ENDOTHELIAL PROGENITOR CELLS, URAEMIC TOXINS, & THE DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION IN CHRONIC KIDNEY DISEASE

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Submitted in fulfillment of the

Degree of Doctor of Philosophy,
University of Adelaide,
June 2012

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Thesis Abstract

Morbidity and mortality rates for cardiovascular disease (CVD) are increased among end stage kidney disease (ESKD) patients receiving dialysis treatment, and not corrected with kidney transplantation (KTx). Classic CVD risk factors do not fully predict the increased risk, with novel factors causing endothelial dysfunction (ED), leading to arteriosclerosis, congestive heart failure (CHF) and sudden death, key to disease pathogenesis.

These novel factors include bone marrow (BM) derived endothelial progenitor cells (EPCs), which have key roles in maintenance, repair and growth of the endothelium. There is limited data about the role of EPCs and CVD in the ESKD population. This uraemic milieu includes p-cresol (sulfate, PC/S) and indoxyl sulfate (IS), toxins associated with CVD in ESKD.

In this thesis, the relationship between CVD and ESKD, and the potential role of EPCs and uraemic toxins was examined from epidemiological, clinical and laboratory perspectives.

Data was obtained for the period between 2002-2007 for all hospital separations in Australia. Analysis was performed based on ICD-9/10 coding. This showed (for the first time in an Australian population): (i) an increase in risk for CVD hospital separations among dialysis and KTx, with higher rates for CHF than acute cardiac events (ACE); (ii) an advantage for KTx recipients in regards to ACE, but not CHF hospital separations, over dialysis recipients, and (iii) for CHF, no increase in in-hospital mortality, or length of stay per separation for any ESKD group compared to controls.

At a clinical level, in groups of haemodialysis (HDx), KTx patients and controls, low peripheral blood (PB) EPC numbers were correlated with surrogate markers of CVD

and ED. No clear relationship of IS and PC/S with ED was seen (although study power was limited).

For *in vitro* studies, techniques were developed for isolation (Flow sort and AutoMACS), enumeration (FACS) and culture expansion of EPCs from BM and umbilical cord blood samples.

The effects of uraemic serum and toxins PC and IS on cultured endothelial cells (ECs) and EPCs *in vitro* was examined, as a model of vascular pathology in ESKD.

Greater HUVEC VCAM-1 expression and reduced tube formation in Matrigel were observed in response to increasing PC concentration than IS. The effect of IS (but not PC) at higher concentration in Matrigel was reduced by the addition of EPCs. Akt/ERK expression by western blot, cell migration to VEGF, and supernatant investigation by FlowCytoMix for soluble cell surface markers, were also performed. Testing of HUVEC function post-exposure to sera from control, transplant and HDx recipients did not replicate the above results on the basis of sera PC and IS levels.

In summary, this thesis has explored the increased burden of CVD in ESKD patients in Australia, the relationship of EPCs, both *in vivo* and *in vitro,* to vascular disease in this setting, and the role of uraemic toxins as agents for CVD. These results underline why certain therapies may not be effective in the ESKD population for CVD prevention, and suggest novel approaches are needed.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Dr Shaundeep Sen and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Acknowledgements

This thesis began as a very loose collection of ideas, which over time, developed into more solid plans. The initial, more grandiose, thoughts were tempered, whilst blind optimism was quickly replaced by pessimistic reality, and then later guarded hope.

I was neither aware nor prepared for what a PhD really required of it's aspirant, until well into my candidature – for a physician, clinical work can be heavy, but usually provides you with regular positive feedback. Basic and clinical research do not provide the same consistent gratification, despite greater effort.

As my principal supervisor, Stephen McDonald has been a mentor, motivator, teacher and friend. He has forged a career that inspires me to do more with my own. I cannot thank him enough, especially for his gentle, and at other times more vigorous, encouragement, as well as his consistent broad vision for the thesis.

Toby Coates I am also privileged to consider a friend, as well as mentor. His balance of a heavy clinical load, whilst directing a large active laboratory is testament to his unlimited energy, whilst his personal generosity is renowned. I thank him in particular for his ongoing enthusiasm for my work, and unwavering optimism.

Claudine Bonder became key to my completion of this PhD. As a scientist, she has had to put up with my non-existent laboratory skills and irregular work hours, without once making me feel out of place in the lab. As a manager, Claudine shows how a very successful research program can be developed in the midst of multiple competing factors for your time. For her friendship, I will be always grateful.

Beyond my supervisors, I received significant support from several different groups of individuals. I wish to acknowledge from the cardiology department at the QEH Dr Alicia Chan for the initial EPC isolation, and Prof John Beltrame and Dr Christine Bell. From Toby's lab Emma Leedham, Clyde Milner, Chris Drogemuller, Claire Jessup, Matthew Stephenson and Svjetlana Kireta, and all the PhD students and staff, who made my time there such a joy. From Claudine's lab, Kiwi Sun (who constantly inspires me with her work ethic), Samantha Escarbe, Sarah Brice, Michaelia Cockshell, Katie Tooley, Kate Parham, Jyotsna Pippal and Jeff Barrett. From the Detmold Imaging Suite, Katherine Pilkington and Ghafar Sarvestani, Bin Tong from the AIHW, and Dr Joachim Jankowski from the Charite.

I would also like to thank all the physicians, nurses, administrators, patients and subjects involved in the clinical study, in particular Inspector Philip Newitt for his recruitment drive, and the staff of the renal units at the QEH, RAH and FMC.

My family has continued to support, encourage and inspire me throughout my life and my career, without question. I gained daily inspiration from the friendship and love given to me by my parents Pradeep and Surekha, sister Rukmi, parents-in-law John and Carole, brother-in-law Andrew, and sisters-in-law Catherine and Benita, who have all individually achieved so much.

Finally, to my wonderful wife Sarah and our gorgeous children Abbie and Aidan, I dedicate this work. They have dealt with my low times, infuriating procrastination, long hours in the lab, and absences from home. They have always supported, encouraged and loved me, and have been a constant reminder of what is valuable and important in life. Without Sarah, and the sacrifices she in particular has made, none of this would have been possible.

Honours and Awards

2010 Queen Elizabeth Hospital

- Departmental Postgraduate Research Scholarship

2009 Transplantation Society of Australia and New Zealand Annual Scientific Meeting

- Kidney Health Australia Prize for the Best Presentation in the Field of Clinical Research
- Young Investigator Award

2009 Queen Elizabeth Hospital Research Day

- Best Poster Prize

2007 Kidney Health Australia

- PhD Biomedical Scholarship

Publications

Penko D, Mohanasundaram D, Sen S, Drogemuller C, Mee C, Bonder CS, Coates PTH, and Jessup CF. Incorporation of endothelial progenitor cells into mosaic pseudoislets. Islets. 3(3): 73-79. May/June 2011

Sen S, McDonald SP, Coates PTH and Bonder CS. Endothelial Progenitor Cells: novel biomarker and promising cell therapy for cardiovascular disease. Clinical Science. 120(7): 263-83, 2011 April

Conference Presentations

2011 – American Society of Nephrology: Renal Week/ASM

- S Sen, PTH Coates, CS Bonder, SP McDonald. "The interaction of uraemic toxins and endothelial progenitor cells in the progression of cardiovascular disease" – Poster

2011 - Australia and New Zealand Society of Nephrology: ASM

- S Sen, PTH Coates, CS Bonder, SP McDonald. "The interaction of uraemic toxins and endothelial progenitor cells in the progression of cardiovascular disease" – Poster

2010 – The Transplantation Society: World Congress

- S Sen, SP McDonald. "Kidney Replacement Therapies are Associated with Increased Rates of Hospital Separation and Mortality for Acute Cardiac Events" – Mini-Oral

2009 – Queen Elizabeth Hospital Research Day

- S Sen, B Tong, SP McDonald. "Kidney Replacement Therapies are Associated with Increased Rates of Hospital Separation and Mortality for Acute Cardiac Events" – Poster

2009 – Australia and New Zealand Society of Nephrology

- S Sen, B Tong, SP McDonald. "Kidney Replacement Therapies are Associated with Increased Rates of Hospital Separation and Mortality for Acute Cardiac Events" – Poster
- S Sen, SP McDonald, PTH Coates, CS Bonder. "In vitro Endothelial Cell Tube-Forming Capacity Reduced By Transplant Sera, But Migration Increased by Haemodialysis" – Poster

2009 – Transplant Society of Australia and New Zealand

- S Sen, SP McDonald, PTH Coates, CS Bonder. "A Novel Protocol for the Expansion of CD133+ Human Endothelial Progenitor Cells in EGM-2 Plus Cytokines" – Oral Presentation
- S Sen, B Tong, SP McDonald. "Renal Transplantation Decreases Rates of Hospital Separation for Both Acute Cardiac Events and Cardiac Failure Compared to Dialysis" – President's Prize Session Oral Presentation

Manufacturers

- Abbott Ireland, Longford, Co. Longford, Ireland
- AtCor Medical Pty Ltd, West Ryde, NSW, Australia
- Baxter Healthcare, Old Toongabbie, NSW, Australia
- Dr B. P. Bailey, Lane Cove, NSW, Australia
- BD, Franklin Lakes, NJ, USA
- BD Biosciences,
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    - North Ryde, NSW, Australia
- Beckman Coulter, Miami, FL, USA
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- Detmold Family Trust Cell Imaging Facility, Hanson Institute, SA Pathology
- Diagnostica Stago Inc., Parsippany, NJ, USA
- Fresnius, Kabi, Norge

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- Institute for Medical and Veterinary Science (IMVS) / SA Pathology, Adelaide, SA, Australia
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- Siemens Healthcare Diagnostics, Deerfield, IL, USA
- Sigma Aldrich, St Louis, MO, USA
- Sigma Pharmaceuticals, Croydon, Vic, Australia
- SonoSite Inc, Bothwell, WA, USA

- Southern Biotech, Birmingham, AL, USA
- StataCorp LP, Texas USA
- SupelCo, Bellafonte, PA, USA
- Sysmex America Inc., Mundelein, IL, USA
- Thermo Scientific, Waltham, MA, USA
- Vital Diagnostics, Bella Vista, NSW, Australia
- Wayne Rasbands, National Institutes of Health, USA
- Worthington Biomedical Corporation, Lakewood, NJ, USA

Abbreviations

AA	ascorbic acid
ABS	Australian Bureau of Statistics
ACE	acute cardiac events
Ac-LDL	acetylated low density lipoprotein
ACEi	angiotensin converting enzyme inhibitor
ADMA	asymmetric dimethylarginine
AI	augmentation index
AIHW	Australian Institute of Health and Welfare
AMI	acute myocardial infarction
Ang-1	angiopoietin I
ANZDATA	Australia and New Zealand Dialysis and Transplant Regsitry
AT I	angiotensin I
ATRA	angiotensin II receptor antagonist
bFGF	basic fibroblast growth factor
BNP	brain natriuretic peptide
BP	blood pressure
^{o}C	degrees Celsius
CAC	coronary artery calcification
CCA	common carotid artery
CCL	CC chemokine ligand
CFDA	carboxy-fluorescein diactetate
CHF	congestive heart failure
cIMT	carotid artery intima-medial thickness

CKD	chronic kidney disease
CK-MB	myocardial creatine kinase
CMV	cytomegalovirus
CPC	circulating progenitor cell
CRP	C-reactive protein
CT	computerised tomography
cTnT	cardiac troponin T
CVD	cardiovascular disease
CXCL	CXC chemokine ligand
CXCR	CXC receptor
Cy5/7	cyanine 5/7
DAPI	4’,6-diamidino-2-phenylindole
DBP	diastolic blood pressure
Dil Ac-LDL	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein
DMSO	dimethylsulfoxide
DPP IV	dipeptidylpeptidase IV
EBCT	electron beam computerised tomography
EC	endothelial cell
ECG	electrocardiograph
ECGF	endothelial cell growth factor
ED	endothelial dysfunction
EDTA	ethylenediaminetetraacetic acid
eNOS	endogenous nitric oxide synthetase
EPC	endothelial progenitor cell

EPO	erythropoietin
ESKD	end-stage kidney disease
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FDR	flow debt repayment
FITC	fluorescein isothiocianate
FLK	Fms-like tyrosine kinase
G-CSF	granulocyte colony stimulating factor
GFR	glomerular filtration rate
GM-CSF	granulocyte macrophage colony stimulating factor
GTN	glycerol trinitrate
HbA1C	haemoglobin A1C
HDL	high density lipoprotein
HDx	haemodialysis
hEGF	human endothelial growth factor
HIF-1α	hypoxia inducible factor-1alpha
HMGB1	high-mobility box group 1
HPLC	high performance liquid chromatography
HT	hypertension
HUVEC	human umbilical vein endothelial cell
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule-1
ICD-10	International Classification of Disease, Version 10
Ig	immunoglobulin

IGF-1	insulin-like growth factor-1
IL	interleukin
IM	intramuscular
IS	indoxyl sulfate
KDR	kinase insert domain receptor
KRT	kidney replacement therapy
KTx	kidney transplant
LDL	low density lipoprotein
LV	Left ventricle
LVEF	left ventricular ejection fraction
LVH	left ventricular hypertrophy
MACS	magnetic activated cell separation
MAPC	multipotent adult progenitor cell
MCP-1	monocyte chemotactic protein-1
MMP	matrix metalloproteinase
MNC	mononuclear cell
MRA	magnetic resonance angiography
MRI	magnetic resonance imaging
m/s	metres per second
NO	nitric oxide
NFκB	nuclear factor kappaB
NIH	National Institutes of Health
NT-proBNP	N-terminal pro-brain natriuretic peptide
oxLDL	oxidised low density lipoprotein

PB	peripheral blood
PC	para-cresol, p-cresol
PCS	para-cresol sulfate, p-cresol sulfate
PDx	peritoneal dialysis
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule-1
PET	positron emission tomography
PMT	photomultiplier tubes
PPARγ	peroxisome proliferator activated receptor gamma
PTH	parathyroid hormone
PVD	peripheral vascular disease
PWA	pulse wave analysis
PWV	pulse wave velocity
RAGE	receptor for advanced glycation end products
RNA	ribonucleic acid
SBP	systolic blood pressure
SCF	stem cell derived factor
SDF-1	stromal cell derived factor-1
sICAM	soluble intercellular adhesion molecule
SMC	smooth muscle cell
SPECT	single photon emission computerised tomography
TGFβ-R1 inhIII	tissue growth factor beta R1 inhibitor III
TNFα	tumour necrosis factor alpha
UCB	Umbilical cord blood

US	ultrasound
USRDS	United States Renal Data Systems
VCAM-1	vascular cell adhesion molecule - 1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGF-R2	vascular endothelial growth factor receptor 2
vWF	Von Willebrand factor

Chapter 1: Introduction

Diseases of the vascular system, including cardiac, peripheral and cerebrovascular disease, pose a major health burden. Ischaemic heart disease and cerebrovascular disease are the leading causes of death in both developed and developing economies[1]. Current therapies for cardiovascular disease (CVD), including lifestyle and pharmacological control of risk factors and surgical revascularisation, do not completely reverse the underlying vascular pathophysiology[2].

Progressive chronic kidney disease (CKD), and end stage kidney disease (ESKD) have been identified as independent risk factors for progressive CVD[3]. Increased rates of incidence and prevalence of cardiovascular disease (CVD) among people with chronic kidney disease (CKD) have been well documented in many developed countries[4, 5] and risk increases as kidney function declines[3, 6, 7].

1.1 Chronic kidney disease is a common problem in Australia

Up to 16% of the Australian population have at least one marker of kidney disease, including reduced glomerular filtration rate (GFR), haematuria or proteinuria [8]. In the AusDiab Survey, approximately 9% of respondents had a GFR of less than 60ml/min, with over 50% of the over 65's in this category [9]. In this subpopulation of 9%, all had significantly increased prevalence of hypertension, hyperlipidaemia and type II diabetes mellitus, as well as self-reported angina, myocardial infarction, stroke and CVD. The vast majority of these patients also did not achieve targets for blood pressure control or cholesterol [10]. Furthermore diabetes mellitus, now the most common cause of end stage kidney disease (ESKD) in Australia[4], continues to increase in prevalence, doubling over 20 years to 7% of the adult Australian

population in 1999. A further 16% had glucose “intolerance”, or “pre-diabetes”, with approximately 50% of both diabetic groups undiagnosed at the time of study[11].

1.1.1 ESKD is Increasing in Australia

Rates of new patients beginning kidney replacement therapy (KRT, either kidney transplantation (KTx) or dialysis) are rising in Australia, with prevalent patient rates increasing from 246 per million in 1983, to 741 per million in 2005[12]. Patients in the age groups 75-84 and 85+ years of age have had the greatest increase in acceptance onto renal replacement therapy. Diabetic nephropathy remains the most prevalent cause of ESKD, at 32% of all new cases [4].

1.1.2 CKD is associated with increased mortality

CKD is an independent risk factors for CVD; after adjustment for other factors, the risk for CVD increases progressively as renal function decreases[3]. Furthermore, in ESKD, having a cardiac event significantly increases one’s risk of mortality from CVD in the future[13].

Death rates in the ESKD population on KRT have remained unchanged over the last 10 years. For dialysis patients, this is approximately 15 deaths per 100 patient years[14]. Transplant patients have an improved survival, with a death rate of 2.3 per 100 patient years. Cardiovascular death accounts for 39% and vascular disease 10% in the dialysis population. For transplants, it was 36% and 7% respectively. These death rates are up to 10-30x the general age-matched population for dialysis, and more pronounced at younger age [14]. Although CVD rates in the general population in Australia have fallen over recent years[15], concurrently with a range of advances in treatment of vascular risk factors and established CVD, these changes have not been seen among the KRT population.

1.2 Aetiology of CVD in ESKD

1.2.1 Different types of CVD seen in KRT

The nature of cardiovascular disease in ESKD patients differs from those in the general population, with an apparent excess of deaths due to congestive heart failure (CHF) and sudden death due to cardiac arrythmia (up to 27% of all-cause mortality)[16], and relatively less angina and acute transmural myocardial infarction. CHF prevalence rates of up to 36% in incident dialysis patients[17], as well as KTx[17] populations are reported, compared to 1.5-2% for CHF in the general population, for those aged over 45 years[18]. For acute cardiac events (ACE), the incidence is up to 7.6% post KTx[19] and up to 38%[20] in dialysis recipients, and 1.8% for the non-kidney replacement therapy (non-KRT) population[18].

1.2.2 CVD risk factors

1.2.2.1 Classical risk factors

The increased rate of incidence of CVD in ESKD is not accounted for by classical risk factors[21-23]. Hypertension (HT), obesity and hypercholesterolaemia have not been consistently shown to be associated with worsened CVD outcomes in the ESKD group[24, 25].

1.2.2.2 “Reverse epidemiology”

The presence of classical risk factor in the short to medium term has been associated with better survival – this has been termed “reverse epidemiology” [25]. This is likely based on a syndrome of malnutrition in the presence of chronic inflammation, and differentiates relationships of these factors with short and long-term outcomes. Evidence for this theory has come with findings such as worsened cardiac outcomes with low albumin[26], anaemia[6], increased interleukin-6 (IL-

6)[27], elevated C-reactive protein (CRP)[28-30], elevated serum calcium and phosphate[31], and elevated tumour necrosis factor alpha (TNF-α)[32].

1.2.2.3 Risk factors specific to KRT

There are several risk factors unique to RRT patients. Dialysis activates the immune system[33], increases basal levels of oxidative stress[34], is associated with increased vascular calcification[35, 36] and large fluid gains between haemodialysis (HDx) sessions[37]. These factors are all associated with increased CVD risk. KTx is complicated by the addition of new risk factors including organ rejection[38], adverse effects of immunosuppressives[39], HT[39], and infections, specifically cytomegalovirus (CMV)[40].

1.2.2.4 Management of risk factors

In contrast to the general population, treatment for classical markers (hypertension, abnormal lipid profile, obesity) has had mixed success. No clear evidence exists for benefit from statins among dialysis patients, with two large trials showing no benefit[41]39], and an increased stroke rate [42]. Conversely, beta-blockers[43] and aspirin[44] have been shown to reduce CVD risk.

1.2.3 Biomarkers and ESKD

There are a large number of biomarkers that have been proposed as predictive of CVD in ESKD. Below is discussion of those agents focused on those with the most evidence for causation, rather than purely association. The latter group includes Troponin T (released upon damage to cardiac myocytes and predicts all-cause and cardiac mortality[29, 45], and homocysteine, which despite increasing in serum concentration in CKD and disrupting NO production, treatment to lower its concentration has no clinical benefit[46, 47].

1.2.3.1 Asymmetric Dimethylarginine (ADMA)

ADMA is a potent endogenous inhibitor of endothelial nitric oxide synthetase (NOS). ADMA is normally either broken down to citrulline, or excreted renally. In ESKD, the associated oxidative stress decreases enzymatic transformation to citrulline, and with the loss of renal excretion, ADMA concentrations may reach six times higher than the general population. Through its negative action on nitric oxide production, ADMA has been found to be a strong independent risk factor for CVD in CKD, and concentrations are higher in those with pre-existing atherosclerosis, including in HDx[33, 48, 49]]. In peritoneal dialysis (PDx), elevated ADMA is also associated with reduced residual kidney function and left ventricular hypertrophy (LVH)[50].

1.2.3.2 N-Terminal Pro-Brain Natriuretic Peptide (NT-proBNP)

Brain natriuretic peptide (BNP) and NT-proBNP are cardiac neurohormones released in response to ventricular stretch and overload[51]. They are elevated in ESKD, and increased concentrations are predictive of CVD events[52]. NT-proBNP is not removed by HDx, and is a consistent marker for left ventricular (LV) dysfunction among ESKD groups receiving HDx[53].

1.2.4 Uraemic Toxins

Given the increased risk of CVD associated with ESKD, a potential role for uraemic toxins (biological breakdown products, normally excreted by the kidney) as contributors to this increased risk has been examined. The European Uremic Toxin Working Group identified and classified 90 compounds retained in uraemia. Of these, 22 were classified as “middle molecules”, with a molecular weight of greater than 500 daltons[54] – these larger molecules are more difficult to remove by regular dialysis techniques, and are not normally measured in clinical practice. Two lower weight, protein-bound agents however, para-cresol (p-cresol, PC) and indoxyl sulfate

(IS), have been recently identified as being independently associated with worsened clinical outcomes[55, 56]. Further research suggests their pathologic role in CVD, rather than mere markers of disease (as discussed below).

1.2.4.1 Para-cresol (PC)

PC (4-methylphenol, $CH_3C_6H_4OH$, 108Da), is a product of bacterial fermentation of L-tryptophan in bowel[57], and is partially conjugated to p-cresol sulfate (PCS; >15% of PC is unconjugated in HDx subjects[58]) at the colonic brush border[59]. Whilst described in the past as predominately protein bound in blood[58], it has been recently shown that PC and PCS have only 13-20% binding to human serum albumin at 37 degrees Celsius (^oC)[60]. This has implications for methods of removal of PC/PCS in the clinical setting, and for use of them in *in vitro* studies in the presence of serum protein.

1.2.4.1.1 Elevated PC and PCS concentrations and disease

Elevated serum concentrations of PC are independently predictive of increased CVD event risk in CKD[55], increased future mortality from cardiac- and all-causes[61, 62], as well as being associated with impaired leukocyte anti-infective ability[63]. Furthermore, increased serum concentration of endothelial cell (EC) microparticles are found with increased PC serum concentration, indicative of direct endothelial damage and dysfunction[64]. Both total and free PC has been measured *in vivo*, with both having similar associations with worsened CVD outcomes at increasing serum concentration[65].

PCS is also associated with an increased concentration of circulating EC microparticles[66]. It has also been shown that free PCS independently predicts future all-cause and CVD mortality in subjects with “mild-to-moderate” CKD, whilst total PCS only predicts CVD mortality[62].

In vitro, PC inhibits EC proliferation[67], as well as decreasing leukocyte adhesion[68, 69]. PCS also directly damages ECs [66] *in vitro*, but has an apparently different effect on leukocytes, by stimulating oxidative burst through inflammatory pathways[70]

1.2.4.1.2 Interventions to decrease serum p-cresol concentration

Decreased serum concentrations are achieved with pro-biotic formulations (e.g. Lactobacillus casei Shirota, and Bifidobacterium breve Yakult)[71], high efficiency HDx or haemodiafiltration[72, 73], activated charcoal in dialysate[74], and high carbohydrate/low protein load to the colon[75]. The clinical benefit of these interventions is as yet unknown.

1.2.4.1.3 Controversies for *in vitro* testing with p-cresol and p-cresol sulfate

Since the *in vitro* studies of the thesis was commenced and completed, further data on PC has emerged. As summarised by Vanholder *et al*[76], previously measured PC from patient sera is actually PCS which had been desulfated during the acidic deproteination of samples for measurement by high performance liquid chromatography (HPLC)[58, 73], and that PC alone is found only in very low concentrations *in vivo*. Vanholder argues that PC only exists either *ex vivo*, or at the brush border in the gut, before it is sulfated. This has implications for extrapolation of results from *in vitro* experiments.

Whilst a difference in effect of PC and PCS on leukocytes *in vitro* has been demonstrated in two studies only, there have been no comparable studies determining the effect of PC and PCS on EC or other cell function. Furthermore, *in vivo* studies suggest similar prognostic capability of both chemicals in regards CVD risk – whilst this provides minimal mechanistic insights, it demonstrates an association with the CV system in humans for both agents.

Protocols for sulfating commercially purchased PC are available, however when this study was commenced, issues pertaining to PC and PCS were not well understood. Local laboratories had not had experience in handling of PC from commercial sources, nor a protocol to measure concentrations of PC/PCS in samples. Therefore, experiments for this thesis were performed with PC (Chapter 2.7.2), understanding its potential clinical limitations. Subsequently, an association was formed with an international laboratory, which was able to measure toxin concentrations from serum and supernatants.

1.2.4.2 Indoxyl sulfate (IS)

IS, ($C_8H_6NO_4SK$, 251.3Da), is a member of the indole group, originating from the bacterial breakdown of the amino-acid tryptophan derived from diet[77]. Indole is metabolised in the liver to IS[78].

1.2.4.2.1 Elevated indoxyl sulfate levels and disease

In *in vitro* and *in vivo* studies, IS is associated with increased production of reactive oxygen species[79, 80], activation of smooth muscle cells (SMC)[81, 82], osteoblast resistance to parathyroid hormone (PTH)[83], direct damage to renal tubules via tissue growth factor beta (TGFβ)[84], and increase in glomerulosclerosis [85], leading to increased rate of decline in GFR. Clinically, higher IS concentrations in CKD and ESKD are independently associated with both surrogate markers of athero and arteriosclerosis, as well as cardiac and all-cause mortality[56].

1.2.4.2.2 Interventions to decrease serum indoxyl sulfate concentration

The oral sorbent AST-120 (spherical charcoal entity) is approved for use in Japan in pre-ESKD patients to reduce the rate of decline in GFR from IS. It acts by binding the IS precursor indole in the gut, and increasing its excretion in faeces. AST-120

has been shown to both reduce IS serum concentration and rate of GFR decline in animal[86, 87] and human[88-91] models of CKD. The beneficial effect of AST-120 (via reduction in IS concentrations) may be through reductions in expression of the tumour suppressor gene p53 in tubular cells[92] and nuclear factor kappaB (NFκB)in the kidney[93], as well as overall reductions in oxidative stress[87, 94].

High efficiency HDx techniques also reduces circulating concentrations of IS, but the long term clinical effect of this remains unknown[95, 96].

The overall CVD benefit of IS reduction (beyond reduction in decline of GFR) is also still unknown – one animal study of AST-120 administration to rats with acquired CKD demonstrated reduced cardiac size, left ventricular volume and fibrosis in the treatment group despite no change in kidney function. The authors suggested the mechanism was reduced oxidative stress as lower urine concentrations of 8-hydroxydeoxyguanosine and acrolein were found, with less staining for both in cardiac myocytes[87].

1.2.5 Interaction of multiple factors in pathology of vascular disease in CKD

There are a number of factors that may explain the differences in vascular pathology seen in renal failure. As well as increasing in prevalence as kidney function declines, all these processes have a deleterious effect on vascular function through inhibiting normal endothelial function.

- Increased oxidative stress secondary to retained uraemic toxins[34].
- Increased production of pro-inflammatory proteins[97].
- Altered production and impaired functional effect of nitric oxide[98].
- Accelerated vascular calcification through altered calcium metabolism[99].
- Salt and water retention [100].
- Sympathetic overactivity [101].

1.3 The Endothelium and Vascular Disease

1.3.1 Normal function of the endothelium

The inner lining of the blood vasculature, formed by endothelial cells (EC), balances a number of diverse functions (see Figure 1), including:

- Regulation and buffering of normal blood flow
- Facilitation of transfer of gases, solutes and cells to underlying tissue
- Aiding in cellular repair[102]

Heterogeneity of ECs at differing anatomical sites provides specialised EC actions throughout the body (reviewed in detail [103, 104]). These differences in ECs reflect blood vessel diversity, from the tightly associated ECs of the major conduit arteries where vessel integrity is paramount, to the fenestrated ECs that populate end organs such as the kidney and liver.

1.3.2 Disease of the Endothelium

Atherosclerosis and arteriosclerosis are the two major causes of occlusive vascular disease.

1.3.3 1. Atherosclerosis

The predominant form of vascular disease in the general population is atherosclerosis, characterized by formation of a fat-based blood vessel plaque overlying the intima, which causes progressive obstruction of a vessel lumen over time. In situations of prolonged inflammation [105], such as in CKD [106], plaques may become unstable and rupture, causing acute obstruction of vessels, leading to distal ischaemia/infarction.

1.3.4 2. Arteriosclerosis

Whilst atherosclerosis primarily affects the intima of blood vessels leading to obstruction, arteriosclerosis refers to stiffening of the vessel wall, from changes in the cellular phenotype and function in the middle layer (media) of the blood vessel wall. It was first described in 1892[107] as a consequence of normal ageing. It predominantly affects larger, capacitance arteries (thoracic aorta and central vessels) rather than peripheral vessels (which do not normally have the capacity to buffer blood pressure).

Figure 1.1 panels B and C show the stepwise progression in endothelial dysfunction during these diseases that occur as a consequence of exposure to risk factors. Importantly, vascular ECs are integral to cardiac function.

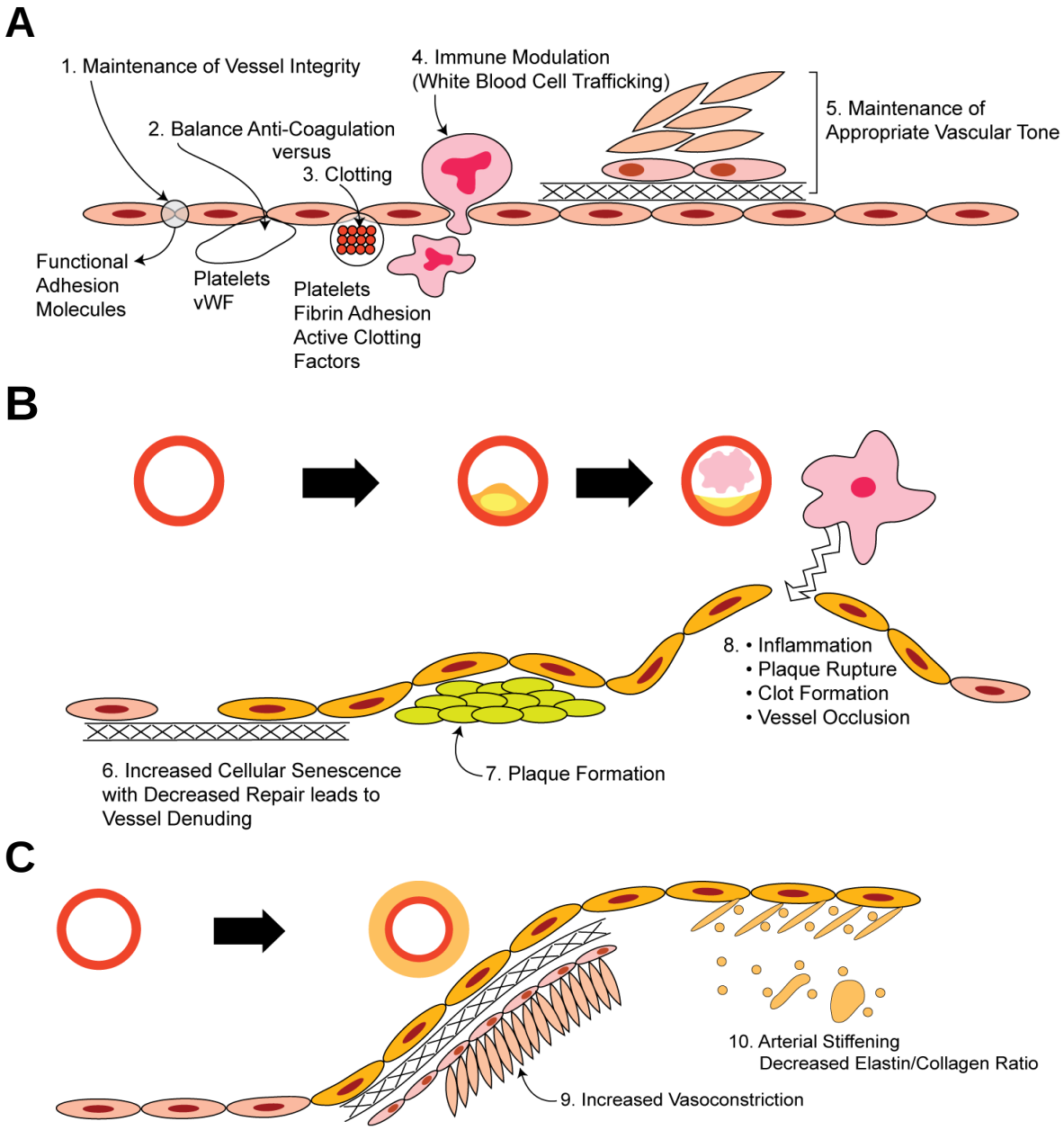

Figure 1.1: Under normal conditions (A), ECs maintain (1) blood vessel integrity, (2) balance anticoagulation and (3) clotting, (4) modulate the immune system by regulating leukocyte trafficking, and (5) maintain vascular tone along with pericytes and smooth muscle cells. In contrast, during disease (B & C), senescent ECs with decreased repair cause (6) vessel denuding, (7) plaque formation, (8) rupture and thrombosis, with vessel occlusion, as well as (9) vasoconstriction and (10) arterial stiffening.

1.3.4.1.1 Pathological changes in arteriosclerosis

Changes observed in arteriosclerotic vessels include increased ground substance and collagen in the vessel media, with breakdown of elastic fibres. The consequence of this is scarring and calcification, especially the latter in ESKD, where there is a strongly pro-calcification milieu arising from deranged calcium phosphate metabolism[108-110]. In severe cases, found predominantly in ESKD, small vessels in subcutaneous tissues have massive calcium deposition, leading to breakdown of blood vessels and overlying skin. This is termed calcific uraemic arteriolopathy[111].

1.3.4.1.2 Arterial stiffness/arteriosclerosis and cardiac disease

London *et al* have shown that, independent of systemic blood pressure, there is significant dilatation of the aorta from root to bifurcation in ESKD [100]. The functional effect of this is an increase in pulse pressure due to increased reflection of the early arterial wave. This has a direct effect on the LV, causing hypertrophy and sub-endocardial ischaemia [112]. LVH in ESKD is hypothesised as one of the major causes of the increased rate of sudden cardiac death. The aetiology for this is presumed increased cardiac arrythmia rates secondary to chronic tissue ischaemia[113].

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1.4 Non-Invasive Markers of Endothelial Dysfunction and CVD Risk in ESKD

Assessment of present and future risk for CVD is required for effective management of individuals in a clinical setting. However, whilst it is appropriate to use invasive techniques such as angiograms, contrast computerised tomography (CT), and intra-vascular ultrasound (US) in an acute clinical setting, this is not practical in the screening or research setting.

Non-invasive techniques, examining surrogate markers of endothelial disease, are an alternative method of defining both structural and functional vascular disease states. In particular, impaired endothelial function may also be assessed through pharmacologic modulation of vessel function.

Whilst there are many surrogate techniques available, there are caveats to their validity in KRT populations, given differences in pathophysiology as well as frequency of disease. Table 1.1 summarises the main techniques used for non-invasive testing of CVD risk, the associations that have been investigated, and potential limitations.

The basis for each technique is summarised below.

1.4.1 Carotid Artery Intima-Medial Thickness (cIMT)

cIMT is the thickness of the inner two layers of the artery wall, measured by ultrasound (see Figure 1.2). Both atherosclerotic and arteriosclerotic processes influence it. Medial thickness is associated with hypertensive and arteriosclerotic change – and increases with age and in males. Thickening is defined as greater than the 75th percentile for age and sex, with normal generally between 0.6 and 1.2mm [114]. cIMT increases over time and therefore with age – the typical yearly increases in cIMT are in the range of 0.014 to 0.02mm [115]).

cIMT has been validated in clinical studies as reproducible [116], and has been used in both renal and non-renal populations. In a separate study, Kanters *et al*[117] reviewed previous studies that had used cIMT alone to confirm the reproducibility of the test, as well as suggesting a standardised protocol. The far wall is suggested for measurement as the near wall can underestimate thickness, due to interference from proximal echos from the denser adventitia. Improved reproducibility is also found with multiple measurements in different directions, measuring common carotid artery (CCA) rather than internal carotid artery (ICA) or carotid bulb, using the mean rather than maximum IMT, and utilising automated edge-tracking methods [117].

cIMT does not directly measure atherosclerosis in the carotid artery, but increased vessel wall thickness that is associated with atherosclerotic burden elsewhere. In fact, plaque must be avoided, as it inappropriately increases IMT measurements. Pitfalls of the technique are operator dependent accuracy and anatomical variation[118]. Presence of plaque in PDx patients, however, is associated with markers of both atherosclerosis and arterial stiffening elsewhere[119].

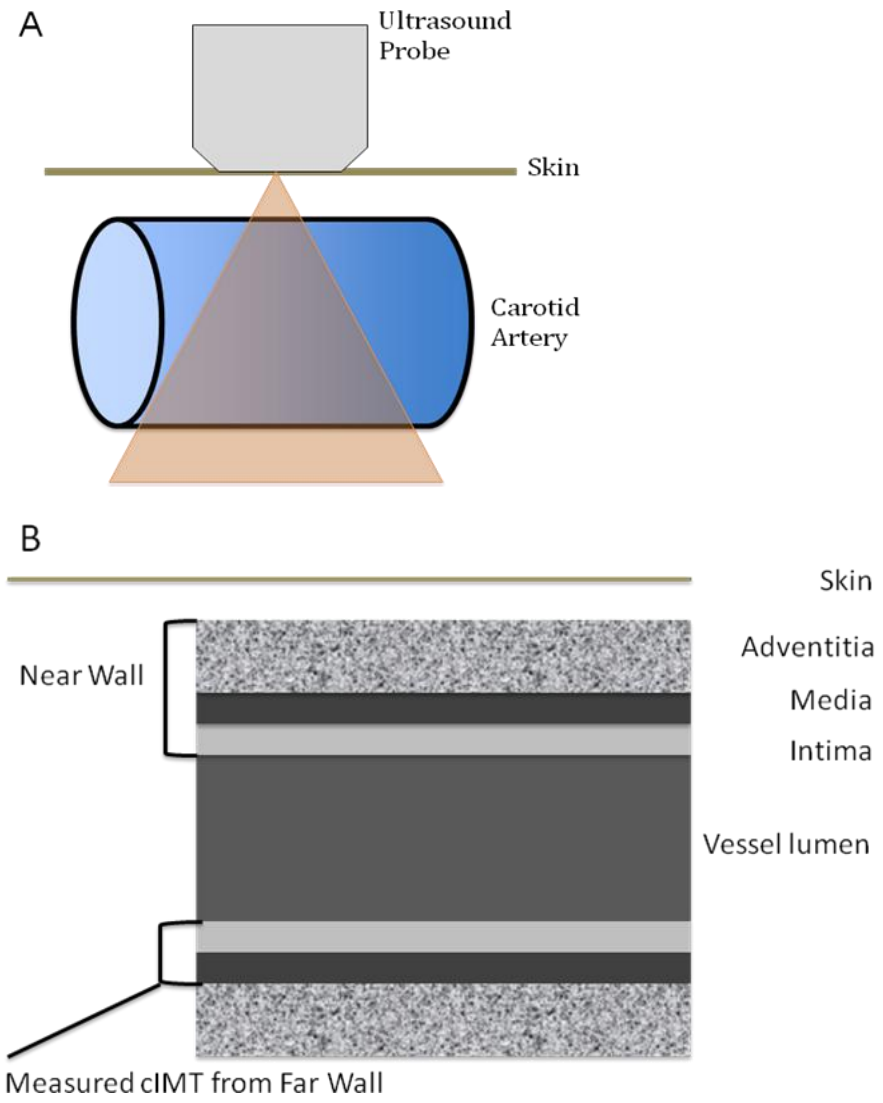

Figure 1.2: representation of ultrasound measurement of cIMT, using the far wall of the carotid artery. A – the probe is held in parallel to the artery for imaging. B – longitudinal view of the vessel obtained showing the measured intima and media, separate to the adventitia.

1.4.2 Large vessel distensibility

The ability of larger vessels to dilate in response to various stimuli has been used as a marker of endothelial function for over 20 years.

1.4.2.1 Brachial artery

Celermayer and colleagues first described the use of high-resolution ultrasound for measurement of brachial artery diameter in response to ischaemic stimulus, as well as sublingual glycerol trinitrate (GTN). Average resting vessel diameters were 3-4mm, with 0-20% changes in vessel diameter to stimulus recorded[120].

Increase in vessel diameter is recorded after ischaemia, and describes “flow debt repayment” (FDR), or the ability of the vasculature to respond to increased tissue oxygen requirement. This is an “endothelial dependent” function, and may be compared to response to GTN, which is “endothelial independent”.

1.4.2.2 Carotid artery

Carotid artery distensibility is also determined by ultrasound measurement of maximal and minimal vessel diameter during systole and diastole. It is not normally performed in response to pharmacologic or other stimulus. A derived measure, the incremental modulus of elasticity, further describes vessel “stiffness”, and is not related to vessel anatomy[121].

1.4.3 Pulse Wave Velocity (PWV)

PWV is the measurement of the speed of the pressure wave originating from the heart during systolic contraction through a specific vessel. The faster the velocity of the pulse wave, the more stiff the vessel being examined is, as the pressure wave is not absorbed by the compliance of the vessel wall. It may be measured across the aorta, brachial and femoral arteries.

In ESKD, only aortic PWV should be measured, as peripheral vessels such as the femoral artery have not been validated for use in this population. This is performed using a hand-held tonometer at the carotid and than femoral arteries, to record the pulse wave. These pressure waves are gated to a 3-lead electrocardiograph (ECG) trace, to determine the time taken for the pressure wave to move from one site to the next. The distance between the two sites is measured, and divided by the time taken to provide a velocity (in metres per second, m/s)[122].

Savage *et al* examined reproducibility of PWV in pre-treatment, dialysis and transplantation groups. 95% of all results were found to be within 2 standard deviations of the mean, and had a markedly superior prognostic capacity compared to peripheral blood pressure [123]. This difference between peripheral blood pressure and PWV most likely relates to the phenomenon of elevated central pressures in ESKD, versus elevated peripheral pressures in other causes of HT[121].

1.4.4 Pulse Wave Analysis (PWA)

Carotid imaging and PWV examine large vessel distensibility and compliance; small/conduit vessel endothelial function must be examined in other ways.

The Augmentation Index (AI), as measured by PWA, is a ratio of the difference between the early and late systolic peaks in the pressure pulse waveform, and the total pulse pressure, expressed as a percentage (%, Figure 1.3).

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Figure 1.3: Augmentation Index is a ratio of the difference between the early and late peaks in the systolic contour, and the total pulse pressure – taken from Kelly *et al*, Circulation 1989[124]. PP = pulse pressure, △P = pressure from shoulder to late peak, AI = △P/PP (as a %).

AI is a measure of the effect of the reflected pressure wave from peripheral vessels. This ordinarily occurs in diastole and does not increase the overall pulse pressure, but with age and other causes of vessel stiffening, may coincide with systole. This causes a significant increase in afterload on the heart[125], and leads to complications including CHF.

PWA, using tonometry readings of the arterial pressure wave from the radial artery compared to blood pressure, has been used to measure a combination of small and large vessel function. PWV is more a measure of aortic compliance, and AIx an indirect measure of stiffness [126].

Using a mathematical transfer function, tonometer readings from the radial artery are integrated with brachial artery blood pressures to calculate the aortic waveform[121]. This generalised transfer function has been shown to be valid for a broad range of blood pressures, although may slightly underestimate AI[127]. Comparison of direct versus indirect measurement of aortic AI showed significant correlation, with $p<0.001$[128].

The advantage of measurement of AI over pulse pressure measured by peripheral blood pressure (BP) with an external cuff, is that the pulse amplitude is greater the further distance from the heart (especially at younger ages)[124]. Therefore, a peripherally recorded BP may not correctly reflect the pulse pressure centrally. Furthermore, AI provides information not only about BP, but also vessel distensibility.

1.4.5 Plethysmography

Plethysmography involves measuring the volume of blood that enters one or more vessels, with dynamic studies measuring how those vessels are able to compensate in response to medications or ischaemia.

Venous occlusion strain gauge plethysmography examines blood flow in forearm vessels. With the hand excluded by a high-pressure cuff on the wrist, 70% of blood

in the forearm will be within the skeletal muscles, and the rest in the skin[129]. A strain gauge is placed around the widest part of the forearm, and blood volumes within the forearm can be calculated in response to stimuli. These results correlate with small vessel capacity to dilate and constrict[130].

Photoplethysmography measures flow of blood in superficial and deeper vessels using laser techniques. This is normally performed at the fingertip, but the technique is also used to measure blood flow in solid organs.

1.4.6 Coronary artery calcium score

Electron beam computerised tomography (EBCT) for identification of coronary artery calcification (CAC) has been used clinically over the past decade for estimation of future CVD risk. It measures intimal calcification, which has been shown to predict future events with greater specificity and sensitivity than Framingham risk stratification[131]. It is not as useful as cardiac nuclear imaging for short-term risk management, as it does not specify degree of vessel obstruction[132].

There are high rates of vascular calcification in ESKD, and CAC scores are generally elevated, especially in HDx[133] – whether this relates to worsened clinical outcomes remains unclear for HDx recipients[134], although elevated scores are associated with increased coronary artery disease burden on formal angiogram[135].

1.4.7 Magnetic Resonance Imaging (MRI)

Various MRI techniques, including contrast, phase contrast and diffusion weighted imaging have been used to identify vessels, vessel walls, and functional characteristics[136, 137]. MRI and previously magnetic resonance angiography (MRA), have been used widely for investigation of vascular abnormalities, but have been limited in ESKD.

MRI is a robust technique for estimation of LV mass in ESKD, and avoids the echo complication of overestimation of mass[138], as well as providing further information on the type of pathology intrinsically affecting the heart muscle[139].

There is some data on vascular function, with small studies of HDx participants confirming poor aortic compliance[140], similar in severity to control patients with severe CVD[141].

The limitations of MRI relate to cost, patient claustrophobia, and the risks associated with the use of gadolinium in late CKD/ESKD (specifically nephrogenic systemic fibrosis, NSF[142]).

Table 1.1: Surrogate Markers of CVD in CKD/ESKD – associations and limitations

Modality	Associations	Limitations
Common carotid artery IMT	• General Population: CVD morbidity and mortality[143], in both middle-age [144] and elderly populations – including all-cause mortality in the latter[145] • HDx: independent risk factor for future all-cause and cardiac mortality for subjects with IMT in the highest tertile[146-148] • PDx: predicts left ventricular hypertrophy and future cardiac death[147] • KTx: associated with present and future CVD risk[149], as well as worsening kidney function[150], independently of classical and novel risk factors • Validated in Dialysis/KTx recipients and the gen. pop. [116]	• Operator variability • Increased accuracy of measurement with multiple measurements and use of edge detection software[117] • Presence of atherosclerotic plaque masking IMT • Requirement for cardiac paced measurement • Elevated IMT is also associated with hypertension, increasing age (0.014 to 0.02mm per year) and male sex[115]. Thickening often defined as above the 75th percentile for age and sex, with 0.6-1.2mm considered normal[114]
Common carotid artery diameter and stiffness/distensibility	• General Population: Increased with atrial fibrillation[151]; Decreased with diabetes[152], hypertension[153], smoking[154], congestive heart failure[155], future CVD risk[156] • HDx: does not acutely affect arterial distensibility[157, 158]; decreased distensibility predicts CV and all-cause mortality[159] • KTx: decreased distensibility associated with future CVD mortality[160]	• Moderately repeatable only[161] • May not be reliable clinically as small changes in diameter significantly affect derived results[162]
Plethysmography		
• Venous Strain Gauge Plethysmography	Moderate CKD (GFR ~ 29ml/min)[163]823] After correcting for classical risk factors, endothelium dependent vasodilatation is reduced in association with creatinine clearance[163] HDx: decreased flow debt repayment associated	Bulky equipment. Requires normal vascular anatomy.

	with other markers of CVD and ED[164]	
• Photo-plethysmography	General Population: Decreased flow associated with rheumatic diseases, HT, DM and known CVD HDx: Decreased ED dependent and independent vasodilatation seen in HT patients, and is assoc with other markers of endothelial damage[165] KTx: Not assessed in this group for CVD risk. May be used for direct investigation of microcirculation of transplanted organ[166]	HDx: may not differentiate between those with and without CVD[167] Reproducibility remains problematic[168, 169], with no standardisation between studies
Flow-Mediated Brachial artery ultrasound	General Population: Associated with classical risk factors for CVD[170] HDx: No acute benefit on ischaemia or GTN response[157]. Decreased flow debt repayment associated with all-cause and CVD mortality[148]. KTx: No correlation of high rates of ED found, with other markers of atherosclerotic load[171]	Highly operator-dependent
Pulse Wave Velocity	General Population: associated with increased CVD mortality in hypertension[172], diabetes [173] and pre-existing vascular disease[174] HDx: increased velocity associated with future cardiac and all-cause mortality[148, 175] KTx: does not return to baseline and correlates with eGFR and CRP[176]	Reproducible, with inbuilt quality assurance indicators. Faster PWV found in those of significantly shorter stature[177]. PWV increases with age regardless of comorbidities.
Pulse Wave Analysis / Augmentation Index	General Population: increased AI predicts future CVD events in high risk patients[178] HDx: increased augmentation associated with history of CVD and LV hypertrophy[179] KTx: AI does not return to baseline and correlates with eGFR and CRP[176]	Good reproducibility in all groups due to inbuilt quality assurance indicators, however ongoing questions over validity of the generalised transfer function[123] – others argue that there are too many factors that influence AI assessment, beyond pulse pressure[180]. Increased AI not consistently shown to be associated with CVD in age groups <45 years of age[181]

CT-Derived Coronary Calcium Score	General Population: greater predictive ability than Framingham score for future CVD events[131] CKD (Pre-Dialysis, Younger Age): Highest scores (>400 Agatston score) associated with future events, and known lesions[182], however can occur in the absence of occlusive vascular lesions[135]. HDx: increased score predicts future CV events[183]. HT and age associated with increased score, but not serum calcium or phosphate[184]. Correlates with cIMT thickness[36] KTx: Increased score with age and total cholesterol. No change after one year post-Tx, and no CVS data[185]	….. In KRT, calcium score is not associated with angiographic evidence of CAD[135, 186], nor of functional coronary blood flow[183]. CT is also unable to differentiate between intimal and medial calcification, with the latter more prevalent in ESKD[187].
Magnetic Resonance Imaging	General Population: able to image for IHD, but unclear of sensitivity in low risk populations[188]; able to image sub-endocardial disease[189]. HDx: able to identify infarcted myocardium with greater sensitivity than nuclear imaging[190]. Able to differentiate different types of pathology in ischaemic myocardium[139].	Expensive, technical/software limitations Risk associated with use of gadolinium[142]. Long term prognostic significance not clear[191].

1.5 Renal Replacement Therapy and Surrogate Markers of CVD Risk

HDx, PDx and KTx have significant effects on factors involved in the progression of CVD in ESKD. Improvements in levels of uraemic toxins and agents of oxidative stress, are balanced by side effects of the therapy itself. These are specific to the therapy, and include issues such as volume overload, haemodynamic changes with HDx, and the partial normalisation of GFR with KTx.

Thus, surrogate markers of CVD cannot be assumed have the same prognostic capacity in KRT as in the general population.

1.5.1 Dialysis effects on arterial function

1.5.1.1 HDx

HDx, through rapid fluid and electrolyte shift, and increases in oxidative stress and inflammation, has an immediate effect on arterial function. AIx may decrease, however PWV is variably affected. The latter measurement is influenced by peripheral small artery compliance, which is known to increase post dialysis [192]. Others have found no acute effect on arterial distensibility if blood pressure and fluid status are considered[157, 158]. This suggests a fixed abnormality in the vessels of HDx recipients.

However, GTN-induced, endothelium-independent dilatation improves post HDx, to levels similar to that of hypertensives with normal renal function. Lack of improvement in GTN-induced, endothelium-independent dilatation is associated with increased left ventricular mass. In contrast, B_2-agonist endothelium-dependent dilatation however remains abnormal pre- and post dialysis [126]. These findings imply the functional abnormality within HDx recipient vessels originates from the endothelium primarily.

1.5.1.2 PDx

PDx is a more haemodynamically gentle process than HDx, given the absence of sudden volume shifts. However, there are vascular complicating factors including high glucose loads, atherogenic lipid accumulation, advanced glycation end-products and chronic fluid overload[193]. Conversely, residual kidney function may be maintained for longer than HDx[194], and is positively correlated with compliance of the aorta[195]. However, the degree of small solute clearance during PDx has not been shown to be associated with vessel stiffness[195].

1.5.2 KTx effects on arterial function

Improvements in endothelial function have been demonstrated in a number of studies post KTx. Covic *et al* showed in a small group of dialysis patients (average 40 years of age) that KTx significantly improved PWV and AI to levels similar of those in a hypertensive age-matched population without CKD or CVD. This change was noted 3 months after KTx, and also included the response to endothelium dependent and independent stimuli [196].

In a double-blind, randomised controlled trial of fluvastatin versus placebo in a KTx population, fluvastatin reduced cholesterol and improved brachial artery flow-mediated dilatation after 6 months therapy. There was however no evidence of improved endothelium-independent vasodilatation (via GTN), indicating no significant change in the elastic properties of the vessel[197]. There was also no follow-up after the six months, so the prognostic significance of this change is unknown.

1.6 The Role of Endothelial Repair in Vascular Health: Endothelial Progenitor Cells

Dysfunction of normal endothelial in CKD leads to increased risk for CVD, including both atherosclerosis and, more prominently in CKD, arteriosclerosis. Direct modulation of the function of the endothelium has been viewed as a possible technique for reducing this CVD risk, however how the endothelium normally regenerates and is maintained is not well understood.

Previously, repair of the endothelium, as well as new vessel formation, was thought to be undertaken by mature ECs in the vessel wall. In 1997, Asahara and colleagues published the first peer-reviewed article of endothelial progenitor cells (EPC), a bone marrow-derived cell involved in both repair of existing vessels, as well as new vessel formation[198]. Despite constituting less than 0.0001-0.01% of peripheral blood (PB) mononuclear cells (MNC) (and 1-5% of the total bone marrow cell (BMC) population)[199] EPCs achieve these roles through both direct cell-to-cell contact, as well as both autocrine and paracrine effects [200].

EPCs can be used as a robust biomarker of CVD, and their inhibition of number and function in disease states helps understanding of vascular pathophysiology further. However, its potential as a biomarker and cellular therapy has not yet been realised, due to factors including:

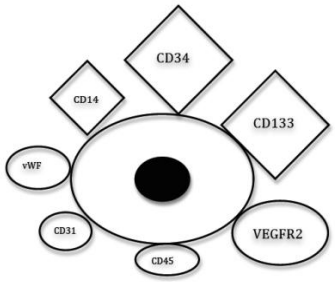
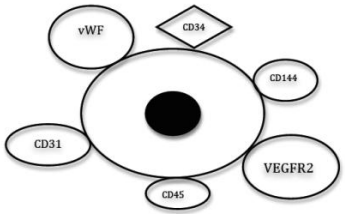
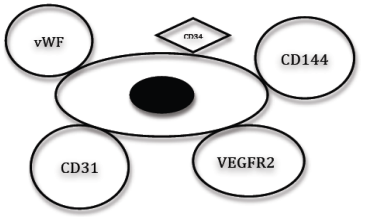
- Lack of standardised identification, isolation and culture techniques, in part related to the maturation process that EPCs undergo from the BM to the peripheral circulation – this has lead to the title EPC being used in the literature for a broad number of different cell types with different properties (see section 1.6.1)

- Variable findings in regards the effect of disease states on circulating concentrations or function of EPCs – in the field of ESKD/KRT, there has been limited research, with minimal understanding of the effects of factors unique to these environments (e.g. uraemia, fluid overload, anaemia)
- There is limited data on the use of EPCs alone as a therapy for ischaemia (the majority of studies to date have utilised unsorted MNCs rather than pure ”EPC” populations)[201].

1.6.1 EPC Characterisation

There is no single cell surface or other marker available for identification of an EPC. The most widely accepted current definition is co-expression of surface markers CD133, CD34 and vascular endothelial growth factor receptor 2 (VEGF-R2)[202]. Whilst these individual markers are not unique to EPCs, their combination characterises a specific progenitor cell at a specific maturation stage. As shown in Figure 1.4, changes in cell surface marker expression occur in parallel to morphological and functional development. These phenotypic markers allow EPCs to migrate from the bone marrow, to peripheral sites.

Figure 1.4: Differentiation of Early vs. Late EPC and Mature EC (*no surface marker listed is specific to any of the cell types known)

	Early EPC	**Late Outgrowth EPC**	**Mature EC**
Culture Characteristics	Rounded Cells Present early (<10 days) Slowly proliferative Adherent	Elongated, "Cobblestone" Present after 14 days Highly proliferative Adherent	Spindle-shaped Loss of function with multiple passage Adherent
Surface Marker Expression*			
Functional Characteristics			
In Vitro			
Ac-LDL Uptake	Capable	Capable	Capable
Tube formation	Capable	Highly Capable	Capable
Migration	Capable	Capable	Capable
In Vivo			
New Vessel Formation	Less Capable	Capable	Unable
Integration in Existing Vessels	Highly Capable	Capable	Unable
Improve Existing Vessel Function	Capable	Capable	Unable

1.6.1.1 CD34

The stem cell marker CD34 was the first used to define EPCs[198]. Its expression defines EPC “stage specificity”, as it decreases in intensity as EPCs differentiate [203, 204]. The function of CD34 is predominantly for cell-to-cell adhesion, as a ligand for E-selectin, with an ability to regulate the tightness of EC adherence to adjoining cells[205]. Notably, CD34 has also been used since the 1980s for identification of bone marrow derived haematopoietic progenitor and stem cells[206].

1.6.1.2 CD133

CD133 is a 5-transmembrane protein[207], found on 20-60% of $CD34^{bright}$ cells in bone marrow and blood, but not mature ECs[208]. Although a function has been ascribed to CD133 as an ‘organizer’ of membrane topology by regulating lipid composition within the plasma membrane (reviewed elsewhere[209]) it is likely to have additional affects yet to be determined.

1.6.1.3 Vascular Endothelial Growth Factor Receptor-2 (VEGF-R2)

VEGF-R2, otherwise known as Kinase insert Domain Receptor (KDR) or Fms-like tyrosine kinase 1 (Flk1), is one of three VEGF receptor family members, including VEGF-R1 (Flt1) and VEGF-R3 (Flt-4)[210]. It was initially identified on cells involved in vasculogenesis, and unlike VEGF-R1, was present on cells with potential to differentiate into mature ECs[211]. VEGF-R2 is a receptor tyrosine kinase for VEGF, which is critical for EC functions including maturation and migration[210].

1.6.1.4 Additional surface markers

Other cell surface markers have been used for EPC identification, such as von Willebrand Factor (vWF)[212], CD31/Platelet Endothelial Cell Adhesion Molecule-1

(PECAM-1)[213] and CD144[214], but these are mature EC markers, whilst CXCR4 is found on numerous cell types (reviewed elsewhere[215]).

1.6.1.5 Functional assessment

The inherent difficulty in EPC identification has lead to the use of functional assays to discriminate EPCs from other cell types (see below). These functional assays also augment the use of EPCs as a biomarker, as impairment of a specific cell function can be associated with specific CVD risk factors.

1.6.2 Activation, Release and Homing of EPCs from Bone Marrow

The functional characterisation of EPCs is based on the process by which these cells are activated, home to sites of need, and exert influence.

1.6.2.1 Bone marrow milieu

EPCs reside within a stem cell niche in the bone marrow characterised by low oxygen tension[216], and high levels of stromal cell derived factor-1/CXC chemokine ligand 12 (SDF-1/CXCL12), a potent chemo attractant for EPCs[217] that binds via the receptor CXCR4[218].

1.6.2.2 Initial mobilisation

1.6.2.2.1 Stimulant factors

EPCs mobilise from bone marrow to peripheral circulation in response to tissue hypoxia and trauma (Figure 1.4A), which cause production and release of EPC activation factors such as hypoxia inducible factor-1alpha (HIF-1α)[219], VEGF, erythropoietin (EPO), oestrogen or CXCL12 to a concentration greater than that in the marrow[220-222]. These agents act via the phosphoinositol-3-kinase/Akt

pathway to activate endogenous nitric oxide synthetase (eNOS) by phosphorylation of serine^{1177} and increase production of (NO) from L-arginine[223-225].

1.6.2.2.2 Discharge from bone marrow environment

The physical release of EPCs from the bone marrow is also dependent on the production of NO and local activity of matrix metalloproteinases (e.g. matrix metalloproteinase 9, MMP-9). MMP-9 causes release of soluble kit ligand from EPCs in the marrow, allowing the cells to move out to the peripheral circulation[226]. Reduction in circulating NO results in reduced MMP-9 levels and reduced mobilisation of EPCs, and reduced *in vivo* capillary formation[227], even in the face of elevated VEGF blood concentration[228]. Following release from marrow, EPCs follow cytokine gradients to activated tissues, then act in one of three ways: paracrine, integration, or new vessel formation (Figure 1.4B).

1.6.2.3 Diurnal variation in EPC

Diurnal variation in peripheral EPC number is seen with lowest levels at the start of the day, little change by 1500hr, then a substantial increase (approximately 25%) by 2200hr[229]. This may be due to parallel diurnal changes in granulocyte macrophage colony stimulating factor (GM-CSF)[230] and granulocyte colony stimulating factor (G-CSF)[231]. Also, reduction in stromal cell production of SDF-1 occurs in response to noradrenaline release by sympathetic nerve fibres embedded in the bone marrow. Noradrenaline release occurs during daylight hours, and, as SDF-1 levels decrease in the marrow niche as increased numbers of haematopoietic stem cells are released into the peripheral circulation[232]. These findings are relevant to clinical investigation, as for comparison, PB EPCs should be collected at the same time of day.

1.6.2.4 Cell surface markers for EPC attachment

Subsequent EPC attachment requires direct cell:cell contact via adhesion molecules. For example, EPC expression of P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and PECAM-1, as well as integrins α4, β1, β2, β3 and β5 facilitate EPC binding to the vascular endothelium[233]. β1 and β2 integrin levels are further increased on EPCs by high-mobility box group 1 (HMGB1), a nuclear protein that is released from damaged cells. HMGB1 acts via Receptor for Advanced Glycation End products (RAGE) on EPCs to significantly increase adhesion to EC associated fibronectin and ICAM-1[234].

1.6.3 Regulation of ECs and vasculature by EPCs

ECs require ongoing autocrine and paracrine survival factors for the maintenance of normal function, in part through the bias of cell survival over senescence or apoptosis. These pro-survival factors, in the form of VEGF, insulin-like growth factor-1 (IGF-1) and SDF-1, can originate from myocardial[235] and skeletal muscle[236], as well as EPCs themselves[200].

1.6.3.1 Nitric oxide

Activated EPCs are a potent source of NO, an essential mediator of normal vessel relaxation, thrombosis and repair[237-240], as well as improving arterial compliance through augmented large artery distensibility[241]. Low levels of NO are associated with endothelial dysfunction[120, 242] and low EPC numbers are associated with reduced NO-dependent small vessel function[243]. In a murine model, infusion of EPCs increases NO production and improved endothelial function[244].

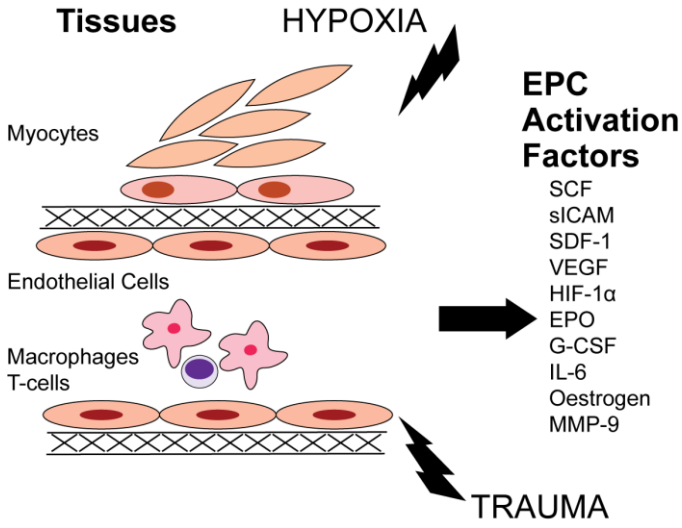
1.6.4 EPCs contribute to new blood vessel formation

Under the influence of VEGF[245], SDF-1[217] and monocyte chemotactic protein-1/CC chemokine ligand 2 (MCP-1/CCL2)[246] EPCs home to sites distal to pre-existing vasculature, and form new vessels, or aid in propagation of new vessels from pre-existing ones[247]. EPCs may also endothelialise vascular grafts both *ex vivo*[248] and *in vivo*[249], as well as vascularise denuded large arteries in a rabbit model[250].

1.6.4.1 EPC tethering and integration

In situ maturation of EPCs is augmented by the presence of platelets and fibrin clots, which act as intermediaries in cell tethering[251]. Proteomic studies implicate thymidine phosphorylase as critical to EPC defense against oxidation-related apoptosis, and also directly influencing ECs in a paracrine fashion to improved migration and tube formation[252]. Vasculature associated with malignancies is fast growing, and often initially distant to pre-existing normal vessels and EPC incorporation is seen in these vessels[253]. It is hypothesised that these newly recruited EPCs produce and release VEGF which causes the internalisation of vascular endothelial-cadherin (VE-cadherin) on resident ECs releasing them from their surroundings for contribution to angiogenesis [254]. Importantly, our current understanding of EPCs is limited by identification and *ex vivo* expansion factors with the two most widely described EPC populations, i.e. early and late outgrowth EPCs differing substantially in phenotype and function (Figure 1.5).

A



B

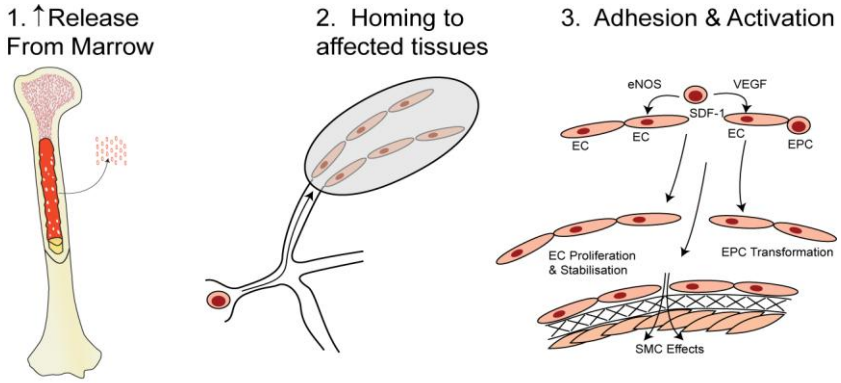


Figure 1.5: Regulation of EPC mobilization, homing and function. (A) Events such as hypoxia and trauma cause production and release of EPC activation factors such that EPCs overcome the hypoxia-related retention of EPCs in their bone marrow niche. (B) On release from marrow, EPCs follow cytokine gradients to activated tissues, then act in one of three ways: paracrine, integration or new vessel formation (SCF – stem cell derived factor; sICAM – soluble intercellular adhesion molecule; SDF-1 – stromal cell derived factor 1; VEGF – vascular endothelial cell growth factor; HIF-1α – hypoxia inducible factor alpha; EPO – erythropoietin; G-CSF – granulocyte colony stimulating factor; IL-6 – interleukin 6; MMP-9 – matrix metalloproteinase 9; eNOS – endogenous nitric oxide synthase; EPC – endothelial progenitor cell; EC – endothelial cell; SMC – smooth muscle cell)

1.7 Issues in EPC Identification and Characterisation

The inconsistency seen in clinical studies of treatment of ischaemic tissue with EPCs is likely due to an inability to isolate a pure population of EPCs, and hence differing functional ambiguities. This reflects a lack of consensus on identifying a "true" EPC.

1.7.1 Alternative EPC nomenclature

A number of different terms have been used to describe cells that have some EPC characteristics or functions in common. These include early colony forming ECs, progenitor derived mature ECs, haematopoietic ($CD45^{low}$ is expressed on early EPCs, then disappears[199]), and myeloid derived progenitors(CD116+ myeloid cells as well as CD14+ monocytes and macrophages[255-257]), all of which have been termed EPCs[258-261].

The "EPC" signature may also be found on cancer cells[262], specifically, the protein AC133-CD133/1 is differentially expressed on some colon cancer cells[263]. Clearly, a novel cell surface marker specific to EPCs would be helpful to unequivocally distinguish these cells from the closely related. In the interim, we and others advocate the use of co-expression of CD133, CD34 and VEGF-R2 as indicative of an early EPC phenotype[264].

1.7.1.1.1 Other cell lines

Beyond nomenclature, other cell lines may act as pseudo-EPCs, or differentiate into mature ECs. Haematopoietic[265] and multi-potential stem cells have this capacity, as well as more mature monocyte and macrophage-derived cells[266]. It is generally agreed that EPCs differentiate from an immature form that expresses CD133+/VEGF-R2[267]. Confusion arises with

myeloid cells expressing CD14+/CD34- and endothelial markers, and which are functionally able to form tube-like structures *in vitro*[267].

Reyes *et al* describe a multipotent adult progenitor cell (MAPC), which is CD34-, CD133+, VEGF-R2+. These cells, under the influence of VEGF, differentiate into CD34+ EPCs, prior to formation of functional mature ECs [268].

Standardised use of cultured “EPC” may therefore be compromised by “pure” EPCs being of different maturation/functional stage, or having the presence of other cell lines with similar actions[269], including tissue resident progenitors. Recent research also highlights the possibility of cultured MNCs picking up debris or microparticles from damaged platelets/ECs which express vWF and PECAM-1. Monocytes in particular can take up and express these markers which then increase their *in vitro* angiogenic potential[270]. None of these issues have been addressed in clinical study, where unsorted MNCs are primarily used. Also to date, late outgrowth EPCs are yet to be used in human clinical trials.

1.7.1.2 Functional capacity

An alternative strategy to characterise EPCs is to assess functional capacity, with a number of *in vitro* assays being developed. Table 1.3 summarises of the most commonly used assays: migration, tube formation, cytokines production and NO production. These characteristics may be used in conjunction with cell surface markers to more specifically define and describe EPCs, especially given the maturation process EPCs undertake in the peripheral circulation.

1.7.2 Culture techniques

The difficulties with identification have led to a lack of consensus on EPC isolation or culture techniques.

1.7.2.1 MNC isolation and seeding

Currently, MNCs are isolated from whole blood by gradient density separation, prior to seeding on fibronectin, vitronectin or rat-tail collagen[198] for 3-72 hours. As EPCs are unable to bind to fibronectin[271], adherent cells are then discarded and the non-adherent fraction cultured.

1.7.2.2 EPC selection

Pre-culture EPC selection enables improved cell purity, and may be achieved using FACS, or magnetic bead separation. Notably, both processes rely on antibody binding - usually one or more of CD34, CD133 and VEGFR2, which are not unique to EPCs.

1.7.2.3 Culture media

Over time, culture media have also evolved from basic Media 199, to proprietary media such as Endothelial Growth Media with added cytokines and supplements (ascorbic acid, steroids, VEGF, β-FGF, IGF-1, Endothelial Cell Growth Factor (ECGF)– most commonly from Lonza, Basel Switzerland), and Stemline Media[272] (Sigma). The aim of the culture environment is to promote retention of an early EPC phenotype, at the expense of other cell lines. No single culture media, proprietary or otherwise, is advertised for EPCs specifically, and the optimal environment for culture is not known.

The appropriate cytokine environment is also unknown. As shown above, a number of factors are involved in EPC survival and maturation, including VEGF, SDF-1, IGF etc. Standardised plating and culture techniques remain an ongoing objective for EPCs[261].

1.8 Endothelial Progenitor Cells as a CVD Biomarker

Damage to endothelial cells from such factors as oxidative and shear stress predates atherosclerotic change[273], and endothelial dysfunction (ED) is an early and integral factor in the progression of atherosclerotic plaques and arteriosclerotic stiffening [274]. Both processes lead to disruption of normal blood flow and subsequent tissue ischaemia [172, 275]. For example, the presence of ED in coronary [276], conduit [277] and resistance [278] arteries is an independent risk factor for both cardiovascular and all-cause mortality[276, 279, 280].

1.8.1 Factors affecting EPC concentration and function

Cardiovascular risk factors which affect endothelial function such as cholesterol[281], diabetes[282], smoking[283] and age[170], in parallel affect EPC function and number (summarised in Table 1.1).

As listed in Table 1.1, vascular trauma[284], acute myocardial infarction[285-287], acute coronary syndrome[288] and surgery or burns[284] cause acute hypoxia and vascular injury and increase EPC concentration in peripheral blood. Guven *et al* found increased expansion capacity of EPCs collected from patients experiencing acute coronary ischaemic events, with greatest growth capacity in EPCs from patients with the worst vascular disease[289]. In comparison to acute events, as shown in Table 1.1, many chronic disease states correlate with deficiencies in concentration or function of EPCs. Lower circulating EPC concentrations are an independent predictor of coronary artery ED[290, 291].

Table 1.2: Human disease states associated with altered circulating EPC number and function (*including migration, tube formation, cytokines production and NO production, as described in the *Comments* column).

Disease	EPC Number	EPC Function*	Comments
Coronary Artery Disease	↓	↓	1. *In vivo*, decreased number of EPCs has been associated with reduced coronary endothelial reactivity[290]. 2. Impaired *in vitro* migration capacity of EPCs is associated with increased cIMT[292], a marker of atherosclerosis and future cardiac risk. 3. EPCs from IHD subjects have reduced or impaired CXCR-4 receptor function[293].
Ischaemic Cardiomyopathy	↑ Early in disease, then ↓ later	↓	1. Decreased migratory capacity to SDF-1[294]. 2. Concentrations increased early, then decrease in the latter stages of CHF [295].
Diabetes	↓	↓	1. For both Type I[296] and Type II Diabetes[297]. 2. In rodent models, association with impaired sympathetic release of noradrenaline into bone marrow resulting it increased bone marrow SDF-1 levels, and reduction in EPC release into the peripheral circulation[298], as well as microangiopathy causing EPC apoptosis[299]. Elevated haemoglobin A1C (HbA1C) also associated with decreased EPC and increased macrophage release from the marrow[300]. 3. In humans, increased numbers of adipocytes at the expense of other cell types are found in the marrow, which may lead to decreased hemangioblasts [301]. Brunner noted decreased circulating EPC numbers in non-proliferative diabetic retinopathy, but markedly elevated levels of mature EPCs in patients with proliferative lesions[302]. Other recent studies in humans suggest the roles of oxidative stress, [303] and advanced glycation end-products[304] causing decreased EPC function due to cell injury.
			…..

Disease	EPC Number	EPC Function*	Comments
Hypercholesterolaemia	↓	↓	1. Reduced numbers and impaired general functional capacity with increasing total cholesterol and LDL [305]. EPC senescence is secondary to oxidised LDL (oxLDL)[306], with in vitro studies showing dephosphorylation of Akt by oxLDL in the presence of VEGF, leading to decreased EPC differentiation [307]. 2. Atorvastatin reverses the effect in both rat [308] and human models [247, 307], including humans with stable IHD [309]. 3. For isolated hypercholesterolaemia dietary change +/- exercise [310] significantly increase EPC numbers.
Hypertension	↓	↓	1. Decreased numbers seen in hypertensive patients with or without diabetes or IHD[311] 2. *In vitro* migratory capacity of human EPCs specifically reduced by HT [312]. HT is more associated with arteriosclerosis than atherosclerosis, and it may be postulated that this specific effect on EPCs explains some of this difference 3. Increased senescence of EPCs in humans with pre-HT and HT has been shown[313, 314], but aetiology is unclear. Reduced release of EPCs from BM is known to be associated with elevated levels of complement C3a, and elevated C3a with reduced circulating EPCs has been found in humans with resistant HT [315]. Futhermore, in patients with resistant HT, low circulating EPC number independently correlates with endothelial dependent vasodilatation[316].
Smoking	↓	↓	1. Decreased number with ongoing use [312], but cessation associated with an increase in numbers [317]. Functions including proliferation, migration, adhesion and tube formation reduced in healthy smokers [318]
Aging	↓	↓	1. Decreased number with increasing age[319], and associated with endothelial dysfunction[320]. 2. In mice, older EPCs have been shown to be less effective than younger EPCs at preventing atherosclerotic lesions in a hypercholesterolaemic state[321]. 3. Chang suggests a decreased response to hypoxia by reduced stabilisation of HIF-1α in peripheral tissues of the elderly causes reduced recruitment rather than a primary EPC problem[322]. …

Disease	EPC Number	EPC Function*	Comments
Rheumatoid Arthritis	↓	↓	1. Reduced Number[323], as well as function, correlating with endothelial dysfunction[324]
Inflammation	↓	↓	1. CRP - Direct reduction in function, through blockade of eNOS[325]. Reductions in CRP also associated with improvement in EPC number[288]. 2. TNFα - Reduced number, possibly related to myelosuppression[295]
Chronic Kidney Disease	↓	↓	1. Decreased number and function in both chronic and end stage kidney disease [326, 327]. 2. HDx is associated with increased EPC number (?from elevated VEGF levels) but reduced migratory function[328] 3. KTx improves migratory capacity and adhesion in vitro of EPCs, but overall cell numbers decrease[329]. Improved EPC number are found with better functioning grafts[330].
Pulmonary Hypertension	↓	↓	1. Reduced function, in particular disabling ability of transplanted cells in a mouse model to respond to ischaemic stimulus[331]. 2. A human pilot study of EPC therapy for pulmonary HT showed significant improvement in 6 minute walk test 12 weeks after a single bolus of EPCs. There was a concurrent improvement in pulmonary vasculature and right heart pressures[332]

1.8.2 The effect of ongoing vascular injury

Further vascular insult, such as thrombosis with vascular occlusion, that occur in acute coronary artery syndromes induces local elevations of VEGF, which then recruit EPCs to the site of injury[285]. The pro-survival effect of early EPCs on resident EC viability, and replacement of dying ECs[235, 333] contributes to enhanced neovascularisation and improved left ventricular function. However, with ongoing hypoxia or injury comes a fall in VEGF/SDF-1 production from target tissue, and an inability of the bone marrow to maintain adequate EPC release[295].

A reduction in nitric oxide production, due to uncoupling of NOS, may also follow. This results in further reduction in release of EPCs from the marrow niche (reviewed by Jarajapu *et al.*[334]). Increased production of reactive oxygen species, which parallels this, further reduces EPC function and survival through kinase pathways[335].

1.8.3 EPCs in clinical studies

Changes in EPC number and function during CVD allow their use as a biomarker [336]. In clinical studies:

(i) Low circulating numbers and reduced functional capacity of EPCs are currently being used to predict future cardiovascular events, independent of other cardiovascular risk factors. This has been validated in high-risk[337, 338] as well as low risk[339], population groups. A reduction in EPC migratory capacity correlates with increased atherosclerotic load in humans (measured by carotid intima media thickness) [292]. In a recent study by Fadini of pooled data from 4 studies of 1057

high risk patients, the lowest tertile of number of circulating progenitor cells (CPCs, defined as CD34+ve ± KDR+ve) was independently predictive of future major cardiac events, to an average of 1.7 years[340]. The benefit of CPC count to predict events was additional to that of classic risk factors, and was enhanced by a concurrent elevation in human serum CRP.

(ii) In humans, restoration of EPC number, or augmentation of function is possible through pharmacologic and other means, and is associated with improved risk profile[341-343]. Furthermore, in a rabbit model of atherosclerosis, infusion of EPCs was associated with reversal of atherosclerotic plaques[250].

1.8.4 Benefit of use of EPC as a CVD biomarker

EPCs provide a clinical advantage over the use of other biomarkers. Measurement of circulating number or function of EPCs correlate directly with endothelial function, whereas other biomarkers only correlate with end tissue damage or stress (myocardial creatine kinase (CK-MB))[344], troponin[345], NT pro-BNP[346, 347] [348, 349]), or the agents which cause the insult (oxLDL)[350] and CRP[351]).

1.8.4.1 EPC function versus concentration as a biomarker

Current use of EPC concentration alone, however, may not fully explain vascular risk in certain settings, including among patients taking agents that alter EPC activity. Rupp investigated the effect of atorvastatin prescription to patients with and without CAD, in regards *ex vivo* EPC ability to differentiate

into “cardiomyogenic” cells, in the presence of rat myocytes. The authors found that EPCs from treated individuals showed a greater differentiative capacity, which did not relate to actual PB EPC concentrations from the patient donor[352]. Numaguchi *et al* demonstrated in a post-acute myocardial infarction (AMI) cohort treated with stenting, that ability of patient EPCs to more efficiently differentiate to a mature EC phenotype and attach (*in vitro*) was associated with better left ventricular (LV) function at 6 months[353]. As shown in Table 1.1, a number of different disease states alter specific EPC functions, such as hypertension and reduced EPC migratory capacity[312].

Given these factors, whist EPCs may more accurately reflect endothelial health compared to other biomarkers, care must be taken in their use in a clinical setting. Disease activity and co-morbid conditions should be taken into consideration, along with identification issues. Furthermore, EPC function assessment may also be required, and may in fact provide a pathway to tailor CVD therapy in individuals.

1.9 Endothelial Progenitor Cells in Trials

With over 150 clinical trials targeting EPCs (www.clinicaltrials.gov, April 2011, search term: endothelial progenitor), there is much activity in exploration of EPC use as a therapeutic tool. One or more of four approaches have been used in studies to date (Figure 1.6).

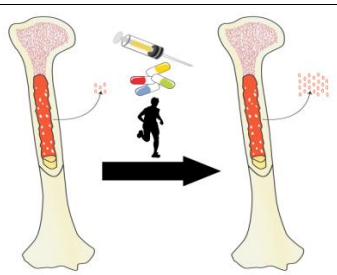
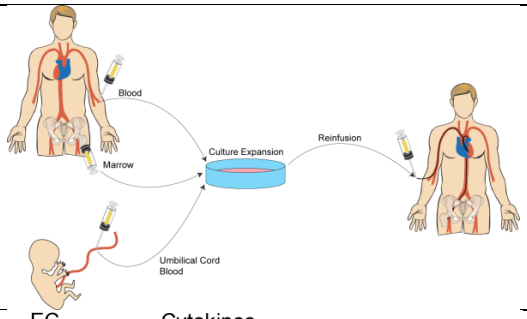
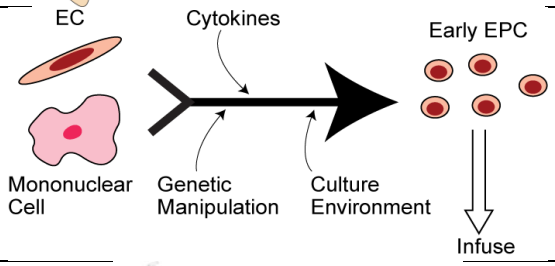
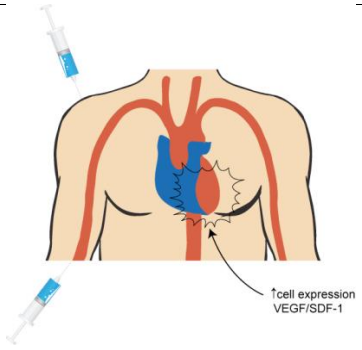
Increase Bone Marrow EPC Mobilisation • Lifestyle – Diet / Weight Loss / Exercise / Smoking Cessation • Immediate Pharmacological – EPO / G-CSF • Long-Term Pharmacological – Statins / PPARγ agonists / ACEi / ATRA	
Ex vivo EPC Expansion and Reinfusion • Autograft – Bone marrow or peripheral blood source +/- pharmacological stimulation • Allograft – ability to build bank of cells for rapid infusion from peripheral, umbilical cord blood or bone marrow • Zenograft	
Reverse Engineer EPC from Differentiated Cell Sources • Cell options including mature ECs, mononuclear cells • Combination of agents likely to be required to drive dedifferentiation	
Increase EPC Homing/Action at Target Site • Direct Injection of EPCs into target tissue • Direct injection of chemoattractants into target tissue • Increase target tissue production of chemoattractants (VEGF, SDF-1) through upregulation of RNA production • Addition of supporting cell types, including smooth muscle cells and pericytes, to EPC infusions	

Figure 1.6: Strategies for increasing EPC numbers and function at target sites. This specifically includes increasing number of EPCs delivered to the target site, and improving function once there (ACEi – angiotensin converting enzyme inhibitor, ATRA – angiotensin II receptor antagonist, PPARγ - peroxisome prolierator activated receptor gamma, RNA – ribonucleic acid).

1.9.1 Increase number or function of endogenous EPCs by pharmacologic or lifestyle intervention

This has been pharmacologically attempted in both the acute and chronic setting. Intervention studies have shown associated improvement in EPC number or function with agents including:

- G-CSF – in patients with known coronary artery disease, circulating EPC numbers increased, as well as the ability of those cells to express CXCR4 and mature into outgrowth EPCs *in vitro* in response to a 5 day course of 10mcg/kg G-CSF[354]. The randomised, double blind, placebo-controlled G-CSF-STEMI trial prescribed G-CSF or placebo to late presentation AMI patients. At three months, the treatment arm showed improved neovascularisation of the infarct area, but no improvement in LV function[355].
- ACEi[337] and ATRA[356] – both may increase EPC number. There are several mechanisms for action, including reduction in oxidative stress by blockade of the effect of angiotensin II on its receptor angiotensin I (AT I), and increasing VEGF release (reviewed elsewhere[357])
- Oestrogen – In animal models, short term oestrogen replacement improved EPC survival via up-regulation of eNOS[222] and MMP-9 activity in bone marrow, increasing EPC release[358].
- Statins – Atorvastatin upregulates NO production by activating the PI3/AKT/NOS pathway [238, 309, 337], leading to increased EPC activation in humans[238] and peripheral differentiation (the latter

demonstrated in a mouse model)[308]. This has been shown in humans for both atorvastatin [309] and rosuvastatin[341]

- PPARγ agonists – Studies have shown their ability to reduce EPC senescence, improve adhesion and increase EPC number in humans [359-361].
- EPO analogues – Lipsic *et al* injected 300mcg of darbepoetin alfa pre-angioplasty following AMI. They showed a 2.8x increase in circulating EPC number by 72hr, but no difference in cardiac outcome, and no adverse side effects were observed[362].

1.9.1.1 Exercise

Increasing exercise duration positively influences EPC number in both low risk [363] and stable coronary artery disease[364] patient groups. Concurrent elevations in VEGF and IL-6 are seen with strenuous activity precedes an increase in peripheral EPC numbers [365]. Conversely, “detraining” is associated with decreased PB EPC concentration, with the latter proportional to worsening in brachial artery flow mediated dilatation[366]. As noted, many of these studies were animal-based, or utilise surrogate markers of CVD risk, rather reporting long-term outcomes.

1.9.2 Infusion of autologous in vitro expanded EPCs for acute therapy of tissue ischemia

Post infusion of EPCs in mice, target tissues show supraphysiologic levels of VEGF[367], SDF-1, IGF-1, basic fibroblast growth factor (bFGF) and angiopoietin I (Ang-1). However, recruitment of endogenous BM EPCs

potentiates the effect of the initial exogenous EPC infusion, further improving clinical outcomes[235].

1.9.2.1 EPC sources

Cell infusion therapy relies on appropriate EPC isolation, phenotypic stability, whilst maintaining phenotype, and subsequent infusion ensuring delivery of cells to target tissues for therapeutic effect. Most recent EPC studies include those collected from peripheral blood, but also from liver[368], spleen[369], umbilical cord blood[370], adipose tissue [371]and bone marrow[247].

1.9.2.2 EPC mobilisation for harvesting

An alternative strategy for human EPC collection includes G-CSF administration to mobilise CD34+ve cells into the peripheral circulation, where they are isolated [372]. However, Honold *et al* demonstrated cleaving and inactivation of CXCR4 on human EPCs collected from peripheral blood following G-CSF mobilisation. This effect caused a decrease in EPC migration *in vitro*, as well as reduced new vessel formation in a mouse model of hind limb ischaemia [373]. How this impacts human clinical trials requires further investigation.

1.9.2.3 Cardiac infusion

In cardiac studies, non-targeted peripheral intravenous infusion of human EPCs into rats has shown poor efficiency of delivery of cells to the heart (approximately 1% of infused EPC). This proportion increases to 2% if active ischaemia is present, and greater than 4% with direct LV injection[374]. Whilst directly injecting EPCs into infarcted tissue may improve clinical

outcomes, including up to 30% reduction in LV end diastolic pressure in a rodent model[375], this is unlikely to be possible in all human clinical settings.

1.9.2.4 Augmented tissue targeting

An alternative to intravenous injection is to inject pro-angiogenic cytokines into target tissue to augment EPC migration. The combination of VEGF and SDF-1 appears most effective in human *in vitro* studies [376]. A novel approach based on this is to pharmacologically improve the half-life of pro-angiogenic cytokines *in vivo*. Cell surface peptidase CD26/dipeptidylpeptidase IV (DPP IV) is found on a sub-population of CD34+ve cells as well as multiple cell types in the periphery. Its action is to cleave and inactivate SDF-1 (at its position two proline), which in turn inhibits CD34+ve migration [377]. Increased activity of DPP IV on CD34+ve cells can be found in some, but not all, diabetic patients. It is associated with decreased cell migration and tube formation *in vitro* [378]. Sitagliptin is a DPP IV inhibitor, and novel treatment for diabetes by increasing incretin hormone levels (reviewed elsewhere [379]). It is postulated that agents such as sitagliptin could be used in conjunction with other agents to augment EPC homing by increasing SDF-1 half-life.

1.9.2.5 Clinical intervention studies

As noted earlier, there are now over 150 clinical trials involving interventions with EPCs or similar cells taking place, investigating diseases such as myocardial infarction, diabetic neuropathy and pulmonary hypertension (www.clinicaltrials.gov, April 2011). Published studies are listed in Table 1.3.

Table 1.3: Comparison of completed EPC studies in humans. A number of different cell types and cell collection techniques were used. There is limited long-term data available, however there appeared to be no significant risk associated with any of the studies at up to 24 (Flores-Ramirez et al.) and 49 (Pasquet et al.) months (IM – intramuscular, PVD – peripheral vascular disease, PET – positron emission tomography, SPECT – single photon emission computerised tomography).

Author/Trial	**Clin Condition**	**Intervention**	**Cell Definitions**	**Outcome**
Kalka[380]	Critical leg ischaemia	IM injection of naked plasmid DNA encoding VEGF	KDR +ve; VE-Cadherin +ve; CD34 +ve; E-Selectin	30x increase in numbers of circulating "EPCs" following increased tissue expression of VEGF. Clinical outcome not reported
Dobert[381]	AMI	Intra-coronary injection of autologous BM or peripherally derived EPCs 4+/-2 days post-AMI		Improved LVEF from 53-58%, with no difference in residual tissue perfusion by PET and SPECT for either cell type.
Lipsic[362]	AMI	Single bolus 300mcg darbepoietin alfa	CD34 +ve; CD45 -ve	8x increase in EPCs at day 3, but no significant increase in LVEF at 4 months.
Wang[332]	Pulmonary Hypertension	Autologous EPC infusion		Significant improvement in 6min walk test, with no side effects
Pasquet[382]	AMI	Intra-coronary injection of G-CSF mobilised PB MNCs	CD34 +ve	Significant improvement in LVEF at 49 months, however with evidence of endothelial and myocardial differentiation of CD34 +ve cells
Kawamoto[383]	Critical limb ischaemia from PVD or Bergers disease	Autologous PB CD34 +ve MNCs collected following G-CSF Mobilisation	CD34 +ve	Significant improvement in symptoms, TBPI and oxygen pressure, without major S/Es related to the infusion at 12 weeks. Moderate S/E reported from G-CSF.

Author/Trial	Clin Condition	Intervention	Cell Definitions	Outcome
Yang[384]	Severe coronary artery disease	Intra-coronary stent +/- adenovector virus coding the human hepatocyte growth factor gene	CD34 +ve; CD117 +ve	Increased CD34 +ve cells in PB, as well as increased levels of MCP-1, IL10
Flores-Ramirez[385]	Patients on Heart Transplant List due to CHF with LVEF<35%	Intra-coronary infusion of autologous EPCs collected after G-CSF mobilisation	CD133 +ve	Significant improvement in LVEF at 24 months, with no side effects related to infusion

A meta-analysis of cell infusion trials for cardiac disease by Lipinski *et al* included both unsorted and sorted bone marrow and peripheral blood MNCs. Cells were infused within 14 days of myocardial injury and follow-up was a minimum of 3 months. Decreased risk of heart failure and death up to 2 years post cell infusion was found, despite only a 3% improvement in LVEF. The authors suggested this modest change in LVEF was similar to other interventions such as angioplasty, and those other factors, such as improved survival of hibernating myocardium may have been involved [386].

1.9.2.5.1.1 Timing of cell infusion

The optimal timing of EPC infusion post tissue infarction remains unclear – cell delivery at 2 days shows no benefit[387], whilst 3-7 days is efficacious[388]. Awad *et al* postulate that CD14+ leukocytes are the first cell type which influence endothelial repair at ischaemic sites[246]: it is possible that the CD14+ cells, with decreased angiogenic capability themselves, prime the environment for EPC activity. The currently recruiting JUVENTUS trial will attempt to circumvent timing problems by repeated autologous bone marrow derived EPC intra-arterial infusion for critical peripheral vascular disease [389].

1.9.2.5.2 Peripheral vascular disease

Fadini and colleagues recently published a meta-analysis of cell therapy trials for severe peripheral arterial disease. They demonstrated two important points: (i) That BM MNCs were superior to peripheral circulation MNCs, and (ii) Direct intramuscular injection of cells gave greater benefit than intra-arterial routes. Both routes of administration groups showed improvement in

subjective and objective outcomes compared to traditional conservative management, with safety of therapy also confirmed [390]. These results may be related to greater EPC percentages found in the bone marrow compared to the periphery, and also the advantage of direct cell infiltration rather than reliance on cell homing that may be limited by reduced chronically ischaemic tissues' production of cytokines.

1.9.3 Gene transfer to endogenous EPCs to enhance survival and longevity of phenotype, or to augment target tissue production of activating cytokines.

Targets for action include increasing VEGF expression from ischaemic tissue to promote endogenous EPC release[380], to increasing the activity of sphingosine kinase (SK), a lipid enzyme recently identified as an EPC control mechanism with increased enzymatic activity retaining bone marrow-derived EPCs in a progenitor phenotype[391]. The current ENACT-AMI trial will attempt to upregulate eNOS activity in autologous EPCs by transfection with a human eNOS-pVAX plasmid before reinfusion, as a means of augmenting EPC function[392]. Others have transduced human EPCs for increased VEGF expression for improved revascularisation of skin flaps in nude mice[393].

Id1 (inhibitor of DNA binding-1) is a transcription factor which supresses the p21 gene, under the influence of hypoxia and VEGF[394]. The effect is increased release of EPC from BM and possibly increased functional capacity. Stimulation or over-expession of Id1 may be a future target for gene therapy, however elevated Id1 has also been associated with atherosclerotic plaque destabilisation (reviewed by Wang *et al.*[395]).

An alternative strategy is to manipulate pro-EPC factors: Feng *et al* increased high density lipoprotein (HDL) production in mice by transfer of the human *apolipoprotein A-1* gene, resulting in increased EPC release from bone marrow, incorporation into ischaemic tissue and improved revascularisation[396]. Balancing these paracrine factors is essential for optimal EPC function and is depicted graphically in Figure 1.4.

1.9.4 Addition of supporting cell lines to augment EPC function

Whilst the endothelial lining is critical to vessel function, the support structure within the vessel wall remains as crucial. As shown in Figure 1.1, dysfunction of vessels relates to problems of both vessel zones.

1.9.4.1 Smooth muscle cells (SMC)

SMC are integral in maintaining vessel tone in response to chemokines released by the endothelium, as well as maintaining the physical structure and shape of the vessel. Conversely, *in vitro* study of human EPCs demonstrated, that under exposure from shear stress, smooth muscle cells promoted differentiation of EPCs to mature ECs via up-regulation of Akt [397]. Murine studies have suggested the role of Ang-1 originating from SMCs activating Tie-2 positive EPCs to improved survival and tube formation [398]. An *in vitro* human study also demonstrated augmented tube formation with the combination of late outgrowth EPCs and SMCs in a scaffold [399].

1.9.4.2 Other cell types

Other pre-clinical studies included human mesenchymal progenitors [400] and adipose stromal cells [401], which were injected into mice along with EPCs. Both additional cell types augmented vessel formation.

Pericytes are another ubiquitous vascular cell that may further augment EPC function. Their complex relationship with the endothelium is reviewed elsewhere, but involves physical support, as well as releasing VEGF [402].

To date, there are no completed human clinical trials of EPCs with additional cell types[201]. These findings do however explain in part the results of whole bone marrow cell infusions. The future study of EPCs as a cellular therapy will certainly require the investigation of the co-infusion of EPCs with pericyte progenitors or smooth muscle cells[398].

1.10 Ongoing issues in EPC clinical trials

Despite their promise, these therapeutic options have not changed clinical practice yet. Substantial unresolved issues include EPC classification, as well as inadequate optimisation of cytokine dosing and timing. Furthermore, unlike unsorted BM-MNC trials, there is limited long-term data on symptoms, survival or side effects (BM trials show variable therapeutic responses to two[403], three[404] and four[405] years, but with no side effects).

Whilst concern has been raised for *de novo* malignancy post EPC infusion, based on the potential role of EPCs in vessel growth in neoplasms (reviewed elsewhere[406, 407]), there have been no reports in EPC studies thus far. One autopsy report on a patient directly injected with unsorted autologous BM-MNCs 11 months prior showed enhanced vasculogenesis and transformation of pericytes towards a myocyte phenotype, with no evidence of abnormal cell activity [408].

Use of G-CSF for peripheral release of BM-MNCs is associated with a number of well-known side effects (eg leukocytosis, pain), but with no reported cases of increased malignancy risk in otherwise normal individuals (Therapeutic Goods Administration Neupogen information).

Pro-arrythmic effects of infused cells in regenerating myocardium have also been raised, but no evidence of this has been found following use of EPCs [409] or unsorted BM MNCs [410].

1.11 EPCs and CKD/KRT

There have been a number of studies examining associations between various KRT groups and EPC concentration and function. These studies have shown variable and, at times, conflicting results. The variability in results is likely to reflect a number of factors:

- Different identification techniques for EPC maturation.
- Counting number of EPC rather than function.
- Different inclusion criteria

In kidney failure, there is conflicting evidence about the association between kidney function and decreased EPC number, and whether improved small solute clearance on HDx may partially reverse this phenomenon[326, 411]. Others have found similar EPC concentrations in controls compared to ESKD on HDx, but decreased functional capacity in the latter[328]. In a PDx study however, there was no association between EPC number and endothelial function, but EPC numbers were decreased with increased CVD risk[412].

De Groot *et al* have shown the negative effects of uraemic serum on EPCs from both normal and ESKD individuals, and that uraemic serum can reduce function, as measured by migration and tube-forming capacity[413]. The effects of uraemic toxins PC and IS on association with PB EPC concentration are currently not known, nor *in vitro* effects on function.

Other studies have also shown that transplantation will improve EPC number and function to that of non-CKD individuals [329, 330], and that whilst immunosuppressives such as prednisolone and cyclosporine have negative in vitro effects, clinically they do not have a significant effect[330].

1.12 Summary

- There is an increased rate of CVD in ESKD, and it is the major cause of mortality in among KRT recipients
- The pathogenesis of CVD in CKD/ESKD is different to that of the non-CKD population, involves multiple factors, and is not well understood
- Both atherosclerosis and arteriosclerosis are involved in the CVD seen in KRT recipients – in particular arteriosclerosis is more prominent in ESKD than the general population
- Normal function of the endothelium is critical to reducing CVD risk, however EC function and repair are limited in CKD and ESKD, in part due to novel risk factors such as uraemic toxins and accelerated vascular calcification
- EPCs regulate normal endothelial function, vascular repair and new vessel formation, but their number and function are limited in CKD/ESKD. The causes for this have not been fully elucidated
- The interaction between vascular function, EPCs, uraemic toxins and other novel CVD risk factors is not well understood. Specifically, the number and function of EPCs in people receiving HD and PD is not clearly established

1.13 Hypotheses

1. Arteriosclerosis predominates over atherosclerotic change in ESKD subjects, and will manifest as significantly altered rates of acute cardiac events and congestive heart failure requiring hospital intervention compared to the general population
2. CD34/CD133/VEGFR2 triple positive EPCs can be isolated from a human source and expanded *ex vivo* whilst retaining an unchanged phenotype.
3. Uraemic toxins PC and IS decrease basic EC function *in vitro*.
4. Cultured human EPCs protect EC function against the deleterious effects of uraemic toxins PC and IS.
5. *In vivo*, PB concentrations of IS, PC and EPCs relate to surrogate markers of endothelial function and arterial stiffening, and other markers of CVD risk in subjects receiving KRTs

1.14 Aims and Thesis Structure

In this thesis, the issues surrounding CVD and its key component, endothelial damage and repair, amongst KRT recipients, are examined from an epidemiological, clinical and laboratory perspective.

Chapter Two describes the methods utilised in the *in vitro* and *in vivo* studies.

Chapter Three characterises the morbidity burden of KRT recipients related to the type of CVD found, and factors that influence CVS outcomes are described.

Chapter Four describes the methodological problems associated with isolation, enumeration and culture of EPCs which have led to a number of contradictory results, and proposes a technique to standardise this process

Chapter Five examines EPC function from a laboratory perspective, in particular examining the effect of key uraemic toxins PC and IS on the function of cultured endothelial cells as well as endothelial progenitor cells, as a model of progressive endothelial dysfunction in ESKD.

Chapter Six continues the *in vitro* study of EC and EPC function, but in response to KRT and control sera, rather than isolated toxins.

Chapter Seven extends the laboratory studies of EPCs into the clinical setting. It describes a cross-sectional study of KRT and control subjects, comparing surrogate markers of endothelial function, with known biomarkers of CVD, as well as measurement of serum concentration of PC, IS and circulating endothelial progenitor cells.

Chapter Eight is the final discussion, and describes how each of the variables investigated may interact, and how this may influence future research and treatment options for ESKD patients with CVD.

Chapter 2: Materials and methods

2.1 Ethics

2.1.1 Epidemiology – Chapter 3

Clearance for the epidemiological study utilising de-identified data from the Australian Institute of Health and Welfare (AIHW), Australian Bureau of Statistics (ABS) and Australian and New Zealand Dialysis And Transplant Registry (ANZDATA), was obtained separately from each institution listed below.

2.1.2 Clinical – Chapters 4-7

Clinical Studies were performed at metropolitan (Queen Elizabeth Hospital (QEH), Royal Adelaide Hospital (RAH), Flinders Medical Centre (FMC), Womens and Childrens Hospital (WCH)) and regional (Maitland Hospital, Berri Hospital) hospitals in South Australia. Ethics clearance was obtained from all individual health services, as well as the University of Adelaide. All subjects gave full, informed, written consent for procedures performed as per the Declaration of Helsinki, and current National Health and Medical Research Council guidelines.

2.1.2.1 Identification and Approach of KRT Subjects

Eligible HDx and KTx subjects were identified from all public renal services within South Australia, via treatment lists obtained from relevant administration services. Treating physician approval was obtained before further action was taken. Subjects were solicited first by introductory letter, and then phone call by the primary investigator, or in outpatient clinic by the treating physician. Formal consent paperwork was given to subjects at this time, and written consent obtained prior to commencement of the study.

2.1.2.2 Identification and Approach of Control Subjects

Control subjects were identified by word of mouth, and email mail-out. The majority were employed full-time by the South Australian Police.

2.2 Cell sources – Chapters 4-7

2.2.1 Endothelial cells

The collection of human umbilical cord and blood for use in this study was given ethical clearance from the Human Research Ethics Committee of the Children, Youth and Women's Health Service (CYWHS), North Adelaide, South Australia and informed written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Human umbilical vein endothelial cells (HUVEC) were used as both control samples, and for functional assays. They were collected by standard technique[414]. Briefly, intact umbilical cords were collected and processed within 24-36 hours of delivery. Under sterile conditions, the umbilical vein was identified (single vessel with patulous walls, compared to 2 arteries of smaller diameter with thick vessel walls) and dilated using a pair of forceps, at both ends of the cord. A stainless steel adaptor was then inserted into each end of the cord and tied into place with twine. A single-use plastic stopcock (BD Connecta luer lock) was then attached to each adaptor. Using a 25ml Terumo syringe warmed PBS was flushed until the effluent was clear. Collagenase I (0.25% in sterile PBS; Worthington Biomedical Corporation) solution was then injected into the cord until the cord was filled to pressure similar to that of a balloon, the stopcocks were closed and the complete cord with syringes, stopcocks and adaptors were placed in a 1L beaker with 500ml sterile saline 37^0C for 7 min (timing critical, as further collagenase digestion leads to contamination with smooth muscle cells (SMC), and damage to HUVEC). ECs were then collected by flushing the cord with HUVE wash and gentle massage performed to loosen any remaining cells.

Cells collected were spun at 1500 RPM for 5 minutes at room temperature, then resuspended in HUVE media – approximately 7ml for every 10cm length of intact cord processed.

2.2.2 Endothelial progenitor cells (EPC)

Four cell sources were available for investigation: normal bone marrow, peripheral blood, umbilical cord blood, and leukapheresed peripheral blood product from subjects with haematological malignancies. The latter source did not yield viable cells, and was not used in further studies.

2.2.2.1 Normal bone marrow (BM)

The haematology department at the RAH maintains a healthy volunteer roster for BM donation for scientific use. Briefly, bone marrow samples were collected by fine needle aspiration from the posterior superior iliac spine of sedated participants under sterile conditions in theatre by a suitably trained haematologist. Samples were collected in 4 x 9ml potassium ethylenediaminetetraacetic acid (EDTA) Vacuette^{TM} blood tubes (Greiner Bio-One). Samples were processed within one hour of collection.

Each bone marrow donor had a formal bone marrow film assessed by a local haematologist as per unit protocol in the previous year to confirm normality of marrow (all were described as normal, specifically no evidence of reduced iron or altered cell ratios).

2.2.2.2 Umbilical cord blood

Due to the known effects of illness and pre-eclampsia on circulating EPC number[415, 416], only otherwise well pregnant women undergoing elective caesarean section were considered for inclusion. Whilst maintaining the sterile theatre field, after the second phase of labour, the umbilical cord was clamped and the umbilical vein needled, with the blood draining directly into a sterile MacoPharma cord blood collection bag (MSC1201DU; MacoPharma) containing citrate phosphate dextrose anti-coagulant.

2.2.2.3 Peripheral blood

Uncuffed PB samples were collected using a 30ml Terumo syringe and 20-gauge needle, into a 9ml EDTA tube. Minimum volume of PB collected was 9ml. Matched peripheral blood samples were collected with some of the bone marrow donors, and full blood counts, including white cell differential counts were performed at the local commercial laboratory (Institute for Medical and Veterinary Science), using a Sysmex xe-2100 (Sysmex America Inc).

2.2.3 EPC/Mononuclear cell isolation from whole blood/bone marrow

MNCs were isolated from marrow and blood samples by density-gradient separation based on current practice[417]. Briefly, under sterile conditions, cell samples were diluted 1:1 with Dulbecco's phosphate-buffered saline (PBS). 30ml of this cell solution was then carefully layered on top of 15ml Lymphoprep^{TM} (Fresenius), density 1.077g/ml, in a 50ml Falcon polypropylene conical tube (BD Biosciences). Samples were then centrifuged at 400G at room temperature for 30min, with no brake.

The band of MNCs, apparent above the Lymphoprep, was then aspirated using a sterile soft pipette. Collected cells were then washed three times with HUVE media.

2.3 EPC isolation from unsorted MNCs

Two techniques were examined for cell isolation, both requiring antibody labeling (Figure 2.1). Non-specific antibody binding was blocked in the same fashion for both techniques: Isolated MNCs were resuspended in 5ml of 5% normal mouse serum in HUVE media with 100μl Fc receptor blocker (Miltenyi Biotech) for 10min at room temperature. Cells were then washed 3 times with HUVE media prior to cell sorting.

2.3.1 Fluorescence activated cell sorting

MNCs were resuspended in 100μl HUVE media, labeled with mouse anti-human CD133/1-PE (phycoerythrin conjugated, clone AC133), and mouse anti-human CD34-PE-Cy7 (phycoerythrin-cyanine 7 conjugated, clone AC136) from Miltenyi Biotech, at 1μg per $1x10^6$ cells, as per manufacturer's instruction. Aliquots of MNCs were also kept for compensation (1 x unlabelled, as well as single antibody stained). Cells were incubated at 4^0C for 30 minutes in a Hera Cell incubator (Thermo Scientific), before being washed three times and resuspended in HUVE media (10^6 cells/ml), and aliquotted into polypropylene FACS tubes for separation.

Dual CD34 and CD133 positive EPCs were sorted using a Becton Dickinson Aria FACS machine, using FACS Diva Software version 6.1.3 (BD Biosciences), or Beckman Coulter Epics Altra HyperSort, using Expo MultiComp Software version 1.2B (Beckman Coulter, Miami, FL, USA). Unstained and single antibody stained target MNCs were used for compensation.

2.3.2 Magnetic bead separation

Separation was performed using either a manual protocol, or autoMacs Pro Separator™ (Miltenyi). Both techniques utilised a positive sort protocol for CD133 cells. Positive cells were bound to the magnetised beads, which were caught in a magnetic field in the separation column. Once the unlabelled cells were washed through, the magnetic field was removed, and the positive cells recovered.

Briefly, MNCs were incubated in 5ml HUVE media with 5% normal mouse serum (IMVS) for 10 minutes at room temperature in a 10ml centrifuge tube (Sarstedt), and then washed twice with HUVE Media. Up to 10^8 cells were then resuspended in 300μl of MACS buffer, and incubated with 100 μl of human FcR blocking reagent and 100 μl of CD133+ microbeads (clone AC133, Miltenyi Biotec) for 30 min at 4^0C, then washed twice in MACS buffer. Cells were then finally resuspended in MACS buffer at a concentration of less than $3x10^7$/ml, for either manual or automatic separation.

2.3.2.1 Manual separation

Under sterile conditions, cell isolation was performed using a MACS (magnetic activated cell separation) metal stand, MiniMACS™ separator, and 1 MACS *MS*™ separation column per $1x10^8$ cells (Miltenyi). MNCs were flushed through the column with 2ml of MACS buffer, the column removed from the magnetic field, and a plunger used to elute captured CD133 positive cells into a 10ml collection tube. Cells were then washed twice in HUVE media prior to phenotyping/culture.

2.3.2.2 autoMACS^{TM} separation

Under sterile conditions, MNCs were diluted to 5x10^{6} cells per 1ml MACS buffer into 10ml centrifuge tubes, and kept on ice till loaded on to autoMACS^{TM}. To increase specificity, the Possel-S separation program was used, which utilises a single column, but at slow flow rate. The autoMACS^{TM} maintained samples at approximately 4^{0}C, and separated positive from negative cells into 2 x 10ml centrifuge tubes.

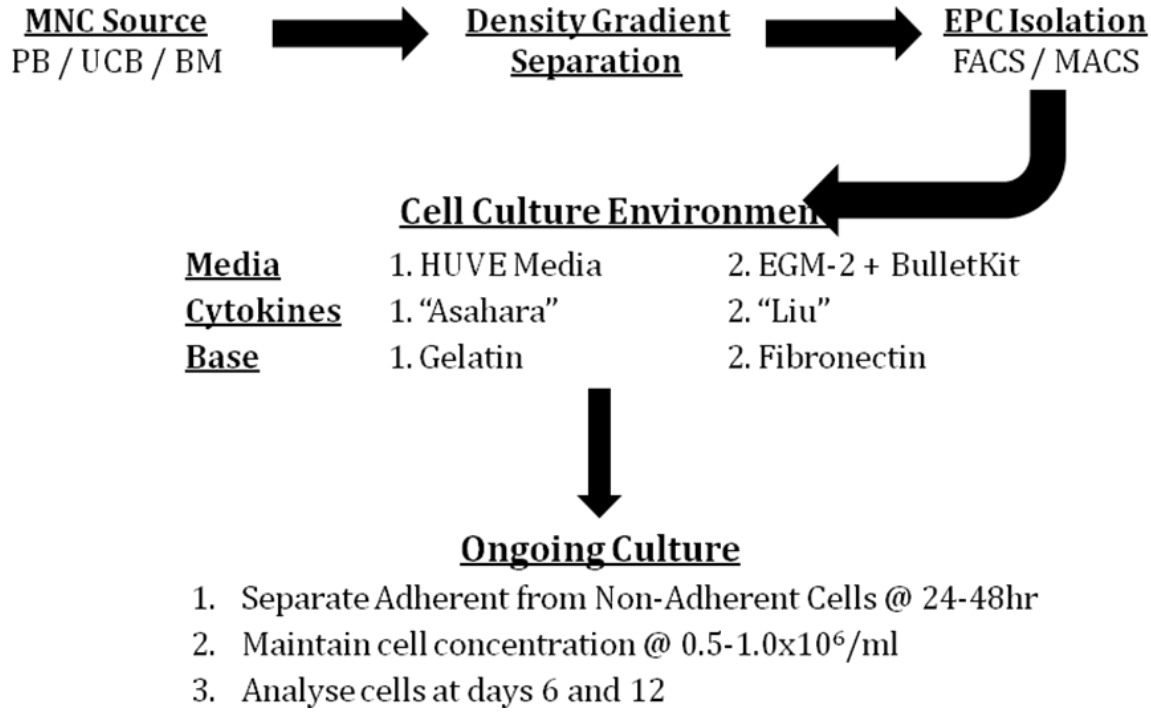


Figure 2.1: Flow chart of EPC isolation and culture techniques investigated. MNCs were obtained from PB, UCB and BM by density-gradient separation, then EPCs isolated using cell surface marker-based techniques. Several different culture environments were investigated. This included HUVE media (Media 199, pen/strep, heparin and ECGF), and EGM-2 + BulletKit with either "Asahara" (VEGF, TPO, rhIL-6, Flt-3 lig, SCF, TGFβ-R1 inhIII) or "Liu" (10% FCS, VEGF, IGF-1, FGFb, AA) added agents. Non-adherent cells were separated after 24-48hr of culture, with media change every 2-3 days, and splitting of cells if concentration was > $1.0x10^6$ cells/ml. Gelatin (used for normal HUVEC culture) or fibronectin (immature EPCs unable to attach to fibronectin) were used as base.

2.4 Cell identification by FAC

As per MACs separation, non-specific binding was reduced by exposing MNCs to 5% normal mouse serum in HUVE media (5ml serum per 10^8 cells) for 10 minutes at room temperature, then washed twice with HUVE media.

Cells were then resuspended in HUVE media (100μl), and 40μl Fc Receptor blocker (Miltenyi) added. Cells were placed at 4^0C for 10 minutes, and then washed twice in HUVE wash.

Cells were then resuspended in HUVE media, at 100μl for test sample, and 15ul for negative control and single, dual and triple antibody compensation tubes.

Primary or primary conjugated antibodies were then added as per manufacturers instruction, at approximately 1-2μg/10^6cells, and incubated at 4^0C in darkness for 30 minutes. Cells were then washed twice in HUVE media.

If secondary antibody was needed, samples were resuspended in 50μl HUVE media, conjugated secondary added as per manufacturers' instructions, and cells incubated for a further 30 minutes at 4^0C in darkness. Cells were then washed as above.

Once fully labeled, samples were resuspended in 300μl of FACS fix, and added to polypropylene FACS tubes. These were sealed and stored at 4^0C in darkness till analysed, all within 4 days.

Analysis was performed on the Cytomics FC500 flow cytometer using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2 (Beckman Coulter).

2.4.1 EC phenotype

Mature ECs were identified by dual positivity for mouse anti-human CD31 (clone 51-6f6, in house antibody) with rat anti-mouse IgM-FITC (clone 1B4B1; Southern Biotech, Birmingham, AL, USA), and goat anti-human VE-cadherin (clone N-14, Santa Cruz) with donkey anti-goat IgG-Cy5 (Rockland). All antibodies were used at 1μg per 10^6 cells, with 30min incubation time at 4^{o}C. HUVEC were used as the positive control.

2.4.2 EPC phenotype

EPCs were identified as triple positive for mouse anti-human CD133/1-PE (clone AC133, Miltenyi), mouse anti-human CD34-PE-Cy7 (clone AC136, Miltenyi), and mouse anti-human VEGFR2 (clone EIC, Santa Cruz), with secondary goat anti-mouse IgG with PE-Cy7 conjugate (Santa Cruz), as well as low expression for mouse anti-human CD45 (clone 2D1) with FITC conjugate (BD Bioscience), all at 1μg per test, as per manufacturers' instructions.

2.5 Cell culture

2.5.1 Human umbilical vein endothelial cells (HUVEC)

Collected HUVEC in HUVE media were seeded onto Costar T25 flasks (Corning Lifescience) coated with gelatin, 2ml per flask, incubated 30min at 37^0C, then excess aspirated),at 2.5-5.0x10^5cells in 4ml per flask, and incubated in a Hera incubator at 37^0C with 5% CO_2. Media was replaced at day 2-3, and cells split 1-2 days following when >90% confluent.

Cells were collected by aspirating and discarding the supernatant, washing the adherent cells once with warmed PBS prior to 5ml of 0.01M EDTA in PBS being added to flasks, and sat for 30 seconds at room temperature. The solution was aspirated off, and 1ml of 0.5% trypsin solution carefully added to the entire cell monolayer prior to incubation for ~20 seconds at 37^0C. The flask was then viewed under 10x magnification to confirm detachment of cells (from elongated to rounded in shape), and if not completely detached, the flask was tapped on the side with an open hand to loosen cells further. The flask was then washed three times with 5ml of HUVE wash to deactivate the trypsin and collect the cells, with solution collected in 50ml tubes. The cells were centrifuged at 1500rpm for 5 minutes at room temperature, and the pellet resuspended and prepared for further culture or use in experiments.

2.5.2 Human endothelial progenitor cells (EPC)

Following isolation, small aliquots of both positive and negative sort cells were collected for identification. The remaining cells were cultured. Four different basic techniques were utilised (Figure 2.1, and as below). Adherent cells were discarded at 24-48 hours, and media changed every 2 days:

1. Mature EC growth media: cells resuspended at $1x10^6$/ml in HUVE media plus Endothelial Cell Growth Factor (ECGF, 3μl/ml; Roche) and heparin (3μl/ml), and added to T25 culture flask (BD Falcon Primaria, BD) coated with gelatin, at 4ml per flask. Media replaced every 2 days.
2. Mature EC growth media + Asahara protocol (personal correspondence): as above, with added VEGF (50ng/ml), thrombopoietin (TPO, 20ng/ml), recombinant human interleukin 6 (rhIL-6; 20ng/ml), Fms-like tyrosine kinase 3 ligand (Flt-3 lig, 100ng/ml), Stem Cell Factor (SCF, 100ng/ml) and tissue growth factor beta R1 inhibitor III (TGFβ-R1 inhIII, 0.4ng/ml). Media replaced every two days.
3. Mature EC growth media + Liu[418] protocol: as per protocol 1, with added FCS 10%, VEGF (5ng/ml), IGF-1 (1pg/ml), AA(0.2μg/ml) and bFGF (1ng/ml; R&D Systems).
4. Commercial media: cells cultured on fibronectin (50ug/ml in sterile PBS) coated 24-well plates at 1x10^6 cells/ml in Endothelial Growth Media-2 (EGM-2, Lonza) with Bulletkit additives (human endothelial growth factor (hEGF), hydrocortisone, gentamicin, amphotericin-B, 2% fetal bovine serum, VEGF, human fibroblast growth factor beta (βFGF), IGF-1, ascorbic acid (AA), heparin – concentrations unknown, under proprietry). The non-adherent cell population was collected at approximately 48 hours, replated onto fibronectin

in the same media, and media changed every 48 hours until cells were harvested. Cell concentration was maintained between 0.5 and $1.5x10^6$/ml/well.

5. Commercial media + Liu Protocol: as protocol 4 with additional agents – FCS 10%, VEGF (5ng/ml), IGF-1 (1pg/ml), AA(0.2μg/ml) and bFGF (1ng/ml).

Cell counts were performed every 2 days, and identification pre-/post- sort and at days 6 and 12.

2.6 Functional cell studies

2.6.1 Matrigel tubule assay

2.6.1.1 Incorporation of EPC into EC tubes – Chapter 4

As previously described[417], 10 mg of Matrigel (BD Biosciences) was added to a pre-cooled 96-well plate (100 µl/well) and incubated at 37ºC for 30 min.

Passage 1 HUVEC were added to a gelatin coated 6-well plate at 5 x 10^5 cells in 2ml HUVE media per well, and incubated at 37^0C, 5% CO_2 overnight. The following morning, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein (DiL-Ac-LDL, Biomedical Technologies) was added at 10µg/ml, in 1ml HUVE media per well, then incubated for 4 hours at 37^0C, 5% CO_2, in the dark. HUVEC were then detached using 0.5% trypsin, and resuspended in HUVE media (as described in Materials and Methods 2.5.1).

Day 6 non-adherent EPCs were added to 1ml HUVE media with 10µM carboxy-fluorescein diactetate (CFDA), succinimidyl ester in dimethylsulfoxide (DMSO), as per manufacturer's instruction (Vybrant© CFDA SE Cell Tracer Kit, Invitrogen). EPCs were then incubated at 37^0C, 5% CO_2 for 15 min, then washed twice with HUVE media.

140μl aliquots of cell solution in HUVE media, with ECGF and heparin (100μg/ml each), were prepared as below, and layered onto Matrigel:

- 3.5×10^4 HUVEC unstained
- 3.5×10^4 HUVEC Dil-Ac-LDL labelled
- 3.0×10^4 HUVEC unstained + 5×10^3 EPC unstained
- 3.0×10^4 HUVEC Dil-Ac-LDL labelled + 5×10^3 EPC unstained
- 3.0×10^4 HUVEC unstained + 5×10^3 EPC CFDA labelled
- 3.0×10^4 HUVEC Dil-Ac-LDL labelled + 5×10^3 EPC CFDA labelled

The plate was then incubated at 37^0C, 5% CO_2, and microscope images and tube counts performed every hour for the first 2 hours, then 2 hourly following. Unstained EPCs (3.5×10^4) alone were also placed in Matrigel. All experiments were done in at least duplicate.

For labeled cells, images were taken at 7hr using the Bio-Rad Radiance 2100 confocal microscope (Olympus, USA) equipped with both Argon ion 488nm and Green HeNe 543nm lasers, and an Olympus IX70 inverted microscope. The objective used was a 20x UAPOW (NA= 0.70). The dual labeled samples were imaged with two separate channels (photomultiplier tubes - PMT) in a sequential setting. The Green fluorescence (CDFA) was excited with an Argon 488 nm laser line and the emission was viewed through a HQ515/30 nm narrow band barrier filter in PMT1. The red fluorescence (Dil-Ac-LDL) was excited with Green HeNe 543 nm laser line and the emission was viewed through a long pass barrier filter (570LP) to allow only red light wavelengths pass through PMT2. In addition the transmission detector was used to collect the unstained background. All PMT signals were automatically merged.

2.6.1.2 Qualitative and quantitative assessment of tube formation – Chapters 5-7

Assay was performed as above, however no cellular stain was used, and HUVEC used were passage 2, and collected by trypsin digestion following a 2 hour serum starvation, to both align all ECs to the same stage of the cell cycle, as well as prime cells to activation when re-exposed to sera.

Initial experiments involved regular photography of wells at 1, 2, 4, 6 and 8 hours. After optimal time for tube formation was ascertained (~7 hours), photos and tube counts were only performed at that one time point. Maximal number of tube branches from a single cell was quantified from saved photos of each well, in a blinded fashion.

2.6.2 Migration Assay

Migratory capacity was analysed using an 8-micron pore polyester HTS-transwell in a 96 well plate (Corning Life Sciences). The inferior surface of the transwell was coated in fibronectin (50μg/ml, Roche) for cell capture, and VEGF at 100ng/ml was added to the reservoir in 100μl Hanks Balanced Salt Solution (HBSS) + 0.5% bovine serum albumin (BSA) + ECGF (100μg/ml). $1x10^4$ EPCs or HUVECs in 50μl HBSS + 0.5% BSA were added to the transwell. HUVECs were used as negative control. Transwells were incubated for 2 hours at 37^0C, 5% CO_2. Membranes were then fixed in paraformaldehyde overnight at 4^0C prior to cell labeling with 4',6-diamidino-2-phenylindole (DAPI) and membranes mounted onto individual microscope slides with fluorescence-stabilised mounting media. Membranes were read on an Olympus E71 inverted fluorescent microscope (Olympus UK Ltd), excitation 360nm and emission 461nm. Three random 10x power microscope fields were photographed, and DAPI positive cells counted manually using the Analysis LifeSciences software package (Olympus) with the average per field calculated.

2.6.3 Vascular cell adhesion molecule-1 expression

2.6.3.1 Cell preparation

HUVEC (passage 2, >90% confluent) were grown in complete HUVE media with ECGF/heparin, and then serum starved for 2 hours prior to experimentation. UCB EPCs were prepared as described in chapter 2.5.2. Cells were used at day 5-7, to maintain stable phenotype.

2.6.3.2 Cell exposures

2.6.3.2.1 TNFα

EPCs at a concentration of 50, 500 and 5000 cells per T25 flask of HUVECs were added. This equated to an EPC concentration of approximately 0.0001, 0.001 and 0.01% respectively (compared to HUVEC), which is comparable to peripheral blood concentrations of EPCs found in humans[199]. This was done in the presence or absence of TNFα (0.5ng/ml; R&D Systems), to mimic profound endothelial inflammation. *In vivo*, TNFα serum concentration correlates with sVCAM-1 concentrations[28]. Flasks were then incubated for 24 hours at 37^0C at 5% CO_2 concentration, and cells collected as per above with EDTA/trypsin.

2.6.3.2.2 Uraemic Toxin

For HUVEC alone, toxins were added alone or in combination, at increasing concentration. For HUVEC + EPC, EPCs were also added at 500 and 5000 cells/ HUVEC flask, with both PC (40.7mg/L) and IS (236mg/L) added. Flasks were then incubated for 24 hours at 37^0C / 5% CO_2 concentration, and all cells subsequently collected with EDTA/trypsin and washed twice.

2.6.3.3 Cell labeling and FACS

Cells were resuspended in HUVE media at 25μl each – negative controls were split into 2 x 12.5μl each.

Mouse anti-human VCAM-1 antibody (IgG, in-house, clone 51.10.C9) was added at 1ng/sample. Mouse anti-human Isotype antibody (IgG, in-house, clone 23.1.F11), was added to the second of the split negative controls at 1ng/sample.

All samples were then incubated at 4^0C for 30 minutes, washed twice in HUVE media, before being resuspended in 25μl HUVE media.

Secondary antibody, goat anti-mouse IgG Alexa 488 labeled, was added (1:1000 dilution in HUVE media, 50μl per sample), and samples incubated for 30 minutes at 4^0C in darkness.

Samples were then washed twice in HUVE media, and resuspended in 300μl FACS Fix, in flow tubes.

Cell analysis was performed on Beckman Coulter Cytomics FC500, using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2. (Beckman Coulter, Miami, FL, USA). Excitation was with Argon laser (488nm excitation), with data collection from FL1. Compensation was performed using the isotype control. Samples were compared by the –fold increase in mean fluorescence intensity (MFI) of VCAM-1 expression between the test samples and the negative control.

2.6.4 Survival factor expression

Akt and MAPK are involved in regulation of the cell cycle, both through blockade of apoptosis and regulation of genes involved in cell proliferation[419, 420]. An increase in the ratio of the phosphorylated to the total Akt or MAPK concentration indicates upregulation of cell survival factors. The effect of uraemic toxins on Akt and MAPK expression in HUVEC was investigated by Western Blot techniques.

2.6.4.1 HUVEC toxin exposure

HUVECs were prepared as previously described. Cells were used in T25 flasks at passage 2, when at least 90% confluent. No serum starvation was performed, as this activates Akt and ERK in its own right[421, 422].

HUVECs were maintained in complete HUVE media with ECGF/heparin, and incubated during exposure at 37^0C, 5% CO_2.

Toxin type and time of exposure is listed in Figure 4.1. Sphingosine-1-phosphate (S-1-P) was used as the positive control, as it increases phosphorylation of both MAPK (at 10min, maximal with 5uM S-1-P), and Akt (at 60min, maximal with 2.5uM S-1-P) (reviewed elsewhere[423]). These agents were added to HUVEC flasks already containing 4ml of HUVE media with ECGF/heparin.

Table 2.1: Exposure times for HUVECs to toxins and S-1-P for investigation of survival factor (p/tERK, and p/tAkt) expression.

	10 minutes	60 minutes	24 hours
Negative control	×	×	✓
Sphingosine-1-phosphate(2.5 or 5uM)	✓	✓	×
PC (40.7mg/L) + IS (236mg/L)	✓	✓	✓
PC (20.1mg/L) + IS (53mg/L)	✓	✓	✓
PC (0.6mg/L) + IS (0.6mg/L)	×	×	✓
IS (236mg/L)	✓	✓	✓
IS (53mg/L)	✓	✓	✓
PC (40.7mg/L)	✓	✓	✓
PC (20.1mg/L)	✓	✓	✓

2.6.4.2 Western blotting

2.6.4.2.1 Lysate Preparation

After exposure to toxin, cells remaining adherent to flasks were washed twice with 5ml cold PBS with 1% phosphatatse inhibitor ($NaVO_4/H_2O_2$). All fluid was then aspirated from the flasks, and 150ul lysis solution added (lysis buffer, 2% protease inhibitor, 1% phosphatase inhibitor), then incubated on ice for 10 minutes. Flasks were then scraped with a rubber policeman with lysate collected. If significant cellular debris was present, samples were sonicated for 30 seconds. Lysates were spun at 13000 RPM for 15 minutes at 4^0C, supernatants collected into a clean Eppendorf tube and stored at -80^0C till analysed.

2.6.4.2.2 Lysate protein concentration estimation

Lysate protein concentration was estimated using the Bradford method[424]. In brief, protein standards were prepared using serial dilution of bovine serum albumin. Bradford reagents (BIO-RAD, Hercules, CA, USA) were used according to the manufacturer's instruction. 5μl of protein standards and 5μl of cell lysates were added to wells in 96 well flat-bottomed plate. 25μl of 50:1 reagent A:S was added, followed by 200μl of reagent B. Samples were then read on an ELISA plate reader (BIO-RAD), at wavelength of 595nm. A standard curve was generated using Microplate Manager software (BIO-RAD) and protein concentrations for the samples derived from this.

2.6.4.2.3 Protein Electrophoresis

Western Blot method for protein electrophoresis was performed using BIO-RAD Protean II setup, as per manufacturer's instruction. 10% Tris Glycine SDS-Polyacrylamide gels (SDS-PAGE) were used.

SDS-reducing buffer was added to cell lysates (1:100), mixed and boiled at 100^0C for 5 minutes. Bradford estimation was used to load 30μg of protein per sample to the stacking gel wells.

The loaded gels were run at 150V for 90min in the Bio-Rad Protean II xi vertical slab electrophoresis instrument, using cooled Running Buffer.

Once run, the gels were transferred by Western Blot method to activated Amersham Hybond-P membranes (GE HealthCare) that had been soaked in methanol for approximately 10 seconds. Sandwich setup for transfer used the following order: sponge – Whatman filter paper – SDS-PAGE – Hybond membrane – filter paper – sponge. This was immersed in cold Transfer Buffer, and added to the Protean instrument, with an added "flea" and ice in the tank to maintain cooling. Transfer was obtained by setting of 100 volts for 75 minutes.

2.6.4.2.4 Antibody Probing

Once transferred, membranes were blocked against non-specific antibody binding by using 5% skim milk powder in PBS-Tween for 60 minutes at room temperature.

Blots were probed for phosphorylated and total MAPK and Akt. Antibodies used were rabbit anti-human p44/42 MAPK antibody, phospho-p44/42 MAPK (Thr202/Tyr204) (197G2) Akt antibody, and phospho-Akt (Ser473) antibody (Cell Signaling), as per the manufacturer's instruction (diluted 1:1000, exposed to membranes overnight at 4^0C, on rocker). Membranes were washed 3 times in PBS-

Tween, then horseradish peroxidase tagged secondary antibody (diluted 1:10000) was used in conjunction with Enhanced Chemoluminescence Plus (GE Healthcare), as per manufacturer's recommendation, to achieve chemiluminescence, by enzymatic generation of an acridinium ester. Images were photographed and processed using the FujiFilm LAS-4000 imaging system (FujiFilm Life Sciences). Varying exposure times were used, to optimise images obtained.

2.6.4.2.5 Data Analysis

Analysis for protein concentrations was performed using ImageJ, version 1.43u, 64-bit (Wayne Rasbands, NIH), as per the website:

http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html.

Briefly, each blot contained an untreated control sample from the specific cell line, so each time point could be compared within each line. Variation in protein loading was eliminated by comparing the ratio of phosphorylated to total Akt or ERK, as well as confirming the presence of β-Actin in all samples.

2.7 Supernatant investigation for uraemic toxins and soluble cell surface markers

Supernatants from T25 flasks were collected and centrifuged at 3000RPM for 10 minutes at 4^0C, and any solid elements discarded. Samples were then separated into 0.5ml aliquots in Eppendorf tubes, and stored at -80^0C till processed further.

2.7.1.1 Uraemic toxin concentrations

Analysis was performed at the Jankowski Laboratory, Charite, Berlin, Germany, using standardised techniques, as summarised below. Serum samples were air-freighted to Germany from Australia on dry ice, with the courier company guaranteeing maximum transport temperature of less than -20^0C. The Jankowski study was supported by a grant from the German Federal Ministry of Education and Research (01GR0807) and by grant FP7-HEALTH-2009-2.4.5-2 to “SYSKID” from the European Union."

2.7.1.1.1 Deproteination

The samples were centrifuged at 2,100 g for 10 min at 4°C. 0.33 µg of a dimethylphenol was added as internal standard. The samples were deproteinized with 0.6 mol/L (final concentration) perchloric acid and centrifuged (2,100 g at 4^0C for 5 minutes). After adjusting pH to 7.0 with 5 mol/L potassium hydroxide (KOH) the precipitated proteins and $KClO_4$ were removed by centrifugation (2,100 g at 4^0C for 5 minutes).

2.7.1.1.2 Reverse phase chromatography

The 10 µl of the deproteinated samples were separated by analytic reverse phase HPLC (high performance liquid chromatography) using di-potassium hydrogen phosphate trihydrate (K_2HPO_4, 20 mmol/l) and tetrabutylammonium hydrogensulfate

(TBA, 4 mmol/l) as the ion-pair reagent (pH 6.5). The samples were injected into a reverse phase HPLC column (Chromolith^{TM} Performance, RP-18e; 100-4.6 mm; Merck, Germany). Ethanol with 0.01%TFA (eluent B) and the following gradient were used for the elution: 0-26 min: 0-60% eluent B; 60-60.5 min: 60-100% eluent B; 60.5-62 min: 100% eluent B; 62-62.5 min= 100-0%; flow: 1 ml/min. UV-absorption was detected at 220 nm. The concentrations of uremic toxins were calculated using calibration curves created with synthetic IS, PC and PCS.

2.7.1.2 Soluble cell surface marker expression enumeration by FlowCytoMix

Human Adhesion 6Plex (Bender MedSystems, Burlington, CA, USA) for soluble E-selectin, ICAM-1, ICAM-3, PECAM-1, P-selectin and VCAM-1, was utilised as per manufacturer's instruction with supernatants.

In brief, FlowCytoMix utilises antibodies to soluble surface markers and cytokines, which are attached to specific fluorescent beads. These beads are differentiated by specific size and concentration of internal fluorescent dye. A PE conjugated secondary antibody is then added for secondary binding. This combination allows multiple different bead sets to be differentiated on a single fluorescence channel using flow techniques. The kit utilises standard solutions to produce a standard curve to calculate actual concentrations of target molecules. A standard curve is produced for each experiment, and analysed using software provided with the kit.

2.8 Toxin preparation – Chapter 5

Both toxins were prepared as per guidelines suggested by the European Toxin Working Group[425].

Toxins were prepared to mimic levels found normal individuals (normal), average ESKD patients (uraemic), and maximal recorded concentration in humans (maximal). The latter was to confirm the presence of an effect of the agent.

Toxins were studied alone, and in combination – pairs of PC and IS at normal, uraemic or maximal concentration.

2.8.1 P-cresol

PC was purchased from Sigma, and diluted to 100x stock concentration in methanol (2.795g/L), under sterile conditions, and stored at -20^{0}C.

This stock was further diluted using cell culture media (HUVE media, EGM-2), giving study concentrations of

- 0.6mg/L (Normal)
- 20.1mg/L (Uraemic)
- 40.7mg/L (Maximal)

2.8.2 Indoxyl sulfate

IS was purchased from SupelCo, and diluted to 10x concentration stock with sterile normal saline (0.9% sodium chloride) and stored in aliquots at -20^{0}C.

This stock was further diluted using cell culture media, giving study concentrations of

- 0.6mg/L (Normal)
- 53mg/L (Uraemic)
- 236mg/L (Maximal)

2.9 Commercial laboratory peripheral blood investigations – Chapters 6-7

2.9.1 Blood collection

Uncuffed blood samples were collected from anterior cubital fossa veins using a 35ml Luer-Lok™ syringe and a 20 gauge PrecisionGlide™ needle (BD) with sterile technique. Blood was immediately separated into the following specimen tubes (Vacuette™), agitated, then placed on ice till processing (all within 4 hours):

- EDTA, 1 x 9ml and 1 x 4ml
- Potassium citrate, 1 x 4ml
- Lithium-Heparin, 1 x 9ml
- Serum separate clot activator (Plain Tube), 1 x 9ml

2.9.2 Laboratory testing

All blood samples were processed at the Institute if Medical and Veterinary Science, Queen Elizabeth Hospital and Frome Road Laboratories (SA). See appendix A for details.

2.10 Clinical sera study – Chapter 6

2.10.1 Study design

A cross-sectional study of the effects of Non-KRT, HDx and KTx subject sera on the function of *in vitro* cultured HUVECs.

2.10.2 Patient Recruitment

2.10.2.1 Inclusion & Exclusion Criteria

As this was a study primarily of sera, there were few criteria for entry. Only limited patient data was available, therefore few clinical criteria were controlled for.

2.10.2.1.1 Inclusion Criteria

- Non-diabetic: HbA1C $\leq$ 6% +/- Fasting BSL $\leq$ 6.1: in compliance with World Health Organisation definition for diabetes[426]
- Stable on current medical treatment and KRT for at least three months: could include change in dosage, but not type of medication
- Able to given informed, written consent, and to follow commands to undertake the study

2.10.2.1.2 Exclusion Criteria

- Evidence in last three months of haematological disorder, malignancy, inflammatory process, new tissue ischaemia, vascular or other major surgical procedure, or any other type of acute illness
- Medication use:
    - sirolimus
    - Hypoglycaemic agents or insulin
    - prednisolone, other than for immunosuppression of renal transplant
    - Non-steroidal use in the previous 2 weeks
    - Trial medication of unknown type/dose

2.10.3 Appointment Logistics

All sera were collected after a minimum 8 hour fast, which allowed ingestion of water, but not morning medications, nor stimulants (including coffee or chocolate) in the prior 24 hours.

Collection occurred in either the dialysis unit or kidney transplant outpatient units of the Queen Elizabeth Hospital, or in the subjects' homes.

2.11 Clinical observational study – Chapter 7

2.11.1 Study Design

Given time constraints of a PhD, difficulties with patient identification, and limited number of appropriate patients within the entire statewide service, a cross-sectional study was performed. Surrogate markers of CVD were used to determine disease burden and risk, given clinical outcomes could not be followed. The initial aim was to have 6 study groups: HDx, KTx and normal controls, each with or without documented cardiac disease, with 8 subjects per study arm. Unfortunately, despite approaching over 200 potential subjects, and obtaining the support of cardiac services at two hospitals, we were unable to recruit enough appropriate patients. As a consequence, the study was decreased to the three treatment groups (non-KRT, HDx, KTx).

2.11.2 Inclusion & Exclusion Criteria

Given the number of potential factors that may affect an individual's risk for CVD, tight criteria were devised to limit subject variability.

2.11.2.1 Inclusion Criteria

- Male sex
- 45-65 years of age at time of testing
- Non-diabetic: HbA1C $\leq$ 6% +/- Fasting BSL $\leq$ 6.1: in compliance with World Health Organisation definition for diabetes[426]
- Stable on current medical treatment and KRT for at least three months: could include change in dosage, but not type of medication
- Able to given informed, written consent, and to follow commands to undertake the study

2.11.2.2 Exclusion Criteria

- Evidence in last three months of haematological disorder, malignancy, inflammatory process, new tissue ischaemia, vascular or other major surgical procedure, or any other type of acute illness
- Known Cardiac Arrythmia
- Severe Hypertension (SBP > 200/-) or Hypotension (SBP < 100/-)
- Known sensitivity to study medication – glycerol trinitrate sublingual, salbutamol inhaled (CFC free)
- Severe valvular or other functionally significant anatomical heart disease
- Bilateral upper and/or lower limb vascular surgery affecting radial, brachial, carotid or femoral arteries

- Use of the following medication:
    - Sirolimus
    - Hypoglycaemic agents or insulin
    - Prednisolone, other than for immunosuppression of renal transplant
    - Non-steroidal use in the previous 2 weeks
    - Cyclic guanosine monophosphate PDE5 inhibitor (eg sildenafil, vardenafil, tadalafil, alprostadil)
    - Trial medication of unknown type/dose

2.11.3 Appointment Logistics

All subjects were examined after a minimum 8 hour fast, which allowed ingestion of water, but not morning medications, nor stimulants (including coffee or chocolate) in the prior 24 hours.

Investigations were undertaken in outpatient settings at the Queen Elizabeth Hospital, Royal Adelaide Hospital, Lyell McEwin Health Service, Berri Hospital and Maitland Hospital. For a number of patients who requested, home visits were accommodated. In all settings, the subject had access to a bed, fully reclining chair or examination couch, with ambient room temperature between 20 and 23^0C. For two patients, electrical interference in the hospital setting did not allow the measurement of pulse wave velocity.

2.11.4 Medical history and examination

Relevant medical history was obtained directly from the patient, as well as from hospital case and electronic records, and treating physician records.

A screening examination was also performed prior to commencement of each study to confirm both patient stability/safety and appropriateness for the study.

Data types obtained are listed in Appendix B.

2.11.5 Carotid intima media thickness assessment

2.11.5.1 Basis of methodology

The protocol chosen for C-IMT measurement was based on literature review[427]. This highlighted the importance of:

- Measurement of both carotid arteries
- Measurement from posterior wall rather than anterior, to limit variation in measurement
- Measurement at commencement of cardiac cycle (end-diastole - immediately prior to P-wave on ECG), to ensure consistency with vessel stretch and relaxation
- Anterolateral, lateral and postero-lateral angle measurements of each artery to reduce variability
- Avoidance of areas of atherosclerotic plaque
- Measurement below the carotid bulb/bifurcation

2.11.5.2 Training

Training in the procedure was undertaken between April 2007 and January 2008 under the guidance of A/Prof Stephen McDonald at the Queen Elizabeth Hospital and Hampstead Centre to confirm technique competency. All images were taken and analysed (in blinded fashion) by the same operator.

2.11.5.3 UltraSound

Images were obtained on a Sonosite Titan Ultrasound (SonoSite Inc) with an L38 hand-held transducer (10-5MHz, 38mm broadband linear array, 6.5cm scan depth), and three lead ECG cable (SonoSite).

2.11.5.4 Patient positioning and confirmation of anatomy

Images were obtained from patients in a supine position, head positioned to the left or right, on a pillow to ensure neck muscles were relaxed. Confirmation that imaging was of the carotid artery was proven by inability to compress the vessel, presence of carotid bulb and bifurcation, and presence of flow on colour Doppler imaging.

2.11.5.5 Image acquisition

Three still images, in anterior, mid and posterior position, from both the left and right common carotid artery, at least 1cm below the bifurcation, were recorded onto the flash card in the Sonosite Titan in digital format (24 bit-per-pixel Microsoft Windows© BMP image file). For standardisation, all images were taken just prior to the commencement of the p-wave on the concurrent 3-lead ECG trace, to coincide with the commencement of diastole. UltraSound settings for gain and depth were determined separately for each individual, to optimise picture quality. The intima and media were defined as the low echo region of the vessel wall: atherosclerotic plaque were excluded by not including any area that encroached on the vessel lumen[428] (Figure 2.2).

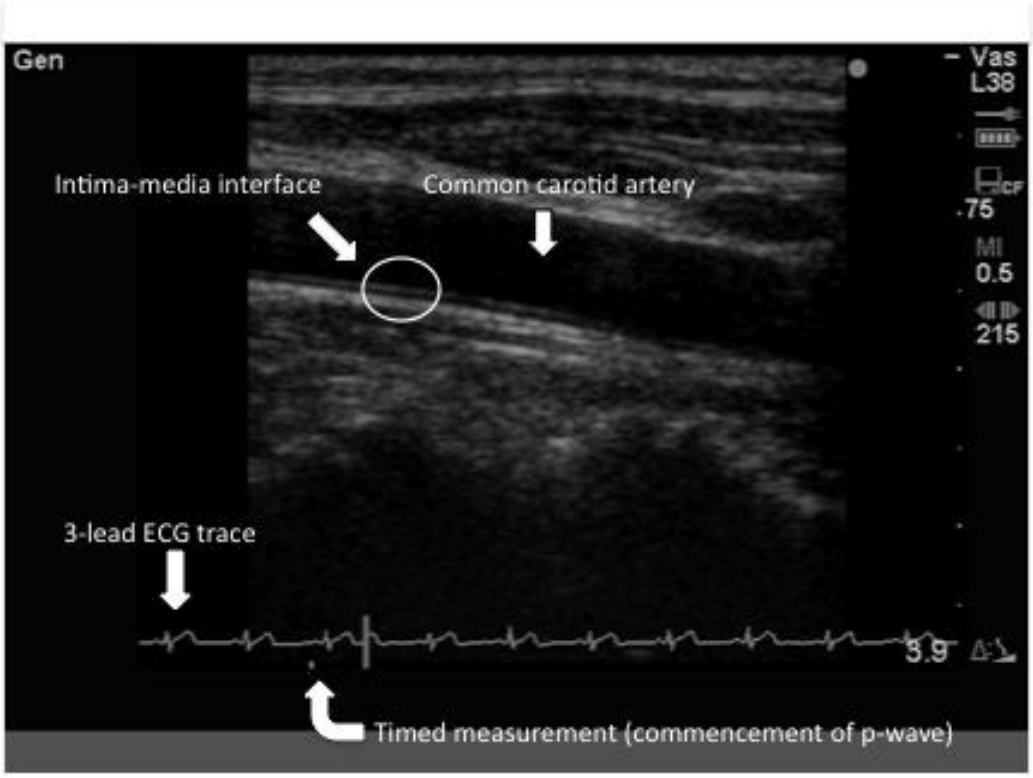


Figure 2.2: screen shot from ultrasound showing common carotid artery, timed ECG trace and area of interest

2.11.5.6 Image collection and preparation for analysis

Images were saved in digital format onto a flash card, and then transferred to a computer at a later date using SiteLink Image Manager software (SonoSite Inc.). Images were then converted to 8-bit (256 grey scale) using ImageJ 64 for Mac and PC (Version 1.43u, Wayne Rasband, NIH, USA).

2.11.5.7 cIMT analysis

Intima media thickness was assessed, in a blinded fashion, using semi-automated edge detection software (Carotid IMT Analysis Program, Version 2.00a, Dr B. P. Bailey, Lane Cove, NSW, Australia). Each image was calibrated for scale using centimetre measurements on the original image. The software would then automatically detect the edges of the intima and media (Figure 2.3). Any outlying edges, or artifact, were manually excluded from analysis, with the automatic rejection threshold not changed for any analyses to maintain consistency.

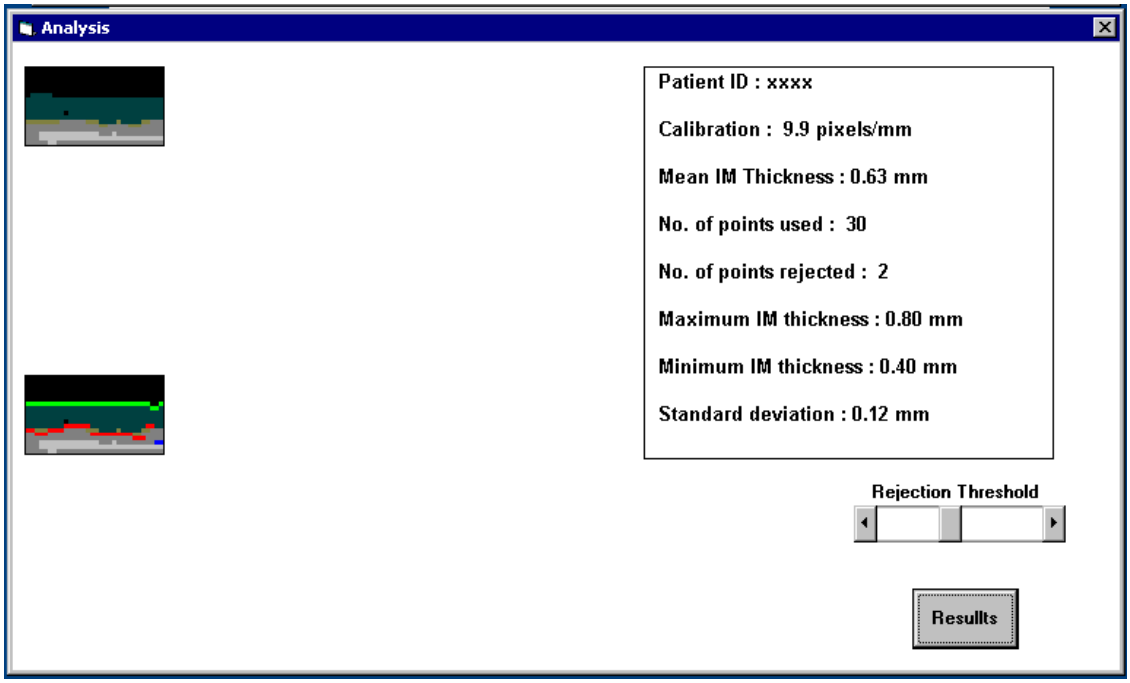


Figure 2.3: screen capture from IMT measurement software. The green line indicates the lumenal interface, and the red line the media-adventitial interface. Obvious outlying measurements were able to be manually removed prior to final analysis.

Results were exported to a spreadsheet for collation and analysis. A minimum of 10 points for measurement was required for analysis from a single image. The final cIMT measurement was defined as the average of all six images from the subject.

2.11.5.8 cIMT reproducibility

All observations were recorded by a single technician (Dr Sen). 7 subjects had repeat testing, at least 24 hours after the initial investigation to confirm degree of intra-operator variability. As cIMT measurement by software was semi-automated, 5 random subject results were repeated. Bland-Altman analysis was used to compare repeat testing. Bias (95%CI) for repeat US measurements was 0.017mm (-0.063 – 0.096), and 0.0017mm (-0.041 – 0.045) for repeat software measurement, and considered adequate clinically.

2.11.5.9 Carotid artery diameter and distensibility

Dynamic vessel characteristics were not assessed, as volume status was not consistent between patients. For HDx patients, data acquisition occurred prior to dialysis.

2.11.6 Aorto-femoral pulse wave velocity (PWV) assessment

2.11.6.1 Device details

An AtCor SphygmoCor SCOR-Px/Vx/Mx (AtCor Medical Pty Ltd) was utilised, with provided software for PC.

2.11.6.2 Patient preparation

The patient was maintained in a recumbent position after cIMT assessment. Blood pressure was reassessed with an automated cuff (Omron). Measurements (in mm) were taken from the sternal notch to right carotid artery, and from the sternal notch to right femoral artery. Measurements were input to PWV software, to determine the distance the pressure wave would be travelling. A 3-lead ECG was also attached to the patient for timing purposes. Height and weight were measured prior to analysis.

2.11.6.3 Data collection

The tonometer was placed over the carotid artery, and positioned so a clear waveform was obtained, with a minimum of 12 consecutive waves measured. The tonometer was then placed over the femoral artery and measurement was repeated. For some patients, there was difficulty identifying the artery, and the ultrasound was used to confirm position. A minimum of 3 measurements was taken for each patient.

2.11.6.4 Quality control

Reproducibility analyses were not required, due to the in-built quality control software of the SphygmaCor Vx. As per the manufacturer's recommendations, a PWV result (in m/s) was only accepted if the ECG trace showed a large r-wave for commencement of timing, a clear base of the pressure wave was evident, at least three pairs of data were present, and the standard error of the mean of the PWV was

≤ 10%, or 10-15% if the previous criteria were reached (see figure 2.4 for example of software results page).

2.11.6.5 Data analysis

The average of the three readings taken was used for the final PWV result for each patient.

Figure 2.4: Screen shot of PWV analysis post measurement, showing carotid and femoral pressure wave traces, timed to cardiac rhythm strip. PWV, in m/s, shown at bottom of the screen, with reference range corrected for age in the bottom right corner.

2.11.7 Radial pulse wave analysis assessment

2.11.7.1 Device details

An AtCor SphygmoCor SCOR-Px/Vx/Mx (AtCor Medical) was utilised, with provided software for PC.

2.11.7.2 Patient preparation

The patient was maintained in a recumbent position after PWV assessment. Blood pressure was measured with an automated cuff before each PWA measurement.

2.11.7.3 Data collection

Tonometer readings were taken from the radial artery, contralateral to the fistula, if one was present. Blood pressures were taken from the contra-lateral arm, or the leg if a fistula was present. Three baseline readings were taken.

2.11.7.4 Pharmacologic intervention

2.11.7.4.1 Endothelial-independent vasodilatation

After baseline was confirmed, 150mcg of glycerol trinitrate (600mcg Anginine tablet, split into quarters with a pill cutter; Sigma Pharmaceuticals, Croydon, Vic, Australia) was placed under the participant's tongue, and allowed to dissolve. PWA readings were then taken every three minutes for 21 minutes (from placement of the tablet).

2.11.7.4.2 Endothelial-dependent vasodilatation

CFC free Salbutamol sulfate, 2 x 100μg puffs via metered dose inhaler (Ventolin inhaler complete, GlaxoSmithKline, Boronia, Vic, Australia) and adult sized spacer (pre-primed) was administered to the subject, and further PWA measurements taken every three minutes for a further 21 minutes.

2.11.7.4.3 Side effects of medication

Apart from transient headaches and dizziness lasting less than 5 minutes in 5 subjects, no significant side effects were reported.

2.11.7.5 Quality control

The tonometer was positioned to the wrist over the radial artery where the strongest pulse could be palpated. Readings were accepted if a reproducible, large amplitude pressure waveform was recorded. A minimum of 10 seconds of recording was required for analysis.

The SphygmoCor has built-in quality control measures in the software package. An operator index is recorded (range 0-100), with a reading of $\geq$ 75 arbitrarily designated as adequate by the software. This score is based on four factors:

- Average pulse height
- Pulse height variation
- Diastolic variation
- Shape deviation

2.11.7.6 Data analysis

To allow comparison, only augmentation index measurements corrected to heart rate of 75 beats/minute (AI@75) were used in the analysis.

An average of baseline AI@75 was calculated. Response to glycerol trinitrate was measured every 3 minutes for 21 minutes, and then repeated post salbutamol.

Baseline AI@75, and lowest AI@75 achieved post GTN and salbutamol were used in analysis.

2.12 Statistical analysis – Chapters 4-7

Data was analysed using Microsoft Excel for Mac 2008 (Microsoft Corporation), and GraphPad Prism version 5.03 (GraphPad Software Inc).

Data was expressed as mean with 95% confidence interval (α=0.05). Comparison between groups was analysed by unpaired 2-tailed T-test, or one-way ANOVA with significance set at $p<0.05$ for continuous variables, or Chi-Square for categorical variables.

All the studies performed involved relatively small subject numbers with multiple variables investigated. This study design allowed hypothesis-generation from results, but not more detailed statistical analysis such as multiple regression.

2.13 Buffers, media and solutions

- 1x PBS was prepared from 10x PBS stock (Gibco) diluted with sterile water (Baxter)
- 0.9% saline for cords was prepared from 9gm sodium chloride dissolved in 900ml distilled water, which was then autoclaved
- HUVE Media was prepared using Media 199 (Sigma); with 20% fetal calf serum (FCS, Hyclone), 1.5% sodium bicarbonate, 2% HEPES buffer solution, 1% Penicillin Streptomycin, sodium pyruvate (Gibco Invitrogen) and non-essential amino acids (Sigma).
- HUVE Wash was prepared as HUVE Media, but with 2% FCS only.
- 0.5M EDTA/PBS stock (pH 7.3) was prepared from 186gm of EDTA dissolved in 1L of 1x PBS, with pellets of sodium hydroxide used to dissolve EDTA, and pH adjusted to 7.3 using hydrogen chloride. The final solution was passed through a 0.22um filter and stored in a sterile bottle at room temperature.
- 0.01M EDTA in PBS was prepared using 16ml of stock 0.5M EDTA in PBS, with 80ml of 10x PBS and 800ml of sterile water.
- FACS Fix was prepared using 1% formaldehyde, 20g/L glucose and 5mM sodium azide in sterile PBS
- MACS buffer was prepared using 2mM EDTA and 0.5% BSA in sterile PBS, which was degassed prior to use.
- 1% phosphatase inhibitor/sodium pervanadate was prepared by boiling equal volumes of 100mM sodium orthovanadate ($NaVO_4$; 0.2759g in 15ml distilled water, stored in aliquots at -20^{0}C) and 100mM hydrogen peroxide (H_2O_2) at 100^{0}C for 3 minutes.

- Lysis solution was prepared from 10ml of 1M tris (hydroxymethyl) aminomethanehydrochloride (Tris/HCl) at pH 7.5, 2ml of nonyl phenoxypolyethoxylethanol (NP40 – to final concentration 1%), 6ml of 5M sodium chloride (NaCl), 2ml of 200mM ethylene glycol tetraacetic acid (EGTA), 40ml of 500mM sodium fluoride (NaF), 20ml of 100mM sodium pyrophosphate and 114ml of distilled water with 1:500 sodium azide. At the time of use, additional 100mM sodium pervanadate (1:100 dilution) and protease inhibitor (1:50 dilution) were added
- 30% acrylamide mix was prepared from 87.6gm acrylamide and 2.4gm N'N'-bis-methylene acrylamide dissolved in total volume of 300ml distilled water, then passed through 0.8μm filter and stored at 4^0C.
- 10% sodium dodecyl sulfate (SDS) solution was prepared from 40gm of SDS dissolved in 400ml of distilled water
- 10% ammonium persulfate (APS) was prepared from 0.6gm APS dissolved in 6ml distilled water. The solution was aliquotted into 50μl volumes in 0.5ml Eppendorf tubes, and stored at -20^0C for single use.
- 0.04% Tetramethylethylenediamine (TEMED) solution was prepared from 40μg TEMED in 100ml distilled water
- 10% Tris Glycine SDS-Polyacrylamide gels (SDS-PAGE) were prepared from distilled water, 30% acrylamide mix, 1.5M Tris (pH 8.8), 10% SDS, 10% APS and 0.04% TEMED, poured into a glass mould, and covered with distilled water to flatten the superior surface. Gels polymerised in 30 minutes, the water drained off, and stacking gel (distilled water, 30% acrylamide, 0.5M Tris (pH 6.8), 10% SDS, 10% APS and 0.02% TEMED) layered on top, and a comb positioned in place. After the stacking gel had polymerised, the comb

was removed, and the wells rinsed with running buffer.

- Running buffer was prepared from 115gm glycine, 22gm Tris Base and 4gm SDS in 4L distilled water. The solution was adjusted to pH 8.3.
- Transfer Buffer was prepared from Tris Base 12.12gm, glycine 57.6gm, methanol 800ml and distilled water 3.2L, all cooled to 4^0C.
- Reducing buffer was prepared from 4ml distilled water, 1ml of 500mM Tris-HCl pH 6.8, 0.8ml glycerol, 1.6ml of 10% SDS, 0.2ml of 0.05% Bromophenol Blue, and 0.4ml 2-β-Mercaptoethanol
- PBS-Tween was prepared from PBS with 0.1% Tween-20.
- Hanks Balanced Salt Solution (HBSS) was prepared from 0.137M NaCl, 5.4mM KCl, 0.25 mM Na_2HPO_4, 1.3mM $CaCl_2$, 1.0mM $MgSO_4$ and 4.2mM $NaHCO_3$, in 100ml of distilled water.
- Collagenase solution was prepared from 0.2gm collagenase cls 1 (Worthington), 2.2ml of 100x $CaCl_2$ and $MgSO_4$, 194ml of sterile water (Baxter), 22ml of sterile 10x HBSS with glucose and 0.4ml FCS. All ingredients were mixed, and filtered through a Millipore 0.22um filter, and stored for no more than 1 week at 4^0C.
- Gelatin was prepared from 750ml sterile EF water, 15.39gm granular gelatin (Sigma), 32.6ml FCS, 19.6ml sterile EF 7.5% $NaHCO_3$, and 65.2ml sterile 10x HBSS with glucose. The water was warmed, and then the gelatin added. The mixture was then gently dissolved over low heat (without a flea), autoclaved then cooled to 4^0C to confirm that it would set. The mixture was then re-thawed and remaining ingredients added.

- Trypsin solution was prepared from 5ml of 10x stock 2.5% trypsin (Invitrogen) 219.7ml sterile Baxter water, with 25ml 10x PBS and 0.3ml 0.5M EDTA in PBS

Chapter 3: Epidemiology of cardiovascular disease and end stage kidney disease

3.1 Introduction

3.1.1 Congestive heart failure and acute cardiac events in KRT

Rates of CHF and acute cardiac events (ACE) differ among those receiving KRT compared to the general population. There is a substantial increase in CHF rates observed in the dialysis cohort, with a lesser increase in ACE – the reported prevalence of CHF is >36% in incident dialysis patients[31, 429]and KTx[17] populations, compared to 1.5-2% for CHF in the general population[18]. In a recent paper by Roberts *et al* investigating CVD mortality patterns in an Australian setting, 37% of CVD deaths in HDx or PDx recipients were attributed to sudden cardiac death or CHF, compared to 9% for the general population. However, the percentage of deaths attributed to AMI was lower in the dialysis compared to the general population (45% vs 53%)[430] – Given the substantially higher overall numbers however, the actual absolute AMI rate remains higher for dialysis recipients.

3.1.2 Differences between KRT modalities

Within KRT modalities, HDx has been associated with a greater relative risk of CVD than PDx[431]. For KTx, the incidence of new diagnosis of CHF increases immediately post surgery, but then decreases below that of those who remain on the wait list[432, 433].

3.1.3 Australian experience

In Australia, the rates of cardiovascular mortality among all dialysis patients vary with age, from 10 to 30 times age-matched controls. Furthermore, as rates of CVD have decreased over the last 10-15 years in the non-KRT population, the relative risk of CVD mortality to KRT recipients has increased[430].

3.1.4 Hospitalisations

While there is substantial information on mortality rates, less is known about the rates and length of hospitalisation for CVD although these are key markers of the burden of morbidity on individuals and important determinants of the cost burden to the health system. A recent sub-analysis of the HEMO study demonstrated increased rates of hospital admission, all-cause and CVD-related mortality with worsening severity of CHF. However, this study was limited by low patient numbers with severe CHF[429].

There is evidence that overall the rate of hospitalisation among HDx, PDx and KTx recipients in Australia is substantially increased[434].

3.1.5 Aims and rationale of study

In this chapter hospital separations (admissions) due to cardiac causes were investigated. The use of hospital separation data has a number of advantages

- The definition of heart failure may be difficult to make formally in the primary care setting[435], whereas Australian hospital coded data has been shown to be robust [436]
- Only events of clinical significance requiring hospital admission were included; this has a number of advantages over primary or outpatient care studies. By use of hospital admission data, illness episodes are selected which are of at least moderate severity (i.e. requiring hospital admission) and clearly indicate a morbidity “cost” (for the patient who is hospitalised) and resource utilization. This complements both primary, case-based studies[31, 437] and mortality studies[6, 16, 438]
- Hospital separation data have direct economic implications, and therefore relevance to health policy. Recent reports of the resource burden of end-stage kidney disease in Australia have only included the direct costs of provision of dialysis therapy, transplantation surgery and associated drugs costs[439]. The burden associated with the increased risk of cardiac disease associated with KRT has not been included

This study examined this burden, both overall and within sub-types of CVD.

3.2 Methods

3.2.1 Source data

Specific summary data of all hospital separations in Australia between 1 July 2002 and 30 June 2007 were obtained from the national hospital morbidity dataset compiled by the Australian Institute for Health and Welfare (AIHW). These separations were coded using the International Classification of Disease Codes, Version 10 (ICD-10). Further population data (age, gender, KRT) from the same time period was obtained from the Australian Bureau of Statistics (ABS) and details of the person-time in appropriate categories checked from the Australian and New Zealand Dialysis and Transplant (ANZDATA) Registry.

Separations were coded on the basis of the principal separation diagnosis into 4 groups – ACE, CHF, other cardiac (COth) and non-cardiac (Non-CVD) (Table 1).

Separations were also classified by whether there were codes indicating a dialysis modality or kidney transplant status, and by age group and gender, using the methodology described in McDonald & Tong[434].

3.2.1.1 Exclusion criteria

Although indigenous status was considered *a priori* as a likely confounder, indigenous identification was not considered reliable by the AIHW in the timeframe examined, and this data was therefore not supplied.

Day-only HDx separations were also excluded, as these were planned events associated with normal patient management, rather than an acute event or complication of treatment.

Table 3.1: ICD 10 codes used for defining type of cardiac events. Pulmonary oedema was not included in heart failure, as in dialysis patients this is not necessarily a primary cardiac condition

Variable	**Details**
Modality	Non-KRT / HD / PD / KTx
Age Group	<15 / 15-24 / 25-34 / 35-44 / 45-54 / 55-64 / 65-74 / ≥75
Gender	Male / Female
Kidney-Related	**Yes / No:** N00-08 – glomerular diseases / N10-16 – renal tubulo-interstitial diseases / N17-19 – renal failure / N20-23 – urolithiasis / N25-29 – other disorders of kidney and ureter / N30-39 – other diseases of urinary system
CVD Type	**None**
	Acute Cardiac Event:I20 – Angina Pectoris / I21 – Acute Myocardial Infarction / I22 – Subsequent Myocardial Infarction / I23 – Certain current complications following AMI / I24 - Other acute ischaemic heart disease
	Heart Failure:I50 – Heart Failure / I110 – Hypertensive Heart disease with heart failure / I130 – Hypertensive Heart and Renal disease with heart failure / I132 – Hypertensive Heart and Renal disease with both heart and renal failure / I255 – Ischaemic cardiomyopathy / I420 – Dilated cardiomyopathy / I426 – Alcoholic Cardiomyopathy
	Other Cardiac:E1071 – Insulin dependent DM with complications / E1171 – Non-Insulin dependent DM with complications / E1371 – Other specified DM with complications / E1471 – Non-specified DM with Complications / E877 – Fluid Overload, excludes oedema / E105 – Insulin dependent DM with peripheral circulatory complications / E115 – Non-Insulin dependent DM with peripheral circulatory complications / E13 – Other specified DM / E145 – Other unspecified DM with other circulatory problems / J81 – Pulmonary Oedema / R00 – Abnormalities of heart beat / R01 – Cardiac murmurs and other cardiac sounds / R03 – Abnormal blood pressure reading, without diagnosis / R072 – Praecordial pain / R09 – Other symptoms and signs involving the circulatory and respiratory systems

3.2.2 Data analysis

Statistical analysis was performed using the Stata version 10 statistical software package (StataCorp LP, Texas USA), and GraphPad Prism version 5.03 (GraphPad Software Inc, California USA). Poisson regression was used to examine the relationship of rates of separation and other endpoints with various covariates for testing significance of separation rates. The exposure term for Poisson models was derived from the ANZDATA Registry (for the number of person-years of treatment provided in Australia for haemodialysis and peritoneal dialysis and transplantation in each age category and calendar year). For the general population, the estimated Australia Resident population was used for each age and year category, less the total KRT person-years (Table 3.2). Results are expressed as age and sex adjusted relative separation rate, compared to the general population (with 95% confidence intervals included). For in-hospital mortality and length of admission, rates per hospitalisation and per patient-year were calculated. For comparison between age groups, the 45-54 year age group was used as the reference point.

3.3 Results

3.3.1 Separation rates

Of a total 31,303,876 hospital separations between July 2002 and June 2007 throughout Australia, non-kidney related separations accounted for 29,411,210 (Table 3.2). Of these, 7.95% had a cardiac cause as principal diagnosis; these were the principal focus of this analysis. Higher rates of hospital separations for cardiac causes were seen in all KRT modalities. However, the degree of increase differed between different KRT modalities and different types of CV separations.

Table 3.2: Number of hospital separations for all patient groups and event types for the five year period from 2002-2007 in all Australian hospitals, excluding separations with a “renal” principal diagnosis and day admission for haemodialysis. Unadjusted event rate, per 1000 person years (pyr), shown with 95% CI in brackets.

	Non-KRT	HD	PD	KTx	TOTAL
Non-CVD events/1000pyr {95% CI}	**27,019,478** 265 {265-266}	**23,427** 740 {732-751}	**6,721** 720 {702-737}	**24,153** 758 {748-768}	**27,073,779**
ACE events/1000pyr	**636,343** 6 {6-6}	**2,751** 87 {84-90}	**610** 65 {60-71}	**665** 21 {19-22}	**640,369**
CHF events/1000pyr	**220,280** 2 {2-2}	**1,455** 46 {44-48}	**255** 27 {24-31}	**304** 10 {8-11}	**222,294**
Other Cardiac events/1000pyr	**1,466,816** 14 {14-14}	**4,591** 145 {141-150}	**1,132** 121 {114-128}	**2,229** 70 {67-73}	**1,474,768**
TOTAL events/1000pyr	**29,342,917** 287 {287-288}	**32,224** 1018 {1001-1039}	**8,718** 933 {914-953}	**27,351** 859 {848-869}	**29,411,210**
Exposure person years	**101,779,340**	**31,585**	**9,339**	**31,856**	**101,852,120**

3.3.1.1 Acute cardiac events

For ACE separations, the relative increase in unadjusted rate was greatest for HDx, followed by PDx, then KTx(Figure 3.1). All, however, remained significantly higher than the rate for the general population. Age and gender adjusted rate compared to the general population showed the same pattern (HDx RR 4.7 {95% CI 4.5-4.9}; PDx RR 3.6 {3.3-3.9}; KTx RR 2.3 {2.1-2.5}).

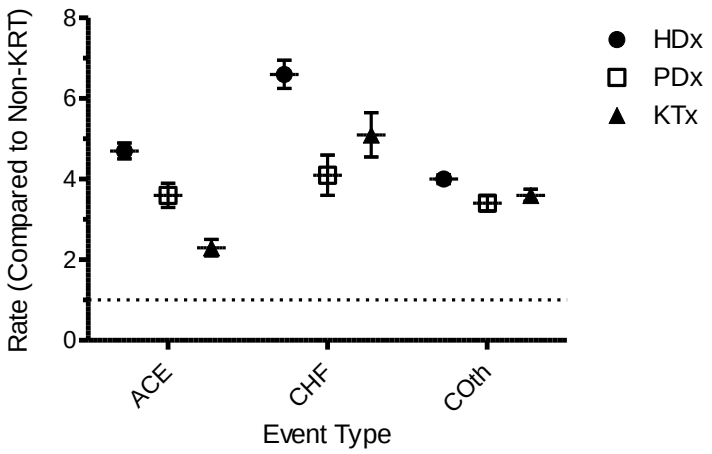


Figure 3.1: Unadjusted relative risk of hospital separation: Rate compared to non-KRT ($p<0.001$ for all groups, 95%CI).

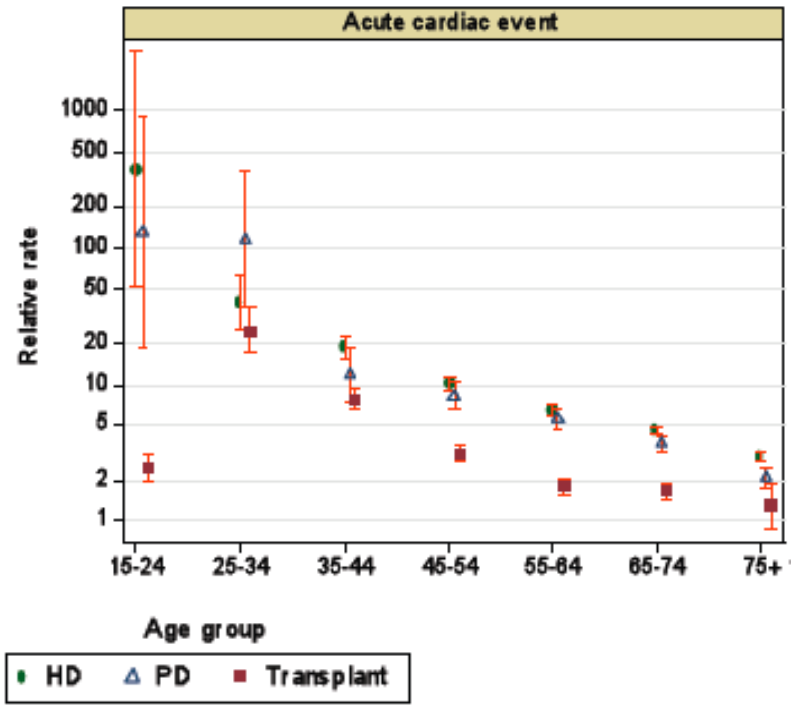
3.3.1.2 Congestive heart failure

Age and sex adjusted relative rates demonstrated the highest rate of hospital separation for HDx recipients, and a lower but similar rate for KTx and PDx (HDx 6.6 {6.3-7.0}; PDx 4.1 {3.6-4.6}; KTx 5.1 {4.6-5.7}) (Figure 3.1). Despite overlapping 95%CIs[440], the adjusted rate of separation for CHF in the KTx group was statistically significantly higher compared to PDx (p=0.01, Wald test). Further, the relative rate of KTx separation for CHF was significantly higher than for ACE (5.1 vs 2.3), as it was also for HDx (6.6 vs 4.7). There was no significant difference in separation rates for CHF or ACE for PDx recipients.

3.3.1.3 Effect of age

There was an interaction between relative hospital separation rates and age, such that the age-specific relative risk for CV hospital separation rates varied for each of ACE, CHF and COth events ($p<0.001$ for all interactions between age and each KRT modality). For each interaction, the direction was that the excess risk of separations was greater among younger people receiving KRT, although the absolute separation rate remained higher among older people (Figures 3.2-3.4).

A



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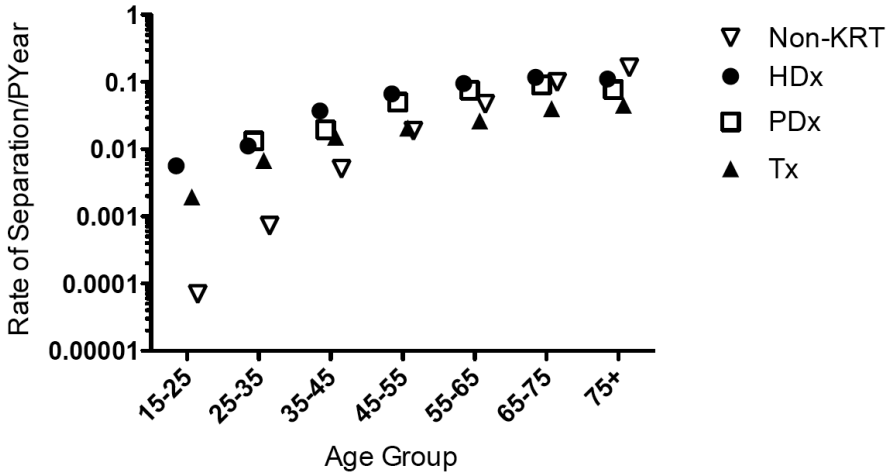
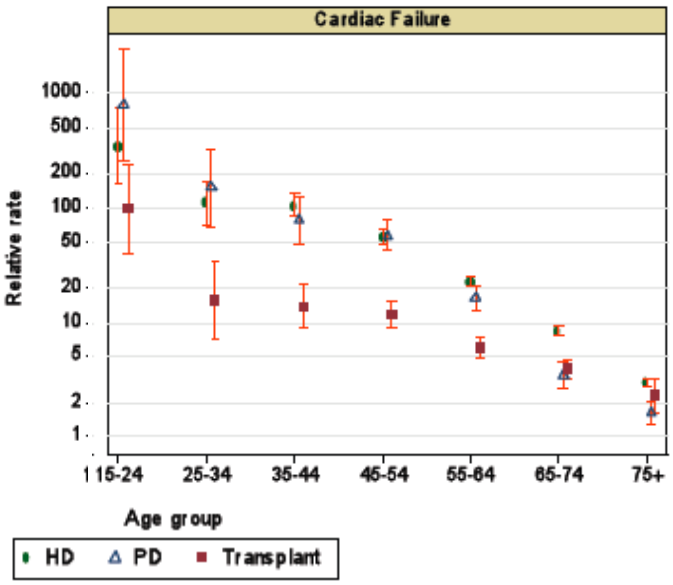


Figure 3.2: A - Relative, and B – Absolute, rate of hospital separations for ACE for KRTs compared to the general population, by age group. Whilst the absolute unadjusted rate of hospital separations for ACE increased with age in all patient groups, the relative risk of hospital separation decreased with age for KRTs compared to the non-KRT group.

A



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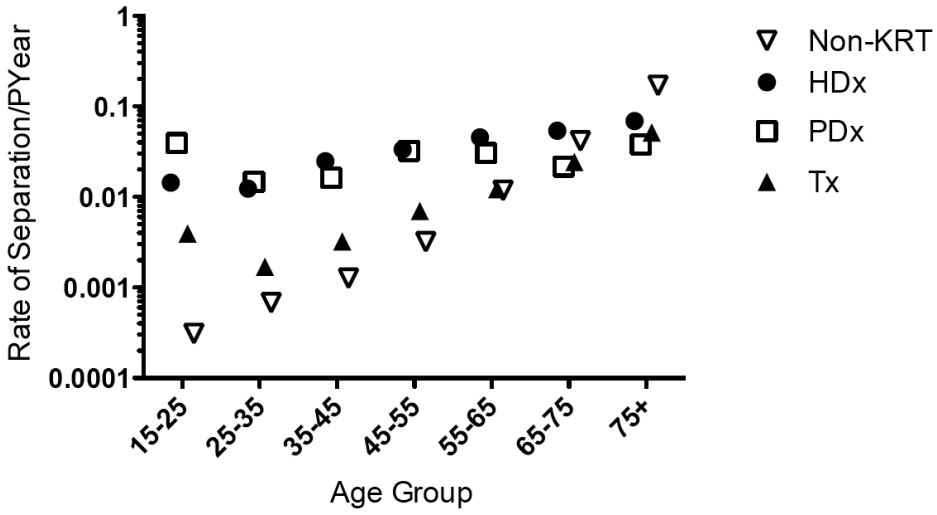
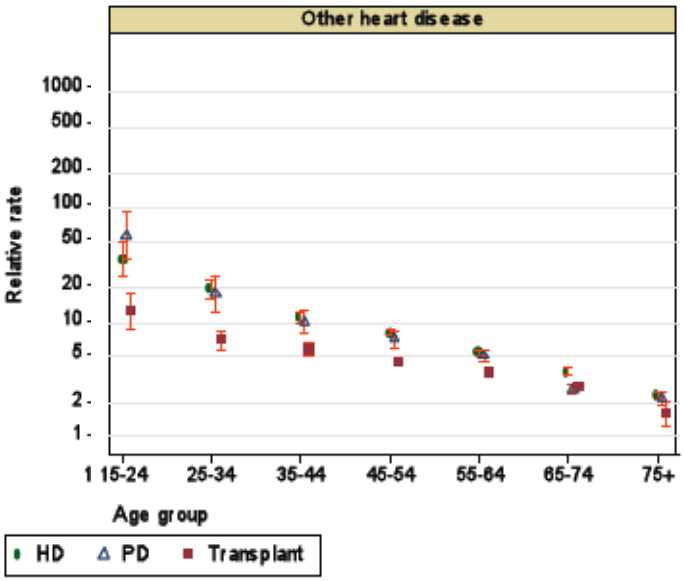


Figure 3.3: A – Adjusted relative, and B – Unadjusted absolute, rates of hospital separations due to CHF for KRT groups compared to the general population, by age group. Whilst absolute unadjusted rates of hospital separation for CHF generally increased with age for all population groups, the adjusted relative risk for separations decreased with for all KRT groups compared to the non-KRT population.

A



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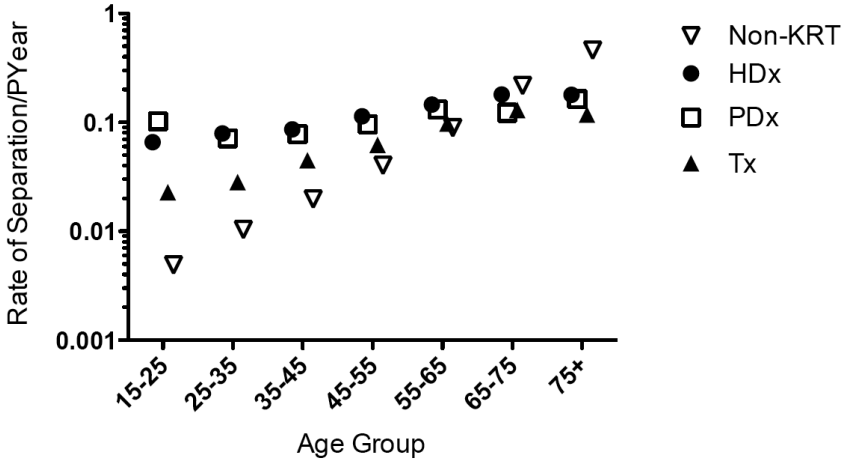


Figure 3.4: A – Adjusted relative, and B – Unadjusted absolute, rates of hospital separation for COth for KRTs compared to the general population, by age group. Whilst the absolute unadjusted rate of hospital separations for COth increased with age in all patient groups, the relative risk of hospital separation decreased with age for KRTs compared to the non-KRT group.

3.3.2 In-Hospital death

The number of in-hospital deaths in each category is shown in table 3.3. Also shown are the rates of in-hospital death per 1000 hospital separations, and per 1000 person-years of the relevant population.

Table 3.3: In-hospital death for all patient groups and event types for the five year period from 2002-2007 in all Australian hospitals, excluding separations with a “renal” principal diagnosis and day admission for haemodialysis. Event rates are unadjusted, with 95% CI in brackets.

	Non-KRT	HD	PD	KTx
Non-CVD				
Total # in-hospital deaths	**245,133**	**1,210**	**390**	**347**
Rate, per 1000 admissions	9 (9-9)	52 (49-55)	58 (52-64)	14 (13-16)
Rate, per 1000 person year	2 (2-2**)**	38 (36-41)	42 (38-47)	11 (10-12)
ACE				
Total # in-hospital deaths	**17,895**	**121**	**52**	**25**
Rate, per 1000 admissions	28 (28-29)	44 (36-52)	85 (62-108)	38 (23-52)
Rate, per 1000 person year	0.2 (0.2-0.2)	4 (3-5)	6 (4-7)	1 (0-1)
CHF				
Total # in-hospital deaths	**17,179**	**69**	**17**	**18**
Rate, per 1000 admissions	78 (77-79)	47 (36-59)	67 (35-98)	59 (32-87)
Rate, per 1000 person year	0.2 (0.2-0.2)	2 (2-3)	2 (1-3)	1 (0-1)
Cardiac Other				
Total # in-hospital deaths	**50,059**	**305**	**89**	**69**
Rate, per 1000 admissions	34 (34-34)	66 (59-74)	79 (62-95)	31 (24-38)
Rate, per 1000 person year	0.5 (0.5-0.5)	10 (9-11)	10 (8-12)	2 (2-3)
TOTAL				
Rate, per 1000 person year	**3 (3-3)**	**54 (52-57)**	**59 (54-64)**	**15 (13-16)**
Exposure				
(person-years)	**101,779,340**	**31,504**	**9,218**	**31,350**

3.3.2.1 Acute and other cardiac events

The absolute rate of death per hospital separation was higher for PDx compared to all other groups, whilst KTx recipients were not significantly different to the general population. The age and gender-adjusted relative risk for in-hospital mortality per separation from an ACE was significantly higher for all KRTs (HDx RR 1.8 {95% CI 1.5-2.1}, PDx 3.7 {2.8-4.5}; KTx 3.0 {2.0-3.9}: p<0.001). This was also the case for other cardiac events (HDx 2.1 {1.9-2.3}; PDx 2.5 {2.0-3.0}; KTx 1.7 {1.4-2.1}: p<0.001) (Figure 3.5).

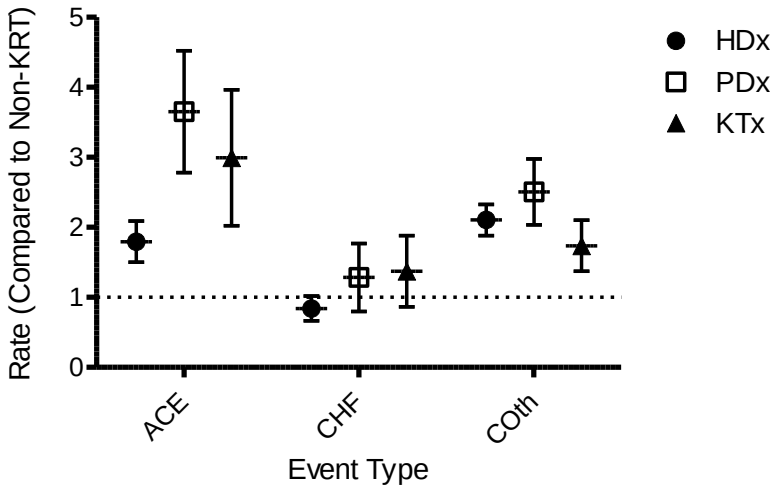


Figure 3.5: Age and gender-adjusted relative risk of in-hospital death per admission, compared to the non-KRT population. A significant increase (p<0.001) in the rate of in-hospital death ACE and Coth for all KRT groups was noted. No increased risk was observed for in-hospital death related to CHF for any of the KRT groups (p>0.1).

3.3.2.2 Congestive heart failure

HDx recipients had a lower unadjusted risk of death from CHF per admission compared to the general population, but not when comparing unadjusted rates of death per person year. Age and gender-adjusted relative risk of in-hospital death per separation from CHF was not increased for KRT groups compared to non-KRT patients (HDx 0.8 {0.7-1.0}; PDx 1.3 {0.8-1.8}; KTx 1.4 {0.9-1.9}: p>0.1) (Figure 3.5). However, the risk of death during a hospitalisation from CHF per person/year was higher than the general population for all KRT groups (HDx 3.8 {3.0-4.9}, PDx 3.4 {2.1-5.5}, KTx 5.6 {3.5-8.9}).

3.3.3 Length of hospital stay

The length of hospital stay per separation (relative to the non-KRT population) was higher for all KRT groups for all cardiac separation types ($p<0.001$) (Figure 3.6A). Statistically significant differences in rates between all KRT modalities for all event types were observed. Clinically, the increase in length of stay for ACE hospital separations (HDx 1.95 {1.93-1.97}; PDx 2.30{2.24-2.36}; KTx 1.56 {1.51-1.61}) was substantial. The length of stay for CHF was prolonged to a lesser extent (HDx 1.06 {1.04-1.08}; PDx 1.31 {1.26-1.36}; KTx 1.12 {1.08-1.18}).

When the length of stay was calculated per patient year, a significant increase in CHF hospital days per year was seen for all KRTs compared to non-KRT (Figure 3.6C).

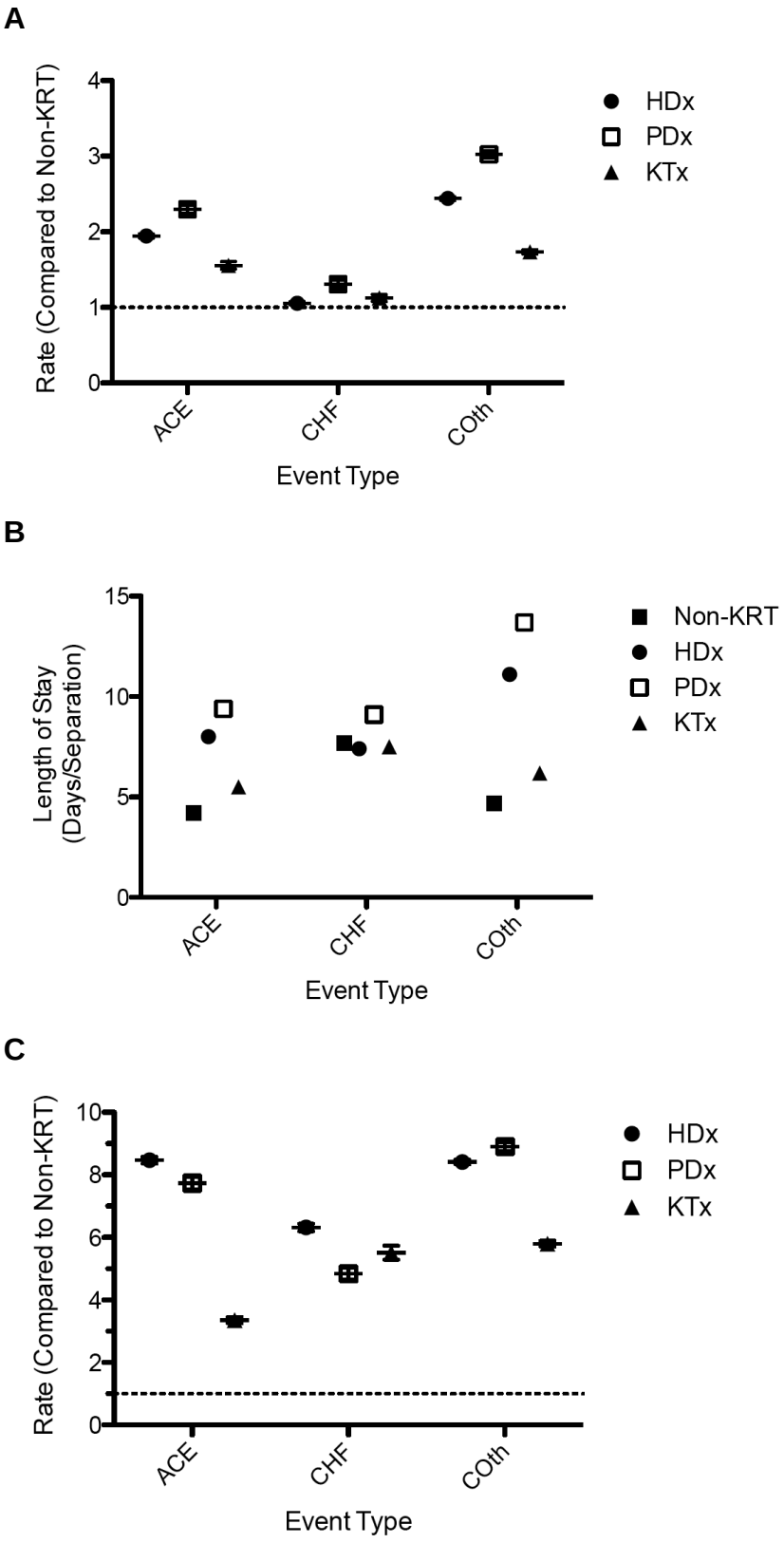


Figure 3.6: Length of stay per hospital separation, A – Relative rate compared to non-KRT; B – Unadjusted absolute rate, as days per separation; C – Relative rate, per person year, compared to non-KRT

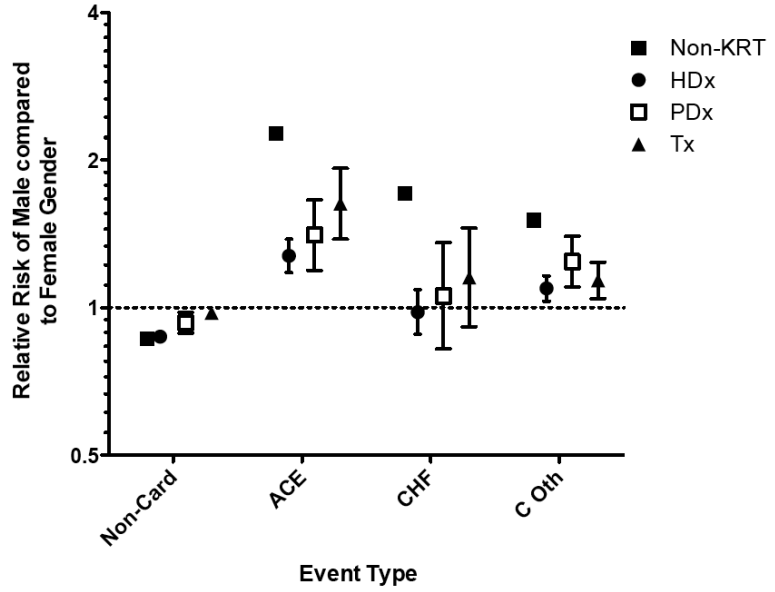
3.3.4 Gender effect

In the non-KRT population female gender had a greater relative risk of hospital separation for non-cardiac events (male to female RR 0.87 {0.86-0.87}), whereas male gender conferred a significantly greater risk for hospital separation for any of the three cardiac types (ACE 2.3 {2.3-2.3}, CHF 1.7 {1.7-1.7}, COth 1.5 {1.5-1.5})(Figure 3.7A)..

In the three KRT groups this gender difference was also observed, but to a lesser degree, for ACE (HDx 1.3 {1.2-1.4}, PDx 1.4 {1.2-1.7}, KTx 1.6 {1.4-1.9}) and COth (HDx 1.1 {1.0-1.2}, PDx 1.2 {1.1-1.4}, KTx 1.1 {1.0-1.2}) hospital separations. However, for CHF, there was no gender effect observed in any of the KRT groups (HDx 1.0 {0.9-1.1}, PDx 1.1 {0.8-1.4}, KTx 1.2 {0.9-1.5}) (Figure 3.7A).

For in-hospital death per admission, male gender was associated with increased risk for all event types in the non-KRT population (Non-CVD 1.8 {1.8-1.8}, ACE 1.9 {1.8-1.9}, CHF 1.7 {1.6-1.7}, COth 1.6 {1.6-1.6}). For Non-CVD or COth deaths, gender had no effect in any of the KRT groups. For ACE deaths, all KRT modalities demonstrated increased relative risk for male gender (HDx 3.5 {1.7-7.3}, PDx 4.0 {1.2-13.7}, KTx 13.7 {4.0-47.5}). For CHF, whilst male HDx (3.9 {1.6-9.3}) and KTx (17.7 {3.9-79.1}) recipients had a significantly increased risk of in-hospital death compared to females, no such difference between genders was observed in the PDx population (1.3 {0.9-2.0}).

A



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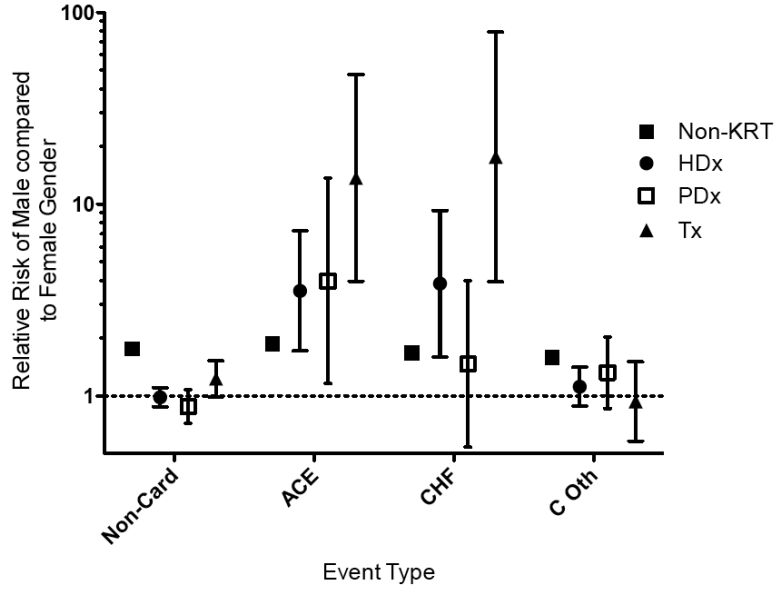


Figure 3.7: Age-adjusted association of gender (relative risk of male compared to female sex) for A – hospital separations by event type, and B – in-hospital death per admission by event type. Differences in patterns of effect of gender are observed between non-KRT and KRT populations, as well as between event types.

3.4 Discussion

CKD, ESKD and KRTs are all known to be associated with increased risk of CVD. The data we have presented highlights the different patterns and severity of CVD experienced by these patient groups compared the general population.

3.4.1 KRT versus non-KRT

KRT recipients had an increased frequency of hospital separation for all event types compared to the general population. Paralleling all-cause mortality in ESKD patients[14], the absolute rate of separations increased with age, whilst the greatest relative risk was seen in the younger age groups. Deaths per admission did no show the same pattern: CHF events were not associated with increased risk of death per separation, or clinically relevant increased length of stay, for any of the KRTs compared to the general population. However, given the increased frequency of separations for CHF, there was an increased overall rate of death in hospital per person-year for HDx and PDx recipients compared to the non-KRT group, but not KTx recipients.

Length of stay per separation was consistent with the above findings, with increased absolute unadjusted and relative length of hospital stay for all KRTs compared to non-KRT for ACE and COth. CHF events were not associated with clinically significant increased LOS (absolute or relative rate per separation) for either HDx or KTx recipients compared to the general population. PDx recipients did have longer LOS per separation compared to non-KRT when calculating both absolute and relative rates. This is not unexpected, given the greater burden of disease in PDx recipients compared to the non-KRT population. Others have also found a higher mortality rate from CHF in PDx compared to HDx recipients[441], which one might

extrapolate to include more severe disease requiring longer LOS for treatment. Increased overall frequency of events again lead to an increased relative rate of LOS per person year for all KRT groups compared to the non-KRT population.

Male gender had a significantly greater effect on increasing hospital separations for ACE and CHF for non-KRT subjects compared to KRT recipients. This difference was not observed when comparing in-hospital death per admission, where PDx recipients showed a significantly greater negative effect of male gender compared to non-KRT recipients.

3.4.2 Differences between KRT modalities

A consistent pattern of improved CVD outcomes accompanying better kidney function with transplantation was not observed. Whilst KTx recipients had lower rates of separation and length of stay per separation for ACE compared to the two dialysis modalities, no such differences were observed for CHF events. Increased relative rates of hospital separation for CHF compared to ACE were documented for all KRT modalities.

For in-hospital death due to CHF, adjusted relative risk per admission was similar for all KRTs. For ACE events, deaths per separation were lower in HDx recipients compared to PDx, with no difference observed with KTx. The effect of event rate however lead to a decreased rate of death per person year in the KTx group for both ACE and CHF compared to the dialysis groups. In regards gender, there was a trend towards greater negative effect of male gender on KTx outcomes from both ACE and CHF for hospital separations and in-hospital death.

3.4.3 Comparison with previous studies

Rates of prevalent and incident CHF post KTx have been investigated in a number of studies, however hospital separations, a key driver of resource utilisation and marker of morbidity, had not been included. A Canadian cohort study found significantly increased rates of *de novo* heart failure in KTx recipients, compared to the general population (1.26 events/100 person years, approximately 2-5x the Framingham rate[438]). From United States Renal Data Systems (USRDS) analyses, Lentine also showed a progressive increase in incidence of CHF post-transplant (up to 18% by 36 months), which decreased in prevalence below that of age/gender matched patients on the transplant waiting list after the first few months. The authors also showed female sex and older age at time of transplantation (adjusted hazard ratio 1.13 for females, and 2.49 for >60 years old) were associated with greater risk[442]. However, these studies reported on both inpatients and outpatients, with the former only reviewing patients greater than one year post transplant.

Severity of CVD type is difficult to compare between studies due to different criteria utilised, and reliance on doctors, nurses, and medical coders for diagnosis to be confirmed. However, our study is internally consistent, insofar as the coding for all groups was performed by the same process.

Further USRDS analysis demonstrated that up to 36% of those on dialysis had a diagnosis of CHF, with risk factors including age and female gender[5]. Our data do not show a negative effect of female gender on CHF outcomes, with this difference between US and Australian populations not able to be explained on the basis of the data available.

Harnett found that more than 30% of those commencing KRT had a diagnosis of CHF, and a further 25% had symptoms consistent with CHF after 41 months' follow-

up. Being diagnosed with CHF significantly increased the risk of death, with median survival decreasing from 62 to 36 months[443].

Our data was not able to clarify the interaction between ACE and future CHF events, but this has been identified as a likely pathway of disease progression[442].

Male gender as a risk factor for CVD has been documented for over 80 years[444]. This gender effect has also been observed for KTx populations[438, 445, 446], but not consistently in HDx recipients[447]. Our data demonstrated an increased relative rate of separation for all CVD events by male gender for non-KRT subjects, but significantly less gender effect for all the KRT modalities. For in-hospital death per admission, male gender had the greatest negative effect for KTx recipients for both ACE and CHF-related deaths. Overall numbers of deaths were low, hence wide 95% CIs, and no difference observed between non-KRT, HDx or PDx recipients.

There are several factors that may underlie the differing trends between KRT modalities for ACE and CHF separation and mortality rates.

1. Management of CHF and ischaemic heart disease differs significantly among KRT patients for a number of reasons:
    a. states of fluid excess or retention are associated with both HDx and PDx. Especially in HDx recipients, there is necessarily a fluctuating state of fluid overload, which also makes neutral fluid balance difficult to achieve due to weight gains between HDx sessions[37];
    b. due to the continuous nature of treatment and maintenance of residual renal function, ideal body weights may be more achievable on PDx, however over time there is a trend towards loss of control of fluid balance whilst on PDx[448];
    c. whilst potentially returning near normal kidney function, KTx is complicated by the addition of new risk factors including:
        i. organ rejection[38];
        ii. adverse effects of immunosuppressives[39];
        iii. hypertension[39]; and
        iv. infections, specifically CMV[40];
2. There is under-prescribing of pharmacologic and other agents for dialysis patients[449] (Beta-blockers[43], angiotensin receptor blockers[450], and implantable cardiac defibrillators[451] all show benefit in dialysis populations). Such sub-optimal medical management may lead to increased frequency of significant cardiac events;

3. Other strategies including cholesterol reduction with statin [41, 42] and combination agents[452], and treatment of homocysteine[453] show variable benefit in ESKD/dialysis groups compared to non-KRT populations;
4. In KTx recipients, calcineurin inhibitors such as cyclosporine or tacrolimus cause vascular constriction and reduced endothelial relaxation[454];

3.4.4 Limitations of the study

3.4.4.1 Disease definitions

Our data is based on ICD coding of separations, determined by hospital-based coders. There is no set criteria laid down in ICD-10, and coders are instructed to base their assignment on the diagnoses recorded in hospital notes. The definition of CHF in clinical and epidemiological studies can be problematic, as it is primarily a clinical diagnosis. In this setting, there is further variability as the hospital notes are recorded by a variety of medical staff, without standard criteria.

Unlike acute cardiac events, there is no clear clinical progression, nor robust biomarkers defining significant cardiac damage. N-terminal pro-brain natriuretic peptide (NT-pro-BNP) has been used in ESKD, but whilst predicting future CVD mortality, can be elevated in the absence of clinical CHF[455]. Efforts have been made to reach consensus on CHF diagnosis in research[456], but this has not yet been formalised. This is especially a problem in clinical trials, when attempts are made to compare studies. However, even with differing clinical criteria used, outcomes are often similar[457]. Further complexity may be present if the type of CHF is being defined, ie diastolic versus systolic disease[458]. However, these types are not distinguished in ICD-10, and thus this study included all “types” of CHF, as defined by coders.

Nevertheless, the use of billings or Medicare reporting of cardiac events has been investigated, and shown to be a robust and relevant measure of events[459], as has the validity of ICD-10 coding in Australia[460].

A recent systematic review of the validity of algorithms used to define CHF using various data sources found positive predictive values of >90%, using the

Framingham Heart Study criteria as the gold standard. The authors found that the highest positive predictive value and specificity was found in studies that used the ICD-9 code 428-X[461]. A number of corresponding ICD-10 codes were incorporated into this study[462]. As noted earlier, pulmonary odema was excluded as in this this group it may simply reflect to dialysis-related fluid overload rather than specific cardiac factors. For this KRT study, the frequency of CHF admissions is therefore likely to be an underestimate – some inherently cardiogenic causes of pulmonary oedema will have been excluded.

There are also possible issues relating to accuracy of the coding data. Deidentified data was provided by two reporting agencies (AIHW, ANZDATA), both of which are not externally validated for coding accuracy. Whilst this limitation must be acknowledged, these are the definitive national datasets in this area, both having a history of providing robust datasets, and are widely used in the published literature.

3.4.4.2 Out-of-hospital events

The nature of our study precluded examination of out-of-hospital events. Therefore we will have underestimated the CV death rate in each modality. Sudden cardiac death occurs with greater frequency in dialysis recipients compared to the general population[16, 463]. In Australia in 2007, approximately 14% of all dialysis recipients' deaths were attributed to cardiac arrest[464] – how many of these were out of hospital is not known.

3.4.4.3 Ethnicity

No information was available on factors including ethnicity, in particular indigenous vs non-indigenous, as coding for indigenous racial origin was considered unreliable. This is likely to be a confounding factor, especially given the higher CVD[465] and

ESKD[466] rates for indigenous Australians. This confounding effect will be greater for HDx, and less for PDx and KTx recipients given the lower use of these modalities among indigenous patients. Primarily, the effect will be greatest for the 40-60 year age group, where the excess risk of both cardiac and renal disease is greatest among those of indigenous origin.

3.5 Conclusions

- KRT recipients all have increased hospital separation rates for all CVD types compared to an age and gender matched general population, with increased rates of CHF separation compared to ACE
- The severity of a specific CHF event leading to admission, as measured by in-hospital death and LOS, was similar between both KRT and non-KRT subjects
- Differences in patterns of CVD were observed between KRT populations. In particular, KTx did not confer an in-hospital mortality advantage over those receiving HDx or PDx
- The increase risk associated with male gender was lesser among KRT patients, for all cardiac separation types.
- Given the hospital-based design of the study, these results have both clinical and financial implications to the delivery of services to KRT patients with CVD
- These results highlight the broad burden of CVD in this population group, but do not provide further information on the specific aetiology of the disease seen

Chapter 4: Endothelial progenitor cell identification, culture and enumeration – development of methods

4.1 Introduction

This chapter describes how methods were developed to address existing inadequacies in the enumeration, isolation and culture of EPCs for use in the clinical and research setting.

4.1.1 Significance and clinical role of EPCs

As described in chapter one, bone marrow-derived EPCs have a role in maintenance of normal vascular function[198, 247, 467], can be used to stratify CVD risk [337, 339, 468], and can be infused to improve vascular function in different clinical settings [388, 469-471].

4.1.2 Pitfalls in use of EPCs

Processing and identification of EPCs for use in research and the clinical setting has been hampered by the lack of a single surface or other marker to unambiguously identify these cells. As a consequence, there has been no consensus reached on the techniques used for EPC isolation, identification, or culture[472]. Combinations of surface markers, with functional testing (given the different capabilities of early and late circulating EPCs[259]) can aid in classification. Until a universally accepted cell process technique, combination of markers, or unique new marker is developed, authors will be required to clearly document what type of cells are being identified as EPCs. Without such structure, monocyte, macrophage and mature EC lines may inadvertently be included in analysis.

Given the different capabilities or early and late maturity EPCs, researchers may also choose to focus on a specific subpopulation, depending on the effect or interaction being investigated.

4.1.3 Methodological aims

The aims of this project were

- To identify a reliable source of human MNCs, from which viable EPCs, of sufficient number for clinical and research use, could be isolated
- Standardise EPC separation protocol
- Standardise EPC identification, including functional assays
- Standardise EPC culture, particularly the use of additional cytokines, and plating techniques, to maintain an early EPC phenotype

Immature EPCs were targeted, due to their greater paracrine, pro-angiogenic capacity[472]. These cells appear early from non-peripheral blood EPC sources, and are characterised by specific functional capabilities (see Figure 1.3) compared to their matured counterparts.

Strict isolation and culture techniques were also investigated to avoid cross-contamination with both later stage EPCs and other cell types.

4.2 Methods

Full methods are described in Chapter 2. Figure 2.1 graphically demonstrates the steps followed, and the different cellular and technique variables investigated.

4.2.1 EPC sources

EPCs have been isolated from multiple human sources, including PB[198], BM[247], UCB[473], heart and skeletal muscle, adipose tissue[371], spleen[474], small intestine[475] and vascular adventitia[476].

To identify the best source of EPCs, we decided to investigate BM, leukapheresed PB, normal PB, and UCB as potentially relevant sources of EPC for clinical testing: these were all relatively uncomplicated to access, and using healthy donors, also allowed for samples to be collected at specific times to aid standardised cell preparation. PB EPC counts vary in a diurnal pattern[229], thus all samples were collected before 10am.

4.2.2 Separation protocols

The technique chosen for EPC isolation has a significant impact on the type of EPC obtained. Focusing on differing combinations of cell surface markers may result in greater or lesser proportions of so-called early EPCs, “late outgrowth” EPCs, haematopoietic stem cells, monocyte/macrophage phenotypes, or even mature endothelial cells[255, 257-261, 477]. The clinical implication of this is significant, given the broad functional characteristics of each group (see Figure 1.3 regarding differences between early and late EPCs and mature ECs).

4.2.2.1 Cell adhesion

Fibronectin and collagen have been the most commonly used plating agents for EPC culture, including unsorted MNCs and surface-marker isolated EPCs. In particular, many therapeutic trials have used fibronection-adherent populations. These adherent populations contain no CD133 positive cells, but instead monocytes and macrophages[271], or mature EC phenotype cells[478]. These cells do not have the paracrine effects of early EPCs, which do not adhere to fibronectin[267]. Adherent cell populations produce colony-forming units, which are potentially an indirect marker of EPC function (as a gross representative of overall angiogenic capacity within an individual), but do not correlate with PB EPC counts, nor VEGF serum concentrations[271]. This study utilised non-adherent cells.

4.2.2.2 Cell surface marker-dependent isolation

Fluorescence activated cell sorting (FACS), or magnetic bead separation can achieve more specific isolation of EPCs. Both rely on appropriate selection of cell surface markers for isolation and invariably use CD133, CD34 or VEGFR2. FACS gives high purity, but increased cell death due to the mechanics of acceleration/deceleration through the flow stream. Bead separation is inherently more gentle on cells, but potential for contamination with other cell types is high[269]. Both techniques were assessed.

4.2.3 EPC identification

A combination of CD133 alone, or CD133 and CD34 dual positivity was used for EPC isolation. EPC identification was confirmed by triple-positivity for CD34, CD133 and VEGFR2. CD45 was investigated to ensure reduced haematopoietic cell contamination. Mature endothelial cells were identified by presence of VE-Cadherin and PECAM-1.

4.2.4 EPC culture

Irrespective of isolation or culture conditions, EPC culture protocols have been unable to maintain expression of CD133 beyond 5-7 days. Loss of CD133 expression is consistent with maturation of EPCs from an early to late phenotype. Up to 50 or more cell doublings are possible from this point, but with progressive maturation of EPCs to outright endothelial cells[272]. As described in Chapter 1.6.2, EPCs are under the influence of numerous cytokines and other variables, including HIF-1∝, VEGF, EPO, SDF-1, and G-CSF. Use of these agents, whilst ensuring survival of EPCs, will also promote their maturation.

There is no unified commercial kit recommended for EPC culture, however Media 199 with antibiotics[339], Stemline II (Sigma-Aldrich)[272] and Endothelial Growth Media-2 (EGM-2)+ BulletKit cytokines (Lonza)[479] have been used.

This study investigated two cytokine combinations for culture, as well as the use of Media 199 vs EGM-2 with Bulletkit cytokines.

The cytokine combinations were based on personal correspondence (Dr C. S. Bonder with Prof T. Asahara and Prof H Masuda, Tokai University, Japan, 2007), publication by Masuda[480], and a publication by Liu *et al*[418], and chosen as they both contained combinations of cytokines known to be important to EPC survival, maturation, release and homing *in vivo*. Neither had previously been used in combination with EGM-2 + Bulletkit.

4.2.5 EPC functional assays

Functional capacity of EPCs was examined by ability to

- Form vascular structures in Matrigel
- Migrate across an angiogenic cytokine gradient (VEGF)

4.3 Results

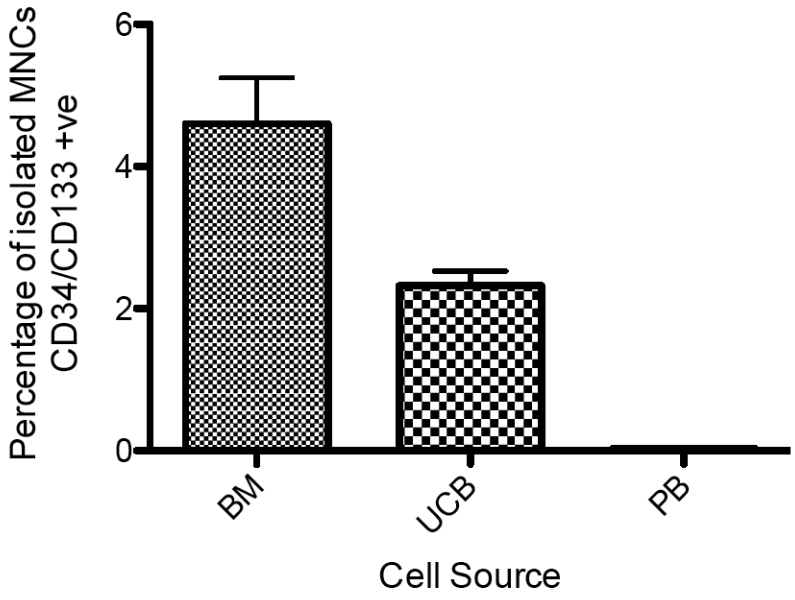
4.3.1 EPC isolation

Initial cell isolation was performed using CD34/133 labeling, and FACS, with BM MNCs. Purity of samples was confirmed at >90% dual positive for CD34/133 post-sort (by FACS analysis). However, given that CD34 is also expressed by mature ECs and to distinguish EPCs from ECs and in the interest of enhanced cell survival during the isolation process, all further EPC isolation was performed by autoMACs, utilising CD133 labeling alone. It has been previously shown that all CD133 positive cells also co-express CD34 from bone marrow[208].

4.3.2 Mature EPC cell surface markers

4.6% of BM MNCs were dual positive for CD34 and CD133, compared to 2.3% for UCB and 0.03% for PB MNCs (Figure 4.1). Leukapheresed blood did not contain sufficient viable CD133 positive cells, and were excluded from further analysis (n=4). This was presumed to have occurred as samples were predominantly from subjects with haematological malignancies, and hence had abnormal blood films. EPCs have been collected by apheresis techniques from such patients in the past, but only after G-CSF mobilisation[481]. Given low concentration of EPCs in PB (0.03%, n=6), consistent with previous literature[199], and limited blood volume collected (~10-20ml), PB samples were only used for enumeration, and not culture, of EPCs.

A



B

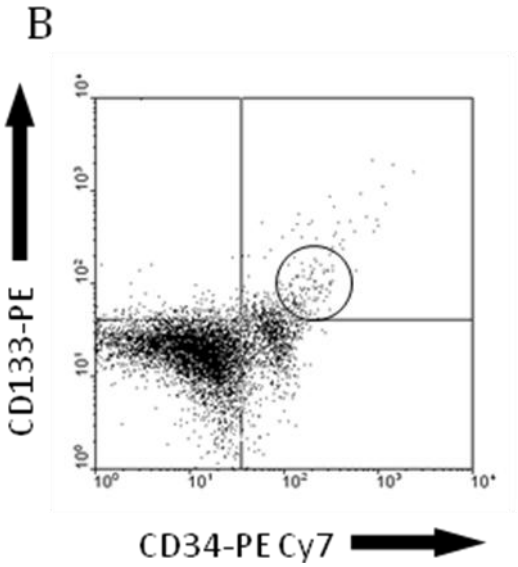


Figure 4.1:A - Percentage of cells dual positive for CD133 and CD34 at time of sample collection, from BM (n=9), UCB (9) and PB (6). A significant difference in percentage of MNCs dual positive for CD34/CD133 ($p<0.01$) was observed between all three groups. B – Representative dot-plot of CD34/CD133 positive MNCs from UCB. Less than 0.5% of all cells were CD133+/CD34-. 3.2% of MNCs were CD34+/CD133+, of which 1.5% from this sample were considered a discrete population.

Approximately 87% (n=3, 68-100%) of all CD133 positive cells were positive for CD34. Concurrent CD133/VEGFR2 positivity was >95% (n=3, 93-100%). (Figure 4.2). Given these preliminary results, EPCs isolated for *in vitro* testing were isolated by CD133 expression alone. CD45 staining was dim for all EPC sources compared to PB MNCs, and was not used for analysis (Figure 4.3).

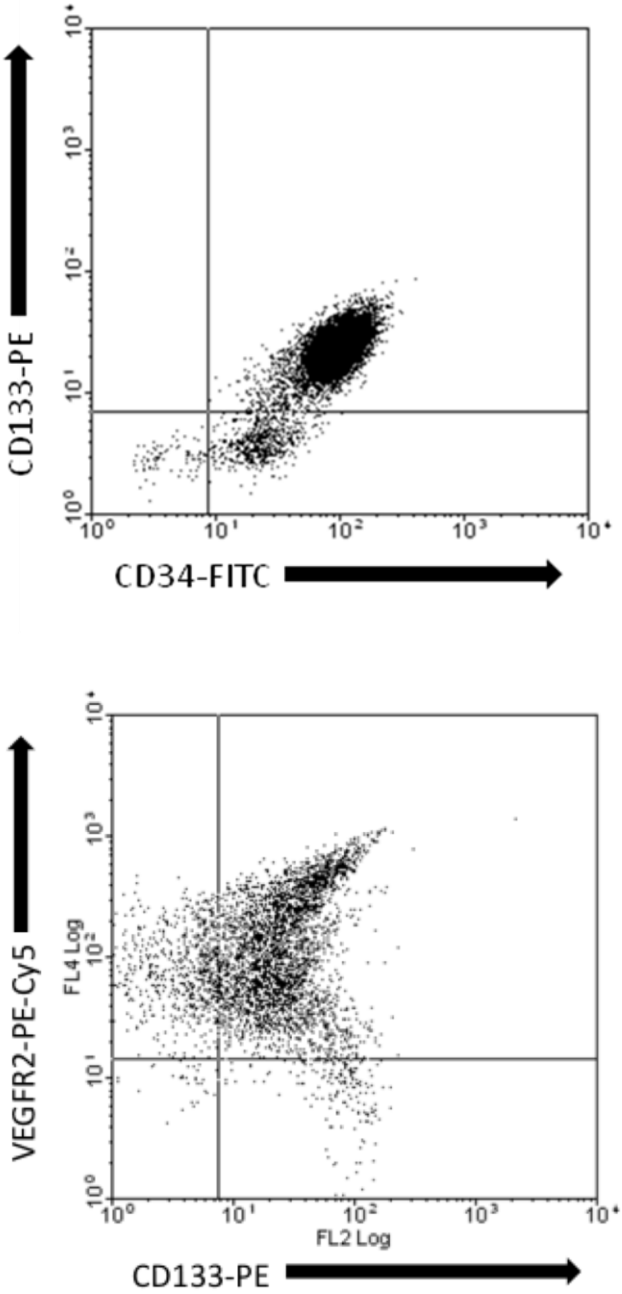


Figure 4.2: Dot plots showing post sort EPCs dual positive for CD133 and CD34 (n=3, 87% {68-100%}), as well as CD133 and VEGFR2 (n=3, >95% {93-100%}). Plots from UCB EPCs, but indicative of BM EPC.

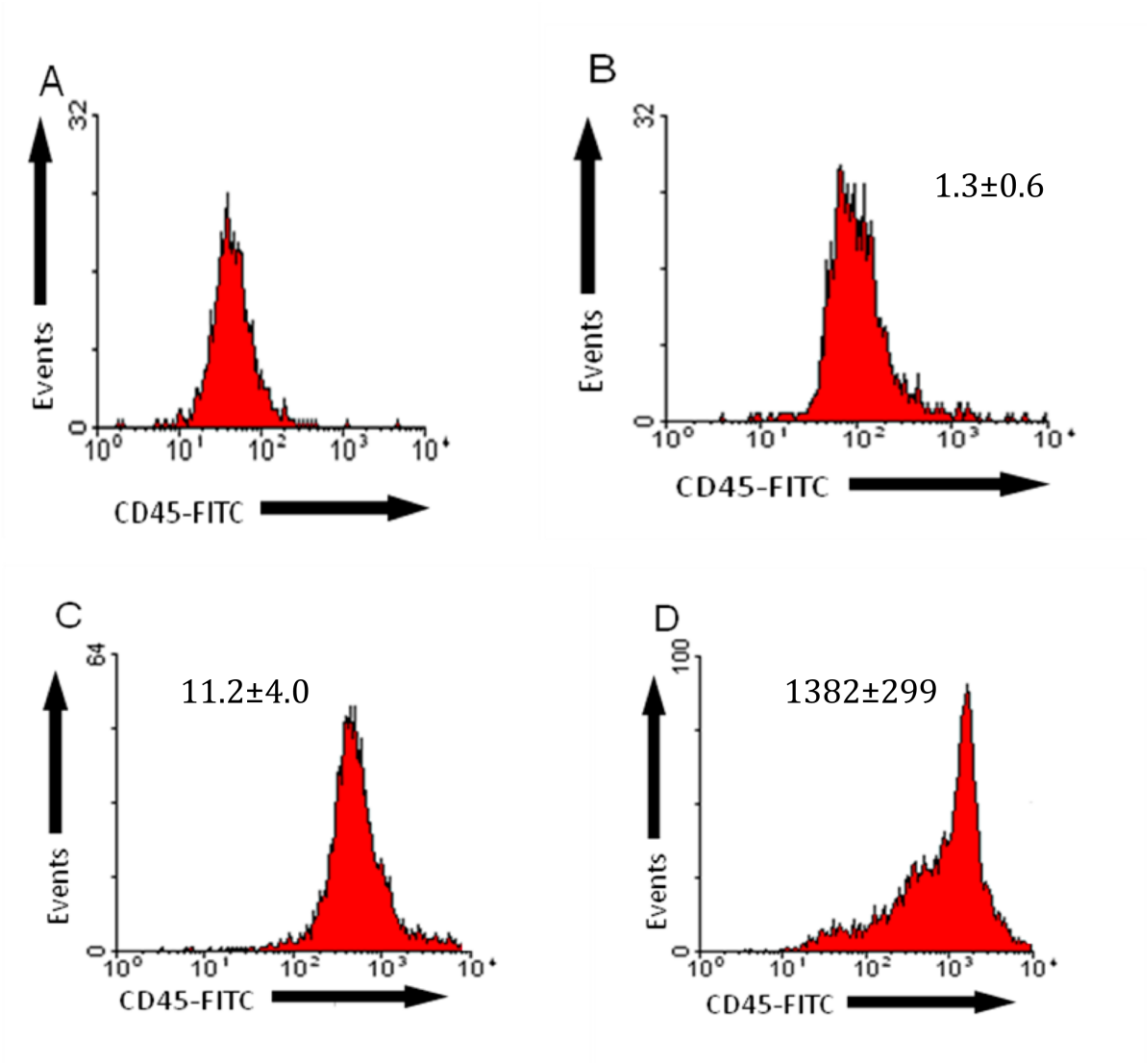


Figure 4.3: Representative CD45 expression from: A – no antibody control; B – HUVEC; C - non-adherent EPC; D - PB MNCs. EPCs demonstrated $CD45^{low}$ expression compared to PB MNCs. Representative figure of n=3, with ratio of sample MFI : no antibody control MFI ±SD, one-way ANOVA $p<0.05$.

4.3.3 Mature EPC/EC surface marker expression

Using EBM-2 + BulletKit + FCS/IGF-1/FGFb/VEGF/AA on fibronectin, BM EPCs were able to be grown out to day 12 (full protocol detailed in Chapter 2.5.2). Non-adherent cells were replated every two to three days in fresh media. There was a progressive increase in CD133 and CD34 expression to day 6 (Figure 4.4). Mature EC markers VE-cadherin and PECAM did not significantly increase in expression over the initial 6 days (Figure 4.5).

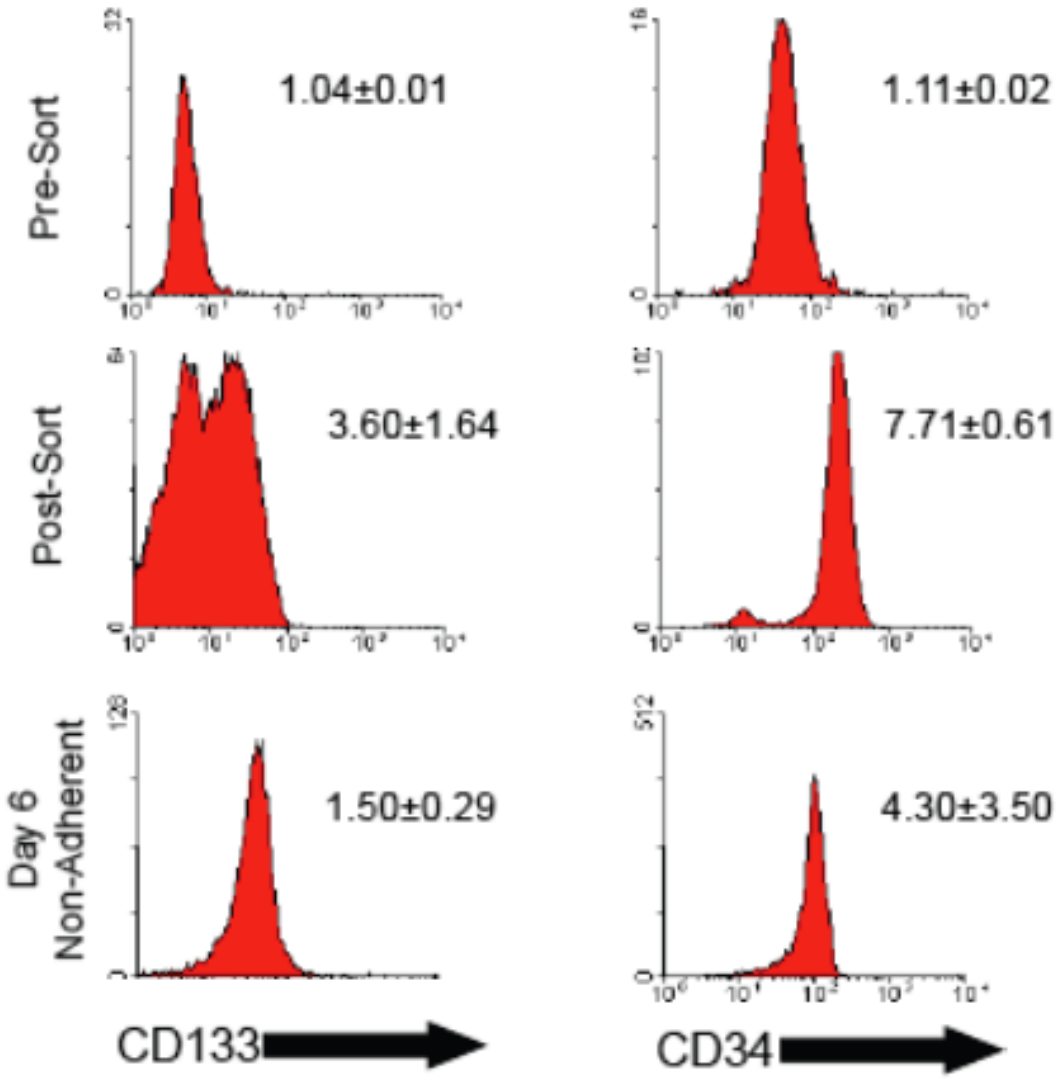


Figure 4.4: Increased expression of CD133 and CD34 from day 0 to day 6 by BM-derived EPCs isolated by AutoMACS and cultured on fibronectin with EGM-2 + Bulletkit + FCS/IGF-1/VEGF/FGFb/AA. (see Chapter 2.5.2). Ratio sample MFI : no antibody control MFI ± SD shown, with representative plots of n=3.

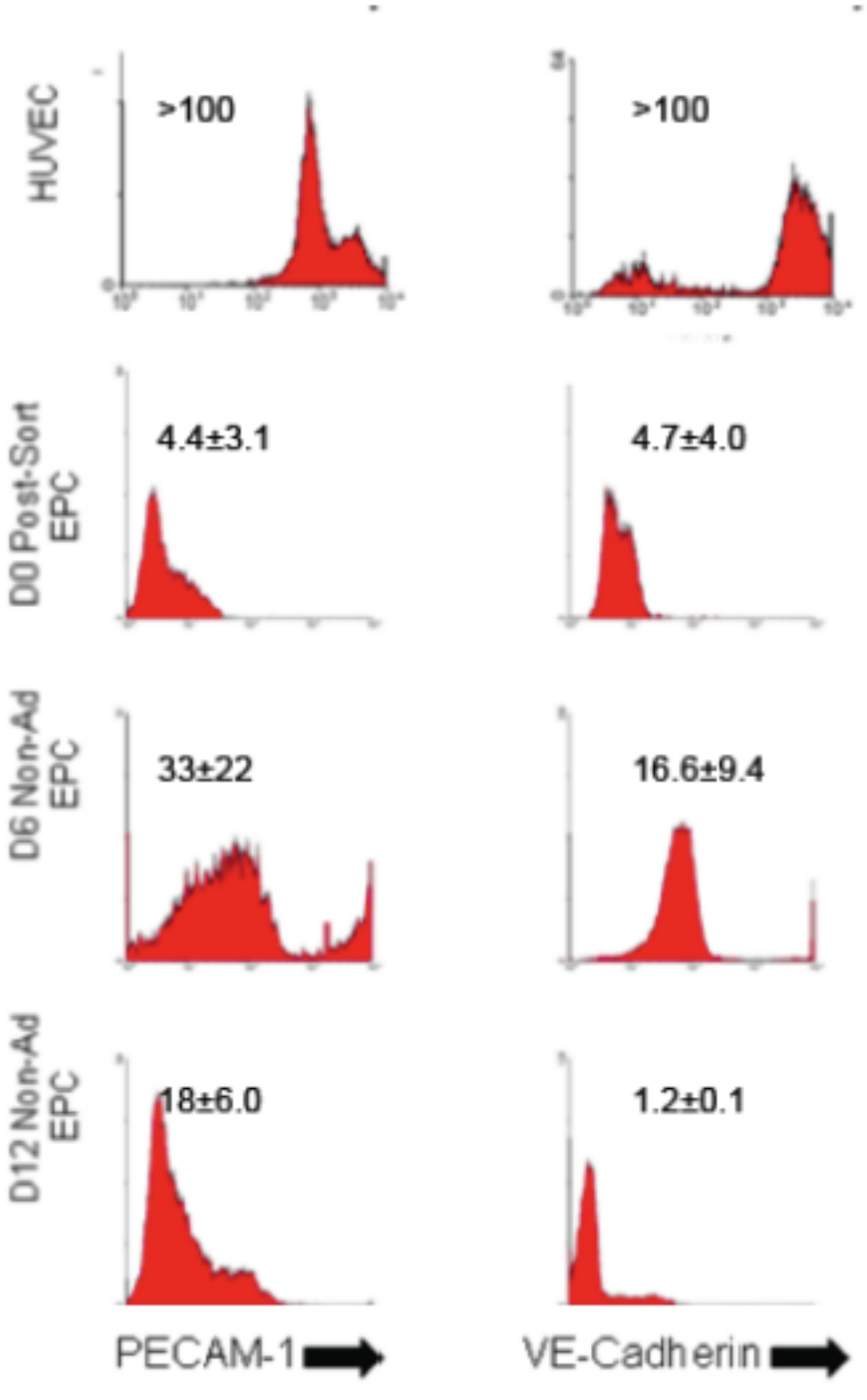


Figure 4.5: Expression pattern of PECAM-1 and VE-Cadherin by BM-derived EPCs isolated by AutoMACS and cultured on fibronectin with EGM-2 + Bulletkit + FCS/VEGF/IGF-1/FGFb/AA (see Chapter 2.5.2), as compared to unstained D0 EPC and HUVEC (passage 2) positive control. Both PECAM-1 and VE-Cadherin expression on EPC increased at day 6, but not to levels of HUVEC, then returned towards D0 levels by D12 (n=2). Representative plots of repeated experiments, with mean of ratio sample : no antibody control MFI ± SD.

4.3.4 Adherent and non-adherent EPCs

Approximately one third of cells in EPC culture were adherent after 12-14 days of culture in EGM-2 + BulletKit + additional cytokines (n=2 each for BM and UCB). As demonstrated in Figure 4.6, adherent EPCs demonstrated increased expression of mature EC surface markers PECAM-1 and VE-Cadherin, as well as reduced expression of CD133 (Figure 4.7).

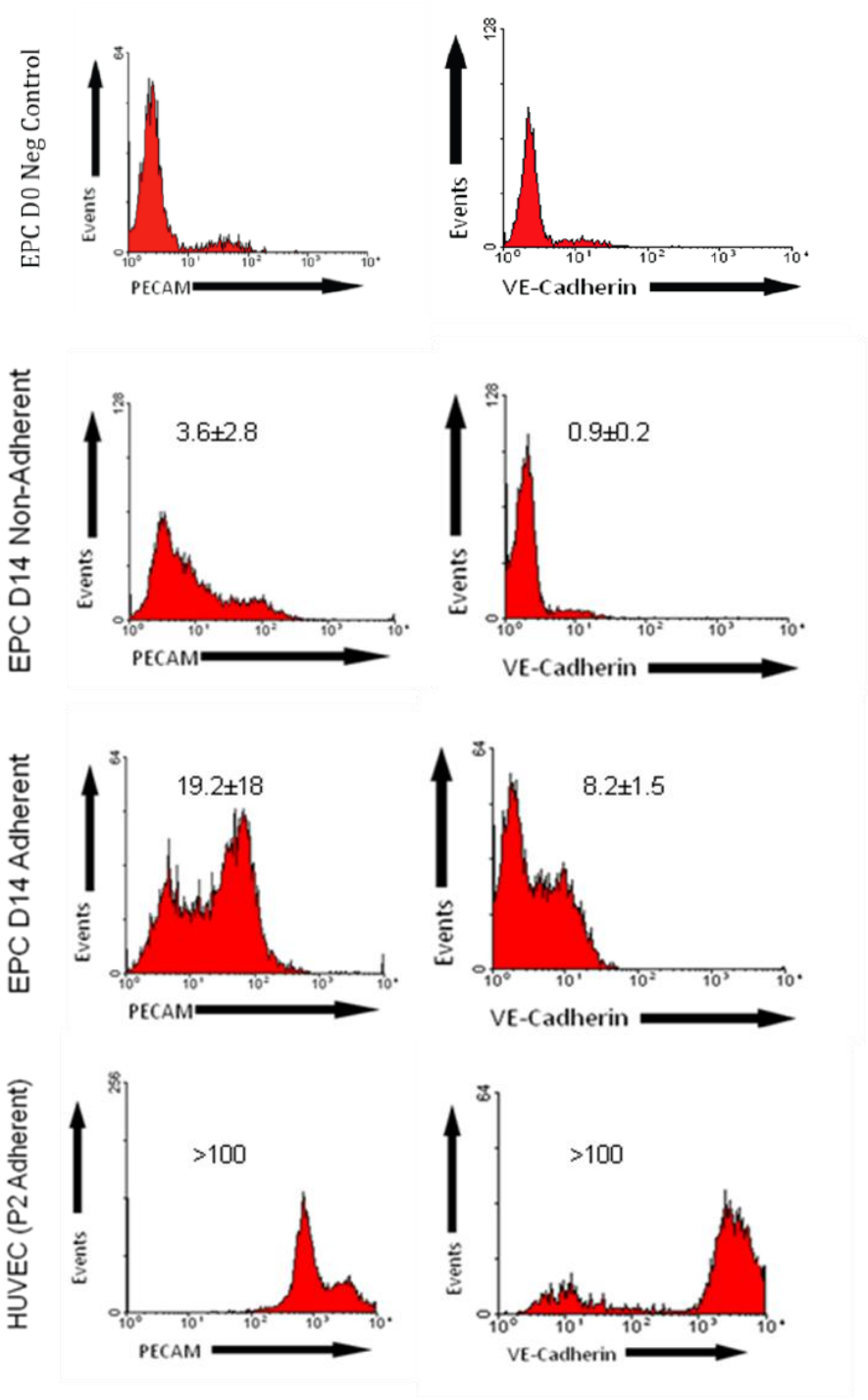


Figure 4.6: Expression of PECAM-1 and VE-Cadherin on adherent and non-adherent EPC in culture post-sort for 14 days. Adherent EPCs demonstrate increased expression of both PECAM-1 and VE-Cadherin compared to non-adherent EPCs, but not to the same degree as HUVEC (representative of UCB and BM EPCs, n=3, ratio sample MFI : no antibody control MFI ± SD).

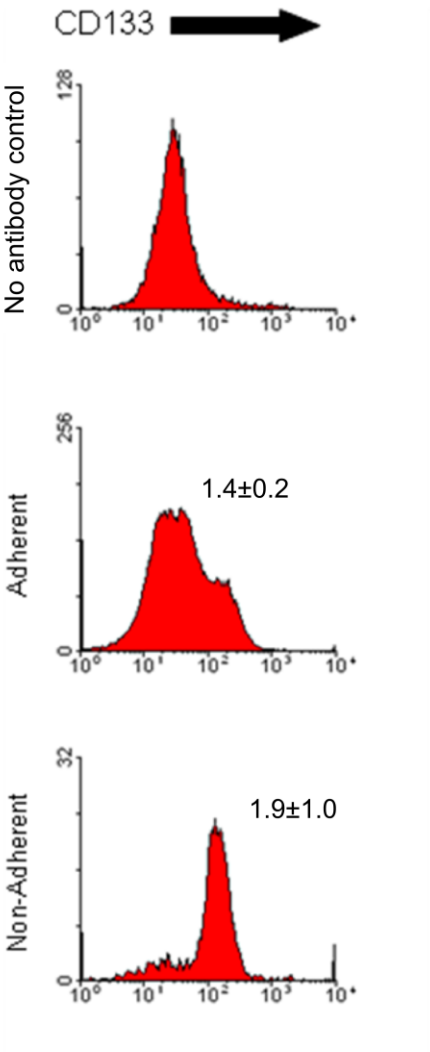


Figure 4.7: Comparison of CD133 expression between adherent and non-adherent EPCs cultured on fibronectin to day 12-14. Adherent cells separated at time of media changes, and maintained in same media type. CD133 expression remained increased in non-adherent cells (representative of UCB and BM EPCs, n=3, ratio sample MFI : no antibody control MFI ± SD).

4.3.5 Relationship between peripheral blood and BM-EPC counts

There is no literature on the association of EPC PB counts and other cell types or haematological parameters. We made comparison between PB examination of haemoglobin, total white cell count, and white cell differentiation, to BM-EPC counts. By linear regression, an association was found between concentration of peripheral blood monocytes (standard diagnostic test, as measured by flow cytometry: forward and side scatter, and lateral fluorescent light, on the Sysmex XE-2100 (Sysmex)[482]) and BM-EPC concentration (n=12, R^2 0.43, p=0.02, Figure 4.8). BM-EPCs were defined as either CD133 alone, or CD34/133 dual positive.

No association was found between BM-EPC counts and any other peripheral blood cell concentration, apart from monocytes.

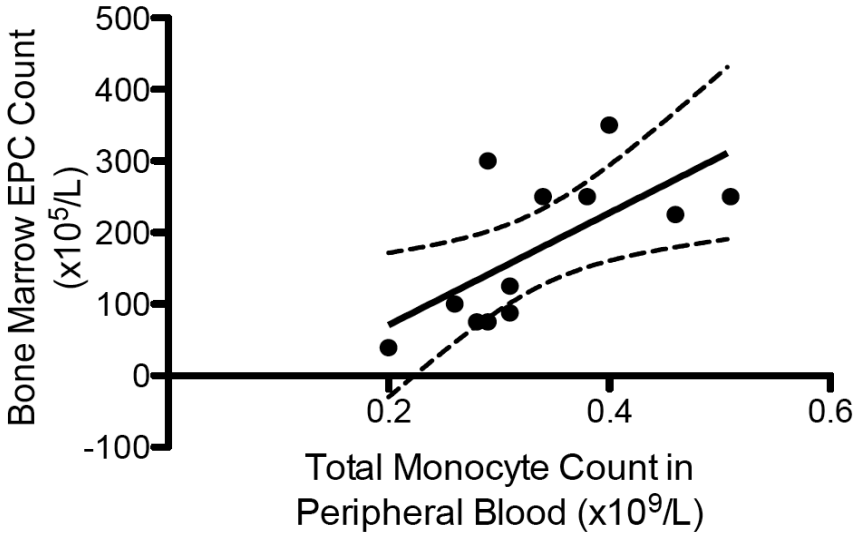


Figure 4.8: Linear regression analysis of relationship between PB-monocytes (as measured by flow cytometry: forward and side scatter, and lateral fluorescent light, on the Sysmex XE-2100) and BM-EPCs, defined as either CD133 positive alone, or CD34/133 dual positive by FACS analysis. n=12, R^2=0.43, p=0.02

4.3.6 Microscopic appearance

Microscopically, both BM and UCB EPCs cultured in EGM-2 + BulletKit + FCS/IGF-1/VEGF/FGFb/AA plated on fibronectin (+ Liu protocol media) appeared confluent, rounded and autofluorescing, as well as non-adherent. This was in contrast to the spindle shape of mature, adherent ECs. Adherent EPCs demonstrated an intermediate pattern, which included tubular shaped structures at day 12 (Figure 4.9).

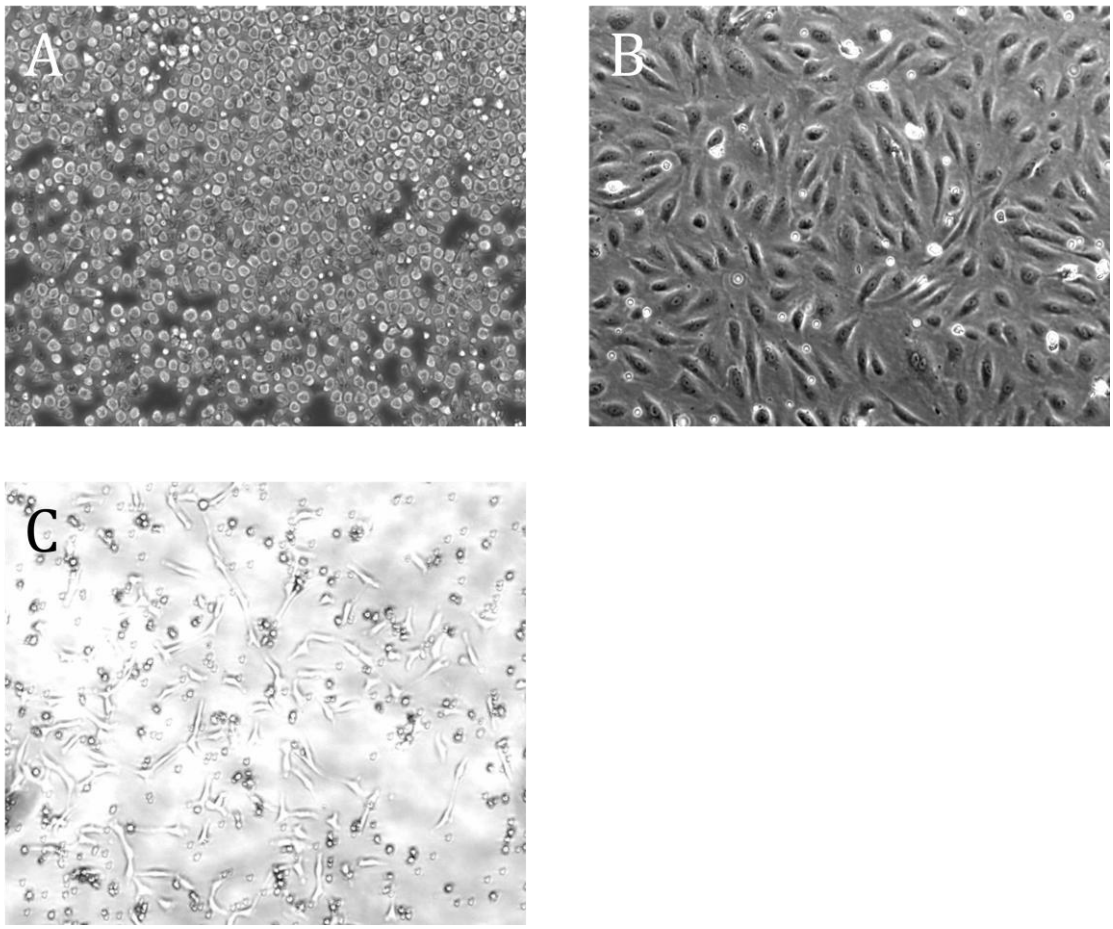


Figure 4.9: Comparison of (A) cultured, non-adherent, autofluorescing BM EPC (mag 20x, on fibronectin, cultured in EGM-2 + BulletKit + FCS/IGF-1/VEGF/FGFb/AA), and (B) passage 2, adherent HUVECs (mag 20x, on collagen, HUVE Media) demonstrating classic spindle-shaped pattern. In (C), Adherent BM MNCs post CD133 EPC sort develop an intermediate phenotype with tubular structures at day 12 (10x, on fibronectin, EGM-2 + BulletKit + FCS/IGF-1/VEGF/FGFb/AA).

4.3.7 Cell expansion in culture

All cell expansion observed occurred with EGM-2 + BulletKit + FCS/VEGF/FGFb/AA/IGF-1. No EPC survival was seen beyond day 3 with any of

- Media-199 +/- VEGF/ECGF
- EGM-2 + BulletKit
- EGM-2 + BulletKit + VEGF, TPO, rhIL-6, Flt-3 lig, SCF, TGFβ-R1 inhIII

Maximal cell expansion occurred for BM-derived EPC compared to UCB sources ($p=0.01$; BM 1700±1100%, UCB 520±420%). However, there was also a significant decline in cell numbers in culture for both sources by day 12 ($p<0.01$; BM 470±1000%, UCB 40±30%) (Figure 4.10).

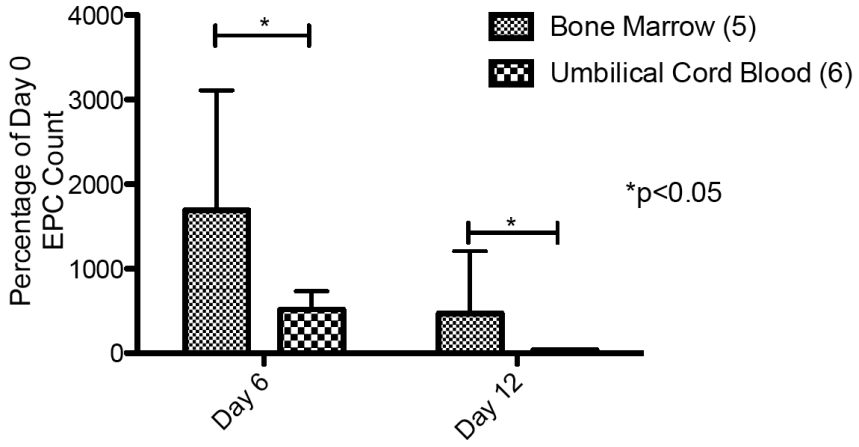


Figure 4.10: EPC growth in EGM-2 + BulletKit + FCS/IGF-1/VEGF/FGFb/AA on fibronectin, at day 6 and 12 post isolation. BM EPCs show significant ($p=0.01$) growth advantage over UCB *in vitro* at both day 6 and 12, but with maximal expansion at day 6. There was a significant decline in EPC count from day 6 to 12 for both cell sources ($p<0.01$).

4.3.8 Tube formation

To investigate the functional capabilities of the expanded EPCs *in vitro*, tube formation was tested. As shown in Figure 4.11, UCB EPCs incorporated into tubular structures formed by HUVEC in Matrigel (using a ratio of $3x10^5$ HUVEC : $5x10^4$ EPC). At this ratio, there was a non-significant increase in number of tubes formed in Matrigel at 7 hours (HUVEC alone 189 tubes, 95%CI 123-254; HUVEC+EPC 199, 118-280; n=6, paired T-Test p=0.7). There was also no difference in maximal number of branches produced (HUVEC alone 4.3 branches, 2.9-5.8; HUVEC+EPC 3.2 branches, 2.5-3.9, n=3, p=0.12).

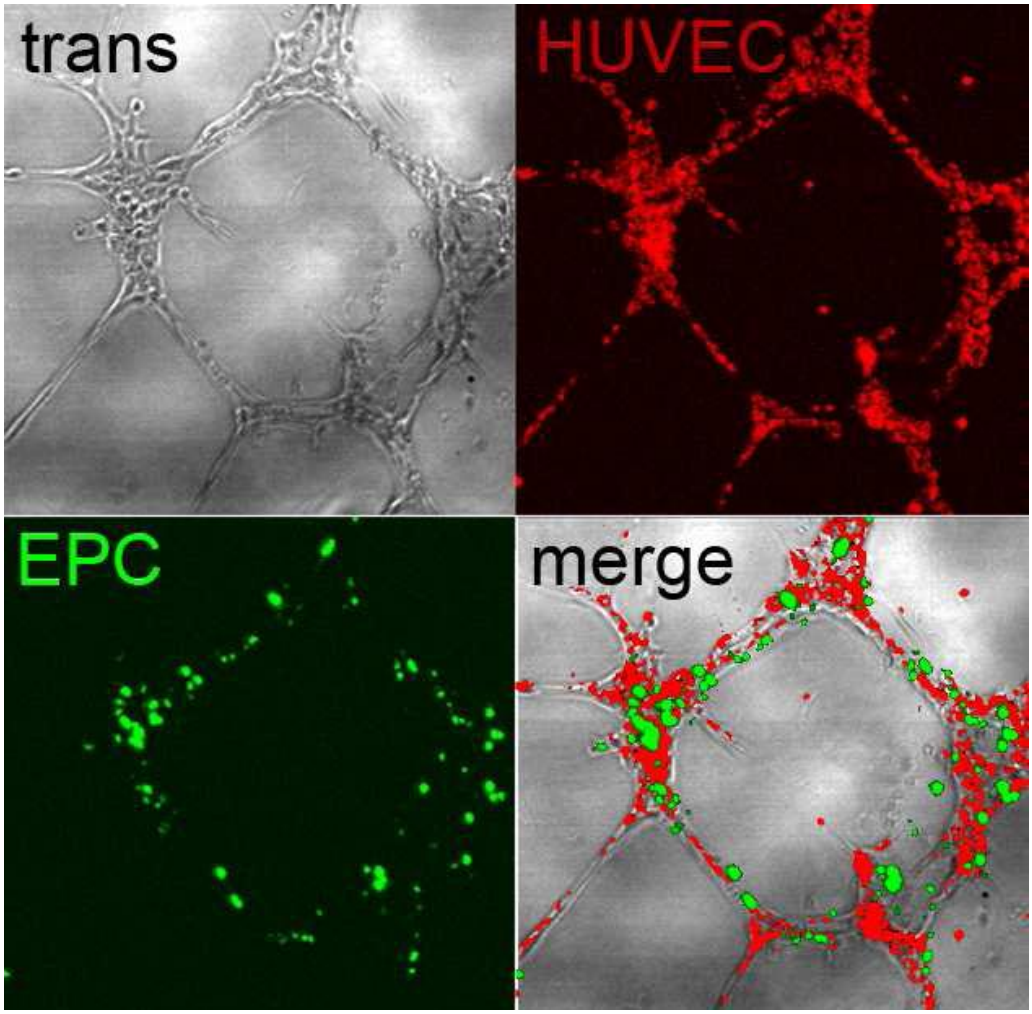


Figure 4.11: Merged image of CDFA (green) labeled Day 6 EPCs co-localising to DiL-Ac-LDL (red) labeled HUVEC, which have formed tubular structures at 6 hours in Matrigel (10x magnification).

EPCs cultured to day 6 from any source (BM or UCB), were however not able to forms tubes in Matrigel on their own. Only one sample of BM-EPCs, cultured to day 12, successfully formed tube-like structures at 10-24 hours, which persisted to 36 hours. This is in comparison to passage 2 HUVECs, which reached maximal tube formation by 6-8 hours in Matrigel, and disintegrated by 24 hours. Tubes appeared similar between HUVECs and EPCs (Figure 4.12).

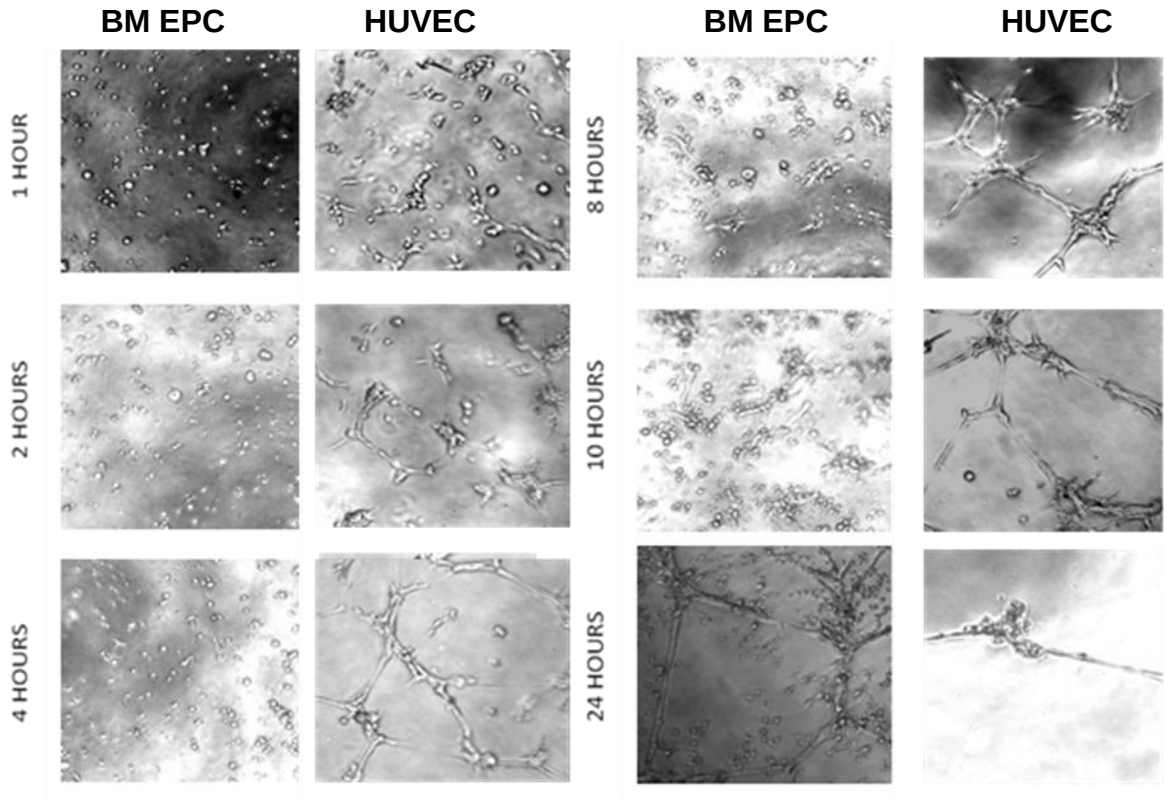


Figure 4.12: Comparison of Day 12 cultured BM-EPC and Passage 2 cultured HUVEC forming tubes in Matrigel assay (20x magnification). HUVEC formed tube-like structures within 2-4 hours, with maximal tube numbers by 8-10 hours, and disintegration over the next 12 hours. Day 12 BM EPC formed tubes after 10 hours of incubation (37^{o}C, 5% CO_2), with maximum number at 24 hours. Tubes persisted to 36 hours (not shown). Tubes were not able to be formed with cultured BM EPC from earlier time points, or UCB-derived EPC.

4.3.9 Migration

Further functional testing was performed utilising an 8 micron pore Transwell, to show the ability of cultured cells to move across the membrane, with or without a 100ng/ml VEGF gradient. The assay was optimised for HUVEC, which readily crossed the membrane, with increased numbers of cells migrating in response to the VEGF gradient – counts were made as average number of cells per three 40x magnification microscope fields after 2 hours incubation (HUVEC Control 38 cells, 95%CI 0-76; with VEGF 54 cells, 9-99; n=4, paired t-test p=0.051). For EPCs, fewer cells migrated in the time frame, with a significantly limited response to VEGF (EPC Control 5 cells, 0-10; with VEGF 8 cells, 0-16; n=3, p=0.22). Figure 4.13 demonstrates DAPI stained HUVEC and EPC that passed through the transwell, and were remaining within the fibronectin layer, as well as the quantified data. Due to cell availability, only UCB-EPCs were used for migration in further studies.

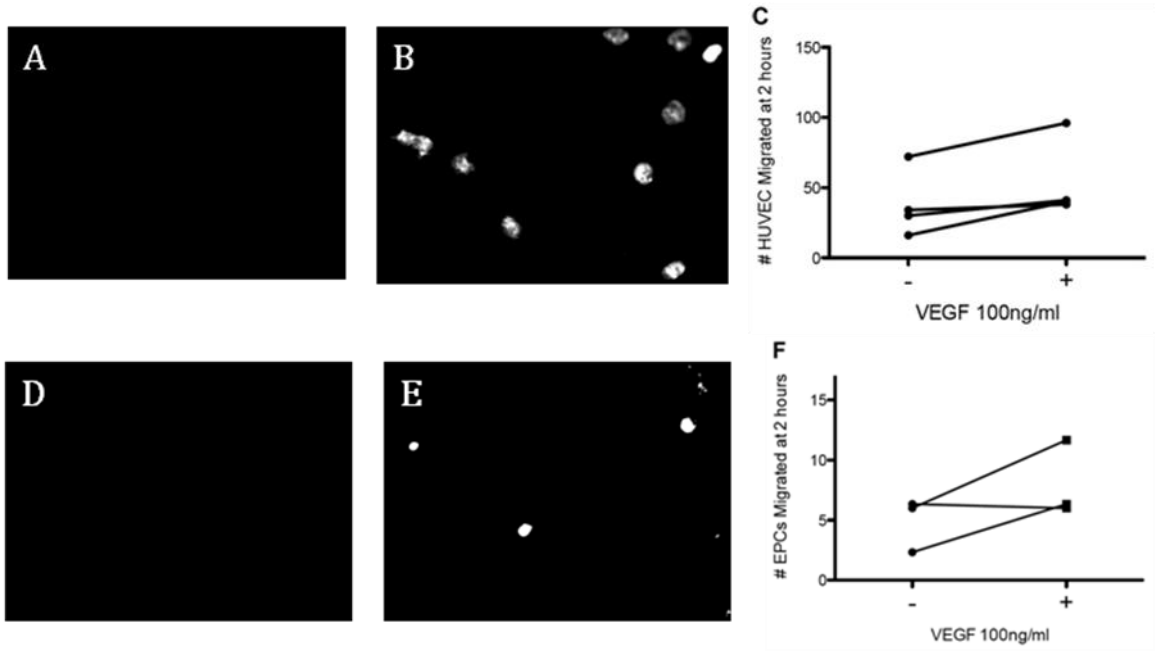


Figure 4.13: Cell migration through 8 micron pore Transwell with or without 100ng/ml VEGF gradient. HUVEC: (A) unstained transwell post migration (40x mag); (B) DAPI stained post migration without VEGF (40x mag); (C) qualitative analysis (paired t-test, p=0.051); EPC: (D) unstained transwell post migration (40x mag); (E) DAPI stained post migration without VEGF(40x mag); (F) qualitative analysis (paired t-test, p=0.22).

4.4 Discussion

We have demonstrated a protocol that enables isolation and expansion by up to 16-fold, of immature phenotype EPCs, to clinically significant numbers (~$1x10^7$ cells) by day 6 of culture, which remain functionally active.

Culture expansion of isolated EPCs was only possible using a combination of EGM-2 + BulletKit, with additional cytokines (FGFb, VEGF, IGF-1, AA) and FCS. This protocol allowed cell expansion with maintenance of cellular expression of CD133/CD34/VEGFR2 to day 6-7. The cytokines utilised are important for normal EPC release from BM *in vivo*, as well as homing and maturation. It is not unexpected, then, that use of cytokines such as VEGF would promote maturation *in vitro*. Cell death is therefore occurring as the new cytokine requirement of maturing EPCs is not met by the original media.

Functional studies confirmed that these cultured cells were biologically active, with ability to interact with ECs forming tubes, as well as migrate to VEGF. Inability to form tubes on their own is a characteristic of immature EPCs[267].

Compared to umbilical cord blood, initial EPC concentration was higher in BM[483], however function post culture was similar between the two groups.

Finally, an association was noted between peripheral blood monocyte concentration, and bone marrow EPC concentration. This is a novel finding, and potentially could be used to screen individuals who are to donate marrow for therapeutic purposes.

4.5 Summary

- EPCs could be isolated at higher concentration from BM rather than UCB.
- There was no difference in the pattern of phenotype, maturation or function of EPCs from UCB or BM.
- Using EGM-2 BulletKit with additional FCS and cytokines (VEGF, FGFb, IGF-1, AA), isolated EPCs could be cultured to 6 days without losing CD34/CD133/VEGFR2 expression.
- Day 6 cultured EPCs could be used for *in vitro* studies.

Chapter 5: *In vitro* Studies of Uraemic Toxins

5.1 Introduction

CKD is associated with increased morbidity and mortality related to CVD. CKD is a unique environment as classical risk factors underestimate the CVD risk, not all therapies used in the general population are effective, and the pattern of CVD itself is altered.

We demonstrated in Chapter 3 that there are significant differences in rates of hospital admission and death related to CHF and ACE between the general population and KRT recipients, with further differences between those on dialysis and KTx recipients.

Based on the current literature, it is believed that the increased incidence of CHF in ESKD is related to endothelial dysfunction[274]. A number of factors have been identified as potential causative agents, including toxins found at increased concentration in uraemia, and dysfunction of EC repair mechanisms, specifically related to the dysfunction of EPCs.

PC(4-methylphenol, $CH_3C_6H_4OH$, 108Da), is a product of bacterial fermentation of L-tryptophan in bowel[57], and is partially conjugated to PCS (>15% of PC is unconjugated in HDx subjects[58]) at the colonic brush border[59].

IS, ($C_8H_6NO_4SK$, 251.3Da), is a member of the indole group, originating from the bacterial breakdown of the amino-acid tryptophan derived from diet[77]. Indole is metabolised in the liver to IS[78].

The rationale for investigating these toxins was based on the following:

- *In vitro*:

    - PC inhibits EC proliferation[67] whilst PCS also directly damages ECs [66].
    - IS is associated with increased production of reactive oxygen species[79, 80], activation of smooth muscle cells (SMC)[81, 82] and osteoblast resistance to parathyroid hormone (PTH)[83].
- *In vivo*, increased serum concentrations of both PC/PCS[55, 61, 62], and IS[56] are associated with increased morbidity and mortality related to CVD
- Both PC/PCS[71-75] and IS[88-91, 95, 96] serum concentrations can be reduced in the clinical setting through dialysis or other interventions.

EPCs are a BM-derived progenitor that are released into the peripheral circulation in response to ischaemic stimulus[284-288], and are involved in both repair and regulation of current vessels, as well as growth of new vessels[198, 200]. Reduced PB concentration of EPCs is associated with ED in coronary vessels[290, 291], as well as other risk factors for vascular disease (DM[296, 297], hypercholesterolaemia[305], HT[311], aging[320], and inflammation[325].

Decreased numbers of PB EPCs are found in CKD/ESKD[326, 327]. Improved kidney function, through KTx, is associated with improved EPC number and function[329, 330]. Increased small solute clearance on HDx has also been associated with improved EPC counts[411], however, the direct effect of uraemic toxins PC/PCS and IS on EPC function is not known.

5.2 Hypotheses

That, *in vitro*, exposure to uraemic toxins PC and IS is associated with EC dysfunction, as represented by the following *in vitro* assays:

- Decreased EC migration to VEGF gradient
- Decreased EC tube formation in Matrigel
- Increased EC expression of surface VCAM-1 as measure of cell activation/inflammation
- Altered expression of survival factors pAkt and pERK
- Increased shedding of cell surface markers and increased concentrations of inflammatory cytokines into EC supernatant

That, *in vitro*, EPCs have a limited ability to improve EC function in the presence of uraemic toxins PC and IS, as measured by the above assays.

5.3 Methods

Methods are described in full in Chapter 2.

5.3.1 Cell sources

ECs and EPCs were collected, isolated and cultured as per Chapter 2.2- 2.5. EPCs were confirmed as triple positive for CD34/CD133/VEGF as per Chapter 4, and used at day 6-8, to maintain standard functional phenotypes. ECs were used at passage 2 only.

5.3.2 Toxin preparation

Both toxins were prepared as per guidelines suggested by the European Toxin Working Group[425] (Chapter 2.7), which mimics levels found in normal individuals (normal), average ESKD patients (uraemic), and maximal recorded concentration in humans (maximal).Toxins were studied alone, and in combination – pairs of PC and IS at normal, uraemic or maximal concentration.

5.3.2.1 Protein binding

PC, in particular, is protein-bound *in vivo* (approximately 90% of PC and PCS[60]), and this was addressed by maintaining FCS concentration at 20% of total cell culture volume. Approximately 90% of IS has also been shown to be protein bound[484], but its affinity for albumin is unclear.

FCS concentration was maintained at 20% of culture volume, as this optimised HUVEC and EPC culture expansion. This compares to normal human serum protein concentration of 5-8%. Whilst differences in protein concentration between the *in vitro* and *in vivo* environment ideally should be minimised, it has been shown *in vitro* that protein binding has minimal effect on EC response to toxin exposure[67]. Furthermore, an *in vivo* study has shown that the actual binding of PC and PCS to serum albumin is of very low affinity[60].

5.3.3 Cell preparation

5.3.3.1 HUVEC

HUVEC were isolated, cultured and collected as previously described. All cells were passage 2 from at least 90% confluent, 25cm^2, gelatin-coated flasks. Prior to use in experiments, cells were starved in serum free HUVE media for 2 hours to increase response to stimulus. Preliminary experiments identified that without the starvation period, the cells under-responded to positive stimulus such as TNFα (5ng/ml, 30min-24hr, data not shown).

Serum starvation is a common technique used with *ex vivo* cultured HUVEC.

5.3.3.2 EPC

All experiments were initially performed with BM-derived EPCs, but all results reported were from day 5-7 cultured UCB-EPC, once a regular UCB supply was obtained. Cell harvesting, isolation and culture were as per Chapter 2.

Serum starvation was not performed on EPCs to augment responses, as these cells did not tolerate low serum states for any length of time.

5.3.4 Cell migration

Movement of cells through a Transwell to VEGF gradient (100ng/ml) was performed as described in Chapter 2.6.2. $1x10^4$ HUVEC or EPCs were added to the 96-well Transwell in the presence of toxin, with untreated cells as controls. Toxins were added approximately 30min prior to cells being placed in the Transwell.

Wells were incubated at 37^0C, 5% CO_2 for 2 hours, then washed, fixed in formaldehyde overnight, and stained with DAPI the next day. Membranes were mounted on slides, stored at 4^0C in darkness, and read within 2 days.

5.3.5 Tube formation

5.3.5.1 HUVEC

Matrigel was performed as described in Chapter 2.6.1. $3.5x10^4$ HUVEC were added to the wells, in the presence of toxins (the latter added a maximum of 15 minutes prior to commencement of the experiment). Toxins were added singly, as well as in combination, with paired toxins at normal, uraemic and maximal concentrations.

5.3.5.2 EPC

$5x10^3$ EPC were added to $3.0x10^4$ HUVEC, and exposed to toxin as per HUVEC alone.

5.3.5.3 Enumeration of tubes

Total number of tubes, as defined by the presence of a cellular projection with a lumen and 2 walls, was counted for each well at approximately 7 hours. The average from the duplicate wells was recorded. Maximal number of branches from a single cell were also counted, and averaged.

5.3.6 Vascular cell adhesion molecule-1 expression

Methods were as per Chapter 2.6.3, for both HUVEC alone, and HUVEC + EPC. Exposure was for PC and IS alone and in combination at normal, uraemic, and maximal concentrations.

5.3.7 Survival factor expression

Methods for HUVEC expression of pAkt/Akt and pERK/ERK in response to uraemic toxins, by Western Blot, were as per Chapter 2.6.4.

5.3.8 Supernatant investigation for uraemic toxins and soluble cell surface markers

Supernatants from T25 flasks were collected and tested for soluble surface markers as per Chapter 2.6.5. Uraemic toxin concentrations were also measured to confirm accuracy of concentration of toxins added.

5.4 Results

5.4.1 Toxin levels in cell media

Negative controls for all experiments used HUVE media or equivalent, with FCS. From 4 HUVEC lines, all but one supernatant gave 0mg/l measurements for PC, PCS and IS. The one line that gave a positive reading was only positive for IS (6mg/l), and was considered an aberration.

Positive controls were media with TNF alpha. Again, all but one line recorded 0μg/ul concentrations for toxins. The same line as the negative control above recorded presence of IS (13mg/l).

Toxin-added supernatants were collected after 24 hour exposure on HUVEC, with added PC and IS at “normal”, “uraemic” and “maximal” concentrations (Table 5.1). There was a significant correlation between added and measured IS (r^2=0.98, p=0.008) and PC (r^2=0.99, p=0.007), but not for added PC to measured PCS (r^2=0.15, p=0.61). Concentrations of toxins at 24hrs were significantly increased compared to baseline, but was considered a consequence of cell death.

Table 5.1: Comparison of added toxin concentration to measured supernatant toxin concentration following 24 hour exposure of HUVEC. (geometric mean, {95%CI})

	Added		Measured @ 24 hours		
Toxin	IS(mg/l)	PC(mg/l)	IS(mg/l)	PC(mg/l)	PCS(mg/l)
Negative control (3)	0	0	1.5 {0-4.4}	0	0
Positive control (3)	0	0	3.3 {0-9.7}	0	0
Normal (3)	0.6	0.6	2.1 {0.8-5.6}	0	0
Uraemic (3)	53	20.1	206 {129-330}	130 {63-271}	1.1 {0.6-1.9}
Maximal (6)	236	40.7	560 {438-717}	196 {170-225}	0

5.4.2 Cell migration

Functional testing was performed to demonstrate the effect of toxins on normal cellular activity. It was expected that both PC and IS would have a negative effect on both HUVEC and EPC migration through direct toxic injury. N=5 for all experiments, with one-way ANOVA used to test for significance between groups. Graphs represent mean of the –fold change in cell number migrated compared to the negative control, with 95%CI.

5.4.2.1 HUVEC

There was decreased migration of HUVECs to VEGF gradient in the presence of increasing PC concentration ($p<0.05$, Figure 5.1A).

For IS alone, and PC and IS in combination, there was no significant difference between groups at increasing concentrations (p=ns, Figure 5.1B and C). Wide variation in cell migration numbers was however seen for maximal concentrations of both toxins.

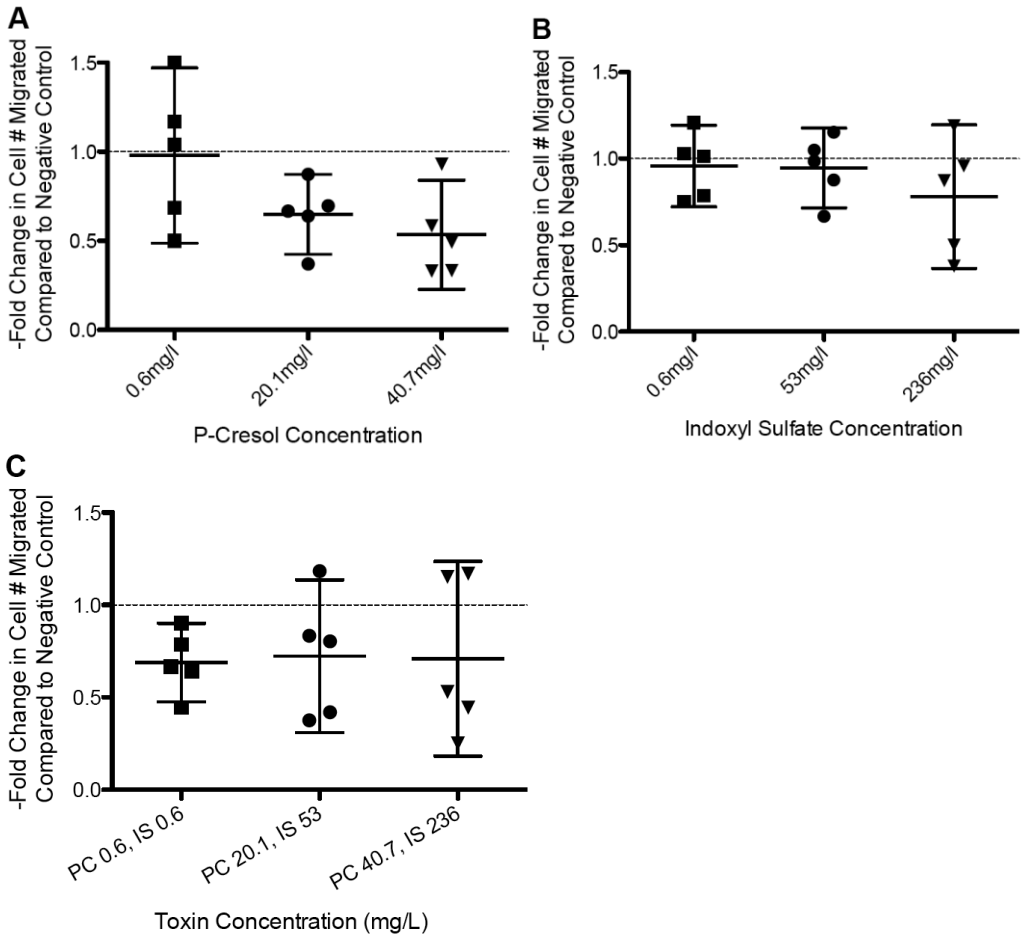


Figure 5.1: HUVEC migration to VEGF (100ng/ml) across Transwell, in the presence of A - PC; B – IS; C – IS + PC. Only increasing concentrations of PC was associated with decreased HUVEC migration ($p<0.05$, one-way ANOVA). N=5 for all experiments.

5.4.2.2 EPC

UCB-EPCs were collected, isolated and cultured as per Chapter 2.2, using EGM-2 + BulletKit + FCS + Cytokines (IGF-1, FGFb, VEGF, AA). There was a large range of migratory rates of EPCs noted within the assay. This included migration counts as low as 1 to maximum 50 cells per 40x magnification field. No significant differences were found between PC, IS, or the combination of the two at increasing concentration (n=3, one-way ANOVA p=ns, Figure 5.2).

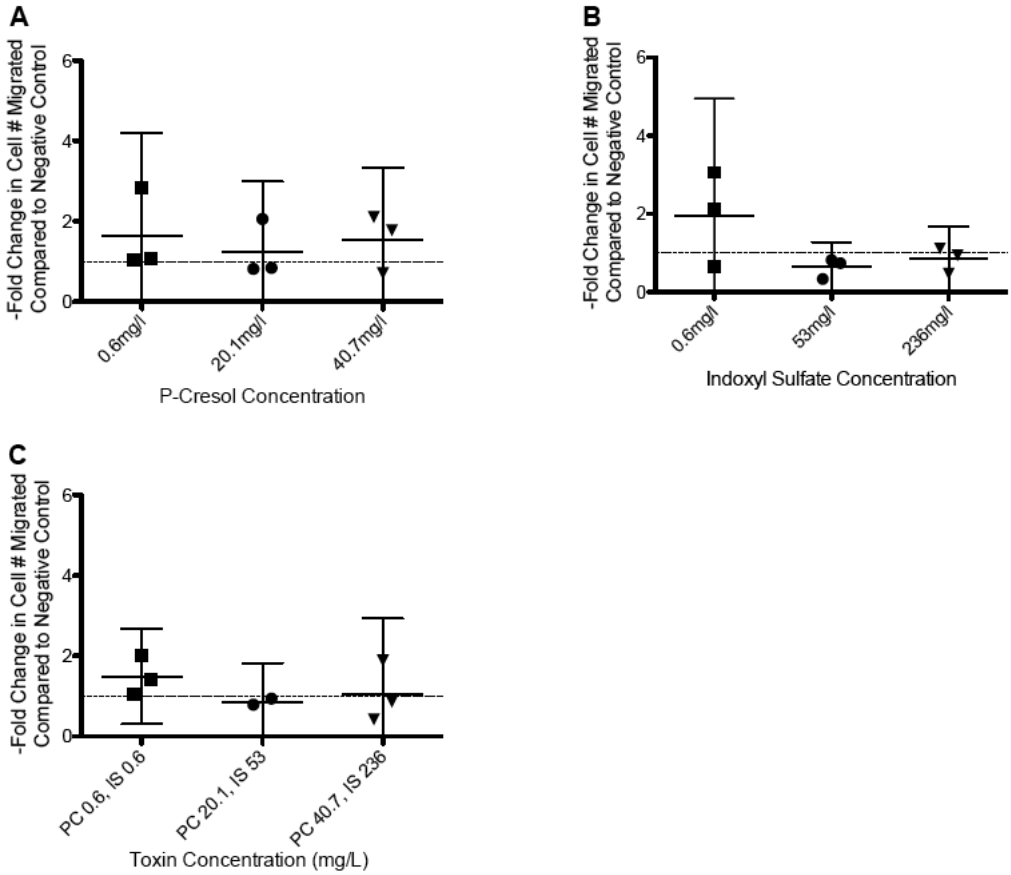


Figure 5.2: EPC migration to VEGF (100ng/ml) across Transwell, in the presence of A - PC; B – IS; C – PC + IS. No statistically significant difference was found between the groups (n=3, one-way ANOVA p=ns).

5.4.3 Tube formation

5.4.3.1 HUVEC

5.4.3.1.1 P-cresol

40.7mg/L PC inhibited both the number of tubes formed by HUVECs and the maximal number of branches produced at a single site (Figures 5.3 A and B), without affecting cell survival. Microscopically, increased HUVEC clumping was noted with increased PC concentration (Figures 5.3 C-F). A statistically significant linear trend was observed for increasing concentrations of PC with both number of branches and tubes formed ($p<0.001$)

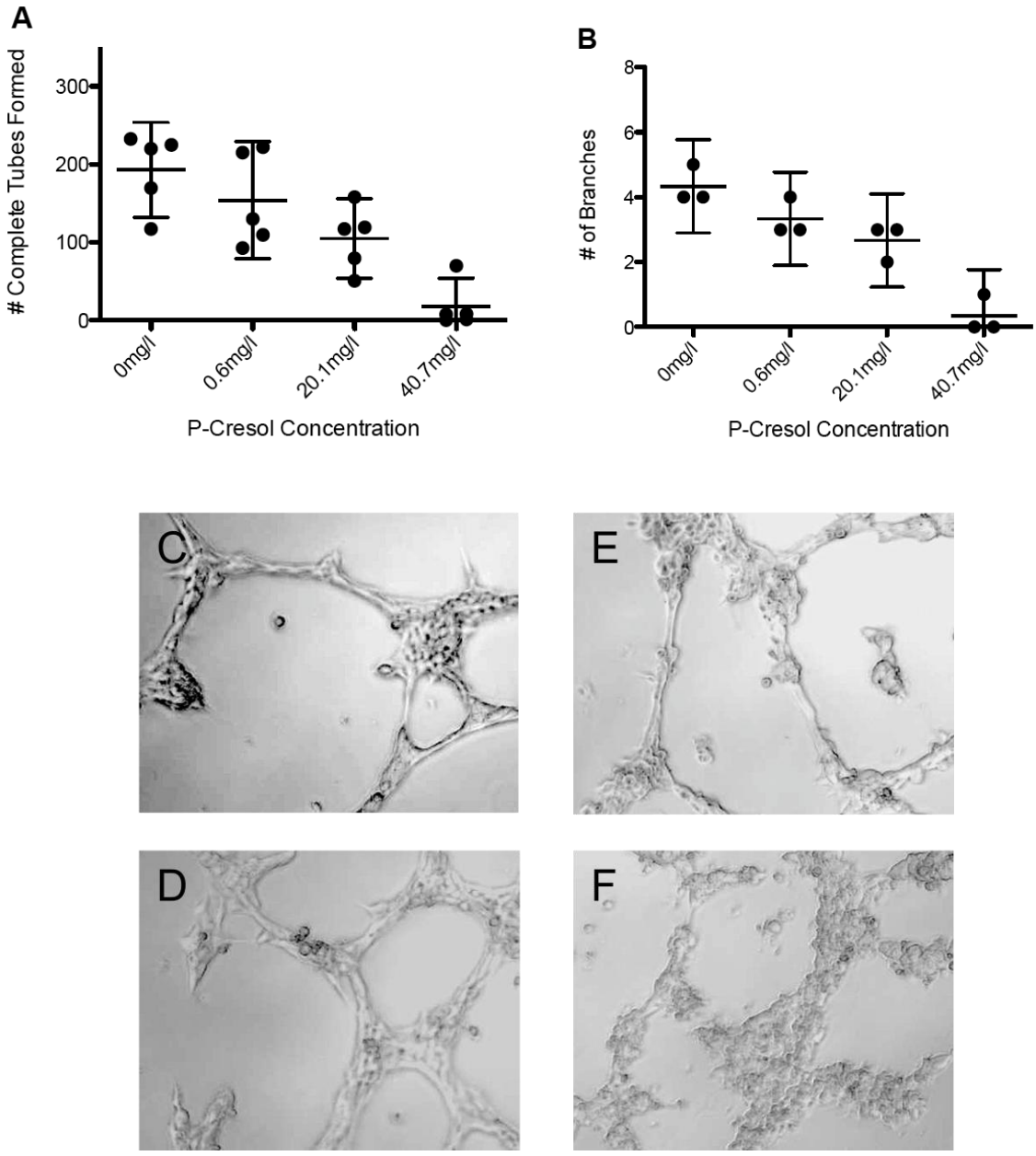


Figure 5.3: Effect of PC on HUVEC in Matrigel after 7hr incubation. A – number of complete tubes formed by HUVECs (n=5, one-way ANOVA $p<0.001$); B – maximal number of branches formed by HUVECs from a single point (n=5, one-way ANOVA $p<0.001$); PC caused a significant reduction in both tubes and branches formed by HUVEC, at increasing toxin exposure. 20x magnification of HUVEC in Matrigel with increasing PC concentration (single representative cell line); C – 0mg/L, D –0.6mg/L, E – 20.1mg/L, F – 40.7mg/L. Increased cellular clumping observed with increased PC concentration.

5.4.3.1.2 Indoxyl sulfate

There was inhibition of both branch and tube formation (One-way ANOVA, $p<0.05$) by HUVEC in Matrigel, with increasing concentrations of IS (Figures 5.4 A and B). The effect did not appear as great as that of PC, and there was no gross microscopic evidence of cell death (Figures 5.4 C-F).

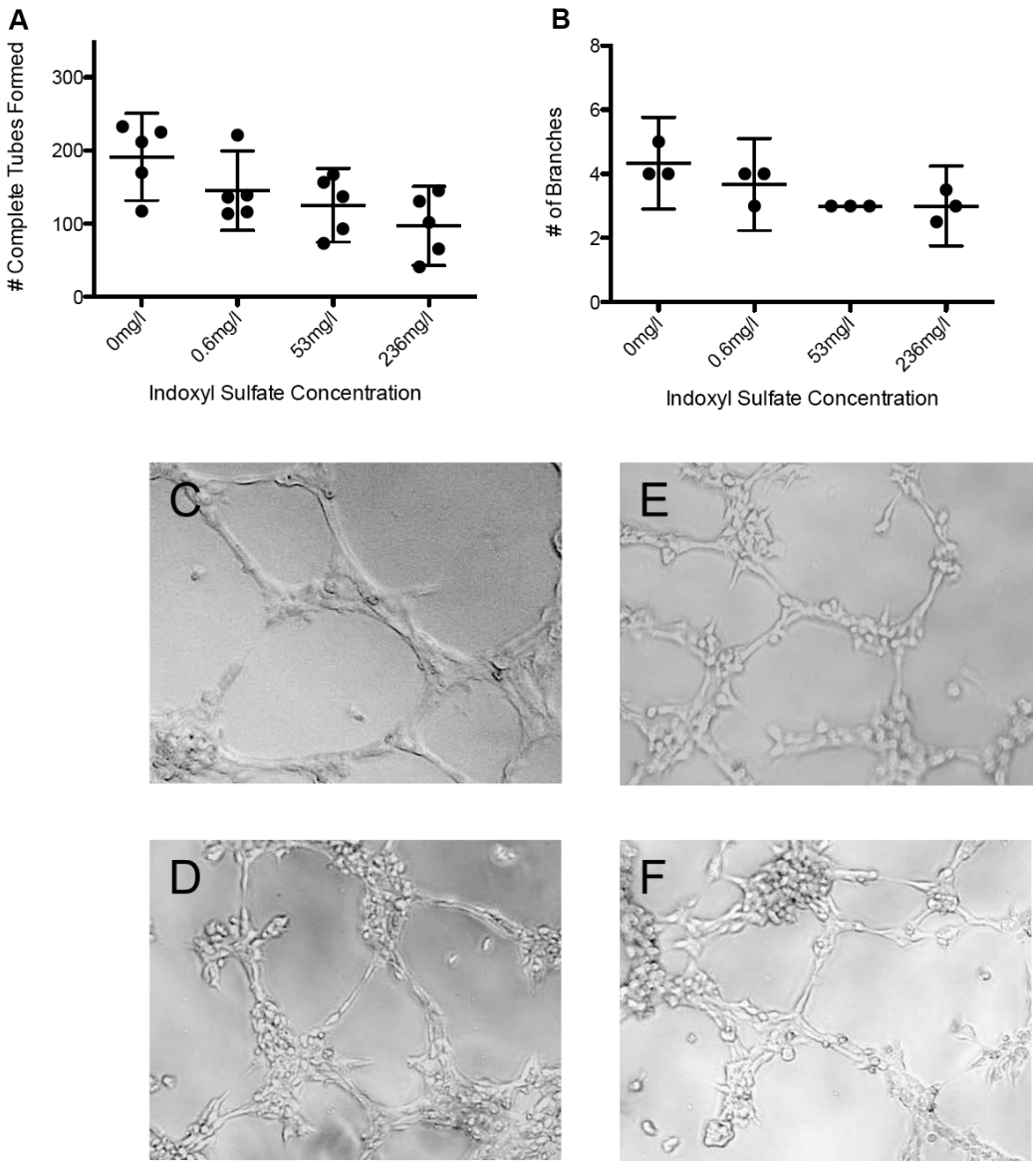


Figure 5.4: Effect of IS on HUVEC in Matrigel after 7hr incubation. A – number of complete tubes formed by HUVECs (n=5, one-way ANOVA $p<0.05$); B – maximal number of branches formed by HUVECs from a single point (n=3, $p<0.05$). IS caused a significant reduction in both tubes and branches formed by HUVEC, at increasing toxin exposure. 20x magnification of HUVEC in Matrigel with increasing IS concentration (single representative cell line); C – 0mg/L, D – 0.6mg/L, E – 53mg/L, F – 236mg/L. There was no gross microscopic evidence of cell death or specific increased cell clumping.

5.4.3.1.3 Combination p-cresol and indoxyl sulfate

The combination of PC and IS at equivalent concentrations also caused significant reduction in branch number and tube formation (Figures 5.5 A and B), in a similar pattern to PC alone. A linear trend between increasing concentrations of PC/IS and decreasing HUVEC tube and branch formation was also observed ($p<0.01$). There was no evidence of gross cell death at increased toxin concentration by basic light microscopic examination (Figures 5.5 C-F).

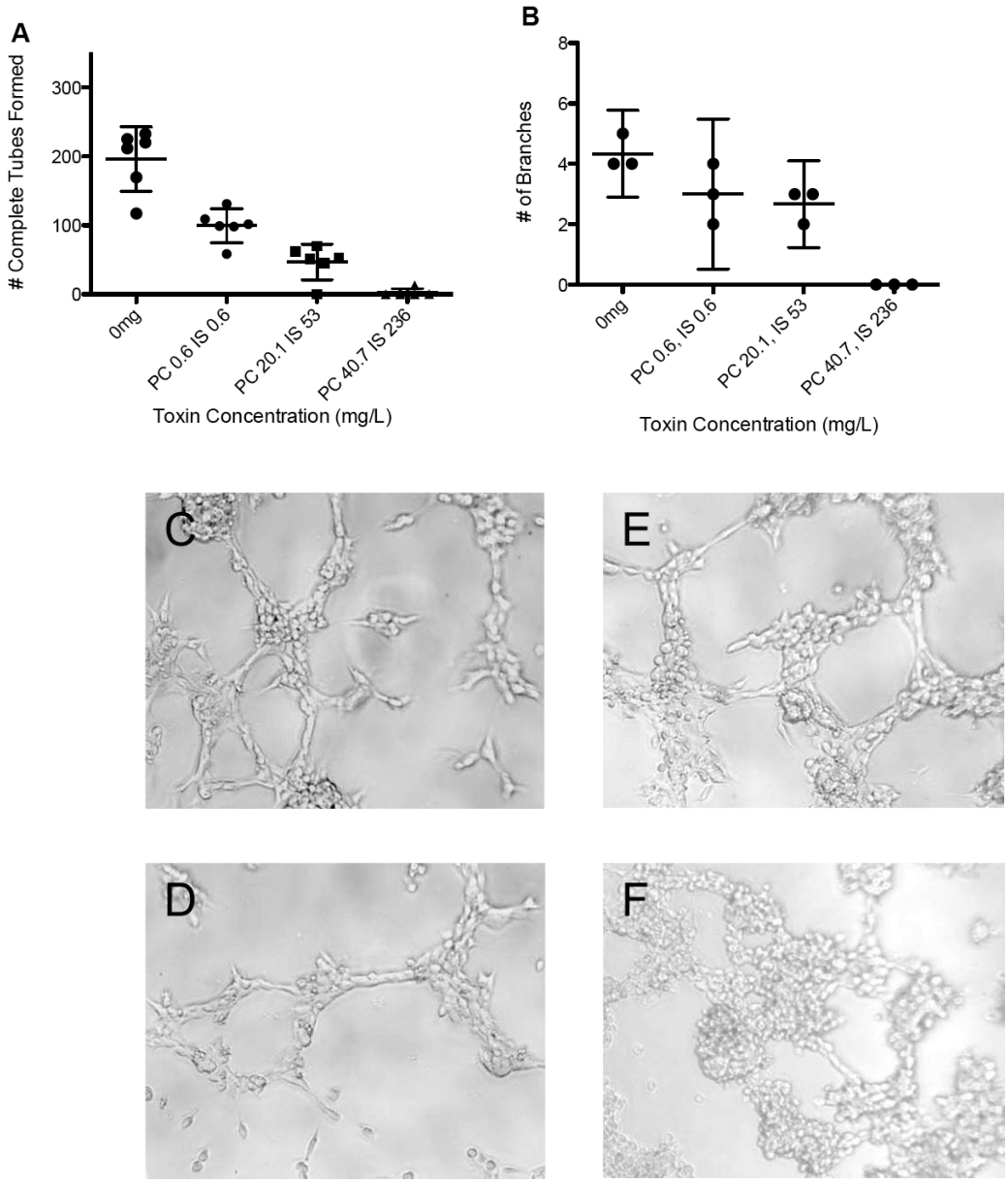


Figure 5.5: Effect of PC+IS on HUVEC in Matrigel after 7hr incubation. A – number of complete tubes formed by HUVECs (n=6, one-way ANOVA $p<0.01$); B – maximal number of branches formed by HUVECs from a single point (n=3, $p<0.001$). The combination of toxins caused a significant reduction in both tubes and branches formed by HUVEC, at increasing toxin exposure. 40x magnification of HUVEC in Matrigel with increasing PC+IS concentration; C – 0mg/L, D – 0.6/0.6mg/L, E – 20.1/53mg/L, F – 40.7/236mg/L.

5.4.3.2 EPC + HUVEC

5.4.3.2.1 P-cresol

Increasing PC concentration inhibited HUVEC tube formation and branching in the presence of EPCs (one-way ANOVA, $p<0.01$), however compared to HUVEC alone, 20.1mg/L PC did not significantly decrease branching from baseline ($p<0.05$) (Figures5.6 A and B). No gross cell death was observed at increased PC concentration by basic light microscopic examination (Figures 5.6 C-F).

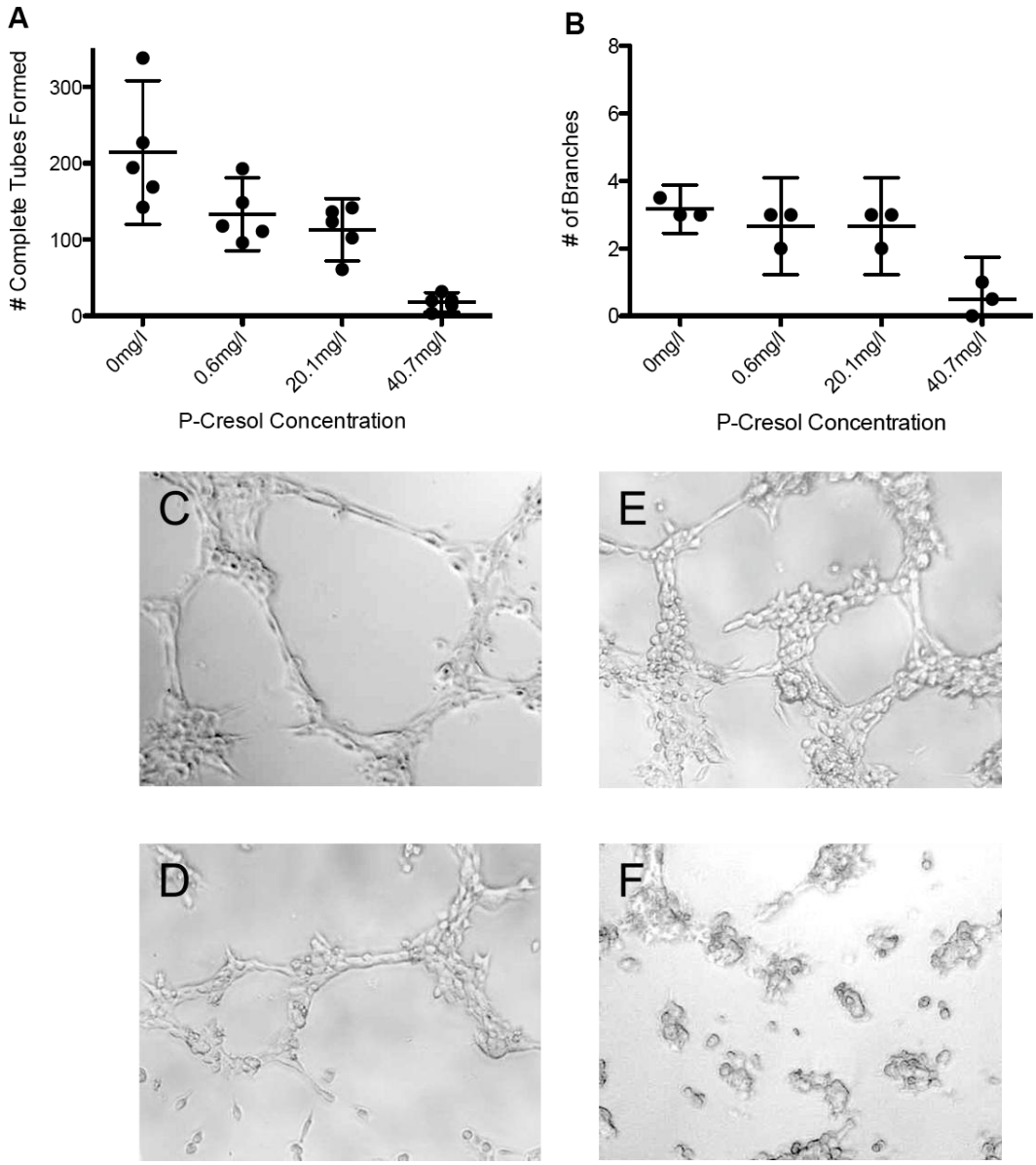


Figure 5.6: Effect of PC on HUVEC ($3.0x10^5$ cells)+EPC ($5x10^4$ cells) in Matrigel post 7hr incubation. A – number of complete tubes formed by HUVEC+EPC (n=6, one-way ANOVA $p<0.01$); B - maximal number of branches formed by HUVEC+EPC from a single point (n=3, $p<0.01$). Addition of EPCs did not prevent overall impairment of HUVEC function at increasing PC concentrations. There was no light microscopic evidence (20x magnification) of gross cell death from C – 0mg/L, D – 0.6mg/L, E – 20.1mg/L, or F – 40.7mg/L concentration PC.

5.4.3.2.2 Indoxyl sulfate

Increasing concentrations of IS had no statistically significant effect on HUVEC tube (n=5, one-way ANOVA p=ns) or branch formation (n=3, one-way ANOVA p=ns), when in the presence of EPCs (Figures5.7 A and B). There was no evidence of cell death at any IS concentration on gross microscopic appearance (Figures 5.7 C-F).

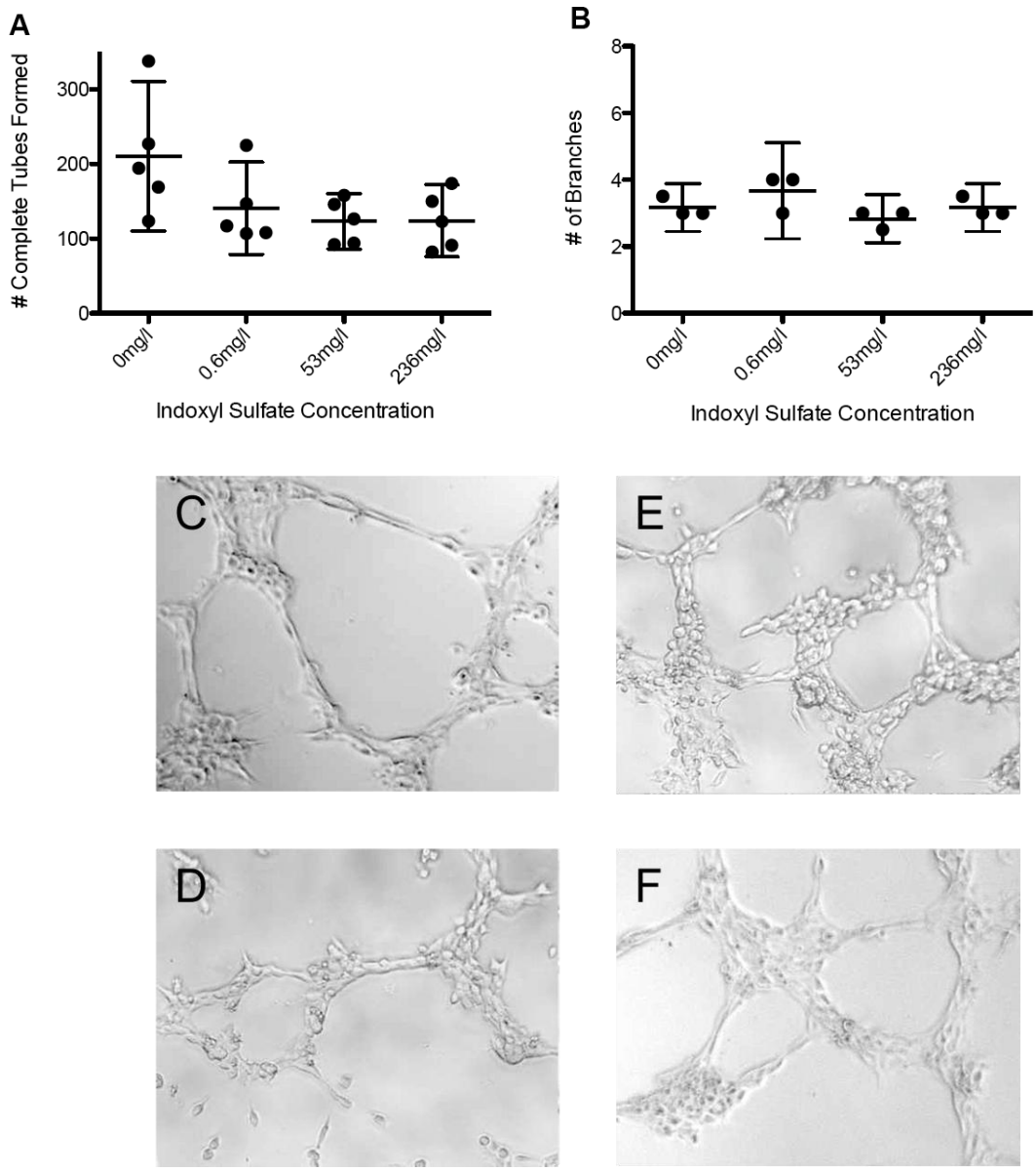


Figure 5.7: Effect of IS on HUVEC ($3.0x10^5$ cells)+EPC ($5x10^4$ cells) post 7hr incubation in Matrigel. A – number of complete tubes formed by HUVEC+EPC (n=5); B – maximal number of branches formed by HUVEC+EPC from a single point (n=3). There was no statistically significant difference between any of the IS groups (one-way ANOVA, p=ns). 40x magnification of HUVEC in Matrigel with increasing IS concentration; C – 0mg/L, D – 0.6mg/L, E – 53mg/L, F – 236mg/L.

5.4.3.2.3 Combination p-cresol and indoxyl sulfate

Increasing concentrations of PC and IS reduced both number of tubes formed and maximal branch formation by HUVECs in the presence of EPCs in Matrigel (one-way ANOVA, $p<0.01$, Figure 5.8 A and B). There was no evidence of cell death at higher toxin concentrations on microscopic appearance (Figures 5.8 C-F).

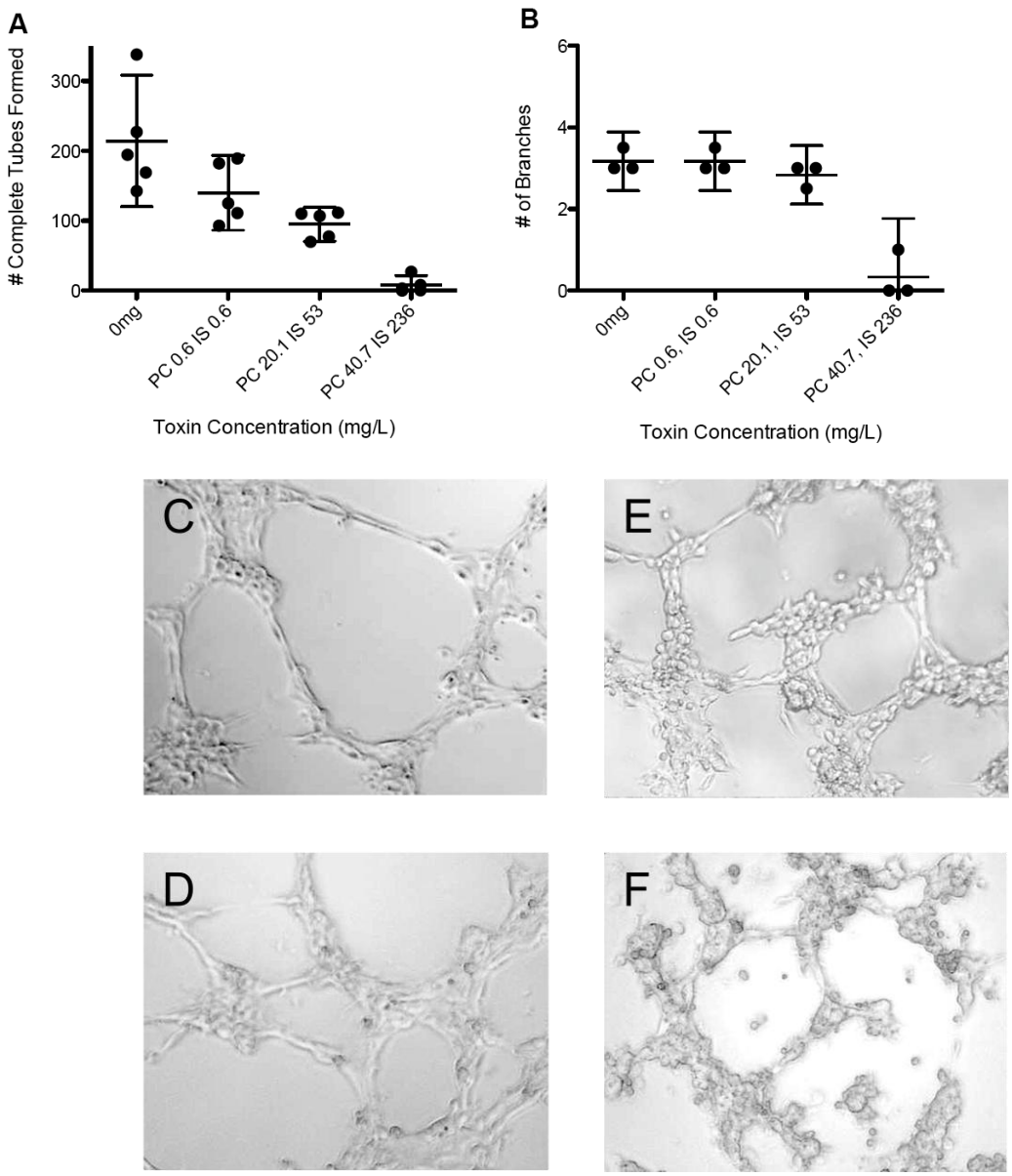


Figure 5.8: Effect of PC/IS on HUVEC ($3.0x10^5$ cells)+EPC ($5x10^4$ cells) post 7hr incubation in Matrigel. A – number of complete tubes formed by HUVEC+EPC (n=5); B – maximal number of branches formed by HUVEC+EPC from a single point (n=3). Both tube numbers and branches formed decreased with increasing toxin concentration (one-way ANOVA, p=0.01). 40x magnification of HUVEC+EPC in Matrigel with increasing PC+IS concentration; C – 0mg/L, D – 0.6/0.6mg/L, E – 20.1/53mg/L, F – 40.7/236mg/L.

5.4.4 VCAM-1 expression

5.4.4.1 HUVEC

A significant increase in VCAM-1 expression was found in all groups for maximal concentrations of PC (n=7, one-way ANOVA $p<0.01$) or IS (n=8, $p<0.05$) alone, or in combination (n=5, $p<0.01$). However, only uraemic concentrations of PC (Bonferroni post-test, $p<0.05$), but not IS (p=ns), were associated with increased expression of VCAM-1 on HUVECs (Figure 5.9 A-C).

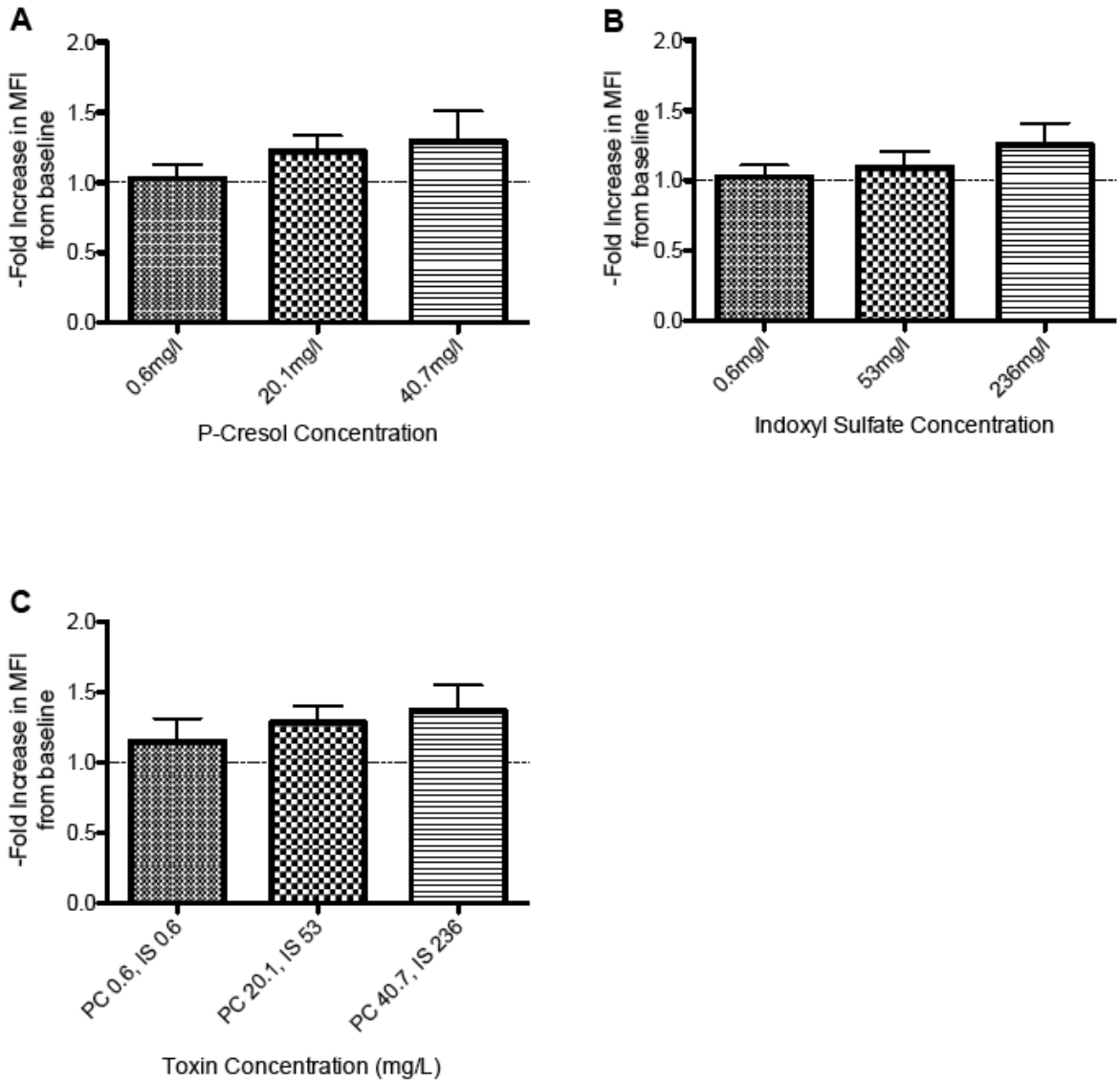


Figure 5.9: Effect of toxins on HUVEC expression of VCAM-1. A – PC (n=7, one-way ANOVA $p<0.01$); B – IS (n=8, $p<0.05$); C – PC + IS (n=5, $p<0.01$). 20.1mg/L PC was also associated with a statistically significant increase in VCAM-1 expression compared to baseline (Bonferroni post-test $p<0.05$), whilst 53mg/L IS was not (p=ns).

5.4.4.2 EPC

Increasing concentrations of EPC did not significantly alter VCAM-1 expression on HUVECs (n=6, one-way ANOVA p=ns). Whilst overall VCAM-1 expression was higher in the presence of TNFα, EPCs also did not change expression patterns within this group (n=6, one-way ANOVA p=ns, Figure 5.10).

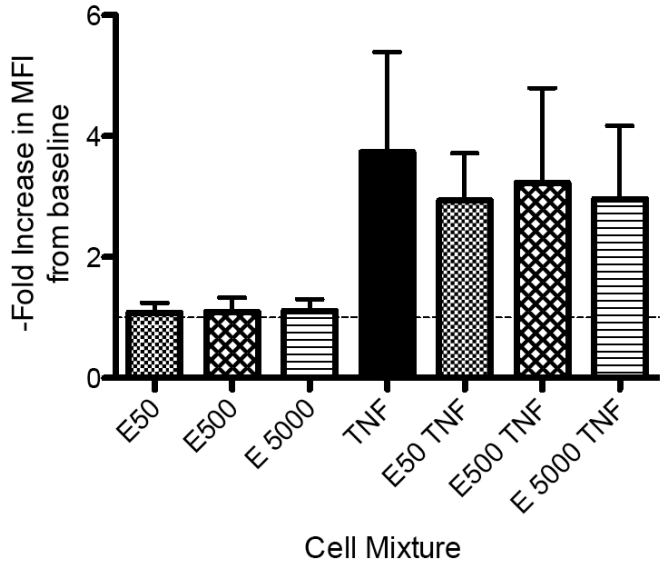


Figure 5.10: HUVEC VCAM-1 expression in the presence or absence of EPCs, and in response to TNFα (0.5ng/ml). There was no difference in VCAM-1 expression related to concentration of added EPCs (E50/500/5000 – 50/500/5000 EPCs added to T25 flask of confluent HUVECs {~$1x10^6$ cells}, n=6, one-way ANOVA p=ns) for either the control or TNFα group.

In the setting of maximal concentrations of PC (40.7mg/L) and IS (236mg/L) in combination, increasing concentrations of EPCs had no effect on VCAM-1 expression (n=4, one-way ANOVA p=ns), which all remained significantly above baseline expression (p<0.01,Figure 5.11).

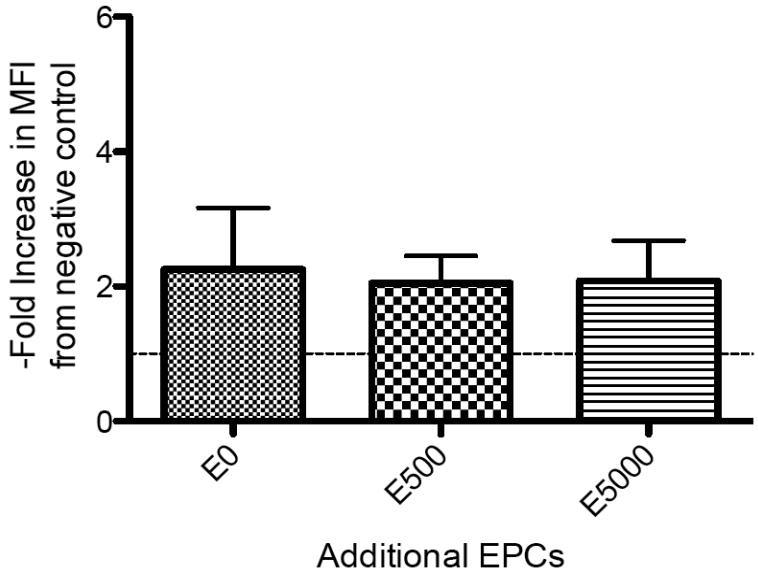


Figure 5.11: HUVEC VCAM-1 expression in the presence or absence of EPCs, with maximal concentrations of PC (40.7mg/L) and IS (236mg/L) in combination. There was a significant increase in VCAM-1 expression with exposure to toxins (p<0.01), but no further change in expression with addition of EPCs, at any concentration (p=ns).

5.4.5 Survival factor expression

Of the fully confluent T25 flasks of HUVEC exposed to toxins at the commencement of the assays, approximately 50% had become detached by the final time point (at 24 hours), compared to the 0, 10min and 60min time points. Cells present in the supernatant were not included for analysis.

5.4.5.1 ERK

There was a significant difference for p/tERK expression at 60 minutes between toxin concentrations (repeated measures ANOVA, p=0.02), but not at 10 minutes or 24 hours.

PC and PC/IS were associated with a non-significant decrease in pERK:tERK at 60 minutes, then an increase by 24 hours. IS exposure alone was conversely associated with a non-significant decrease in pERK:tERK over time (Figure 5.12).

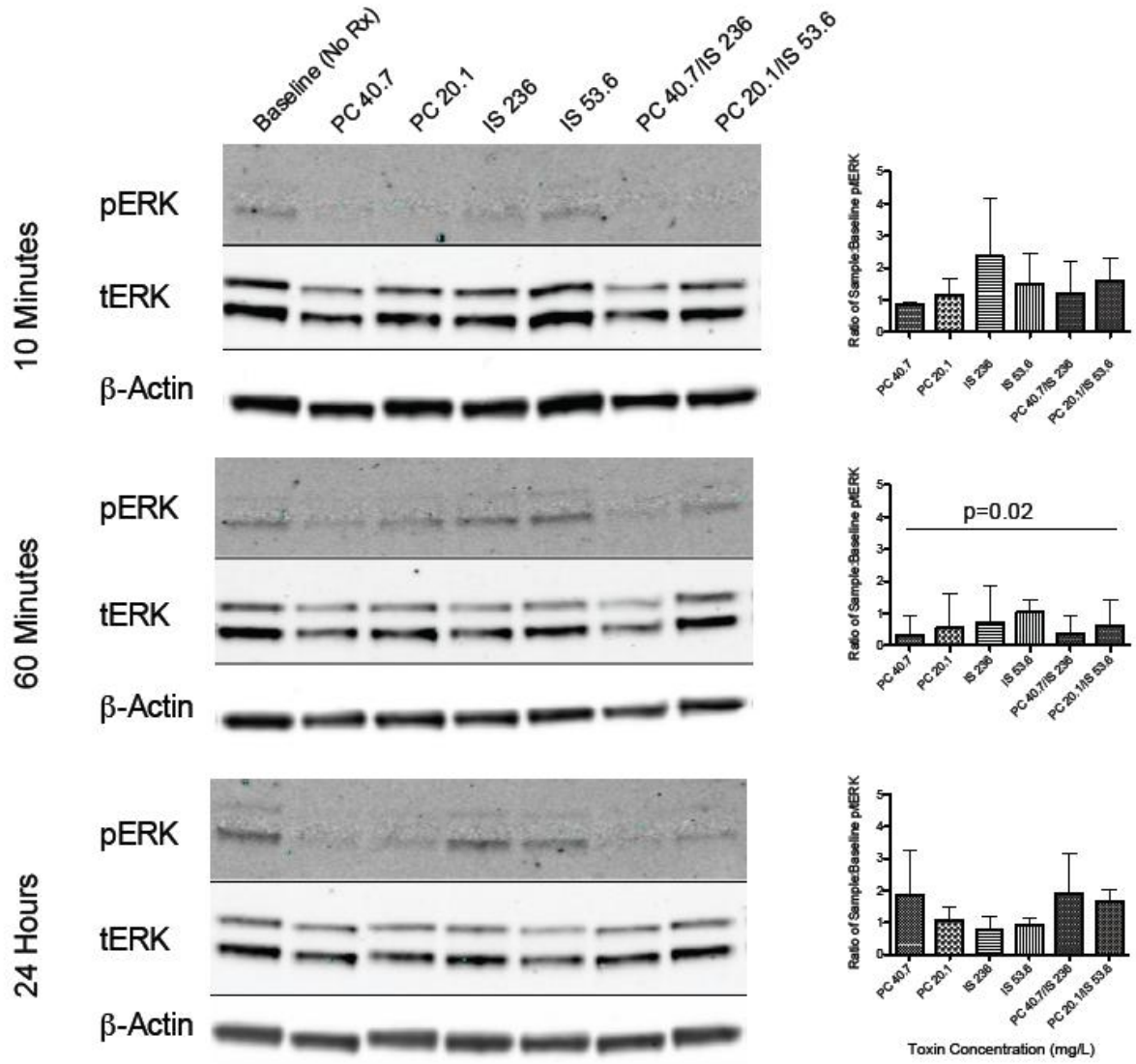


Figure 5.12: Ratio of pERK:tERK at 10min, 60min and 24 hours in response to toxins. Representative Western Blots, with corresponding bar graphs of ratio sample:baseline p/tERK (n=3), corrected for protein loading between samples. There was no significant difference between time points for any of the toxin concentrations (p=ns). There was a significant difference for p/tERK expression at 60 minutes between toxin concentrations (repeated measures ANOVA, p=0.02).

5.4.5.2 Akt

Compared to the control samples, all toxins caused a reduction in the pAkt:tAkt ratio at 10 and 60 minutes, which then returned to baseline levels by 24 hours. These results did not, however, reach statistical significance (Figure 5.13).

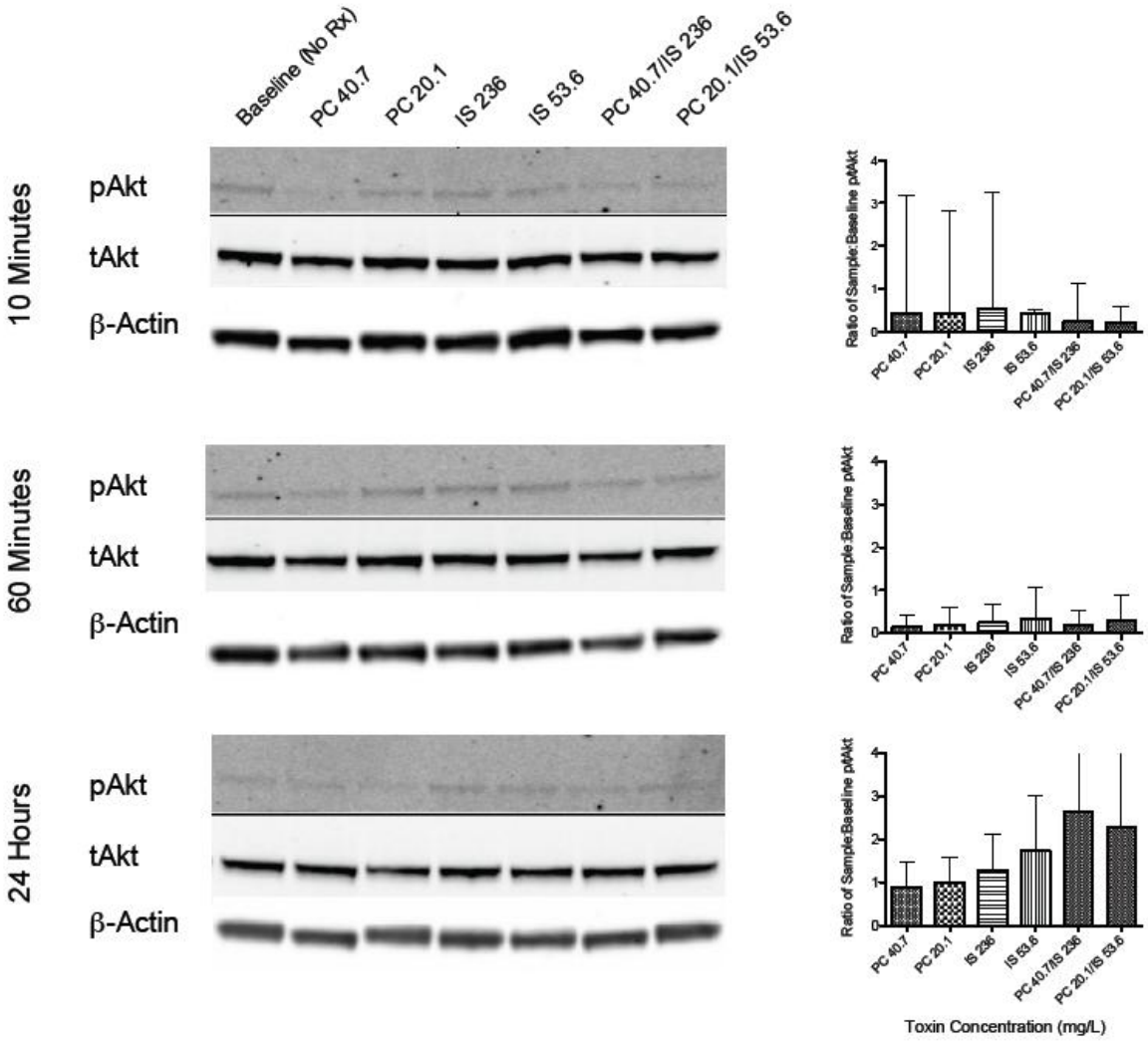


Figure 5.13: Ratio of p/tAkt at 10min, 60min and 24 hours in response to toxins. Representative Western Blots, with corresponding bar graphs of ratio sample:baseline p/tAkt (n=3), corrected for protein loading between samples. There were no significant differences between time points for any of the toxin concentrations, or between toxins at either 10 minutes, 60 minutes or 24 hours (p=ns).

5.4.6 FlowCytoMix of soluble surface markers

FlowCytoMix assay was used to identify the concentrations of multiple soluble agents (soluble E-selectin, ICAM-1, ICAM-3, PECAM-1, P-selectin and VCAM-1) that may have been associated with EC activation by toxins. sPECAM, sICAM-1, sICAM-3, sE-Selectin and sP-Selectin results fell below the level of detection in the assay and as such were excluded from analysis. Only sVCAM-1 supernatant concentrations were significantly increased from HUVECs exposed to TNFα (0.5ng/ml, 24 hours, $p<0.01$), but not for any other group, compared to the negative control (Table 5.2). Interestingly, sVCAM-1 concentration negatively correlated with HUVEC VCAM-1 expression (as a percentage of baseline MFI, slope -0.049, $r^2=0.49$, $p=0.02$; Table 5.2) which suggests that increased expression of VCAM-1 remains associated with the cells and may imply an increased capacity to bind integrins (eg LFA-4) commonly expressed by leukocytes. There was no correlation between VCAM-1 expression from HUVEC with EPCs alone or with TNFα, compared to sVCAM-1 concentrations from supernatants from the same cells ($p>0.3$).

Table 5.2: Raw data of supernatant sVCAM-1 concentration compared to HUVEC expression of VCAM-1 (average±SE). There was no association between sVCAM-1 concentration in supernatants, and VCAM-1 expression on HUVEC exposed to increasing concentrations of PC or IS, alone or in combination (p=ns).

	sVCAM-1 (ng/ml)	VCAM-1 expression (% baseline MFI)
Control (no added toxin)	6.1±1.3	100
PC 0.6mg/L	4.8±1.9	106±9
PC 20.1mg/L	3.9±1.1	119±12
PC 40.7mg/L	3.8±1.0	122±22
IS 0.6mg/L	4.0±0.3	103±11
IS 53.6mg/L	4.5±1.7	101±8
IS 236mg/L	4.0±1.2	115±5
PC 0.6 / IS 0.6 mg/L	3.1±0	114±12
PC 20.1 / IS 53.6 mg/L	3.4±0	128±8
PC 40.7 / IS 236 mg/L	3.5±0.3	136±13

5.5 Discussion

This result shows a difference in the effects between PC and IS on *in vitro* HUVEC function, with or without EPCs. Specifically, EPCs had limited, if any, ability to curtail the deleterious effects of increasing PC concentration on HUVEC function, across multiple assays. Conversely, in the presence of IS, EPCs were able to show modest benefit regarding maintenance of normal HUVEC function. These are important results, given the interest in the use of EPCs as a therapeutic tool in vascular disease, and the potential limitations therefore of this approach for therapy in subjects with CKD.

5.5.1 Migration assay

Whilst we were unable to show a difference, others have found decreased *in vitro* migration of leukocytes across ECs in the presence of PC, by decreasing EC membrane expression of junctional adhesion molecule A (JAM-A) and fractalkine[69]. Using a more direct marker of cell migration, Dou *et al* caused disruption of layers of ECs, by forming a denuded area, then measuring the rate of return of ECs in the presence of uraemic toxins. Both PC and IS significantly reduced migration of ECs[67]. Given these previous findings, we expected to observe:

1. Migratory capacity of EPCs (reviewed elsewhere[485]): The transwell type used (PET membrane, 8 micron pore), cell concentration, time for migration and VEGF gradient were optimised in early experiments. However, given limited supply of EPCs, further optimisation was not performed. This included using additional SDF-1 as a chemo attractant[376, 486, 487] (although VEGF has been used successfully on it's own in the literature[326]), adding fetal calf serum to media to improve EPC viability, and increasing or decreasing time of exposure to toxins

prior to migration. Any of these factors may have attenuated EPC migration to the levels seen.

2. A difference in migration between cells exposed to different toxin concentrations: There is significant data available describing the multiple effects *in vitro* and *in vivo* for PC and IS, but none describing specific EC or EPC migratory capacity. Altered migratory capacity of leukocytes across ECs has been documented for increasing PC concentrations[69]. Given this, and the increased oxidative stress associated with IS[488], a difference was expected.

   The expectation was for reduced migration for increasing concentrations of both IS and PC.

   Concomitant with leukocyte migration, HUVECs demonstrated a trend towards decreased migration with increasing PC concentration, but did not reach significance difference between any of the groups.

5.5.2 Tube formation in Matrigel

Choi *et al* previously showed decreased incorporation of EPCs from uraemic patients into tubes formed by HUVEC in matrigel, as well as decreased complexity of the tubules[326]. We have demonstrated that uraemic toxins PC and IS both reduce tube formation by HUVEC alone, and by HUVEC in the presence of normal EPCs, as well as reduce the complexity of the structures formed.

The deleterious effect of PC was greater than that of IS at higher concentration, and was not improved by the presence of EPCs. With the relative lower inhibition of tube formation by high concentration IS however, EPCs were able to show improved tube formation numbers per well. At 0.6mg/L PC, EPCs were able to increase the number of tubes formed.

This data shows:

1. PC has a more significant negative effect on HUVEC tube formation ability than IS at physiologically comparable concentrations
2. The negative effect of PC extends to EPCs, which are unable to counteract its effect at physiologic, uraemic or maximal concentrations – this has implications for near ESKD and dialysis recipients.
3. EPCs do show an ability to promote tube formation by HUVEC *in vitro,* despite the presence of maximal concentration of IS.

The literature clearly shows the difference in effect of PC[61, 63, 65, 69] and IS[56, 80], on generation of oxygen radicals, leukocyte activation and clinical outcomes. This was expected, given that PC and IS are members of different chemical families, and are generated in the gut from separate substrates. Furthermore, serum concentrations of PC and IS uraemic subjects do not necessarily correlate, despite similar patterns of removal by conventional dialysis[489].

That EPCs are able to overcome the effect of IS on tube formation to some degree, suggests that targeting reduction in PC rather than IS in serum by therapeutic means may be a more effective way of improving vascular function. Further *in vitro* and *in vivo* studies would be required to confirm this, including repeating assays with PCS, separate treating of EPC or HUVECs with toxins for varying lengths of time, and animal models of toxin addition.

5.5.3 VCAM-1 and sVCAM-1

VCAM-1 expression on HUVEC increased in response to increasing toxin levels, comparably between groups. VCAM-1 is an important cell membrane receptor on ECs, as it is upregulated in CVD[242], and allows tethering of leukocytes[490] involved in atherosclerotic plaque formation[491]. It is also expressed on smooth muscle cells and fibroblasts, and is implicated in the progression of cardiac hypertrophy[492] and heart failure[493].

Dou *et al* found that PC had no effect on HUVEC expression of VCAM-1, and furthermore, that ≥10mg/L PC *in vitro* inhibited the upregulation of VCAM-1 by TNF alpha[68]. Ito *et al* found that IS enhanced adhesion of monocytes to HUVECs, but through E-selectin, rather than VCAM-1[494]. Our results show a significant increase in VCAM-1 expression for both toxins at increasing concentrations. However, whether a 20-40% increase in VCAM-1 expression is clinically significant for increased CVD risk is not known.

EPCs had no effect on expression of VCAM-1 in the setting of either toxins or TNF alpha. There is no literature available on this interaction. *In vivo*, Kong *et al* demonstrated reduced VCAM-1 expression from mouse coronary ECs two weeks after balloon injury, if EPCs had been mobilised using G-CSF[495]. The *in vivo* study does not mechanistically give an answer to the EPC – VCAM-1 interaction: their findings may instead only show that vessels were repaired more quickly in the presence of increased EPC concentration.

sVCAM-1 concentrations from supernatants of HUVEC exposed to toxin negatively correlated with HUVEC VCAM-1 expression in the presence of toxins. This was an unexpected finding, as sVCAM-1 has been used as a marker of endothelial activation and has been shown to correlate with cellular expression of VCAM-1[496]. The time

point used (24 hours) may have been too early to see increased sVCAM-1 levels. Conversely, it was noted that significant numbers of HUVEC had become detached and disintegrated over the 24 hour period when exposed to high levels of toxin (high levels of PCS are associated with increased shedding of endothelial microparticles both *in vivo* and *in vitro*[66]). The elevated sVCAM-1 supernatant concentration may have peaked earlier, and then decreased by 24 hours. If the sVCAM-1 level was truly reduced, then the mechanism requires further elucidation – one mechanism may be that *in vivo*, a significant portion of the circulating VCAM-1 may originate from SMCs or fibroblasts that have become exposed after endothelial damage.

5.5.4 Survival factors

Both PC and IS at increasing concentration suppressed cell survival factors at 10 and 60 minutes, but by 24 hours, ratio of phosphorylated to total Akt and ERK had returned to baseline. There was broad variation in the results gained, leading to wide confidence intervals – statistical significance was therefore unable to be demonstrated.

Rat vascular SMCs have been shown to be activated by IS through ERK/MAPK expression[82]. Conversely, IS has also been demonstrated to inhibit human tubular cells through the ERK/MAPK pathway[497].

These findings reiterate the differences in responses of varying cell types in the body to distinctive noxious stimuli. There is no published data on the relationship between Akt phosphorylation and PC or IS.

Our findings would suggest that there is also variation within specific cell types – as described previously, a significant proportion of HUVECs exposed to high levels of toxin for 24 hours were sloughed off their gelatin base, and therefore not considered in the Western Blot. Perhaps the remaining cells had a greater tolerance to the toxins, and were able to survive the initial insult and ongoing elevated toxin levels.

Whilst these HUVECs were able to survive, the earlier results show that they do so at the expense of normal function.

5.6 Summary

Uraemic toxins PC and IS, which are known to be associated with increased CVD risk and endothelial dysfunction in a clinical setting, are further shown here to impair the regulation of normal EC function *in vitro*. Furthermore, that PC, and to a lesser degree IS, impair the ability of EPCs to normalise EC function.

This is the first study to examine these specific functional aspects of EC function *in vitro*, as well as investigate the interaction between HUVEC, uraemic toxins, and EPCs.

Chapter 6: *In vitro* Human Sera Studies

6.1 Introduction

Progressive CKD is associated with increased morbidity and mortality related to CVD[3, 6, 7], with an increase in CHF compared to IHD in ESKD(see Chapter 3 and Herzog *et al*[16]). Novel risk factors unique to CKD/ESKD have been implicated in the pathogenesis of CVD in this patient population, such as increased oxidative stress[34] and vascular calcification[35, 36] in HDx recipients, and organ rejection[38] and immunosuppressive use[39] in KTx recipients.

In Chapter 5 we demonstrated the deleterious *in vitro* effects of uraemic toxins PC and IS on HUVEC and EPC function. However, the result also highlighted the differences in effects of PC and IS compared to each other. Specifically, increasing PC concentrations had a greater negative effect on EC and EPC function than IS. The effect of IS was such, that some of its negative effect on EC function (*e.g.* tube formation) was able to be abrogated by the addition of EPCs.

Investigation of uraemic toxins as agents of CVD risk is difficult, as there are over 90 recognised chemicals of increased concentration in CKD/ESKD[54] – the vascular effect of all these chemicals is not known, nor is the effect of interaction between toxins.

In this chapter we investigated these relationships further by looking at whether sera from CKD/ESKD subjects, with known concentrations of PC/PCS and IS, would have a similar effect on *in vitro* function of HUVEC that synthetic PC and IS demonstrated in Chapter 5. EPCs were excluded from the following study, due to the limited effect they demonstrated in Chapter 5.

6.2 Hypothesis

- That human sera from HDx and KTx subjects with specific PC/PCS and IS concentrations, will have similar *in vitro* effects to synthetic PC and IS on cultured HUVEC function.

6.3 Methods

Methods were as per Chapter 2.10 for study design, patient recruitment and blood sample collection and analysis. The latter included routine laboratory testing for biochemistry and haematology, and specific analysis for PC, PCS and IS serum concentration (Chapter 2.7).

All blood samples were transported on ice, and processed within 4 hours of collection. Saved sera were stored at -80^{0}C in separate aliquots, and did not undergo more than one freeze-thaw cycle.

6.3.1 *In vitro* HUVEC studies

HUVEC matrigel, migration and VCAM-1 expression were performed as per Chapter 2.6, with 2 hour serum starvation of HUVEC prior to assays.

Test sera were added at 20% of total supernatant volume, with FCS used as control.

With timelines previously established and detailed in Chapter 5, Matrigel plates were enumerated at 7 hours, migration at 2 hours, and VCAM-1 at 24 hours.

6.4 Results

6.4.1 Baseline characteristics

Table 6.1 summarises the baseline characteristics between the three patient groups. The HDx recipients were of significantly older age, as well as having increased serum concentrations of creatinine, CRP, PC, PCS and IS. They also demonstrated lower concentrations of total cholesterol, Hb and serum calcium.

No difference was observed in serum PTH or potassium concentrations between groups, however Figure 6.1 demonstrates the “outliers” within the HDx group.

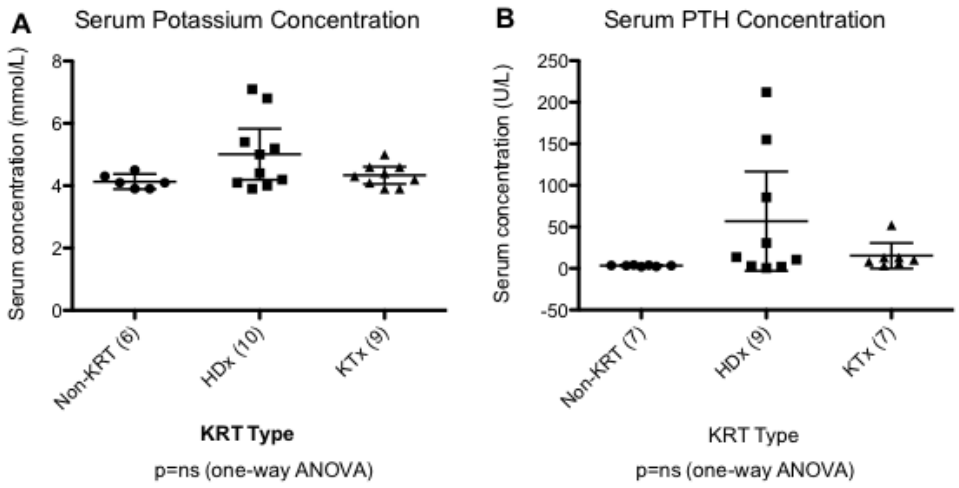


Figure 6.1: Range of baseline measurements for A – serum potassium concentration; B – serum PTH concentration. A wider variation in results was observed for HDx recipients, but there was no overall difference between patient groups.

Table 6.1: Comparison of baseline characteristics of the three patient groups (average ± SE). One-way ANOVA with Bonferroni post-test analysis used (ns=not significant). ☼Kruskal-Wallis test used for non-parametric data, with Dunn's post-test.

	Non-KRT	HDx	KTx	
#Subjects	7	10	9	
Age (years)	34±12*	72±10*	55±17	*p<0.001
Male Gender	5 (71%)	6 (67%)	4 (44%)	ns
Creatinine (μmol/L)	82±15	664±120*	110±25	*p<0.001 HDx compared to Non-KRT and KTx
Potassium serum concentration (mmol/L)	4.1±0.2	5.0±0.8	4.3±0.3	ns
Calcium serum concentration (mmol/L)	2.48±0.08*	2.27±0.13*	2.43±0.08	*p<0.05 between Non-KRT and HDx
Phosphate serum concentration (mmol/)	1.15±0.18	1.63±0.35*	0.94±0.23*	*p<0.01 between HDx and KTx
Haemoglobin (g/L)	141±14	112±11*	143±14	*p<0.01 HDx compared to Non-KRT and KTx
Total serum cholesterol concentration (mmol/L)	5.2±0.8	3.5±0.5*	4.8±0.7	*p<0.01 HDx compared to Non-KRT and KTx
C-reactive protein serum concentration (mg/L)	3.0±0.9	39.0±34.9*	5.2±5.1	*p<0.01 HDx compared to Non-KRT and KTx☼
Parathyroid Hormone (U/L)	3.3±0.7	57.0±60	15.3±15	ns
PC serum concentration (mg/L)	0	0.36±0.34*	0	*p<0.05 HDx compared to Non-KRT and KTx
PCS serum concentration (mg/L)	1.2±0.9	20.8±9.0*	1.9±1.8	*p<0.001 HDx compared to Non-KRT and KTx
IS serum concentration (mg/L)	0.9±0.7	44.7±21.1*	1.8±0.9	*p<0.001 HDx compared to Non-KRT and KTx

6.4.1.1 Uraemic toxins

PC, PCS and IS serum concentrations all correlated closely with each other (Table 6.2 and Figure 6.2). As noted in Table 6.1, PC was only detectable in HDx recipients. PCS and IS remained correlated with each other, even in non-HDx recipients.

Differences were observed between the toxins in regards correlation to other baseline characteristics (Table 6.2). In particular, PC alone was not associated with age, Hb or total serum cholesterol. IS was also the only toxin that correlated with elevated serum phosphate. For non-HDx subjects, serum creatinine did not correlate with PC or IS concentrations.

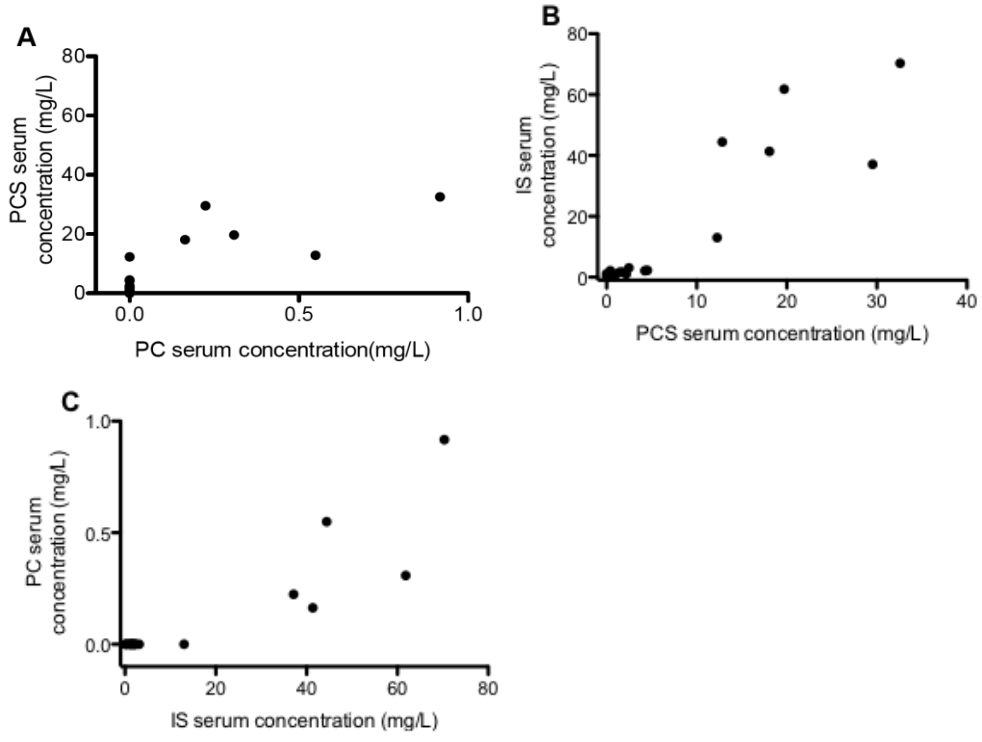


Figure 6.2: Correlation between uraemic toxins. A – PC-PCS; B – PCS-IS; C – IS-PC. All grouped results were obtained from Non-KRT and KTx recipients. All other individually visible data points (6) originated from HDx recipients.

Table 6.2: Correlation of serum concentrations of PC, PCS and IS to baseline characteristics of study participants sera. Whilst toxin concentrations were closely correlated with each other, differences were observed between toxins for other measured indices.

	PC	PCS	IS
Age (years)	ns	$r^2 = 0.28$, $p = 0.02$	$r^2 = 0.29$, $p = 0.02$
Male Gender	ns	ns	ns
Creatinine (μmol/L)	$r^2 = 0.50$, $p = 0.001$	$r^2 = 0.68$, $p < 0.0001$	$r^2 = 0.85$, $p < 0.0001$
Creatinine (μmol/L) - Not HDx	N/A (PC only detectable in HDx)	ns	ns
Phosphate (mmol/L)	ns	ns	$r^2 = 0.37$, $p = 0.009$
Calcium serum (mmol/L)	ns	ns	ns
Hb (g/L)	ns	$r^2 = 0.41$, $p = 0.004$	$r^2 = 0.31$, $p = 0.02$
Total serum chol (mmol/L)	ns	$r^2 = 0.27$, $p = 0.03$	$r^2 = 0.23$, $p = 0.049$
CRP (mg/L)	ns	ns	ns
PTH (U/L)	ns	ns	ns
PC (mg/L)	-	$r^2 = 0.63$, $p < 0.0001$	$r^2 = 0.79$, $p < 0.0001$
PCS (mg/L)	$r^2 = 0.63$, $p < 0.0001$	-	$r^2 = 0.83$, $p < 0.0001$
PCS (mg/L) - Not HDx	N/A	-	$r^2 = 0.39$, $p < 0.05$
IS (mg/L)	$r^2 = 0.79$, $p < 0.0001$	$r^2 = 0.83$, $p < 0.0001$	-
IS (mg/L) - Not HDx	N/A	$r^2 = 0.39$, $p < 0.05$	-

6.4.2 Tube formation

Sera from all patient groups reduced HUVEC tube formation in Matrigel, compared to the 20% FCS negative control (Non-KRT 68% {95% CI 46-90}, HDx 58% {48-68}, KTx 32% {12-51}, $p<0.05$). However, KTx recipients showed significantly reduced tube formation ability compared to both other groups ($p<0.05$) (figure 6.3). No correlation was found between Tube formation and any other measured factor ($p>0.05$ for age, gender, serum creatinine, Hb, phosphate, albumin, CRP, or uraemic toxins).

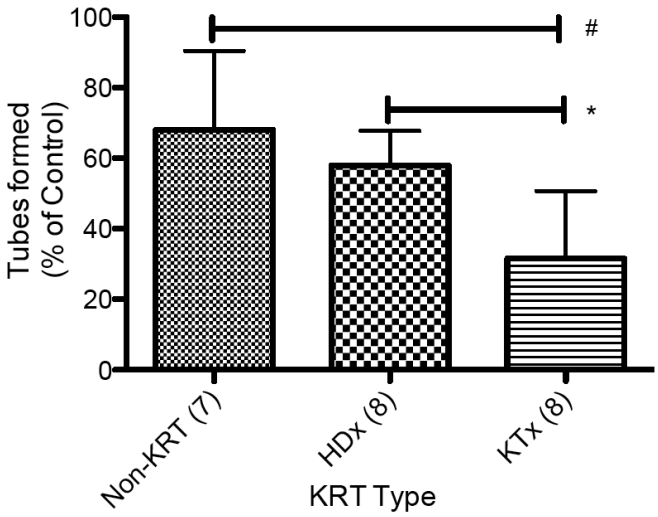


Figure 6.3: HUVEC Matrigel tube formation after 7 hour exposure to patient sera. Compared to the 20% FCS control, all patient groups demonstrated reduced tube formation ($p<0.05$), with KTx sera suppressing tube formation to a greater degree than both non-KRT ($^{\#}p<0.01$) and HDx ($^{*}p<0.05$) sera.

6.4.3 Migration

HUVEC migration was performed across an 8 micron pore Transwell to a 100ng/ml VEGF gradient. Addition of 20% HDx sera significantly increased the percentage of HUVEC migrating across the Transwell 2 hours after incubation, compared to all other sera types, and the negative control (as % of the negative control {95% CI}: Non-KRT 116% {74-159}, HDx 279% {186-373}, KTx 110% {66-154}, $p<0.05$) (Figure 6.4).

This increased migration observed with HDx sera-exposed HUVEC significantly correlated with factors associated with ESKD/HDx, including creatinine ($r^2 = 0.76$, $p<0.001$), phosphate ($r^2 = 0.47$, $p=0.017$), IS ($r^2 = 0.62$, $p = 0.02$), PC ($r^2 = 0.48$, $p = 0.012$), PCS ($r^2 = 0.666$, $p = 0.0014$) and PTH ($r^2 = 0.3$, $p = 0.04$). PTH itself was not significantly elevated in HDx compared to Non-KRT or KTx.

Migration was also negatively correlated with total cholesterol ($r^2 = 0.28$, $p = 0.04$), calcium ($r^2 = 0.34$, $p = 0.017$) and haemoglobin ($r^2 = 0.25$, $p = 0.047$), which were all significantly decreased in HDx subjects.

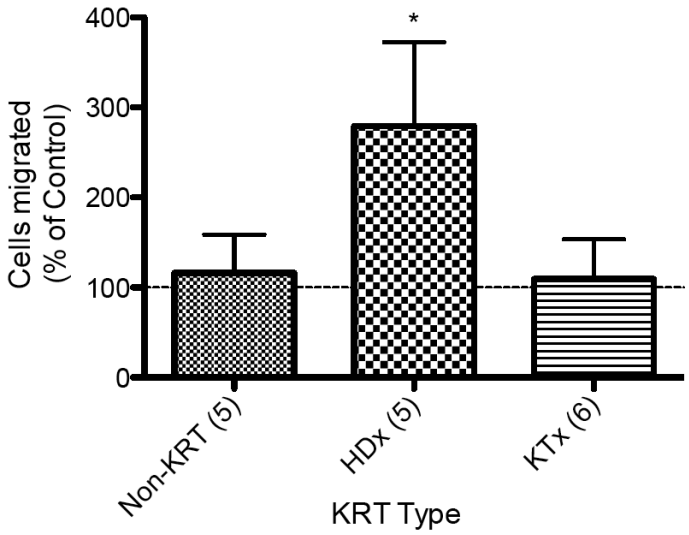


Figure 6.4: HUVEC migration to 100ng/ml VEGF across 8 micron pore Transwell, in the presence of human sera. Addition of 20% HDx sera was significantly associated with increased HUVEC migration across the Transwell, compared to control, non-KRT and KTx sera exposed HUVEC (*p<0.001, one way ANOVA).

6.4.4 VCAM-1 expression

VCAM-1 expression on HUVEC at 24 hours was not influenced by exposure to different sera types (MFI as % of negative control: Non-KRT 92±39%, HDx 95±19%, KTx 96±21%, p=ns) (figure 6.5). There was also no difference noted at a 4 hour time point, nor in the presence of TNF alpha (p=ns).

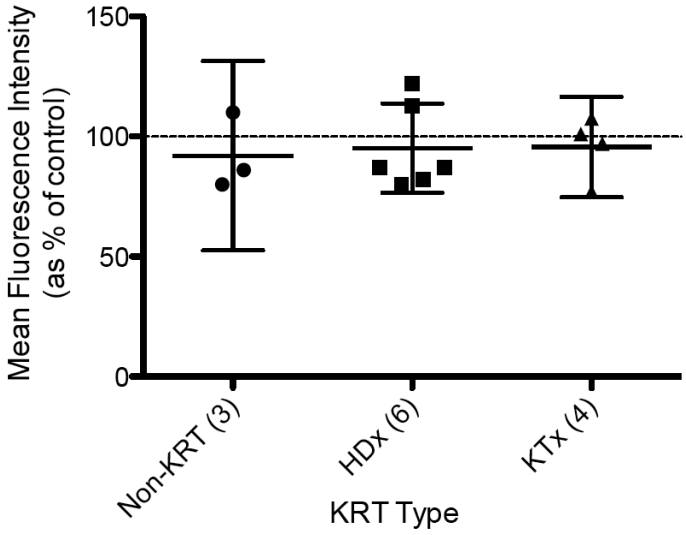


Figure 6.4: VCAM-1 expression on HUVEC post exposure to 20% human sera from non-KRT, HDx or KTX patients after 24 hour incubation. There was no significant difference between sera groups compared to the 20% FCS negative control (p=ns, one way ANOVA).

6.5 Discussion

These results highlight the difference between the *in vitro* effects of PC or IS, alone or in combination, compared to whole sera from different patient groups.

6.5.1 Uraemic toxins

Clinically significant elevations in PCS and IS were only observed in HDx recipient sera, with PC only detectable in this group (if PCS concentration was >10mg/L – see Figure 6.2). However, PCS and IS serum concentrations correlated in non-HDx recipient sera.

As discussed previously, the use of PC rather than PCS in *in vitro* testing has been raised as potentially not representing normal physiology[76]. This certainly must be taken into consideration when extrapolating *in vitro* effects of PC to the clinical setting. This was partly addressed by measuring both PC and PCS in our subjects. The serum concentrations were not comparable, with PCS concentration approximately 100x higher than that of PC. This may suggest that the processes which sulfate PC can potentially be overwhelmed, leading to presence of “free” PC in the circulation. Others have not been able to detect PC when using non-heat, non-acid methods for measurement of PC/PCS[58, 73]. It is difficult to compare assays for PCS, as there are subtle differences in the techniques used, and also the cut-offs for detection used.

PC and PCS concentrations also did not equally correlate with other biochemical indices in our subjects, specifically total serum cholesterol and Hb concentrations. PC concentrations were proportionally lower than their counterpart PCS, with a ratio approaching 1:100 between the two.

Further direct comparison of the effect of PC and PCS, with and without protein binding, on cultured HUVEC and other cell lines is required to determine which is

more significant. However, a recent *in vivo* study suggests that it is only the unbound fraction of PCS that has association with vascular dysfunction and CVD mortality[62]. This observation adds further weight to the use of PC *in vitro* for mechanistic studies, as low levels of “overflow” PC with elevated free PCS *in vivo* may be the pathologic agents in CVD. Our study measured both PCS and PC as separate entities.

6.5.2 Tube formation

KTx derived sera significantly reduced tube formation by HUVEC in matrigel compared to all other groups. There was no correlation between this finding, and any other measured factor.

It is well known that agents such as cyclosporine A through endothelin[498], and sirolimus and tacrolimus via cell cycle inhibitor p27[499] reduce EC survival and function. Wilasrusmee *et al* investigated the effect of immunosuppressives on tube formation by ECs – they demonstrated the marked inhibitory effect of cyclosporine A (via endothelin), and mycophenolate mofetil (specific mechanism unknown), but noted that tacrolimus, sirolimus and dexamethasone had limited effect[500].

This finding for rapamycin is in contrast to its known *in vitro* effect on ECs of marked reduction in function and cell survival[501], as well as the senescent effect the agent has on EPCs via telomerase inactivation[502].

None of the KTx subjects in this study were prescribed sirolimus, and were instead on a combination of cyclosporine or tacrolimus, plus mycophenolate mofetil and prednisolone.

It would have been expected to observe an effect of HDx sera compared to that derived from non-KRT subjects, but no such difference was observed. Both sera types suppressed tube formation compared to the FCS control, but not to the same degree as KTx derived sera. The reason for this is beyond the scope of this study, although it would be reasonable to speculate that unmeasured factors in HDx sera also have influence on EC tube formation, in both a retardant and stimulant fashion. Alternatively, degradation of particular agents (e.g. pro-inflammatory cytokines), normally elevated in HDx sera, may have occurred despite the rigorous processing and storage protocol used for sera in this study. Finally, HDx sera may have

required a longer incubation period with HUVEC to exert a greater effect on tube formation, *e.g.* longer duration of pre-treatment with sera prior to the assay being performed.

6.5.3 Migration

HUVEC migration was significantly augmented by the presence of HDx sera. Multiple HDx-dependent factors were also associated with increased migratory capacity. This included the three measured uraemic toxins, and is in contrast to the findings in Chapter 5, where increasing toxin concentrations did not affect EC migration.

As discussed previously, the migration assay was potentially not fully optimised – time of cell exposure to toxin/sera is likely to be of critical importance, given the changes in pAkt and pERK observed with HUVEC exposed to toxins. It could be postulated that very early exposure (0-60 minutes) leads to initial cell "stunning", followed by up-regulation within a few hours (our assay exposed cells for approximately 2.5 hours). Beyond 12-24 hours, cellular senescence and death are likely to occur, with surviving cells not able to complete normal functions, either due to impairment, or loss of cell-to-cell contact.

In CKD, KTx or ESKD on peritoneal dialysis, there is a steady state of uraemic toxins, with small variations only likely related to meals. However, for those on HDx, there is a significant (approximately 20%) drop in serum concentration in protein bound toxin[73]. This cyclical change in toxin concentration, along with the oxidative milieu of HDx could lead to greater EC injury, as cells are unable to reach equilibrium with their environment.

6.5.4 VCAM-1

No difference in VCAM-1 expression by HUVEC was seen with exposure to human sera. This included early and late time points, as well as in the setting of TNF alpha. We expected to find upregulation of VCAM-1, especially at the earlier time point, on HUVEC. Serum starvation of HUVEC also had no effect on outcomes.

Unlike direct addition of high concentrations of PC and IS, human sera did not cause widespread cell destruction, therefore it is unlikely that the activated cells were "missed".

An alternative possibility is that there are other suppressive agents in human sera that reduce the immediate response of HUVECs to noxious stimuli, such as IL10 and nitric oxide, which were not measured.

VCAM-1 is one of the important surface molecules on ECs involved in interactions with mononuclear cells, and its expression in ESKD requires further investigation.

6.5.5 Conclusions

These results highlight the complex interaction between factors present in human sera that may have an effect on normal vascular function. The lack of consistency between the results generated in Chapters 5 and 6 demonstrates that our *in vitro* environment was not the same as the as *in vivo* milieu, with results obtained from the latter a consequence of the increased complexity.

Given these *in vitro* findings, correlation between surrogate markers of CVD and biochemical/cellular markers of CVD risk is needed to further understand the role of uraemia in vascular disease.

Chapter 7: Human Clinical Observational Study

7.1 Background and aims:

The increased incidence of CVD in ESKD has been clearly demonstrated in the literature, as well as the understanding that the risk factor profile in ESKD leads to a number of different processes affecting vascular function in general. The interplay between these processes results in different patterns of CVD, response to therapeutic intervention, and overall poorer clinical outcomes. This was in part shown in Chapter 3 of this thesis.

Chapter 5 investigated the role of uraemic toxins PC and IS with cultured HUVEC and human EPCs in an *in vitro* model. This demonstrated the greater inhibitory effect of PC than IS on HUVEC tube formation, and the inability of EPCs to protect against addition of PC. Increased VCAM-1 expression was also observed with increased toxin concentration.

Chapter 6 demonstrated the complexity of the uraemic environment, whereby HDx sera, with documented increased serum concentrations of PC, PCS and IS, enhanced HUVEC migration to a VEGF gradient(approx 180% above the FCS control). Conversely, all serum groups demonstrated reduced tube formation compared to fetal calf serum, with KTx sera inhibiting tube formation to the greatest degree(Non-KRT 68% {95% CI 46-90}, HDx 58% {48-68}, KTx 32% {12-51}, $p<0.05$).

Given the findings in chapters 5 and 6 of altered CVD outcomes in KRT patient groups, and differences between the effects *in vitro* of uraemic toxins and human sera, an observational study was performed to compare serum concentrations of uraemic toxins, EPCs and other markers of CVD risk, with known surrogate markers of CVD. These markers included PWV, AIx (pre and post endothelial dependent and independent stimulation), and cIMT.

Whilst there is significant data on the interaction of EPCs with other markers of CVD risk, less is known about PC, PCS and IS. In the most complete study to date, Jourde-Chiche *et al* did not show any correlation of human serum concentrations PC, PCS or IS with CD34/CD133 or CD34/KDR positive cell counts from peripheral blood. Only indole-3 acetic acid and β2-microglobulin were negatively associated with CD34/133 PB counts, whilst indole-3 acetic acid also induced CD133 positive cell apoptosis *in vitro*. The study also failed to demonstrate an association between PWV and CD133 positive PB cell counts[503].

Uraemic toxins are not all increased to the same extent in patients with CKD. IS itself has been shown to be independently associated with serum creatinine and HDL concentration[504], In HDx, Meijers found no association between IS and PCS[489]. In Chapter 6, we were able to show a significant positive correlation between serum concentrations of IS, PC and PCS. IS concentration was also associated with serum phosphate concentration, whilst both IS and PCS concentrations were associated with serum total cholesterol and whole blood Hb concentrations.

The relationship between PWV and kidney function has been variable between studies. Covic *et al* found no difference in PWV in young HDx recipients[181], whilst Shinohara found a decrease in PWV in those on HDx compared to participants with CKD[505]. Marchais *et al* suggested it was not uraemic toxins, but increased serum calcium concentration that increased PWV[506].

AIx is associated with CVD in both the general[178] and KRT populations[176, 179]. Endothelial-dependent and independent function is assessed post administration of

the inhaled β-agonist salbutamol (which stimulates EC release of NO[507]) and sublingual GTN respectively (GTN is administered first, with maximal effect reached within 3-6 minutes, and return to baseline haemodynamics by 20 minutes)[508]. In the general population, CVD is associated with a decreased reduction in AIx post salbutamol compared to controls (-2.4% versus -13.2%), but no difference with GTN (-27.6% versus -38.9%)[507]. For KRT subjects, Covic *et al.* demonstrated significantly increased baseline AIx measurements for HDx and PDx recipients compared to KTx and control subjects. Reduced responses post GTN were seen for the same groups, whilst only PDx recipients had no response to salbutamol challenge[509].

Confirmation of association of IS, PC and PCS with markers of CVD is important, as levels of all three toxins can be reduced in early CKD through to ESKD on KRT, by diet[510, 511], pharmacologic[91, 512], and specific (but not standard) dialysis techniques[95, 513].

How EPCs fit in the model of vascular disease seen in CKD remains unclear. The postulate is that the endothelial dysfunction present in CKD, which causes changes in blood vessels leading to CVD, is in part due to impaired function of EPCs. Further, that the reduced EPC capability is secondary to the effects of increased serum concentrations of uraemic toxins IS and PC/PCS in CKD individuals.

7.2 Hypotheses

- That surrogate markers of risk of atherosclerosis (cIMT) and arteriosclerosis (PWV and AIx) correlate with increasing serum concentrations of PC, PCS and IS
- That low PB concentrations of EPCs relate to increased CVD risk in KRT recipients (as measured by increased aortic PWV, increased cIMT by US, and increased baseline radial artery AIx with reduced response to glycerol trinitrate and salbutamol) and are associated with increased PB concentrations of PC, PCS and IS

7.3 Methods:

An observational study was performed on males aged 45-65 years without diabetes. The three patient groups included HDx, KTx and normal controls. A history of significant CVD was used to stratify subjects further. Markers of CVD were assessed by measurement of cIMT, arteriosclerotic change by aortic PWV, and endothelial dysfunction by endothelial dependent and independent arterial pulse wave analysis for calculation of aortic augmentation index.

Ethics, target patients, inclusion and exclusion criteria, and methods for data collection are described in Chapter 2.10. Details of medical history and physical examinations performed are listed in Appendix B.

Investigations performed on all participants were:

- Medical History and Examination
- Blood collection
    - Commercial laboratory testing
    - IS, PC and PCS serum concentration measurement
    - FlowCytoMix for soluble surface markers
    - EPC PB concentration (defined as triple positive for CD34, CD133 and VEGFR2)
- Ultrasound for cIMT
- Tonometry
    - Radial PWA for AIx
    - Carotid-femoral PWV

Significant difficulties were found in patient recruitment, despite a statewide approach, due to tight inclusion and exclusion criteria. In particular, only one non-KRT subject with a history of CVD consented to the study, and no further KRT

recipients were available for investigation in the local and regional area surrounding the primary investigation site, despite frequent and repeated efforts at identifying appropriate subjects.

Specific subject characteristics are described in detail in Section 7.4.3. Tight patient criteria limited subject recruitment, but also allowed several major confounding factors (diabetes, gender, age) to be excluded. Whilst it would have been ideal to have a specific control non-KRT group with a CVD history, the protocol used allowed confidence to be had in the control subjects included.

7.4 Data preparation and analysis

7.4.1 Distribution

To test for normal Gaussian distribution of data, the D'Agostino & Pearson omnibus normality test was performed on EPC and surrogate marker results. If the $p < 0.05$, then the data was log10 transformed (with a constant added if values ≤ 0 were present – post GTN/salbutamol AIx) (see table 7.1).

Based on raw data, EPC counts were skewed to the right (K2 = 15.6). However, on log10 transformation, a bimodal distribution was demonstrated (Figure 7.1), with a highly significant difference between the means ($p<0.0001$).

For troponin T (whole blood) and PC (serum) concentrations, results were categorised between low (concentration at level below detection ability of test), and high. Comparison with previous results in KRT patients (to ascertain baseline levels) was not done, as any patient with active cardiac disease was already excluded from the study.

Table 7.1: Test for normal distribution of all measured subject variables, using D'Agostino & Pearson omnibus normality test. Log(10) transformation performed if $p<0.05$, and test repeated. EPC count did not conform to a normal distribution.

	# of Values	Mean	Standard deviation	Normality Test -K2	p value	Passed normality? ($\alpha>0.05$)
EPC (% PB MNCs)	25	0.013	0.014	16.0	<0.001	×
Log_{10} (EPC, %)	26	-2.56	1.17	7.7	0.02	×
AIx 0min (%)	25	3.0	14	2.3	0.31	✓
AIx GTN max (%)	25	3.0	14	2.3	0.31	✓
AIx salbutamol max (%)	25	13.5	13.68	2.7	0.25	✓
cIMT (mm)	25	0.71	0.11	7.8	0.02	×
Log_{10}(cIMT, mm)	25	-0.15	0.07	3.0	0.23	✓
PWV (m/s)	25	6.6	1.2	9.2	0.01	×
Log_{10}(PWV, m/s)	25	0.81	0.08	3.3	0.19	✓
BMI (kg/m^2)	26	27.4	4.37	6.8	0.03	×
Log_{10}(BMI, kg/m^2)	26	1.43	0.07	2.1	0.35	✓
SBP (mmHg)	27	145	27	6.1	0.048	×
Log_{10}(SBP, mmHg)	27	2.2	0.08	3.0	0.22	✓
DBP (mmHg)	27	80	15	55.5	<0.001	×
Log_{10}(DBP, mmHg)	27	1.9	0.08	4.9	0.08	✓
Urate serum conc. (mmol/L)	27	0.38	0.13	6.1	0.047	×
Log_{10}(urate, mmol/L)	27	-0.44	0.14	0.62	0.73	✓
Ca x P product ($mmol^2/L^2$)	27	2.7	1.3	7.1	0.03	×
Log_{10}(Ca x P, $mmol^2/L^2$)	27	0.39	0.20	1.6	0.44	✓
Homocysteine (mmol/L)	21	18.3	11.7	13.8	<0.001	×
Log_{10}(Homocysteine, mmol/L)	21	1.2	0.22	5.2	0.08	✓
TG (mmol/L)	27	1.6	1.1	13.8	0.001	×
Log_{10}(TG, mmol/L)	27	0.14	0.26	0.30	0.86	✓
PTH (pmol/L)	27	19.9	33.1	36.1	<0.001	×
Log_{10}(PTH, pmol/L)	27	0.89	0.59	1.6	0.45	✓
						....

	# of Values	Mean	Standard deviation	Normality Test -K2	p value	Passed normality? (α>0.05)
CRP (mg/L)	27	3.4	4.1	13.2	0.0013	×
Log_{10}(CRP, mg/L)	27	0.25	0.51	4.2	0.12	✓
IS (mg/L)	26	0.022	0.034	9.4	0.009	×
Log_{10}(IS, mg/L)	26	-2.4	0.84	5.0	0.08	✓
PCS (mg/L)	26	0.01	0.014	20.4	<0.001	×
Log_{10}(PCS, mg/L)	26	-2.3	0.51	2.7	0.27	✓

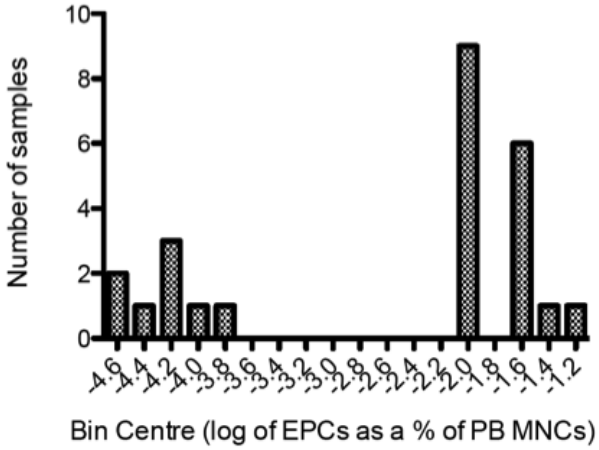


Figure 7.1: Frequency of distribution of log10 transformed raw EPC count from all subjects, showing two distinct populations. EPCs were subsequently analysed as a dichotomous variable.

7.4.2 Comparison of groups and assessment of significance

Categorical variables were compared using 2x2 or 2x3 contingency tables. Statistical significance was tested by two-sided Fisher's Exact or Chi-Square test ($p<0.05$).

Continuous variables were shown as average $\pm$ 95% CI, with differences between 3 or more groups calculated by one-way ANOVA, $p<0.05$(with Bonferroni post-test analysis for differences between groups). For categorical variables, the significance of the differences was calculated using Chi-Square Tests ($p<0.05$).

7.4.3 Baseline characteristics between KRT groups

Nine subjects were recruited for each of the 3 groups. Baseline characteristics and observations are described in Table 7.2.

Table 7.2: Comparison of subject characteristics, dependent on KRT modality. Continuous variables shown as geometric average (95%CI), with differences between groups calculated by one-way ANOVA (alpha<0.05). For categorical variables, total number (%), significance of differences between groups calculated using Chi-Square. $^{*,\#}p<0.05$, $^{**,\#\#}p<0.01$, $^{***,\#\#\#}p<0.001$

	Non-KRT	HDx	KTx
Number of Subjects	9	9	9
Age (years)	51 (47-55)	55 (51-59)	50 (46-54)
Paid Employment	9/9**	3/9**	6/9
History of Significant Cardiac Disease	1/9*	6/9*	3/9
Hypertension	1/9*	7/9	7/9
Hyperlipidaemia	1/9*	6/9	5/9
Smoker (current/former)	2/9	5/9	7/9
Alcohol intake	8/9	5/9	7/9
History of Gout	0/9	5/9**	0/9
Statin use	2/9	3/9	3/9
ESA use	0/9	9/9**	0/9
Beta-blocker use	0/9*	5/9*	2/9
ACEi or ARB use	1/9	4/9	3/9
Aspirin or Warfarin use	1/9	6/9	4/9
Duration KRT (yrs) - Dialysis - KTx	 - -	 1.6 (0.6-4.2)	 1.4 (0.8-2.7) 3.9 (2.1-7.2)
Body Mass Index (kg/m^2)	25 (23-27)*	30 (27-33)*	27 (24-31)
Systolic Blood Pressure (mmHg)	128 (123-134)***	158 (133-188)***	144 (128-163)
Diastolic Blood Pressure (mmHg)	74 (68-81)	81 (66-100)	82 (75-90)
Haemoglobin (g/dL)	157 (151-163)	118 (109-127)***	152 (141-164)
Creatinine (μmol/L)	79 (70-89)*	N/A	126 (89-177)*
Potassium serum concentration (mmol/L)	4.3 (4.0-4.6)*	4.9 (4.5-5.4)*	4.5 (4.1-5.0)
Urate serum concentration (mmol/L)	0.31 (0.25-0.37)*	0.34 (0.29-0.41)	0.47 (0.37-0.59)*
Calcium-phosphate serum product ($mmol^2/L^2$)	2.2 (2.0-2.6)	3.2 (2.0-5.3)*	2.1 (1.7-2.6)
Parathyroid hormone serum conc (pmol/L)	2.6 (1.7-3.8)**	19.5 (5.2-72.7)**	9.6 (5.9-15.6)
			…..

	Non-KRT	HDx	KTx
Albumin serum concentration (g/L)	39 (37-42)	33 (30-35)**	37 (35-39)
Total cholesterol:HDL serum ratio	3.5 (2.8-4.3)	3.9 (3.0-5.0)	3.3 (2.8-4.0)
C-reactive protein (mg/L)	0.6 (0.4-1.0)**	3.1 (1.3-7.2)	2.9 (1.3-6.7)
Triglycerides (mmol/L)	1.0 (0.7-1.5)	1.6 (1.0-2.4)	1.7 (1.1-2.7)
Homocysteine (mmol/L)	10.9 (8.9-13.4)**	19.2 (13.9-26.5)**	19.7 (10.1-38.4)
Troponin T High (≥0.02U/L)	0/7	8/9***	1/7
Fibrinogen (mg/L)	2.8 (2.4-3.3)**	3.9 (3.4-4.6)**	3.5 (3.2-3.8)

7.4.3.1 History and examination

Cause of kidney failure was glomerulonephritis in 6 participants, polycystic kidney disease in 4, and the remainder unknown, reflux nephropathy, vascular or drug-related.

Cardiac disease was predominantly represented in the HDx group, however similar rates of HT, hypercholesterolaemia and smoking were found in both KRT groups. Gout was only reported in HDx patients, however there was no statistically significant difference in urate levels between those and other subjects.

Six KTx recipients were receiving tacrolimus, two were receiving cyclosporine, and one was not receiving a calcineurin inhibitor. Seven were taking mycophenolate mofetil, and one was taking azathioprine. Five were taking prednisolone (dose range 1-10mg/day). Only one patient was on mono-therapy for immunosuppresion, consisting of tacrolimus.

Of the Non-KRT subjects, only one patient, who had a history of cardiac disease, was prescribed antihypertensives, aspirin and a statin agent. There was no difference between the HDx and KTx groups in prescription of these agents. Only HDx subjects were prescribed an ESA.

KTx recipients had been on HDx or PDx for 1.4 years prior to transplantation. The time since transplantation was 3.9years. HDx recipients had been on KRT for 1.6years.

BMI ($kg/m^{2)}$ was increased in HDx subjects compared to Non-KRT, but no difference was observed with KTx recipients.

Systolic, but not diastolic, blood pressure was increased in HDx subjects compared to Non-KRT. This equated with an increased pulse pressure (mmHg) between HDx

recipients and non-KRT subjects, but no difference when compared to KTx recipients.

7.4.3.2 Routine laboratory testing

Haemoglobin concentrations were significantly lower in HDx recipients compared to both other groups. All HDx recipients, and none of the other groups, were prescribed an ESA.

HDx recipients had a significantly higher serum potassium concentration compared to Non-KRT subjects, but there was no difference to KTx recipients.

KTx recipients had a significantly higher serum concentration of urate compared to both Non-KRT and HDx recipients.

Calcium-phosphate product was significantly higher in HDx recipients, compared to both on Non-KRT and KTx recipients. This was based on an increased serum concentration of phosphate in HDx recipients compared to the other patient groups, whilst serum calcium concentrations were similar.

There was no significant difference between any of the groups for the ratio of total cholesterol to HDL in serum. Both total cholesterol and HDL concentrations in serum were significantly lower in HDx compared to Non-KRT subjects. As noted above, there was no difference in the use of statin agents between the groups.

There was no significant difference in serum CRP concentration between any of the subject groups.

Troponin T concentration was in the high group for 8/9 HDx recipients, but only 1/9 KTx recipients, and none of the non-KRT subjects.

HDx subjects had a higher fibrinogen serum concentration than both Non-KRT and KTx, but only significantly so compared to Non-KRT.

7.4.4 Extended laboratory investigation - results between KRT groups

The geometric means of serum sVCAM-1 concentrations, a measure of endothelial cell activation, were not significantly different between the three patient groups (p=ns), with broad confidence intervals noted (Table 7.3).

PC (measured by HPLC) was not detected in any of the non-KRT subjects, and only one of eight KTx recipients. It was, however, detected in seven of nine HDx serum samples.

Both IS and PCS serum concentrations (measured by HPLC) were significantly higher in HDx recipients compared to both non-KRT subjects and KTx recipients ($p<0.001$). There was no statistically significant difference in PCS or IS serum concentrations between non-KRT and KTx recipients.

Table 7.3: Comparison of measured factors between patient groups based on KRT type (geometric metric, 95%CI). Statistically significant increases in PC, PCS and IS demonstrated in HDx recipients compared to other patient groups, with no difference between non-KRT and KTx subjects. No difference in PWV noted between groups, however baseline and post-GTN/salbutamol AIx measurements lower in non-KRT subjects compared to HDx recipients. Non-KRT subjects also demonstrated significantly higher PB EPC counts than KRT recipients. *p<0.05, **p<0.01, ***p<0.001.

	Non-KRT (9)	HDx (9)	KTx (9)
sVCAM-1 (ng/ml)	2414 (1534-3799)	3718 (1682-8219)	4704 (1156-19142)
PC detected (mg/L)	0/9	7/9*** (0.69±0.34)*	1/8 (0.22)
PCS (mg/L)	0.7 (0-9.9)	17.9*** (9.1-35.1)	1.1 (0-13.3)
IS (mg/L)	1.4 (0.8-2.4)	41.8*** (16.0-108.9)	1.3 (0.5-3.2)
PWV (m/s)	6.3 (5.5-7.2)	5.1 (2.7-9.5)	6.5 (5.7-7.5)
AIx - baseline (%)	7.0 (2.2-21.6)	26.0* (18.0-37.4)	15.5 (5.4-39.5)
AIx – minimum level post GTN (%)	-14.1 (-19.5 - -1.0)	11.1* (5.3-18.2)	2.2 (-7.0-17.6)
ΔAIx post GTN (%)	18.6 (12.1-28.7)	4.0 (0.3-45.7)	11.6 (4.3-27.2)
AIx – minimum level post salbutamol (%)	1.0 (-6.0 - 11.0)	17.5* (9.7-27.3)	8.7 (-6.6 – 36.9)
ΔAIx post salbutamol (%)	2.3 (0.1-44.1)	2.6 (0-8.4)	0.7 (-1.7 – 25.9)
Carotid IMT (mm)	0.66 (0.58-0.75)	0.74 (0.65-0.83)	0.70 (0.62-0.79)
EPC Count “Low” (<0.01% of PBMNCs)	0/9	4/8*	4/8*

7.4.4.1 EPC PB concentration

Four of eight HDx and four of eight KTx recipients had “low” (<0.01% of total PB MNC count) EPC concentrations as determined by FAC analysis (EPCs identified as triple positive for CD34, CD133 and VEGFR2), whereas all (9/9) non-KRT subjects had “high” levels ($p<0.05$).

7.4.4.2 Pulse wave velocity

There was no difference in PWV between groups, with all measurements below 10m/s (Figure 7.2).

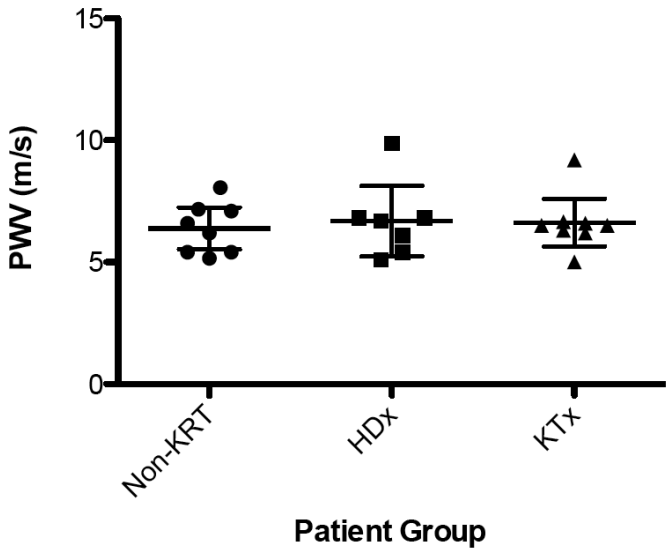


Figure 7.2: Vertical scatter plot of PWV by patient group, with average ± 95% CI shown. There was no difference between any of the groups, with all measurements below 10m/s (one-way ANOVA, p=ns).

7.4.4.3 Augmentation index

HDx recipients had significantly higher AIx measurements (%) at baseline, and post endothelial-dependent and endothelial-independent stimulation, compared to non-KRT subjects.

Post-GTN, AIx was significantly lower in the non-KRT group compared to HDx recipients. There was no difference in the actual reduction in AIx percentage between groups however, indicating the response to GTN was the similar for all.

Post-salbutamol, AIx was again significantly lower in the non-KRT group compared to HDx recipients. There was again no difference in the actual reduction in AIx percentage between groups.

Specifically investigating KTx recipients further, linear regression demonstrated no difference between the slopes of baseline-GTN (1.1 ± 0.2, r^2 0.77, $p<0.01$) and baseline-salbutamol AIx (1.3 ± 0.2, r^2 0.84, $p<0.01$), however the y-intercept for baseline-GTN was significantly lower ($p<0.05$) (Figure 7.3).

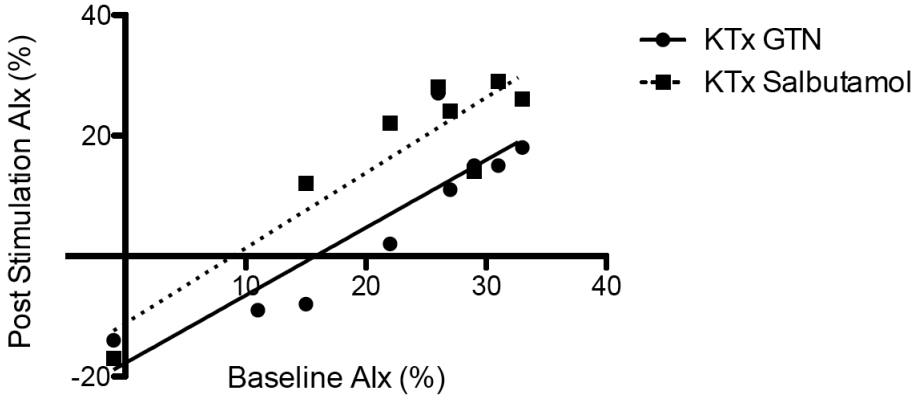


Figure 7.3: Comparison of slopes of AIx baseline-GTN and baseline-salbutamol in KTx recipients. There was no statistically significant difference between the two slopes (p=0.7).

7.4.4.4 cIMT

No difference was seen in cIMT measurements between groups ($p>0.05$). A range of measurements was observed for each patient group, as demonstrated in Figure 7.4.

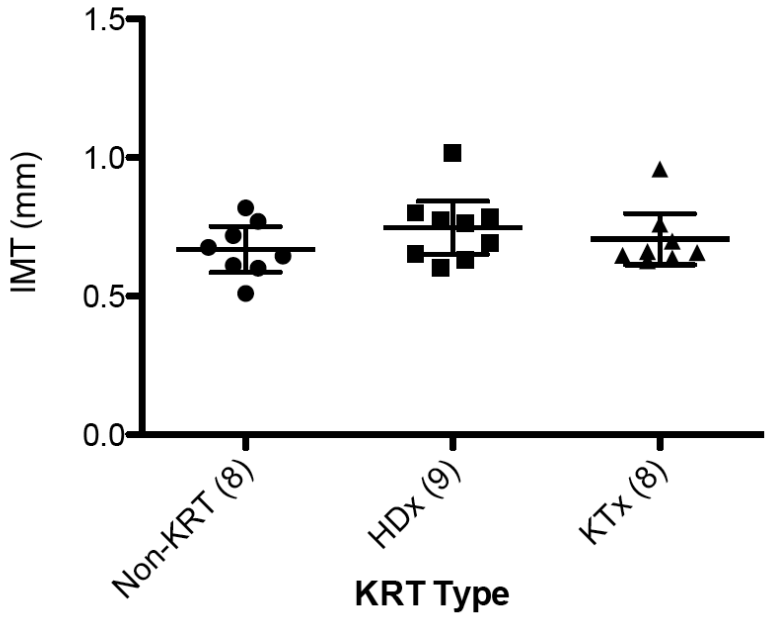


Figure 7.4: Scatter plot of cIMT measurements (mm) for each KRT type. No significant difference was found between the groups (Non-KRT 0.67±0.08mm, HDx 0.75±0.1, KTx 0.71±0.09; p=ns, one-way ANOVA).

7.4.4.5 EPC peripheral blood count

All non-KRT recipients had EPC counts ≥ 0.01% of the peripheral blood mononuclear cell count, whereas only half of HDx and KTx recipients had “high” EPC counts (Table 7.4).

A wide variation in EPC counts was observed for non-KRT subjects, whereas KRT recipients demonstrated two groups of EPC counts: those between approximately 0.01-0.02% of PB MNC, and a second group with counts less than 0.0001% (Figure 7.5).

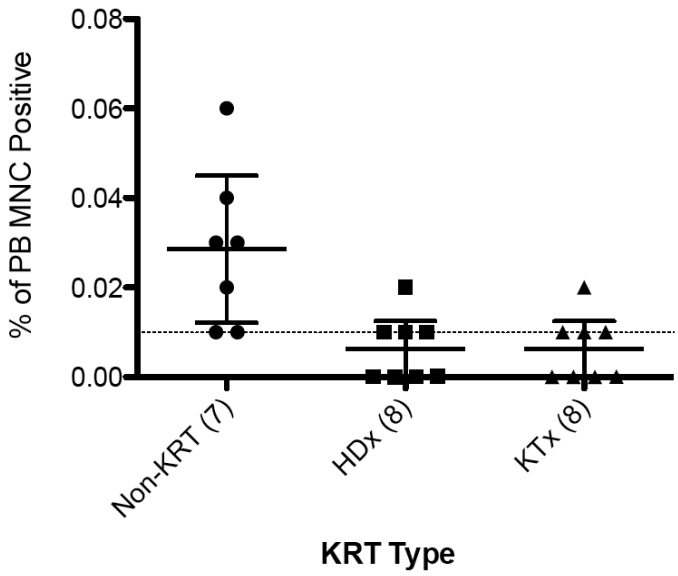


Figure 7.5: Distribution of PB EPC counts as % of MNCs (including all cells triple positive for CD34, CD133 and VEGFR2). Dotted line indicates cut-off for “high” or “low” EPC count (at 0.01% of PB MNCs)

7.4.5 Characteristics between subjects with and without a history of ischaemic heart disease

There was no control group specifically with CVD, with a history of CVD primarily represented in the HDx group. Therefore, associations with IHD were likely confounded by the presence of KRT. Table 7.4 lists baseline measures of subjects by presence or absence of IHD. Increased age, hypertension, current or former smoker, duration of KRT, gout, and hyperlipidaemia were associated with IHD. Regarding medication prescribed, statins, ESAs, beta-blockers and aspirin/warfarin were all more likely to be prescribed to those with a history of IHD. ACEi/ARBs however showed no statistically significant difference between groups. On clinical examination, increased BMI ($p<0.05$), but not systolic or diastolic BP, was associated with IHD.

Blood indices with association included increased potassium, creatinine, calcium-phosphate product, CRP and fibrinogen, and high troponin T. However, lipid and cholesterol indices, homocysteine, urate and sVCAM-1 were not associated with IHD history.

For uraemic toxins, increased PC and IS concentrations showed significant association with IHD, whilst PCS concentration approached statistical significance in its relationship to IHD ($p=0.05$).

Regarding surrogate markers of endothelial function PWV demonstrated no difference between groups, whilst cIMT, a measure of coronary atherosclerosis, was greater in the IHD positive group. Baseline, post-GTN and post-salbutamol AIx measurements were not statistically different between IHD and non-IHD groups. The raw change in AIx between baseline and post-GTN was however statistically greater

in the non-IHD group, consistent with increased endothelial-independent vessel dilatation.

High EPC counts were found in 14/15 non-IHD, but only 3/10 subjects with known IHD.

Table 7.4: Comparison of characteristics of subjects and measured variables, dependent on presence or absence of IHD. Significance calculated by two-tailed T-test for continuous variables, and two-sided Fisher's Exact Test for categorical variables. Continuous variables shown with geographic mean (95%CI), categorical variables with (% of total). *p<0.05, **p<0.01, ***p<0.001.

	IHD	No IHD
Number of subjects	11	16
Non-KRT	1	8
HDx	7	2
KTx	3	6
Age (years)	56 (52-59)**	49 (47-52)**
Hypertension	9/11*	6/16*
Hyperlipidaemia	9/11**	4/16**
Smoker (current/former)	8/11	6/16
Alcohol intake	7/11	13/16
History of Gout	5/11**	0/16**
Statin use	7/11*	2/16*
ESA use	7/11*	2/16*
Beta-blocker use	6/11**	1/16**
ACEi or ARB use	5/11	3/16
Aspirin or Warfarin use	10/11***	1/16***
Body Mass Index (kg/m^2)	29.3 (26.5-32.2)*	25.6 (23.7-27.6)*
Systolic Blood Pressure (mmHg)	152 (133-174)	137 (126-148)
Diastolic Blood Pressure (mmHg)	75 (66-85)	82 (75-90)
Creatinine serum concentration (μmol/L), excluding HDx	150 (54-419)**	89 (78-101)**
Potassium serum concentration (mmol/L)	4.8 (4.4-5.3)*	4.4 (4.2-4.7)*
Urate serum concentration (mmol/L)	0.38 (0.29-0.49)	0.36 (0.31-0.41)
Calcium-phosphate serum product	3.3 (2.6-4.3)**	2.2 (1.9-2.6)**
Albumin serum concentration (g/L)	35 (32-38)	37 (36-39)
Total cholesterol:HDL serum ratio	3.6 (3.0-4.5)	3.5 (3.0-4.1)
Triglycerides (mmol/L)	1.7 (1.2-2.3)	1.2 (0.9-1.7)
Homocysteine (mmol/L)	18.2 (14.1-23.3)	14.6 (10.0-21.1)
		….

	IHD	No IHD
Parathyroid Hormone (pmol/L)	13.3 (4.5-39.5)	5.4 (3.1-9.5)
C-reactive protein (mg/L)	3.5 (1.7-7.6)**	1.1 (0.7-1.9)**
Troponin T High (≥0.02 U/L)	7 (64%)*	2 (17%)*
Fibrinogen	4.1 (3.5-4.7)***	3.0 (2.7-3.3)***
sVCAM-1 (ng/ml)	2841 (1438-5612)	3083 (2033-4674)
PC detectable (mg/L)	6/10 (60%)* 0.003 (0-0.4)	3/16 (19%)* $1x10^{-5}$ ($0-2x10^{-4}$)
PCS (mg/L)	9.3 (4.1-21.0)	0.4 (0.02-10.3)
IS (mg/L)	19.4 (6.8-55.1)***	1.8 (0.7-4.2)***
PWV (m/s)	6.7 (5.8-7.8)	6.3 (5.7-6.8)
AIx - baseline (%)	13 (4-34)	11 (4-25)
AIx – minimum level post GTN (%)	6 (-3 – 19)	-8 (-14 – 4)
ΔAIx post GTN (%)	8 (3-18)*	17 (13-22)*
AIx – minimum level post salbutamol (%)	11 (-3 – 37)	8 (1-17)
ΔAIx post salbutamol (%)	6 (3-11)	9 (6-13)
Carotid IMT (mm)	0.78 (0.71-0.85)**	0.65 (0.61-0.69)**
EPC Count “High” (≥0.01% of PBMNCs)	3/10 (30%)**	14/15 (93%)**

7.4.6 EPC associations

7.4.6.1 Baseline characteristics

History of HT and SBP, and history of hyperlipidaemia were significantly associated with low PB EPC counts. Diastolic BP and serum lipids however showed no association (Table 7.5).

History of gout and elevated measured serum urate concentrations demonstrated a trend to a statistically significant association with low EPC counts. History of smoking also showed a similar trend.

Prescription of ESAs or statin agents had no association with EPC count. However, ACEi/ARB use was associated with lower EPC counts, whilst aspirin or warfarin use was associated with high EPC counts.

Markers of inflammation CRP and fibrinogen, as well as increased serum creatinine were associated with reduced EPC counts ($p<0.01$), whilst albumin concentration was not.

Table 7.5: Comparison of baseline characteristics of subjects, dependent on EPC count, as a percentage of total mononuclear cells (high ≥ 0.01%, low < 0.01%). Significance calculated by two-tailed T-test for continuous variables, and Two-sided Fisher's Exact Test for categorical variables. Continuous variables shown with geographic mean (95%CI), categorical variables with (% of total). *p<0.05, **p<0.01, ***p<0.001.

	EPC Count High	EPC Count Low
Number of subjects	17	8
Age (years)	51 (48-53)	54 (50-59)
Hypertension	6 (35%)*	7 (88%)*
Hyperlipidaemia	4 (24%)**	7 (88%)**
Smoker (current/former)	6 (35%)	6 (75%)
Alcohol intake	12 (71%)	6 (75%)
History of Gout	1 (6%)	3 (38%)
History of IHD or cerebrovascular disease	3 (18%)**	7 (88%)**
Statin use	4 (24%)	4 (50%)
ESA use	4 (24%)	4 (50%)
Beta-blocker use	2 (12%)	3 (38%)
ACEi or ARB use	2 (12%)***	7 (88%)***
Aspirin or Warfarin use	10 (91%)***	1 (6%)***
Body Mass Index (kg/m^2)	26 (24-28)	28 (24-33)
SBP (mmHg)	136 (126-148)*	160 (135-189)*
DBP (mmHg)	80 (73-87)	79 (65-94)
Creatinine serum concentration (μmol/L), HDx excluded	87 (76-100)**	159 (68-373)**
Potassium serum concentration (mmol/L)	4.5 (4.2-4.8)	4.7 (4.3-5.2)
Urate serum concentration (mmol/L)	0.34 (0.30-0.39)	0.42 (0.30-0.58)
Ca-P serum product ($mmol^2/L^2$)	2.4 (2.0-2.8)	2.5 (1.5-4.2)
Albumin serum concentration (g/L)	37 (35-39)	34 (31-39)
Total cholesterol:HDL serum ratio	3.8 (3.3-4.3)	3.1 (2.3-4.1)
C-reactive protein (mg/L)	1.2 (0.7-2.1)**	5.3 (2.7-10.2)**
Troponin T High (U/L)	5/15 (33%) 0.015 (0.01-0.02)	4/8 (50%) 0.031(0.01-0.09)
Fibrinogen (g/L)	3.2 (2.8-3.5)**	4.4 (3.6-5.4)**

7.4.6.2 Surrogate markers of EC function and uraemic toxins

sVCAM-1 showed no association with EPC counts.

PCS serum concentration demonstrated a substantial, but non-significant (p=0.1) trend towards an association with EPC counts. PC and IS showed no association.

However, lower PWV, and lower AIx measurements, including at baseline and after GTN or salbutamol were associated with high EPC counts. There was also an association of decreased cIMT thickness with increased EPC count (Table 7.6).

Table 7.6: Comparison of characteristics of subjects (including surrogate markers), divided by EPC count, as a percentage of total mononuclear cells (high ≥ 0.01%, low < 0.01%). Significance calculated by two-tailed T-test for continuous variables, and Two-sided Fisher's Exact Test for categorical variables. Continuous variables shown with geographic mean (95%CI), categorical variables with (% of total). *p<0.05, #p=0.01.

	EPC Count High (17)	EPC Count Low (8)
sVCAM-1 (ng/ml)	2347 (1690-3260)	3259 (1369-7758)
PC detectable (mg/L)	5/15 (33%) 0.004 (0-0.1)	4/8 (50%) 0.008 (0-0.5)
PCS (mg/L)	2.9 (1.1-7.3)#	4.5 (3.0-67.4) #
IS (mg/L)	3.4 (1.3-9.1)	10.5 (1.4-78.7)
PWV (m/s)	6.1 (5.6-6.7)*	7.3 (6.2-8.7)*
AIx - baseline (%)	13 (6-23)*	20 (7-49)*
AIx – minimum level post GTN (%)	-4 (-10 – 4)*	8 (-3 – 24)*
ΔAIx post GTN (%)	14 (9-21)	9 (3-24)
AIx – minimum level post salbutamol (%)	7 (1-15)*	11 (-6 – 49)*
ΔAIx post salbutamol (%)	5 (3-9)	4 (-1 – 13)
Carotid IMT (mm)	0.67 (0.63-0.72)*	0.77 (0.66-0.89)*

7.4.7 Correlation of surrogate markers with blood indices

Analysis was performed using Pearson correlation coefficient. Results expressed as the coefficient of determination (R square, r^2), with significance (alpha < 0.05), calculated by two-tailed P value, not corrected for multiple comparisons, in brackets. For variables that did not follow a Gaussian distribution, Spearman's rank correlation coefficient was calculated to measure level of association (Table 7.7).

7.4.7.1 cIMT

There was a significant negative association of cIMT with DBP and Hb. A positive association was demonstrated with age, CRP, troponin T and fibrinogen, all of which are known risk factors for IHD. Further association was noted with PWV, and AIx at baseline and post-GTN. There was a trend only for association of cIMT with AIx post-salbutamol (p=0.06).

7.4.7.2 Pulse wave velocity

There was a significant positive association of PWV with SBP (a component of the algorithm used to determine aortic PWV) and trend only to association with PB EPC count (as a continuous variable).

7.4.7.3 Augmentation index

Lower baseline AIx was associated with increased serum albumin and Hb concentrations. Marker of cardiac damage troponin T, and inflammation-CRP were positively associated with increased baseline AIx.

AIx post-GTN demonstrated significant correlation with SBP, CRP, troponin T, PC and PCS. Negative correlations were also documented with albumin, Hb, and EPC counts.

AIx post-salbutamol however, did not show the same relationship with IS or PCS, nor with CRP or troponin T.

Baseline and post stimulation AIx measurements all significantly correlated with each other.

Table 7.7: Analysis of correlation between surrogate markers of vascular function and independently measured variables. *For variables that did not follow a Gaussian distribution, Spearman's rank correlation coefficient was calculated to measure level of association.

	cIMT (log)	PWV (log)	AIx baseline	AIx GTN	AIx Ventolin
Age	0.44 (0.03)	0.17 (0.44)	0.16 (0.45)	0.37 (0.066)	0.34 (0.101)
BMI (log)	0.289 (0.17)	0.16 (0.47)	0.27 (0.19)	0.40 (0.056)	0.51 (0.015)
SBP (log)	-0.008 (0.97)	0.54 (0.008)	0.15 (0.46)	0.43 (0.031)	0.35 (0.088)
DBP (log)	-0.47 (0.017)	0.09 (0.67)	0.04 (0.85)	0.19 (0.37)	0.21 (0.31)
Ca x P (log)	0.22 (0.30)	0.15 (0.50)	0.028 (0.89)	0.28 (0.18)	0.10 (0.65)
PTH (log)	0.12 (0.58)	-0.20 (0.59)	0.33 (0.10)	0.33 (0.11)	0.30 (0.14)
Albumin	-0.38 (0.065)	-0.22 (0.31)	-0.50 (0.009)	-0.51 (0.009)	-0.38 (0.063)
Tot Chol : HDL	-0.16 (0.45)	0.089 (0.69)	-0.09 (0.66)	0.14 (0.50)	0.15 (0.47)
TG (log)	-0.18 (0.40)	-0.24 (0.27)	0.17 (0.41)	0.11 (0.60)	0.24 (0.24)
Homocysteine (log)	-0.06 (0.80)	-0.26 (0.31)	0.24 (0.31)	0.41 (0.083)	0.38 (0.10)
Hb	-0.54 (0.006)	-0.37 (0.09)	-0.50 (0.011)	-0.65 (0.001)	-0.47 (0.022)
CRP (log)	0.42 (0.035)	0.11 (0.61)	0.40 (0.045)	0.41 (0.04)	0.34 (0.099)
**Trop T*	*0.47* *(0.028)*	*0.15* *(0.54)*	*0.43* *(0.048)*	*0.46* *(0.037)*	*0.29* *(0.19)*
Fibrinogen	0.48 (0.04)	0.056 (0.84)	0.32 (0.18)	0.46 (0.057)	0.45 (0.063)

	cIMT (log)	PWV (log)	AIx baseline	AIx GTN	AIx Ventolin
Ferritin	0.29 (0.18)	-0.21 (0.36)	0.29 (0.18)	0.20 (0.36)	0.39 (0.073)
IS (log)	0.29 (0.17)	0.15 (0.50)	0.23 (0.27)	0.40 (0.051)	0.27 (0.51)
**PC*	*0.26* *(0.23)*	*0.092* *(0.68)*	*0.48* *(0.014)*	*0.60* *(0.002)*	*0.51* *(0.012)*
PCS (log)	0.32 (0.28)	-0.001 (1.0)	0.37 (0.086)	0.46 (0.031)	0.28 (0.20)
**EPC (% of MNC) (log)*	*-0.33* *(0.127)*	*-0.41* *(0.066)*	*-0.37* *(0.076)*	*-0.46* *(0.028)*	*-0.42* *(0.039)*
cIMT (log)	- -	0.44 (0.039)	0.47 (0.021)	0.45 (0.032)	0.40 (0.061)
PVW (log)	0.44 (0.039)	- -	0.16 (0.49)	0.29 (0.20)	0.28 (0.22)
AIx baseline	0.47 (0.021)	0.16 (0.49)	- -	0.81 (<0.001)	0.82 (<0.001)
AIx post GTN	0.45 (0.032)	0.29 (0.20)	0.81 (<0.001)	- -	0.85 (<0.001)
AIx post salbutamol	0.40 (0.061)	0.28 (0.22)	0.82 (<0.001)	0.85 (<0.001)	- -

7.4.8 Correlation of factors with uraemic toxins PC, PCS and IS

Serum concentrations of PCS and IS were significantly correlated with each other (Pearson correlation of log10 transformed data: r=0.54, two-tailed p=0.004). Figure 7.6 demonstrates the association, with subject groups defined separately – 8 of 9 HDx subjects had IS serum concentrations higher than all other patients. The correlation remained significant even with these subjects excluded (r=0.34, p=0.01)

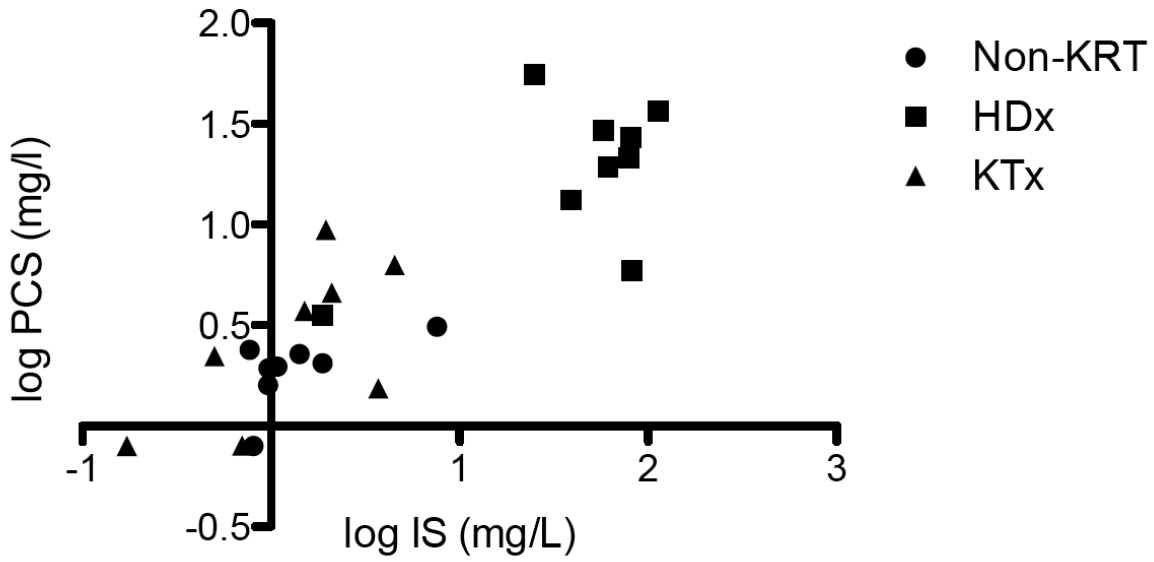
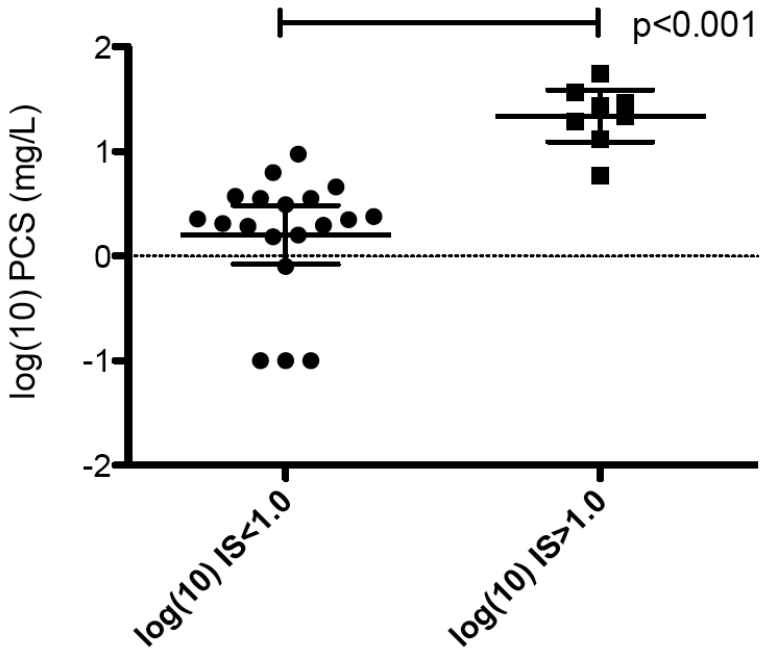


Figure 7.6: Linear regression of log10 transformation of measured PCS and IS (both mg/l). There was a significant correlation between the two (p<0.001), however with a dichotomous appearance, especially of log IS results.

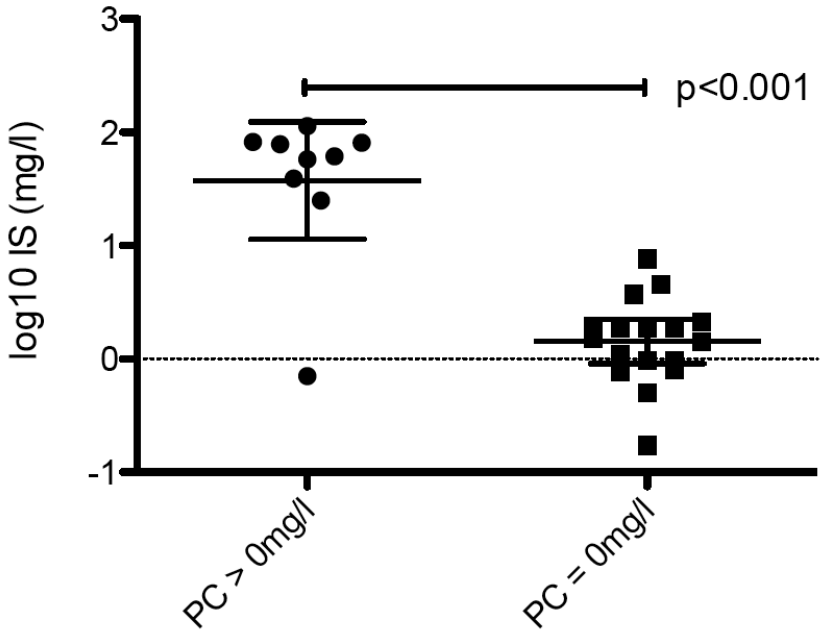
Given the dichotomous appearance of log(10) IS, the data was re-analysed using log(10) IS as a categorical variable, “high”≥1.0, and “low”<1.0 (Figure 7.7). There was a significant (p<0.001) difference between the IS groups for PCS concentration:



Log(10) IS	**<1.0**	**≥ 1.0**
Number of subjects	19	8
PCS concentration, mg/L {95%CI}	5.8 {0-13.8}	11.3 {4.1-18.5}

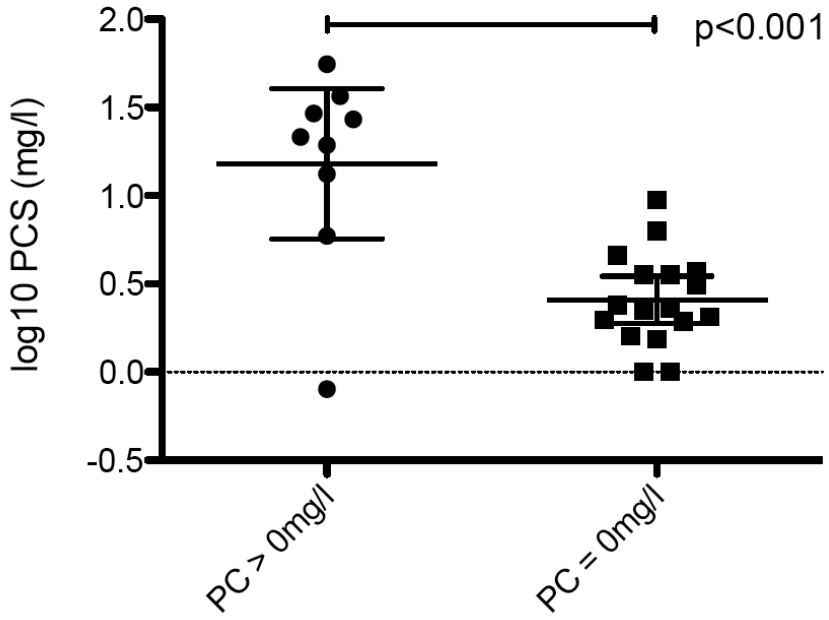
Figure 7.7: Comparison of high and low IS serum concentrations from subjects on the basis of PCS serum concentration. Log(10) transformation used to normalise data – due to dichotomous distribution, IS was considered as a categorical variable, demonstrating a significant association between the high IS group, and elevated (log) PCS concentrations (p<0.01).

PC was classified as a categorical variable, either detectable or undetectable. One KTx recipient showed reduced IS and PCS serum concentrations in the setting of detectable PC (Figures 7.8 and 7.9). This patient did not have any other outlier results – this suggests an issue with measurement of PC alone. Otherwise, elevated concentrations of both IS (PC detectable: 1.6mg/l {1.1-2.1}, PC undetectable 0.15mg/l {0-0.3}), and PCS (1.2mg/l {0.8-1.6}; 0.4mg/l {0.3-0.5}) were significantly associated with detectable PC ($p<0.001$).



PC	> 0mg/l	0mg/l
Number of subjects	9	17
IS concentration, mg/l {95%CI}	1.6 {1.1-2.1}	0.2 {0-0.3}

Figure 7.8: Comparison of serum IS concentrations (log transformation, from mg/L) in the presence of detectable or undetectable serum PC (mg/L) in study subjects. Detectable PC was significantly associated with elevated serum concentrations of IS (two-tailed T-test, p<0.001).



PC	> 0mg/l	0mg/l
Number of subjects	9	17
IS concentration, mg/l {95%CI}	1.2 {0.8-1.6}	0.4 {0.3-0.5}

Figure 7.9: Comparison of serum PCS concentrations (log transformation, from mg/L) in the presence of detectable or undetectable serum PC (mg/L) in study subjects. Detectable PC was significantly associated with elevated serum concentrations of PCS (two-tailed T-test, p<0.001).

All toxins demonstrated a positive association with PTH, CRP, troponin T, fibrinogen and creatinine ($p<0.05$), and a negative association with Hb and albumin (Table 7.8). These factors are largely CKD related, and highlight the accumulation of these toxins with worsening renal function.

IS also demonstrated a clinically significant association with increasing BMI and SBP, both also increased in the HDx group. IS was further increased with tot chol:HDL, whilst PC and PCS were associated with increased homocysteine concentrations.

Table 7.8: Pearson correlation of uraemic toxins PC, PCS and IS with subject examination and blood laboratory results. Correlation coefficient with p-value recorded. For non-Gaussian distributed Troponin T, Spearman correlation was used.

	IS (mg/l)	PC (mg/l)	PCS (mg/l)
BMI (kg/m^2)	0.43 (0.031)	0.38 (0.060)	0.32 (0.13)
SBP (mmHg)	0.50 (0.009)	0.38 (0.059)	0.30 (0.15)
DBP (mmHg)	0.12 (0.55)	0.053 (0.80)	0.20 (0.36)
Potassium (mmol/l)	0.38 (0.059)	0.37 (0.063)	0.29 (0.17)
Creatinine (μmol/l)	0.79 (<0.001)	0.71 (<0.001)	0.68 (<0.001)
Urate (μmol/l)	-0.049 (0.81)	0.00 (1.0)	-0.078 (0.72)
Ca x P ($mmol^2/l^{2)}$	0.38 (0.055)	0.42 (0.033)	0.22 (0.29)
Albumin (g/l)	-0.50 (0.009)	-0.55 (0.003)	-0.70 (<0.001)
Total Chol : HDL (mmol/l)	0.39 (0.049)	0.27 (0.18)	0.14 (0.50)
Triglycerides (mmol/l)	0.20 (0.32)	0.24 (0.24)	-0.029 (0.89)
Homocysteine (mmol/l)	0.25 (0.29)	0.50 (0.025)	0.58 (0.011)
PTH (U/l)	0.56 (0.003)	0.40 (0.045)	0.70 (<0.001)
Hb (g/l)	-0.80 (<0.001)	-0.70 (<0.001)	-0.74 (<0.001)
CRP (g/l)	0.50 (0.018)	0.47 (0.016)	0.52 (0.009)
Troponin T	0.70 (<0.001)	0.69 (<0.001)	0.60 (0.004)
Fibrinogen	0.68 (0.001)	0.62 (0.005)	0.62 (0.005)
Ferritin	0.21 (0.33)	0.25 (0.25)	0.013 (0.96)

7.5 Discussion

This study was performed to investigate, for the first time, the interaction between PC/PCS, IS and EPCs in the peripheral circulation of humans, including their effect on endothelial function and vascular risk factors. This is on a background of the preceding chapters first highlighting the altered cardiovascular outcomes in patients depending on KRT modality, then demonstrations of the negative functional consequence *in vitro* on ECs and EPCs of exposure to PC, IS, and to serum from KRT recipients. The *in vivo* study in this chapter provides a link between the observations of the population-based investigations of Chapter 3, and the laboratory studies of Chapters 5 and 6.

This study was designed as a pilot investigation and involved limited patient numbers. We therefore cannot exclude that a larger study would demonstrate statistically significant associations between factors additional to those described herein.

7.5.1 EPCs

This study population clearly demonstrated that a low concentration of circulating PB EPCs was associated with both KTx and HDx therapies, as compared to the non-KRT population. Low PB EPC concentration was also associated with increased cIMT measurement, a strong surrogate marker of coronary artery atherosclerotic burden (increased cIMT measurement was also associated with a history of IHD, but not KRT modality), and classical IHD risk factors, including a past history of IHD, systolic hypertension and hyperlipidaemia. Associations with novel risk factors included increased CRP and fibrinogen concentrations. We also demonstrated an association of low EPC counts with increased aortic PWV, a marker of arteriosclerotic change[503]. Finally, low EPC counts were associated with increased AIx, at baseline and also post-endothelial dependent and independent stimulation. This association has not been previously reported in the literature, and adds further context to the influence of EPCs on normal vascular function, including the potential role they play interacting with ECs (atherosclerosis, and endothelial-dependent AIx measurements), as well as with cells of the intima and media (arteriosclerosis and endothelial-independent function).

7.5.2 EPCs and uraemic toxins

It is well understood that EPC number and function are affected by CKD[269, 326] and KRTs[328]. Factors such as medications[238, 361] and transplant graft function[330] have been implicated, with effects proposed via nitric oxide pathways, or increasing VEGF release[357]. On the basis of the *in vitro* results of Chapters 5 and 6, we postulated that increased serum concentrations of uraemic toxins PC and IS would be associated with both surrogate markers of vascular disease, as well as decreased concentrations of PB EPCs. However, we found no association between any of the measured uraemic toxin serum concentrations and PB EPC concentration, despite increased toxin concentrations having the same association with surrogate markers of vascular function as low EPC counts. There was also a difference in association between toxin and EPC concentrations when considering other clinical and biochemical markers of risk (Table 7.10). To this time, there has been no literature published regarding the relationship between uraemic toxins, EPCs and overall endothelial function– this is the first evidence suggesting a separation of effect between these factors.

The lack of association between EPC counts and measured toxins was not wholly unexpected – this is consistent with a recent study that only showed an association between concentrations of PB CD 34/133+ cells and β_2-microglobulin and indole-3 acetic acid, but not IS or PCS[503]. Conversely, a trend towards association of PCS serum concentration and PB EPC counts was observed – a significant association would be likely to be observed in an appropriately powered study.

A limitation of this study was that function of EPCs derived from subjects was not tested. On the basis of the findings from Chapters 5 and 6, significant differences between KRT groups and association with toxin serum concentrations would have

been expected. This study was unable to be performed, as the technique used for EPC isolation and culture (Chapter 4) did not successfully expand PB EPCs to numbers needed for investigation. This would have required donated blood volumes of over 150ml, which were not appropriate for this study. The next step in EPC studies will be to develop culture techniques that require small volume blood donation only, to generate meaningful EPC numbers *in vitro*.

Table 7.9 demonstrates the lack of a pattern of conformity between the toxins themselves and EPCs, compared to biochemical and surrogate markers. PC/PCS[57, 59] and IS[77, 78] are derived from different sources, and belong to different chemical families. What the specific causative relationships are between the toxins and these other variables is not clear, and beyond the scope of this study. This also highlights that measuring PB EPC concentrations alone to identify a relationship with uraemic toxins *in vivo* may not be sensitive enough an assay.

Interestingly, whilst EPC counts were associated with surrogate markers of atherosclerotic and arteriosclerotic risk, the toxins were not. All were however associated with a history of IHD – this may be a manifestation of an underpowered study.

Regardless of the lack of association between EPC counts and toxin concentrations, PB EPC enumeration alone was associated with all surrogate markers tested. These investigations included measures of both arterio- and athero- sclerotic risk, and highlight the possible role of EPCs in any disease affecting the vascular endothelium.

Table 7.9: Comparison of different associations (✓ = $p<0.05$) between surrogate markers of vascular function/CVD risk, and increased uraemic toxins and decreased EPC concentrations in peripheral blood. Whilst PCS and IS concentrations positively relate to each other in a given individual, IS is not associated with any of the measured surrogate markers, whilst increased PCS concentration is associated with endothelial-independent AIx. Whether this is a result of a β-type error is not known.

Associations: ✓ / ×	IS High	PC High	PCS High	EPC count Low
cIMT (log, mm)	×	×	×	✓
PWV (log, m/sec)	×	×	×	✓
AIx baseline	×	×	×	✓
AIx post-GTN	×	×	✓	✓
AIx post-salbut.	×	×	×	✓

7.5.3 Arteriosclerosis versus atherosclerosis

Tight entry criteria were used for this study, to avoid confounding by factors (age, gender, diabetes and specific medications) known to affect EPC counts. The consequence of this was small subject numbers, however the control subjects all had higher EPC counts than KTx and HDx recipients, as well as lower AIx measurements pre and post stimulation compared to HDx. KTx recipients had impaired endothelial independent AIx measurements only, which is consistent with the hypothesis that KTx may only partially reverse the vascular damage incurred during ESKD/HDx.

There was no difference between the patient groups for PWV. There is a strong association between increasing age and increased PWV – this variable was accounted for by the age entry criteria. Covic *et al* have also suggested that in HDx recipients, PWV is more a measure of age and co-morbid conditions rather than CVD risk[181]. With diabetics and the over 65s excluded, the disease burden in the aorta of subjects may not have been enough to reveal a statistically significant difference. All measured PWVs in this study were <9m/s, whereas others use 10m/s as a cut-off for increased risk[514].

There was also no difference in cIMT measurements between patient groups. Like PWV, cIMT measurement is increased by age in CKD[515], and may be a factor in why our age-controlled groups showed no significant difference.

These results suggest worsened small and medium vessel endothelial dysfunction in HDx recipients, consistent with our hypothesis regarding the aetiology of vascular disease in CKD.

In comparison, subjects with IHD had increased cIMT measurements, a known robust marker of future CVD risk[516]. A cIMT measurement of >0.75mm in ESKD pre-KTx is associated with significant coronary artery disease[517]. This study gave an average measurement of 0.78±0.08mm for cIMT in those with IHD. Neither PWV nor AIx were elevated in subjects with known IHD – an association between CVD mortality and increased AIx has been well documented[179, 518], however not all studies have found the same results[519].

Finally, increased PC, PCS and IS serum concentrations were associated with a history of IHD. These toxins were in part chosen for investigation on the basis of their known association with future CVD events and mortality[55, 56, 61]: our data is consistent with this literature.

The above results are consistent with our hypothesis that endothelial dysfunction explains the increased rate of overall cardiac disease observed in KRT recipients compared to the general population. This endothelial change does not specifically result in marked increased risk of occlusive vascular disease, but rather increased evidence of arterial stiffening, leading to heart failure and sudden death.

Our findings demonstrate an association between these endothelial changes and uraemic toxins PC/PCS and IS, as well as EPCs, but does not clarify the *in vivo* relationship between them. Chapters 5 and 6 highlighted the effect of uraemic toxins and whole serum on the function of HUVEC *in vitro*. Specifically, that toxins and uraemic sera affect this function, and that EPCs do exert a protective effect against increasing concentrations of IS, but not PC. This link between low EPC counts *in vivo* with increased risk for vascular disease (as measured by surrogate markers), and the findings of Chapters 5 and 6 requires further investigation.

7.5.4 Further study

Further study is required, with a larger prospective study based on this observational study. With increased subject numbers, the effect of increasing age, gender and diabetes could be addressed, as well as documenting future events as they arise, rather than relying on surrogate markers. A study of the function of EPCs obtained from the same participants, rather than purely PB EPC counts may also demonstrate the relationship *in vivo* EPCs have with uraemic toxins.

7.5.5 Conclusions

- PB EPC counts are associated with a known history of IHD, as well as surrogate markers of both atherosclerotic burden and arteriosclerosis in small and large vessels
- Despite being associated with known IHD and having significant deleterious effects on EC and EPC function in vitro, elevated serum concentrations of PC/PCS or IS are not associated with PWV/PWA, cIMT, or EPC counts
- This study was underpowered to demonstrate significant associations between EPC counts, toxin concentrations and markers of vascular risk, and requires further investigation

Chapter 8: Conclusions and future directions

CVD that presents in patients with ESKD receiving KRT is the consequence of exposure to a unique combination of risk factors that is not present in the general population. This requires a different understanding of the pathophysiology of vascular disease, as both the natural history and response to therapy for CVD in ESKD differs to other patient groups.

Many potential factors have been highlighted as contributing to the increased vascular risk seen in ESKD: this thesis specifically addressed the varying increased risk for different types of CVD hospital admissions, and the role of EPCs, PC/PCS and IS in the progression of vascular dysfunction. We hypothesised that dysfunction or reduced circulating numbers of PB EPCs, and increased serum concentrations of PC and IS worsened endothelial function and hence accelerated arteriosclerotic change.

Chapter 1 reviewed the currently identified risk factors for CVD in ESKD, which includes both classical and novel agents. This highlighted the rationale for further investigating uraemic toxins PC/PCS and IS, given the data on their association with both CVD and all-cause mortality, as well as *in vitro* studies demonstrating effects on ECs and WBCs. We also introduced the EPC as a cell type being more widely understood as key to the normal functioning of the vascular endothelium, and potentially crucial to the progression of CVD in those with CKD.

Chapter 3 demonstrated an increased relative rate for CVD hospital separations for KRT recipients compared to the general population. Underlining the difference in pattern of CVD, rather than an across-the-board increase in rate, the relative rate of admission for CHF events was greater than for ACE compared to the non-KRT population. In addition, whilst relative in-hospital death rates due to ACE were higher for all KRT groups, CHF admissions did not confer any greater mortality risk (per admission) in the KRT group. Finally, our results confirmed that despite improvement in kidney function, KTx recipients do not return to non-KRT patterns of CVD.

CHF is associated with endothelial dysfunction and arteriosclerosis, rather than the classic atherosclerosis of ACE/IHD. Having documented a population level difference for CVD patterns in KRT recipients, with an excess of CHF, the remainder of the thesis was directed at investigating potential factors (PC/PCS, IS and EPCs) causing worsened endothelial dysfunction in ESKD/KRT recipients.

Chapter 4 described a new isolation and culture protocol for EPCs from BM and UCB, which both expanded cell numbers, and maintained an early EPC phenotype. This allowed the *in vitro* studies of Chapters 5 and 6 to be performed, using EPCs that were standardised to a specific level of maturation and function. Lack of an agreed, standardised protocol for *ex vivo* expansion of EPCs has been a major stumbling block to the use of these cells, both in basic science, and in clinical therapy. The current explosion in research and publication numbers exemplifies the importance and dedication to this. To this end, very recent publications have

described EPC expansion protocols and the immortalisation of primary EPCs to generate cell lines for scientific research purposes.

Chapter 5 investigated the *in vitro* effects of EPCs, PC and IS on normal cultured HUVEC, as a model of endothelial function. Both PC and IS had deleterious effects on a number of HUVEC functions, but with a greater negative effect of PC observed. This suggests targeting PC/PCS reduction *in vivo* may yield greater clinical benefit. The addition of EPCs to the toxin environment had limited effect on HUVEC function, except at low toxin concentrations, or moderate IS concentrations. These findings implied that native EPCs alone might not have the capacity to overcome uraemic toxin-induced endothelial dysfunction. However, at disease initiation, when toxin levels are low in concentration, EPCs may act as a first line of defence. They may then continue to be effective until toxin levels become too high and render the EPCs non-functional, or be negatively influenced by the dysfunction of other cell types (*e.g.* SMCs, pericytes, macrophages) around them.

Chapter 6 further investigated *in vitro* HUVEC function in the setting of sera from patients, rather than isolated toxins. There are over 80 documented uraemic “toxins” which might mediate these effects, as well as differences in KRT therapies. Highlighting that these interactions are not well understood, compared to control and KTx sera, HDx sera increased HUVEC migration. This increased migration rate was associated with increased serum concentrations of IS and PC/PCS. No such association was demonstrated with isolated toxins in Chapter 5 – furthermore, the literature suggests a negative effect of these toxins on migration, whilst we only showed a negative effect of increasing concentration of PC *in vitro*. For tube

formation, it could have been expected that HDx sera again, with higher toxin concentrations would have the greater inhibitory effect, whereas it was KTx derived sera that demonstrated the most inhibition - immunosuppressive use or other unrecorded factors may explain this finding.

The clinical observation study described in chapter 7 was undertaken to marry the *in vitro* findings with clinical outcomes. Given the time constraints of a PhD, surrogate markers of CVD risk were utilised instead of specific clinical outcomes. The study was hampered by limited patient recruitment: stratification by gender, age and diabetes reduced the available pool of potential recipients, but also removed three major potential confounders in the progression of CVD. Regardless, the study was able to demonstrate that KRTs were associated with worsened small vessel endothelial function (as measured by AIx). In addition, that both high serum concentrations of PC/PCS/IS and low EPC PB concentrations were significantly, or showed trends towards, association with AIx. This included baseline AIx, and AIx following endothelial-dependent and endothelial–independent stimulation. EPC PB concentration further demonstrated a trend towards a negative association with PWV measurements, but neither EPC or toxin concentrations correlated with cIMT measurements, a marker specifically of occlusive coronary artery disease risk, rather than arterial reactivity or stiffness.

These findings describe a complex interaction in which the combination of risk factors, rather than the presence of specific agents at a particular concentration, is more important to CVD risk in ESKD/KRT. This supports the theory that there is not

one “magic bullet” for the prevention or treatment of CVD in ESKD, and that all known risk factors need to be considered in patient management.

This thesis augments the current published literature regarding the increased risk and prevalence of vascular endothelial dysfunction in ESKD/KRT, and their specific relation to PC, PCS, IS and EPC PB concentrations. This includes the association of low EPC counts with a history of, and markers for, vascular disease, and the more complex interaction of toxins with CVD risk, and *in vitro* effects on ECs and EPCs.

Concentrations of IS, PC/PCS and EPCs can all be altered in the clinical setting, by lifestyle, dietary, pharmacologic or other means, and serial measurement of their PB concentrations may provide a technique for monitoring response to CVD therapies.

Beyond disease monitoring, EPCs have been touted as a potential cell therapy. However, the findings of this thesis would suggest that merely infusing EPCs into an uraemic environment would have limited benefit due to the inhibitory effect of toxins alone. Improving the function of endogenous EPCs is potentially a more pertinent focus for future research. This could include increasing EPC release from BM, improve homing to target tissues, and potentiating either early or late EPC phenotypes depending on the clinical requirement. Improving the survival and function of other cell types, including SMCs and other circulating MNCs would also benefit vascular function. For ESKD/KRT recipients, direct targeting of toxin concentrations would be justifiable: this could include altered HDx membranes, dietary intervention, and novel agents for reducing toxin generation and absorption from the gut. A caveat to these potential therapies, is that it remain unclear if EPCs

from uraemic donors maintain normal function, nor if allogeneic EPCs from a normal donor would maintain normal function in a uraemic environment.

This thesis focused on the epidemiology of CVD in ESKD, functional changes in large and small vessels, as well as the altered function of EPCs and HUVEC *in vitro,* in the uraemic environment. The findings to this point could be taken forward in a number of ways

1. Extending the epidemiological study to include further patient data, including presence of comorbidities, as well as number of events and outcomes per specific individual
2. Execute HUVEC/EPC functional studies using sera from subjects in clinical observational study and directly correlate findings with surrogate markers of CVD, as well as comparing the effect of PC and PCS in parallel.
3. Spiking normal serum with PC/PCS and IS and examine function of HUVEC/EPC. This would help refine whether it is these agents in particular, or whether there are other factors in uraemic serum.
4. Examine an animal model of vascular function (e.g. a mouse model of vessel permeability), and the response to increased concentrations of PC / PCS / IS with or without allogeneic EPCs
5. Directly culturing EPC from study subjects (would require significantly greater blood volume to be collected, *e.g.*>150ml) and investigating function directly
6. Increase the number of subjects enrolled in the clinical observation study, and follow them over a defined time period to determine rates of cardiovascular morbidity and mortality

7. Perform an intervention study to decrease IS/PC/PCS serum concentrations, and measure effect on vessel and *in vitro* cellular function. This may be a lifestyle (*e.g.* diet), pharmacologic (AST120), or dialysis (HDF vs. HDx) intervention.

Longer-term, these studies could be extended to subjects receiving peritoneal dialysis, and importantly, to those individuals with CKD not yet requiring KRT. The greatest benefit of intervention to reduce or prevent progression of CVD (and CKD) could be seen in this group. This could include dietary, and possibly pharmacologic, intervention at a population level.

Australia and other developed countries are witnessing significant increases in rates of CKD, and those requiring KRT. Many who require dialysis or KTx are older, diabetic, or already have a significant CVD burden. Novel strategies for management will be increasingly important to improve outcomes for these individuals. Reducing PC/PCS and IS serum concentrations, as well as augmenting endogenous EPC function may become significant adjunctive treatment goals in the future.

This thesis has demonstrated the burden of CVD in ESKD/KRT subjects at a population level in Australia, at the individual level through surrogate markers of vascular disease, and at a cellular level by examining cell function. Future investigation and clinical management of this complex problem will require consideration of novel factors such as EPCs, but with the understanding that a multifactorial approach to both is required.

Appendix A: Commercial laboratory testing for blood samples

Pathology requested from separate tubes

EDTA 1 x 4ml

- Haemoglobin (Hb, g/L)
- Mean Cell Volume (MCV, fl)
- Total White Blood Cell Count (WCC, $x10^9$/L)
- Platelet Count (Plt, $x10^9$/L)
- Serum Folate (nmol/L)
- NT-ProBNP (ng/L) – not all subjects had NT-ProBNP performed due to lab costing issues, and it was eventually removed from the analysis.

EDTA 1 x 9ml

- Whole blood used within 30min to four hours of collection for EPC isolation and enumeration (Masouleh showed a decline in EPC number after 4 hours storage of whole blood[520]).

EDTA 1 x 9ml

- Serum separated within 30min to 4 hours and frozen at maximum of minus 20^0C for future assessment of uraemic toxin and circulating surface marker concentrations.

Potassium citrate 1 x 4ml

- Activated Partial Thromboplastin Time (APTT, seconds)
- International Normalised Ratio (INR) – as calculated by the ratio of subject prothrombin time to a "normal" standard
- Fibrinogen (g/L)
- Erythrocyte Sedimentation Rate (ESR, mm)

Lithium-Heparin 1 x 9ml

- Glucose (gluc, mmol/L)
- Glycohaemoglobin (HbA1C,
- Electrolytes: Sodium (Na, mmol/L), Potassium (K, mmol/L), Bicarbonate (Bic, mmol/L), Urea (mmol/L), Creatinine (creat, μmol/L), Urate (mmol/L)
- Liver Function Tests (LFTs): Albumin (Alb, g/L), Globulin (Glob, g/L), Bilirubin (bili, μmol/L), gamma glutamyl transpeptidase (GGT, U/L), Alkaline phosphatase (ALP, U/L), Alanine Aminotransferase (ALT, U/L), Aspartate Aminotransferase (AST, U/L), Lactate Dehydrogenase (LD, U/L)
- Calcium (Ca, mmol/L)
- Phosphate (Phos, mmol/L)
- Magnesium (Mg, mmol/L)
- Creatine Kinase (CK, U/L)
- Cardiac Troponin T (TropT, μg/L)
- Total Cholesterol (tChol, mmol/L))
- High Density Lipoprotein Cholesterol (HDL, mmol/L))
- Low Density Lipoprotein Cholesterol (LDL, mmol/L)
- Total Triglycerides (TG, mmol/L)
- Plasma Homocysteine (Hcy, μmol/L)

Plain Tube 1 x 9ml

- Vitamin B12 (B12, nmol/L)
- Parathyroid Hormone (PTH, pmol/L)
- Thyroid Stimulating Hormone (TSH, mIU/L)
- Thyroxine (FT4, pmol/L)
- C-Reactive Protein (CRP, mg/L)
- Ferritin (Ferr, μg/L)
- Transferrin Saturation (TSat, %)

Routine Laboratory testing methods

Routine laboratory testing was performed by the Institute of Medical and Veterinary Science (Frome Road, Adelaide, SA, Australia) using automated diagnostic machines as below

- Advia 2400 Chemistry System (Siemens Healthcare Diagnostics)
    - Electrolytes; Gluc; Ca; Phos; Mg; LFT's; urate; Ferr; TSat; CRP; CK; tChol; HDL; LDL TG
- Advia Centaur Immunoassay System (Siemens Healthcare Diagnostics)
    - Hcy; TSH; FT4
- Architect i2000SR (Abbott Ireland)
    - B12; folate
- Elecsys E170 (Roche Diagnostics GmbH)
    - Trop T; PTH; NT-ProBNP
- Sysmex xe-2100 (Sysmex America Inc)
    - Hb; MCV; WCC; Plt
- StarRRsed Compact ESR Analyser (Vital Diagnostics)
    - ESR
- STA-R Evolution (Diagnostica Stago Inc)
    - INR; APTT; Fibrinogen
- Variant II (Bio-Rad Laboratories)
    - HbA1C

Appendix B: History and physical examination – Chapter 7

Medical history

Age (on day of study)

Employment (sedentary/active)

Smoking history

Alcohol intake

Kidney disease

Diagnosis

Duration/Type KRT

Ideal body weight (if on HDx)

Concurrent comorbid conditions

Hypertension

Hypercholesterolaemia / Hypertriglyceridaemia

Gout

Peripheral Vascular Disease

Cerebrovascular Disease

Ischaemic Heart Disease, defined as either history of:

- acute myocardial infarction with elevated cardiac enzymes/ECG changes
- angioplasty or coronary artery bypass graft
- objectively positive cardiac angiogram, stress echo, or MIBI scan

Current medication use

HMG Co-A reductase inhibitor

Erythropoiesis stimulating agent

Anti-Hypertensives

Beta blocker

Calcium channel blocker

Angiotensin converting enzyme inhibitor

Angiotensin II receptor antagonist

Aspirin or Warfarin

Supplemental vitamins

Phosphate binders

Immunosuppresive agents

Prednisolone

Tacrolimus
Cyclosporine
Mycophenolate
Azathioprine

Physical Examination

Height (metres) and weight (kilograms), for body mass index (BMI, kg/m2) estimation
Radial pulse rate (beats/min)
Resting Blood pressure (BP, mmHg; automated BP cuff – OMRON Model IA2)
Measured by automated device in millimetres of mercury (mmHg):
OMRON Intelli-SenseTM model IA2 (OMRON Healthcare Co LTD, Kyoto, Japan)
Diastolic BP defined by disappearance of fifth Korotkoff sound
Measured in non-dominant, or non-fistula arm
Repeated three times over 5 minute period, after 10 min rest, and average taken
Cardiac auscultation
To exclude obvious flow murmurs
Vascular examination
Presence of peripheral pulses to palpation
Carotid, brachial, radial, femoral, dorsalis pedis and posterior tibial
Presence of vascular bruits to auscultation
Carotid, abdominal aortic, femoral

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