

Subcutaneous resistance vessels from abdominal fat from patients with CKD, collected at the time of live kidney transplant, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=12; high phosphate, n=9) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁹ to 1x10⁻⁵M) to induce contraction. Carbachol was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and carbachol for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

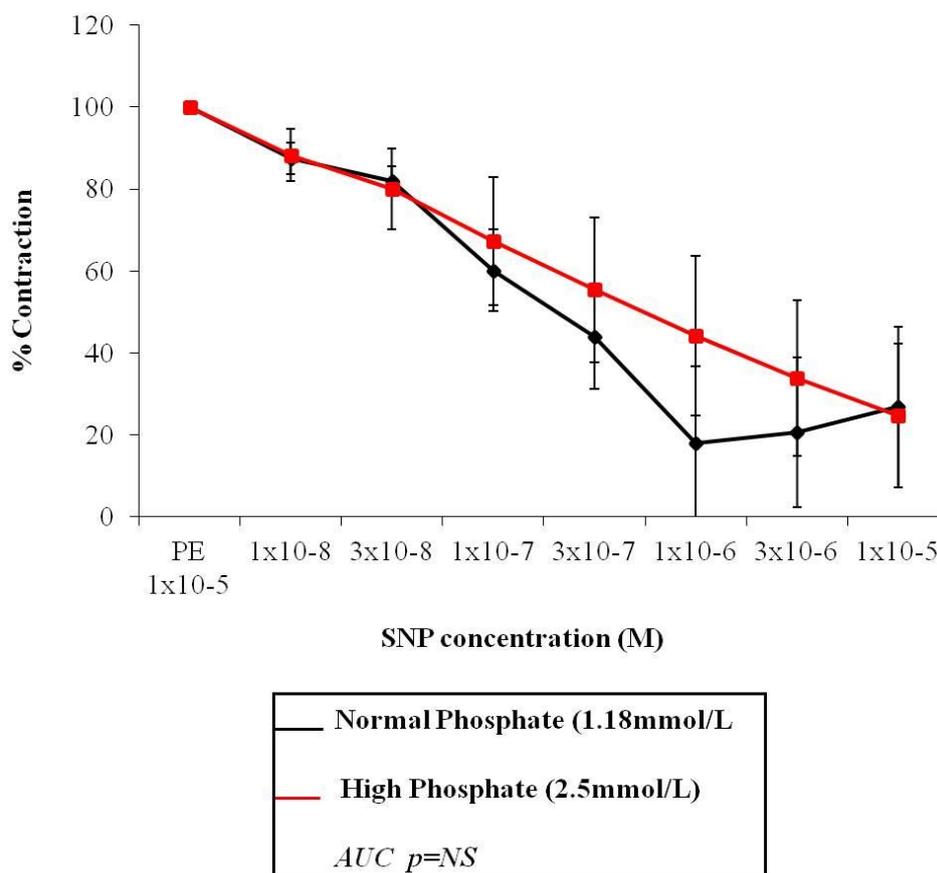


Figure 5-7: Vasodilatation to increasing concentrations of SNP expressed as a % of maximal contraction (100%) with PEP 1x10⁻⁵M (CKD).

Subcutaneous resistance vessels from abdominal fat from patients with CKD, collected at the time of live kidney transplant, were incubated in normal (1.18mM) or high (2.5mM) phosphate concentration PSS for 16 hours. Vessels (normal phosphate, n=12; high phosphate, n=9) were then mounted on a wire myograph and set, in stages, to 0.8g of tension. PEP was added in increasing concentrations in a stepwise fashion (1x10⁻⁹ to 1x10⁻⁵M) to induce contraction. SNP was then added in increasing concentrations (1x10⁻⁸ to 3.5x10⁻⁵M) to induce relaxation. Vessels were exposed to each concentration of PEP and SNP for three minutes or until a peak response at that concentration was reached. Cumulative concentration-response curves were constructed. Response expressed as mean \pm SEM and comparison made between AUC of the two groups by the Student's T test.

5.3.4 Superoxide measurement

O_2^- measurements were made in whole blood samples from living kidney donors and CKD patients. The demographics of these patients are shown in Table 5-1. The rate of superoxide production (expressed as pmol superoxide/ng of protein/minute) was slightly higher in the patients with CKD compared with the live donors: $3.93 \pm 1.5 \times 10^4$ and $3.38 \pm 1.6 \times 10^4$. This did not reach statistical significance (Figure 5-8). The patients were of a similar age and those with CKD had a higher phosphate and FGF-23 as would be expected with a lower eGFR. There were more males in the CKD group.

HUVECs cultured in 0.5mM phosphate concentration medium appear to produce less O_2^- (corrected for protein content) than cells cultured in 3mM phosphate medium but this does not reach statistical significance (Figure 5-9). Similar results were obtained from rat ECs (data not shown).

5.3.5 Immunoblotting for eNOS and nitrotyrosine

There was significantly less phospho and total eNOS (normalised to GAPDH) present in the HUVECs cultured in high phosphate medium (Figure 5-11 shows total eNOS expression and Figure 5-12, phospho eNOS expression). Figure 5-13 shows nitrotyrosine expression; cells cultured in high phosphate expressed higher quantities of nitrotyrosine (normalised to GAPDH) than cells cultured in normal phosphate concentration medium.

5.3.6 Griess reaction in HUVECs

As illustrated in Figure 5-13, there were significantly lower levels of NO in the cells cultured in high phosphate ($16.9 \pm 1.6 \mu\text{M}$ vs $21.7 \pm 1.3 \mu\text{M}$; $p=0.03$).

5.3.7 Intra-cellular calcium measurements

In HUVECs, the intra-cellular calcium measurements were not significantly different between cells cultured in high compared with normal phosphate concentration medium ($749.5 \pm 13.9 \text{nM}$ vs $752 \pm 54.2 \text{nM}$). In rat ECs and VSMCs, there was also no difference in the intra-cellular calcium content of cells cultured in normal compared with high phosphate concentration medium (Figure 5-14 illustrates the HUVEC and the rat VSMC data).

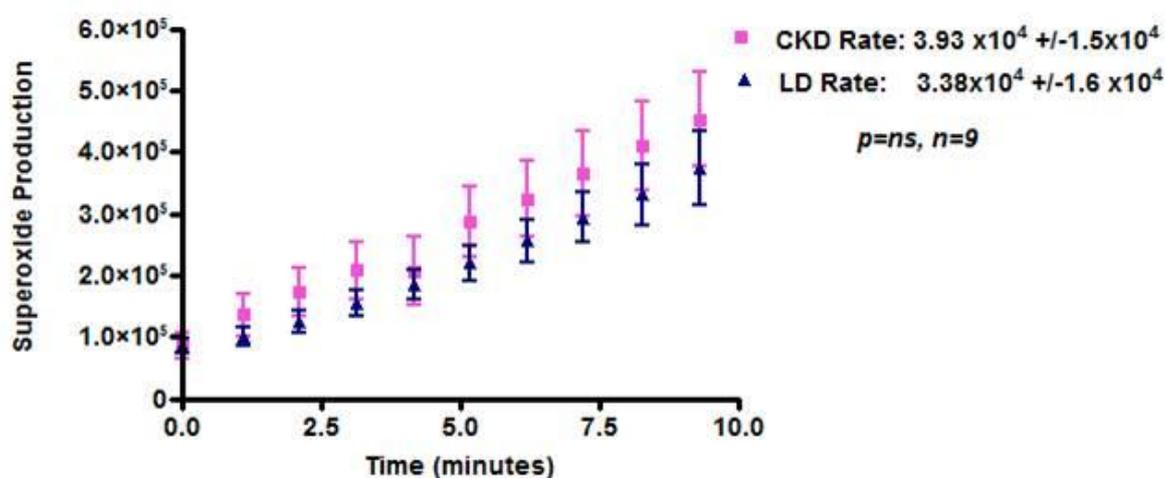


Figure 5-8: The rate of superoxide production (expressed as superoxide production/ng of protein/minute) in patients with and without CKD.

This was undertaken by Ms Elaine Friel (Technician, Institute of Cardiovascular and Medical Sciences). Whole blood samples were collected from patients with and without CKD ($n=9$ in each group). The samples were placed on ice immediately after collection. The Bruker e-scan EPR machine was calibrated using a reduced form of the spin probe, CP (3-carboxyl-proxyl radical) at concentrations of $1\mu\text{M}$, $5\mu\text{M}$ and $10\mu\text{M}$. Each was read ten times and the mean, standard deviation (SD) and coefficient of variation were calculated from these results. A HEPES buffer containing 100mM DTT and 20% (v/v) Tween-20 was used to keep the pH of the solutions constant at 7.4. DF and DTEC) were used as chelators at final concentrations of $25\mu\text{M}$ and $5\mu\text{M}$ respectively. CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine) was then used as a spin probe, labelling the superoxide present in the sample (final concentration 1mM). Everything was kept on ice throughout. Blood was added to HEPES buffer and chelators and finally CPH was added immediately before processing. The solution was drawn up by a $15\mu\text{l}$ capillary tube and sealed with 'cristoseal' sealant. After ten minutes on the EPR machine, the sample was removed and disposed of. Each blood sample was processed three times. Results were analysed in Microsoft excel using a linear equation and plotted in GraphPad Prism. Values are mean \pm SEM

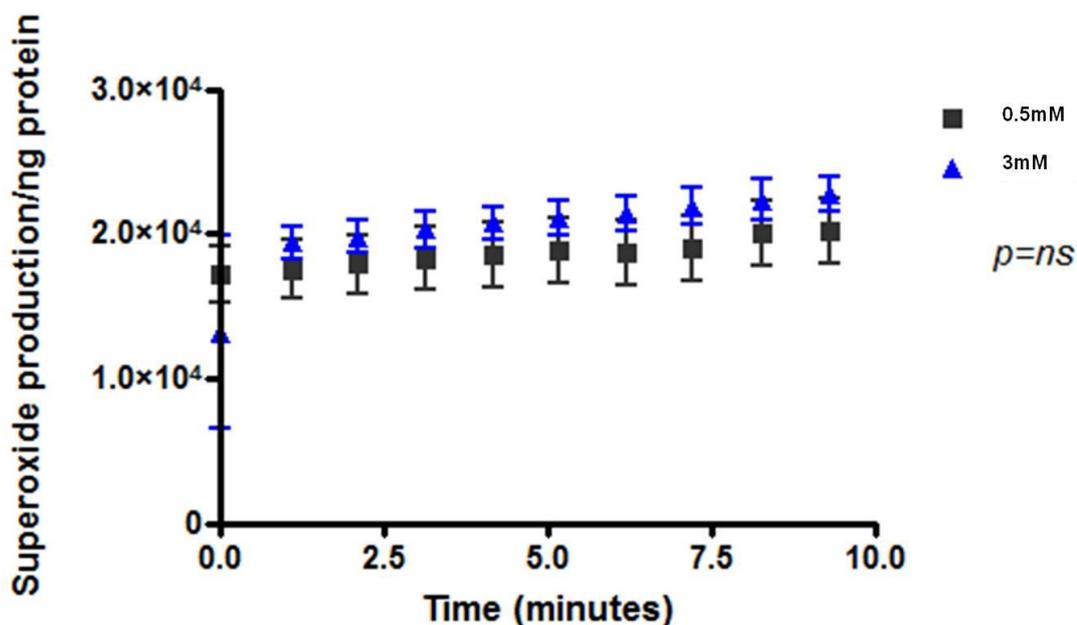


Figure 5-9: Superoxide production from HUVECs.

HUVECs were cultured and plated onto six well plates at a cell density of 1×10^4 cells/well. Upon reaching 90% confluence, the cells were washed twice with PBS and then incubated with a Krebs-HEPES buffer and the CPH spin probe for one hour at 37°C. The CPH spin probe and Krebs-HEPES buffer were also incubated in a well without cells for the same time period. The supernatant was then removed and immediately put onto ice before the solution was drawn up by a 15 μ l capillary tube and sealed with 'cristoseal' sealant and then run through the EPR machine in triplicate. The CPH without exposure to cells was used to assess the natural oxidation of the spin probe. The protein content of each well was quantified using the Bradford's assay. The figures obtained from the spin probe alone were subtracted from those attained with cells present and then normalised to the protein content of each well. Thus the final value is pmol superoxide/ng of protein/minute. Values are mean \pm SEM and represent replicates from four separate experiments.

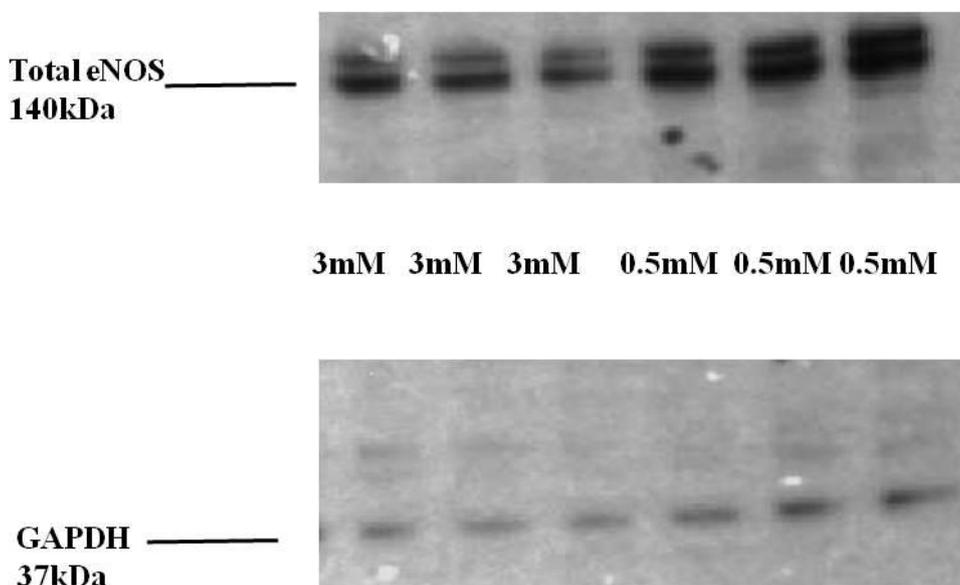


Figure 5-10: Total eNOS expression in HUVECs.

HUVECs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells were cultured until 90% confluence and then cells were placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a total eNOS, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top panel shows the cells cultured in 3mM phosphate concentration medium, in triplicate, followed by the cells cultured in 0.5mM phosphate concentration medium, in triplicate. The bottom panel shows GAPDH. The panels were all taken from a single gel and each band represents one well from three separate six well plates.

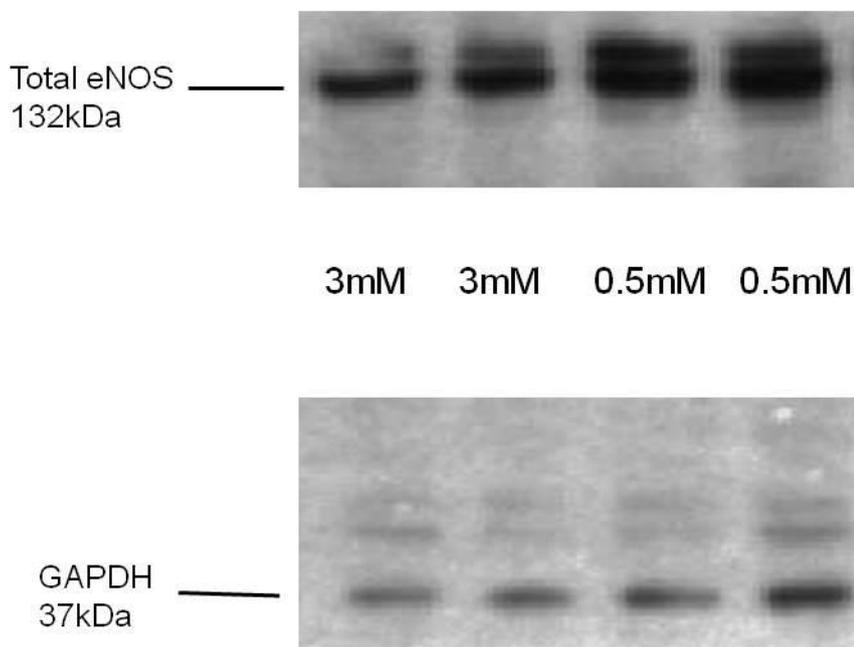


Figure 5-11: Phospho eNOS expression in HUVECs.

HUVECs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells were cultured until 90% confluence and then cells were placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a phospho eNOS, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top panel shows the cells cultured in 3mM phosphate concentration medium, in duplicate, followed by the cells cultured in 0.5mM phosphate concentration medium, in duplicate. The bottom panel shows GAPDH. The panels were all taken from a single gel and each band represents one well from three separate six well plates.

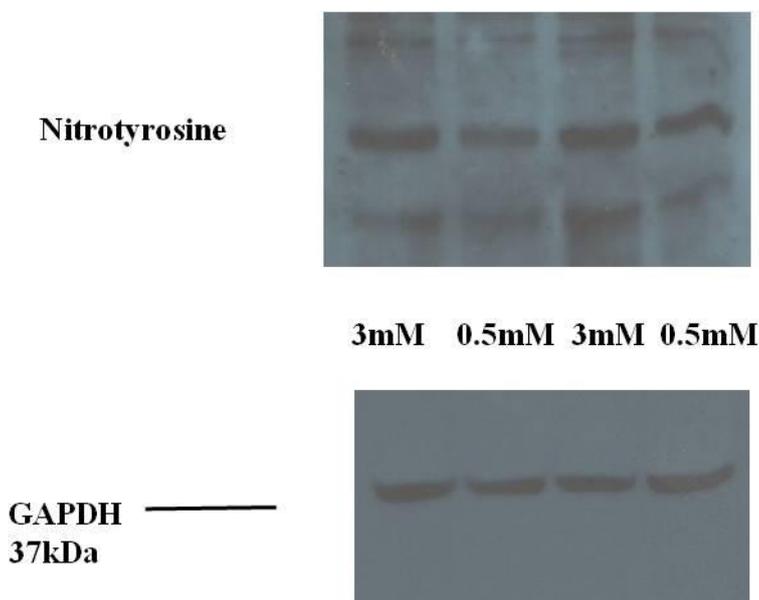


Figure 5-12: Nitrotyrosine expression in HUVECs. The antibody detects nitrotyrosine adducts on proteins.

HUVECs were cultured on six well plates at a cell density of 1×10^4 cells/well in 0.5mM and 3mM phosphate concentration medium. The cells were cultured until 90% confluence and then cells were placed on ice, washed with PBS and lysed using a RIPA buffer, made in house. The Bradford assay was used to quantify protein concentration. Proteins were resolved using the NuPAGE 4-12% Bis-Tris pre-cast gel system. Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS sample buffer heating to 70°C for 10 minutes. In order to reduce proteins, 50mM DTT was added immediately prior to sample denaturing. Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and the manufacturer's transfer buffer which was supplemented with 20% (v/v) methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS containing 0.1% (v/v) Tween 20. The nitrocellulose membranes were then blocked in 5% (w/v) BSA for 1 hour at room temperature. Blots were incubated with a nitrotyrosine, primary antibody at a 1/1000 concentration in 5% BSA (w/v) overnight at 4°C. Five washes of five minutes with TBST were then carried out prior to incubation with anti-rabbit HRP conjugated secondary antibody (1/2000) for two hours in 5% (w/v) BSA at room temperature. Blots were washed with TBST for five minutes five times. Proteins were visualised by enhanced chemiluminescence (Amersham, GE Healthcare, UK) and autoradiography. The blots were also probed for GAPDH (1/2000) as a primary antibody with anti-mouse HRP (1/2000) conjugated secondary antibody. The top panel shows nitrotyrosine, with high phosphate and normal phosphate cells alternating, in duplicate, and the bottom panel shows GAPDH. The panels were all taken from a single gel and each band represents one well from three separate six well plates.

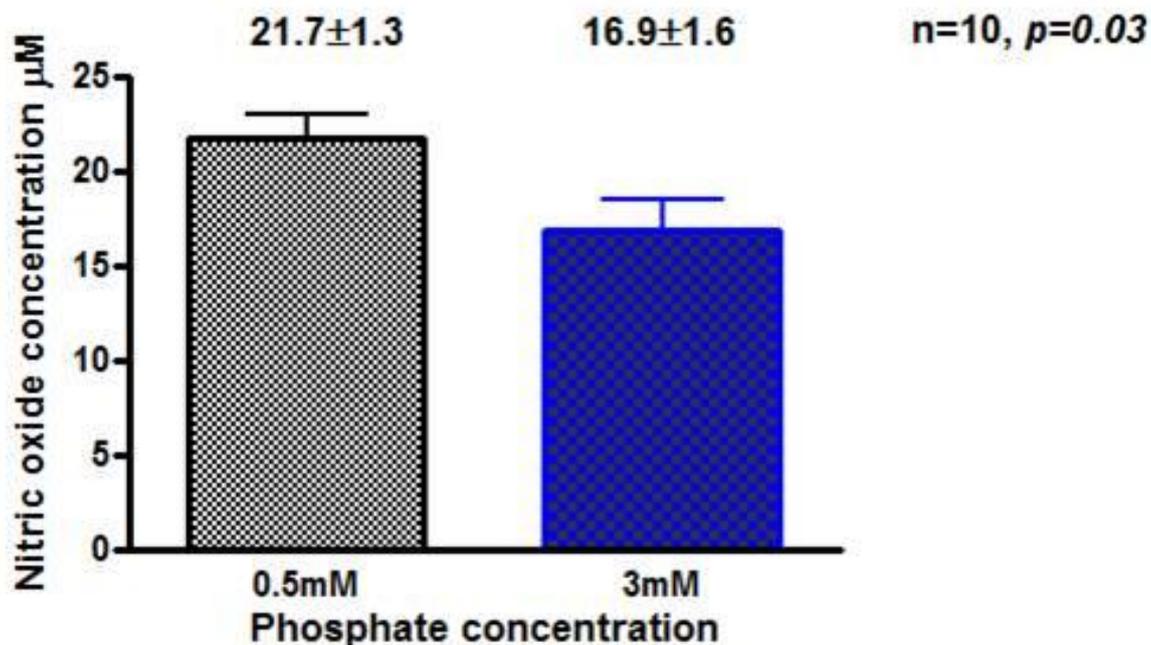


Figure 5-13: Relative NO concentration in HUVECs.

On day one, HUVECs were plated onto 12 well plates at a cell density of 1×10^5 cells/well. After 24 hours fresh media was added to each well. The media was replaced daily until day four when a Griess reaction was performed. Sodium nitrite was used to generate a standard curve in each plate at a concentration of 0-100µM. 50µl of Griess was added to each well, the plate was then wrapped in tinfoil and replaced in the incubator for 30 minutes before being read at an absorbance of 540nm on a microplate reader. This was performed in duplicate, an average taken and 10 replicates were used for each condition. Values are mean \pm SEM

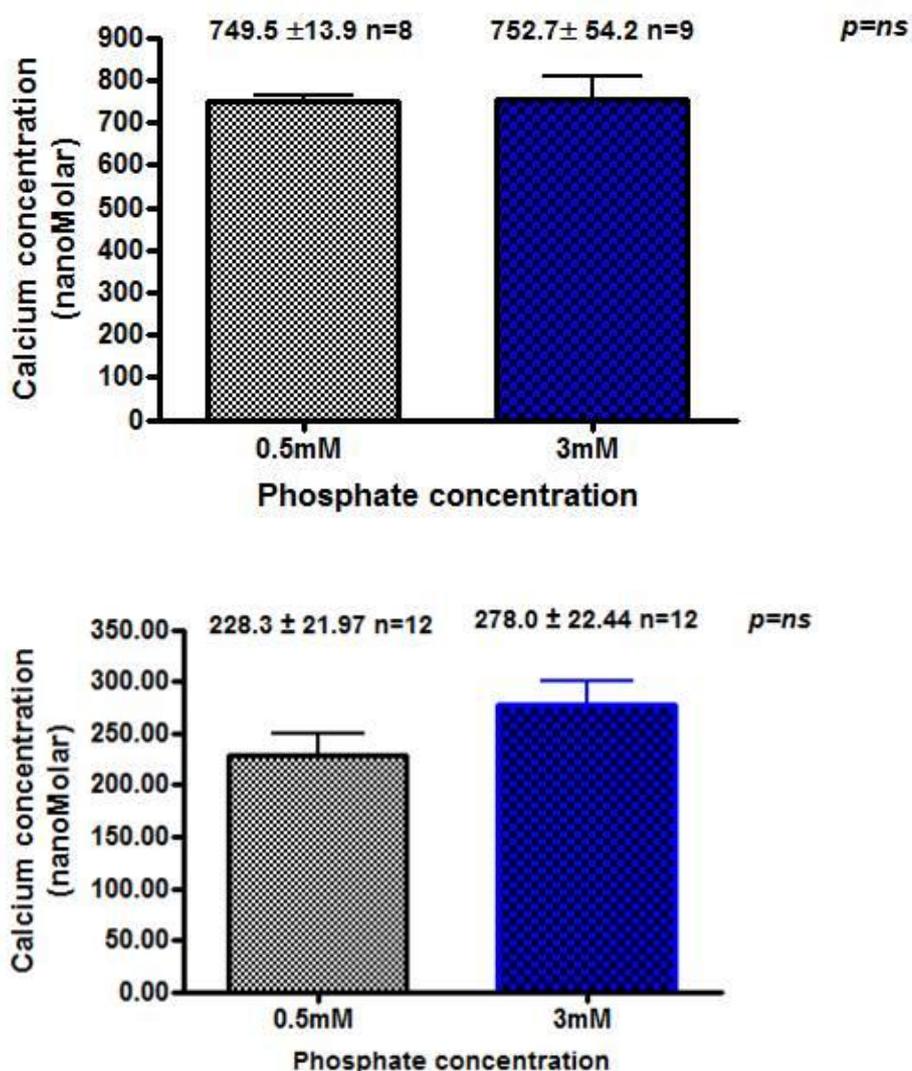


Figure 5-14: Intracellular calcium content of HUVECs (top) and rat VSMCs (bottom) cultured in normal (0.5mM) and high (3mM) phosphate concentration medium.

Cells were plated in glass bottomed plates and allowed to adhere overnight. The following day, cytosolic loading of FURA-2-AM was achieved by incubating the cells with FURA-2-AM (10 μ M endothelial cells and 5 μ M SMCs) at 37 $^{\circ}$ C for 40 minutes (ECs) or 15 minutes (VSMCs). The cells were then washed with 1ml of media and incubated at 37 $^{\circ}$ C for a further 15 minutes. Plates were mounted on an inverted microscope and isolated cells were imaged. Using an oscilloscope and the Clampex data acquisition software, baseline fluorescence was recorded. 2 μ M calcium ionophore was added to prove that a calcium sensitive signal could be generated. On addition of calcium ionophore, the 340/380 fluorescence ratio rises and plateaus. Following this the cell lyser, saponin was added (10 μ M) to trigger cell lysis. A recording was made until the 380 nm wavelength began to fall. The Clampfit programme (Molecular Devices LLC, USA) was used to analyse the data and to determine the baseline fluorescence and the R_{max} values. Values are mean \pm SEM and are from at least 3 experiments with the number of cells included indicated by (n).

5.3.8 Summary of results

In human resistance vessels, from live donors without CKD, incubated in high phosphate concentration PSS (2.5mmol/L) for 16 hours, using wire myography to assess vessel function there is evidence of:

- Impaired maximal contraction to PEP.
- Impaired endothelium dependent vasodilatation to carbachol.
- Impaired endothelium independent vasodilatation to SNP.

In vessels from patients with CKD, there is evidence of:

- Impaired endothelium dependent vasodilatation to carbachol.
- Improved endothelium dependent vasodilatation (similar to that of live donor vessels) to carbachol in the presence of a normal phosphate concentration PSS.
- Intact endothelium independent vasodilatation to SNP.

In whole blood samples from patients with CKD compared to those without CKD, there is a non-significant trend towards increased O_2^- production.

In HUVECs cultured in high phosphate medium (3mM) compared with standard phosphate medium (0.5mM):

- There is a non-significant trend towards increased O_2^- production.
- There is reduced expression of total and phospho eNOS (Ser 1177).
- There is significantly reduced NO production by the Griess reaction in cells cultured in high phosphate compared with normal phosphate.

Intracellular calcium is unchanged in cells (HUVECs, rat endothelial cells and rat SMCs) cultured in normal compared with high concentration phosphate medium.

5.4 Discussion

The *in vitro* study in human resistance vessels presented here demonstrates, for the first time, that exposing resistance vessels to elevated phosphate for a sustained period of time causes impaired endothelium dependent vasodilatation both in patients with and without CKD. This suggests a direct effect of phosphate on the endothelium and on the vascular smooth muscle cell and separates an effect of phosphate from other effects of uraemia. These findings are in keeping with the results of the *in vitro* study presented in chapter four. Thus, the endothelium in the high phosphate environment is less able to produce NO, upon stimulation. I confirm this in a human endothelial cell line by demonstrating reduced expression of eNOS and reduced levels of NO in cells which have been cultured, long term, in a high phosphate medium.

Morris et al described impaired endothelium dependent relaxation in human uraemic resistance vessels and therefore we expected to see impaired endothelium dependent relaxation in the vessels from our patients with CKD, incubated in normal phosphate concentration PSS, when compared with the live donor vessels also incubated in normal phosphate concentration PSS (95). The CKD vessels in our study relaxed as well as the live donor vessels when exposed to a normal phosphate environment. This suggests two things:

- There may be a circulating factor which is ‘washed out’ and removed when the CKD vessels are exposed, overnight, to a normal phosphate environment. Possibilities include asymmetric dimethylarginine (ADMA), ROS (including O_2^- and nitrotyrosine), advanced glycation end products, indoxyl sulphate and p-cresyl sulphate (377;378).
- The endothelium dependent dysfunction seen on exposure to a high phosphate environment is potentially reversible. This agrees with our findings in rat SMCs in chapter 5 and also strengthens the need to identify clearly how phosphate results in its adverse effects.

The possible circulating factors, mentioned above, are known to disrupt the NO pathway at different points and thus could also help to explain the findings of reduced eNOS expression and NO production in the HUVECs cultured in high phosphate medium.

Phosphate might increase ROS which may serve as a circulating factor and I show increased nitrotyrosine expression in HUVECs cultured in high phosphate medium.

There was a non-significant trend towards increased rate of production of the ROS, O_2^- in whole blood from the patients with CKD. The lack of a discernible difference is unclear but may simply rate to the small numbers of patients included. Similarly in HUVECs cultured in high phosphate medium, I also saw a non-significant trend towards an increase in O_2^- production. Possible explanations for this include too few replicates and difficulties with the natural degradation of the spin probe with the EPR technique. O_2^- has a short half life and a high reactivity which makes it difficult to measure. Additionally, I measured O_2^- in the cell supernatant and not intracellularly.

The aim was to separate the effects of phosphate from other effects of the uraemic environment. The significantly worse endothelium dependent vasorelaxation observed in the CKD vessels exposed to high phosphate concentration PSS when compared with those in normal phosphate PSS and the live donor vessels supports a directly detrimental effect of phosphate, separate from other uraemic factors including pH and potassium.

The similar level of relaxation seen in the CKD and live donor vessels incubated in normal phosphate PSS conflicts with results from Morris et al. There are several differences in method between the two studies which may explain the difference.

- Morris et al did not include potassium dihydrogen orthophosphate (KH_2PO_4) in the PSS which they used and thus the vessels were exposed to an artificial environment which contained no phosphate at all.
- The drugs utilised were different: noradrenaline and acetylcholine. Noradrenaline acts on both α_1 and α_2 adrenoceptors and acetylcholine was used at a higher dose by Morris et al, possibly inducing further relaxation. Additionally, in our study we pre-constricted with higher doses of PEP, which may have affected the relaxation ability of vessels.
- Morris et al normalised their vessels using Mulvany's method; I chose not to do this.

The vessels from living kidney donors demonstrated impaired endothelium independent relaxation to SNP when exposed to high phosphate PSS. This suggests disruption further down the NO pathway as a result of phosphate, for example alterations in guanylate cyclase expression or cGMP level. In chapter four we support this hypothesis by demonstrating reduced cGMP level in vessels incubated in high phosphate PSS and reduced PKG expression in cells cultured in a high phosphate medium.

In the CKD vessels, endothelium independent relaxation was worse in the vessels incubated in normal phosphate PSS when compared with the live donor equivalent vessels. This suggests a role for altered cGMP handling in the uraemic environment. However when comparing CKD vessels in high and normal phosphate PSS, there was not a difference in endothelium independent relaxation. This hints at other aspects of the uraemic environment, rather than phosphate, as the culpable factors for the observed differences between the endothelium independent vasorelaxation in CKD and live donor vessels.

One explanation for these observations is that chronic exposure to an altered phosphate environment, as is the case in CKD, allows adaption such that an additional phosphate load (as occurs with the high phosphate concentration PSS) does not have an additive detrimental effect on the cGMP pathway over uraemia alone.

Intra-cellular calcium is closely involved in the process of endothelium relaxation and one important alternative explanation for the results I demonstrate in rat and human resistance vessels is an alteration in the intracellular calcium level in a high phosphate environment. Using cell lines rather than vessels, I demonstrate that the intra-cellular calcium content is unchanged between cells cultured in normal and high phosphate concentration medium. The observed effects of phosphate are, therefore, independent of changes in calcium. To observe a difference of approximately 10% in tone, the difference in intracellular calcium content would need to be roughly 200nM (379).

The series of experiments presented in this chapter provide further evidence to support the following in the context of a hyperphosphataemic environment:

- Reduced NO and/or alterations in eNOS expression.
- Increased levels of ROS.

- Changes seen in the hyperphosphataemic environment can be modified.

5.5 Conclusion

Exposing human resistance vessels, from patients with and without CKD, to high phosphate concentration PSS leads to impaired endothelium dependent relaxation. The altered expression of eNOS and nitrotyrosine, the reduced production of NO seen in cells cultured in high phosphate medium, together with the trend towards higher O_2^- levels in the uraemic environment support the concept that phosphate disrupts the NO pathway and it is this which results in ED.

In CKD vessels, the improved relaxation seen upon exposure to normal phosphate concentration PSS suggests that a circulating factor which is ‘washed away’ is part of the pathogenesis of the ED seen with hyperphosphataemia. This implies reversibility of the effects of hyperphosphataemia which is crucial if we are to be able to reduce CV risk attributable to elevated phosphate.

The findings here support those presented in chapter four where I demonstrated disruption to the NO pathway and that similar abnormalities in rat resistance vessels could be at least partially reversed with the addition of an antioxidant or PDE5I. To further investigate the reversible nature of the impaired endothelium dependent relaxation seen in human resistance vessels, these experiments should be repeated in human resistance vessels, in the presence of PDE5I, from patients with and without CKD.

My goal was to study the effects of longer term exposure to elevated phosphate than in previous studies and to separate the effects of phosphate from other effects of the uraemic environment. Importantly, I show in three cell lines that any observed effects of phosphate are not the result of alterations in intra-cellular calcium content.

In attempting to tease out the underlying mechanism of action of phosphate as a cardiovascular risk factor, I provide strong evidence in both chapters four and five of ED as a result of disruption to the NO pathway. In chapter six, I describe further cell studies designed to investigate the effects of reduced available NO on cells cultured in a high phosphate concentration medium.

6 Chapter Six – Studies of the effect of phosphate on the growth and proliferation of cultured cells.

6.1 Introduction

In chapters four and five, I presented a series of studies providing evidence that exposure to high phosphate disrupts the NO pathway. Whilst undertaking these studies, simple observation suggested that the cells cultured in high phosphate concentration media seemed to proliferate more quickly and appeared bigger than the cells cultured in standard phosphate concentration medium. This was an interesting observation which I elected to explore further to ascertain if there was any measurable difference in the proliferation of the cells cultured in the two different phosphate concentrations.

NO has been studied extensively, particularly in the setting of cancer, and there is significant evidence of an inhibitory effect on growth (380-382). This provides a possible explanation for the effect of phosphate on growth, given its effects on NO production. Increased growth of cells in high phosphate conditions, for example VSMCs, might provide a further link between phosphate, NO and CVD.

In CKD, any effect of NO on growth in the presence of elevated phosphate may be of particular relevance to the heart. LVH is an independent risk factor for the development of development of cardiac failure, arrhythmias and sudden death and it is highly prevalent in patients with CKD. Up to 40% of pre-dialysis patients and 75% of dialysis patients (232;383;384) have LVH. Strategies to reduce CV risk in CKD therefore should include prevention of, and regression of LVH. The aetiology of LVH is multi-factorial, and incompletely understood, contributing to problems in its management (385). Serum phosphate and its regulators have been linked, independently of traditional risk factors like hypertension, to the development of LVH in CKD (268;385). In the myocardium, high phosphate may cause reduced NO production and associated cell proliferation, offering a potential pathophysiological link between hyperphosphataemia and CVD in CKD.

The series of studies presented in this chapter were therefore designed to test the hypothesis that elevated phosphate concentration alters NO production and cell proliferation in a high phosphate environment. Specifically these studies sought to examine:

- Differences in proliferation in cells cultured in standard and high phosphate medium by the MTT proliferation assay (HUVECs, rat ECs, rat VSMCs and cardiac myocytes).

- Differences in gene expression with particular focus on genes involved in cell cycle regulation and cell growth by Microarray and RT-PCR (HUVECs).

6.2 Materials and Methods

6.2.1 Staining and imaging cells

This is described in section 2.4.3.

6.2.2 MTT proliferation assay

The basic assay is described in section 2.4.4. This was performed in rat VSMCs, rat ECs, HUVECs and cardiac myocytes, cultured in normal and high phosphate concentration media. In HUVECs, rat VSMCs and cardiac myocytes, the experiments were repeated in the presence of L-NAME. In HUVECs and cardiac myocytes, the experiments were repeated in the presence of FGF-23 and Klotho. Lastly, the HUVEC assays were performed in the presence of IL1 β .

6.2.3 Microarray and PCR

These techniques are described fully in sections 2.5.1 to 2.5.4.

6.3 Results

6.3.1 Cell staining and imaging

The cells cultured in high phosphate appeared bigger on simple staining and imaging. This was true of all four cell lines. Example images of rat VSMCs and human cardiac myocytes are shown in Figures 6-1 and 6-2.

6.3.2 Qualitative PCR

This confirmed that ECs, cultured in standard phosphate concentration medium, express eNOS, the FGFR, Klotho and the type two sodium phosphate co-transporter (Figure 6-3).

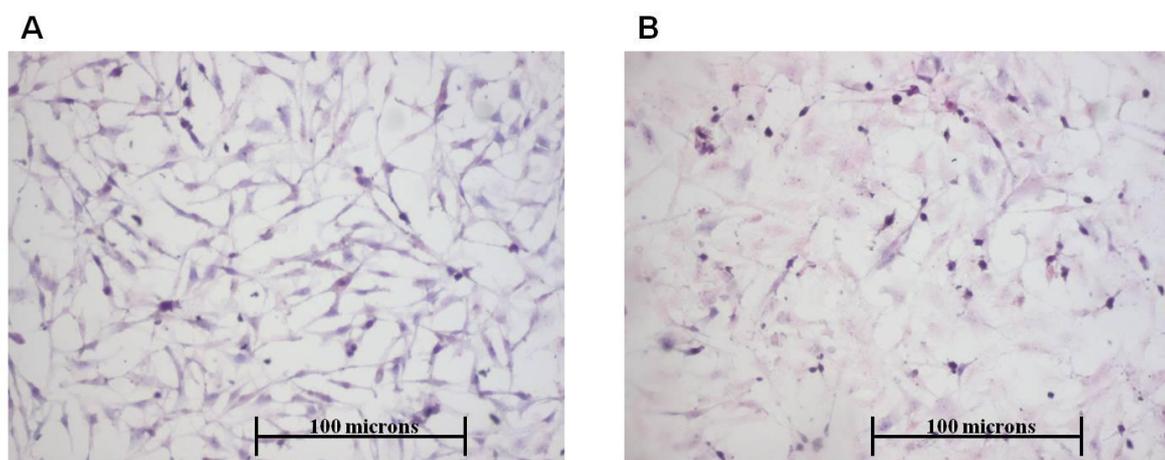


Figure 6-1: Rat smooth muscle cell cultured in A, normal phosphate (0.5mM) and B, high phosphate (3mM) concentration media.

Cells were plated onto four chamber glass slides at a density of 1.8×10^3 cells per chamber. The following day the cells were washed with PBS and then fixed with paraformaldehyde 4% (w/v). The cells were washed in PBS and then stained with haematoxylin (0.1% w/v) and eosin (0.2% w/v). The slides were washed in 70% (v/v), 90% (v/v) and then 100% (v/v) ethanol and placed in histoclear. A cover slip was placed into the slide and the cells were then image and photographed with an Olympus BX40 microscope and a Micropublisher RTV camera using Image Pro Software.

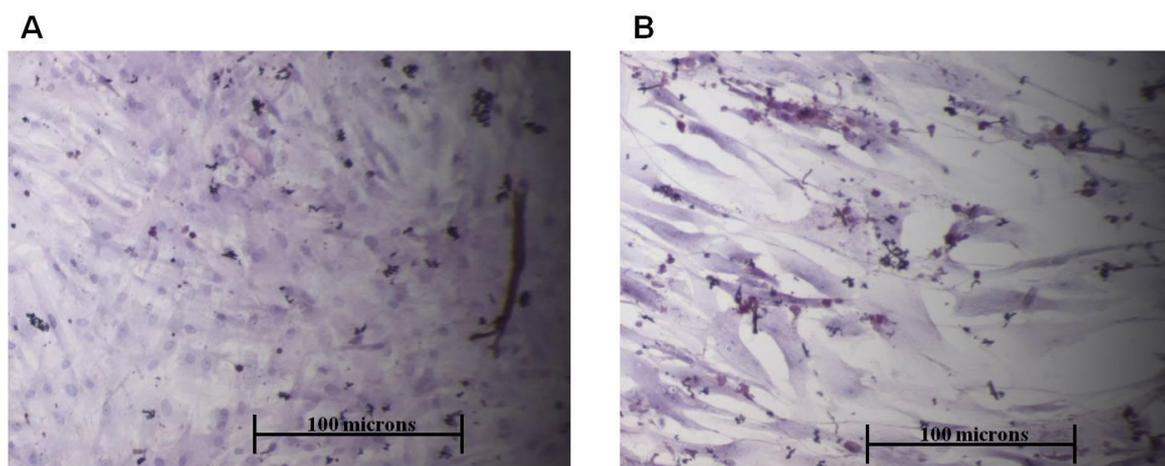


Figure 6-2: Human cardiac myocytes, cultured in A, normal phosphate (0.5mM) and B, high phosphate (3mM) concentration media.

Cells were plated onto four chamber glass slides at a density of 1.8×10^3 cells per chamber. The following day the cells were washed with PBS and then fixed with paraformaldehyde 4% (w/v). The cells were washed in PBS and then stained with haematoxylin (0.1% w/v) and eosin (0.2% v/v). The slides were washed in 70% (v/v), 90% (v/v) and then 100% (v/v) ethanol and placed in histoclear. A cover slip was placed into the slide and the cells were then image and photographed with an Olympus BX40 microscope and a Micropublisher RTV camera using Image Pro Software.

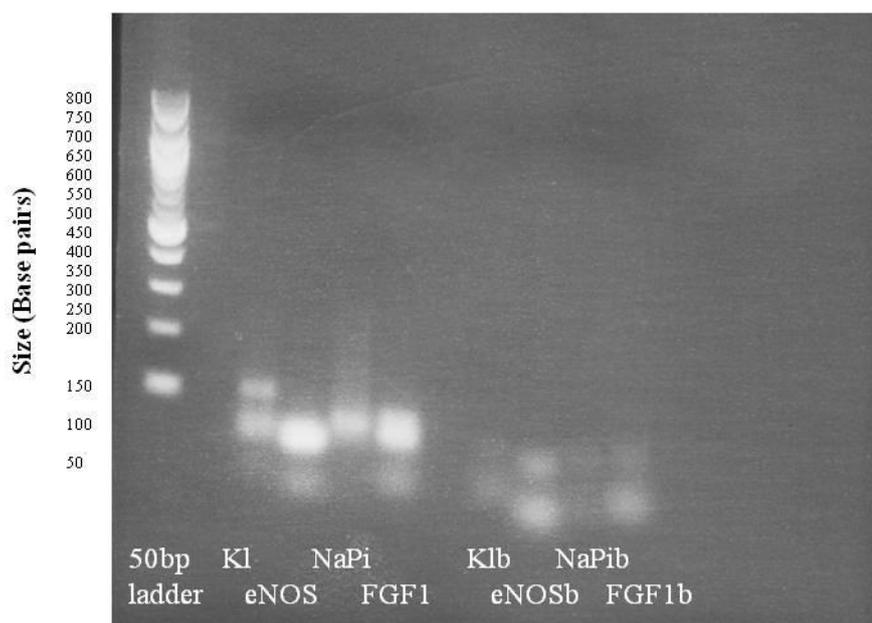


Figure 6-3: Qualitative PCR showing expression of Klotho (KL), eNOS, the FGFR (FGF1) and NaPi2b in HUVECs. Control bands run with water and not DNA are also shown (suffix b).

HUVECs were cultured in standard phosphate concentration medium (0.5mM).on 6 well plates at a cell density of 1×10^4 cells per well. Cells were trypsinised at 90% confluence and cytoplasmic RNA was extracted using the Qiagen RNeasy Mini Kit, cDNA was then synthesised using the Invitrogen M-MLV reverse transcriptase kit and PCR was performed using a PTC-100 programmable thermal controller with the Invitrogen Superscript III™ first strand system for RT PCR. A $1 \mu\text{l}$ of template (0.2ng), $0.25 \mu\text{l}$, 100nM forward and reverse primers, 0.5mls of 10mM dNTPs, $0.25 \mu\text{l}$ Taq polymerase (5U/ μl), $5 \mu\text{l}$ 10x buffer (200mM Tris-HCl (pH 8.4), 500mM KCl) and PCR-grade water were combined to give a $25 \mu\text{l}$ reaction volume. Samples were denatured at 94°C for 30 seconds then primers annealed at 55°C for 45 seconds and DNA extended at 68°C for 1 minute. This thermal cycling profile was used for 35 cycles. The DNA samples were mixed with blue loading buffer and $15 \mu\text{l}$ of sample/loading buffer mix was loaded onto the agarose gel. The DNA samples were run at 100V for 1 hour with TAE buffer (0.04M Tris-acetate, 0.001M EDTA). The 2% Agarose gels were made using 0.8g agarose for molecular biology, 40ml of TAE buffer and $4 \mu\text{l}$ ethidium bromide. An ultraviolet transilluminator was used to visualise bands and images were recorded on a BioRad ChemiDoc XRS digital imaging system.

6.3.3 MTT proliferation assays

6.3.3.1 Endothelial cells

HUVECs cultured in high phosphate proliferated more than those cultured in normal phosphate concentration media ($p < 0.001$). When cells cultured in 0.5mM phosphate concentration medium were exposed to L-NAME, proliferation increased such that there was a significant difference between these cells and cells cultured in 0.5mM media alone ($p < 0.001$) and no difference between these cells and cells cultured in high phosphate medium (3mM). This is illustrated in Figure 6-4. Adding L-NAME to cells cultured in high phosphate medium did not alter proliferation rate (data not shown). When the cultured cells were exposed to FGF-23 and Klotho, there was no added effect on proliferation in either 0.5mM or 3mM phosphate concentration media (Figure 6-4).

Lastly, the addition of IL1 β made no significant difference to the proliferation effect of phosphate; there was a trend to increased proliferation in the cells cultured in normal phosphate concentration medium. The differences in proliferation between cells cultured in normal and high phosphate concentration media alone were maintained (data not shown).

In rat ECs, cells cultured in high phosphate also proliferated significantly more than those in normal phosphate ($p < 0.05$; data not shown).

6.3.3.2 Human Cardiac Myocytes

Cells cultured in high phosphate medium proliferated significantly more than those cultured in normal phosphate medium ($p < 0.001$). The addition of L-NAME increased proliferation, although not significantly, in the cells cultured in 0.5mM phosphate. L-arginine had no effect (Figure 6-5). In cells cultured in 3mM phosphate, the addition of L-arginine (data not shown) or L-NAME did not affect proliferation (Figure 6-5).

6.3.3.3 Rat VSMCs

Rat VSMCs cultured in high phosphate proliferated significantly more than those cultured in normal phosphate ($p < 0.05$). The addition of L-NAME had no effect on proliferation at either phosphate concentration (data not shown).

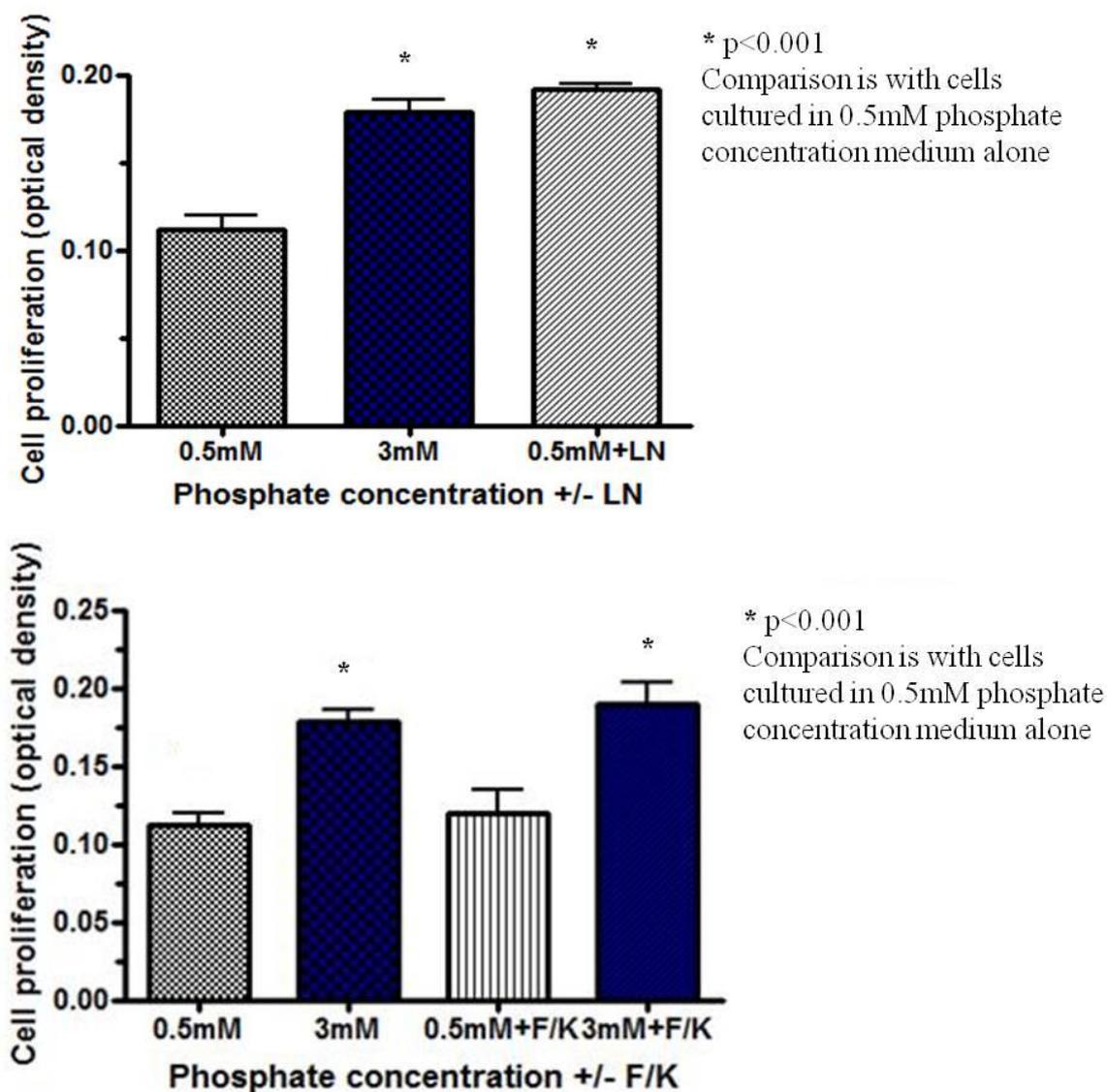


Figure 6-4: Cell proliferation in HUVECs +/- L-NAME (top) and +/-FGF-23 and Klotho (bottom).

Cells were cultured, in either normal (0.5mM) or high (3mM) phosphate concentration media, on 12 well plates at a cell density of 1×10^5 cells per well. Cells were plated on day one and the media was changed daily. On days two and three when the media was changed, L-NAME ($1 \times 10^{-4}M$) or FGF-23 ($3.5 \times 10^{-7}M$) and Klotho ($2 \times 10^{-6}M$) were added to selected wells. On day four, MTT was added (final concentration 1mg/ml) and the cells were incubated for three and a half hours. The media/MTT solution was removed and MTT solvent was added (1:1 ethanol/DMSO mixture). The plate was covered and agitated on an orbital shaker before being read on a microplate reader. There were control wells in each plate which contained media but no cells. The figures are the combined results from six separate experiments. For each experiment, there were two control wells per phosphate concentration, two wells containing cells only and two wells containing either LNAME or FGF-23 and Klotho. Values are mean \pm SEM.

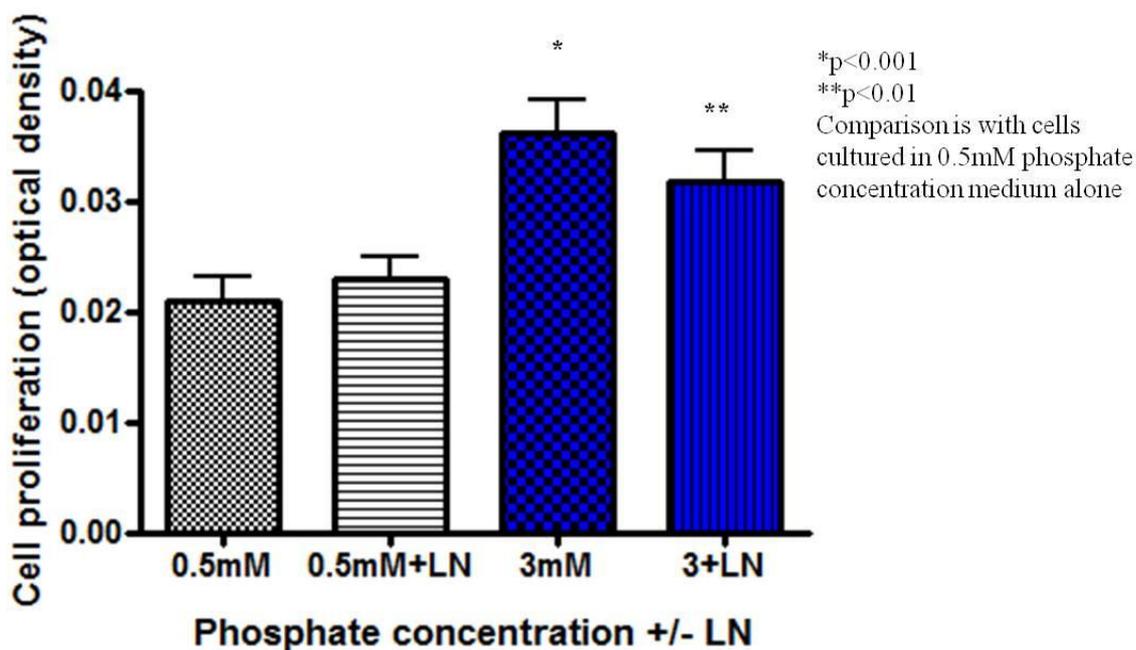


Figure 6-5: Cell proliferation in cardiac myocytes +/- L-NAME.

Cells were cultured, in either normal (0.5mM) or high (3mM) phosphate concentration media, on 12 well plates at a cell density of 1×10^5 cells per well. Cells were plated on day one and the media was changed daily. On days two and three when the media was changed, L-NAME ($1 \times 10^{-4}M$) was added to selected wells. On day four, MTT was added (final concentration 0.5mg/ml) and the cells were incubated for one hour. The media/MTT solution was removed and MTT solvent was added (1:1 ethanol/DMSO mixture). The plate was covered and agitated on an orbital shaker before being read on a microplate reader. There were control wells in each plate which contained media but no cells. The figure shows the combined results from four separate experiments. For each experiment, there were two control wells per phosphate concentration, two wells containing cells only and two wells containing LNAME. Values are mean \pm SEM.

6.3.4 Gene expression studies

6.3.4.1 Microarray

Prior to cDNA synthesis, HUVEC total RNA quality was confirmed by electrophoresis of the total RNA samples on the Agilent Bioanalyzer 2100 (Dr Jing Wang, Glasgow Polyomics centre, University of Glasgow). Figure 6-6 shows a representative result. There is a marker peak evident at 25 nucleotides and defined bands for 28S and 18S rRNA species. Below the 18S band, between 25 and 200 nucleotides, there is a very small hump which is 5S and transfer RNA. RNA Integrity Numbers (RINs) were consistently above 9.5. Following IVT to synthesise biotinylated cRNA, cRNA quality was also assessed by electrophoresis on the Agilent Bioanalyzer 2100. Results demonstrated approximately normal distribution and equal concentrations of cRNA for all samples (Figure 6-7).

6.3.4.2 Microarray Data Quality Control and Beadstudio analysis

Direct Hyb Control Plots obtained for samples were consistent with BeadStudio example control plots, verifying the validity of microarray gene expression data. Using Illumina® BeadStudio software, a total of 131 probes were found to be differentially expressed by a minimum of 2 fold between HUVECs cultured in normal or high phosphate concentration medium. Expression of 58 probes was up-regulated in HUVECs cultured in high phosphate whilst 73 probes were down-regulated. A complete list of differentially expressed probes is shown in Table A1 in the Appendix (section 9.2).

6.3.4.3 MetaCore™ Analysis

Using MetaCore™ software, analysis of BeadStudio microarray data identified aspects of the cell cycle as being significantly affected in cells cultured in high phosphate; seven of the ten most significantly affected pathways included the cell cycle (Figure 6-8). Significance is based upon the total number of genes with differentially expressed probes which map to the particular pathway. The two most significant pathways were ‘The Role of APC in cell cycle regulation’ and ‘Chromosome condensation in prometaphase’ (Figures 6-9 and 6-10). From these pathways, I identified candidate genes for further study to confirm differential expression: *CCNA2*, *CCNB2* (encoding Cyclin A, CyclinB; generic binding proteins), *AURKA* and *AURKB* (encoding Aurora-A and Aurora B; protein kinases). Each of these genes was upregulated in high phosphate conditions (Table A1, appendix).

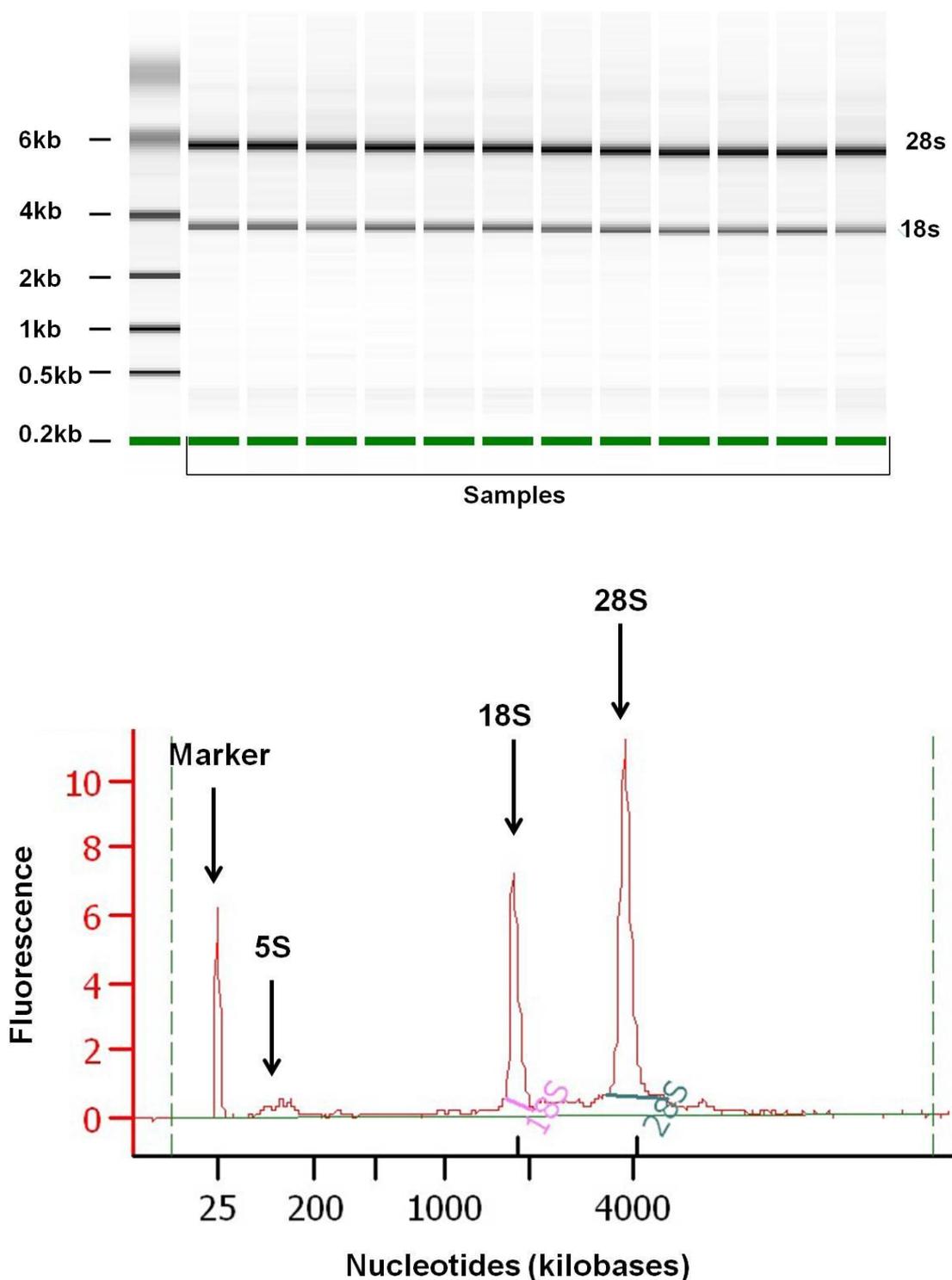


Figure 6-6: Agilent Bioanalyzer 2100 Assessment of Total RNA Quality.

The top figure shows electrophoresis of a single representative total RNA sample with defined bands for 28S and 18S rRNA. The green bands represent a 200 base pair size marker (to align samples and controls). The bottom figure is a representative individual electropherogram with the marker peak and defined peaks for 28S and 18S rRNA. No RNA species smaller than 18S rRNA were observed - indicative of intact RNA. This was undertaken by Dr Jing Wang, Glasgow Polyomics centre, University of Glasgow.

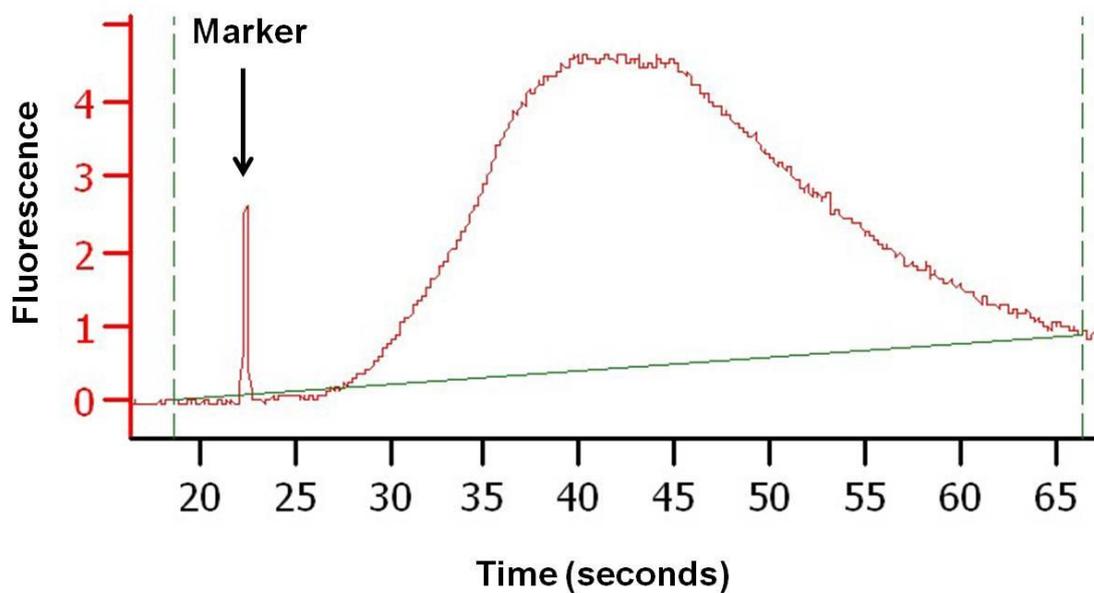


Figure 6-7: Agilent Bioanalyzer 2100 Assessment of cRNA Quality.

This shows a single representative electropherogram showing approximately normal distribution of cRNAs. The 200 bp size marker is shown. This was undertaken by Dr Jing Wang, Glasgow Polyomics centre, University of Glasgow

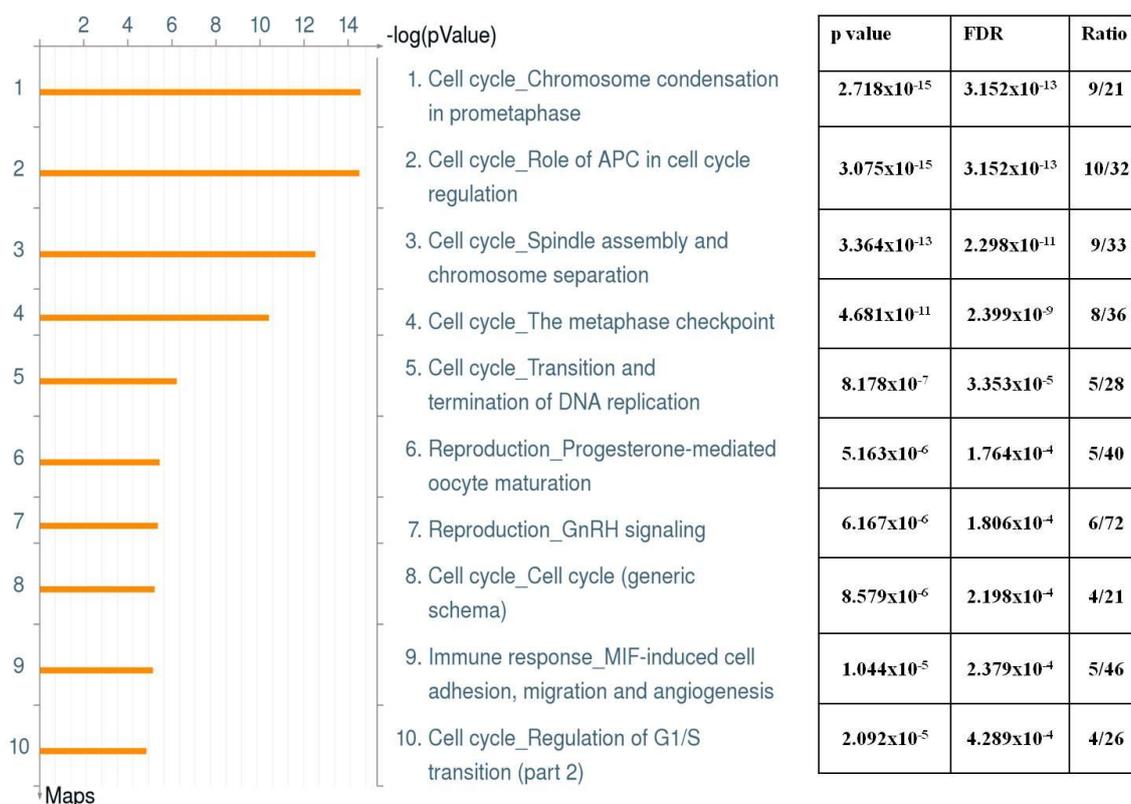


Figure 6-8: MetaCore™ pathway analysis of BeadStudio Microarray data.

Pathway maps significantly affected by the presence of high phosphate in cell growth medium were observed to cross the threshold value ($p \leq 0.05$). Significance is based on the number of differentially expressed genes in the pathway. The table to the right shows the actual p value and the false discovery rate (FDR). The ratio is the number of differentially expressed genes mapping to the pathway divided by the total number of genes mapping to the pathway. Differentially expressed genes involved in the cell cycle were identified as candidate genes for further investigation.

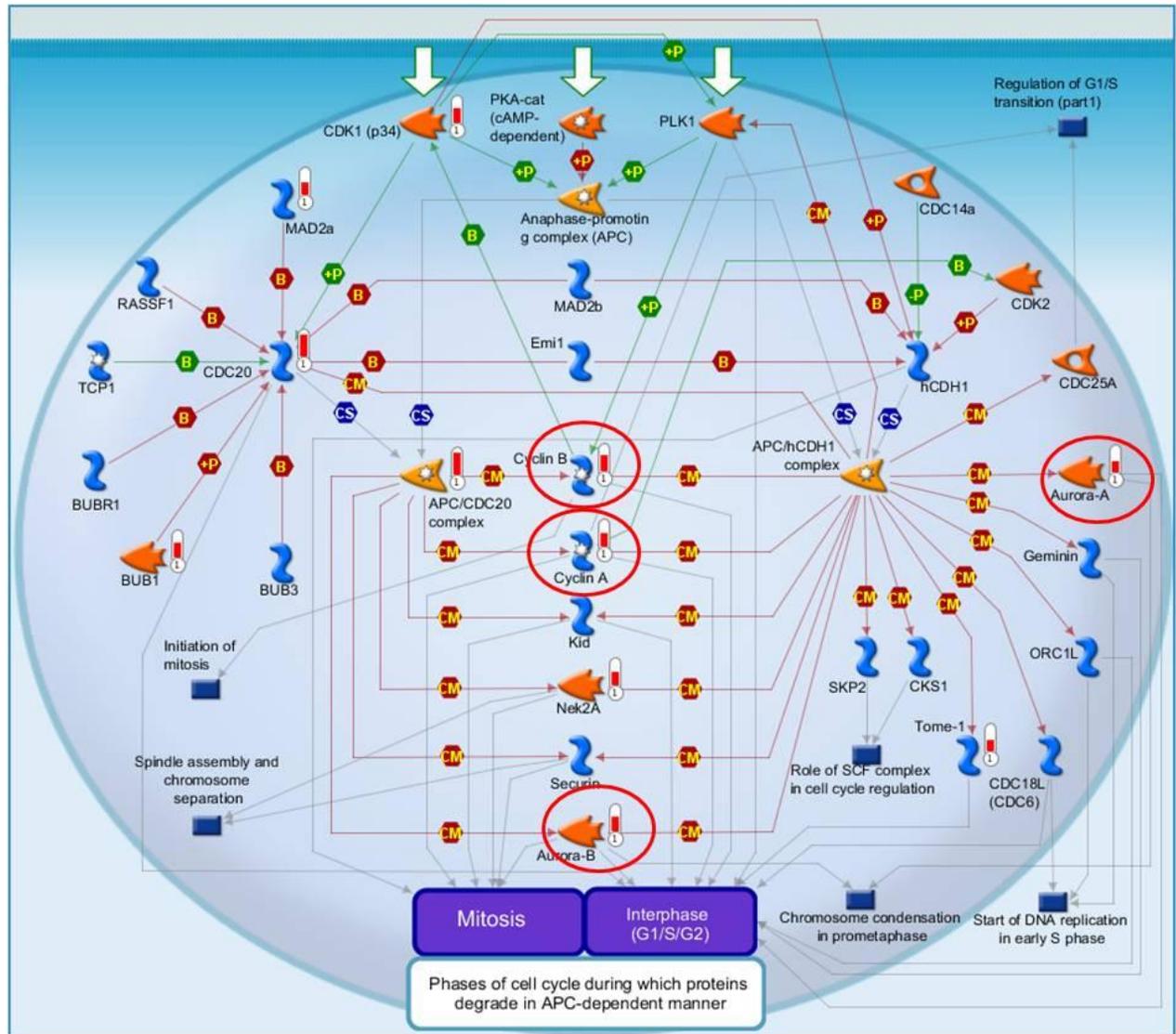


Figure 6-9: The Role of APC in cell cycle regulation.

The products encoded by candidate genes are circled in red and the adjacent small red and white bars indicate up-regulation in high phosphate conditions. For legend see Figure 6-11.

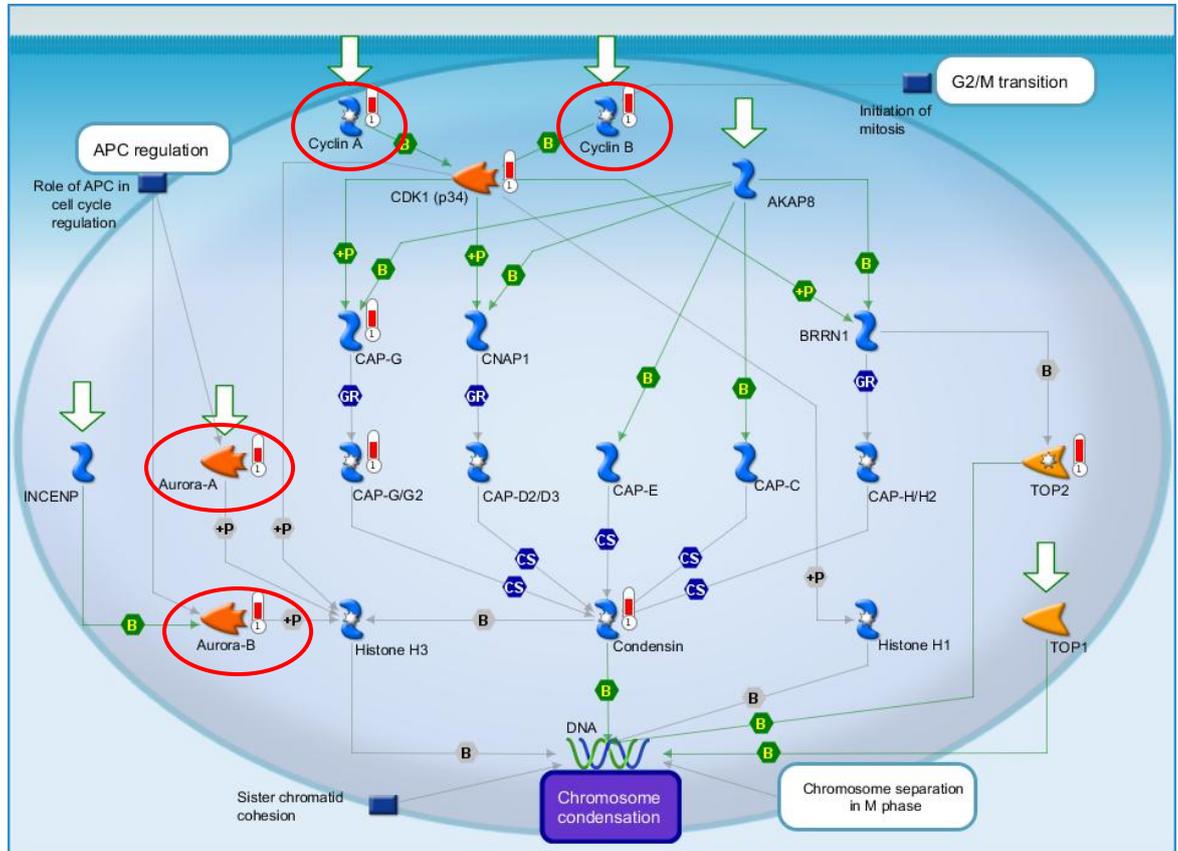


Figure 6-10: Chromosome condensation in prometaphase.

The products encoded by candidate genes are circled in red and the adjacent small red and white bars indicate up-regulation in high phosphate conditions. The legend is shown in Figure 6-11.

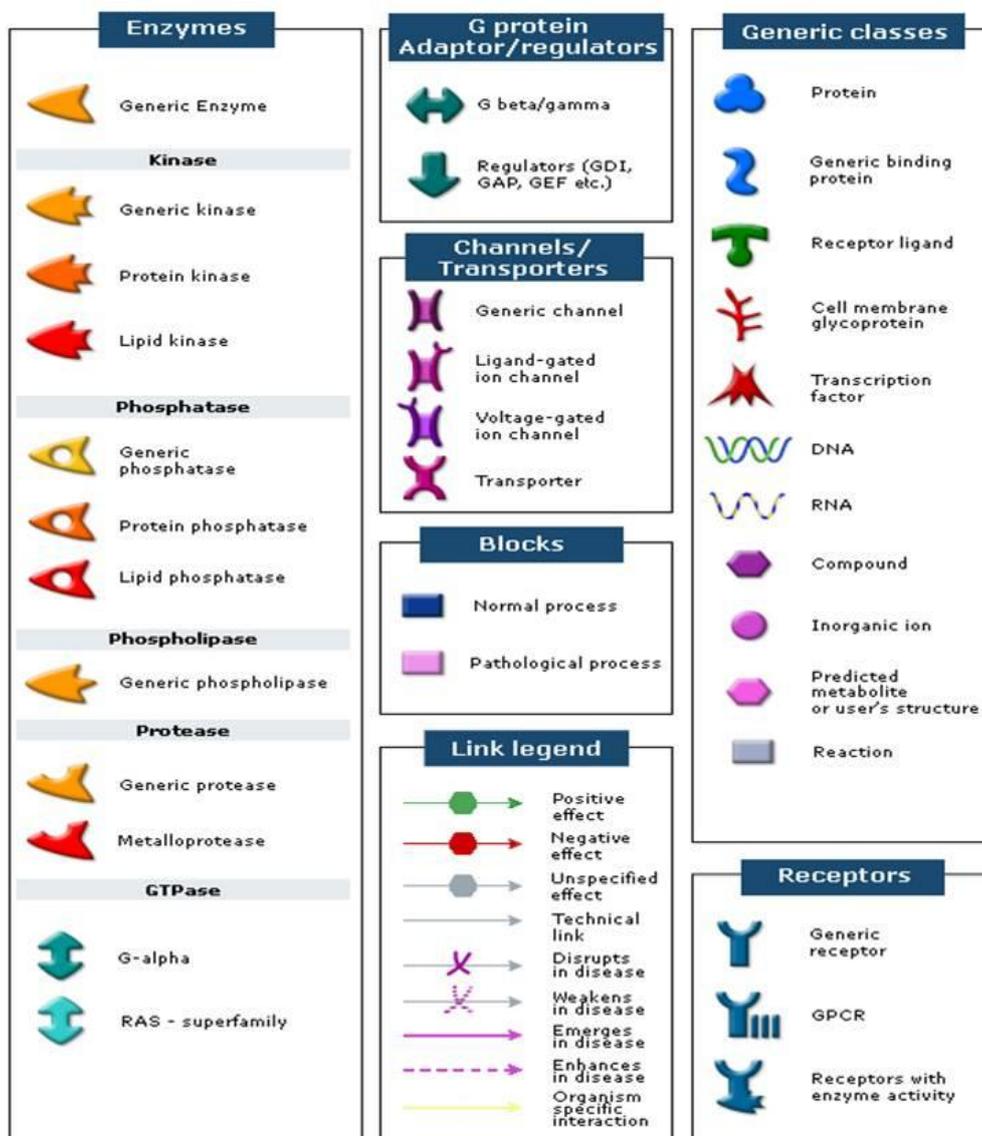


Figure 6-11: Legend for Figures 6-9 and 6-10.

In addition to any influence on the cell cycle and cell growth, I have shown that phosphate and its regulators affect the NO pathway and cell adhesion (chapters 3, 4 and 5) and thus I also selected to study the following genes: *E-selectin*, *ADORA2A* (encodes the adenosine 2A receptor), *NOS 2* (the inducible form of NOS 2), Nitric oxide synthase-interacting protein (*NOSIP*), *PiT 1* and *PiT2*. *E-selectin* and *ADORA-2A* play a role in inflammation and ED (386). In chapter four of this thesis, FGF-23 in combination with a hyperphosphataemic environment was found to stimulate production of cell adhesion molecules including *E-selectin* in HUVECs. *NOSIP* encodes the enzyme of the same name which modulates eNOS activity and plays a role in cell-cycle related actions of eNOS (387). *PiT 1* and *PiT 2* are type 3 phosphate transporters responsible for transporting phosphate into the cell; alterations in expression may affect the quantity of phosphate entering an individual cell.

6.3.4.4 Confirmation of differential expression of candidate genes

TaqMan® RT-PCR confirmed the differential expression of *CCNA2*, *CCNB2*, *AURKA* and *AURKB* seen in the microarray. Each of the four genes was up-regulated in cells cultured in high phosphate medium. This is illustrated in Figure 6-12. Expression of *ADORA2A*, *NOS2*, *NOSIP*, *PiT1* and *PiT2* was not significantly different in HUVECs cultured in high phosphate medium and *E-selectin* was not expressed at either phosphate concentration (data not shown). However, in cells stimulated with IL1 β for 6 hours, there was significantly increased expression of *E-selectin* and *ADORA2A* in HUVECs cultured in standard compared with high phosphate medium (Figure 6-13).

6.3.4.5 Metacore™ network process analysis

Using Metacore™, it is possible to produce a series of ‘network processes’. These identify possible relationships between ‘objects’ of interest (including genes, enzymes, protein, transcription factors). The larger the number of ‘objects’ included then the more complex the resultant network becomes. It is possible to specify which ‘objects’ are to be included and also the degrees of separation between ‘objects’. I compiled several network processes and included ‘objects’ of interest from the microarray analysis: *CCNA2*, *CCNB2*, *AURKA* and *AURKB*, *HSP6A* (which encodes heat shock protein (HSP) 70 and was down-regulated in high phosphate conditions). Additionally, I elected to include additional ‘objects’ of interest to identify any possible associations: these included eNOS and PKG. Figure 6-14 is an example of one of the obtained network processes and illustrates possible connections between ‘objects’ of interest in my microarray dataset and aspects of the NO pathway.

HSP 70 is down-regulated which may reduce activation of HSP 90, protein kinase B (Akt) and of transcription factor, CREB1 and thus of eNOS. Cyclin A is up-regulated which may enhance activation of transcription factor, SP1 and thus inhibition of PKG which is reduced in expression in high phosphate conditions (chapter 4). SP1 activates eNOS but this may not be its pre-dominant effect. Similarly, Aurora-A is up-regulated and activates Cyclin B which has an inhibitory effect on SP1, again this may not be a dominant effect. Up-regulation of Aurora-A and B may enhance inhibition of transcription factor, p53 leading to reduced PKG. PP1-cat (a protein phosphatase) activity may also be reduced via Aurora-A with a knock on effect leading to reduced eNOS. The inhibitory effect of HSP 70 on Aurora-A is reduced.

Activation of the epidermal growth factor receptor (EGF-R) may be the stimulus for enhanced expression of Aurora-A and thus the trigger for the sequence of events described above.

6.3.5 Summary of Results

- HUVECs, rat ECs, human cardiac myocytes and rat VSMCs proliferate more when cultured in high phosphate concentration medium.
- The addition of L-NAME, a NOS inhibitor, increases proliferation in HUVECs cultured in standard phosphate medium. FGF-23 and Klotho has no effect on proliferation.
- Microarray analysis of HUVECs cultured in high phosphate concentration medium revealed that there was differential expression in 131 probes and the most significantly affected pathways were those involving the cell cycle.
- RT-PCR confirmed differential expression of candidate genes involved in the cell cycle and showed no difference in expression of *PiT1*, *PiT2*, *iNOS*, *NOSIP*, *E-selectin* or *ADORA2A*. When cells were stimulated with IL1 β , cells cultured in high phosphate expressed significantly less *E-selectin* and *ADORA2A*.

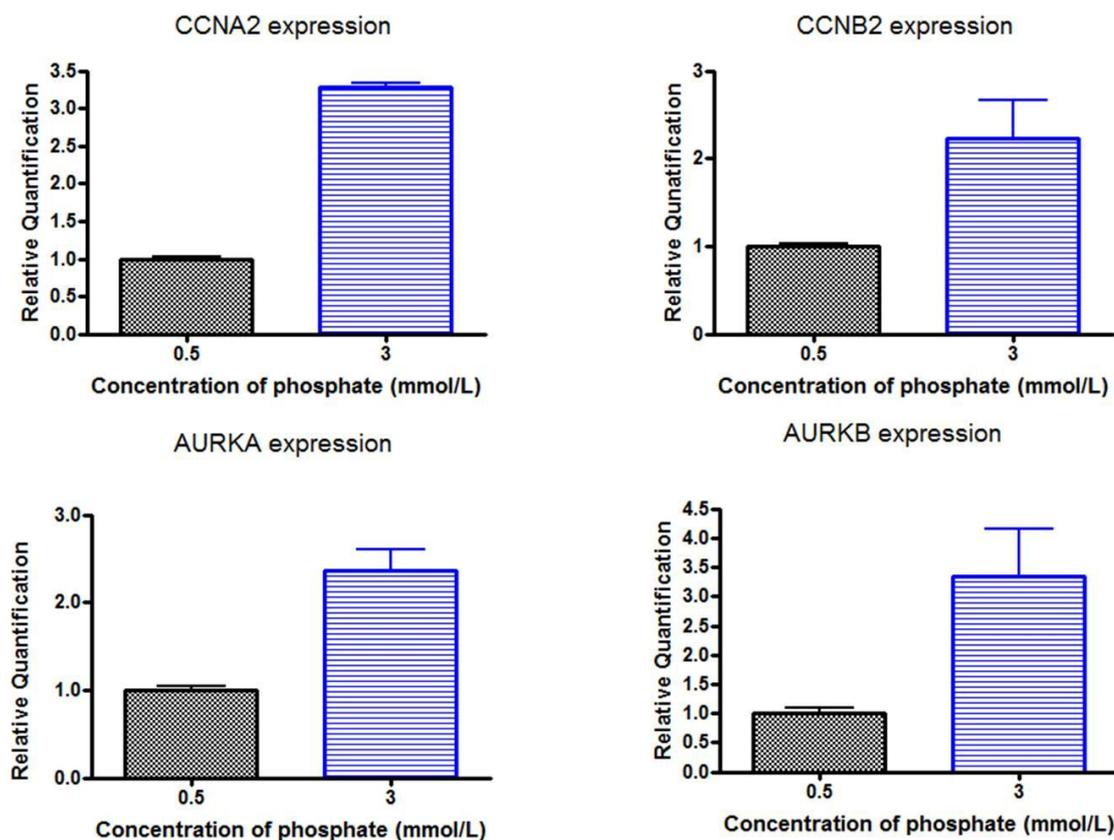


Figure 6-12: BeadStudio candidate gene mRNA expression relative to GAPDH.

(TaqMan® qRT-PCR). CCNA2 (top left), CCNB2 (top right), AURKA (bottom left) and AURKB (bottom right) mRNA expression was compared in HUVECs cultured in normal and high phosphate concentration medium ($n=3$). (CCNA2, ΔCt 1.86 ± 0.05 vs., 0.15 ± 0.02 ; $p=0.02$. CCNB2, ΔCt 2.30 ± 0.04 vs., 1.14 ± 0.31 ; $p=0.02$. AURKA ΔCt 2.73 ± 0.07 vs., 1.49 ± 0.13 ; $p=0.001$. AURKB, ΔCt 3.64 ± 0.12 vs., 1.9 ± 0.31 ; $p=0.006$).

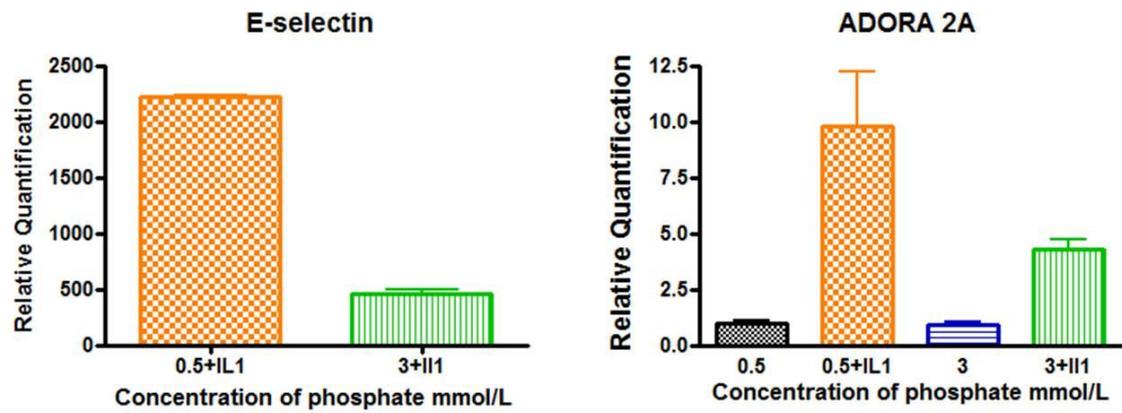


Figure 6-13: Candidate gene mRNA expression relative to GAPDH.

(TaqMan® qRT-PCR). *E-selectin* (left) and *ADORA-2A* (right) mRNA expression was compared in HUVECs cultured in normal and high phosphate concentration medium alone and in the presence of IL1 β (n=3). (Δ Ct values are for normal phosphate stimulated with IL1 β vs., high phosphate stimulated with IL1 β : *E-selectin*, Δ Ct -5.6 ± 0.01 vs -3.3 ± 0.09 ; $p < 0.001$ and *ADORA2A*, Δ Ct 4.9 ± 0.32 vs., 6.1 ± 0.15 ; $p = 0.03$.)

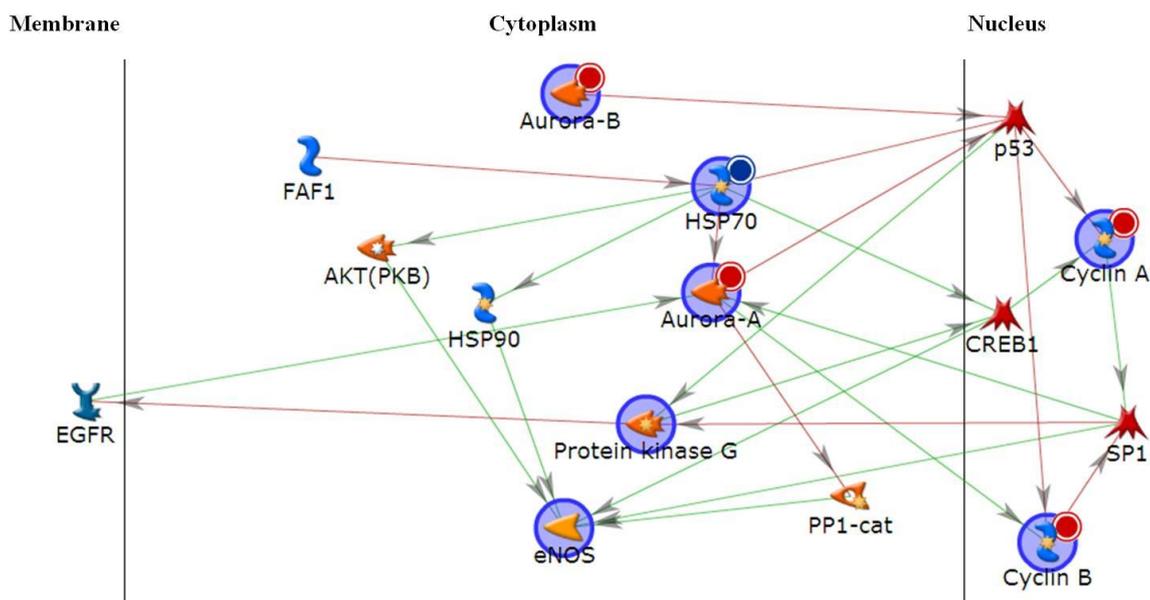
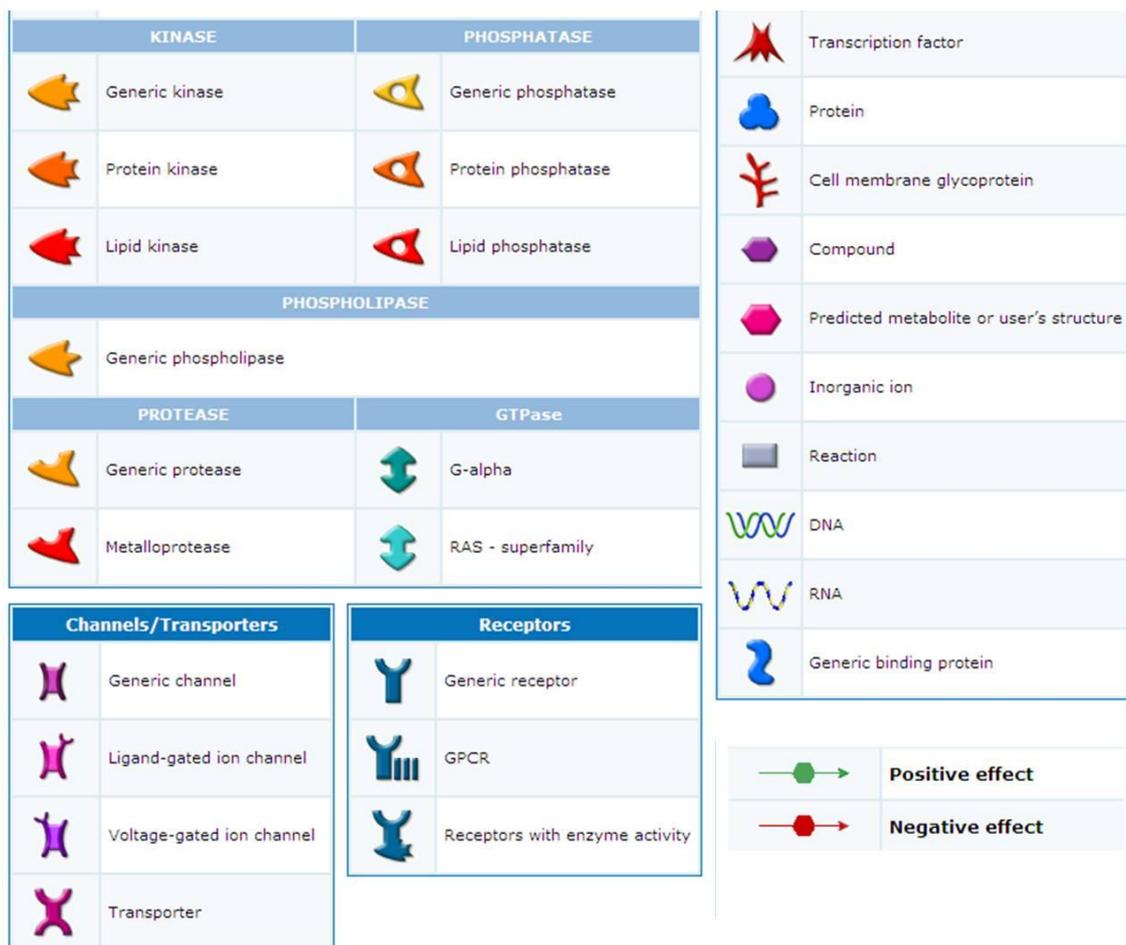


Figure 6-14: An example of a network process identifying possible relationships between objects of interest.

The blue circles around objects identify objects of interest and the small red/blue circles identify genes which were up/ down regulated significantly in my dataset. Location of the objects of interest within the cell is indicated at the top of the figure. The legend is shown above; thin green arrows indicate activation of an object; thin red arrows inhibition.

6.4 Discussion

LVH is a risk factor for cardiac arrhythmia and sudden cardiac death and it affects a significant proportion of patients with CKD (232;383;384). Phosphate and FGF-23 have been linked, independently and through an unknown mechanism, to the development of LVH in CKD (268;385). The work presented in this chapter lends support to the notion that phosphate may have direct effects on cell growth, rather than the prevailing view that such effects are mediated by FGF-23. Moreover, I have identified a number of sub-cellular mechanisms through which phosphate may mediate cell growth.

6.4.1 Direct effects of phosphate on cell growth

Cells cultured in high phosphate medium are bigger and proliferate more rapidly than those cultured in normal phosphate concentration medium. This may reflect reduction of NO, and NO dependent growth inhibition, in high phosphate conditions. In support of this hypothesis, inhibition of NO synthesis by addition of L-NAME increased proliferation in HUVECs cultured in normal phosphate concentrations. There are several possible mechanisms by which the effect of phosphate on NO, and its actions, may result in increased growth. One possibility is that phosphate increases ROS and this inhibits NO. In chapter five, I showed higher levels of superoxide and nitrotyrosine and lower levels of NO in ECs cultured in high phosphate. The MTT assay has also been used to suggest the presence of ROS. Superoxide has been shown to reduce tetrazolium salts and this principle has been used to measure superoxide production in cellular assays (388;389). Thus the higher readings identified in high phosphate conditions in all cell lines might represent not only an increase in proliferation but also in superoxide generation.

In high phosphate conditions, gene expression studies confirm up-regulation of genes involved in the cell cycle. Whilst analysis did not identify alterations in expression of genes directly involved in the NO pathway, microarray analysis did identify relationships between the NO pathway and the cell cycle. Possible connections are illustrated in Figure 6-13. There are several identified transcription factors which connect the 'objects' of interest in the NO pathway and the microarray analysis. These include p53, CREB1 and SP1. Further exploration of the effect of phosphate on these factors is necessary by activity assays, gene and protein expression.

Additionally the EGFR may be of importance; it has enzymatic activity and is activated through ligand binding including TGF α and EGF. High phosphate conditions may

stimulate production of TGF α and EGF; both are produced by macrophages. If in high phosphate conditions, there is a state of chronic inflammation with increased cell adhesion molecules (chapter 3) and down-regulation of ADORA2A, which is known to be protective against inflammation, there may be an increase in production of these molecules from activated macrophages. Further study of this receptor including studies where the receptor is blocked in the presence of high phosphate would be useful and provide insight into the mechanism of action of phosphate and also if the effects can be reversed.

The phosphate transporters PiT1 and PiT2 are expressed on ECs but I did not demonstrate increased expression of either of these transporters in high phosphate conditions. This does not exclude the possibility of receptor dysfunction.

6.4.2 FGF-23, Klotho and cell growth and proliferation.

FGF-23 and Klotho are conventionally felt to mediate vascular cell growth and hypertrophy in CKD and phosphate has an acknowledged but undefined role. I have demonstrated that phosphate itself causes direct effects on cell growth. This leads to three key questions:

1. Do FGF-23 and Klotho cause these same effects on growth *in vitro*?
2. What are the precise mechanisms through which phosphate causes direct effects on growth?
3. Do the pathophysiological effects of phosphate require the interaction between phosphate, Klotho and FGF-23?

I provide some answers to question two in section 6.4.1 above. Further work is necessary but it is likely that the growth effects I demonstrate relate to effects of phosphate on gene and protein expression leading to disruption of the NO pathway, ED, inflammation and as a consequence dysregulation of cell growth.

Question one is interesting. I confirmed that HUVECs express Klotho and the FGFR but I did not find an independent effect of FGF-23 or Klotho on proliferation of ECs or cardiac myocytes. I did not look at differences in expression of Klotho and FGFR between cells cultured in standard and high phosphate conditions. Until recently, ECs were not thought to express Klotho but my findings agree with other groups who have found Klotho to be

expressed on HUVECs. Klotho is down-regulated in CKD (390) and specifically it may be down regulated in the hyperphosphataemic environment, thereby contributing to the observed hypertrophy and proliferation via reduced NO. The secretion of Klotho by ECs is thought to induce NO production and thus reduce ROS release and oxidative stress (391;392) Expression of membranous and secreted Klotho should be quantified in all cell lines cultured in both normal and standard phosphate concentration medium and MTT assays should be repeated in the presence of FGF-23 and Klotho alone.

I checked that the FGF-23 used was active by performing Western blot for MAPK (ERK1 and 2) in ECs, cultured in standard and serum free medium, stimulated with FGF-23 for different time periods. This revealed an increase in ERK expression in standard conditions compared with serum free and maximal expression after 30 minutes of stimulation with FGF-23; thus confirming FGF-23 does have an effect on ECs (data not shown). Contrary to my findings, others have demonstrated that FGF-23 stimulates cell proliferation in ECs, via ERK (390).

In observational human studies, FGF-23 is independently associated with LVH (sections 1.7.2 and 1.7.3) and in animal studies there is some mechanistic evidence of an effect on cardiac cell growth and size; causing LVH in mice, mediated via the PLC γ pathway (201). This effect of FGF-23 was independent of Klotho which is not thought to be expressed on myocardial cells.

Finally question three, do the pathophysiological effects of phosphate require interaction between phosphate, FGF-23 and Klotho? One key aim of this thesis was to attempt to separate the effects of chronic phosphate overload from those of other aspects of disordered homeostasis, eg elevated FGF-23, and the uraemic environment. This has been achieved and I have demonstrated in this chapter and the preceding chapters that phosphate disrupts the NO pathway, alters expression of cell adhesion molecules, causes ED and affects cell proliferation. In reality, however, the effects of phosphate on CV risk in CKD and the precise relationship between FGF-23, Klotho and phosphate is likely to be much more complex than we yet appreciate.

Evidence of the complex interaction between these factors is seen when looking at effects on inflammation. ADORA2A is protective against inflammation and reduces ROS (393). In HUVECs cultured in high phosphate conditions, expression was unaltered. However when cells were stimulated, there was significantly reduced expression of ADORA-2A

suggesting a failure of innate protective mechanisms. Similarly E-selectin expression was significantly elevated in stimulated cells cultured in a normal phosphate concentration medium. In chapter three, I showed that in the high phosphate environment alone, E-selectin expression was not increased but when cells were stimulated with FGF-23 in addition to IL1 β , E-selectin expression was significantly higher in cells cultured in high phosphate medium. The addition of Klotho significantly reduced expression E-selectin, supporting an anti-inflammatory and anti-ageing role for Klotho.

The directly detrimental and isolated effects seen in hyperphosphataemia may change in the presence of FGF-23 and Klotho and the predominant effect may differ between individuals and in the uraemic environment.

7 Chapter seven – Sustained phosphate loading and endothelial function: a cross over study

7.1 Introduction

In vitro studies have been presented in the preceding chapters demonstrating that exposure to elevated concentrations of phosphate results in ED. This is evident both in cells and in resistance vessels from animals and from humans, with and without CKD. Furthermore, exposure to a high phosphate environment affects cell growth and exerts additional detrimental effects independently of any effect on the endothelium. This work adds to the mounting evidence that phosphate has direct, independent, detrimental effects on the function of the endothelium *in vitro* (6;249;250;368). The observed effects of phosphate on endothelial function are not limited to those with CKD but are also evident in the healthy population supporting a role for chronic long term phosphate overloading resulting in a detrimental effect on cardiovascular risk even in the face of a normal serum phosphate level (6).

The details of the association between ED, serum phosphate and CV risk, in CKD and in other populations are not well understood. If exposure to elevated phosphate is sustained either through faulty homeostasis (CKD) or continued high exposure (elevated consumption; health and CKD) this may lead to a self-perpetuating cycle including oxidative stress, an imbalance in redox and thus ED. Phosphate may have direct effects *in vitro* and *in vivo* or other factors involved in phosphate homeostasis (eg FGF-23) may be involved. More detailed information may allow us to target increased CV risk more effectively.

Clinical studies have tended to focus on the cardiac aspect, for example LVH, of the elevated cardiovascular risk seen with phosphate rather than the vascular effects (3;8;239;278). Since vascular effects by definition are widespread and not confined to one organ, these should have similar, if not predominant, focus in research studies with endothelial and vascular function as clinical endpoints.

Shuto et al., looked at the effect on endothelial function of a single high phosphate containing meal (1200mg) compared with a standard phosphate containing meal (400mg) and found that the meal containing high phosphate significantly reduced flow mediated dilatation in 11 healthy male volunteers (6). This is of interest but the relationship between ED, CV risk and serum phosphate is more complex than the effects seen following the ingestion of one phosphate rich meal. The effect of sustained phosphate loading on the

endothelium has never been studied. Therefore, the primary aims of the present study were in healthy volunteers:

- To assess the effect of sustained phosphate loading on endothelial function.
- To assess the effect of sustained phosphate loading on vascular function and vascular stiffness.

Secondary aims were:

- To study the relationships between endothelial and vascular function, sustained phosphate loading, serum phosphate, serum FGF-23 level and urinary phosphate and FGF-23 excretion.
- To assess the effect of phosphate binding medication on serum and urinary phosphate and FGF-23 levels in the absence of CKD.
- To assess the effect of sustained phosphate loading on endothelium independent vasodilatation.

7.2 Materials and Methods

These are explained in detail in section 2.6.2. Briefly, this was a single blind cross over study. Healthy volunteers attended for three visits. Prior to visits two and three, volunteers were randomised to receive phosphate supplements (three tablets daily, equivalent to 48.3 mmol additional phosphate/day) or 3000mgs lanthanum carbonate daily for two weeks. After a two week wash out period, volunteers took the other drug. At each visit identical tests were carried out. Endothelial function was measured with FMD and vascular stiffness with PWV and PWA. Each FMD was performed and analysed by me and I was blinded to the order in which volunteers took each of the two drugs. PWV and PWA were carried out by one of a group of four trained individuals, also blinded to the order of drug ingestion.

7.3 Results

7.3.1 Sample size and demographic data

There were 20 participants. One was incidentally found to have CKD and was therefore excluded, leaving 19 volunteers, all of whom completed the study. 63% (n=12) were female and the mean age was 42.2 ± 14.3 years. Participant demographics at baseline are illustrated in Table 7-1. At the baseline visit, mean eGFR was 102 ± 10 mls/min, serum phosphate level was 1.05 ± 0.18 mmol/L and fractional excretion of urinary phosphate (FeP) was $14.3 \pm 3.4\%$. Participants were normotensive and normoglycaemic with mean lipid values (triglycerides, HDL and LDL cholesterol) within the normal range. Median FMD was 8.4, 6.2-11.6% post cuff inflation and median % vessel dilatation post GTN was 17.7, 13.4-23.2%.

7.3.2 Demographic data following each intervention

The effect of each intervention on demographic data is presented in Table 7-2. Following phosphate supplementation and lanthanum carbonate there was no significant difference in mean serum phosphate, PTH, vitamin D, adjusted calcium or magnesium levels compared with values obtained at the baseline visit. Serum FGF-23 levels increased significantly following phosphate supplementation and fell, although not significantly, following lanthanum compared with the baseline value. Urinary phosphate levels increased significantly from baseline values following phosphate and reduced although not significantly with lanthanum. Figure 7-1 A, B and C illustrates serum phosphate, serum FGF-23 and FeP at baseline and following each intervention.

Post cuff FMD decreased significantly following both interventions, although the effect was strongest following phosphate. There was a trend towards lower values for post GTN vessel dilatation, PWV and AI@75 following each intervention when compared with baseline values; this was particularly true for AI@75. These differences were not, however, statistically significant.

Parameter	Baseline
Age (years)	42.2±14.3
Male Sex	36.8% (n=7)
BMI	26.0±4.1
Creatinine (µM)	66.4±6.3
Systolic BP (mmHg)	123.1±15.8
Diastolic BP (mmHg)	74.5±10.5
Adj. calcium (mmol/L)	2.35±0.07
Phosphate (mmol/L)	1.05±0.18
Vitamin D3 (nmol/L)	48.2±23.3
PTH (pg/ml)	5.9±2.1
FGF-23 (RU/ml)	49.7 (45.9-69.1)
FeP (%)	14.3±3.4
Urinary cGMP (nmol/L)	472.8 (312-645.4)
Urinary FGF-23 (RU/ml)	46.1 (26.6-288.2)
FMD post cuff (%)	8.4 (6.2-11.6)
FMD post GTN (%)	17.7 (13.4-23.2)
PWV (m/s)	7.4±1.9
AI@75	12.8±12

Table 7-1: Baseline demographics.

Parameter	Baseline	Post lanthanum	Post phosphate	p value
Age (years)	42.2±14.3			
Male Sex	36.8% (n=7)			
BMI	26.0±4.1	26.3±3.9	26.3±3.8	NS
Creatinine (µM)	66.4±6.3	65.8±6.8	65.8±6.6	NS
Systolic BP (mmHg)	123.1±15.8	122.9±10.3	119.8±16.8	NS
Diastolic BP (mmHg)	74.5±10.5	75.2±9.4	74.1±12.1	NS
Adj. calcium (mmol/L)	2.35±0.07	2.36±0.05	2.34±0.09	NS
Phosphate (mmol/L)	1.05±0.18	1.03±0.18	1.06±0.16	NS
Vitamin D3 (nmol/L)	48.2±23.3	40.3±20.6	45.6±25.8	NS
PTH (pg/ml)	5.9±2.1	5.8±1.4	6.4±2.3	NS
FGF-23 (RU/ml)	49.7 (45.9-69.1)	59.1 (38.2-73.4)	66.6 (50.0-84.9)	0.028
FGF-23 % change		-1 (-19.8-21.7)	19.6 (3.1-38.9)	0.004
FeP (%)	14.3±3.4	11.4±4.3	28.4±9.2	<0.001
Urinary cGMP (nmol/L)	472.8 (312-645.4)	530.6 (288.2-756.4)	501.1 (274.9-674.0)	NS
Urinary FGF-23 (RU/ml)	46.1 (26.6-288.2)	139.5 (31.3-360.6)	227.9 (39.4-405.8)	NS
FMD post cuff (%)	8.4 (6.2-11.6)	6.6 (3.4-10.3)	3.4 (2.6-5.3)	<0.001
FMD post cuff % change		-23.5 (-59 --0.2)	-58.1 (-71.9 --43.4)	<0.001
FMD post GTN (%)	17.7 (13.4-23.2)	17.2 (12.3-23.7)	16.3 (12.1-17.7)	NS
PWV (m/s)	7.4±1.9	7.3±1.7	7.1±1.6	NS
AI@75	12.8±12	12.5±13.1	9.9±12.6	NS

Table 7-2: Participant demographics at baseline, following lanthanum and phosphate.

Statistically significant differences ($p < 0.05$) between baseline and subsequent measures are indicated in bold. % change refers to the % change in a particular parameter when compared with the value obtained at baseline.

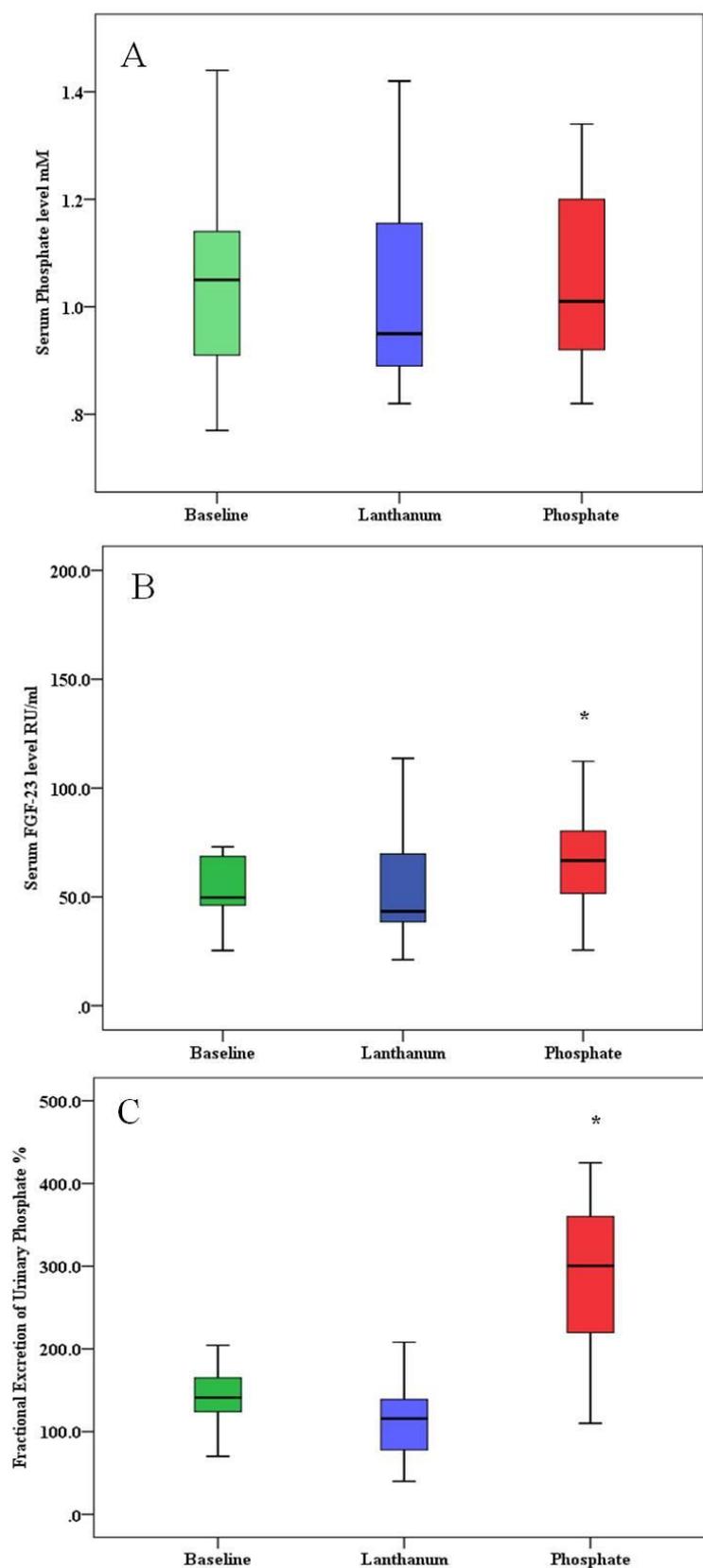


Figure 7-1: Levels of serum phosphate (A) and FGF-23 (B) and fractional excretion of urinary phosphate (C) at baseline (green) and after lanthanum (blue) and phosphate (red) in the study population. Values are median and IQR. * denotes a significant change ($p < 0.05$) compared with baseline values.

7.3.3 Determinants of vascular stiffness, endothelial function

The following demographics were entered into a correlation matrix with PWV, AIx@75bpm, post cuff FMD and post GTN vessel dilatation: age, sex, BMI, systolic and diastolic blood pressure, eGFR, serum phosphate, serum FGF-23, triglycerides, total cholesterol, HDL, PTH, vitamin D, FeP, urinary sodium (fractional excretion), urinary FGF-23, and urinary cGMP. The results are presented in Table 7-3.

There was significant correlation between PWV and age, sex, BMI, systolic and diastolic blood pressure and AIx@75bpm. There was a significant correlation between AIx@75bpm and age, systolic and diastolic blood pressure, eGFR, serum phosphate, HDL and PWV.

Post cuff FMD correlated with urinary sodium, urinary phosphate and triglycerides.

Serum FGF-23 correlated with sex, BMI, systolic blood pressure, eGFR, urinary FGF-23 and post GTN vessel dilatation. Serum phosphate correlated with sex, BMI, systolic blood pressure, eGFR, triglycerides, HDL, AIx@75bpm, urinary FGF-23 and urinary cGMP.

7.3.4 Flow mediated dilatation

To assess reliability of image analysis, an intra-class correlation coefficient (ICC) was calculated on a selection of images from the total cohort; the raw data are presented alongside the ICC in Table 7-4.

Using only data from the baseline visit (ie prior to any intervention), univariate analysis was performed by creating a general linear model (GLM) with baseline brachial artery diameter as the dependent variable and sex, age systolic and diastolic blood pressure as co-variates. Women have smaller brachial arteries with a mean diameter of 3.69mm \pm 0.6mm compared with 4.77mm \pm 0.8mm in the male subjects. Blood pressure and age do not affect vessel diameter. The model was repeated with post cuff FMD and post GTN FMD as dependent variables and age, sex and blood pressure did not affect the ability of a vessel to dilate following cuff inflation or GTN administration.

Increased phosphate intake was associated with a significant reduction in post cuff FMD. (3.38 (2.57-5.26)%, $p < 0.001$) and with lanthanum, post cuff FMD also fell (6.6 (3.4-10.3)%, $p = 0.033$). There was no significant effect on post GTN FMD. This is illustrated in Figure 7-2.

Demographic	PWV	AIx@75bpm	Post cuff FMD	Serum Phosphate	Serum FGF-23
Age (years)	0.807, <0.001	0.612, <0.001			
Male gender	0.430, 0.001			-0.378, 0.004	-0.264, 0.05
BMI	0.387, 0.003			-0.377, 0.004	0.270, 0.04
Systolic blood pressure (mmHg)	0.379, 0.004	0.366, 0.005		-0.261, 0.05	-0.367, 0.005
Diastolic blood pressure (mmHg)	0.363, 0.006	0.630, <0.001			
Triglycerides (mmol/L)			-0.305, 0.02	-0.289, 0.03	
AIx@75bpm	0.485, <0.001			0.329, 0.01	
PWV (m/s)		0.485, <0.001			
HDL (mmol/L)		0.360, 0.006		0.377, 0.004	
eGFR (mls/min/1.73m ²)		-0.305, 0.02		-0.402, 0.002	-0.283, 0.033
Serum phosphate mmol/L		0.329, 0.01			
Fe sodium (%)			-0.379, 0.004		
FeP(%)			-0.314, 0.02		0.273, 0.04
Urinary FGF-23 (RU/ml)				0.306, 0.02	0.425, 0.001
Urinary cGMP (nmol/L)				-0.392, 0.003	
Post GTN FMD (%)					0.334, 0.011

Table 7-3: Significant correlations between PWV, AIx@75bpm, post cuff FMD, serum phosphate and serum FGF-23.

Values are r, p. p<0.05 = significant. Fe sodium, fractional excretion of urinary sodium.

Patient (visit)	Baseline diameter (cm)	Post cuff diameter (cm)	FMD (%)	Baseline diameter (cm)	Post cuff diameter (cm)	FMD (%)
01 (2)	4.02	4.48	11.53	4.10	4.56	11.22
01 (3)	3.79	3.91	3.08	3.86	3.99	3.37
02 (1)	4.85	5.07	4.53	4.83	5.06	4.76
02 (3)	4.67	4.75	1.86	4.63	4.69	1.30
03 (3)	3.49	3.56	2.10	3.38	3.48	2.96
04 (2)	4.84	5.03	3.85	4.88	5.06	3.69
05 (1)	3.84	4.50	17.00	3.84	4.51	17.35
06 (3)	3.76	3.88	3.37	3.74	3.87	3.48
07 (1)	2.96	3.16	6.88	2.92	3.11	6.51
10 (1)	5.56	5.90	6.24	5.56	5.91	6.36
10 (2)	5.61	5.76	2.61	5.61	5.75	2.50
13 (3)	3.71	4.05	9.25	3.72	4.08	9.68
15 (3)	4.47	4.97	11.26	4.47	4.98	11.41
17 (2)	3.66	3.82	4.56	3.65	3.83	4.93
17 (3)	3.73	4.08	9.29	3.77	4.11	9.02
18 (2)	4.24	4.38	3.38	4.24	4.38	3.38
19 (1)	4.37	4.55	4.12	4.37	4.57	4.66
19 (2)	3.06	3.27	6.98	3.16	3.38	6.96
20 (3)	2.90	3.09	6.56	2.92	3.13	7.19

Measure	F	Significance	Intraclass correlation coefficient	95% Confidence Interval
Baseline diameter	0.52	0.47	0.998	0.996, 0.999
Post cuff diameter	1.9	0.18	0.998	0.996, 0.999
FMD	2.74	0.11	0.996	0.989, 0.998

Table 7-4: The raw data used to calculate the ICC coefficient (top).

The repeat measurements for baseline diameter, post cuff diameter and FMD are highlighted in yellow. ICC and confidence intervals are presented in the bottom table.

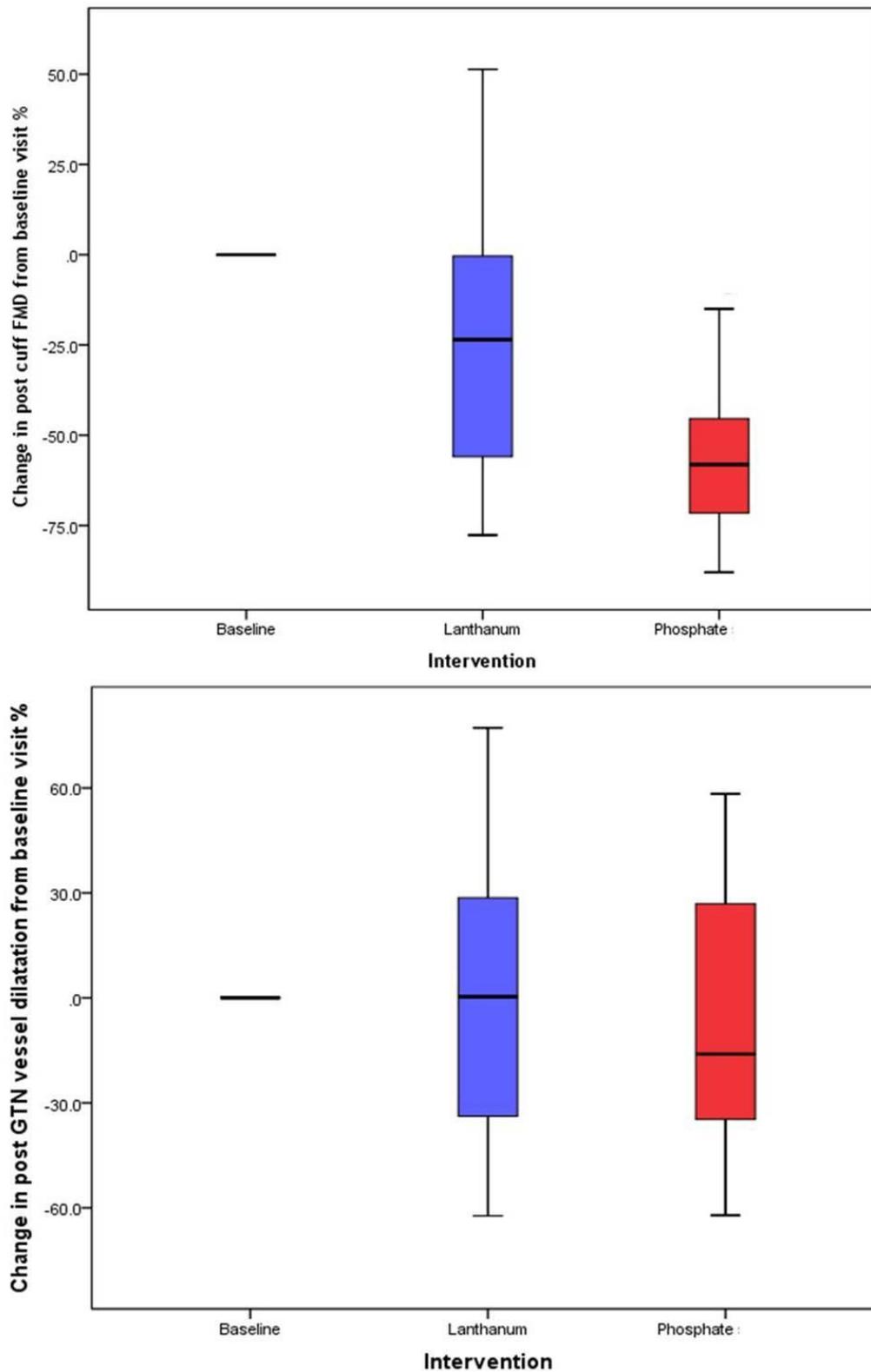


Figure 7-2: Change in post cuff FMD (top) and post GTN vessel dilatation (bottom).

Following intervention with lanthanum (blue) and phosphate (red) expressed as a % change from the baseline visit.

To determine whether randomisation order had any effect, models were created using the entire dataset with post cuff FMD and post GTN FMD as the dependent variables. Various co-variables were added to the models including age, blood pressure, sex and measures of serum and urinary phosphate and FGF-23. Randomisation order was not a predictor of either post cuff FMD or post GTN vessel dilatation regardless of which co-variables were included in the model.

On univariate analysis, by GLM, the change in serum phosphate level from baseline visit, the change in serum FGF-23 from baseline and fractional excretion of urinary phosphate were significant predictors of post cuff FMD. There were no significant predictors of post GTN vessel dilatation. A multiple regression model was created and is presented in Table 7-5. The inclusion of serum FGF-23 and urinary FeP statistically significantly predicted a change in post cuff FMD compared with the baseline visit, $F(2, 54) = 10.07$, $p < 0.001$, $r^2 = 0.28$. Together both variables add statistically significantly to predict the change in FMD ($p < 0.05$). Several models were tried to include the following variables: systolic blood pressure, serum phosphate, age, sex, urinary FGF-23, urinary calcium and urinary cGMP levels. None of these factors were significant predictors of the change in post cuff FMD after an intervention compared with the measures from the baseline. Including drug as a variable in the model increased the r^2 value to 0.53 ($F(4,52) = 15.2$, $p < 0.001$). However, this cancelled the predictive effect of FeP; since the study was not powered to examine the effect of the drugs on FMD, drug was excluded from the final model to produce a more statistically valid conclusion. However, following phosphate, post cuff FMD reduced significantly (3.38% (IQR 2.57-5.26%), $p < 0.001$ ANOVA) and with lanthanum, post cuff FMD also fell (6.6% (IQR 3.4-10.3%), $p = 0.033$ ANOVA). By recoding the three drug categories (no drug, lanthanum, phosphate) into two rather than three variables, it was possible to add the effects of lanthanum on FMD compared to baseline measures and the effect of phosphate on FMD compared to baseline to a multivariate model with the change in post cuff FMD as a % of the baseline visit FMD as the dependent variable. The change in the FeP as a % from baseline and serum FGF-23 were included as co-variables. Now all four variables significantly predict the change in post cuff FMD compared with the baseline visit post cuff FMD, $F(4,52) = 18.31$ $p < 0.001$ $r^2 = 0.59$.

Considering serum FGF-23 and fractional excretion of urinary phosphate in tertiles reveals that those in the highest tertiles for each measure had the lowest post cuff FMD. This relationship is illustrated in Figure 7-3.

Variable	B	Confidence Interval		p value
		Lower Bound	Upper Bound	
Fractional excretion of phosphate	-1.1	-1.9	-0.2	0.014
Serum FGF-23	-0.5	-0.7	-0.2	0.002

Table 7-5: Multiple regression model with post cuff FMD as the outcome measure.

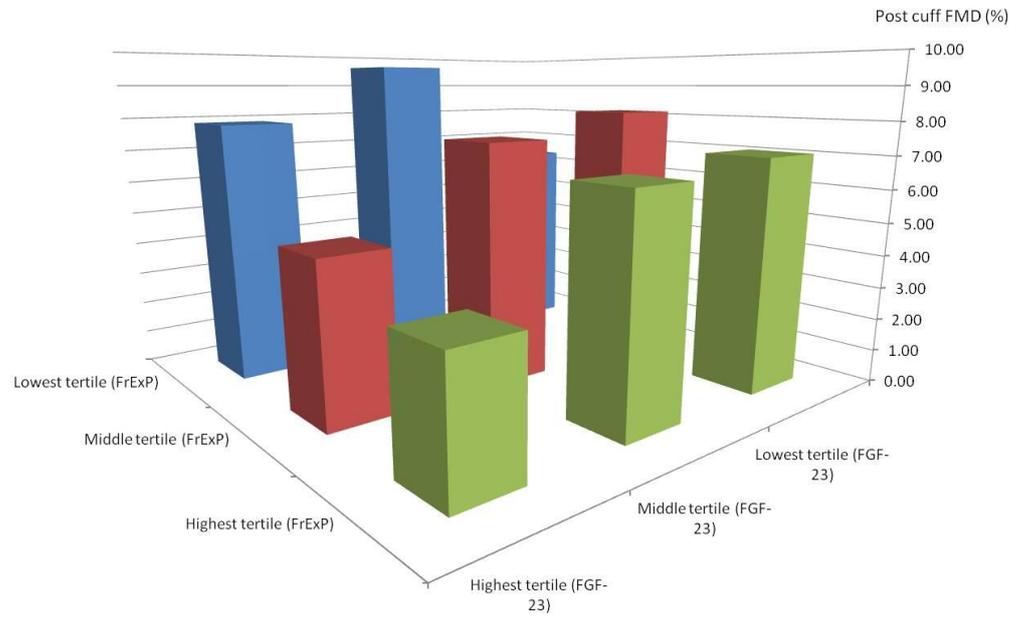


Figure 7-3: The relationship between FeP (FrExP), FGF-23 and post cuff FMD.

7.3.5 Urinary cGMP

Urinary cGMP correlated negatively with serum phosphate ($r = -0.392$, $p = 0.003$) as illustrated in Table 7-3. There was also a correlation with male sex ($r = 0.329$, $p = 0.006$). On univariate analysis, serum phosphate, age, male sex and FeP were predictive of urinary cGMP levels. A multivariate GLM was constructed to assess independent predictors of urinary cGMP. Serum phosphate was the only statistically significant predictor of urinary cGMP $F(5,51) = 2.5$, $p = 0.043$, $r^2 = 0.196$.

7.3.6 Side effects of lanthanum and phosphate

Individuals were asked to comment on the taste and palatability of both drugs and on the presence of any gastrointestinal side effects (nausea, altered bowel habit, bloating). Five of the 19 participants commented on gastrointestinal side effects with phosphate supplementation, mostly abdominal bloating. Six participants commented on the salty taste and two individuals appreciated the orange flavour; otherwise it appeared to be well tolerated. Only one individual did not experience gastrointestinal side effects with lanthanum and eight participants commented on the large size of the tablets. Twelve participants commented on the chalk like taste. Interestingly eight participants volunteered that taking lanthanum with a meal rather than immediately after minimised the abdominal side effects when the predominant side effect was nausea. The manufacturer's advice is that it be taken after a meal.

7.3.7 Summary of results

- Serum phosphate levels do not change significantly in healthy volunteers following lanthanum carbonate or phosphate supplementation.
- Fractional excretion of urinary phosphate increases significantly following phosphate and falls significantly with lanthanum.
- Serum FGF-23 rises by almost 20% when compared to baseline values following phosphate. It falls, although not significantly, following lanthanum.
- Post cuff FMD reduces significantly compared to baseline measures following both phosphate and lanthanum. The effect was more marked with phosphate.

- FeP and serum FGF-23 level are independent predictors of post cuff FMD. The higher the serum FGF-23 and the higher the urinary excretion of phosphate, the lower the % FMD.

7.4 Discussion

This study has demonstrated for the first time that sustained phosphate loading causes ED; this effect is independent of serum phosphate level. Only around 1% of total body phosphate is found in the serum and thus it is perhaps not surprising that used alone this is not necessarily an accurate reflection of total body phosphate.

7.4.1 Phosphate homeostasis and total body phosphate level

The subjects did not have CKD and thus it is reasonable to expect that the homeostatic mechanisms to control serum phosphate within the physiological range should be intact. If, as the evidence increasingly suggests, FGF-23 is the first regulator to act when body phosphate increases, it is reasonable to expect that serum FGF-23 levels should rise following phosphate loading and as a consequence urinary phosphate excretion should increase; PTH would not be expected to rise significantly and vitamin D levels should be maintained.

Against this background, in my study, serum phosphate was unchanged from baseline, serum FGF-23 level rose by around 20% compared with baseline visit measures following phosphate, and urinary phosphate excretion increased by 50% compared with baseline visit mean. This suggests compliance with the study medication and demonstrates phosphate homeostasis. Moreover, it highlights the inadequacies of serum phosphate as a measure of total body phosphate and suggests that other indices of phosphate metabolism may provide additional information pertaining to actual body phosphate level. In early CKD, serum phosphate is maintained within the reference range and rises consistently only when eGFR drops below 30ml/min/1.73m². However, in CKD and in other populations, even in the face of a normal serum phosphate, CV risk seems to be increased and is linked independently to serum phosphate level. This may be explained by large elevations in total body phosphate which are not accurately represented by a single serum measure and it may be possible to further stratify and more precisely quantify risk by identifying those with higher total body phosphate using FGF-23 level and urinary phosphate levels. This is

supported by my findings associating elevated serum FGF-23 and urinary phosphate excretion with poorer endothelium function. Twenty-four hour urinary phosphate level is likely to be superior reflection of phosphate intake and whole body phosphate, and thus increase when body phosphate levels rise in response to a rise in FGF-23. In the clinical setting, it may be possible to use a phosphate:creatinine ratio or a spot FeP to provide additional information about body phosphate levels. This would require to be validated in a clinical study.

7.4.2 Associations with phosphate and FGF-23

Several small studies have demonstrated an association between FGF-23 and dietary phosphate intake in healthy volunteers with increased phosphate intake over a few days resulting in increased levels of serum FGF-23 (394;395). Increased levels of FGF-23 have been shown to correlate with increased serum phosphate, increased urinary excretion of phosphate and increased vitamin D levels (394). We found that higher serum FGF-23 correlated with female sex, higher BMI and lower systolic blood pressure. We also found that higher serum phosphate levels correlate with female sex and lower systolic blood pressure. Women have been shown to have lower systolic blood pressure than men and if phosphate levels are higher, this will trigger an increase in FGF-23 levels. In turn this will reduce the expression of the type 2 sodium phosphate co-transporter in the proximal tubule of the kidney and along with increasing urinary phosphate excretion, fewer sodium molecules will be absorbed, thus offering one possible mechanism for the observed association between higher serum phosphate, FGF-23, lower systolic blood pressure and female sex.

Both higher serum phosphate and higher FGF-23 correlate with lower eGFR and with higher urinary FGF-23 excretion. Serum FGF-23 also correlates positively with fractional excretion of urinary phosphate. Yet there was no significant correlation between serum phosphate and FGF-23; one explanation may be that serum phosphate is not an adequate marker of the total body phosphate content. There was no correlation between serum and urinary phosphate measures which lends weight to this argument. Urinary phosphate excretion alone or in combination with measures of FGF-23 may be a better surrogate for total body phosphate than serum phosphate level.

The lack of a relationship between serum phosphate and serum FGF-23 and urinary phosphate, is an important observation regardless of the root cause. FGF-23 is being touted

as the earliest biomarker for excess total body phosphate and it is associated in my study with increased urinary phosphate excretion but not serum phosphate levels. This supports the notion that lowering CV risk may be dependent upon an intervention prior to a discernible rise in serum phosphate (even within the normal range).

7.4.3 Vascular stiffness

Phosphate loading did not alter PWV and thus vessel stiffness. PWV did correlate with several other demographic factors including age and BMI and this is similar to several other studies which have shown that male sex increasing BMI, age and blood pressure correlate closely with vessel stiffness (396). This suggests that my study population is representative of the general population.

AIx@75bpm is a further measure of arterial stiffness and is independently associated with CV risk. In my study population, increased arterial stiffness measured with AIx@75bpm did correlate with a higher serum phosphate level (within the physiological range) as well as with increasing age and blood pressure. The latter observations are in keeping with findings by other studies but a correlation between serum phosphate and AIx@75bpm has not previously been observed (397). Houston et al., studied the relationship between AIx@75bpm and indices of phosphate metabolism, including serum phosphate and FGF-23 level and urinary phosphate excretion in patients with CKD (398). They found no association between these measures of phosphate metabolism and arterial stiffness and confirmed that traditional measures including age and blood pressure are predictors of arterial stiffness in the CKD population. Their results may be explained by an inadequate study sample (n=74), the wide range of CKD (74% of study population had an eGFR ≥ 30 ml/min/1.73m² and of them 30% had an eGFR >60 ml/min/1.73m²). They also included subjects of black race, a factor which seemed to be associated with reduced urinary phosphate excretion and may contribute to the lack of relationship between measures of phosphate metabolism and AIx@75bpm. Other studies have linked serum phosphate and serum FGF-23 with other measures of vascular stiffness including ankle brachial index both in the presence and absence of CKD (399).

In univariate and multivariate analysis, there was no relationship between serum phosphate, indices of phosphate metabolism and measures of vascular stiffness after either intervention.

7.4.4 Endothelial dysfunction

Higher triglyceride levels, higher fractional excretion of urinary sodium and higher fractional excretion of urinary phosphate correlated with worse endothelial function. On univariate analysis, serum FGF-23 level, FeP and endothelial function differed between baseline and following intervention with phosphate and lanthanum. That lanthanum should also impair FMD was an unexpected finding and whilst the study was not powered to detect differences in the drugs themselves, it is an interesting observation. Further work is necessary to establish if this is an effect of lanthanum and if so the mechanism behind the observation. One explanation may be that enough lanthanum is absorbed to have a detrimental effect on the endothelium. The manufacturers quote up to 10% absorbance and there is increasing evidence that lanthanum accumulates in bone with long term usage. It is also possible that this effect would not be seen in CKD, the population of patients who are prescribed this medication.

FeP and serum FGF-23 fell with lanthanum and rose with phosphate. On multivariate analysis, elevated serum FGF-23 and FeP were significant predictors of worse FMD and if examined in tertiles, those patients with the highest FGF-23 and highest FeP had the lowest FMD. Several observations can be made from these results:

- Elevated serum FGF-23 and urinary phosphate excretion can be linked to ED.
- Serum phosphate does not correlate with ED in my study population.
- Phosphate loading results in ED and this is likely to be the result of increased total body phosphate.
- I did not measure total body phosphate but it is likely that serum FGF-23 and FeP important and additive markers of total body phosphate and this hypothesis requires to be tested.
- It may be that indices of phosphate metabolism are more important to the link between phosphate and CV risk than initially appreciated.
- In the early stages of chronic phosphate overload (from diet or from reduced excretion), FGF-23 may be directly toxic to the endothelium thereby increasing CV

risk. If phosphate excess becomes chronic, then phosphate rises initially within the normal range and as it rises it may then become directly toxic.

I measured urinary cGMP as an indirect marker of NO production. When ED is present, less NO is produced and plasma and urinary cGMP may fall. Urinary cGMP did not correlate with FMD but I did observe a correlation between urinary cGMP and serum phosphate levels. In a multivariate model, higher serum phosphate remained an independent predictor of lower urinary cGMP levels. That is to say serum phosphate may be related to low NO production and therefore ED. That there may be reduced NO produced in the presence of higher phosphate is in agreement with our observations from the *in vitro* work presented in chapters four, five and six. However, if we continue to assert that serum phosphate is not a good marker of total body phosphate excess, why then does it predict lower urinary cGMP levels? There are a few possibilities, the most plausible of which is that urinary cGMP may not be as sensitive a marker of ED as other measures – FMD is the “gold standard” measure and there was no correlation between urinary cGMP and FMD.

Whilst serum phosphate alone is perhaps not a good indicator of total body phosphate levels, this suggests there is an association between the serum phosphate values and adverse effects. In order to fully assess the effects of total body phosphate excess, it would be pragmatic to accept that we need to consider urinary phosphate losses and serum phosphate measures in combination. Ideally, serum FGF-23 measures should also be taken into consideration. I did not consider the type of phosphate consumed in our study but this may also be of relevance; specifically, phosphate from additives and processed foods compared with that in non-processed foods.

I also looked at the effects of phosphate loading on endothelium independent function (post GTN vessel dilatation). There were no differences between the interventions and the baseline values for this. Preserved endothelium independent vasodilation is in agreement with my myography data in rats and human vessels without CKD (chapters four and five). Interestingly, serum FGF-23 correlated positively with post GTN vessel dilatation. It may be that in a high phosphate environment which stimulates FGF-23 and perhaps reduces the level of available NO, the vessel becomes hyper-responsive to an exogenous source of NO.

The relationships between serum and urinary phosphate and other indices of phosphate metabolism are exceptionally complex and poorly understood. However, I demonstrate for

the first time that sustained phosphate loading is associated with ED not linked to serum phosphate but to urinary phosphate losses and serum FGF-23 level. This supports the concept that total body phosphate excess cannot be accurately quantified from a serum measure alone and that perhaps in the quest to modify CV risk by altering phosphate levels, we should focus on indices of phosphate metabolism, certainly initially, rather than on serum phosphate itself.

7.4.5 Strengths and limitations

This was a prospective cross over trial and was appropriately powered to detect a meaningful difference in the outcome measure - FMD. I performed and analysed all images in a single blind manner. The reproducibility of the analysis was excellent. Individuals were fasted and abstained from caffeine for 12 hours prior to study visits and attended for visits at the same time of day. The study participants have been closely phenotyped.

Both sexes were included and it may be helpful to repeat this work in a single sex study to ameliorate possible effects of the female sex hormones. Dietary phosphate intake was not quantified. Urinary cGMP was utilised as an indirect marker of NO production and therefore endothelial function, it may have been advantageous to have measured ROS directly in the serum of patients rather than make use of a by product.

7.4.6 Conclusion

Sustained phosphate loading results in ED, measured by post cuff FMD. Urinary phosphate excretion and serum FGF-23 are significant predictors of worse endothelial function and are likely to be useful markers of total body phosphate, providing more information in combination than serum phosphate or either measure alone. Whilst the study was not powered to study the direct effects of either phosphate supplementation or lanthanum, it is therefore difficult to draw a firm conclusion with regards to the effect of lanthanum on endothelial function. It is however worth noting that lanthanum certainly did not improve endothelial function and may in fact have a detrimental effect. I did not detect any independent effect of sustained phosphate loading on vascular stiffness measures in our study population.

7.5 Further work

It is possible that patients with CKD may respond differently to sustained phosphate loading and thus these results cannot be extrapolated to the CKD population. This study should be repeated in a population with CKD, both pre dialysis and dialysis patients, to ascertain if the same relationships are present.

8 Chapter Eight – General discussion

8.1 Summary of findings

The aim of this thesis was to study the mechanism of action of phosphate as a CV risk factor, particularly teasing out isolated effects of chronic exposure to phosphate, separate from other abnormalities of the uraemic environment. Whilst the prevalent view is that elevated serum phosphate increases CV risk by promoting vascular calcification, an alternative possibility is phosphate directly causes endothelial dysfunction. I have investigated this hypothesis by examining vascular function *in vitro* and *in vivo*, examining gene expression, and quantitative and functional changes in signal transduction pathways in the following systems: cell lines cultured in standard and high phosphate concentration medium; rat and human vessels, exposed to standard or high phosphate concentration PSS, in wire myography studies; and in a cross-over clinical study (with phosphate supplements and binders) with endothelial function as the outcome measure. The main findings were:

- Evidence of disruption of the NO pathway, in the high phosphate environment, at several levels:
 - Reduced basal NO, reduced expression of phospho and total eNOS, reduced expression of PKG, lower concentrations of cGMP, increased expression of peroxynitrite and a trend towards increased superoxide levels. Some of these effects may be reversible.
 - Impaired function, *in vitro*, of human resistance vessels exposed to high phosphate. These effects differ between patients with and without CKD and suggest a degree of adaptability and reversibility of the effects of phosphate in CKD.
 - In rat resistance vessels, the impaired endothelium dependent and independent function observed in high phosphate conditions can be improved with ascorbic acid and ameliorated with the addition of zaprinast, a PDE5I.
 - *In vivo*, in the face of a normal serum phosphate, phosphate loading results in impaired post cuff FMD measures.

- Evidence that phosphate has direct effects on cell growth:
 - This may relate to reduced NO and increased ROS production. There is up-regulation of several genes involved in the cell cycle and this may have a directly inhibitory effect on the NO pathway mediated by inhibition of transcription factors.
 - The relationship between phosphate and its regulators is complex. In cell culture, the addition of FGF-23 and Klotho did not have an additive effect on cell proliferation. However, FGF-23 is associated with LVH, measured by cardiac MRI, in CKD.
- Evidence, in a high phosphate environment, of inflammation and impaired defence response in cells exposed to additional stress:
 - VEGF concentration in cells cultured in high phosphate does not increase significantly as would be expected on exposure to an hypoxic environment.
 - ADORA2A expression reduces significantly in cells cultured in high phosphate and stimulated with IL1 β .
 - In combination with a high phosphate environment, FGF-23 increases expression of the cell adhesion molecules, E-selectin and VCAM.

8.2 Strengths and limitations of these studies

The described studies successfully identify isolated effects of phosphate *in vitro* and *in vivo* and particularly concentrate on the effects of sustained exposure to a high phosphate environment. This differs from other studies in this area where the focus has been on short term stimulation with elevated phosphate. In CKD, patients are exposed to higher phosphate concentrations for long, not short, time periods and thus laboratory and clinical studies looking at sustained exposure to high phosphate are more comparable with 'real life'. In my studies, exposure to the high phosphate environment leads to inflammation, the generation of ROS, cell proliferation and impaired relaxation and each of these detrimental

effects contributes to ED in CKD. The mechanism underlying this is complex and is likely to involve multiple pathways. Figure 8-1 suggests possible pathways through which phosphate elevates CV risk, based upon the work presented in this thesis.

FMD is the most accurate non-invasive measure of endothelial function and the patients in the clinical cross-over study underwent detailed analyses. This study provided a unique opportunity to study the direct effects of sustained phosphate loading on endothelial and vascular function *in vivo* without confounders from the uraemic environment.

The studies presented provide strong evidence of an effect of phosphate on endothelial function at every level from cell through to *in vivo* and suggest that the mechanism underlying the adverse effects and increased CV risk relates to effects on the NO pathway. The cell based studies consider both gene and protein expression as well as different aspects of the NO pathway, resulting in robust study of the whole NO pathway. The use of different cell lines also serves to provide more detailed and clinically relevant information on the effects of phosphate.

Cultured cell lines may not represent the true *in vivo* phenotype and there is criticism of the HUVEC cell line in particular. However, HUVECs have been widely utilised in this area of research and thus I have adopted a pragmatic approach. Of interest, the concentration of phosphate in cell media varies enormously. The standard concentration in the media used in these experiments was 0.5 mM. This would represent a low phosphate *in vivo* although as discussed in chapter seven, the validity of serum phosphate as a marker of total body phosphate is unclear. Increasing to 3 mM for the high phosphate experiments seemed to be logical to mimic the *in vivo* environment. However, using a range of phosphate concentrations may have provided further information. Additionally, RPMI cell media is very widely used and it contains approximately 5mM phosphate, casting aspersions on the clinical applicability of studies making use of this media.

Rat VSMCs are a myoblast cell line and share characteristics with VSMCs but they are not VSMCs and thus it is not possible to extrapolate the findings seen in my studies to VSMCs. Additionally, I did not use a human VSMC line which is a limitation. One option would have been to culture human VSMCs from the human resistance vessels and to undertake gene and protein expression studies in these cells.

In the rat resistance vessel work in chapter four, the rationale behind exposing the vessels to an elevated phosphate concentration was to try to tease out the isolated effects of phosphate from other effects of the uraemic environment. An alternative method would have been to have performed these experiments in rats fed on a normal and high phosphate diet: this may provide a more accurate reflection of the effects of long term exposure to phosphate on endothelial and vascular function. I demonstrate that the addition of PDE5I ameliorates the observed detrimental effects of the high phosphate environment. It would have been useful to have performed the same experiments in the presence of different concentrations of PDE5I and also to have incubated the drug for different time periods and compared these results. I measured cGMP in the rat vessels and I went on to use cell lines to measure eNOS, VEGF and NO. Culturing endothelial and smooth muscle cells from the actual resistance vessels and using these cells would have been more informative.

Additionally, failure to measure guanylate cyclase activity and expression is an important omission. I used the Griess reaction to measure NO and did not observe any difference in the rat VSMCs; one explanation for this might be that the cells were cultured on 96 well plates and thus the numbers of cells may have been too small. Furthermore, it is important to remember that these are rat resistance vessels from healthy rats, without CKD, and the results cannot therefore be extrapolated to CKD or to humans.

In chapter five, I describe studies in human resistance vessels and although the results are interesting, there are a number of limitations. The small numbers of vessels is a major limitation. It is also impossible to allow for the many different confounders including mode and length of time on RRT of the patients included in the study. The observed improvement in the function of the CKD vessels when exposed to a normal concentration PSS is an interesting one and I hypothesise that this may relate to the presence of a circulating factor which is 'washed out'. There are several possibilities including ADMA and ROS. Measuring the level of these both in the study participants and in the vessels would be informative. The linear nature of the relaxation curves (to both carbachol and SNP) seen in the vessels from living kidney donors was not expected and is difficult to explain but may relate to the length of time the vessels were *ex-vivo*. The studies should be repeated in vessels incubated for shorter time periods. Similarly to the rat studies in chapter four, performing the cell culture experiments from cells cultured from the resistance vessels themselves would have been more meaningful than using the HUVEC cell line.

One main aim of this thesis was to study the isolated effects of phosphate, separate from other aspects of the uraemic environment. Whilst this has been achieved, in reality any

effects of phosphate, particularly in the setting of CKD, are in the presence of other abnormalities and are very likely to be intimately connected to phosphate regulators including FGF-23 and Klotho. Thus, more in depth study of the effects of FGF-23 and Klotho is desirable. Repetition of the cell studies described in chapters four and five in the presence of FGF-23 and Klotho, particularly with regards to eNOS expression would be of value. Similarly performing the myography experiments in the presence of FGF-23 and Klotho would be informative and may offer additional insight into the relationship between these factors, phosphate and endothelial function. I demonstrate in chapter six, that endothelial cells cultured in a normal phosphate environment express Klotho and the FGF receptor. Studying expression in cells cultured in high phosphate should also be undertaken and quantification and comparison of expression between the different phosphate concentrations would be informative. Measuring Klotho is difficult but it is possible to measure both the membrane bound and secreted forms of Klotho and this should have been attempted in blood samples from the human vessel study participants and also in extracted DNA. Similarly failure to measure eNOS gene expression in DNA from study participants is a flaw.

In terms of the clinical studies undertaken, in chapter three, I studied the association of FGF-23 and vascular structure in CKD with additional *in-vitro* studies on the underlying mechanism. The study population comprised patients with CKD secondary to diabetic or IgA nephropathy and thus the findings of FGF-23 as an independent predictor of LVH cannot be extrapolated to patients with CKD from other aetiologies including inherited conditions. Furthermore this was a single centre pilot study and numbers were small and I did not prove causality with elevated FGF-23 and LVH. Whilst, I provide some evidence that in the hyperphosphataemic environment, FGF-23 stimulates the production of the cell adhesion molecules, E-selectin and VCAM. I measured these by ELISA and not Western blot. My findings require to be confirmed by Western blot. I did not perform gene expression studies and this is also necessary to ascertain what difference, if any, hyperphosphataemia and FGF-23 make to gene expression. Furthermore I used the HUVEC cell line and the above experiments should be performed in a cardiac myocyte cell line to ascertain if similar direct effects to those observed could be seen.

In chapter seven, I describe a cross over trial in healthy volunteers without CKD looking at the effect of sustained phosphate loading on endothelial function. Both sexes were included and it may be helpful to repeat this work in a single sex study to ameliorate possible effects of the female sex hormones. Dietary phosphate intake was not quantified

and this can vary hugely depending upon the types of food consumed, particularly the consumption of foods containing preservatives. Urinary cGMP was utilised as an indirect marker of NO production and therefore endothelial function, it may have been advantageous to have measured ROS directly in the serum of patients rather than make use of a by product. I demonstrate that urinary phosphate excretion and serum FGF-23 are significant predictors of worse endothelial function and are likely to be useful markers of total body phosphate, providing more information in combination than serum phosphate or either measure alone. However I did not measure total body phosphate nor intracellular phosphate and it would be informative to do this to determine if serum FGF-23 and urinary phosphate excretion correlate and are indeed markers of total body phosphate. Lastly, it is possible that patients with CKD may respond differently to sustained phosphate loading and thus these results cannot be extrapolated to the CKD population. This study should be repeated in a population with CKD, both pre dialysis and dialysis patients, to ascertain if the same relationships are present.

8.3 Future work

In vivo, the interaction between phosphate, FGF-23, Klotho and other aspects of the uraemic environment is complex. Furthermore, it is likely that effects differ between individuals and between organ systems. Phosphate causes ED and I provide strong evidence that this is why it is associated with CV risk. I have demonstrated that some of the effects of phosphate seem to be reversible and this may be a means to lower CV risk in CKD. Serum phosphate may not be a good marker of total body phosphate and other markers, including FeP should be considered. Phosphate binding medication is not well tolerated and there may be better ways in which to lower total body phosphate, e.g. a phosphate channel blocker, which would prevent phosphate entering an individual cell. There are several identified areas to expand upon the work contained within this thesis.

Cell based studies focussing on transcription factor expression (p53, SP1 and CREB1) and effects of the high phosphate environment on expression of Klotho, the FGF-R and the EGF-R in the high phosphate environment. Intracellular phosphate should be measured and more detailed study of ROS is also necessary.

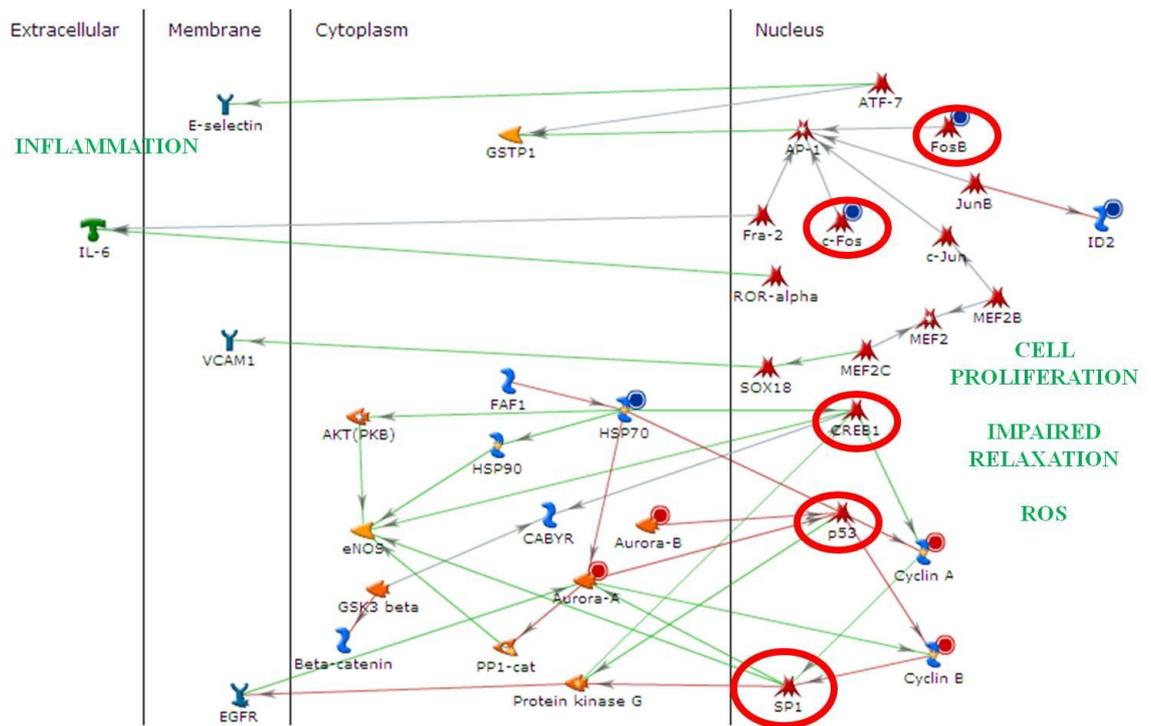


Figure 8-1: Effects of phosphate leading to ED, based on the findings in this thesis.

Transcription factors which are likely to be key to the detrimental effects of phosphate are circled in red. Phosphate probably affects multiple pathways, with some overlap, causing inflammation via E-selectin, VCAM, perhaps mediated via c-Fos; cell proliferation and impaired relaxation mediated via down-regulation of the NO pathway with the transcription factors SP1, CREB1, p53 and c-Fos being of importance. Phosphate also stimulates ROS mediated via the NO pathway. Green arrows indicate an activation effect, red an inhibitory effect and grey an undefined interaction.

In human resistance vessels, studies with larger numbers of vessels and to look at the effect of zaprinast, ascorbic acid and phosphate channel blockers are of interest.

A repeat cross-over study in patients with CKD should be performed and additional measurements undertaken including intracellular phosphate, secreted Klotho and gene and protein expression studies following each intervention.

A larger observational study looking at FeP and its correlation with serum phosphate and with CV outcome is of interest and ultimately an outcome study demonstrating that lowered phosphate translates to CV risk reduction and survival benefits.

8.4 Conclusion

Figure 8-2 shows possible mechanism of action as phosphate based on the main findings in this thesis. Long term exposure to elevated phosphate is associated with direct ED. It is likely to be ED which contributes to the elevated CV risk seen in those with CKD and not as convention has dictated, VC. In the high phosphate environment, endothelial and vascular dysfunction is evident in individual cell lines, in blood vessels and in humans exposed to prolonged oral phosphate loading. Strategies to block phosphate transport and reduce intracellular phosphate may have therapeutic potential.

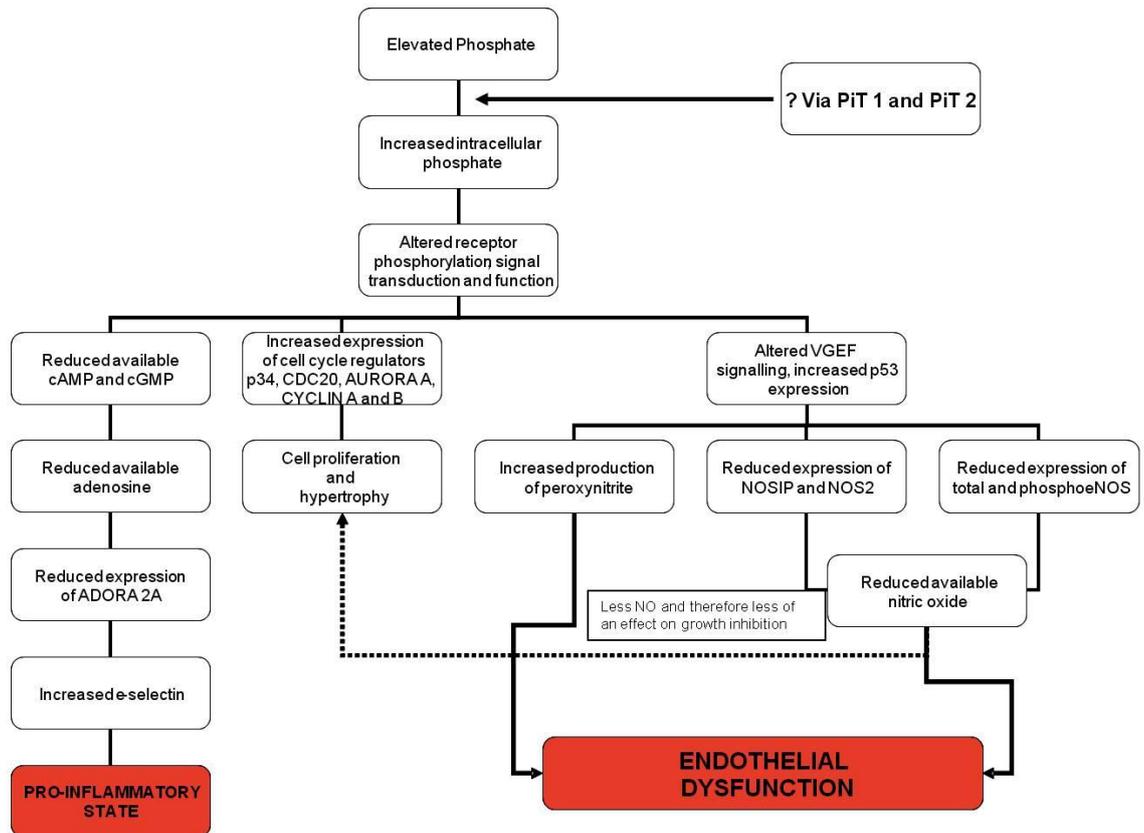


Figure 8-2: Possible mechanism of action of phosphate resulting in endothelial dysfunction and a pro-inflammatory state based on the main findings in this thesis.

9 Appendix

9.1 Publications, awards and presentations

Full Publications

1. **K.K. Stevens**, I.R. Morgan, R.K.Patel, P.B. Mark, C.C. Geddes, A.G.Jardine, C.Delles. **Serum Phosphate and outcome at one year post deceased donor renal transplantation.** Clin Transplant. 2011 Mar-Apr;25(2):E199-204.
2. **K.K. Stevens**, E.P McQuarrie, W. Sands, D.Z Hillyard, R.K.Patel, P.B. Mark and A.G. Jardine. **Fibroblast growth factor 23 predicts left ventricular mass and induces cell adhesion molecule formation.** Int J Nephrol. 2011;2011:297070.
3. **K.K.Stevens**, R.K. Patel, S. Methven, M.J. Clancy, J.G. Fox, A.G.Jardine and C.C.Geddes. **Proteinuria and Outcome After Renal Transplantation: Ratios or Fractions?** Transplantation. 2013 Jul 15;96(1):65-9.

Published abstracts

1. **KK Stevens**, RK Patel, CC Geddes, AG Jardine, C Delles **Phosphate Level, Graft Failure and Mortality in Renal TransplantRecipients.**The Scottish Medical Journal 2010 55(2): 45-53
2. **K.K. Stevens**, R.K.Patel, C.C.Geddes, A.G.Jardine and C.Delles **Serum phosphate and outcome at one year post deceased donor renal transplant.** *NDT Plus* (2010) 3(suppl.3): iii541
3. **K.K.Stevens**, E.C. Beattie, W.Sands, C.Delles and A.G. Jardine **Hyperphosphatemia impairs function of resistance vessels.** *NDT Plus* (2011) 4(suppl 2): doi:10.1093/ndtplus/4.s2.3
4. **K.K. Stevens**, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine **Hyperphosphataemia Impairs Relaxation in Resistance Vessels; an Effect Which Is Partially Reversed in the Presence of a Phosphodiesterase Inhibitor.** J. Am. Soc. Nephrol 22: 2011
5. **K.K. Stevens**, W. Sands, D.Z. Hillyard, R.K. Patel, P.B. Mark and A.G. Jardine **Hyperphosphataemia Increases Endothelial Cell Size, Granularity and Rate of Proliferation.** J. Am. Soc. Nephrol 22: 2011
6. **K.K. Stevens**, E.P. McQuarrie, W. Sands, D.Z. Hillyard, R.K. Patel, P.B. Mark and A.G. Jardine **Fibroblast Growth Factor 23 Predicts Left Ventricular Mass and Induces Cell Adhesion Molecule Formation.** J. Am. Soc. Nephrol 22: 2011
7. **K.K. Stevens**, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine **Zaprinast can correct phosphate induced impaired relaxation in rat resistance vessels.** *NDT* (2012) 27(suppl 2): ii36-ii37 doi:10.1093/ndt/gfs193
8. **K.K. Stevens**, R.K. Patel, S. Kettlewell, M.J. Clancy, C. Delles G.L.Smith and A.G. Jardine **Phosphate has deleterious effects on vascular function.** J. Am. Soc Nephrol. 23: 2012
9. **K.K.Stevens**, R.K. Patel, S. Kettlewell, C.Delles, G.L. Smith and A.G. Jardine **Toxic effect of phosphate on the vasculature can be reversed.** J. Am. Soc Nephrol. 23: 2012
10. **K.K. Stevens**, E.C. Beattie, C. Delles and A.G. Jardine **Phosphate impairs endothelial function – a mechanism for increased cardiovascular risk?** *NDT* (2013) 28(suppl 1): i3-i4 doi:10.1093/ndt/gft134
11. **K.K. Stevens**, R.K. Patel, G.L.Smith, M.J. Clancy, C. Delles and A.G. Jardine **Deleterious effects of phosphate on vascular function.** *NDT* (2013) 28(suppl 1): i66 doi:10.1093/ndt/gft177

12. **K.K. Stevens, R.K. Patel, P.B. Mark, C. Delles and A.G. Jardine Sustained phosphate loading impairs endothelial function: a single blind cross over trial.** NDT (2013) 28(suppl 1): i331-i351 doi:10.1093/ndt/gft129

Awards

1. Nominated for the ERA-EDTA Young Investigator's award 2013/2014
2. One of eight best abstracts by young presenters, European Renal Association, Istanbul 2013
3. ERA-EDTA Travel Grant for abstract, European Renal Association, Istanbul 2013
4. Poster of Distinction, The Renal Association, Bournemouth 2013
5. BHF Junior Clinical Fellowship (£107 000), 2012
6. ERA-EDTA Travel Grant for abstract at European Renal Association, Paris 2012
7. John Robertson Bequest award, University of Glasgow (£1390.40), 2012

Additional presentations

1. **K.K. Stevens, E.P. McQuarrie, R.K. Patel, P.B. Mark and A.G. Jardine FGF-23 is an independent predictor of LVH, measured with cardiac MRI, in patients with CKD.** Oral presentation, The Scottish Renal Association, Inverness 2011
2. **K.K. Stevens, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine Hyperphosphatemia impairs relaxation in resistance vessels.** Poster presentation The World Congress of Nephrology, Vancouver 2011
3. **K.K. Stevens, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine. Hyperphosphatemia alters both endothelial dependent and independent relaxation in resistance vessels.** Poster presentation The Renal Association, Birmingham, 2011
4. **K.K. Stevens, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine. Hyperphosphatemia impairs endothelial function in cells and in resistance vessels – a mechanism for increased cardiovascular risk?** Oral presentation Scottish Renal Association, Dumfries, 2012
5. **K.K. Stevens, E.C. Beattie, W. Sands, C. Delles and A.G. Jardine Hyperphosphatemia impairs relaxation in resistance vessels – a mechanism for increased cardiovascular risk?** Poster Presentation The Royal Medical and Chirurgical Society, Glasgow 2012
6. **K.K. Stevens, E.C. Beattie, R.K. Patel, C. Delles and A.G. Jardine Phosphodiesterase 5 inhibitor can correct phosphate induced impaired relaxation in rat resistance vessels.** Poster presentation The Renal Association, Newcastle, 2012
7. **K.K. Stevens, E.C. Beattie, S. Kettlewell, D. Hillyard, W. Sands, C. Delles, G. Smith and A.G. Jardine Deleterious effects of hyperphosphatemia *in vitro* – a novel mechanism for increased cardiovascular risk?** Oral presentation The Physiological Society, Edinburgh 2012
8. **K.K. Stevens, R.K. Patel, P.B. Mark, C. Delles and A.G. Jardine Oral Phosphate Loading and Endothelial Function: a Single Blind, Cross Over Trial.** Poster presentation The Royal Medical and Chirurgical Society, Glasgow 2013
9. **K.K. Stevens, R.K. Patel, P.B. Mark, C. Delles and A.G. Jardine. Long term phosphate loading and endothelial function: a single blind cross over trial.** Poster presentation The Renal Association, Bournemouth, 2013.

9.2 Supplementary Material

1. Patient information sheet (Chapter five)
2. Consent form (Chapter five)
3. Patient information sheet (Chapter seven)
4. Consent form (Chapter seven)
5. GP letter (Chapter seven)
6. Recruitment email (Chapter seven)
7. Table A1



The University of Glasgow, charity number SC004401
Dr Kate Stevens
BHF Glasgow Cardiovascular Research Centre
University of Glasgow
126 University Avenue
Glasgow G12 8TA
Phone: +44 (0) 141 330 2409
Email: k.stevens@clinmed.gla.ac.uk

Patient information sheet for patients undergoing abdominal surgery including nephrectomy

Study Title

Phosphate and endothelial function in human blood vessels

Invitation

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

What is the Purpose of the Study?

Patients with kidney disease are at increased risk of heart disease. Traditional risk factors for heart disease for example smoking do not adequately explain this increased risk. Phosphate, a salt naturally present in the body, is often elevated in patients with kidney disease because it is more difficult to excrete it in the urine. Whilst we know that high levels of phosphate are a risk factor for heart disease, we don't understand why this should be. High levels of phosphate may interfere with the function of the body's blood vessels making it more difficult for them to contract and relax appropriately. Increased phosphate may also cause different substances to be produced by cells within the blood vessel walls and may activate different genes causing the cells to behave differently to normal. All of this may increase the risk of heart disease. Some risk factors for heart disease for example sex cannot be altered but it is possible to modify phosphate levels in the body both with dietary measures and by taking tablets. It is important therefore that we try to establish why higher

levels of phosphate are linked with heart disease so that we can look at targeting phosphate as a way to lower the risks of heart disease.

Why have I been chosen?

We are planning to examine blood vessels from 20 patients who are known to have kidney disease and 20 patients who do not have kidney disease. You have been selected either because you have kidney disease or because you do not and because you are having an operation on your abdomen which involves your surgeon making an incision through adipose tissue (fat tissue).

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign two consent forms. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

We would like to use any left-over blood vessels from your operation for research. When the surgeon is performing your operation, it is necessary to cut through small amounts of adipose tissue (fat tissue) under the skin. This tissue is usually discarded during the operation. However, there are many small blood vessels contained within this tissue which we would like to use. Surgeons will **not remove any extra tissue** for this study. There will be no change to your operation or your treatment in any way. We would like to use your spare blood vessels for a number of examinations. For example we will examine how your blood vessels contract and relax when exposed to different levels of phosphate and which substances are produced by different layers of your vessels. We will also look at different genes within your blood sample to see if these are turned on or off when exposed to different levels of phosphate.

We would also like to look at your medical notes and copy some of the information about any other medical conditions which you may have. Where available we will also take a copy of recent findings including blood results and other clinical data such as a current blood pressure reading.

What do I have to do?

To participate, we will ask you to sign three copies of a consent form; 1 copy of which you will be able to keep. We will take a blood sample from you and a urine specimen. During your operation if your surgeon has excess fat tissue, he will contact us. There will be no change to your operation or other treatment in any way.

What are the side effects of taking part?

Your participation in this study will make no difference to your clinical treatment.

What are the possible benefits of taking part?

There is no direct benefit for you from taking part in this study. However, the information we get from this study may help us in the future to have a better understanding of the role phosphate plays in heart disease and it may allow us to better treat patients with kidney disease and heart disease.

What if something goes wrong?

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

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. The tissue specimens will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Results of this study will be published in scientific journals and thereby made available to the public. At BHF Glasgow Cardiovascular Research Centre we will have events to inform the public about our ongoing research and about results from this and other studies.

What will happen to any samples I give?

You will donate blood vessel samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples or parts of them for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research. Similarly the

blood and urine specimens will also be examined straight away and a small amount will be stored for future analysis.

Who is organising and funding the research?

This study is organised by scientists from the BHF Glasgow Cardiovascular Research Centre at Glasgow University. The study is funded by charities and researchers will not receive any payment for conducting this research.

Who has reviewed the study?

The West of Scotland Research Ethics Committee 4 has reviewed this study.

Contact for Further Information

Should you have any further questions please feel free to call Dr K Stevens at the BHF Glasgow Cardiovascular Research Centre, telephone 0141 330 2409.



University of Glasgow | BHF Glasgow Cardiovascular Research Centre

Contact: Dr Kate Stevens, Rm 312, 126 University Place, University of Glasgow, G12 8TA

Tel: +44(0)141-330-2409, email: k.stevens@clinmed.gla.ac.uk

CONSENT FORM

Version: 2.0 Date: 25 May 2010

Title of Project:

Phosphate and endothelial function in human blood vessels

Name of Researcher:

Dr K. Stevens

Patient Identification Code for this Study:

--	--	--	--	--	--	--	--

- I confirm that I have read and understand the information sheet dated 31/03/2010 (v1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
- I understand that sections of any of my medical notes and data collected during the study, may be looked at by members of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- I agree to take part in the above project and to give any surplus pieces of adipose (fat) tissue removed during my operation for medical research. I agree to donate samples of blood and urine, for the purposes of the research project. I understand that samples will be examined for genes and proteins that may be related to the kidney and phosphate regulation. I agree that my samples will be retained for future use.
- I understand that my surgeon will **NOT** be removing any extra tissue other than what is necessary as part of my care

Name of Participant

Date

Signature

Name of Person taking consent

Date

Signature

1 for participant; 1 for researcher; 1 for casenotes



The University of Glasgow, charity number SC004401
 Dr Kate Stevens
 BHF Glasgow Cardiovascular Research Centre
 University of Glasgow
 Glasgow G12 8TA
 Phone: 0141 330 2409. Email: Kate.Stevens@Glasgow.ac.uk

***For DRUG RELATED queries, please contact Professor Alan Jardine. Address as above. Phone: 0141-330-2705. Email: Alan.Jardine@Glasgow.ac.uk**

Patient information sheet

Study Title: The physiological effects of altering phosphate concentration on the function of human blood vessels.

Invitation

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

What is the Purpose of the Study?

Phosphate, a salt naturally present in the body, is known to be linked to an increased risk of cardiovascular disease. We know that higher levels of phosphate, even within the normal range, are a risk factor for heart disease but we don't understand why. Phosphate may interfere with the function of blood vessels and may cause genes to be activated causing blood vessels to behave differently. This may increase the risk of heart disease. It is possible to reduce phosphate levels with dietary changes and medication. It is important to establish why higher levels of phosphate are linked with heart disease and then we can look at ways to reduce phosphate levels as a way to lower the risks of heart disease. We would like to alter the level of phosphate within the body to see how this affects blood vessel function.

Why have I been chosen?

We are planning to look at the effects of phosphate on blood vessels from 24 patients who have no known health problems.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign three consent forms. If you decide to take part you are still

free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

You will be invited to our research centre at The University of Glasgow for 3 90 minute appointments over a 6 week period. A taxi will pick you up and take you home.

You are not required to do anything prior to attending for your initial visit (visit 1). Between visit 1 and visit 2 you will be given a phosphate binding medication (Lanthanum) **or** a phosphate supplement (Phosphate Sandoz) and asked to take 1 tablet three times daily for two weeks. After visit 2, you will be given the other tablet to take for 2 weeks. You will then attend for visit 3. You will know which of the 2 drugs you have been taking but the doctor who sees you will not. This is to avoid any bias during the analysis of the results. We ask that you do not divulge which drug you have been taking. **IF YOU HAVE ANY CONCERNS/QUESTIONS ABOUT THE DRUGS, PLEASE CONTACT PROFESSOR ALAN JARDINE (CONTACT DETAILS ABOVE)**

On each occasion, we ask you to come fasted and not to take any caffeine for 12 hours beforehand. We will provide a urine specimen container and ask you to collect your urine for 24 hours before attending the appointment. At each visit, we will carry out the following tests:

1. Height and weight and blood pressure ask you some simple questions about your health and the medication you are taking. This will take no more than 10 minutes.
2. Blood samples – about 2 tablespoons in total. We will do a number of different tests including phosphate level and kidney function. Some of the blood will be stored in our lab and we will use it to look at genes involved in the regulation of phosphate. We will also analyse and store your urine specimen.
3. Examination of the blood vessels in your wrist with a pencil-like probe. It takes 1 to 2 minutes and gives us useful information about the blood vessels.
4. Function of the blood vessels. We will measure changes in blood flow with a probe on your upper arm. A blood pressure cuff will be placed around your forearm and inflated for 5 minutes and then deflated. We can then measure the changes in the size of and blood flow to the upper arm blood vessels. The final part of the test involves a spray of a medication called GTN spray under the tongue. GTN is a very short acting medication which dilates the blood vessels

and gives us additional information about the blood vessel function. The whole test takes about 20 to 25 minutes.

What are the risks of taking part?

We will take blood from the vein in your arm which in rare cases results in a small bruise. The amount of blood taken for this research does not place you at any risk. The other tests are 'non-invasive' – the probes are attached to the skin and no needles are involved. Measurement of the function of the blood vessels may lead to some numbness in the fingers when the blood pressure cuff is inflated. This will disappear when the cuff is deflated. A small bruise on your forearm may result from the cuff but will disappear within a couple of days. GTN spray is very short acting. In some people it can cause a headache will disappear within a few minutes.

Lanthanum and Phosphate Sandoz are well tolerated tablets with few side effects. Lanthanum can cause constipation; diarrhoea; nausea; stomach pain; vomiting. Serious side effects are uncommon but include allergic reactions (rash; hives; itching; difficulty breathing; swelling of the mouth, face, lips, or tongue), dizziness and weakness Phosphate sandoz are well tolerated; apart from nausea and diarrhoea, very few side effects have been reported.

What are the possible benefits of taking part?

There is no direct benefit for you from taking part in this study. However, the information we get from this study may help us in the future to have a better understanding of the role phosphate plays in heart disease and it may allow us to better treat patients who have heart disease or who are at risk of developing it.

What if something goes wrong?

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

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. The blood and urine specimens will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the

course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Results of this study will be published in scientific journals and thereby made available to the public. At BHF Glasgow Cardiovascular Research Centre we will have events to inform the public about our ongoing research and about results from this and other studies.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away. Other examinations will be done at a later stage when we collect more samples from other patients. We will also store some of your samples or parts of them for up to 10 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research.

Who is organising and funding the research?

This study is organised by scientists from the BHF Glasgow Cardiovascular Research Centre at Glasgow University. The study is funded by charities and researchers will not receive any payment for conducting this research.

Who has reviewed the study?

The West of Scotland Research Ethics Committee 2 has reviewed this study.

Contact for Further Information

Should you have any further questions please call Dr K Stevens at the BHF Glasgow Cardiovascular Research Centre, telephone 0141 330 2409. **FOR DRUG RELATED QUERIES, PLEASE CONTACT PROFESSOR JARDINE, TELEPHONE 0141-330-2705.**

If you wish to discuss the study with an independent contact person who is not involved in the study, please contact Dr Marie Freel via email on Marie.Freel@Glasgow.ac.uk or by telephone 0141-330-3412.



University of Glasgow | BHF Glasgow Cardiovascular Research Centre

Contact: **FOR DRUG RELATED QUERIES:** Prof Alan Jardine, BHF GCRC, 126 University Place, G12 8TA. Tel:0141-330-2705 .Email: Alan.Jardine@Glasgow.ac.uk For all other queries: Dr Kate Stevens, BHF GCRC, 126 University Place, G12 8TA. Tel:0141-330-2409. Email Kate.Stevens@Glasgow.ac.uk

CONSENT FORM

Version: 1.0 Date: 13 September 2011

Title of Project: The physiological effects of altering phosphate concentration on the function of human blood vessels. **Name of Researchers:** Prof. A. Jardine and Dr K. Stevens

Patient Identification Code for this Study:

--	--	--	--	--	--	--	--

- I confirm that I have read and understand the information sheet dated 13/09/2011 (v1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
- I understand that sections of my medical notes and data collected during the study, may be looked at by members of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- I agree to take part in the above project and undergo the tests described in the information sheet. I agree to donate samples of blood and urine. I understand that samples will be examined for genes and proteins and agree that my samples will be retained for future use.
- I understand that I will be asked to take a phosphate binder and a phosphate supplement during the study.
- I agree to my General Practitioner being informed of my participation.

Name of Participant

Date

Signature

Name of Person taking consent

Date

Signature

1 for participant; 1 for researcher; 1 for casenotes



GP Letter Version 1 Date 30/06/2011

Study title: The physiological effects of altering phosphate concentration on the function of human blood vessels

Date_____

Dear_____

RE:_____

The above patient, who is not known to have chronic kidney disease, has agreed to participate in a research study looking at the effects of phosphate on endothelial function. Patients with and without chronic kidney disease will undergo a number of different tests on 3 separate occasions including non invasive pulse wave analysis, endothelial function testing and measures of vascular stiffness. Blood and urine will also be analysed. As part of the study patients will take a phosphate binder and phosphate supplement; each for a two week period.

We will not routinely report results back to you. However should you wish further information please do not hesitate to contact me on the telephone number below.

Yours Sincerely,

Dr Kate Stevens

Dear Colleague

The physiological effects of altering phosphate concentration on the function of human blood vessels.

I would be very grateful if you would consider volunteering for the above study. This projects aims to examine the effects of phosphate on the function of blood vessels and in particular the lining of the blood vessels. Phosphate is taken in through the diet and we have some evidence that levels at the high end of the normal range might be associated with an increased risk of cardiovascular disease. We do not fully understand why phosphate should increase cardiovascular risk but believe that it might be related to damaging effects on the blood vessel lining. We would like to alter your phosphate level with tablets and measure the effects this has on blood vessel function. Ultimately, we are looking to clarify the mechanism of phosphate as a cardiovascular risk factor because it can be modified with dietary intervention and drug therapy and therefore

Further information is attached. **If you would like to discuss the study further (with no obligation) or participate please contact me.**

Best Wishes,

Dr Kate Stevens

0141 330 2409

Kate.Stevens@glasgow.ac.uk

The ethics approval number for the study is 11/AL/0035

R&D management approval number GN10RE236

ILLUMINA® PID	Gene Symbol	FC	ILLUMINA® PID	Gene Symbol	FC
ILMN_1806165	HSPA6	-25.3139	ILMN_1743445	FAM107A	-2.03453
ILMN_1789074	HSPA1A	-11.4314	ILMN_2374865	ATF3	-2.02714
ILMN_1729216	CRYAB	-9.41668	ILMN_1764709	MAFB	-2.02531
ILMN_3235964	HSPA7	-7.65947	ILMN_1739428	IFIT2	-2.02522
ILMN_1660436	HSPA1B	-7.47329	ILMN_1738335	TNFSF18	-2.02355
ILMN_1739423	RN7SK	-7.24565	ILMN_1801584	CXCR4	-2.01862
ILMN_3234762	RN5S9	-5.20804	ILMN_1712913	UNC5A	-2.0181
ILMN_3246273	RNU1-3	-5.17103	ILMN_1811472	KIF23	2.00272
ILMN_3244646	RNU1G2	-5.04368	ILMN_1886655		2.00757
ILMN_1658383	HSPA6	-4.80295	ILMN_1671843	PSRC1	2.00877
ILMN_2086095	ID2	-4.77679	ILMN_1724407	TACC3	2.01981
ILMN_3236653	RNU1-5	-4.704	ILMN_2222008	KIFC1	2.02306
ILMN_3243644	LOC100132564	-4.51724	ILMN_2392472	CENPA	2.02325
ILMN_1814221	NPTX1	-4.28743	ILMN_2409298	NUSAP1	2.03674
ILMN_3239574	SNORD3A	-4.24576	ILMN_1796949	TPX2	2.03907
ILMN_1793990	ID2	-4.1334	ILMN_1700337	TROAP	2.06598
ILMN_3242315	SNORD3D	-4.09294	ILMN_3236160	C4orf49	2.07398
ILMN_3241034	SNORD3C	-3.95073	ILMN_1768097	RPGR	2.07443
ILMN_1655595	SERPINE2	-3.93494	ILMN_1672611	CDH11	2.08778
ILMN_3309453	RNU4-1	-3.77473	ILMN_2225718	CENPE	2.08879
ILMN_1692938	PSAT1	-3.68627	ILMN_1801246	IFITM1	2.10152
ILMN_1728785	GPR116	-3.56191	ILMN_1666305	CDKN3	2.10514
ILMN_3240220	RNU1F1	-3.46815	ILMN_1680955	AURKA	2.12336
ILMN_3245678	RNU1A3	-3.35049	ILMN_1753063	KIF15	2.14086
ILMN_1746517	KYNU	-3.24605	ILMN_2409220	HMMR	2.1558
ILMN_2341229	CD34	-3.10609	ILMN_2163723	KRT7	2.17037
ILMN_1751607	FOSB	-3.01094	ILMN_1739645	ANLN	2.17495
ILMN_1796417	ASNS	-2.93579	ILMN_1685916	KIF2C	2.18083
ILMN_1732799	CD34	-2.92934	ILMN_1786125	CCNA2	2.18388
ILMN_3305273	LOC729779	-2.8901	ILMN_1747911	CDC2	2.18961
ILMN_1669523	FOS	-2.88802	ILMN_1737728	CDCA3	2.2138
ILMN_2074860	RN7SK	-2.88655	ILMN_1664516	CENPF	2.216
ILMN_2150851	SERPINB2	-2.74696	ILMN_1788166	TTK	2.22291
ILMN_1687757	AKR1C4	-2.71868	ILMN_2077550	RACGAP1	2.23268
ILMN_1704537	PHGDH	-2.67736	ILMN_1781943	FAM83D	2.25605

Table A1: Differentially expressed probes identified by Illumina BeadStudio software in HUVECs culture in high phosphate.

PID, probe identification; FC, fold change where the fold change is in cells cultured in high phosphate compared with cells cultured normal phosphate medium. Candidate genes are highlighted.

ILLUMINA® PID	Gene Symbol	FC	ILLUMINA® PID	Gene Symbol	FC
ILMN_1651498	GADD45G	-2.58816	ILMN_1751776	CKAP2L	2.2712
ILMN_1666733	IL8	-2.58615	ILMN_1753449	CST1	2.31416
ILMN_1775304	DNAJB1	-2.56957	ILMN_1794539	KIF11	2.36294
ILMN_3241878	RNU6ATAC	-2.56502	ILMN_1793025	OSAP	2.37514
ILMN_2120695	TSPAN7	-2.54602	ILMN_1716279	CENPE	2.37678
ILMN_2184373	IL8	-2.51539	ILMN_2143155	KIF11	2.40801
ILMN_2150856	SERPINB2	-2.44167	ILMN_1684217	AURKB	2.41338
ILMN_1908824		-2.43835	ILMN_1751444	NCAPG	2.44288
ILMN_1809291	TSPAN7	-2.418	ILMN_2162860	SLFN11	2.50444
ILMN_1768139	RNU12	-2.37141	ILMN_1726720	NUSAP1	2.52074
ILMN_2139761	LIMCH1	-2.36854	ILMN_1703906	HJURP	2.55119
ILMN_1796179	HIST1H2BK	-2.35807	ILMN_2072101	C4orf49	2.56007
ILMN_2173611	MT1E	-2.35039	ILMN_1747016	CEP55	2.56808
ILMN_1798360	CXCR7	-2.33758	ILMN_1752520	SLFN11	2.58088
ILMN_1775170	MT1X	-2.32448	ILMN_1728934	PRC1	2.63054
ILMN_1813314	HIST1H2BK	-2.28837	ILMN_1801939	CCNB2	2.63694
ILMN_1698725	FRMD3	-2.25119	ILMN_1749829	DLGAP5	2.66043
ILMN_2371458	CXCR7	-2.21337	ILMN_2051373	NEK2	2.66356
ILMN_3310351	RNU6-15	-2.19738	ILMN_2301083	UBE2C	2.67029
ILMN_1910180		-2.17005	ILMN_2202948	BUB1	2.69462
ILMN_2186137	RRAD	-2.15489	ILMN_1801257	CENPA	2.74712
ILMN_3247458	LOC652750	-2.15003	ILMN_1695658	KIF20A	2.7828
ILMN_3308335	RNU6-1	-2.14885	ILMN_1714730	UBE2C	2.83813
ILMN_1770152	ADAMTS18	-2.13135	ILMN_3239771	DLGAP5	2.94993
ILMN_2412336	AKR1C2	-2.11502	ILMN_1673639	ABI3BP	3.07325
ILMN_2398107	ASNS	-2.0654	ILMN_1663390	CDC20	3.29585
ILMN_1667081	CCND2	-2.05733	ILMN_1652826	LRRC17	3.48163
ILMN_1662419	COX7A1	-2.0552	ILMN_1686097	TOP2A	3.48996
ILMN_1651343	ITGA11	-2.05311	ILMN_2406815	LRRC17	3.85814
ILMN_3268403	LOC100128252	-2.04976	ILMN_1815184	ASPM	3.99805
ILMN_1694671	ZFAND2A	-2.04516			

Table A1 continued

Reference List

- (1) Hsu CY, Chertow GM. Elevations of serum phosphorus and potassium in mild to moderate chronic renal insufficiency. *Nephrol Dial Transplant* 2002 Aug;17(8):1419-25.
- (2) Kestenbaum B, Sampson JN, Rudser KD, Patterson DJ, Seliger SL, Young B, et al. Serum phosphate levels and mortality risk among people with chronic kidney disease. *J Am Soc Nephrol* 2005 Feb;16(2):520-8.
- (3) Achinger SG, Ayus JC. Left ventricular hypertrophy: is hyperphosphatemia among dialysis patients a risk factor? *J Am Soc Nephrol* 2006 Dec;17(12 Suppl 3):S255-S261.
- (4) Giachelli CM. The emerging role of phosphate in vascular calcification. *Kidney Int* 2009 May;75(9):890-7.
- (5) Giachelli CM, Speer MY, Li X, Rajachar RM, Yang H. Regulation of vascular calcification: roles of phosphate and osteopontin. *Circ Res* 2005 Apr 15;96(7):717-22.
- (6) Shuto E, Taketani Y, Tanaka R, Harada N, Isshiki M, Sato M, et al. Dietary phosphorus acutely impairs endothelial function. *J Am Soc Nephrol* 2009 Jul;20(7):1504-12.
- (7) Abramowitz M, Muntner P, Coco M, Southern W, Lotwin I, Hostetter TH, et al. Serum alkaline phosphatase and phosphate and risk of mortality and hospitalization. *Clin J Am Soc Nephrol* 2010 Jun;5(6):1064-71.
- (8) Foley RN, Collins AJ, Herzog CA, Ishani A, Kalra PA. Serum phosphorus levels associate with coronary atherosclerosis in young adults. *J Am Soc Nephrol* 2009 Feb;20(2):397-404.
- (9) Tonelli M, Pannu N, Manns B. Oral phosphate binders in patients with kidney failure. *N Engl J Med* 2010 Apr 8;362(14):1312-24.
- (10) KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009 Aug;(113):S1-130.
- (11) Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J Am Soc Nephrol* 2004 Aug;15(8):2208-18.
- (12) Crowe E, Halpin D, Stevens P. Early identification and management of chronic kidney disease: summary of NICE guidance. *BMJ* 2008;337:a1530.
- (13) Stevens PE, O'Donoghue DJ, de LS, Van VJ, Klebe B, Middleton R, et al. Chronic kidney disease management in the United Kingdom: NEOERICA project results. *Kidney Int* 2007 Jul;72(1):92-9.
- (14) Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in

Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Hypertension* 2003 Nov;42(5):1050-65.

- (15) Keith DS, Nichols GA, Gullion CM, Brown JB, Smith DH. Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. *Arch Intern Med* 2004 Mar 22;164(6):659-63.
- (16) Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004 Sep 23;351(13):1296-305.
- (17) Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol* 1998 Dec;9(12 Suppl):S16-S23.
- (18) Baboolal K, McEwan P, Sondhi S, Spiewanowski P, Wechowski J, Wilson K. The cost of renal dialysis in a UK setting--a multicentre study. *Nephrol Dial Transplant* 2008 Jun;23(6):1982-9.
- (19) Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med* 1999 Dec 2;341(23):1725-30.
- (20) Schnuelle P, Lorenz D, Trede M, Van Der Woude FJ. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence for reduced mortality risk compared with hemodialysis during long-term follow-up. *J Am Soc Nephrol* 1998 Nov;9(11):2135-41.
- (21) Kasiske BL. Risk factors for accelerated atherosclerosis in renal transplant recipients. *Am J Med* 1988 Jun;84(6):985-92.
- (22) Cohen B, Smits JM, Haase B, Persijn G, Vanrenterghem Y, Frei U. Expanding the donor pool to increase renal transplantation. *Nephrol Dial Transplant* 2005 Jan;20(1):34-41.
- (23) Hutchison AJ, Smith CP, Brenchley PE. Pharmacology, efficacy and safety of oral phosphate binders. *Nat Rev Nephrol* 2011 Oct;7(10):578-89.
- (24) Cancer Research UK. <http://www.cancerresearchuk.org/cancer-info/cancerstats/types>. 6-2-2013.

Ref Type: Generic

- (25) Byrne C, Ford D, Gilg J, Ansell D, Feehally J. UK Renal Registry 12th Annual Report (December 2009): Chapter 3: UK ESRD incident rates in 2008: national and centre-specific analyses. *Nephron Clin Pract* 2010;115 Suppl 1:c9-39.
- (26) Webb L, Casula A, Ravanan R, Caskey F. UK Renal Registry 13th Annual Report (December 2010): Chapter 3: demographic and biochemistry profile of kidney transplant recipients in the UK in 2009: national and centre-specific analyses. *Nephron Clin Pract* 2011;119 Suppl 2:c53-c84.
- (27) Bengt Fellstrom, Alan G.Jardine, Hallvard Holdaas, Roland Schmieder, Mattis Gottlow, Eva Johnsson, et al. The effects of rosuvastatin versus placebo on cardiovascular outcomes in patients with end stage renal disease on haemodialysis - results of the AURORA study. *NDT Plus* 2 (suppl2), ii1514. 2009.

Ref Type: Generic

- (28) Jardine AG, Fellstrom B, Logan JO, Cole E, Nyberg G, Gronhagen-Riska C, et al. Cardiovascular risk and renal transplantation: post hoc analyses of the Assessment of Lescol in Renal Transplantation (ALERT) Study. *Am J Kidney Dis* 2005 Sep;46(3):529-36.
- (29) Kopple JD, Zhu X, Lew NL, Lowrie EG. Body weight-for-height relationships predict mortality in maintenance hemodialysis patients. *Kidney Int* 1999 Sep;56(3):1136-48.
- (30) Zager PG, Nikolic J, Brown RH, Campbell MA, Hunt WC, Peterson D, et al. "U" curve association of blood pressure and mortality in hemodialysis patients. *Medical Directors of Dialysis Clinic, Inc. Kidney Int* 1998 Aug;54(2):561-9.
- (31) Tonelli M, Pfeffer MA. Kidney disease and cardiovascular risk. *Annu Rev Med* 2007;58:123-39.
- (32) Connolly GM, Cunningham R, McNamee PT, Young IS, Maxwell AP. Elevated serum phosphate predicts mortality in renal transplant recipients. *Transplantation* 2009 Apr 15;87(7):1040-4.
- (33) K.K.Stevens, I.R.Morgan, R.K.Patel, P.B.Mark, C.C.Geddes, A.G.Jardine, et al. Serum phosphate and outcome at 1 year after deceased donor renal transplantation. *Clinical Transplantation* 2010 (in press) .

Ref Type: Generic

- (34) Cirillo M, Botta G, Chiricone D, De Santo NG. Glomerular filtration rate and serum phosphate: an inverse relationship diluted by age. *Nephrol Dial Transplant* 2009 Jul;24(7):2123-31.
- (35) Covic A, Kanbay M, Voroneanu L, Turgut F, Serban DN, Serban IL, et al. Vascular calcification in chronic kidney disease. *Clin Sci (Lond)* 2010;119(3):111-21.
- (36) Juppner H. Phosphate and FGF-23. *Kidney Int Suppl* 2011 Apr;(121):S24-S27.
- (37) Hallan SI, Ritz E, Lydersen S, Romundstad S, Kvenild K, Orth SR. Combining GFR and albuminuria to classify CKD improves prediction of ESRD. *J Am Soc Nephrol* 2009 May;20(5):1069-77.
- (38) Faqah A, Jafar TH. Control of blood pressure in chronic kidney disease: how low to go? *Nephron Clin Pract* 2011;119(4):c324-c331.
- (39) Taal MW, Brenner BM. Renal risk scores: progress and prospects. *Kidney Int* 2008 Jun;73(11):1216-9.
- (40) Ruggenti P, Perna A, Remuzzi G. Retarding progression of chronic renal disease: the neglected issue of residual proteinuria. *Kidney Int* 2003 Jun;63(6):2254-61.
- (41) Breyer JA, Bain RP, Evans JK, Nahman NS, Jr., Lewis EJ, Cooper M, et al. Predictors of the progression of renal insufficiency in patients with insulin-dependent diabetes and overt diabetic nephropathy. The Collaborative Study Group. *Kidney Int* 1996 Nov;50(5):1651-8.

- (42) Kaplan B, Schold J, Meier-Kriesche HU. Poor predictive value of serum creatinine for renal allograft loss. *Am J Transplant* 2003 Dec;3(12):1560-5.
- (43) Nauta FL, Bakker SJ, van OW, Navis G, van der Heide JJ, van GH, et al. Albuminuria, proteinuria, and novel urine biomarkers as predictors of long-term allograft outcomes in kidney transplant recipients. *Am J Kidney Dis* 2011 May;57(5):733-43.
- (44) Halimi JM, Buchler M, Al-Najjar A, Laouad I, Chatelet V, Marliere JF, et al. Urinary albumin excretion and the risk of graft loss and death in proteinuric and non-proteinuric renal transplant recipients. *Am J Transplant* 2007 Mar;7(3):618-25.
- (45) Lin CC, Chen CC, Kung PT, Li CI, Yang SY, Liu CS, et al. Joint relationship between renal function and proteinuria on mortality of patients with type 2 diabetes: The Taichung Diabetes Study. *Cardiovasc Diabetol* 2012;11:131.
- (46) Matsushita K, van d, V, Astor BC, Woodward M, Levey AS, de Jong PE, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 2010 Jun 12;375(9731):2073-81.
- (47) van Ree RM, Oterdoom LH, de Vries AP, Homan van der Heide JJ, van Son WJ, Navis G, et al. Circulating markers of endothelial dysfunction interact with proteinuria in predicting mortality in renal transplant recipients. *Transplantation* 2008 Dec 27;86(12):1713-9.
- (48) Turin TC, Tonelli M, Manns BJ, Ahmed SB, Ravani P, James M, et al. Proteinuria and Life Expectancy. *Am J Kidney Dis* 2012 Dec 7.
- (49) Obeidat M, Obeidat M, Ballermann BJ. Glomerular endothelium: a porous sieve and formidable barrier. *Exp Cell Res* 2012 May 15;318(9):964-72.
- (50) Erkan E. Proteinuria and progression of glomerular diseases. *Pediatr Nephrol* 2012 Nov 3.
- (51) Bohle A, Mackensen-Haen S, von GH, Grund KE, Wehrmann M, Batz C, et al. The consequences of tubulo-interstitial changes for renal function in glomerulopathies. A morphometric and cytological analysis. *Pathol Res Pract* 1990 Feb;186(1):135-44.
- (52) Agrawal V, Marinescu V, Agarwal M, McCullough PA. Cardiovascular implications of proteinuria: an indicator of chronic kidney disease. *Nat Rev Cardiol* 2009 Apr;6(4):301-11.
- (53) Paisley KE, Beaman M, Tooke JE, Mohamed-Ali V, Lowe GD, Shore AC. Endothelial dysfunction and inflammation in asymptomatic proteinuria. *Kidney Int* 2003 Feb;63(2):624-33.
- (54) Stroes ES, Joles JA, Chang PC, Koomans HA, Rabelink TJ. Impaired endothelial function in patients with nephrotic range proteinuria. *Kidney Int* 1995 Aug;48(2):544-50.
- (55) Brenner BM, Cooper ME, de ZD, Keane WF, Mitch WE, Parving HH, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001 Sep 20;345(12):861-9.

- (56) Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, et al. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 2001 Sep 20;345(12):851-60.
- (57) Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). *Lancet* 1997 Jun 28;349(9069):1857-63.
- (58) Hou FF, Xie D, Zhang X, Chen PY, Zhang WR, Liang M, et al. Renoprotection of Optimal Antiproteinuric Doses (ROAD) Study: a randomized controlled study of benazepril and losartan in chronic renal insufficiency. *J Am Soc Nephrol* 2007 Jun;18(6):1889-98.
- (59) Siragy HM, Carey RM. Role of the intrarenal renin-angiotensin-aldosterone system in chronic kidney disease. *Am J Nephrol* 2010;31(6):541-50.
- (60) Bomback AS, Kshirsagar AV, Amamoo MA, Klemmer PJ. Change in proteinuria after adding aldosterone blockers to ACE inhibitors or angiotensin receptor blockers in CKD: a systematic review. *Am J Kidney Dis* 2008 Feb;51(2):199-211.
- (61) Wu MT, Tung SC, Hsu KT, Lee CT. Aliskiren add-on therapy effectively reduces proteinuria in chronic kidney disease: An open-label prospective trial. *J Renin Angiotensin Aldosterone Syst* 2012 Dec 7.
- (62) Ibels LS, Alfrey AC, Haut L, Huffer WE. Preservation of function in experimental renal disease by dietary restriction of phosphate. *N Engl J Med* 1978 Jan 19;298(3):122-6.
- (63) Neves KR, Gracioli FG, Dos Reis LM, Pasqualucci CA, Moyses RM, Jorgetti V. Adverse effects of hyperphosphatemia on myocardial hypertrophy, renal function, and bone in rats with renal failure. *Kidney Int* 2004 Dec;66(6):2237-44.
- (64) Barsotti G, Morelli E, Giannoni A, Guiducci A, Lupetti S, Giovannetti S. Restricted phosphorus and nitrogen intake to slow the progression of chronic renal failure: a controlled trial. *Kidney Int Suppl* 1983 Dec;16:S278-S284.
- (65) Norris KC, Greene T, Kopple J, Lea J, Lewis J, Lipkowitz M, et al. Baseline predictors of renal disease progression in the African American Study of Hypertension and Kidney Disease. *J Am Soc Nephrol* 2006 Oct;17(10):2928-36.
- (66) Schwarz S, Trivedi BK, Kalantar-Zadeh K, Kovesdy CP. Association of disorders in mineral metabolism with progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2006 Jul;1(4):825-31.
- (67) Voormolen N, Noordzij M, Grootendorst DC, Beetz I, Sijpkens YW, van Manen JG, et al. High plasma phosphate as a risk factor for decline in renal function and mortality in pre-dialysis patients. *Nephrol Dial Transplant* 2007 Oct;22(10):2909-16.
- (68) Bellasi A, Mandreoli M, Baldrati L, Corradini M, Di NP, Malmusi G, et al. Chronic kidney disease progression and outcome according to serum phosphorus in mild-to-moderate kidney dysfunction. *Clin J Am Soc Nephrol* 2011 Apr;6(4):883-91.

- (69) Van Bortel LM, Duprez D, Starmans-Kool MJ, Safar ME, Giannattasio C, Cockcroft J, et al. Clinical applications of arterial stiffness, Task Force III: recommendations for user procedures. *Am J Hypertens* 2002 May;15(5):445-52.
- (70) O'Rourke MF, Staessen JA, Vlachopoulos C, Duprez D, Plante GE. Clinical applications of arterial stiffness; definitions and reference values. *Am J Hypertens* 2002 May;15(5):426-44.
- (71) Laurent S, Cockcroft J, Van BL, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. *Eur Heart J* 2006 Nov;27(21):2588-605.
- (72) Ziemann SJ, Melenovsky V, Kass DA. Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler Thromb Vasc Biol* 2005 May;25(5):932-43.
- (73) Benetos A, Safar M, Rudnichi A, Smulyan H, Richard JL, Ducimetieere P, et al. Pulse pressure: a predictor of long-term cardiovascular mortality in a French male population. *Hypertension* 1997 Dec;30(6):1410-5.
- (74) Forette F, Seux ML, Staessen JA, Thijs L, Birkenhager WH, Babarskiene MR, et al. Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. *Lancet* 1998 Oct 24;352(9137):1347-51.
- (75) Blacher J, Safar ME, Guerin AP, Pannier B, Marchais SJ, London GM. Aortic pulse wave velocity index and mortality in end-stage renal disease. *Kidney Int* 2003 May;63(5):1852-60.
- (76) Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 1999 May 11;99(18):2434-9.
- (77) Laurent S, Boutouyrie P. Recent advances in arterial stiffness and wave reflection in human hypertension. *Hypertension* 2007 Jun;49(6):1202-6.
- (78) London GM, Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME. Arterial wave reflections and survival in end-stage renal failure. *Hypertension* 2001 Sep;38(3):434-8.
- (79) Ford ML, Tomlinson LA, Chapman TP, Rajkumar C, Holt SG. Aortic stiffness is independently associated with rate of renal function decline in chronic kidney disease stages 3 and 4. *Hypertension* 2010 May;55(5):1110-5.
- (80) Taal MW, Sigrist MK, Fakis A, Fluck RJ, McIntyre CW. Markers of arterial stiffness are risk factors for progression to end-stage renal disease among patients with chronic kidney disease stages 4 and 5. *Nephron Clin Pract* 2007;107(4):c177-c181.
- (81) Wang MC, Tsai WC, Chen JY, Huang JJ. Stepwise increase in arterial stiffness corresponding with the stages of chronic kidney disease. *Am J Kidney Dis* 2005 Mar;45(3):494-501.
- (82) Ilyas B, Dhaun N, Markie D, Stansell P, Goddard J, Newby DE, et al. Renal function is associated with arterial stiffness and predicts outcome in patients with coronary artery disease. *QJM* 2009 Mar;102(3):183-91.

- (83) Verberckmoes SC, Persy V, Behets GJ, Neven E, Hufkens A, Zebger-Gong H, et al. Uremia-related vascular calcification: more than apatite deposition. *Kidney Int* 2007 Feb;71(4):298-303.
- (84) Moody WE, Edwards NC, Madhani M, Chue CD, Steeds RP, Ferro CJ, et al. Endothelial dysfunction and cardiovascular disease in early-stage chronic kidney disease: cause or association? *Atherosclerosis* 2012 Jul;223(1):86-94.
- (85) Gross ML, Meyer HP, Ziebart H, Rieger P, Wenzel U, Amann K, et al. Calcification of coronary intima and media: immunohistochemistry, backscatter imaging, and x-ray analysis in renal and nonrenal patients. *Clin J Am Soc Nephrol* 2007 Jan;2(1):121-34.
- (86) Kendrick J, Chonchol M. The role of phosphorus in the development and progression of vascular calcification. *Am J Kidney Dis* 2011 Nov;58(5):826-34.
- (87) Kapustin A, Shanahan CM. Targeting vascular calcification: softening-up a hard target. *Curr Opin Pharmacol* 2009 Apr;9(2):84-9.
- (88) Janner JH, Godtfredsen NS, Ladelund S, Vestbo J, Prescott E. Aortic augmentation index: reference values in a large unselected population by means of the SphygmoCor device. *Am J Hypertens* 2010 Feb;23(2):180-5.
- (89) Mensah GA. Healthy endothelium: the scientific basis for cardiovascular health promotion and chronic disease prevention. *Vascul Pharmacol* 2007 May;46(5):310-4.
- (90) Montezano AC, Touyz RM. Reactive oxygen species and endothelial function--role of nitric oxide synthase uncoupling and Nox family nicotinamide adenine dinucleotide phosphate oxidases. *Basic Clin Pharmacol Toxicol* 2012 Jan;110(1):87-94.
- (91) Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol* 1998 May;18(5):677-85.
- (92) De CC, Amiri F, Brassard P, Endemann DH, Touyz RM, Schiffrin EL. Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin II-infused macrophage colony-stimulating factor-deficient mice: evidence for a role in inflammation in angiotensin-induced vascular injury. *Arterioscler Thromb Vasc Biol* 2005 Oct;25(10):2106-13.
- (93) Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 2003 Aug;285(2):R277-R297.
- (94) Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000 Nov 10;87(10):840-4.
- (95) Morris ST, McMurray JJ, Spiers A, Jardine AG. Impaired endothelial function in isolated human uremic resistance arteries. *Kidney Int* 2001 Sep;60(3):1077-82.
- (96) Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993 Apr 29;362(6423):801-9.

- (97) Panza JA, Quyyumi AA, Brush JE, Jr., Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 1990 Jul 5;323(1):22-7.
- (98) Johnstone MT, Creager SJ, Scales KM, Cusco JA, Lee BK, Creager MA. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation* 1993 Dec;88(6):2510-6.
- (99) Taddei S, Viridis A, Mattei P, Ghiadoni L, Sudano I, Salvetti A. Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. *Circulation* 1996 Sep 15;94(6):1298-303.
- (100) Hyseni A, Roest M, Braun SL, Barendrecht AD, de Groot PG, Ndrepepa G, et al. Chronic dysfunction of the endothelium is associated with mortality in acute coronary syndrome patients. *Thromb Res* 2012 Dec 26.
- (101) Halcox JP, Donald AE, Ellins E, Witte DR, Shipley MJ, Brunner EJ, et al. Endothelial function predicts progression of carotid intima-media thickness. *Circulation* 2009 Feb 24;119(7):1005-12.
- (102) Shechter M, Marai I, Marai S, Sherer Y, Sela BA, Feinberg MS, et al. The association of endothelial dysfunction and cardiovascular events in healthy subjects and patients with cardiovascular disease. *Isr Med Assoc J* 2007 Apr;9(4):271-6.
- (103) Viridis A, Ghiadoni L, Taddei S. Effects of antihypertensive treatment on endothelial function. *Curr Hypertens Rep* 2011 Aug;13(4):276-81.
- (104) Kampoli AM, Tousoulis D, Tentolouris C, Stefanadis C. Novel agents targeting nitric oxide. *Curr Vasc Pharmacol* 2012 Jan;10(1):61-76.
- (105) Wray DW, Nishiyama SK, Harris RA, Zhao J, McDaniel J, Fjeldstad AS, et al. Acute reversal of endothelial dysfunction in the elderly after antioxidant consumption. *Hypertension* 2012 Apr;59(4):818-24.
- (106) Naka KK, Kalantaridou SN, Kravariti M, Bechlioulis A, Kazakos N, Calis KA, et al. Effect of the insulin sensitizers metformin and pioglitazone on endothelial function in young women with polycystic ovary syndrome: a prospective randomized study. *Fertil Steril* 2011 Jan;95(1):203-9.
- (107) Kung CF, Moreau P, Takase H, Luscher TF. L-NAME hypertension alters endothelial and smooth muscle function in rat aorta. Prevention by trandolapril and verapamil. *Hypertension* 1995 Nov;26(5):744-51.
- (108) Hamilton CA, Berg G, McIntyre M, Mcphaden AR, Reid JL, Dominiczak AF. Effects of nitric oxide and superoxide on relaxation in human artery and vein. *Atherosclerosis* 1997 Aug;133(1):77-86.
- (109) Fleming I, Bauersachs J, Busse R. Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J Vasc Res* 1997 May;34(3):165-74.
- (110) Dattilo JB, Makhoul RG. The role of nitric oxide in vascular biology and pathobiology. *Ann Vasc Surg* 1997 May;11(3):307-14.

- (111) Burgoyne JR, Prisyazhna O, Rudyk O, Eaton P. cGMP-dependent activation of protein kinase G precludes disulfide activation: implications for blood pressure control. *Hypertension* 2012 Nov;60(5):1301-8.
- (112) Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, del SP. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A* 2001 Mar 27;98(7):4202-8.
- (113) Bates DO. Vascular endothelial growth factors and vascular permeability. *Cardiovasc Res* 2010 Jul 15;87(2):262-71.
- (114) Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003 Mar;111(5):649-58.
- (115) Hossain M, Qadri SM, Liu L. Inhibition of nitric oxide synthesis enhances leukocyte rolling and adhesion in human microvasculature. *J Inflamm (Lond)* 2012;9(1):28.
- (116) Czucz J, Cervenak L, Forhecz Z, Gombos T, Pozsonyi Z, Kunde J, et al. Serum soluble E-selectin and NT-proBNP levels additively predict mortality in diabetic patients with chronic heart failure. *Clin Res Cardiol* 2011 Jul;100(7):587-94.
- (117) Bouma MG, van den Wildenberg FA, Buurman WA. Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells. *Am J Physiol* 1996 Feb;270(2 Pt 1):C522-C529.
- (118) Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002 Jan;82(1):47-95.
- (119) Gutteridge JM, Halliwell B. Antioxidants: Molecules, medicines, and myths. *Biochem Biophys Res Commun* 2010 Mar 19;393(4):561-4.
- (120) Rada B, Leto TL. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib Microbiol* 2008;15:164-87.
- (121) Gutteridge JM, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci* 2000;899:136-47.
- (122) Bertram G.Katzung, Susan B.Masters, Anthony J.Trevor. *Basic and Clinical Pharmacology*. 1-2-2012. McGraw-Hill.
- Ref Type: Serial (Book,Monograph)
- (123) Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN. Abnormalities of endothelial function in patients with predialysis renal failure. *Heart* 2000 Feb;83(2):205-9.
- (124) Annuk M, Soveri I, Zilmer M, Lind L, Hulthe J, Fellstrom B. Endothelial function, CRP and oxidative stress in chronic kidney disease. *J Nephrol* 2005 Nov;18(6):721-6.
- (125) Annuk M, Zilmer M, Lind L, Linde T, Fellstrom B. Oxidative stress and endothelial function in chronic renal failure. *J Am Soc Nephrol* 2001 Dec;12(12):2747-52.

- (126) Segal MS, Baylis C, Johnson RJ. Endothelial health and diversity in the kidney. *J Am Soc Nephrol* 2006 Feb;17(2):323-4.
- (127) Filer AD, Gardner-Medwin JM, Thambyrajah J, Raza K, Carruthers DM, Stevens RJ, et al. Diffuse endothelial dysfunction is common to ANCA associated systemic vasculitis and polyarteritis nodosa. *Ann Rheum Dis* 2003 Feb;62(2):162-7.
- (128) Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrangé D, et al. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 1995 Nov 1;26(5):1235-41.
- (129) Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. 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 Jan 16;39(2):257-65.
- (130) Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 2011 Jan;300(1):H2-12.
- (131) Kuczmarski JM, Darocki MD, DuPont JJ, Sikes RA, Cooper CR, Farquhar WB, et al. Effect of moderate-to-severe chronic kidney disease on flow-mediated dilation and progenitor cells. *Exp Biol Med (Maywood)* 2011 Sep 1;236(9):1085-92.
- (132) John Emsley. *The Shocking History of Phosphorus. A biography of the Devil's Element.* 1-1-2000. MacMillan.
Ref Type: Serial (Book,Monograph)
- (133) <http://www.webelements.com/phosphorus/>. 25-11-2012.
Ref Type: Generic
- (134) Bansal VK. *Serum Inorganic Phosphorus.* 1990.
- (135) D.D.Perrin, Boyd Dempsey. *Buffers for pH and Metal Ion Control.* 1-1-1974. Science Paperbacks.
Ref Type: Serial (Book,Monograph)
- (136) Edited by R.Hainsworth. *Acid-base balance.* 1-1-1986. Manchester University Press.
Ref Type: Serial (Book,Monograph)
- (137) Blumsohn A. What have we learnt about the regulation of phosphate metabolism? *Curr Opin Nephrol Hypertens* 2004 Jul;13(4):397-401.
- (138) Bergwitz C, Juppner H. Phosphate sensing. *Adv Chronic Kidney Dis* 2011 Mar;18(2):132-44.
- (139) Hsieh YJ, Wanner BL. Global regulation by the seven-component Pi signaling system. *Curr Opin Microbiol* 2010 Apr;13(2):198-203.
- (140) Mouillon JM, Persson BL. New aspects on phosphate sensing and signalling in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2006 Mar;6(2):171-6.
- (141) www.thermpos.com/en/Brochures/Brochure_08.aspx. 1-12-2012.
Ref Type: Generic

- (142) Hutchison AJ, Smith CP, Brenchley PE. Pharmacology, efficacy and safety of oral phosphate binders. *Nat Rev Nephrol* 2011 Oct;7(10):578-89.
- (143) Takeda E, Yamamoto H, Yamanaka-Okumura H, Taketani Y. Dietary phosphorus in bone health and quality of life. *Nutr Rev* 2012 Jun;70(6):311-21.
- (144) Hruska KA, Mathew S, Lund R, Qiu P, Pratt R. Hyperphosphatemia of chronic kidney disease. *Kidney Int* 2008 Jul;74(2):148-57.
- (145) Sabbagh Y, Giral H, Caldas Y, Levi M, Schiavi SC. Intestinal phosphate transport. *Adv Chronic Kidney Dis* 2011 Mar;18(2):85-90.
- (146) Forster IC, Hernando N, Biber J, Murer H. Proximal tubular handling of phosphate: A molecular perspective. *Kidney Int* 2006 Nov;70(9):1548-59.
- (147) Farrow EG, White KE. Recent advances in renal phosphate handling. *Nat Rev Nephrol* 2010 Apr;6(4):207-17.
- (148) Capuano P, Radanovic T, Wagner CA, Bacic D, Kato S, Uchiyama Y, et al. Intestinal and renal adaptation to a low-Pi diet of type II NaPi cotransporters in vitamin D receptor- and 1 α OHase-deficient mice. *Am J Physiol Cell Physiol* 2005 Feb;288(2):C429-C434.
- (149) Corut A, Senyigit A, Ugur SA, Altin S, Ozcelik U, Calisir H, et al. Mutations in SLC34A2 cause pulmonary alveolar microlithiasis and are possibly associated with testicular microlithiasis. *Am J Hum Genet* 2006 Oct;79(4):650-6.
- (150) Prie D, Urena TP, Friedlander G. Latest findings in phosphate homeostasis. *Kidney Int* 2009 May;75(9):882-9.
- (151) Segawa H, Kaneko I, Takahashi A, Kuwahata M, Ito M, Ohkido I, et al. Growth-related renal type II Na/Pi cotransporter. *J Biol Chem* 2002 May 31;277(22):19665-72.
- (152) Bergwitz C, Roslin NM, Tieder M, Loredó-Ostí JC, Bastepe M, Abu-Zahra H, et al. SLC34A3 mutations in patients with hereditary hypophosphatemic rickets with hypercalciuria predict a key role for the sodium-phosphate cotransporter NaPi-IIc in maintaining phosphate homeostasis. *Am J Hum Genet* 2006 Feb;78(2):179-92.
- (153) Beck L, Leroy C, Beck-Cormier S, Forand A, Salaun C, Paris N, et al. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. *PLoS One* 2010;5(2):e9148.
- (154) Beck L, Leroy C, Salaun C, Margall-Ducos G, Desdouets C, Friedlander G. Identification of a novel function of PiT1 critical for cell proliferation and independent of its phosphate transport activity. *J Biol Chem* 2009 Nov 6;284(45):31363-74.
- (155) Salaun C, Leroy C, Rousseau A, Boitez V, Beck L, Friedlander G. Identification of a novel transport-independent function of PiT1/SLC20A1 in the regulation of TNF-induced apoptosis. *J Biol Chem* 2010 Nov 5;285(45):34408-18.
- (156) Mannstadt M, Juppner H, Gardella TJ. Receptors for PTH and PTHrP: their biological importance and functional properties. *Am J Physiol* 1999 Nov;277(5 Pt 2):F665-F675.

- (157) Parfitt AM. The actions of parathyroid hormone on bone: relation to bone remodeling and turnover, calcium homeostasis, and metabolic bone disease. Part IV of IV parts: The state of the bones in uremic hyperparathyroidism--the mechanisms of skeletal resistance to PTH in renal failure and pseudohypoparathyroidism and the role of PTH in osteoporosis, osteopetrosis, and osteofluorosis. *Metabolism* 1976 Oct;25(10):1157-88.
- (158) Sung CC, Liao MT, Lu KC, Wu CC. Role of vitamin D in insulin resistance. *J Biomed Biotechnol* 2012;2012:634195.
- (159) Christakos S, Ajibade DV, Dhawan P, Fechner AJ, Mady LJ. Vitamin D: metabolism. *Endocrinol Metab Clin North Am* 2010 Jun;39(2):243-53, table.
- (160) Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nat Genet* 2000 Nov;26(3):345-8.
- (161) Schiavi SC, Kumar R. The phosphatonin pathway: new insights in phosphate homeostasis. *Kidney Int* 2004 Jan;65(1):1-14.
- (162) Alizadeh Naderi AS, Reilly RF. Hereditary disorders of renal phosphate wasting. *Nat Rev Nephrol* 2010 Nov;6(11):657-65.
- (163) Seiler S, Heine GH, Fliser D. Clinical relevance of FGF-23 in chronic kidney disease. *Kidney Int* 2009 Dec;76(S114):S34-S42.
- (164) Yamashita T. Structural and biochemical properties of fibroblast growth factor 23. *Ther Apher Dial* 2005 Aug;9(4):313-8.
- (165) Shimada T, Muto T, Urakawa I, Yoneya T, Yamazaki Y, Okawa K, et al. Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo. *Endocrinology* 2002 Aug;143(8):3179-82.
- (166) Berndt TJ, Craig TA, McCormick DJ, Lanske B, Sitara D, Razzaque MS, et al. Biological activity of FGF-23 fragments. *Pflugers Arch* 2007 Jul;454(4):615-23.
- (167) Andrukhova O, Zeitz U, Goetz R, Mohammadi M, Lanske B, Erben RG. FGF23 acts directly on renal proximal tubules to induce phosphaturia through activation of the ERK1/2-SGK1 signaling pathway. *Bone* 2012 Sep;51(3):621-8.
- (168) Cavalli L, Mazzotta C, Brandi ML. Phosphatonins: physiological role and pathological changes. *Clin Cases Miner Bone Metab* 2012 Jan;9(1):9-12.
- (169) Kuro O. Phosphate and Klotho. *Kidney Int Suppl* 2011 Apr;(121):S20-S23.
- (170) Torres PU, Prie D, Molina-Bletry V, Beck L, Silve C, Friedlander G. Klotho: an antiaging protein involved in mineral and vitamin D metabolism. *Kidney Int* 2007 Apr;71(8):730-7.
- (171) Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, et al. Suppression of aging in mice by the hormone Klotho. *Science* 2005 Sep 16;309(5742):1829-33.

- (172) Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, et al. Regulation of oxidative stress by the anti-aging hormone klotho. *J Biol Chem* 2005 Nov 11;280(45):38029-34.
- (173) Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997 Nov 6;390(6655):45-51.
- (174) Razzaque MS, Sitara D, Taguchi T, St-Arnaud R, Lanske B. Premature aging-like phenotype in fibroblast growth factor 23 null mice is a vitamin D-mediated process. *FASEB J* 2006 Apr;20(6):720-2.
- (175) Stubbs JR, Liu S, Tang W, Zhou J, Wang Y, Yao X, et al. Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. *J Am Soc Nephrol* 2007 Jul;18(7):2116-24.
- (176) Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP, et al. Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem* 2006 Mar 10;281(10):6120-3.
- (177) Maekawa Y, Ishikawa K, Yasuda O, Oguro R, Hanasaki H, Kida I, et al. Klotho suppresses TNF-alpha-induced expression of adhesion molecules in the endothelium and attenuates NF-kappaB activation. *Endocrine* 2009 Jun;35(3):341-6.
- (178) Farrow EG, Davis SI, Summers LJ, White KE. Initial FGF23-mediated signaling occurs in the distal convoluted tubule. *J Am Soc Nephrol* 2009 May;20(5):955-60.
- (179) Hu MC, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B, et al. Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J* 2010 Sep;24(9):3438-50.
- (180) Huang CL. Regulation of ion channels by secreted Klotho. *Adv Exp Med Biol* 2012;728:100-6.
- (181) Nakamura T, Saito Y, Ohyama Y, Masuda H, Sumino H, Kuro-o M, et al. Production of nitric oxide, but not prostacyclin, is reduced in klotho mice. *Jpn J Pharmacol* 2002 Jun;89(2):149-56.
- (182) Yang J, Matsukawa N, Rakugi H, Imai M, Kida I, Nagai M, et al. Upregulation of cAMP is a new functional signal pathway of Klotho in endothelial cells. *Biochem Biophys Res Commun* 2003 Feb 7;301(2):424-9.
- (183) Saito Y, Nakamura T, Ohyama Y, Suzuki T, Iida A, Shiraki-Iida T, et al. In vivo klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. *Biochem Biophys Res Commun* 2000 Sep 24;276(2):767-72.
- (184) John GB, Cheng CY, Kuro-o M. Role of Klotho in aging, phosphate metabolism, and CKD. *Am J Kidney Dis* 2011 Jul;58(1):127-34.
- (185) Ishizaka N, Mitani H, Nagai R. [Angiotensin II regulates klotho gene expression]. *Nihon Rinsho* 2002 Oct;60(10):1935-9.

- (186) Malmqvist K, Wallen HN, Held C, Kahan T. Soluble cell adhesion molecules in hypertensive concentric left ventricular hypertrophy. *J Hypertens* 2002 Aug;20(8):1563-9.
- (187) Kumar R. Tumor-induced osteomalacia and the regulation of phosphate homeostasis. *Bone* 2000 Sep;27(3):333-8.
- (188) BRICKER NS, MORRIN PA, KIME SW, Jr. The pathologic physiology of chronic Bright's disease. An exposition of the "intact nephron hypothesis". *Am J Med* 1960 Jan;28:77-98.
- (189) BRICKER NS, MORRIN PA, KIME SW, Jr. The pathologic physiology of chronic Bright's disease. An exposition of the "intact nephron hypothesis". *J Am Soc Nephrol* 1997 Sep;8(9):1470-6.
- (190) Levin A, Bakris GL, Molitch M, Smulders M, Tian J, Williams LA, et al. 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 Int* 2007 Jan;71(1):31-8.
- (191) Ghosh B, Brojen T, Banerjee S, Singh N, Singh S, Sharma OP, et al. The high prevalence of chronic kidney disease-mineral bone disorders: A hospital-based cross-sectional study. *Indian J Nephrol* 2012 Jul;22(4):285-91.
- (192) Block GA, Hulbert-Shearon TE, Levin NW, Port FK. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998 Apr;31(4):607-17.
- (193) Cannata-Andia JB, Rodriguez-Garcia M. Hyperphosphataemia as a cardiovascular risk factor -- how to manage the problem. *Nephrol Dial Transplant* 2002;17 Suppl 11:16-9.
- (194) Gossmann J, Wilhelm A, Kachel HG, Jordan J, Sann U, Geiger H, et al. Long-term consequences of live kidney donation follow-up in 93% of living kidney donors in a single transplant center. *Am J Transplant* 2005 Oct;5(10):2417-24.
- (195) Gutierrez OM. Fibroblast Growth Factor 23 and Disordered Vitamin D Metabolism in Chronic Kidney Disease: Updating the "Trade-off" Hypothesis. *Clin J Am Soc Nephrol* 2010 May 27.
- (196) Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H, et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* 2011 Jun;79(12):1370-8.
- (197) Larsson T, Nisbeth U, Ljunggren O, Juppner H, Jonsson KB. Circulating concentration of FGF-23 increases as renal function declines in patients with chronic kidney disease, but does not change in response to variation in phosphate intake in healthy volunteers. *Kidney Int* 2003 Dec;64(6):2272-9.
- (198) Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. *N Engl J Med* 2008 Aug 7;359(6):584-92.
- (199) Shimada T, Urakawa I, Isakova T, Yamazaki Y, Epstein M, Wesseling-Perry K, et al. Circulating fibroblast growth factor 23 in patients with end-stage renal disease

treated by peritoneal dialysis is intact and biologically active. *J Clin Endocrinol Metab* 2010 Feb;95(2):578-85.

- (200) Nakatani T, Ohnishi M, Razzaque MS. Inactivation of klotho function induces hyperphosphatemia even in presence of high serum fibroblast growth factor 23 levels in a genetically engineered hypophosphatemic (Hyp) mouse model. *FASEB J* 2009 Nov;23(11):3702-11.
- (201) Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. *J Clin Invest* 2011 Nov;121(11):4393-408.
- (202) Marsell R, Krajisnik T, Goransson H, Ohlsson C, Ljunggren O, Larsson TE, et al. Gene expression analysis of kidneys from transgenic mice expressing fibroblast growth factor-23. *Nephrol Dial Transplant* 2008 Mar;23(3):827-33.
- (203) Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, et al. The parathyroid is a target organ for FGF23 in rats. *J Clin Invest* 2007 Dec;117(12):4003-8.
- (204) Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T, et al. Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun* 2001 Feb 2;280(4):1015-20.
- (205) Krajisnik T, Olauson H, Mirza MA, Hellman P, Akerstrom G, Westin G, et al. Parathyroid Klotho and FGF-receptor 1 expression decline with renal function in hyperparathyroid patients with chronic kidney disease and kidney transplant recipients. *Kidney Int* 2010 Nov;78(10):1024-32.
- (206) Aizawa H, Saito Y, Nakamura T, Inoue M, Imanari T, Ohyama Y, et al. Downregulation of the Klotho gene in the kidney under sustained circulatory stress in rats. *Biochem Biophys Res Commun* 1998 Aug 28;249(3):865-71.
- (207) Yamazaki Y, Imura A, Urakawa I, Shimada T, Murakami J, Aono Y, et al. Establishment of sandwich ELISA for soluble alpha-Klotho measurement: Age-dependent change of soluble alpha-Klotho levels in healthy subjects. *Biochem Biophys Res Commun* 2010 Jul 30;398(3):513-8.
- (208) Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M, et al. Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2011 Jan;22(1):124-36.
- (209) Yokoyama K, Imura A, Ohkido I, Maruyama Y, Yamazaki Y, Hasegawa H, et al. Serum soluble alpha-klotho in hemodialysis patients. *Clin Nephrol* 2012 May;77(5):347-51.
- (210) Seiler S, Wen M, Roth HJ, Fehrenz M, Flugge F, Herath E, et al. Plasma Klotho is not related to kidney function and does not predict adverse outcome in patients with chronic kidney disease. *Kidney Int* 2012 Aug 15.
- (211) Hu MC, Shi M, Zhang J, Quinones H, Kuro-o M, Moe OW. Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* 2010 Dec;78(12):1240-51.

- (212) Mitani H, Ishizaka N, Aizawa T, Ohno M, Usui S, Suzuki T, et al. In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. *Hypertension* 2002 Apr;39(4):838-43.
- (213) Gutierrez O, Isakova T, Rhee E, Shah A, Holmes J, Collerone G, et al. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. *J Am Soc Nephrol* 2005 Jul;16(7):2205-15.
- (214) Tsujikawa H, Kurotaki Y, Fujimori T, Fukuda K, Nabeshima Y. Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. *Mol Endocrinol* 2003 Dec;17(12):2393-403.
- (215) Saji F, Shiizaki K, Shimada S, Okada T, Kunimoto K, Sakaguchi T, et al. Regulation of fibroblast growth factor 23 production in bone in uremic rats. *Nephron Physiol* 2009;111(4):59-66.
- (216) Arking DE, Krebsova A, Macek M, Sr., Macek M, Jr., Arking A, Mian IS, et al. Association of human aging with a functional variant of klotho. *Proc Natl Acad Sci U S A* 2002 Jan 22;99(2):856-61.
- (217) KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009 Aug;(113):S1-130.
- (218) The Renal Association. <http://www.renal.org/Clinical/GuidelinesSection/CKD-MBD.aspx>. online source . 2012. 12-12-2012.
- Ref Type: Generic
- (219) Bia M, Adey DB, Bloom RD, Chan L, Kulkarni S, Tomlanovich S. KDOQI US commentary on the 2009 KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Kidney Dis* 2010 Aug;56(2):189-218.
- (220) Atkins D, Best D, Briss PA, Eccles M, Falck-Ytter Y, Flottorp S, et al. Grading quality of evidence and strength of recommendations. *BMJ* 2004 Jun 19;328(7454):1490.
- (221) Tentori F, Hunt WC, Rohrscheib M, Zhu M, Stidley CA, Servilla K, et al. Which targets in clinical practice guidelines are associated with improved survival in a large dialysis organization? *J Am Soc Nephrol* 2007 Aug;18(8):2377-84.
- (222) NICE guideline for the management of hyperphosphataemia in CKD. 30-3-2013.
- Ref Type: Internet Communication
- (223) Narang R, Ridout D, Nonis C, Kooner JS. Serum calcium, phosphorus and albumin levels in relation to the angiographic severity of coronary artery disease. *Int J Cardiol* 1997 Jun 27;60(1):73-9.
- (224) Naves-Diaz M, Passlick-Deetjen J, Guinsburg A, Marelli C, Fernandez-Martin JL, Rodriguez-Puyol D, et al. Calcium, phosphorus, PTH and death rates in a large sample of dialysis patients from Latin America. The CORES Study. *Nephrol Dial Transplant* 2010 May 31.

- (225) Chue CD, Edwards NC, Moody WE, Steeds RP, Townend JN, Ferro CJ. Serum phosphate is associated with left ventricular mass in patients with chronic kidney disease: a cardiac magnetic resonance study. *Heart* 2012 Feb;98(3):219-24.
- (226) Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G. Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005 Oct 25;112(17):2627-33.
- (227) Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB, Sr., Gaziano JM, et al. Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007 May 14;167(9):879-85.
- (228) Covic A, Kothawala P, Bernal M, Robbins S, Chalian A, Goldsmith D. Systematic review of the evidence underlying the association between mineral metabolism disturbances and risk of all-cause mortality, cardiovascular mortality and cardiovascular events in chronic kidney disease. *Nephrol Dial Transplant* 2009 May;24(5):1506-23.
- (229) Stevens LA, Djurdjev O, Cardew S, Cameron EC, Levin A. Calcium, phosphate, and parathyroid hormone levels in combination and as a function of dialysis duration predict mortality: evidence for the complexity of the association between mineral metabolism and outcomes. *J Am Soc Nephrol* 2004 Mar;15(3):770-9.
- (230) Noordzij M, Korevaar JC, Bos WJ, Boeschoten EW, Dekker FW, Bossuyt PM, et al. Mineral metabolism and cardiovascular morbidity and mortality risk: peritoneal dialysis patients compared with haemodialysis patients. *Nephrol Dial Transplant* 2006 Sep;21(9):2513-20.
- (231) de Boer IH, Rue TC, Kestenbaum B. Serum phosphorus concentrations in the third National Health and Nutrition Examination Survey (NHANES III). *Am J Kidney Dis* 2009 Mar;53(3):399-407.
- (232) Middleton RJ, Parfrey PS, Foley RN. Left ventricular hypertrophy in the renal patient. *J Am Soc Nephrol* 2001 May;12(5):1079-84.
- (233) Ayus JC, Mizani MR, Achinger SG, Thadhani R, Go AS, Lee S. Effects of short daily versus conventional hemodialysis on left ventricular hypertrophy and inflammatory markers: a prospective, controlled study. *J Am Soc Nephrol* 2005 Sep;16(9):2778-88.
- (234) Yamamoto KT, Robinson-Cohen C, de Oliveira MC, Kostina A, Nettleton JA, Ix JH, et al. Dietary phosphorus is associated with greater left ventricular mass. *Kidney Int* 2013 Jan 2.
- (235) Garland JS, Holden RM, Groome PA, Lam M, Nolan RL, Morton AR, et al. Prevalence and associations of coronary artery calcification in patients with stages 3 to 5 CKD without cardiovascular disease. *Am J Kidney Dis* 2008 Nov;52(5):849-58.
- (236) Gutierrez OM, Januzzi JL, Isakova T, Laliberte K, Smith K, Collerone G, et al. Fibroblast growth factor 23 and left ventricular hypertrophy in chronic kidney disease. *Circulation* 2009 May 19;119(19):2545-52.

- (237) 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 Sep;18(9):1731-40.
- (238) Russo D, Corrao S, Miranda I, Ruocco C, Manzi S, Elefante R, et al. Progression of coronary artery calcification in predialysis patients. *Am J Nephrol* 2007;27(2):152-8.
- (239) Adeney KL, Siscovick DS, Ix JH, Seliger SL, Shlipak MG, Jenny NS, et al. Association of serum phosphate with vascular and valvular calcification in moderate CKD. *J Am Soc Nephrol* 2009 Feb;20(2):381-7.
- (240) Russo D, Miranda I, Ruocco C, Battaglia Y, Buonanno E, Manzi S, et al. The progression of coronary artery calcification in predialysis patients on calcium carbonate or sevelamer. *Kidney Int* 2007 Nov;72(10):1255-61.
- (241) Speer MY, Giachelli CM. Regulation of cardiovascular calcification. *Cardiovasc Pathol* 2004 Mar;13(2):63-70.
- (242) Davies MR, Lund RJ, Mathew S, Hruska KA. Low turnover osteodystrophy and vascular calcification are amenable to skeletal anabolism in an animal model of chronic kidney disease and the metabolic syndrome. *J Am Soc Nephrol* 2005 Apr;16(4):917-28.
- (243) Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, et al. Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol* 2004 Nov;15(11):2857-67.
- (244) Suliman ME, Garcia-Lopez E, Anderstam B, Lindholm B, Stenvinkel P. Vascular calcification inhibitors in relation to cardiovascular disease with special emphasis on fetuin-A in chronic kidney disease. *Adv Clin Chem* 2008;46:217-62.
- (245) Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebbersold R, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of *Cbfa1* and downregulation of smooth muscle lineage markers. *Circ Res* 2001 Dec 7;89(12):1147-54.
- (246) Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. *Circ Res* 2006 Apr 14;98(7):905-12.
- (247) Mathew S, Tustison KS, Sugatani T, Chaudhary LR, Rifas L, Hruska KA. The mechanism of phosphorus as a cardiovascular risk factor in CKD. *J Am Soc Nephrol* 2008 Jun;19(6):1092-105.
- (248) Takeda E, Taketani Y, Nashiki K, Nomoto M, Shuto E, Sawada N, et al. A novel function of phosphate-mediated intracellular signal transduction pathways. *Adv Enzyme Regul* 2006;46:154-61.
- (249) Peng A, Wu T, Zeng C, Rakheja D, Zhu J, Ye T, et al. Adverse effects of simulated hyper- and hypo-phosphatemia on endothelial cell function and viability. *PLoS One* 2011;6(8):e23268.

- (250) Di Marco GS, Hausberg M, Hillebrand U, Rustemeyer P, Wittkowski W, Lang D, et al. Increased inorganic phosphate induces human endothelial cell apoptosis in vitro. *Am J Physiol Renal Physiol* 2008 Jun;294(6):F1381-F1387.
- (251) Koobi P, Vehmas TI, Jolma P, Kalliovalkama J, Fan M, Niemela O, et al. High-calcium vs high-phosphate intake and small artery tone in advanced experimental renal insufficiency. *Nephrol Dial Transplant* 2006 Oct;21(10):2754-61.
- (252) Pauriah M, Khan F, Lim TK, Elder DH, Godfrey V, Kennedy G, et al. B-type natriuretic peptide is an independent predictor of endothelial function in man. *Clin Sci (Lond)* 2012 Sep;123(5):307-12.
- (253) Van TV, Watari E, Taketani Y, Kitamura T, Shiota A, Tanaka T, et al. Dietary phosphate restriction ameliorates endothelial dysfunction in adenine-induced kidney disease rats. *J Clin Biochem Nutr* 2012 Jul;51(1):27-32.
- (254) Al M, I, Patel R, Murrow J, Morris A, Rahman A, Fike L, et al. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. *J Am Coll Cardiol* 2011 Jul 5;58(2):186-92.
- (255) Skaaby T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Fenger M, et al. Vitamin D status and incident cardiovascular disease and all-cause mortality: a general population study. *Endocrine* 2012 Sep 27.
- (256) Pilz S, Iodice S, Zittermann A, Grant WB, Gandini S. Vitamin D status and mortality risk in CKD: a meta-analysis of prospective studies. *Am J Kidney Dis* 2011 Sep;58(3):374-82.
- (257) Weishaar RE, Kim SN, Saunders DE, Simpson RU. Involvement of vitamin D3 with cardiovascular function. III. Effects on physical and morphological properties. *Am J Physiol* 1990 Jan;258(1 Pt 1):E134-E142.
- (258) Weishaar RE, Kim SN, Saunders DE, Simpson RU. Involvement of vitamin D3 with cardiovascular function. III. Effects on physical and morphological properties. *Am J Physiol* 1990 Jan;258(1 Pt 1):E134-E142.
- (259) O'Connell TD, Berry JE, Jarvis AK, Somerman MJ, Simpson RU. 1,25-Dihydroxyvitamin D3 regulation of cardiac myocyte proliferation and hypertrophy. *Am J Physiol* 1997 Apr;272(4 Pt 2):H1751-H1758.
- (260) Wu J, Garami M, Cheng T, Gardner DG. 1,25(OH)₂ vitamin D₃ and retinoic acid antagonize endothelin-stimulated hypertrophy of neonatal rat cardiac myocytes. *J Clin Invest* 1996 Apr 1;97(7):1577-88.
- (261) Park CW, Oh YS, Shin YS, Kim CM, Kim YS, Kim SY, et al. Intravenous calcitriol regresses myocardial hypertrophy in hemodialysis patients with secondary hyperparathyroidism. *Am J Kidney Dis* 1999 Jan;33(1):73-81.
- (262) Tentori F, Blayney MJ, Albert JM, Gillespie BW, Kerr PG, Bommer J, et al. Mortality risk for dialysis patients with different levels of serum calcium, phosphorus, and PTH: the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Am J Kidney Dis* 2008 Sep;52(3):519-30.

- (263) Chartsrisak K, Vipattawat K, Assanatham M, Nongnuch A, Ingsathit A, Domrongkitchaiporn S, et al. Mineral metabolism and outcomes in chronic kidney disease stage 2-4 patients. *BMC Nephrol* 2013;14(1):14.
- (264) Custodio MR, Koike MK, Neves KR, Dos Reis LM, Graciolli FG, Neves CL, et al. Parathyroid hormone and phosphorus overload in uremia: impact on cardiovascular system. *Nephrol Dial Transplant* 2012 Apr;27(4):1437-45.
- (265) Isakova T, Xie H, Yang W, Xie D, Anderson AH, Scialla J, et al. Fibroblast growth factor 23 and risks of mortality and end-stage renal disease in patients with chronic kidney disease. *JAMA* 2011 Jun 15;305(23):2432-9.
- (266) Mirza MA, Larsson A, Lind L, Larsson TE. Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community. *Atherosclerosis* 2009 Aug;205(2):385-90.
- (267) Balci M, Kirkpantur A, Gulbay M, Gurbuz OA. Plasma fibroblast growth factor-23 levels are independently associated with carotid artery atherosclerosis in maintenance hemodialysis patients. *Hemodial Int* 2010 Oct;14(4):425-32.
- (268) Kirkpantur A, Balci M, Gurbuz OA, Afsar B, Canbakan B, Akdemir R, et al. Serum fibroblast growth factor-23 (FGF-23) levels are independently associated with left ventricular mass and myocardial performance index in maintenance haemodialysis patients. *Nephrol Dial Transplant* 2011 Apr;26(4):1346-54.
- (269) Gutierrez OM, Wolf M, Taylor EN. Fibroblast growth factor 23, cardiovascular disease risk factors, and phosphorus intake in the health professionals follow-up study. *Clin J Am Soc Nephrol* 2011 Dec;6(12):2871-8.
- (270) Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 2006 Jun;69(11):1945-53.
- (271) Mac WF, Lessard M, Lafage-Proust MH. Pathophysiology of chronic kidney disease-mineral and bone disorder. *Joint Bone Spine* 2012 Dec;79(6):544-9.
- (272) Yalin AS, Altiparmak MR, Trabulus S, Yalin SF, Yalin GY, Melikoglu M. Calciphylaxis: a report of six cases and review of literature. *Ren Fail* 2013;35(1):163-9.
- (273) Block GA. Control of serum phosphorus: implications for coronary artery calcification and calcific uremic arteriopathy (calciphylaxis). *Curr Opin Nephrol Hypertens* 2001 Nov;10(6):741-7.
- (274) Jimbo-Saito R, Ubara Y, Kadoguchi H, Suwabe T, Nakanishi S, Higa Y, et al. A case of primary hyperparathyroidism with severe bone and renal changes. *J Bone Miner Metab* 2009;27(6):727-32.
- (275) Lockhart ME, Robbin ML, McNamara MM, Allon M. Association of pelvic arterial calcification with arteriovenous thigh graft failure in haemodialysis patients. *Nephrol Dial Transplant* 2004 Oct;19(10):2564-9.
- (276) Lezaic V, Tirmenstajn-Jankovic B, Bukvic D, Vujisic B, Perovic M, Novakovic N, et al. Efficacy of hyperphosphatemia control in the progression of chronic renal

- failure and the prevalence of cardiovascular calcification. *Clin Nephrol* 2009 Jan;71(1):21-9.
- (277) Raggi P, Bellasi A, Gamboa C, Ferramosca E, Ratti C, Block GA, et al. All-cause mortality in hemodialysis patients with heart valve calcification. *Clin J Am Soc Nephrol* 2011 Aug;6(8):1990-5.
- (278) Bellasi A, Ferramosca E, Ratti C, Block G, Raggi P. Cardiac valve calcification is a marker of vascular disease in prevalent hemodialysis patients. *J Nephrol* 2012 Mar;25(2):211-8.
- (279) Drueke TB, Massy ZA. Atherosclerosis in CKD: differences from the general population. *Nat Rev Nephrol* 2010 Dec;6(12):723-35.
- (280) Claes KJ, Heye S, Nuyens D, Bammens B, Kuypers DR, Vanrenterghem Y, et al. Impact of vascular calcification on corrected QT interval at the time of renal transplantation. *Am J Nephrol* 2012;35(1):24-30.
- (281) Zoccali C, Ruggenenti P, Perna A, Leonardis D, Tripepi R, Tripepi G, et al. Phosphate may promote CKD progression and attenuate renoprotective effect of ACE inhibition. *J Am Soc Nephrol* 2011 Oct;22(10):1923-30.
- (282) Volpe M. Should all patients at high cardiovascular risk receive renin-angiotensin system blockers? *QJM* 2012 Jan;105(1):11-27.
- (283) Sigrist MK, Chiarelli G, Lim L, Levin A. Early initiation of phosphate lowering dietary therapy in non-dialysis chronic kidney disease: a critical review. *J Ren Care* 2009 Mar;35 Suppl 1:71-8.
- (284) Campbell KL, Ash S, Bauer JD. The impact of nutrition intervention on quality of life in pre-dialysis chronic kidney disease patients. *Clin Nutr* 2008 Aug;27(4):537-44.
- (285) Khalil AA, Darawad M, Al GE, Hamdan-Mansour AM, Abed MA. Predictors of dietary and fluid non-adherence in Jordanian patients with end-stage renal disease receiving haemodialysis: a cross-sectional study. *J Clin Nurs* 2013 Jan;22(1-2):127-36.
- (286) Hsu HJ, Yen CH, Chen CK, Wu IW, Lee CC, Sun CY, et al. Association between uremic toxins and depression in patients with chronic kidney disease undergoing maintenance hemodialysis. *Gen Hosp Psychiatry* 2013 Jan;35(1):23-7.
- (287) Slatopolsky E, Rutherford WE, Rosenbaum R, Martin K, Hruska K. Hyperphosphatemia. *Clin Nephrol* 1977 Apr;7(4):138-46.
- (288) Navaneethan SD, Palmer SC, Vecchio M, Craig JC, Elder GJ, Strippoli GF. Phosphate binders for preventing and treating bone disease in chronic kidney disease patients. *Cochrane Database Syst Rev* 2011;(2):CD006023.
- (289) Cannata JB, Briggs JD, Junor BJ, Fell GS. Aluminium hydroxide intake: real risk of aluminium toxicity. *Br Med J (Clin Res Ed)* 1983 Jun 18;286(6382):1937-8.
- (290) Hutchison AJ. Improving phosphate-binder therapy as a way forward. *Nephrol Dial Transplant* 2004 Mar;19 Suppl 1:i19-i24.

- (291) Caglar K, Yilmaz MI, Saglam M, Cakir E, Acikel C, Eyileten T, et al. Short-term treatment with sevelamer increases serum fetuin-a concentration and improves endothelial dysfunction in chronic kidney disease stage 4 patients. *Clin J Am Soc Nephrol* 2008 Jan;3(1):61-8.
- (292) Yilmaz MI, Sonmez A, Saglam M, Yaman H, Kilic S, Eyileten T, et al. Comparison of calcium acetate and sevelamer on vascular function and fibroblast growth factor 23 in CKD patients: a randomized clinical trial. *Am J Kidney Dis* 2012 Feb;59(2):177-85.
- (293) Peres AT, Dalboni MA, Canziani ME, Manfredi SR, Carvalho JT, Batista MC, et al. Effect of phosphate binders on oxidative stress and inflammation markers in hemodialysis patients. *Hemodial Int* 2009 Jul;13(3):271-7.
- (294) Brandenburg VM, Jahnhen-Dechent W, Ketteler M. Sevelamer and the bone-vascular axis in chronic kidney disease: bone turnover, inflammation, and calcification regulation. *Kidney Int Suppl* 2009 Dec;(114):S26-S33.
- (295) Suki WN. Effects of sevelamer and calcium-based phosphate binders on mortality in hemodialysis patients: results of a randomized clinical trial. *J Ren Nutr* 2008 Jan;18(1):91-8.
- (296) Di IB, Bellasi A, Russo D. Mortality in kidney disease patients treated with phosphate binders: a randomized study. *Clin J Am Soc Nephrol* 2012 Mar;7(3):487-93.
- (297) Oliveira RB, Cancela AL, Gracioli FG, Dos Reis LM, Draibe SA, Cuppari L, et al. Early control of PTH and FGF23 in normophosphatemic CKD patients: a new target in CKD-MBD therapy? *Clin J Am Soc Nephrol* 2010 Feb;5(2):286-91.
- (298) Pierce D, Hossack S, Poole L, Robinson A, Van HH, Martin P, et al. The effect of sevelamer carbonate and lanthanum carbonate on the pharmacokinetics of oral calcitriol. *Nephrol Dial Transplant* 2011 May;26(5):1615-21.
- (299) Sonikian MA, Pani IT, Iliopoulos AN, Koutala KG, Marioli SI, Vlassopoulos DA. Metabolic acidosis aggravation and hyperkalemia in hemodialysis patients treated by sevelamer hydrochloride. *Ren Fail* 2005;27(2):143-7.
- (300) Chue C.D, Townend J.N., Moody W.E, Edwards N.C., Steeds R.P., Ferro C.J. Effects of Sevelamer Carbonate on Arterial Stiffness and Left Ventricular Mass in Patients with Stage 3 Chronic Kidney Disease: Results of a Randomized Controlled Trial. *Circulation* 2013;126:A517.
- (301) Sprague SM, Ross EA, Nath SD, Zhang P, Pratt RD, Krause R. Lanthanum carbonate vs. sevelamer hydrochloride for the reduction of serum phosphorus in hemodialysis patients: a crossover study. *Clin Nephrol* 2009 Oct;72(4):252-8.
- (302) Arenas MD, Rebollo P, Malek T, Moledous A, Gil MT, Alvarez-Ude F, et al. A comparative study of 2 new phosphate binders (sevelamer and lanthanum carbonate) in routine clinical practice. *J Nephrol* 2010 Nov;23(6):683-92.
- (303) Persy VP, Behets GJ, Bervoets AR, De Broe ME, D'Haese PC. Lanthanum: a safe phosphate binder. *Semin Dial* 2006 May;19(3):195-9.

- (304) Zaichick S, Zaichick V, Karandashev V, Nosenko S. Accumulation of rare earth elements in human bone within the lifespan. *Metallomics* 2011 Feb;3(2):186-94.
- (305) Soriano S, Ojeda R, Rodriguez M, Almaden Y, Rodriguez M, Martin-Malo A, et al. The effect of phosphate binders, calcium and lanthanum carbonate on FGF23 levels in chronic kidney disease patients. *Clin Nephrol* 2013 Feb 8.
- (306) Spiegel DM, Farmer B, Smits G, Chonchol M. Magnesium carbonate is an effective phosphate binder for chronic hemodialysis patients: a pilot study. *J Ren Nutr* 2007 Nov;17(6):416-22.
- (307) Spiegel DM. The role of magnesium binders in chronic kidney disease. *Semin Dial* 2007 Jul;20(4):333-6.
- (308) Isakova T, Gutierrez OM, Chang Y, Shah A, Tamez H, Smith K, et al. Phosphorus binders and survival on hemodialysis. *J Am Soc Nephrol* 2009 Feb;20(2):388-96.
- (309) Chiu YW, Teitelbaum I, Misra M, de Leon EM, Adzize T, Mehrotra R. Pill burden, adherence, hyperphosphatemia, and quality of life in maintenance dialysis patients. *Clin J Am Soc Nephrol* 2009 Jun;4(6):1089-96.
- (310) Arenas MD, Malek T, Gil MT, Moledous A, Alvarez-Ude F, Reig-Ferrer A. Challenge of phosphorus control in hemodialysis patients: a problem of adherence? *J Nephrol* 2010 Sep;23(5):525-34.
- (311) Tangri N, Wagner M, Griffith JL, Miskulin DC, Hodsmann A, Ansell D, et al. Effect of bone mineral guideline target achievement on mortality in incident dialysis patients: an analysis of the United Kingdom Renal Registry. *Am J Kidney Dis* 2011 Mar;57(3):415-21.
- (312) Sampaio MS, Ruzany F, Dorigo DM, Suassuna JH. Phosphate mass removal during hemodialysis: a comparison between eKT/V-matched conventional and extended dialysis. *Am J Nephrol* 2012;36(2):121-6.
- (313) Isakova T, Xie H, Barchi-Chung A, Smith K, Sowden N, Epstein M, et al. Daily variability in mineral metabolites in CKD and effects of dietary calcium and calcitriol. *Clin J Am Soc Nephrol* 2012 May;7(5):820-8.
- (314) Kooienga L. Phosphorus balance with daily dialysis. *Semin Dial* 2007 Jul;20(4):342-5.
- (315) Sawin DA, Himmele R, Diaz-Buxo JA. Phosphate clearance in peritoneal dialysis: automated PD compared with continuous ambulatory PD. *Adv Perit Dial* 2012;28:120-5.
- (316) Molony DA, Stephens BW. Derangements in phosphate metabolism in chronic kidney diseases/endstage renal disease: therapeutic considerations. *Adv Chronic Kidney Dis* 2011 Mar;18(2):120-31.
- (317) Wang Z, Cui M, Tang L, Li W, Wei Y, Zhu Z, et al. Oral activated charcoal suppresses hyperphosphatemia in hemodialysis patients. *Nephrology (Carlton)* 2012 Jun 15.
- (318) Grundy SM, Vega GL, McGovern ME, Tulloch BR, Kendall DM, Fitz-Patrick D, et al. Efficacy, safety, and tolerability of once-daily niacin for the treatment of

dyslipidemia associated with type 2 diabetes: results of the assessment of diabetes control and evaluation of the efficacy of niaspan trial. *Arch Intern Med* 2002 Jul 22;162(14):1568-76.

- (319) Berge KG, Canner PL. Coronary drug project: experience with niacin. Coronary Drug Project Research Group. *Eur J Clin Pharmacol* 1991;40 Suppl 1:S49-S51.
- (320) ALTSCHUL R, HOFFER A, STEPHEN JD. Influence of nicotinic acid on serum cholesterol in man. *Arch Biochem Biophys* 1955 Feb;54(2):558-9.
- (321) Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K, et al. PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nat Med* 2003 Mar;9(3):352-5.
- (322) Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M, et al. Molecular identification of high and low affinity receptors for nicotinic acid. *J Biol Chem* 2003 Mar 14;278(11):9869-74.
- (323) Eto N, Miyata Y, Ohno H, Yamashita T. Nicotinamide prevents the development of hyperphosphataemia by suppressing intestinal sodium-dependent phosphate transporter in rats with adenine-induced renal failure. *Nephrol Dial Transplant* 2005 Jul;20(7):1378-84.
- (324) Katai K, Tanaka H, Tatsumi S, Fukunaga Y, Genjida K, Morita K, et al. Nicotinamide inhibits sodium-dependent phosphate cotransport activity in rat small intestine. *Nephrol Dial Transplant* 1999 May;14(5):1195-201.
- (325) Takahashi Y, Tanaka A, Nakamura T, Fukuwatari T, Shibata K, Shimada N, et al. Nicotinamide suppresses hyperphosphatemia in hemodialysis patients. *Kidney Int* 2004 Mar;65(3):1099-104.
- (326) Muller D, Mehling H, Otto B, Bergmann-Lips R, Luft F, Jordan J, et al. Niacin lowers serum phosphate and increases HDL cholesterol in dialysis patients. *Clin J Am Soc Nephrol* 2007 Nov;2(6):1249-54.
- (327) Kempson SA, Colon-Otero G, Ou SY, Turner ST, Dousa TP. Possible role of nicotinamide adenine dinucleotide as an intracellular regulator of renal transport of phosphate in the rat. *J Clin Invest* 1981 May;67(5):1347-60.
- (328) Young DO, Cheng SC, Delmez JA, Coyne DW. The effect of oral niacinamide on plasma phosphorus levels in peritoneal dialysis patients. *Perit Dial Int* 2009 Sep;29(5):562-7.
- (329) Edalat-Nejad M, Zameni F, Talaie A. The effect of niacin on serum phosphorus levels in dialysis patients. *Indian J Nephrol* 2012 May;22(3):174-8.
- (330) Savica V, Calo LA, Granata A, Caldarera R, Cavaleri A, Santoro D, et al. A new approach to the evaluation of hyperphosphatemia in chronic kidney disease. *Clin Nephrol* 2007 Oct;68(4):216-21.
- (331) Savica V, Calo LA, Monardo P, Davis PA, Granata A, Santoro D, et al. Salivary phosphate-binding chewing gum reduces hyperphosphatemia in dialysis patients. *J Am Soc Nephrol* 2009 Mar;20(3):639-44.

- (332) Calo LA, Savica V, Piccoli A, Fusaro M, D'Angelo A, Davis PA. Reduction of hyperphosphatemia is related with the reduction of C-reactive protein in dialysis patients. Study in sevelamer-resistant dialysis patients treated with chitosan chewing gum as salivary phosphate binder. *Ren Fail* 2011;33(1):11-4.
- (333) Patel RK, Mark PB, Johnston N, McGeoch R, Lindsay M, Kingsmore DB, et al. Prognostic value of cardiovascular screening in potential renal transplant recipients: a single-center prospective observational study. *Am J Transplant* 2008 Aug;8(8):1673-83.
- (334) Mulvany MJ, Aalkjaer C. Structure and function of small arteries. *Physiol Rev* 1990 Oct;70(4):921-61.
- (335) Bevan JA, Osher JV. A direct method for recording tension changes in the wall of small blood vessels in vitro. *Agents Actions* 1972;2(5):257-60.
- (336) Mulvany MJ. Procedures for investigation of small vessels using small vessel myograph. Danish Myo Technology, Aarhus, Denmark. December 1988 (Revised August 2004); 1988.
- (337) Mulvany MJ, Halpern W. Mechanical properties of vascular smooth muscle cells in situ. *Nature* 1976 Apr 15;260(5552):617-9.
- (338) Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* 1977 Jul;41(1):19-26.
- (339) Hogestatt ED, Andersson KE, Edvinsson L. Effects of nifedipine on potassium-induced contraction and noradrenaline release in cerebral and extracranial arteries from rabbit. *Acta Physiol Scand* 1982 Feb;114(2):283-96.
- (340) Aalkjaer C, Mulvany MJ. Functional and morphological properties of human omental resistance vessels. *Blood Vessels* 1981;18(6):233-44.
- (341) Wareing M, Myers JE, O'hara M, Kenny LC, Warren AY, Taggart MJ, et al. Effects of a phosphodiesterase-5 (PDE5) inhibitor on endothelium-dependent relaxation of myometrial small arteries. *Am J Obstet Gynecol* 2004 May;190(5):1283-90.
- (342) Slezak P, Waczulikova I, Balis P, Puzserova A. Accurate normalization factor for wire myography of rat femoral artery. *Physiol Res* 2010;59(6):1033-6.
- (343) van den Akker J, Schoorl MJ, Bakker EN, Vanbavel E. Small artery remodeling: current concepts and questions. *J Vasc Res* 2010;47(3):183-202.
- (344) Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 2005;11:127-52.
- (345) Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* 1993 Jun;303(2):474-82.

- (346) Privat C, Lantoine F, Bedioui F, Millanvoeye van BE, Devynck J, Devynck MA. Nitric oxide production by endothelial cells: comparison of three methods of quantification. *Life Sci* 1997;61(12):1193-202.
- (347) Ahluwalia A, Tarnawski AS. Critical role of hypoxia sensor--HIF-1alpha in VEGF gene activation. Implications for angiogenesis and tissue injury healing. *Curr Med Chem* 2012;19(1):90-7.
- (348) Eisner DA, Nichols CG, O'Neill SC, Smith GL, Valdeolmillos M. The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J Physiol* 1989 Apr;411:393-418.
- (349) McQuarrie EP, Patel RK, Mark PB, Delles C, Connell J, Dargie HJ, et al. Association between proteinuria and left ventricular mass index: a cardiac MRI study in patients with chronic kidney disease. *Nephrol Dial Transplant* 2011 Mar;26(3):933-8.
- (350) Patel RK, Oliver S, Mark PB, Powell JR, McQuarrie EP, Traynor JP, et al. Determinants of left ventricular mass and hypertrophy in hemodialysis patients assessed by cardiac magnetic resonance imaging. *Clin J Am Soc Nephrol* 2009 Sep;4(9):1477-83.
- (351) Alfakih K, Plein S, Thiele H, Jones T, Ridgway JP, Sivananthan MU. Normal human left and right ventricular dimensions for MRI as assessed by turbo gradient echo and steady-state free precession imaging sequences. *J Magn Reson Imaging* 2003 Mar;17(3):323-9.
- (352) http://www.bellybytes.com/foodfacts/soft_drink_facts.html. Internet 10 A.D. November 12
- (353) Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R. Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. *Circulation* 1986 Dec;74(6):1399-406.
- (354) Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, et al. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *J Appl Physiol* 2001 Aug;91(2):929-37.
- (355) Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T, et al. Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun* 2001 Feb 2;280(4):1015-20.
- (356) Patel TV, Mittal BV, Keithi-Reddy SR, Duffield JS, Singh AK. Endothelial activation markers in anemic non-dialysis chronic kidney disease patients. *Nephron Clin Pract* 2008;110(4):c244-c250.
- (357) McIntyre CW, Odudu A, Eldehni MT. Cardiac assessment in chronic kidney disease. *Curr Opin Nephrol Hypertens* 2009 Nov;18(6):501-6.
- (358) Motz W, Scheler S. [Hypertrophy and coronary reserve]. *Dtsch Med Wochenschr* 2008 Dec;133 Suppl 8:S257-S260.
- (359) Cuspidi C, Meani S, Valerio C, Fusi V, Catini E, Sala C, et al. Prevalence and correlates of advanced retinopathy in a large selected hypertensive population. *The*

Evaluation of Target Organ Damage in Hypertension (ETODH) study. *Blood Press* 2005;14(1):25-31.

- (360) Bregman R, Lemos C, Pecoits FR, Abensur H, Draibe S, Bastos MG, et al. [Left ventricular hypertrophy in patients with chronic kidney disease under conservative treatment.]. *J Bras Nefrol* 2010 Mar;32(1):85-90.
- (361) Matsui Y, Ishikawa J, Eguchi K, Shibasaki S, Shimada K, Kario K. Maximum Value of Home Blood Pressure: A Novel Indicator of Target Organ Damage in Hypertension. *Hypertension* 2011 May 2.
- (362) Gjata M, Nelaj E, Collaku L, Gjergji Z, Tase M. Left ventricular hypertrophy in chronic kidney disease. Is pulse pressure an independent risk factor? *Med Arh* 2011;65(1):30-1.
- (363) Jurgensen JS, Grimm R, Benz K, Philipp S, Eckardt KU, Amann K. Effects of anemia and uremia and a combination of both on cardiovascular structures. *Kidney Blood Press Res* 2010;33(4):274-81.
- (364) Larsson TE. The role of FGF-23 in CKD-MBD and cardiovascular disease: friend or foe? *Nephrol Dial Transplant* 2010 May;25(5):1376-81.
- (365) Paoletti E, Bellino D, Gallina AM, Amidone M, Cassottana P, Cannella G. Is left ventricular hypertrophy a powerful predictor of progression to dialysis in chronic kidney disease? *Nephrol Dial Transplant* 2011 Feb;26(2):670-7.
- (366) Wang Y, Sun Z. Klotho gene delivery prevents the progression of spontaneous hypertension and renal damage. *Hypertension* 2009 Oct;54(4):810-7.
- (367) Cancela AL, Oliveira RB, Gracioli FG, Dos Reis LM, Barreto F, Barreto DV, et al. Fibroblast growth factor 23 in hemodialysis patients: effects of phosphate binder, calcitriol and calcium concentration in the dialysate. *Nephron Clin Pract* 2011;117(1):c74-c82.
- (368) Di Marco GS, Konig M, Stock C, Wiesinger A, Hillebrand U, Reiermann S, et al. High phosphate directly affects endothelial function by downregulating annexin II. *Kidney Int* 2013 Feb;83(2):213-22.
- (369) Wang D, Iversen J, Strandgaard S. Contractility and endothelium-dependent relaxation of resistance vessels in polycystic kidney disease rats. *J Vasc Res* 1999 Nov;36(6):502-9.
- (370) Thuraishingham RC, Raine AE. Maintenance of normal agonist-induced endothelium-dependent relaxation in uraemic and hypertensive resistance vessels. *Nephrol Dial Transplant* 1999 Jan;14(1):70-5.
- (371) Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr* 2003 Feb;22(1):18-35.
- (372) Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J Biol Chem* 2001 Jan 5;276(1):40-7.

- (373) Beckman JA, Goldfine AB, Gordon MB, Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* 2001 Mar 27;103(12):1618-23.
- (374) Keswani AN, Peyton KJ, Durante W, Schafer AI, Tulis DA. The cyclic GMP modulators YC-1 and zaprinast reduce vessel remodeling through antiproliferative and proapoptotic effects. *J Cardiovasc Pharmacol Ther* 2009 Jun;14(2):116-24.
- (375) Sebkhii A, Strange JW, Phillips SC, Wharton J, Wilkins MR. Phosphodiesterase type 5 as a target for the treatment of hypoxia-induced pulmonary hypertension. *Circulation* 2003 Jul 1;107(25):3230-5.
- (376) Angus JA, Wright CE. Techniques to study the pharmacodynamics of isolated large and small blood vessels. *J Pharmacol Toxicol Methods* 2000 Sep;44(2):395-407.
- (377) Ando R, Ueda S, Yamagishi SI, Miyazaki H, Kaida Y, Kaifu K, et al. Involvement of advanced glycation end product-induced asymmetric dimethylarginine generation in endothelial dysfunction. *Diab Vasc Dis Res* 2013 Jun 13.
- (378) Brunet P, Gondouin B, Duval-Sabatier A, Dou L, Cerini C, Dignat-George F, et al. Does uremia cause vascular dysfunction? *Kidney Blood Press Res* 2011;34(4):284-90.
- (379) Most P, Remppis A, Weber C, Bernotat J, Ehlermann P, Pleger ST, et al. The C terminus (amino acids 75-94) and the linker region (amino acids 42-54) of the Ca²⁺-binding protein S100A1 differentially enhance sarcoplasmic Ca²⁺ release in murine skinned skeletal muscle fibers. *J Biol Chem* 2003 Jul 18;278(29):26356-64.
- (380) Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The yin and yang of nitric oxide in cancer progression. *Carcinogenesis* 2013 Mar;34(3):503-12.
- (381) Moncada S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol* 2002 Mar;3(3):214-20.
- (382) Reynolds MM, Witzeling SD, Damodaran VB, Medeiros TN, Knodle RD, Edwards MA, et al. Applications for nitric oxide in halting proliferation of tumor cells. *Biochem Biophys Res Commun* 2013 Feb 22;431(4):647-51.
- (383) Foley RN, Parfrey PS, Harnett JD, Kent GM, Martin CJ, Murray DC, et al. Clinical and echocardiographic disease in patients starting end-stage renal disease therapy. *Kidney Int* 1995 Jan;47(1):186-92.
- (384) Levin A, Thompson CR, Ethier J, Carlisle EJ, Tobe S, Mendelssohn D, et al. Left ventricular mass index increase in early renal disease: impact of decline in hemoglobin. *Am J Kidney Dis* 1999 Jul;34(1):125-34.
- (385) Taddei S, Nami R, Bruno RM, Quatrini I, Nuti R. Hypertension, left ventricular hypertrophy and chronic kidney disease. *Heart Fail Rev* 2011 Nov;16(6):615-20.
- (386) Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 2001 Dec 20;414(6866):916-20.

- (387) Schleicher M, Brundin F, Gross S, Muller-Esterl W, Oess S. Cell cycle-regulated inactivation of endothelial NO synthase through NOSIP-dependent targeting to the cytoskeleton. *Mol Cell Biol* 2005 Sep;25(18):8251-8.
- (388) Madesh M, Balasubramanian KA. Microtiter plate assay for superoxide dismutase using MTT reduction by superoxide. *Indian J Biochem Biophys* 1998 Jun;35(3):184-8.
- (389) Burdon RH, Gill V, Rice-Evans C. Reduction of a tetrazolium salt and superoxide generation in human tumor cells (HeLa). *Free Radic Res Commun* 1993;18(6):369-80.
- (390) Lim K, Lu TS, Molostvov G, Lee C, Lam FT, Zehnder D, et al. Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* 2012 May 8;125(18):2243-55.
- (391) Ikushima M, Rakugi H, Ishikawa K, Maekawa Y, Yamamoto K, Ohta J, et al. Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells. *Biochem Biophys Res Commun* 2006 Jan 20;339(3):827-32.
- (392) Rakugi H, Matsukawa N, Ishikawa K, Yang J, Imai M, Ikushima M, et al. Anti-oxidative effect of Klotho on endothelial cells through cAMP activation. *Endocrine* 2007 Feb;31(1):82-7.
- (393) Charles BA, Conley YP, Chen G, Miller RG, Dorman JS, Gorin MB, et al. Variants of the adenosine A(2A) receptor gene are protective against proliferative diabetic retinopathy in patients with type 1 diabetes. *Ophthalmic Res* 2011;46(1):1-8.
- (394) Burnett SM, Gunawardene SC, Bringhurst FR, Juppner H, Lee H, Finkelstein JS. Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women. *J Bone Miner Res* 2006 Aug;21(8):1187-96.
- (395) Antonucci DM, Yamashita T, Portale AA. Dietary phosphorus regulates serum fibroblast growth factor-23 concentrations in healthy men. *J Clin Endocrinol Metab* 2006 Aug;91(8):3144-9.
- (396) Zhang Y, Agnoletti D, Protogerou AD, Topouchian J, Wang JG, Xu Y, et al. Characteristics of pulse wave velocity in elastic and muscular arteries: a mismatch beyond age. *J Hypertens* 2013 Mar;31(3):554-9.
- (397) Shimizu M, Kario K. Role of the augmentation index in hypertension. *Ther Adv Cardiovasc Dis* 2008 Feb;2(1):25-35.
- (398) Houston J, Smith K, Isakova T, Sowden N, Wolf M, Gutierrez OM. Associations of dietary phosphorus intake, urinary phosphate excretion, and fibroblast growth factor 23 with vascular stiffness in chronic kidney disease. *J Ren Nutr* 2013 Jan;23(1):12-20.
- (399) Ix JH, de Boer IH, Peralta CA, Adeney KL, Duprez DA, Jenny NS, et al. Serum phosphorus concentrations and arterial stiffness among individuals with normal kidney function to moderate kidney disease in MESA. *Clin J Am Soc Nephrol* 2009 Mar;4(3):609-15.