Chemokines and macrophage migration inhibitory factor in Diabetic Nephropathy

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Declaration of Originality

I confirm that the work herein is my own and where there has been the use of data from other parties this has been referenced and declared so. Permission to use data from my predecessor Dr Qureshi has been given and a letter confirming this is included in Appendix 1.

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Dedication

Through God everything and anything is possible with an open heart and mind in the search to help others.

For my friends and family whom I have been blessed with and whose support throughout this period has led to the completion of this journey and the beginning of others.

With Love

ABSTRACT

Introduction: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the Western world.

Aim: To determine whether macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1) or CC chemokine ligand 18 (CCL18) have a causative role in the development of renal inflammation and fibrosis in DN and are useful biomarkers of disease progression.

Methods: Urine and plasma samples were collected from 115 DM and 116 Non-DM at baseline, previously analysed for MCP-1 and CCL18 ELISA by Dr Qureshi. I measured MIF in these samples and collected 107 DM and 114 Non-DM data points (GFR, ACR/UPCR and clinical parameters) at >18 months and >3 years. MIF, MCP-1 and CCL18 urine, plasma and serum analysis was performed in 42 DM and 60 Non-DM at >3 years follow up. Intrinsic renal cells were cultured and stimulated with diabetic conditions. These cytokines and fibronectin were measured in tubuloepithelial cells and podocytes.

Results: Baseline plasma CCL18 and MIF predicted a decline in GFR in DM at >18 months but not at >3 years. Cytokine production varies over time with significant correlations at baseline that are not maintained. Cytokines correlate differently with GFR, ACR/UPCR in DM versus Non-DM proteinuric renal diseases. Plasma and serum cytokine levels correlated significantly with no correlation between these and urinary levels. All intrinsic renal cells were able to produce MIF, MCP-1 and CCL18 following stimulation. The interaction of these cytokines and their effects on fibronectin vary in diabetic conditions and following recombinant cytokine stimulation. The diabetic environment appears to orchestrate cytokine signals according to cell type.

Conclusion: These results suggest cytokines may play a key role in the pathogenesis and or progression of DN. The clinical study suggests cytokines may predict progression; however, larger studies are needed with samples taken at different time points.

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ABBREVIATIONS

A2	glycated albumin
Ab	Antibody
ACEi	Angiotensin Converting Enzyme Inhibitors
ACR	Albumin/Creatinine Ratio
AGEP	Advanced Glycation End Products
ALTITUDE	Aliskiren Trial in T2DM Using cardio-renal endpoints
Ang2	Angiotensin II
ARB	Angiotensin 2 Receptor Blockers
AST-120	Antifibrotic
B prefix	Baseline
BCA	Bovine calf protein assay
BEACON	trial
BMI	Body Mass Index
BMP7	Bone Morphogenic Protein-7
BP	Blood Pressure
BSA	Bovine serum albumin
CC	Chemokine Family
CCL2	Alternate name for MCP-1
CCL18	Alternate name for PARC/MIP-4/AMAC-1/DCCK1/SYCA-18
CCR	Chemokine Receptors
CCN2	Alternate name for Connective Tissue Growth Factor (CTGF)
CD74	MIF Receptor

CFGF	Connective Tissue Growth Factor
CKD	Chronic Kidney Disease
Col1	Type I Collagen
Col3	Type III Collagen
Col4	Type IV Collagen
Col5	Type V Collagen
Cr	Creatinine
CRP	C-Reactive Protein
CVA	Cerebrovascular accident
CXC	Chemokine Family
CXCR	Chemokine Receptors
d prefix	rate of change
dACR	rate of change of Albumin/Creatinine ratio
dGFR	rate of change of Glomerular filtration rate
DN	Diabetic Nephropathy
DNA	Deoxyribose Nucleic Acid
DM	Diabetes Mellitus
dUPCR	rate of change of Urinary protein/Creatinine ratio
ECM	Extracellular Matrix
eGFR	Estimated Glomerular Filtration Rate
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme Linked Immunosorbant Assay
EMT	Epithelial Mesenchymal Transition

EPS	Encapsulating Peritoneal Sclerosis
ERK1/2	Extracellular Signal Regulated Kinase 1/2, alternate name p44/42 MAPK
ESRD	End-Stage Renal Disease
ESTHER	trial
FCS	Fetal Calf Serum
FG-3019	Phase 1 anti-CTGF monoclonal antibody
GBM	Glomerular Basement Membrane
GFR	Glomerular Filtration Rate
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GN	Glomerulonephritis
GS	Glomerulosclerosis
GTP	Guanine 5’ Triphosphate
H	High glucose
HbA1c	Haemoglobin A1c
HD	Haemodialysis
HK-2	Human Kidney-2 cells
HPV16	Human Papilloma Virus
hMIF	human Macrophage migration inhibitory factor
IDNT	Irbesartan Diabetic Nephropathy Trial
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry

IL-1	Interleukin Factor 1
IL-4	Interleukin Factor 4
IL-6	Interleukin Factor 6
IL-8	Interleukin Factor 8
IL-10	Interleukin Factor 10
IL-13	Interleukin Factor 13
IL-18	Interleukin Factor 18
LogMIF	Logarithmic base Macrophage migration inhibitory factor
LPS	Lipopolysaccharide
M	Mannitol
MAP	Mitogen activated protein
MAPK	Mitogen Activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein-1; Alternate name CCL2
MC+S	Microscopy, Culture and Sensitivity
MDRD	Modification of Diet in Renal Disease
mid-MD	middle of MD (Res) thesis report
MI	Myocardial Infarction
MIF	Macrophage Migration Inhibitory Factor
MMF	Mycophenolate Mofetil
MMP	Matrix Metalloproteinases
mRNA	messenger RNA
MTT	3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide
n	number of ...

N	normal glucose
NADPH	Nicotinamide Adenine Dinucloetide Phosphate Reduced Oxidase
NASH	Non-Alcoholic Steatotic Hepatitis
NF-κB	Nuclear Factor Kappa B
NO	Nitric oxide
Non-DM	Non Diabetes Mellitus renal disease
OD	Optical Density
ONTARGET	Ongoing Telmisartan alone and in combination with Ramipril Global Endpoint Trial
P prefix	Plasma
PAI-1	Plasminogen Activator Inhibitor-1
PARC	CC chemokine; Alternate name CCL18
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PD	Peritoneal dialysis
Phospho p38	Activated phosphorylated form of p38 MAPK
Phospho p44/42	Activated phosphorylated form of p44/42 MAPK; Alternate name ERK1/2
PITPNM3	G-protein receptor for CCL18
PKC	Protein kinase C
PREDIAN	Pentoxifylline for Renoprotection in DN
PVDF	Polyvinylidene difluoride
PVD	Peripheral Vascular Disease
RAGE	Receptor for Advanced Glycation End Products
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted (CCL5)

RCT	Randomised Control Trials
ROS	Reactive Oxygen Species
RAS	Renin Angiotensin Aldosterone System
RENAAL	trial
REIN	Rampiril Efficacy In Nephropathy trial
RPM	Revolutions per minute
rMIF	Recombinant MIF
rMCP-1	Recombinant MCP-1
rCCL18	Recombinant CCL18
RNA	Ribonucleic Acid
RPMI 1640	cell culture media
RRT	Renal replacement therapy
RT	Renal transplant
RT-PCR	Real-Time Polymerase Chain Reaction
S prefix	Serum
SD	Standard Deviation
SFM	Serum Free Media
Smad1	Transcription Factor
Smad3	Transcription Factor
Src	gene
STZ	Streptozotocin
SV40-T	gene
TAM	Tumour Associated Macrophages

TGF-β	Transforming Growth Factor-Beta
TIMPS	Tissue Specific Inhibitors Metalloproteinases
TIN	Tubulointerstitial Nephritis
TNFα	Tumour Necrosis Factor Alpha
TNFR	TNF Receptors
TRAIL	TNF Related Apoptosis Inducing Ligand
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Tx	Transplant
tCD59	Complement Receptor on tubular cells
tHMC	Transformed Human Mesangial Cells
U prefix	Urinary
UACR	Urinary Albumin/Creatinine ratio
UCCL18	Urinary CC- chemokine ligand 18
UKPDS64	trial
UMIF	Urinary Macrophage migration inhibitory factor
UMCP-1	Urinary Monocyte chemoattractant protein-1
UPCR	Urinary Protein/Creatinine ratio
UTI	Urinary Tract Infection
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1.0 – INTRODUCTION

1.1 Diabetes

The inability of the body to maintain glucose homeostasis results in diabetes mellitus (DM). Type 1 DM (T1DM) is an autoimmune disease whereby the pancreatic islet cells are destroyed. The mechanism for this is thought to involve T lymphocytes (Nokoff and Rewers 2013) and presents in childhood or early adulthood. In contrast Type 2 DM (T2DM) arises following insulin resistance and beta cell failure. The mechanism for this is uncertain, however, the inability to maintain glucose homeostasis in both T1DM and T2DM, results in the macrovascular and microvascular complications of DM. Macrovascular complications include: cerebrovascular disease, ischaemic heart disease and peripheral vascular disease. Macrovascular complications lead to the high morbidity and mortality of DM that is often silent in presentation (Stratton, Adler et al. 2000). Microvascular complications include; retinopathy, neuropathy and nephropathy.

A total of 2.9 million in a UK population of 60 million people are diagnosed with DM (Office of National Statistics 2011). This is predicted to increase to 5 million cases in the UK by 2025. The prevalence of DM is 4.45% in adults in the UK with 10% T1DM and 90% T2DM. There are more male than female T2DM, 6.3% and 5.3% respectively. DM has been reported to be the 5th cause of morbidity and mortality worldwide (Roglic, Unwin et al. 2005). The incidence of T2DM is increasing and is occurring within a younger population, due to a combination of the rising rate of obesity and the metabolic syndrome (Fagot-Campagna, Pettitt et al. 2000).

1.2 Diabetic nephropathy

Diabetic nephropathy (DN) is a common microvascular complication of both T1DM and T2DM. 30% of those with T1DM (Rossing, Rossing et al. 1995) and 20-30% of T2DM (Chong, Keng et al. 2012) go on to develop DN. DN is the commonest cause of end-stage renal disease (ESRD) in the Western World (Mauer, Steffes et al. 1984; Makino, Kashihara et al. 1996; Gross, de Azevedo et al. 2005; Dronavalli, Duka et al. 2008). The combination of genetic and environmental effects, trigger complex pathophysiological events leading to DN (Navarro-Gonzalez, Mora-Fernandez et al. 2011). A genetic component is probable as not all diabetics develop nephropathy and the mechanisms

behind this are poorly understood. An early sign of the development of DN is the presence of microalbuminuria (>30mg but <300mg albumin in the urine per day) (Dronavalli, Duka et al. 2008).

From the diagnosis of DM, modelled data from the UKPDS64 shows that microalbuminuria develops at 2% rate/yr, with macroalbuminuria developing at 2.8% rate/yr and ESRD occurs in macroalbuminurics at a 2.3% rate/yr (Adler, Stevens et al. 2003). The development of microalbuminuria has been associated with disease progression and the development of cardiovascular disease (Jarrett, Viberti et al. 1984; Lane 2004). Macroalbuminuric diabetics have been reported to have a higher mortality before progressing on to ESRD (Adler, Stevens et al. 2003). In addition, the development of systolic hypertension has also been reported to be associated with the progression of DN (Dronavalli, Duka et al. 2008).

The development of microalbuminuria does not, however, always progress to macroalbuminuria and the development of DN (Steinke, Sinaiko et al. 2005). Thus, there is a large variability in the course of progression that is influenced by glycaemic control, blood pressure (BP) control and the use of antagonists of the renin-angiotensin system (RAS), whereby progression is slowed but not necessarily curtailed by good control (1998; Brenner, Cooper et al. 2001; Lewis, Hunsicker et al. 2001; Adler, Stevens et al. 2003; Perkins, Ficociello et al. 2007). There is recent evidence from a randomised controlled trial (RCT) that combination of RAS blockade may not be of additional benefit in those with T2DM (Fernandez Juarez, Luno et al. 2013).

A worse outcome is seen in patients with DM with increased creatinine and the presence of albuminuria. It is well established that cardiovascular events are increased in diabetics with raised creatinine (Adler, Stevens et al. 2003). It is thought that increased albumin excretion may lead to increased cardiovascular disease or may signify an underlying abnormality in platelet aggregation, penetration of the endothelium by atherogenic lipoprotein particles, or an effect on autonomic neuropathy in DM (Adler, Stevens et al. 2003).

1.3 Pathophysiological changes in Diabetic Nephropathy

In DN the structure of the glomerulus undergoes a number of changes so that the kidney is unable to function and subsequently fails, leading to ESRD; requiring the need for dialysis or renal

transplantation (Thrailkill, Nimmo et al. 2009). Changes include glomerular hyperfiltration, hyperperfusion and subsequently the development of histological features: thickening of the glomerular basement membrane (GBM), glomerular hypertrophy, mesangial expansion and glomerulosclerosis (GS). These histological features are currently used to diagnose DN on renal biopsy tissue. There has been a recent change in classification, in order to account for interstitial and vascular changes seen in DN, see Table 1.0 (Tervaert, Mooyaart et al. 2010).

Table 1.0 Histological classification of DN adapted from Tervaert et al 2010

Renal biopsy	Lesion
Glomerular Class I II III IV	 Glomerular basement thickening a - Mild mesangial expansion b - Severe mesangial expansion Nodular sclerosis (Kimmelstiel-Wilson lesion) Advanced glomerulosclerosis
Interstitial	0-3 Fibrosis 0-2 Inflammation
Vascular	0-2 Arteriolar hyalinosis 0-2 Large vessel arteriosclerosis

Mesangial cells maintain glomerular capillary structure and modulate glomerular filtration via smooth muscle activity (Dronavalli, Duka et al. 2008). Hyperglycaemia is known to result in mesangial cell proliferation, increased matrix production and GBM thickening (Dronavalli, Duka et al. 2008). Hyperglycaemia has also been shown to increase extracellular matrix production from mesangial cells *in vitro*. Increased intracellular glucose is thought to be a critical step. Overexpression of upregulated glucose transporters (GLUT1/GLUT4) in normal glucose concentration increases glucose entry to cells and also upregulates the synthesis of extracellular matrix by mesangial cells *in vitro* (Heilig, Concepcion et al. 1995). There may also be an upregulation of vascular endothelial growth factor (VEGF) expression on podocytes thus causing increased vascular permeability (Wolf and Ziyadeh 2007). These all contribute to the histological changes seen.

No difference has been seen in the degree of mesangial expansion between normoalbuminuric and microalbuminuric T1DM/T2DM (Doi, Mima et al. 2008). The glomerular filtration rate (GFR),

however, is seen to decrease and correlate with mesangial expansion rather than GBM thickening with GS occurring at the glomerular capillary wall (Abbate, Zoja et al. 2006).

Recently there has been an increase in understanding of the role of the glomerular endothelium in the development and progression of DN. The glomerular endothelium glycocalyx is thought to be lost and this affects the fenestrations in the glomerular endothelium that have high water permeability. These changes contribute to thickening of the GBM and widening of the podocyte foot processes leading to podocyte loss with disruption of the slit diaphragm (Satchell 2012). The state of the glomerular endothelium correlates more strongly with decreasing GFR and increasing albumin/creatinine ratio (ACR) than seen with a decline in podocytes. It is being increasingly reported that the decline in renal function seen in DM may be independent of proteinuria (Kramer, Nguyen et al. 2003).

A study into Pima Indians with T2DM showed significantly higher levels of podocyte detachment in macroalbuminurics compared with normoalbuminurics and microalbuminurics (Weil, Lemley et al. 2012). Podocyte detachment correlated with albuminuria however, the percentage of endothelial cell fenestration correlated significantly with GBM thickness and GFR, indicating a relationship between the histopathological findings and renal function decline in DN.

α1 and α2 chains of type IV collagen (Col4) are the main components of GS and are affected by a transcription factor (Smad1) critical for its development (Doi, Mima et al. 2008). Col4 is upregulated by advanced glycation end-products (AGEPs) and Smad1. In mesangial cells, Angiotensin II (Ang2) induces Col4 synthesis and increases the expression of Smad 1 and the phosphorylated Src gene that encodes tyrosine kinases involved in cell growth, movement and proliferation. Angiotensin 2 receptor blockers (ARB) are effective at blocking this pathway in the streptozotocin (STZ) rat model and hence may induce these beneficial effects in DN if treatment is commenced early (Doi, Mima et al. 2008).

Tubulointerstitial fibrosis occurs with glomerular changes and with proteinuria (Mauer, Steffes et al. 1984). Fibroblasts, macrophages and lymphocytes are found within the tubulointerstitium during the

process of fibrosis however, the effects of their individual roles remain uncertain, whether causative or beneficial in the phagocytosis of cellular components (Iwano and Neilson 2004). Different theories have been proposed for the origin of fibroblasts in the kidney. Fibroblasts may be derived by epithelial mesenchymal transition (EMT) in their activation following tissue injury via cytokines and cell signalling mechanisms. Thus, fibroblasts may be produced by tubuloepithelial cells (Wada, Sakai et al. 2007). Alternatively, bone marrow derived cells have been implicated in the ureteric obstructive mouse model to be the source of fibroblasts resulting in fibrosis (Jang, Kim et al. 2013).

EMT may arise from the loss of epithelial cells' ability to adhere, thereby allowing cells to infiltrate into the interstitium, or be washed into the tubular fluid. Their effects are then exerted by downstream, orchestrating signals to induce renal fibrosis (Conway and Hughes 2012). Transforming growth factor-beta (TGF-β) together with activation of transcription factors such as Smad3 contribute to the disruption of the tubular epithelial membrane and inhibit proteases that maintain the function of cell adhesion molecules such as matrix metalloproteinases (MMPs). Bone morphogenic protein-7 (BMP7) inhibits TGF-β induced EMT and when administered systemically has led to the repair of severely damaged renal tubular epithelial cells and reversed renal fibrosis (Iwano and Neilson 2004). A decrease in E-Cadherin has been associated with a rise in fibronectin and MMP2. Once fibroblasts are present they proliferate rapidly and produce a number of collagens and fibronectin exacerbating fibrosis. Downregulation of BMP7 in STZ mice was seen to be related to an increase in Tamm-Horsfall protein, that has been described as an early event in DN (Qu, Du et al. 2012).

Production of Ang2 and inhibition of nitric oxide (NO) occur following haemodynamic changes in DN that have been shown in diabetic mouse models (Zhang, Wang et al. 2012). This leads to longstanding vasoconstriction with chronic tissue ischaemia and hypoxia that may contribute to the formation of tubulointerstitial fibrosis (Nakagawa, Sato et al. 2007).

1.4 Proteinuria

Proteinuria has been established as a strong independent predictor of renal outcome in both diabetics and non-diabetics (Breyer, Bain et al. 1996). The Ramipril Efficacy In Nephropathy (REIN) trial showed the baseline urinary protein excretion correlated significantly with GFR decline and progression of non-diabetic proteinuric renal diseases to ESRD (1997, GISEN).

The significance of the development of proteinuria is amplified by the toxic effects of protein on the renal tubular epithelial cells (Navarro-Gonzalez, Mora-Fernandez et al. 2011). This interaction induces chemokines, adhesion molecules and pro-inflammatory cytokines that lead to interstitial infiltration with monocytes, macrophages and lymphocytes, resulting in renal cell and tubulointerstitial damage and fibrosis (Abbate, Zoja et al. 2006; Galkina and Ley 2006; Navarro-Gonzalez, Mora-Fernandez et al. 2011).

Albumin forms the greatest proportion of proteinuria in DM (McIntyre and Taal 2008). Urinary ACR is the preferred method of monitoring in DN (McIntyre and Taal 2008). Albuminuria is not a marker of the earliest phase of DN, even with GS formation therefore; earlier phase sensitive markers are required. GS may occur in normoalbuminuric and microalbuminuric DN and can occur following hyperfiltration, hyperperfusion and thickening of the GBM. Previous reports show there may be no significant structural changes of the glomerulus in diabetics, with or without microalbuminuria, unless this was combined with a raised blood pressure or a change in serum creatinine (Chavers, Bilous et al. 1989; Fioretto, Steffes et al. 1994). The process of loss of renal function therefore can occur before the initiation of proteinuria (Rosolowsky, Niewczas et al. 2008).

Normoalbuminurics have been found to have established changes of DM that would normally be seen in proteinuric DM who go onto progress. In contrast, microalbuminuric DM have been reported thus ACR does not predict progression (Chavers, Bilous et al. 1989; Fioretto, Steffes et al. 1994). A decline in GFR with glomerular changes may occur without proteinuria (Najafian, Alpers et al. 2011). The presence of microalbuminuria may be predictive of decline; however, microalbuminurics do not always progress as previously reported. It is plausible that different genotypes of diabetic renal disease may exist that will influence or predetermine who will progress, thereby emphasising the need for more sensitive and specific markers. Alternatively proteomics may be a more sensitive technique (Zurbig, Jerums et al. 2012).

There is no established step progression from no nephropathy to microalbuminuria to macroalbuminuria and ESRD, as patients may go from any stage to ESRD at any point (Adler, Stevens et al. 2003; Zoppini, Targher et al. 2012). In addition, Perkins showed there may be regression of microalbuminuria that may reflect the introduction of angiotensin converting enzyme

inhibitors (ACEi), (Perkins, Ficociello et al. 2003). The use of albuminuria as a marker of progression of DN is not robustly associated with progression and does not reflect underlying pathological stages associated with progression. Better markers are required that are more sensitive and reflective of disease stage and progression (Perkins, Ficociello et al. 2003; MacIsaac, Tsalamandris et al. 2004).

1.5 Traditional theory

Genetic and environmental factors contribute to the haemodynamic and metabolic changes thought to result in the progression of DN (Cooper 2001). The traditional theory describes renal injury secondary to metabolic and haemodynamic alterations, see Figure 1.0 (Scott and King 2004; King 2008). This causes deposition of extracellular matrix (ECM) within the kidney resulting in fibrosis.

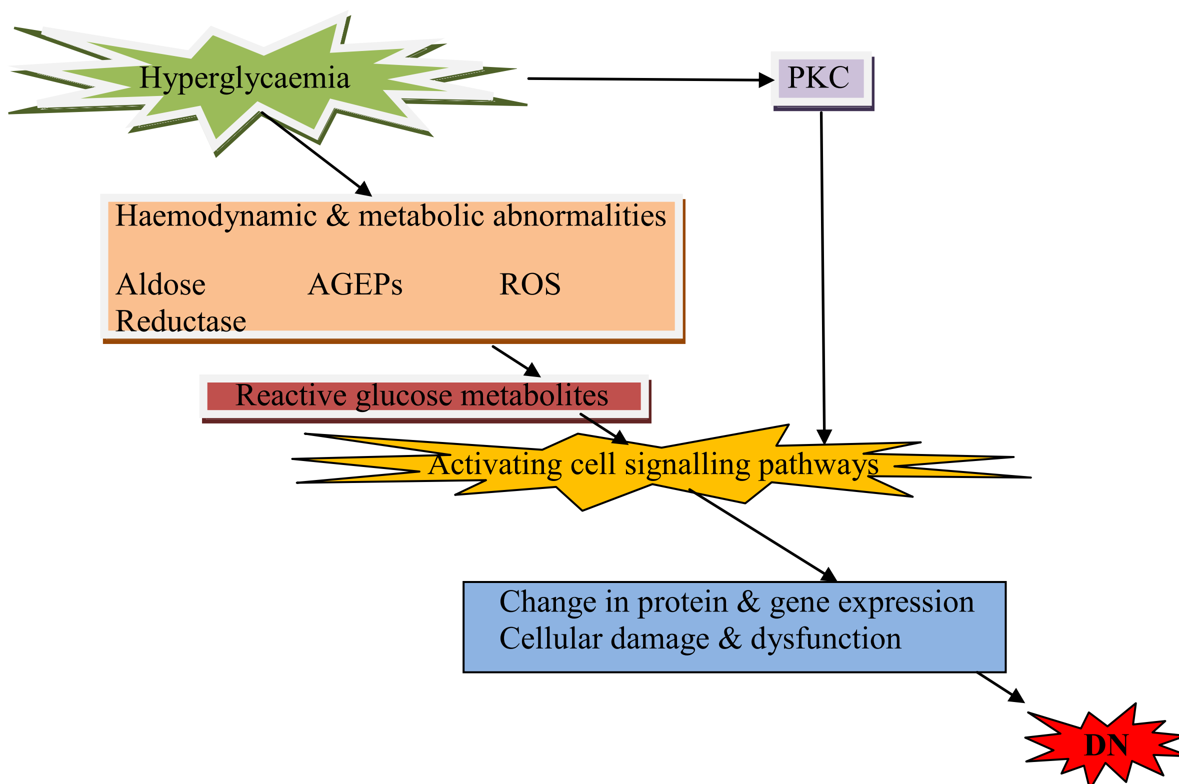


Figure 1.0 Illustration of Traditional theory adapted from King 2008. Hyperglycaemia causes haemodynamic and metabolic abnormalities that involve a number of different pathways. These induce reactive glucose metabolites that activate cell signalling pathways that affect gene expression and protein function. Protein kinase C is increased with hyperglycaemia and further activates cell signalling pathways that contribute to changes in protein and gene expression. Cellular damage and dysfunction occurs, leading to DN.

Hyperglycaemia is a major risk factor for the development of DN (Krolewski, Laffel et al. 1995). This alters gene expression and protein function leading to further cellular dysfunction and damage. Hyperglycaemia alone however, is insufficient to cause the changes found in DN (Mauer, Steffes et al. 1983).

Renal haemodynamic changes arise from defective autoregulatory pathways including: prostanoids, NO, VEGF (Wolf and Ziyadeh 2007), AGEPs (Makita, Radoff et al. 1991), protein kinase C (PKC), (Yamagishi, Fukami et al. 2007), acceleration of the aldose reductase pathway (Porte and Schwartz 1996; Friedman 1999), TGF- β and Ang2 (Sharma, Eltayeb et al. 1999; Hilgers and Veelken 2005; Nagai, Yao et al. 2005). Hyperglycaemia causes formation of reactive glucose metabolites that activate cell signalling molecules. These haemodynamic changes result in albumin leakage through the defective glomerular barrier. This is associated with mesangial expansion and thickening of the GBM, with additional injury to podocytes through these defective autoregulatory mechanisms (Ziyadeh and Wolf 2008). The RAS is thought to contribute to glomerular hypertension, hyperfiltration and renal fibrosis (Zhang, Wang et al. 2012). Antagonists halt the profibrotic effects of Ang2 thereby decreasing the stimulation of TGF-β (Hilgers and Veelken 2005) and renal fibrosis.

Excess glucose binds to free amino acids on tissue proteins or those in the circulation. The nonenzymatic glycosylation that occurs, results in the formation of AGEPs. Initially, these bonds are reversible and attach to the matrix components of the glomerulus or the GBM; later, these bonds become irreversible. The advanced products then interfere with signal transduction. This may occur by changing soluble signals such as cytokines, hormones and free radicals however, more studies looking into this would need to be done. The AGEPs can accumulate throughout the body's tissues as they are unable to be excreted due to the glomerular damage. Increasing amounts of AGEPs in tissues results in microvascular complications (Makita, Radoff et al. 1991). NO concentrations are reduced in a dose-dependent manner with the formation of AGEPs, exacerbating hypertension (Zurbig, Jerums et al. 2012).

Mesangial cell stretch arising from intraglomerular hypertension stimulates the synthesis and deposition of ECM resulting in mesangial expansion and GS (Cortes, Riser et al. 1999; Ingram, Ly et al. 1999). Activation of PKC results in vasodilatory prostanoids that induce glomerular

hyperfiltration. PKC activates TGF-β and may contribute to ECM production in mesangial cells (Wu, Peng et al. 2009). Hyperglycaemia activates PKC to cause formation of diacylglycerol and oxidative stress. PKC also induces the activity of mitogen activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. There is also an acceleration of the aldose reductase pathway and activation of the polyol pathway.

High glucose, TGF-β and VEGF stimulate the synthesis of endothelial NO (Noh, Ha et al. 2002; Vega, Puebla et al. 2009; Kemeny, Figueroa et al. 2013). NO promotes vasodilatation and hyperfiltration found in the early processes of DN. VEGF-A, however, has recently been reported to be important in maintaining the glomerular endothelium (Baelde, Eikmans et al. 2007). VEGF-A (produced by podocytes) induces α3 chain Col4; the importance of this is uncertain, as less VEGF-A is detected with the decreased numbers of podocytes in DN.

In STZ induced rats, TGF-β is upregulated in DN with hyperglycaemia. TGF-β contributes to the cellular hypertrophy and increased synthesis of collagen that occurs in DN (Sharma and Ziyadeh 1995; Sharma, Eltayeb et al. 1999). Connective tissue growth factor (CTGF or CCN2) is upregulated through TGF-β dependent and independent mechanisms. Both are found in the renal glomeruli (Murphy, Godson et al. 1999; Blom, van Dijk et al. 2001; Huang, Matavelli et al. 2011). It has fibrogenic effects on kidneys of diabetics and may play an important role in the development of tubulointerstitial fibrosis.

Oxidative stress is another pathway that results in reactive oxygen species (ROS) produced by many sources, such as mitochondria of cells subjected to hyperglycaemia, autoxidation of glucose and the stimulation of other enzymatic pathways such as nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (Droge 2002). These can go onto cause renal vasoconstriction, damage to DNA, oxidation of proteins and peroxidation of cell membrane lipids. ROS also activates PKC and leads to AGEPs formation (Scivittaro, Ganz et al. 2000; Tan, Forbes et al. 2007).

1.6 Progression

The Reduction of End points in T2DM with the ARB Losartan (RENNAL) study, (Brenner, Cooper et al. 2001), showed Losartan to have a significant benefit beyond that of BP control in preserving renal function whilst decreasing proteinuria. Further analysis of this data (Keane, Brenner et al. 2003) revealed four independent risk factors for the progressive loss of renal function in those with T2DM. These were: proteinuria, serum creatinine, serum albumin and haemoglobin. Age, gender, BP control and smoking status were also found to impact on the degree of progression, however, these were not independently associated with progression (Keane, Brenner et al. 2003). Raised serum cholesterol was also found to be an independent progression promoter in DN (Hovind, Rossing et al. 2001) together with retinopathy, whereby 30% of those with retinopathy go on to have DN. Macroalbuminurics with raised creatinine have a higher mortality (19.2% annual incidence per year) compared with those progressing on to ESRD (2.3% annual incidence per year) (Adler, Stevens et al. 2003).

In the UKPDS 2003 study the median time from microalbuminuria to the development of macroalbuminuria was found to be 19 years, whereas other studies suggest this to be approximately 11 years. These differences may relate to the presence of pre-existing metabolic abnormalities prior to the diagnosis of T2DM being made, together with the introduction of ACEi that resulted in a longer time before progressing. There is no step-wise development from microalbuminuria to macroalbuminuria as the different levels of albuminuria may lead to RRT without a quantitative change in albuminuria (Adler, Stevens et al. 2003). More recently Ogawa reported that a reduction in albuminuria in association with an increase in urinary excretion of reactive oxygen species causes a reduction in blood pressure and reduces albuminuria that is independent of the level of albuminuria in those with DN (Ogawa, S., H. Kobori, et al. 2009).

A further understanding of the molecular mechanisms underlying the progression of DN is needed to provide new therapeutic targets as DN continues to increase, despite near normalisation of blood glucose levels and intensive therapy with ACEi/ARBs. Regression of microalbuminuria particularly in T1DM has also been seen in some studies that are associated with less progression of renal function decline. Regression of microalbuminuria is reportedly more common than progression to ESRD (Perkins, Ficociello et al. 2007). Serum Cystatin C is a non-glycosylated protease inhibitor

that occurs endogenously. Using Cystatin C with microalbuminuria shows that progressive renal decline is an early phenomenon in T1DM. Regression of microalbuminuria tends to occur in individuals with reversible factors such as lowering total cholesterol levels, triglycerides, HbA1c and systolic BP (Perkins, Ficociello et al. 2007) hence, the value of making changes to the reversible elements with a view that progression may turn to regression of microalbuminuria. Progression has been thought to be affected by systemic BP control, albuminuria, glycaemic control, smoking and hyperlipidaemia (Parving 1998).

1.7 Inflammatory theory

Over the past 10 years there is an increasing body of evidence that supports an inflammatory process, occurring at the cellular and molecular level, that is a key factor in the progression of DN (Navarro-Gonzalez, Mora-Fernandez et al. 2011). The theory suggests that metabolic and haemodynamic factors are insufficient to explain the progression and variability of DN.

The inflammatory theory involves innate immunity, whereby the diabetic milieu induces the production of inflammatory molecules that stimulate signalling cascades within the renal cells. Immune mediated inflammation results in increased numbers of chemokines, macrophages and monocytes infiltrating into renal tissue causing ECM changes and DN (van Lieshout, van der Voort et al. 2006; Rivero, Mora et al. 2009; van Lieshout, Vonk et al. 2009). Some of these pathways lead to fibrosis (Figure 1 from Navarro-Gonzalez 2011) and subsequently DN and proteinuria.

Cytokines, including chemokines, are soluble proteins released by cells that act as signals between cells of the immune system and may also act on other cells of the body (Brostoff J, Male DK. 1994 Clinical Immunology, an illustrated outline. Mosby). These have previously been reported to be upregulated in high glucose environments *in vivo* and *in vitro* (Ha, Yu et al. 2002; Tam, Riser et al. 2009). Increased cytokines can cause monocyte/macrophage cell migration into the kidney (Tesch 2007). This has been thought to contribute to a persistent low grade activation of chronic inflammation that leads to DN. The additional insult of haemodynamic and metabolic abnormalities induces further inflammation and activation of immune mediators resulting in DN (Rivero, Mora et al. 2009), see Figure 1.1.

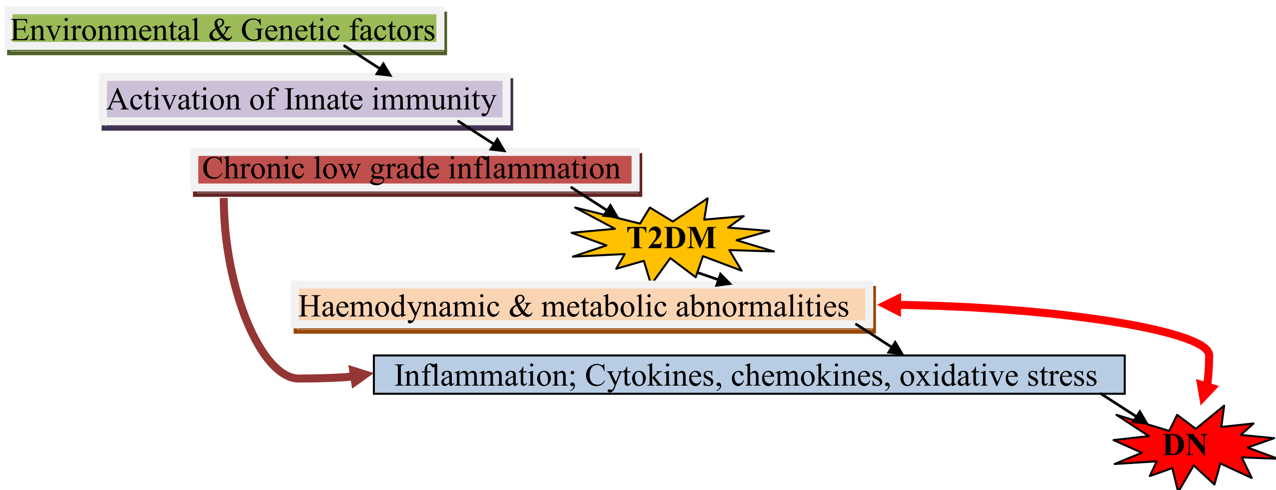


Figure 1.1 Illustration of Inflammatory theory adapted from Rivero A 2009. Environmental and genetic factors activate the innate immunity that continues in the form of a low grade chronic inflammation. This induces T2DM and contributes to the production of cytokines and further oxidative stress. The development of T2DM causes haemodynamic and metabolic abnormalities that further induce cytokines and oxidative stress resulting in DN. DN in turn exacerbates the haemodynamic and metabolic abnormalities that continue the inflammatory response resulting in progressive DN.

Mycophenolate Mofetil (MMF) is a relatively selective inhibitor of lymphocyte infiltration that works through guanine 5'triphosphate (GTP) depletion of target cells. Experimental models have shown when MMF was given to STZ induced diabetic rats, glomerular injury and albuminuria were prevented (Utimura, Fujihara et al. 2003; Rodriguez-Iturbe, Quiroz et al. 2005). Treatment with MMF had no effect blood pressure or blood glucose, hence its effects on reducing albuminuria were not secondary to changes in haemodynamic or metabolic changes. There was a reduction in lymphocyte and macrophage proliferation, with a decrease in expression of adhesion molecules. These anti-inflammatory effects suggest a role for inflammation in DN. These laboratory findings have not been translated to human DN.

There is increasing evidence to support the role of cytokines in microvascular complications of DM (Navarro and Mora 2005; Alexandraki, Piperi et al. 2006; Navarro-Gonzalez and Mora-Fernandez 2008). The current main regulators of inflammation thought to be contributing to the pathogenesis of DN are; Interleukin-1 (IL-1), IL-6, IL-18 and tumour necrosis factor alpha (TNFα) (Alexandraki, Piperi et al. 2006; Navarro-Gonzalez and Mora-Fernandez 2008). These factors have also been shown to cause renal tubular damage, with further production of pro-inflammatory cytokines and attraction of immune cells that contribute to the progression of DN to ESRD (Tang and Lai 2012).

IL-1 has been found to be increased in animal models of DN (Sassy-Prigent, Heudes et al. 2000). In addition, a number of studies have found the synthesis of prostaglandin E and the release of phospholipase A2 increased with IL-1, thereby affecting intraglomerular haemodynamic abnormalities (Pfeilschifter and Muhl 1990).

The presence of albuminuria in DN may itself be causing an inflammatory cascade to occur as albumin has been reported to induce IL-6 release from primary human proximal tubular epithelial cells (Pearson, Colville-Nash et al. 2008). In addition, albumin increases the expression of fibronectin within this cell type (Coleman and Ruef 1992). IL-6 has been reported to increase the ECM with increased expression in mesangial cells and podocytes (Nosadini, Velussi et al. 2000). IL-6 has also been reported in the pathogenesis of DN linking inflammation, glycaemic control and microalbuminuria (Choudhary N, 2008 Iran J Kid Dis). Tubular epithelial cells express IL-18 that induces the release of interferon$_{\gamma}$ that in turn induces more inflammatory signals that lead onto fibrosis and further glomerular and tubulointerstitial injury (Miyauchi, Takiyama et al. 2009).

TNFα is an inflammatory cytokine that is thought to have a central role in the inflammatory pathways and the haemodynamic and metabolic pathway. Disruptions in this, lead on to DN (Navarro-Gonzalez, Mora-Fernandez et al. 2011). A rise in TNFα and IL-1β has been reported in STZ model of T1DM to increase macrophage infiltration in the kidney (Soetikno, Sari et al. 2011). Recent studies show that macrophages accumulate in a number of tissues in diabetes, leading to complications such as DN (Shikata and Makino 2001; Tesch 2007). Proteinuria is thought to induce mononuclear cell infiltrates via: cytokines and complement activation in the tubular epithelial cells that stimulate signalling pathways, transcription factors and further cytokine production. Toll-like receptor 4 antagonists have recently been reported to protect against DN with decreases in Monocyte chemoattractant protein-1 (MCP-1), osteopontin, TGF-β and NF-κB activation in STZ mice (Lin M 2013). MCP-1 and Osteopontin are found to be increased in rat tubuloepithelial cells exposed to albuminuria (Eddy and Giachelli 1995), together with a rise in NF-κB activity (Gomez-Garre, Largo et al. 2001; Sanchez-Nino, Bozic et al. 2011). In human DN, activation of NF-κB in tubuloepithelial cells was reported to correlate with proteinuria and the degree of interstitial cell infiltration of the kidney (Schmid H, 2006 Diabetes).

1.8 Fibrosis

Fibrosis arises from accumulation of ECM with alterations occurring to its structure, and the development of angiogenesis (Ban and Twigg 2008). Abnormal angiogenesis has been reported to occur in early phases of DN in experimental models and in humans (Osterby and Nyberg 1987; Kelly, Buck et al. 2007). Neoangiogenesis contributes to glomerular hypertrophy and hyperplasia (Nyengaard 1993). This is thought to lead to a chronic hypoxia that leads to tubulointerstitial fibrosis, inflammation and further tubular injury with subsequent decline in renal function (Osterby and Nyberg 1987; Fine and Norman 2008). Angiogenesis has also been described following apoptosis that stimulates the neointima of blood vessels within the kidney to acquire an apoptotic resistance that is seen with the development of fibrosis. This has been described in a number of models of fibrosis and within the blood vessels of renal allograft vasculopathy (Cailhier, Laplante et al. 2006).

ECM consists of collagens, structural glycoproteins, elastins, and proteoglycans-hyaluronans. Col4, fibronectin and laminin are constituents of the mesangium and GBM. In GS Col4, Col5, laminin and fibronectin are increased in the mesangial matrix and GBM. Late GS results in increased levels of Col1 and Col3 and is associated with Kimmelstiel-Wilson nodules rather than diffuse mesangial expansion (Ban and Twigg 2008). This suggests there is an increase in collagen synthesis that contributes to the formation of GS. However, some people with T2DM may have slight thickening of the GBM only, with microalbuminuria/proteinuria and tubulointerstitial/arteriolar abnormalities, see section 1.3 (Tervaert, Mooyaart et al. 2010; Kolset, Reinholt et al. 2012).

Hyperglycaemia affects ECM production (Ban and Twigg 2008) through a number of mechanisms, see section 1.5, traditional theory. In addition, AGEPs have been reported to form following hyperglycaemia, (Goldin, Beckman et al. 2006) thereby stimulating the production of ECM directly. AGEPs interact with the RAS, affecting cell signalling, and interact with a number of receptors including the receptor for advanced glycation end-products (RAGE). AGEPs are profibrotic, causing the disruption of matrix-matrix and matrix-cell interactions and further production of Col4 (Doi, Mima et al. 2008). The slowing of progression in proteinuric diabetics is thought to occur with the use of ACEIs or ARBs due to the inhibition of profibrotic stimulus and systolic blood pressure control (Ban and Twigg 2008), see section 1.6.

ECM formation and degradation are continuous processes that occur for the normal structure and function of tissues and organs. There is a balance of matrix formation and degradation. Growth factors including TGF-β and CTGF are responsible for regulating the ECM formation. Degradation of ECM and remodelling arises from the balance of MMPs and tissue specific inhibitors metalloproteinases (TIMPS). MMPs are zinc dependent enzymes that digest all ECM proteins. An excess of MMPs in tissues of those with diabetes has been reported to be associated with the development of fibrosis (Tan and Liu 2012), whereas a lack of these is associated with poor wound healing. MMP2 and 9 break down Col4 from GBM. MMPs are regulated by their TIMPs and may further regulate fibrosis (Thrailkill, Bunn et al. 2007).

TGF-β has been implicated as a major growth factor in the development of DN and fibrosis. TGF-β expression is upregulated in the diabetic environment in mesangial cells, podocytes and tubular epithelial cells (Wahab, Schaefer et al. 2005). The use of TGF-β inhibitors in STZ induced diabetic mice, results in the prevention of glomerular hypertrophy and decreased excess matrix production via the reduction in type Col4 and fibronectin mRNA (Sharma, Jin et al. 1996; Ziyadeh, Hoffman et al. 2000). ARB treatment reduces the expression of TGF-β. TGF-β should not be directly inhibited in humans in view of its anti-proliferative and anti-inflammatory effects that are important for survival.

CTGF is downstream from TGF-β in the fibrotic process and has TGF-β dependent and independent pathways (Murphy, Docherty et al. 2008). CTGF is produced by mesangial cells, podocytes and tubular epithelial cells. CTGF mRNA levels are known to be upregulated in microalbuminuric diabetic patients and in those with DN. Albuminuria correlates with CTGF levels. CTGF overexpression in podocytes results in GBM thickening. CTGF and VEGF-A site specific downregulation, results in podocyte loss in DN (Baelde, Eikmans et al. 2007). CTGF may upregulate its gene expression once induced and is able to initiate changes to the ECM by increasing the expression of fibronectin, Col1, Col3 and Col4, thereby facilitating further deposition of ECM proteins (Wahab, Yevdokimova et al. 2001). This provides a strong chemotactic effect on peripheral blood mononuclear cells, thereby contributing to further inflammation and late fibrosis.

1.9 Common Final Pathway for Traditional and Inflammatory Theories

The traditional and the inflammatory pathways are likely to occur together in the development of fibrosis and DN. Progression of DN to ESRD is by gradual inexorable scarring of renal glomeruli and tubulointerstitial compartments. Increased glucose drives recruitment of monocytes and macrophages (Furuta, Saito et al. 1993; Sassy-Prigent, Heudes et al. 2000). These accumulate in the glomeruli and tubulointerstitium of patients with DN (Nguyen, Ping et al. 2006). Activated macrophages can stimulate fibrosis in both compartments causing long-term damage. The impact of the degree of proteinuria, progression of DN and the cause of proteinuria also needs to be elucidated further.

Monocytes and macrophages contribute to fibrosis by the release of TGF-β that stimulates myofibroblast formation and collagen deposition and possibly EMT (Abbate, Zoja et al. 2006). A decrease in proteinuria was seen in DM rat model with a complement inhibitor together with additional decrease in glomerular deposition of IgG showing that complement may have a role in DN (Fujita, Ohi et al. 1999). Complement may also activate fibrosis, as the inhibition of a complement receptor on tubular cells (tCD59) resulted in less fibrosis (Brown and Turner 2004). In addition, hypoglycaemia has been reported to induce ROS and immune complex deposition that activates complement, thus it has been suggested that changes in glycaemia may equally contribute to DN and not only with persistent hyperglycaemia (Ostergaard, Hansen et al. 2005; Saad, Virella et al. 2006).

Accumulation of ECM proteins in the interstitium together with tubulointerstitial changes result in irreversible damage to the kidney and ESRD (Wada, Sakai et al. 2007; Ban and Twigg 2008; Brosius 2008; Rivero, Mora et al. 2009). Understanding the role of cytokines in these mechanisms may provide a way to predict those at risk of development of DN and have potential to act as new therapeutic targets.

1.10 Biomarkers

Proteinuria suggests there maybe underlying damage to the glomerulus together with tubulointerstitial injury secondary to the abnormal filtration of proteins (Zandi-Nejad, Eddy et al. 2004; Abbate, Zoja et al. 2006), see section 1.3. Albuminuria is sensitive but not specific to DN. A change in albuminuria combined with a change in eGFR is predictive of progression in late

nephropathy, however, these markers are not sensitive enough to detect the initial deterioration and predict progression at the early stage (Jerums, Premaratne et al. 2008). Tsalamandris, et al., showed in almost 30% T2DM patients there was a progressive decline in renal function whilst the rate of albuminuria remained constant (Tsalamandris, Allen et al. 1994). Cystatin C correlates well with GFR and is not affected by age, body mass and gender, unlike creatinine (Rosolowsky, Niewczas et al. 2008). Early renal function decline in DN is detected earlier with Cystatin C compared with creatinine.

TGF-β and CTGF are known to be increased in the urine of DM patients, with CTGF also being detected in the plasma (Sharma and Ziyadeh 1997; Nguyen, Tarnow et al. 2006). It is unclear whether serum, plasma or urine levels reflect tissue levels and whether these would indicate the degree of fibrosis occurring at that time. Col4 and CTGF urinary and plasma levels may be suggestive of the degree of underlying diabetic renal disease (Ban and Twigg 2008). Urinary mRNA levels of CTGF, MCP-1 and PAI-1 were also found to increase with DN progression (Zheng, Lv et al. 2012). Recently serum TGF-β and urinary MCP-1 levels were seen to correlate in T2DM with progressive nephropathy and were suggested to be good prognostic factors; however, this was a small study and these findings have not been validated (Shaker and Sadik 2013).

Urinary excretion of podocytes following their injury has recently been thought to be critical in the pathogenesis and progression of DN (Nakamura, Ushiyama et al. 2000). Staging of DN has been thought to be possible with mRNA quantification of the profile of urinary podocytes. Many genes have been associated with the progression of DN and quantification of a number of different podocyte associated molecules have been postulated to be potential, reliable, future biomarkers.

Urinary proteomics show that those with DN progressing to macroalbuminuria have more collagen fragments. These could be used as biomarkers as they were found 3-5 years prior to progression (Zurbig, Jerums et al. 2012). The understanding in this field requires further development and large studies before they can be validated.

Recently it has also been found, in an 8-12 year follow-up study, that TNF receptors (TNFRs) 1 and 2 predict the progression of DN to ESRD in T2DM (Niewczas, Gohda et al. 2012). Raised serum concentrations of TNFRs at baseline of patients with T2DM, strongly predicts subsequent progression regardless of the presence of proteinuria. TNFRs bind circulating TNFα that may also circulate freely in plasma. The use of TNFRs as a biomarker has currently not been validated.

None of the above have been validated as biomarkers for progression of DN, as the initial findings need to be reproducible, sensitive and specific in large trials. The need to determine more accurate biomarkers illustrates the paucity of knowledge regarding who goes on to progress and hence further research is required.

1.11 Current treatments

Parving in 1983 showed that early aggressive BP control is a key target in preventing progression of DN (Parving, Andersen et al. 1983). Parving studied 10 patients with T1DM prospectively over 29 months whose GFR had decreased whilst the urinary albumin excretion rate and BP rose. On commencing treatment with beta blockers and thiazides he managed to decrease the urinary albumin excretion and the rate of decline in GFR.

Additional benefit has been seen with the use of ACEi, thereby affecting the RAS. 409 patients with T1DM were given captopril in a double-blind RCT. Those on captopril had a 50% decrease in the combined end point of death, dialysis or transplantation. The difference was independent of that of BP in the groups, indicating a renoprotective effect of ACEi (Lewis, Hunsicker et al. 1993). Normotensive patients with T2DM with microalbuminuria who were given ACEi showed long term stabilisation of albuminuria and serum creatinine (Ravid, Savin et al. 1993), see section 1.6. These studies illustrate the beneficial effect of ACEi in both T1DM and T2DM irrespective of BP. Current optimal medical therapy includes the use of ARB following trials such as the Irbesartan Diabetic Nephropathy Trial (IDNT) (Lewis, Hunsicker et al. 2001). There were three arms to this trial with different anti-hypertensives but those given ARB showed a significant delay in the progression of proteinuria in T2DM. The RENAAL (Brenner, Cooper et al. 2001) trial also looked at ARB in T2DM in slowing the progression of DN. In all these studies, the use of treatment which modulates the RAS slows the disease, but does not stop it, leaving a large number of diabetics progressing to ESRD.

Aliskiren, a direct renin inhibitor, also inhibits the expression of TGF-β in animal studies, and has established reduction in ACR in humans (Parving, Persson et al. 2008). The ALTITUDE study (Aliskiren Trial in T2DM Using cardio-renal endpoints) was stopped following an increased incidence of nonfatal stroke, hyperkalaemia and renal complications after 1.5 years of follow up when given in combination with ACEi (Parving, Brenner et al. 2009). Doxycycline, a tetracycline antibiotic, inhibits MMPs. A small RCT in DM taking RAS inhibitors, anti-hyperglycaemic agents and doxycycline, showed a decrease in proteinuria at 6 months. Proteinuria returned to their original levels on cessation of doxycycline (Aggarwal, Jain et al. 2010).

Very recent reports have suggested, from the Ongoing Telmisartan alone and in combination with Ramipril Global Endpoint Trial (ONTARGET) study and the ALTITUDE study, that the combination of RAS inhibitors should no longer be recommended for T2DM until larger RCTs or meta-analysis are performed. Both these studies thus far suggest an increase in commencement of RRT, deterioration of GFR and death in T2DM during follow up (St Peter, Odum et al. 2013). Interestingly ONTARGET trial update reports there were no increases in strokes or major cardiovascular outcomes on T2DM receiving combination therapy, but states adverse events such as dialysis, hyperkalaemia and hypotension were frequently seen in those on dual therapy (Mann, Anderson et al. 2013).

Bardoxolone methyl is an antioxidant inflammatory modulator that can be taken orally that has undergone a phase 2 randomised double blinded placebo-controlled trial. It activates the Keap1-Nrf2 pathway that maintains the structure and function of the kidney. It is thought to exert its effect via the inhibition of the pro-inflammatory NF-KB pathway. Those with advanced CKD and T2DM taking bardoxolone, had an improvement in the GFR at 24 weeks that persisted until 52 weeks suggesting this may be a new therapy to slow progression of renal disease (Pergola, Krauth et al. 2011). This supports an inflammatory role in the progression of DN. However, a recent Phase 3 BEACON clinical trial for bardoxolone methyl in patients with stage 4 CKD and T2DM (NCT01351675) has recently been stopped due to concerns of increased serious adverse events and mortality in the bardoxolone treated patients (reference:
http://www.ukmi.nhs.uk/applications/ndo/record_view_open.asp?newDrugID=5113;
http://www.reatapharma.com/investors-media/news/news-timeline/2012/company-statement-termination-of-beacon-trial.aspx). In a recent study, in Zucker diabetic rats with overt experimental

T2DM, synthetic analogues of bardoxolone worsen DN with additional side effects (Zoja, Corna et al. 2013).

A number of new therapeutic therapies are emerging as inflammatory and fibrotic pathways are targeted with variable effects (Shepler, Nash et al. 2012). The antifibrotic Pirfenidone mediates its effects through its inhibition of TGF-β that has been seen in animal models (RamachandraRao, Zhu et al. 2009). A small RCT with Pirfenidone showed an improvement in GFRs compared to controls after 1 year, however, a higher dose resulted in a higher adverse effect rate of gastrointestinal symptoms and fatigue (Sharma, Ix et al. 2011).

Pentoxifylline is a methylxanthine derivative, a non-selective phosphodiesterase inhibitor with TNF-α activity, has been shown to decrease proteinuria in DN since the 1980s (Solerte, Fioravanti et al. 1987). The PREDIAN trial (Pentoxifylline for Renoprotection in DN) is a RCT that is currently ongoing, whose outcome measures will look at the difference in GFR between DM groups; the results are awaited (Navarro-Gonzalez, Muros et al. 2011). Protein kinase C inhibitor ruboxistaurin appeared to reduce urinary TGF-β in T2DM (Beckman, Goldfine et al. 2010). This study reported a significant decrease in ACR with no change in GFR but was not sufficiently powered (Tuttle, Bakris et al. 2005), thus further studies are needed.

In addition, FG-3019 is an anti-CTGF monoclonal antibody that was given to 24 microalbuminuric T1DM or T2DM patients. Their ACR significantly decreased and was maintained up to 1 year later (Adler, Schwartz et al. 2010). The number of patients in the study would need to be increased to determine the effect and when this treatment should be given in DN. AST-120 is an antifibrotic that reduces TGF-β expression predominantly studied in Asian countries as an oral formulation that binds harmful toxins. There are a few human reports of slowed DN progression (Sanaka, Akizawa et al. 2004), with variable effects on GFR that were reported over 6 months. A double blind RCT, of 40 T2DM patients were given allopurinol and followed up for 4 months. A significant decrease was seen in proteinuria at this time point with no effect on GFR (Momeni, Shahidi et al. 2010). Further studies are currently being carried out for the use of paricalcitol and vitamin D, as these are thought to affect the immune system and thereby affect fibrosis (Fernandez Fernandez, Elewa et al. 2012).

The increasing number of treatments that are being tested, reflect the need for more work in this area as the understanding of progression of DN becomes unveiled.

1.12 Cytokines/Chemokines

Cytokines are inflammatory molecules that orchestrate communication amongst different cell types. They may have autocrine and paracrine functions. These encompass previously named groups of cytokines with specific functions such as interleukins, inducing the effects of lymphocytes and chemokines that have chemoattractant properties.

Chemokines are usually 8kDa molecules and are mainly soluble factors which mediate intercellular communication and cell migration (Gunther, Zimmermann et al. 2011). Chemokines have more than 50 ligands with approximately 17 identified receptors. The chemokine families use the prefix C, CC, CXC or C'C to represent the residue at the amino terminus of the protein. CXC is the largest of these families, and has two amino-terminal cysteines that are separated by a nonconserved amino acid. CC chemokines are where the two amino-terminal cysteines are juxtaposed. All chemokine receptors (CCR 1-10 and CXCR 1-5) have been found in glomerular mRNA; however, this includes many cell types including infiltrating cells (Huber, Reinhardt et al. 2002). Human podocyte culture has shown expression of CXCR subtypes 1, 3, 4, 5 and CCR types 4, 8, 9, 10. Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2; and CCL18 also known as PARC are CC chemokines.

The role of chemokines in DN and progression are increasingly being described. MCP-1, fractalkine and cytokine TNF-α, are all associated with progression of DN (Navarro-Gonzalez, Mora-Fernandez et al. 2011). Chemokines and cytokines are able to attract a number of immune cells including macrophages. Macrophages have been found in the interstitium of DN biopsies in those who had disease progression or advanced DN (Lewis, Steadman et al. 2008) hence these molecules may play an important role in orchestrating the progression of DN. In addition, coronary atherectomy specimens from diabetics have larger areas of monocyte-macrophage infiltration compared to those without diabetes (Moreno, Murcia et al. 2000). Chemokines have been found in atherosclerotic tissue and DM. MCP-1 has been shown to attract monocytes to vascular smooth muscle cells, contributing to the progression of atherosclerosis (Isoda, Folco et al. 2008). Recent clinical RCTs have looked into the benefit of TNF-α as a therapeutic target with Pentoxifylline, see section 1.11

(Navarro-Gonzalez, Jarque et al. 2009). An initial study has shown a decrease in proteinuria and a slowing of disease progression with ACEi and ARBs (Navarro, Mora et al. 2003; Navarro, Mora et al. 2005).

Tubular epithelial cells are known to produce/overexpress MCP-1 and on activation regulate normal T cell expressed and secreted (RANTES or CCL5), when proteinuria occurs (Abbate, Zoja et al. 2006). Albumin upregulates IL-6 expression in tubular cells and is chemoattactant for lymphocytes and neutrophils. The proximal tubule has the receptor for megalin thereby facilitating internalisation and intracellular trafficking of cubulin. Cubulin binds albumin and transfers immunoglobulin G light chains. It has no transmembrane domain. Endocytosis may induce activation of transcription factors and gene expression that affect the haemodynamic and metabolic pathways identified in DN (Abbate, Zoja et al. 2006). Table 1.1 below shows the chemokines causing macrophage accumulation associated with tubulointerstitial injury that have been described since 2006 (Chow, Nikolic-Paterson et al. 2006).

Table 1.1 Cytokine effects in DN

Cytokine	***Effects***
IL-1	Alters expression of chemotactic factors, adhesion molecules, intraglomerular haemodynamics via prostaglandin synthesis. May increase vascular endothelial cell permeability and increase hyaluron production by renal tubular epithelial cells that increase glomerular cellularity (Jones and Phillips 2001). Polymorphisms in IL-1R and IL-1β reported increased risk of ESRD in T2DM (Lee, Ihm et al. 2004) that was not seen in T1DM (Tarnow, Pociot et al. 1997).
IL-6	Has been seen to be involved in the development of thickening of GBM. Possible effect on endothelial permeability and mesangial expansion (Navarro-Gonzalez and Mora-Fernandez 2008).
IL-18	Induces production of IL-1, interferon gamma (IFN-γ), TNFα and may be associated with endothelial cell apoptosis (Navarro-Gonzalez, Mora-Fernandez et al. 2011). Increases TGF-β via MAPK in tubuloepithelial cells (Miyauchi, Takiyama et al. 2009).
TNFα	Causes direct renal injury, cellular apoptosis, endothelial permeability, glomerular haemodynamics and cell-cell adhesion. Contributes to early hypertrophy and hyperfiltration in DN (DiPetrillo, Coutermarsh et al. 2003).

1.12.1 Monocyte Chemoattractant protein – 1/CCL2 (MCP-1)

MCP-1 is a CC chemokine attracting monocytes/macrophages and T cells (Yoshimura and Leonard 1992). MCP-1 is localised on chromosome 17q11-21 with a molecular weight between 9-17kDa and a mature peptide of 76 amino acids with its signal precursor of 23 amino acids (Yoshimura, Yuhki et al. 1989). Mesangial cells, podocytes and tubular epithelial cells have been found to produce MCP-1 when exposed to high glucose concentrations and AGEPs (Ha, Yu et al. 2002; Zhang, Nguyen et al. 2006). MCP-1 has previously been found in the urine of humans with DN (Banba, Nakamura et al. 2000). MCP-1 knockout mice were found to be protected from STZ induced DN (Chow, Nikolic-Paterson et al. 2006). In high glucose, MCP-1 can be induced in cultured mesangial cells without mechanical strain (Ihm, Park et al. 1998).

In a prospective study of patients with renal biopsies for CKD, high levels of proteinuria correlated with MCP-1 induced interstitial damage indicating a common pathway in the presence of proteinuria (Eardley, Zehnder et al. 2006). NF-κB activation and MCP-1 upregulation in the proximal tubular cells has been reported in DM (Mezzano, Aros et al. 2004).

Urinary MCP-1 has previously been reported to be significantly raised in macroalbuminuric diabetic patients (Tam, Riser et al. 2009). In microalbuminuric diabetic patients this relationship is reversed with low urinary levels of MCP-1 in DM. Urinary MCP-1 levels are not raised in proteinuric renal diseases such as nephrotic syndrome secondary to minimal change (Wada, Yokoyama et al. 1996). Urinary MCP-1 has been seen to correlate with DN tubulointerstitial lesions and fibrosis (Wada, Furuichi et al. 2000) and with glomerular injury (Banba, Nakamura et al. 2000); thus the literature of MCP-1 in DN is well established.

1.12.2 Macrophage Migration Inhibitory Factor (MIF)

MIF is a pleiotropic cytokine that has been previously described in the recruitment of macrophages and T-cell activation (Metz and Bucala 1997). MIF is known to be produced by mesangial, glomerular epithelial, glomerular endothelial and tubular cells (Tesch, Nikolic-Paterson et al. 1998; Matsumoto, Maruyama et al. 2005). MIF receptor CD74 has been found on podocytes in DN (Sanchez-Nino, Sanz et al. 2009). Recent phase 1 clinical trials are underway with anti-MIF antibodies in the treatment of prostate cancer following tumour regression in a mouse model using

these antibodies (Hussain, Freissmuth et al. 2013). Previous studies with MIF knockout mice and MIF transgenic animals have demonstrated that MIF is an essential mediator in the development of renal disease (Leung, Chan et al. 2004; Sasaki, Nishihira et al. 2004). Recently MIF has been described as a candidate for inducing microalbuminuria in diabetic mice; however, db/db mice that do not develop microalbuminuria had higher urinary MIF levels than ob/ob mice that have microalbuminuria (Watanabe, Tomioka et al. 2013). Both diabetic mice strains had significantly increased MIF protein with significantly higher MIF gene expression in the db/db strain. The study suggests that MIF could be responsible for initiating microalbuminuria in DN.

MIF is preformed and stored within the cytoplasm of macrophages, pancreatic islet cells, myocytes, cardiomyocytes and intrinsic renal cells – glomerular/tubular epithelial cells and rat mesangial cells (Tesch, Nikolic-Paterson et al. 1998; Toso, Emamaullee et al. 2008). MIF has also been described in rejecting kidney allografts (Lan, Yang et al. 1998). Inhibition of MIF does not ameliorate acute renal allograft rejection in mice (Jose, David et al. 2003). MIF is known to prevent inhibition of NF-κB activation induced by glucocorticoids. In addition, MIF inhibits glucocorticoid induction of the mitogen activated protein kinase (MAPK) pathway (Lan 2008).

MIF receptor CD74 is found in podocytes and tubular cells in human DN. Receptor CD74 recruits CD44 to form a receptor complex activating the extracellular signal regulated kinase 1/2 (ERK1/2) pathways and MAPK p38 that promotes cell proliferation and the synthesis of prostaglandin E in leukocytes and fibroblasts (Leng, Metz et al. 2003). This is thought to be another pathway by which MIF induces its effects. MAPK p38 is a pathway involved in regulating proinflammatory cytokines, cell survival together with apoptosis, and maintaining the cytoskeleton (Adams, Badger et al. 2001). ERK1/2 pathways are involved in transmitting signals from extracellular factors to regulate cellular processes (Koshikawa, Mukoyama et al. 2005). MAPK p38 and ERK1/2 pathways are reported to be needed for MIF induction of TNF related apoptosis inducing ligand (TRAIL) and MCP-1 in both podocytes and tubular epithelial cells (Benito-Martin, Ucero et al. 2009).

Ang2 has been reported to increase the synthesis of MIF from tubular epithelial cells whilst recruiting and activating leukocytes (Rice, Nikolic-Paterson et al. 2003). MIF receptor was found to be increased in podocytes stimulated with high glucose, TNFα and MCP-1 (Sanchez-Nino, Sanz et

al. 2009). Urinary MIF is elevated in proliferative glomerulonephritis (GN) with no change detected in serum MIF levels of healthy controls or those with GN (Brown, Nikolic-Paterson et al. 2002). The levels of urinary and serum MIF in the DN population are currently unknown.

MIF serum levels were found to be high in those with DN (Herder, Kolb et al. 2006). MIF was not found to increase apoptotic cell death, but to activate MAP kinases and increase TRAIL expression (Sanchez-Nino, Benito-Martin et al. 2010). TRAIL expression has previously been reported to be increased in patients with DN (Schneider, Thome et al. 1997). An increase in TRAIL has also been correlated with interstitial fibrosis, cell death, tubular atrophy and interstitial inflammation; hence activation of this cytokine by MIF could lead to progression in DN (Lorz, Benito-Martin et al. 2008).

The ESTHER study recently reported an increased risk of cardiovascular outcomes in DM with or without DN, proinflammatory cytokines and adiponectin. The study looked at serum MIF, IL-6, IL-18, adiponectin and leptin. In those with high MIF and IL-6 levels that had DN, an association was found with higher cardiovascular morbidity that was not seen in the absence of renal dysfunction. High adiponectin levels were associated with higher risk of a primary cardiovascular event.

1.12.3 CC-Chemokine Ligand 18 (CCL18)

Cytokine CCL18 also known as PARC/MIP-4/AMAC-1/DCCK1/SYCA-18 is an 89 amino acid polypeptide on chromosome 17q11.2. No CCL18 is found in rodents. CCL18 is chemotactic for naïve T lymphocytes, CD38 mantle zone B lymphocytes and immature dendritic cells (van der Voort, Kramer et al. 2005). CCL18 has been described in a number of diseases such as Idiopathic pulmonary fibrosis, Systemic sclerosis, Rheumatoid arthritis, Gaucher's disease, Atopic dermatitis, Bullous pemphigoid, Childhood acute lymphoblastic leukaemia and in particular ovarian and gastric malignancies (Atamas, Yurovsky et al. 1999; Struyf, Schutyser et al. 2003; Vulcano, Struyf et al. 2003; Leung, Yuen et al. 2004; Schutyser, Richmond et al. 2005; Boot, Verhoek et al. 2006; Luzina, Papadimitriou et al. 2006; Auer, Blass et al. 2007; Babu, Kumaraswami et al. 2009; Gunther, Carballido-Perrig et al. 2009). Expression of CCL18 is known to increase in antigen presenting cells exposed to IL-4/IL-10/IL-13 and vitamin D (Babu, Kumaraswami et al. 2009) Th2 lymphocyte cytokines. Expression of CCL18 in antigen presenting cells is decreased with Th1 lymphocyte cytokines, IFN-γ and with Lipopolysaccharide (LPS) (Gunther, Carballido-Perrig et al. 2009). The role of CCL18 in DN is unknown. More recently CCL18 has been found in adipocytes in people

with non-alcoholic steatotic hepatitis (NASH). Those with fibrosis and NASH had a higher level of CCL18 messenger RNA (mRNA) in their adipocytes compared with those with NASH without fibrosis (Estep, Baranova et al. 2009). The role of CCL18 in insulin resistance and DN is unknown. There is increasing evidence that insulin resistance has been linked with obesity and the metabolic syndrome and these are known risk factors for the development of T2DM. The finding of CCL18 in adipocytes together with its link to fibrosis makes CCL18 in DN an interesting area to examine.

CCL18 has previously been described to have a T-lymphocyte TGF-β dependent fibrotic pathway producing Col1 in resident fibroblasts, together with a TGF-β independent pathway where CCL18 directly acts on resident fibroblasts to produce Col4 (Luzina, Papadimitriou et al. 2006; Prasse, Pechkovsky et al. 2006; Pochetuhen, Luzina et al. 2007). Production of Col1 has been directly seen in response to stimulation of fibroblasts with CCL18 independent of TGF-β1 (Atamas, Luzina et al. 2003).

In Dr Tam and Dr Frankel's research laboratory, (Ahmad, North et al. 2010) high levels of CCL18 were found in the peritoneal fluid of renal patients on peritoneal dialysis who developed encapsulating peritoneal sclerosis (EPS), a fibrotic condition affecting the peritoneum. Multivariate analysis showed that the amount of peritoneal CCL18 correlated significantly with the amount of glucose exposure in the patient's dialysis regimen.

A prospective cohort study examining diabetic urine in Dr Tam and Dr Frankel's research group (Qureshi A 2007 ASN abstract #551921) showed urinary CCL18 in T2DM with macroalbuminuria correlated positively with urinary ACR (Spearman's correlation $r = -0.36$ $p < 0.0005$) and negatively with estimated glomerular filtration rate (eGFR), Spearman's correlation $r = -0.36$, $p = 0.0003$). Urinary CCL18 was raised and correlated with the severity of DN. These results were taken from 107 diabetic participants and 121 non-diabetic participants with other proteinuric renal diseases. There was no significant difference in plasma CCL18 among three groups of diabetic participants with different severity of albuminuria. There was no correlation between urinary and plasma CCL18 suggesting CCL18 may be locally produced in the kidney.

Recently a receptor for CCL18 has been described in tumour associated macrophages (TAM) that promote breast cancer metastasis (Bonecchi, Locati et al. 2011). It is a phosphatidyl-inosytol transfer protein that has an acidic region with a calcium binding domain and a 6 transmembrane domain with a C-terminal that interacts with PYK2 that is downstream from PKC pathways. The functional G-protein receptor is known as PITPNM3. It is unknown whether this receptor is required for CCL18 to exert all its effects or whether it uses a paracrine or autocrine method to exert its effects in addition to the use of this receptor. PITPNM3 has not been found on gastric invasive cancer tissue margin TAMs and may be tissue/organ specific. A role for serum CCL18 as a predictor for worsening lung fibrosis seen in systemic sclerosis has been proposed (Tiev, Hua-Huy et al. 2011).

1.13 Study proposal

The proposed study will determine whether urinary MIF, MCP-1 and/or CCL18 increase with the progression of DN. The cohort of participants initially used in the study, will be followed prospectively for a minimum of 3 years. The micro-environment affecting synthesis of CCL18, together with the cellular and molecular mechanisms of CCL18 in DN, will be investigated as they are currently unknown. The interaction between MIF, MCP-1 and CCL18 will also be determined.

1.14 Project scope

This project will look at urinary, plasma and serum cytokines including pro-fibrotic growth factors and chemokines, specifically MIF, MCP-1 and CCL18 from clinical samples. These will be compared with the effects of high glucose and AGEPs on the synthesis of these cytokines *in vitro*. In addition, attempts to understand how and whether there is communication between these cytokines in the different renal cell types will be investigated. This will help determine what role cytokines play in the fibrosis found in DN and whether these markers are potentially therapeutic targets.

1.15 Hypothesis

MIF, MCP-1 and CCL18 are causative factors in the development of renal inflammation and fibrosis in DN and are useful biomarkers of disease progression.

1.16 Aims

- *Clinical prospective cohort study* examining; Urinary, plasma and serum cytokines (MIF, MCP-1 and CCL18) in diabetic (DM) and non-diabetic (Non-DM) proteinuric renal disease groups. Baseline samples will be used to determine predictability of deteriorating GFR or increasing ACR/UPCR respectively at >18 months and >3 years.
- *Determine whether the levels baseline cytokines changes with time.* This will be done prospectively, with collection of baseline samples and collecting new samples from the same person at >3 years. Any changes seen will be correlated with clinical outcome to determine whether there is a cytokine profile that may suggest disease progression.
- *Determining the effects of MIF with clinical parameters collected*. Baseline urinary and plasma MIF will be measured in the healthy population and comparison made with DM and Non-DM groups. Baseline MIF levels in DM and Non-DM will be correlated to clinical parameters such as age, gender, BMI.
- *Determining difference in serum and plasma levels of cytokines* (MIF, MCP-1, CCL18) and their prognostic value as markers in DM and Non-DM proteinuric populations.
- *Determining the presence of CCL18* in renal tissue biopsies from participants and in intrinsic renal cells *in vitro*.
- *Investigating the effects of MIF, MCP-1 and CCL18* in the production of profibrotic growth factor fibronectin using human intrinsic renal cells *in vitro*.
- *Determining the interaction between MIF, MCP-1 and CCL18*. This will be done using human intrinsic renal cells *in vitro* and how these differ with the diabetic environment.
- *Determining what cell signalling pathways MIF, MCP-1 and CCL18 activate* when stimulated in the diabetic environment.
- *Determining whether apoptotic pathways are activated* in intrinsic renal cells in diabetic environments stimulated with recombinant cytokines.

CHAPTER 2.0 – METHODOLOGY

2.1 Prospective Clinical Cohort

All patients from the baseline cohort were initially recruited from Imperial College Healthcare NHS Trust at Imperial College Renal and Transplant Centre by Dr Qureshi. Dr Qureshi has given me permission to use the baseline data from the cohort (see Appendix 1.0). The patients had originally attended clinics for diabetes, general nephrology, vasculitis and systemic lupus erythematosus. Some of these patients had been discharged or lost to follow up. Using the hospital computer system the original cohort was identified, and dates when they would be attending clinic were recorded. These patients were approached at that time to invite them to participate again in the second part of the study. Consent was requested again although the original ethics and consent included the follow up study. Those without clinic appointments were invited to participate in the follow up study by letter, with a consent form and a letter reminding them of the details of the study. In the letter they were informed I would telephone them to formalise a time and date convenient for them to attend either Charing Cross Hospital or Hammersmith Hospital. A contact number was provided to enable patients to make direct contact for the study. The patients that had died were documented on the database, and the last data entry of results relevant to the study endpoint was taken from the hospital computerised system as original consent had included the follow up period.

2.1.1 Ethics and consent

Patients had been consented at the initial joining of the study and were approached for re-recruitment and invited to participate again. Consent was re-taken and consent forms were signed to show their continued agreement. The original study had been reviewed and approved by the research ethics committee at Hammersmith Hospital, Queen Charlotte's and Chelsea Hospital and Acton Hospital. When patients attended they were provided with a further copy of the patient information sheet explaining the study and the reason for approaching them in view of the follow up study.

2.1.2 Patients re-recruitment

All patients' identifiable data were anonymised on the database. A copy of the data sheet used to collect the follow up data is included in Appendix 3.0 together with a copy of the consent form and patient information sheet provided. Urine and blood samples were obtained from the patients at their

convenience or during their scheduled clinic appointments if they re-consented. Urine and blood samples were taken for the same laboratory analysis done at baseline to allow comparisons to be made, see Table 2.0. Those participants on RTT were approached to see if they continued to pass urine, thus allowing analysis to be made. Those who had undergone renal transplant (RT) were also approached to request urine and serum samples. The details taken in Table 2.0 were following consent and were at >3 years follow up. The questionnaire used is seen in Appendix 3.1.0.

Table 2.0 Data collected for 3 years follow up

Demographics	**Laboratory investigations**	**Study samples**
Gender Ethnicity Age Height Weight Body mass index (BMI) Smoking status Blood pressure Medication list Past medical history Details of renal biopsy, if applicable Details of complications of DM, if applicable	*Haematology;* Haemoglobin White blood count *Biochemistry;* Serum; sodium, potassium, urea, creatinine, corrected calcium, albumin, phosphate, parathyroid hormone, vitamin D, c-reactive protein (CRP) *Estimation of GFR*; calculated with the results above using Modification of diet in renal disease (MDRD) equation 7 (Levey et al 1999) *Urine;* urinary albumin, total protein and creatinine, ACR and UPCR *Microbiology;* Urine microscopy culture and sensitivity (MC+S)	Plasma Serum Urine

2.1.3 Sample collection

Urine and blood (serum and plasma) samples were collected from patients who consented for the follow up study. Height and weight together with laboratory parameters previously taken were recorded. Medication lists were updated together with clinical diagnosis that may have affected interpretation of data. Patients that had started HD, PD or had a RT were included if the data points for these patients were within the correct follow up time points used prior to commencing RRT i.e. GFR and urinary albumin/ creatinine ratio before starting dialysis or transplantation were used for assessment of progression of renal disease. Anyone with a urinary tract infection at the time of sample collection was excluded. A urinary infection was determined by the sample being sent to the microbiology laboratory. The sample sent to the clinical biochemistry laboratory quantified the urinary albumin, total protein and creatinine levels.

Those patients followed up with non diabetic renal disease (Non-DM), had a variety of renal diagnoses as per the original database. Some of these were on immunosuppression. See Table 2.1 for details of diagnosis with varying degrees of proteinuria and diagnoses that may require immunosuppression as treatment for their disease depending on activity of the underlying disease. The study is too small to undergo a subgroup analysis into Non-DM on immunosuppression and how this differs from those without immunosuppression. This is to be kept in mind during the analysis that follows as it is likely that cytokine levels may be affected with immunosuppression. In addition, some patients were on immunosuppression for reasons unrelated to their underlying renal condition and this was also documented.

Table 2.1 Possibility of immunosuppression in control group

Non Diabetic proteinuric renal disease	**Immunosuppression as possible treatment**
Vasculitis	Yes
Lupus	Yes
Minimal change disease	Yes
Focal segmental glomerulosclerosis	No
Membranous glomerulonephritis	Yes
IgA nephropathy	Yes

Acute kidney injury with heavy proteinuria	No
Polycystic kidney disease	No
Renal tumours	No
Reflux nephropathy	No
Granulomatous interstitial nephritis	Yes
Renal artery stenosis	No
Hypertensive nephropathy	No

2.1.4 Sample aliquots

2.1.4.1 Prospective Urine Samples

A midstream urine sample was requested from all patients agreeing to partake in the follow up. 20mls of urine was centrifuged at 3000 revolutions per minute (rpm) for 10 minutes at 4^0C using CR 312 centrifuge (1107g). Urinary sediment formed a pellet at the base, the urine above this was removed and 10 aliquots of 2000µl were placed in coded labelled tubes. These were stored at -80^0C. Any sample found to have a urinary tract infection was discarded. Samples were coded to differentiate baseline and prospective samples from the same patient. All samples were stored in the -80^0C freezers renal research lab at Hammersmith Hospital.

2.1.4.2 Prospective Plasma/Serum Samples

Both plasma and serum were collected as there is evidence suggesting that clotting factors may affect levels of cytokines found in blood. The original consent form allowed 10ml of blood in total to be collected i.e. 10mls of blood (4mls EDTA (Ethylene-diamine-tetra-acetic acid) tube plasma, 6mls serum tube for serum). The samples however, were taken in 2 distinct tubes to allow for the analysis. The initial work thought to be done with serum samples was in fact plasma samples, hence doing both allowed a comparison to be made from different time points of cytokines in the same person whilst allowing comparison between cytokine levels in plasma and serum and whether these are comparable. Both samples were centrifuged at 3000rpm for 10 minutes at 4^0C using CR 312 centrifuge (1107g). The samples were all stored in the -80^0C freezers renal research lab at Hammersmith Hospital.

2.1.4.3 Stored Baseline Urine Samples

The patient urine sample was identified by a code and an aliquot taken out from the -80^{0}C freezer to defrost at room temperature. The samples were mixed in the rotar mixer prior to use. A total volume of 200μl was used for the MIF specific sandwich ELISA. The samples were then placed back into the -80^{0}C freezer after marking the tube with a dot to signify the sample had been defrosted. This provided a record of how many times a sample was defrosted. Samples were rearranged and stored in a systematic manner such that they could be found again for another user. Any sample on the ELISA that was above the standard curve would be re-identified, defrosted to room temperature and subsequently diluted according to the ELISA diluents in various dilutions. The samples were analysed in duplicate.

2.1.4.4 Stored Baseline Plasma Samples

The plasma sample was be identified by a code from an aliquot from the -80^{0}C freezer. Samples where there was insufficient volume to perform an ELISA were excluded however, if there was sufficient volume to allow a dilution of 1:2 these were included, providing the result was detected on the standard curve. The samples were defrosted to room temperature and any dilutions made using the ELISA solution S (see MIF ELISA protocol). Any samples with remaining volume were returned to the -80^{0}C freezer with a dot on the tube to signify defrosting of the sample.

2.1.4.5 Data Entry

The original database was changed to ensure ease of coding and consistency of data. Data entry was solely done by me to prevent any inconsistencies. Clinical parameters were updated. Cytokine analysis of baseline plasma and urine levels together with prospective samples of plasma, serum and urine levels were entered according to the anonymised codes on the database. 18 months and >3 years clinical time points were entered.

2.2 Immunohistochemistry (IHC)

IHC was used to detect the antigen of interest in a histological section using a specific antibody (Ab) with blocking and incubation steps. The Ab of choice was amplified with a biotinylated marker that could be seen in the histological section where the antigen was found. The literature review of IHC of CCL18 showed clear staining with CCL18 rabbit polyclonal Ab (Peprotech NJ, USA), (Leung, Yuen et al. 2004). CCL18 staining was visible at the margin of gastric tumour invasion into normal gastric tissue. Renal tissue has never been stained for CCL18 in the literature. Gastric biopsy tissue

with the margin of tumour invasion was requested from Charing Cross Hospital histopathology archive. The paraffin embedded tissue was used to stain CCL18 with IHC. Controls included 0.01% rabbit serum and PBS/Tween. CCL18 Ab was diluted to determine the correct concentration on gastric tissue for staining. Refer to Appendix 2.1.0 for details of IHC.

Once the CCL18 positive control gastric tissue was optimised, renal biopsy tissue with DN was requested from the renal archive. The paraffin embedded tissue was cut from blocks with the microtome and placed onto frosted slides. These slides were then used for IHC with positive controls as; gastric tissue, 0.01% rabbit serum on renal biopsy tissue and renal biopsy tissue with PBS/Tween. Renal biopsy tissue with histological findings of tubulointerstitial nephritis (TIN), were also stained as a positive control for CCL18 as high urinary levels of CCL18 were found in participants with TIN. IHC was optimised for renal biopsy tissue with different microwave times, incubation times and concentrations of CCL18 Ab (see Appendix 2.1.0). Limitations arose because gastric tissue was used as a control for CCL18 as renal tissue had never been stained for CCL18 before.

2.3 Enzyme Linked Immunosorbant Assay (ELISAs) for Cytokine quantification

All specific sandwich ELISAs were performed on 96 well NUNC-immunoplate (thermofisher, scientific Roskilde). The protocols for each cytokine ELISA are in the Appendix 2.0. The principle of the ELISAs used is below. All cytokine Capture, Standards and Detection Ab were aliquoted and stored in the -80^{0}C freezer. The Ab and the concentrations used for the ELISAs are specified in Table 2.2.

Table 2.2 Details of Ab concentrations for ELISAs

ELISA	[Capture Ab]	[Highest Standard]	[Detection Ab]	Sensitivity
MIF	2µg/ml	30000pg/ml	0.1µg/ml	31.25pg/ml
MCP-1	2µg/ml	2500pg/ml	0.1µg/ml	9.8pg/ml
CCL18	2µg/ml	5000pg/ml	0.1µg/ml	19.5pg/ml
Fibronectin	2µg/ml	2000pg/ml	0.1µg/ml	1.95pg/ml

Each ELISA plate was coated with the capture Ab of the respective cytokine being measured. 72 of the 96 wells plate were coated. The top and bottom row of the plate were left empty to decrease the chance of any spurious results due to contamination or optical artefacts of the outer wells. The plates were left overnight in the 4^0C fridge with a plate cover to ensure no loss of sample.

The following day the plate was washed with washing buffer three times (360µl per well). 300µl of blocking solution was added to each well. The plate was placed on the plate shaker at 78rpm (Labnet ORBIT 1000) and incubated at room temperature for 1 hour. Recombinant cytokine standards were defrosted to room temperature from the -80^0C freezer and a rotar mixer was used to ensure the antibodies were mixed before opening. The standards were diluted from the stock solution into a 1:2 fold serial dilutions in diluent or solution S depending on the analysis of the ELISA of urine or serum/plasma respectively. Samples were prepared and diluted accordingly. The plate was washed three times with washing buffer and the standards and samples applied in 100µl volume in duplicates accordingly. A sheet with a plate layout was labelled in order to ensure the samples were identifiable and a record kept for data entry. Once the samples were applied there was a further 2 hours incubation on the shaker at room temperature.

The wash step was repeated and 100µl of the biotinylated detection Ab was added per well and incubated at room temperature for a further 2 hours. Following this there was a wash step followed by the addition of 100µl/well of Streptavidin conjugated to horseradish peroxidase. After 20 minutes the wash step was repeated and 100µl of the reaction substrate was added to each well and placed away from direct sunlight to allow the reaction to occur. This time was approximately 5 to 30 minutes for all the ELISAs, with a shorter interval required for serum/plasma samples compared to urine samples. When the deep blue colour was reached in the highest standard 30,000pg/ml MIF, 2500pg/ml MCP-1 or 5000pg/ml CCL18 50µl of Stop Solution was added to each well and gently mixed to produce a yellow colour. The absorbance of the wells, was measured at a wavelength of 450nm and analysed using ANthos HII ELISA plate reader (Lab Tech International, Ringmer, UK). Later experiments used the plate reader BioTek EL800 (SN242457) that replaced the older plate reader pre-mentioned. The corrected optical density (OD) for each well was given a number. The numbers were entered into a Microsoft excel sheet to establish a standard curve and thereby allow quantification of the OD with log concentration of cytokine. This analysis was done through Graph Pad Prism software (version 4 Graph Pad Software Inc, San Diego, CA). The log was placed back

into the spreadsheet that calculated the anti-log of the data to give an actual concentration. This number was then multiplied according to the dilution factor i.e. 1:10 results in the anti-log multiplied by 10 to give the actual concentration of cytokine in pg/ml. The standard curve needed to have an r^2 <1 for the ELISA to be accepted with the OD varying less than 10% between duplicates of the same sample. Any samples with a greater variability of 10% were repeated. Samples that were at the extremes of the standard curve were repeated to avoid bias. Each cytokine had an individualised excel sheet as the standard curve varied between cytokine ELISAs as seen in Table 2.2 with varying highest standard concentrations for each ELISA.

The Fibronectin ELISA has two overnight steps; capture Ab for coating and incubate overnight. The following day wash step, blocking, wash step and placing standards and samples on a plate for further incubation overnight. The next day there is a further wash step followed by detection, wash step and development with substrate following streptavidin stage. Fibronectin uses a different substrate to allow development (see Appendix 2.2.3 for protocol). Fibronectin ELISAs require readings to be done at 492nm wavelength on the plate reader.

All cytokine Abs were bought from R&D systems (Abingdon, UK). Fibronectin Abs were purchased from Sigma (UK). Details of the Ab of the ELISA are found in table 2.3.

Table 2.3 Details of ELISA Ab

ELISA	**Details**
MIF duo set	Murine monoclonal anti-human MIF Ab for capture and biotinylated goat anti-human MIF Ab for detection. E.coli derived recombinant human MIF from R&D systems was used as the antigen for the standard curve. The standard curve ranged from 31.25-30000pg/ml.
MCP-1	Murine monoclonal anti-human MCP-1 Ab for capture and biotinylated goat anti-human MCP-1 Ab for detection. E.coli derived recombinant human MCP-1 from R&D systems was used as the antigen for the standard curve. The standard curve ranged from 9.8-2500pg/ml.
CCL18	Murine monoclonal anti-human Ab for capture and biotinylated goat anti-human CCL18 Ab for detection. E.coli derived recombinant human anti CCL18 from R&D systems was used as the antigen for the standard curve. The standard curve ranged from 19.5-5000pg/ml.

Fibronectin	Rabbit anti-human fibronectin polyclonal Ab for capture and biotinylated murine anti-human fibronectin monoclonal Ab for detection. Fibronectin derived from human plasma from Sigma was used as the antigen for the standard curve. The standard curve ranged from 1.95-2000pg/ml.

2.3.1 ELISA Intra- and inter-assay variability

The intra-assay variability is the difference between the results of the same sample on different parts of the same plate. The inter-assay variability is the difference between the results of the same sample on a different day, thereby ensuring the results are reproducible. The results of 20 samples were taken to determine the mean and standard deviations (SD) for the variability of each assay performed. The concentrations were taken from the mid portion of the standard curve to ensure accuracy. Table 2.4 shows the intra and inter-plate variability of urinary and serum/plasma samples in all the ELISAs performed and the intra and inter-plate variability seen in the fibronectin ELISA on cell culture supernatants. Cell culture supernatants were treated like urinary samples and had the same intra and inter-variability in MIF, MCP-1 and CCL18. MIF ELISAs included baseline and prospective samples.

Table 2.4 Intra- and inter-variability of ELISAs

ELISA	Urinary intra-variability	Urinary inter-variability	Serum intra-variability	Serum inter-variability	Plasma intra-variability	Plasma inter-variability
MIF	6.6%	11.9%	5.2%	11.3%	11.5%	14.7%
MCP-1	7.3%	9.4%	6.8%	12.7%	7.9%	8.9%
CCL18	6.4%	11.5%	9.1%	10.0%	9.1%	11.7%
	Cell culture	**Cell culture**				
Fibronectin	5.4%	8.7%	n/a	n/a	n/a	n/a

2.3.2 MCP-1 ELISA

MCP-1 ELISAs were performed on prospective urine, plasma and serum samples. MCP-1 ELISA was also used to detect MCP-1 in cell culture supernatants. See Appendix 2.2.0 for MCP-1 protocol.

2.3.3 MIF ELISA

MIF ELISAs were performed on baseline and prospective urine, plasma and serum samples. In addition, cell culture supernatants were analysed with MIF ELISA. See Appendix 2.2.1 for protocol.

2.3.4 CCL18 ELISA

CCL18 ELISAs were performed on prospective urine, plasma and serum samples. CCL18 ELISA was also used to quantify CCL18 in cell culture supernatants. See Appendix 2.2.2 for CCL18 protocol.

2.3.5 Fibronectin

This ELISA detects the whole Fibronectin protein found in the extracellular matrix (ECM). This protein has a number of binding sites that may bind surfaces, collagen and heparin. See Appendix 2.2.3 for Fibronectin protocol. This ELISA was used to detect the ability of intrinsic renal cells to produce fibrotic markers and whether this was influenced by diabetic conditions or cytokine stimulation.

2.4 MTT Assay

MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide] assay, functions on the basis of the mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals. These crystals are largely impermeable to cell membranes thereby resulting in its accumulation within healthy cells. A detergent is added to solubilise the cells thereby releasing the crystals that are solubilised. The number of surviving cells is directly proportional to the formazan product formed. The colour is quantified on the ELISA plate reader 550nm (See Appendix 2.3.0 for MTT Assay protocol). This assay was used to determine cell survival and to see whether the conditions or the treatment with cytokines affected cell survival. This is important as any differences detected in cytokines or fibronectin production need to be differentiated from those occurring from a decrease in cell survival within that condition. Each well was seeded with the same number of cells, hence allowing comparison between conditions as well as that of cytokine stimulation.

2.5 Cell culture

A 4 week cell culture experiment was performed by a lab colleague using transformed human mesangial cells (tHMC) and human podocytes. The cells were exposed to normal glucose (4mM D-glucose), mannitol (26mM + 4mM D-glucose) or high glucose (30mM D-glucose) over this period and supernatant was collected at weekly intervals. I analysed the supernatant of these experiments with MCP-1 and CCL18 specific sandwich ELISAs. These results were presented in my MD progress report and showed that tHMC consistently produced small amounts of CCL18 over the 4 week period irrespective of the conditions. Podocytes were able to produce more CCL18 compared to tHMC regardless of the conditions and this also remained constant over a 4 week period (Data previously shown in MD progress report). No great difference was seen in the production of cytokines over time in this preliminary experiment and thus a shorter period of exposure time with the conditions was sought, as the cells were able to produce these cytokines in the above conditions.

Human cell lines; tHMC, Podocytes and proximal tubule epithelial cells (HK-2), were used to determine whether these intrinsic renal cells were able to produce cytokines. The cell lines were used to ascertain basal cytokine production, cytokine production in diabetic milieu with the appropriate controls (normal glucose (4mM D-glucose), mannitol (26mM+4mM D-glucose), high glucose (30mM D-glucose), low dose glycated albumin (A1) (Sigma, UK) (100µg/ml+4mM D-glucose), high dose glycated albumin (A2) (500µg/ml+4mM D-glucose). The production of cytokines following stimulation with individual cytokines in the diabetic milieu was also done. Each cell culture condition was done in duplicate in this pilot study that also determined a dose response curve for the different cytokine stimulations. Following the pilot study human podocytes and HK-2 cells were used to ensure the initial results were reproducible in specific conditions (normal glucose, mannitol, high glucose and high dose glycated albumin 500µg/ml (A2)) and at 0, 10 and 20ng/ml cytokine stimulation dose. Time limitations allowed ELISA analysis to be done on the 0 and 20ng/ml dose.

2.5.1 Transformed Human Mesangial Cells (tHMC)

tHMC used for these experiments were a kind gift from Professor Roger Mason, Imperial College, London. Normal human mesangial cells were transformed with simian virus 40 large T antigen using the Immortalex kit instructions from Novus Molecular (San Diego, CA, USA). The cells were then cultured to immortalisation termed tHMC (Wahab, Yevdokimova et al. 2001). Cells were

counted, seeded and grown in RPMI 1640 medium (Life Technologies, Grand Island, NY). Media was supplemented with 1000U/ml of Penicillin, 100µg/ml Streptomycin and 2mM glutamine with 5% heat-inactivated fetal calf serum (FCS) (See Appendix 2.4.0). The cells were cultured at 37˚C in 5% CO_2 atmosphere.

T75 flasks were used to grow the cells until there were sufficient cells to seed into 6 well plates. Each of the 5 conditions were done in duplicate and the plates were clearly labelled to determine those that would undergo stimulation with 0, 2, 10 or 20ng/ml of cytokine in addition to the background condition. Equal number of cells, were placed in each well following counting cell number per flask per ml with Trypan blue and a hemacytomatometer. Approximately $5x10^6$ cells per well were used with 70% confluent as tHMC have no growth cycle arrest. The cells were placed in maintenance media for 24 hours prior to the experiment. The cells were exposed to the conditions above (diabetic milieu) in the serum free stage of the experiment. After 24 hours serum free media (SFM) the supernatant, was removed and the serum free conditioned media was added with the variable doses of cytokine stimulation. After 48 hours in this condition the supernatant was collected and the cells lysed for ribonucleic acid (RNA) extraction and subsequent real-time polymerase chain reaction (RT-PCR) (See Appendix 2.7.0).

Supernatant was collected into eppendorf 1500µl tubes on ice and was centrifuged at 14000rpm for 10 minutes 4ºC in eppendorf centrifuge 5417R (10958g). The supernatant was removed, added to new labelled eppendorf tubes and stored at -20ºC for further analysis with cytokines. 1ml of Hanks solution was added to the pellets in the tube and further centrifuge step 1500rpm (128g) 5 minutes 4ºC. The supernatant was removed and the cell pellet resuspended in 1ml of Hanks solution with a repeat of the centrifuge step followed by removal of the Hanks solution and the addition of 1ml of Trysol to the pellet (See Appendix 2.7.0). The tubes were stored in -80ºC with accurate labelling of the conditions on the tube and cell type. The samples were stored for future PCR, however, the timescale of this project did not allow time for analysis of these samples to determine whether there was an upregulation of cytokine production following the stimuli. The cells were reviewed daily to look for any morphological changes.

2.5.2 Human Kidney-2 cells (HK-2 cells)

HK-2 cells are an immortalised proximal tubule epithelial cell line from normal adult human kidney. These cells are dependent on epidermal growth factors that maintain a well differentiated phenotype of proximal tubule epithelial cells. Transduction of recombinant retrovirus with human papilloma virus (HPV16) E6/E7 genes was introduced into a primary normal human proximal tubule epithelial culture. This was first done in 1994 (Ryan MJ, KI 45(1):48-57 HK-2: an immortalised proximal tubule epithelial cell line from normal adult human kidney).

HK-2 cells were maintained in keratinocyte media with bovine pituitary extract and epidermal growth factors (Life Technologies, GIBCO), supplemented with 1000U/ml of Penicillin, 100µg/ml Streptomycin and 5% FCS (See Appendix 2.5.0). $5x10^6$ cells were seeded to 70% confluence in 6-well plates for 24 hours. The cells were left 24 hours prior to changing the media to RPMI 1640 media supplemented with 1000U/ml of Penicillin, 100µg/ml Streptomycin and 2mM glutamine without serum but with the diabetic conditions. The supernatants were collected 24 hours later and the above SFM with the diabetic conditions and cytokine stimulation were placed in the relevant wells for 48 hours. The supernatant was collected and cells collected for PCR.

2.5.3 Human Podocytes

The podocytes used for these experiments were a kind gift from Professor M Saleem, Bristol, UK. A conditionally immortalized human podocyte cell line with; nephrin and podocin expression. This cell line was developed by transfection with temperature sensitive SV40-T gene and a telomerase gene. Cells proliferate at 33ºC and stop proliferating when transferred to 37ºC as they enter cell growth arrest and begin to differentiate into vivo podocytes with established podocyte markers; nephrin, podocin. These markers were stained by a colleague in the laboratory to ensure the cells differentiated into podocytes. Podocytes were seeded at $5x10^6$ per well and incubated at 37ºC. After 2 weeks at 37ºC in maintenance media; RPMI 1640 supplemented with 1000U/ml of Penicillin, 100µg/ml Streptomycin and 2mM glutamine with 25% FCS (See Appendix 2.6.0). The cells were then exposed to serum free conditions initially and this resulted in cell death. On repeating the experiment I used 5% FCS rather than serum free, together with the conditions and cytokine stimulations done in this media thereby allowing interpretation of increase or decrease of cytokine production under the different conditions. All cells that were not required for the experiments were frozen down and stored in liquid nitrogen (See Appendix 2.7.3).

Following interim analysis of the pilot cell culture experiments, HK-2 cells stimulated with CCL18 and MCP-1 in normal glucose, mannitol, high glucose and glycated albumin A2 were chosen to be repeated in 6 wells (each condition n=6). This experiment was repeated 3 times on different days to ensure the initial findings were reproducible. Podocytes stimulated with MCP-1 and MIF in normal glucose, mannitol, high glucose and glycated albumin A2 and were also repeated in 6 wells with 3 different experiments to ensure the initial findings were true. This approach allowed statistical analysis to be performed on these results while mechanistically attempting to try and explain the clinical findings. The pilot experiments were done in duplicates in order to establish any novel finding and determine the appropriate dose to induce these findings i.e. 10ng/ml and 20ng/ml cytokine stimulation dose. Time limitations unfortunately did not allow ELISAs analysis of the 10ng/ml stimulation of the repeated cell culture experiments.

2.6 Western blot

Western blotting allows identification and quantification of protein from cell lysates. The amount of protein in the cell lysates of the cell culture experiments were quantified using the BCA protein assay (Pierce Biotechnology, ThermoScientific, USA). This was to ensure the same amount of protein was loaded in the gels for Western blotting thereby allowing correct interpretation of band width. (See Appendix 2.8.0 for protocol). The cell lysates from HK-2 cell and podocyte experiments were analysed with Western blot. The cell lysates stored in the -20ºC freezer were heated in a block and the Western blot performed using a semi-dry transfer. The Western blots were performed on the samples with the following antibodies for cell signalling and apoptosis. (See Table 2.5 for Ab details). The Abs were used at 1:1000 dilution and bought from Cell Signalling Technology, UK. Anti-rabbit IgG HRP Ab was used. Phospho-p44/42 MAPK (also known as ERK1/2) and Phospho-p38 were used to determine whether different conditions in HK-2 cells or Podocytes activated these cell signalling pathways. The addition of cytokine stimulation in the same condition, was compared to see whether there was further activation of these pathways or if unaffected this may suggest different pathways are employed in order to exert the cytokine effect. MIF has previously been described to use ERK1/2 pathways with MCP-1 using MAPK-p38. It is unknown how CCL18 exerts its affects in these cell types. Actin (Santa Cruz Biotechnology Inc, USA) was initially used to ensure there was approximately the same amount of protein within each sample thereby ensuring the BCA was accurate at a dilution of 1:200. Caspase 3, 7 and 9 were used to determine whether there was an increase in apoptotic pathway proteins in the different conditions under different cytokine stimulation. These findings can correlate with that of the MTT assay to see whether a decreased cell survival was a reflection of increased caspase and apoptosis.

Table 2.5 Details of Ab for Western blot

Antibody	Molecular Weight	Isotype
Phospho-p44/42 MAPK	42,44 kDa	Rabbit IgG
Phospho-p38 MAPK	40 kDa	Rabbit IgG
Actin	40 kDa	Mouse IgG
Caspase 3	17, 19, 35 kDa	Rabbit IgG
Caspase 7	20, 35 kDa	Rabbit IgG
Caspase 9	17, 35, 37, 47 kDa	Rabbit IgG

Samples were separated by SDS-PAGE and semi-dry transfer to polyvinylidene difluoride (PVDF) membranes. Blocked with PBS containing 0.1% Tween-20, 7.5% dry skimmed milk for 1hr at room temperature and incubated in the same buffer with different primary Ab overnight at 4ºC. After washing the membranes with peroxidase-conjugated secondary Ab and developed them using an ECL chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were quantified in each sample using the BCA method (See Appendix 2.7.2). 50µg of protein per sample was loaded in each lane. The membranes were re-stained for Actin to ensure similar amounts of protein from each sample were loaded. See Appendix 2.8.1 for re-blot protocol.

The autoradiographs were scanned using ImageQuant300 Capture with ImageQuantTL to provide band densities and allow comparison of protein detection between different treatments. Ideally the total and cleaved proteins would be re-blotted to further quantify this however time and funding were limiting factors to this step.

2.7 Statistical analysis

2.7.1 Statistical analysis for Clinical cytokine study

All statistical analysis other than the development of the multivariant regression mixed model was done by myself. Graph Pad Prism software was used for my analysis (version 4 Graph Pad Software Inc, San Diego, CA). Column statistics were initially performed to determine the distribution of the data with normality testing with Shapiro-Wilk and Kolmogorov-Smirnov test. Log transformation of the data did not change its distribution and hence data was analysed in its collected form with non-

parametric testing. Non-parametric data were analysed with Mann-Whitney 2-tailed testing for the comparison of independent samples. Comparison of more than three groups, were tested with Kruskal-Wallis with Dunn's post analysis for multiple comparison test. Wilcoxon signed rank test was used for comparison of related cytokine samples at >3 year time point.

Univariant analysis of the baseline urinary and plasma MIF has been included to determine its correlation with baseline, >18 months and >3 years GFR, ACR and UPCR. Spearman's correlation was used to determine whether there was a difference between serum and plasma cytokine levels in all the cytokines analysed. Spearman's correlation was also used to determine whether there was any correlation between urinary and plasma levels of different cytokines i.e. urinary MIF with urinary MCP-1.

2.7.2 Statistical analysis for Prospective Clinical cytokine study

An Imperial College Statistician Dr Fabiana Gordon helped develop a model for the analysis of the clinical cohort in order to determine whether the baseline samples were able to predict progression over time. The model accounts for variables with the use of residuals that increase with each variable included. The model modulates the findings according to the data entered. Without developing a model to account for the variables that occur with time incorrect conclusions may be reached if this data were analysed with multiple univariant analysis alone. All baseline urinary and plasma cytokines were included and looked at the following factors to see whether independently they could predict progression of DN. The change of; GFR, ACR and UPCR were used at 3 years to see whether this predicted outcome. The outcome of RRT and death were also taken as end-points of the study. Loss of follow up, reaching end-point or decline of re-entry into the study contributed to missing data that significantly reduced the power of the model and subsequently may not determine significances due to the multiple variables.

2.7.3 Statistical analysis for Pilot cell culture experiments

The pilot cell culture experiments were done in duplicates (n=2) hence statistical analysis was not performed on these very small numbers. Analysis is presented in graphs as the mean of each condition to appreciate the dose response curves in the different basal conditions following stimuli with different doses of the same cytokine. This has been done for each of the cell lines used. This informed the decision to repeat the experiments of interest for statistical evaluation.

2.7.4 Statistical analysis for HK2 and Podocyte cell culture experiments

Subsequent cell culture experiments were repeated 3 times with each experiment having the same condition repeated in 6 wells (n=6) and hence analysed with non-parametric statistical analysis. Kruskal-Wallis and post analysis Dunn's multiple comparison test allowed comparison of selected control data, i.e. Normal glucose compared with its control glycated albumin and Mannitol with High glucose. See table 2.6 for cell culture controls. In addition, the effect of cytokine stimulation (20ng/ml) within a certain condition was detected by the difference in levels from the condition without stimulation (0ng/ml). This change is analysed with Kruskal-Wallis and Dunns post analysis multiple comparisons with e.g. normal glucose 20ng/ml with normal glucose 0ng/ml. The effect of cytokine and fibronectin levels can then be determined. Analysis was performed on 0ng/ml and 20ng/ml in view of time limitations preventing ELISA analysis of 10ng/ml.

Table 2.6 Cell culture controls

Cell culture diabetic condition	**Control used for statistical analysis**
Glycated Albumin	Normal glucose
High glucose	Mannitol

The experiments were repeated three times, thus each condition had a total of 18 wells (n=18) over all three experiments. The results of these wells were not pooled together as this would introduce bias to the results, therefore, each experiment with 6 wells per condition was analysed (n=6) and the results reviewed to establish whether the findings were consistent. For ease of understanding the results of a single experiment have been shown throughout Chapter 4, for each cell type, conditions and cytokine stimulations.

CHAPTER 3.0 – RESULTS – PROSPECTIVE CLINICAL COHORT

This chapter will determine what the demographics are for the clinical cohort at baseline, 18 months and >3 year time points. It will explore the relationship of baseline urinary and plasma MIF with demographics and outcomes; GFR, ACR and UPCR in DM and Non-DM groups. The baseline relationship between MCP-1 and CCL18 with outcomes; GFR, ACR and UPCR will be analysed to allow direct comparison of these between the DM and Non-DM groups. The latter data has previously been analysed in a different manner by Dr Qureshi MD 2012 who began the database for this cohort and gave me permission to use data from the original database. Clinical correlations between MCP-1 and CCL18 have been previously analysed by Dr Qureshi. I collected further data points for all patients in the cohort over time and amended the database to allow for the new data. I performed MIF ELISAs on all the available baseline urine and plasma samples from the original cohort.

The relationship between baseline urinary and plasma MIF, MCP-1 and CCL18 will be analysed with GFR, ACR and UPCR at the different time points. The relationship between GFR and ACR or UPCR in the different groups will be analysed at baseline and at >3 years' time point to determine whether this changes over time. Comparison will be made between those in DM or Non-DM groups. The cytokines relationship at >3 years with GFR, ACR and UPCR will also be analysed to determine whether these change over time. Comparison of the urinary and plasma cytokine measurements at baseline to cytokines at >3 years urinary and plasma samples will also be made. This will determine the direction of change and whether this is different in DM or Non-DM patients.

I performed a univariant analysis to determine the effects of the cytokines in rate of change in GFR, ACR or UPCR in DM and Non-DM groups, respectively. This looks to see whether baseline cytokines are related to the outcome measures above, over time. There are limitations in using univariant analysis as this does not consider changes due to different variables such as age, gender, BMI. Mixed models that account for these variables also have limitations as they do not allow identification of those in a group that may have faster deteriorating GFRs. The results of these analyses are presented herein with their limitations. The mixed model of statistics was performed by Dr Gordon (Imperial College Statistician) on the data points and samples I collected and analysed during this study. This will help determine whether a single test at baseline will be able to predict a worse outcome in the DM group.

Finally this chapter will look at the differences between plasma and serum cytokines in all the samples collected at >3 years. This will determine whether samples can be taken in the same tubes as other blood tests without affecting the levels of different cytokines present. In addition, the profile of the cytokines at baseline and at >3 years will be compared and whether being on RRT affects MIF, MCP-1 or CCL18 levels regardless of DM or Non-DM groups. The latter is a small analysis and requires interpretation with caution, as larger sample size would be required and the effects of RRT on cytokines are multifactorial.

3.0.1 Demographics for Baseline

The baseline cohort had 231 people; 115 DM and 116 Non-DM patients. Those with urinary tract infections (UTIs) were excluded from the initial cohort. 102 urine and 94 plasma DM samples remained from the original cohort for the analysis of MIF cytokine with 106 urine and 93 plasma samples from Non-DM patients. MIF ELISAs were performed on all samples available. A flow diagram depicting those analysed from the cohort is seen in Fig 3.0.1.0.

The age distribution of this cohort shows both DM and Non-DM populations to be normally distributed with a mode 61-80 years in DM, mean age 62.7 years (median 65 years) and 41-60 years in Non-DM, mean age 55 years (median 53 years) Fig 3.0.1.2. There is a significant difference in age between DM and Non-DM ($p<0.001$, Mann-Whitney U 4600).

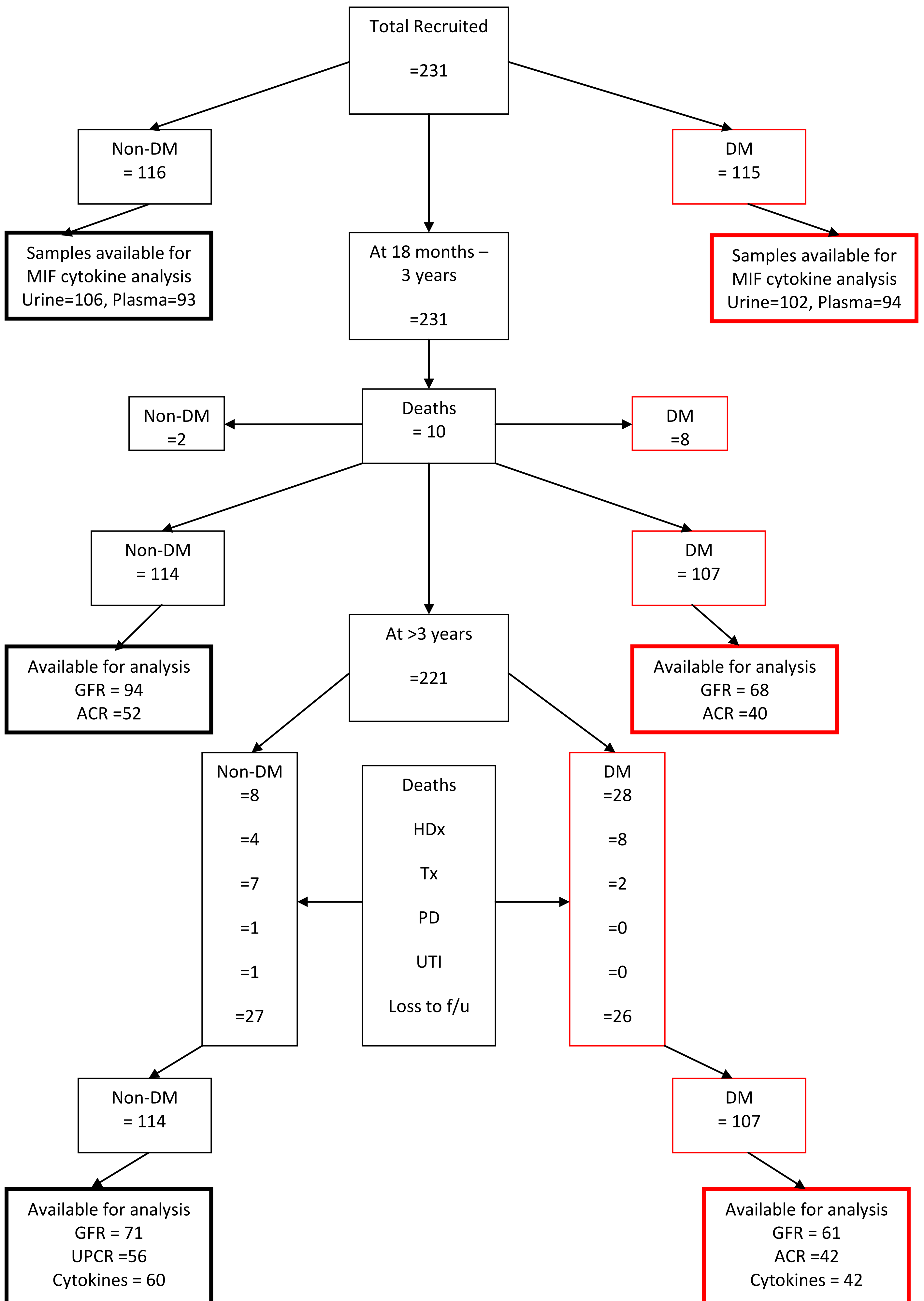


Fig 3.0.1.0 Flow diagram of Prospective Cohort Study

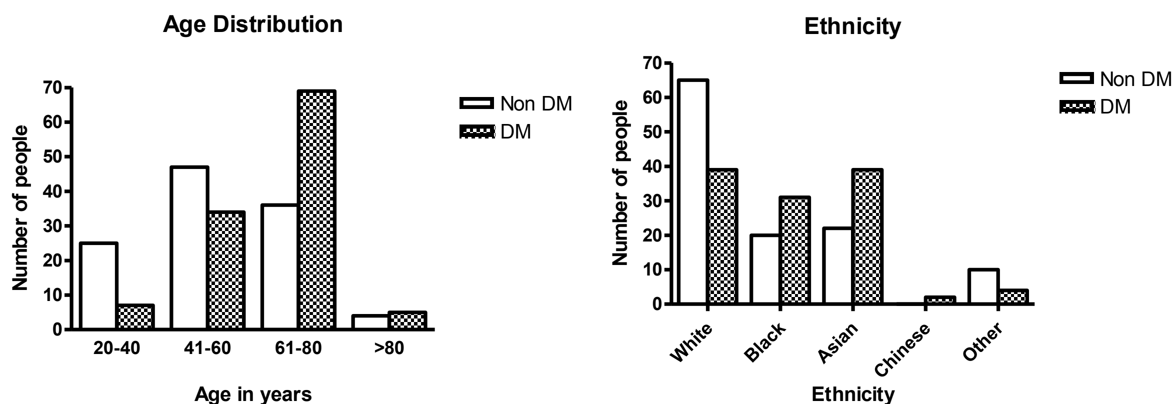


Figure 3.0.1.1 Age and Ethnicity distribution amongst the clinical cohort

There were 135 Male and 96 Females in the study; in DM group (79M, 36F), Non-DM (56M, 60F). There were significantly more females than males in Non-DM than in DM group ($p<0.01$, Mann-Whitney U 5310). The distribution of ethnicity is seen in Fig 3.0.1.1.

There were approximately equal number of white, black and Asian people in the DM group. In contrast there were more white people with Non-DM proteinuric renal disease in this cohort. Table 3.0 shows the number of Non-DM patients with different primary proteinuric renal disease and those who were on immunosuppression. Immunosuppression is an important factor to bear in mind on reviewing the cytokine production in the Non-DM group as the following results are presented. Immunosuppression status was documented for the entire cohort and reflects treatment of renal and non renal pathology.

Table 3.0 Primary Non-DM proteinuric renal diagnosis

Primary renal diagnosis	***Number of non-diabetics with this condition***	***Number on Immunosuppression***
Vasculitis	24	20
IgA Nephropathy	10	5
Renal artery stenosis	5	2
Lupus	19	12
Minimal change disease	1	
Membranous glomerulonephritis	5	3
Reflux nephropathy	2	
Renal Tumours	1	
Polycystic kidney disease	6	3
Granulomatous interstitial nephritis	2	2
Focal segmental glomerulosclerosis	4	2

Hypertensive nephropathy	13	5
Acute kidney injury with heavy proteinuria	2	1
Chronic GN	1	1
Obstruction	4	3
Renal stones	2	2
CKD	2	2
MGUS	1	
Single kidney	1	

The BMI, MAP and HbA1c were all significantly higher in DM than in Non-DM groups ($p<0.05$, Mann-Whitney U 1010, $p<0.0001$, Mann-Whitney U 3330, $p<0.0001$ Mann-Whitney U 180). GFR and the number of patients on ACEi/A2RB were similar in DM and Non-DM group at baseline. Cholesterol levels were higher in the Non-DM and may be a reflection of the DM group being more intensively managed with statins ($p<0.0001$, Mann-Whitney U 3370). These results are summarised in Table 3.1. There were 36 smokers in the Non-DM group and 34 in DM group.

Table 3.1 Baseline comparators between DM and Non-DM.

Comparator for Non-DM vs DM	**Statistical test**	**p value**
BMI	1010 Mann-Whitney U (DM 30(21-55), Non-DM 27(18-45))	p<0.05*
MAP	3330 Mann-Whitney U (DM 87.5(18-220), Non-DM 65.1(24.7-175))	p<0.0001****
HbA1c	180 Mann-Whitney U (DM 7.9(4.8-12.2), Non-DM 5.6(4.6-6.0))	p<0.0001****
Baseline GFR	6250 Mann-Whitney U (DM 40.2(11.1-117), Non-DM 50(6.1-99.5))	ns
Baseline Cr	6060 Mann-Whitney U (DM 145(59-472), Non-DM 128(67-661))	ns
Baseline Cholesterol	3370 Mann-Whitney U (DM 4.4(2-10.6), Non-DM 4.9(2.7-9.3))	p<0.01**
GFR vs UPCR Non-DM	Spearman's r=-0.25	p<0.05*
GFR vs ACR DM	Spearman's r=-0.28	p<0.01**

ns= non-significant, * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

See section 3.0.4 for GFR and ACR/UPCR in DM and Non-DM respectively over the time points used in this study.

3.0.2 Demographics for <3 years analysis

From the original cohort there were 10 deaths in <3 years (8 DM and 2 Non-DM patients). A total of 221 people in the cohort were analysed: 107 DM (73M, 34F) and 114 Non-DM (56M, 58F) excluding the deaths from each group (129M, 92F total 221).

GFRs were collected and analysis performed on those from the initial cohort that had results available on the computer at >18 months to <3years. Those who had commenced RRT at these time points were excluded from the analysis. Those with data points during this time period included; GFR analysis on 68 DM (59%) and 94 Non-DM (81%); ACR analysis 40 (35%); and UPCR analysis 52 (45%), (see Table 3.2).

Table 3.2 >18 months comparators between DM and Non-DM.

Comparator for Non-DM and DM	Statistical test	p value
18 months GFR Non-DM vs DM	1630 Mann-Whitney U (DM 33.3(6.2-106), Non-DM 49.5(6.8-111))	$p<0.001$***
GFR vs UPCR Non-DM	Spearman's r=-0.163	ns
GFR vs ACR DM	Spearman's r=-0.383	ns

ns= non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.0.3 Demographics for >3 years analysis

The prospective cohort at >3 years had a total of 221 people (129M, 92F), 107 DM (73M, 34F) and 114 Non-DM (56M, 58F). This is summarised in the flow chart Fig 3.0.1.0. From those in the cohort, 36 of Non-DM at >3 years were immunosuppressed; that was less than the original cohort as some patients had their immunosuppression stopped over this period. Those on immunosuppression were included in the analysis of Non-DM group as the group was not sufficiently large enough to be powered for a subgroup analysis. Those who were on RRT were excluded from the analysis; however, the samples for cytokine analysis were collected. Two DM and 7 Non-DM patients had renal transplants (RT), 8 DM and 4 Non-DM were on haemodialysis (HD) and 1 Non-DM was on peritoneal dialysis (PD). 22 in total were excluded for RRT. One Non-DM patient had a UTI and

was not included in the analysis. There were 46 deaths in total by >3 years follow up in the cohort (36 DM, 10 Non-DM). More males died in the DM group compared with females (20M, 16F) with the reverse occurring in the Non-DM group (2M, 8F).

Cytokine analysis was further limited by numbers lost to follow up despite written invitations to patients inviting them to attend. There were fewer samples for cytokine analysis than those with predictive GFR, ACR and UPCR as this data was available on the computer system. Cytokines collected at >3 years were analysed in 42 DM and 60 Non-DM patients. 26 DM and 27 Non-DM patients were lost to follow up (in each group 24% were lost to follow up).

GFR analysis at >3 years from baseline was available in 61 DM (53%) and 71 Non-DM (61%) patients. ACR analysis on 42 DM (37%), and UPCR analysis on 56 Non-DM (48%). See Table 3.3 for analysis of clinical comparators between DM and Non-DM. Those who had results on the computer at >3 years prior to commencing RRT, death or lost to follow up were included. All those re-recruited for cytokine analysis had paired GFR and ACR/UPCR. The cytokine analysis for RRT has been excluded from the main analysis.

All DM patients on HD died by >3 years follow up while 50% of those Non-DM patients on HD survived. All those with RT were alive at this time point with 2 DM and 7 Non-DM. The Non-DM patients on PD had survived >3 years. Over the 3 year period 3 Non-DM developed DM, however the HbA1C continued to be significantly lower than those in DM group (p<0.0001).

Table 3.3 >3 years comparators between DM and Non-DM.

Comparator for Non-DM and DM 3 years	**Statistical test (Median range)**	**p value**
BMI	2260 Mann-Whitney U (DM 31(21.9-55), Non-DM 28.1(19.5-50.7))	p<0.05*
MAP	1310 Mann-Whitney U (DM 87.1(33.7-220), Non-DM 66.7(26.3-163))	p<0.0001****
HbA1c	321 Mann-Whitney U (DM 8(5.2-12.7), Non-DM 5.6(4.7-*9.2)*	p<0.0001****
Cholesterol	1870 Mann-Whitney U (DM 4(2.2-10.4), Non-DM 4.6(2.8-8.6))	p<0.0001****
3 years GFR	1920 Mann-Whitney U (DM 38.4(2.5-102), Non-DM 49(8.5-114))	p<0.05*

GFR vs UPCR Non-DM	Spearman's r=-0.432	p<0.001***
GFR vs ACR DM	Spearman's r=0.057	Ns

ns= non-significant, * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

All of the above factors were taken into account when developing the mixed model detailed in Section 3.1.3, to determine the predictive value of baseline cytokines with outcome; GFR, ACR, UPCR, RRT or death.

Summary

BMI, MAP, HbA1c and cholesterol were significantly different between DM and Non-DM groups with all levels being higher in the DM group at baseline and over time. GFR was significantly lower >18 months from baseline in the DM group, and that continued at >3 years' time point. A lower GFR correlated with a higher UPCR in Non-DM patients at baseline and this strengthened at>3 years. The higher the ACR the lower the GFR at baseline, however, this did not continue over time and is illustrated in section 3.0.4.

3.0.4 Clinical correlation GFR, ACR and UPCR

GFR significantly correlated with ACR in DM group at baseline (Spearman's r=-0.28 p<0.01) see Figure 3.0.4.0, however, this was not seen at >18 months or >3 years' time point (Spearman's r=-0.69 p=0.07, Spearman's r=0.06 p=0.76, respectively).

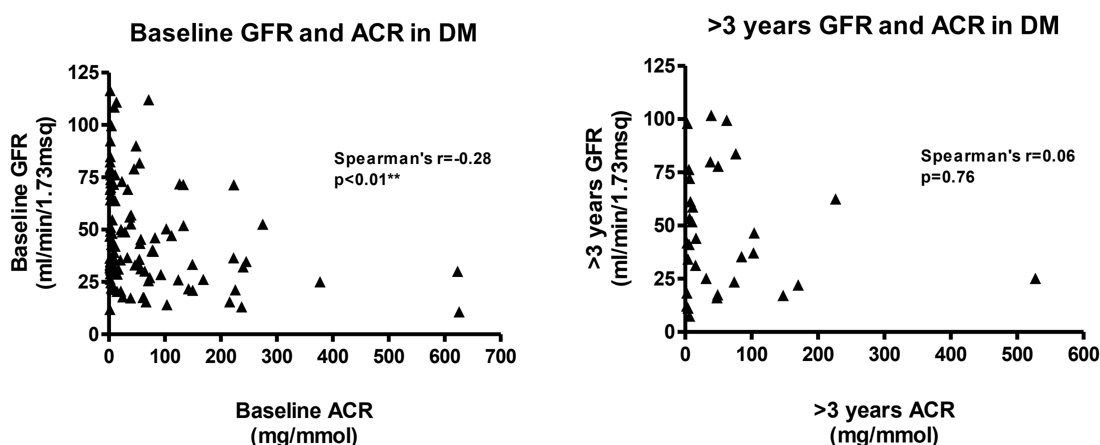


Figure 3.0.4.0 Correlation of GFR and ACR in DM

GFR did not correlate significantly with UPCR at >18 months in Non-DM group (Spearman's r=-0.163, p=0.33). The correlation was significant at baseline and at >3 years as seen in Figure 3.0.4.1.

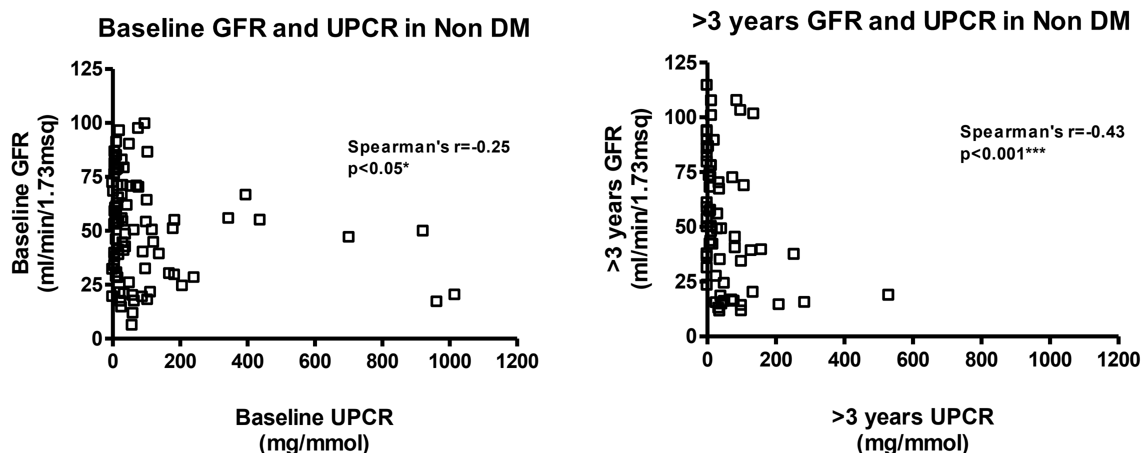


Figure 3.0.4.1 Correlation of GFR and UPCR in Non-DM

A rising UPCR is associated with a decline in GFR at baseline and >3 years. The relationship between GFR and UPCR is strengthened at >3 years. This is in accordance with other studies regarding declining renal function with large amounts of proteinuria. In DM patients the degree of albuminuria trends to a decline in GFR, however, this is not significant. This may reflect the sample size but also that a decline in GFR does not always occur with a simultaneous increase in albuminuria, thus reflecting the need for a more sensitive biomarker than ACR in DM group. The use of ACEi/ARB in DM may also contribute to the lack of correlation seen.

Summary

GFR correlated significantly with ACR at baseline, that was not sustained at >18 months or >3 years. In contrast UPCR maintained its relationship with GFR with a higher UPCR correlated to a low GFR. This however was not seen at >18 months but returned in >3 years.

3.1 MIF

Baseline urinary and plasma MIF were analysed using ELISA on initial baseline samples taken from the clinical cohort. Normal levels of urinary and plasma MIF were determined using healthy control samples that had been stored at the same time as the DM and Non-DM samples to measure MIF, see section 3.1.1. Factors looked at in DM and Non-DM group include; gender, ethnicity, age, GFR, ACR, UPCR, BP, BMI, Smoking, ACEi/ARB, HbA1C, Cholesterol, see section 3.1.2. Spearman's correlation was used as the non-parametric test of correlation in view of the small numbers with non-Gaussian distribution. Baseline urinary MIF was used and converted to a ratio with Cr to take into account the individuals Cr and allow comparisons to be made between individuals.

3.1.1 Baseline Healthy volunteers

In order to be consistent urinary and plasma MIF levels were measured on the healthy volunteers taken from the original baseline cohort collection. This accounts for any changes attributed to storage of the plasma and urine samples in -80ºC over time.

3.1.1.1 Healthy Urinary MIF/Cr ratio

The normal urinary MIF/Cr ratio range in healthy volunteers is between 2.31-182.32ng/mmol with a median of 55.48ng/mmol. Median for Non-DM group was 274.8ng/mmol (0.33-11200) and DM group 84.35ng/mmol (0.08-5100). Healthy urinary MIF/Cr ratio levels did not correlate with GFR (p=0.34, Spearman's r=-0.275) or ACR (p=0.24, Spearman's r=0.338).

There is a significant difference between the levels of urinary MIF/Cr ratio in healthy volunteers compared to Non-DM and DM groups (Kruskal-Wallis p<0.0001, see above for medians and range). Dunn's comparison showed the healthy and DM urinary MIF/Cr ratio levels to be significantly different to Non-DM group (p<0.001 for both), see Fig 3.1.1.1.

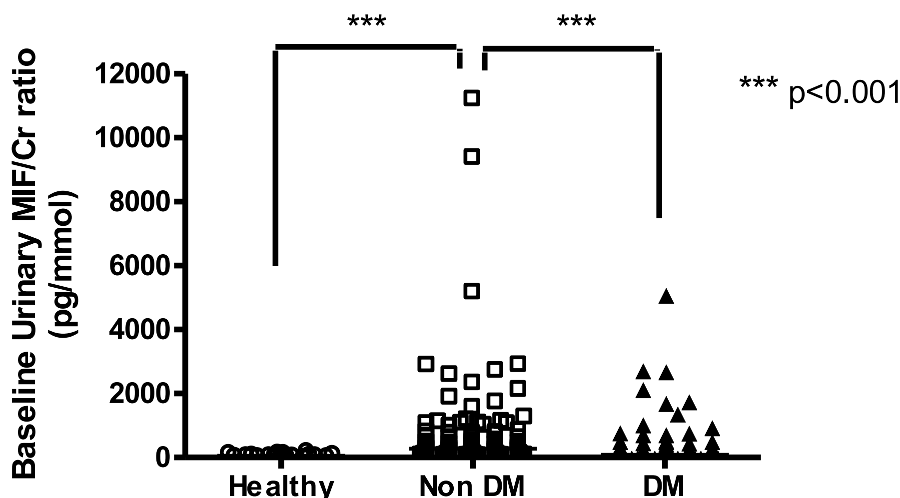


Figure 3.1.1.1 Baseline urinary MIF/Cr ratio of healthy, Non-DM and DM groups.

3.1.1.2 Healthy Plasma MIF

The normal plasma MIF range in healthy volunteers is 10-4176pg/ml with a median of 1710pg/ml. Non-DM group median was 3900pg/ml (13.9-10300), with DM group median 1750pg/ml (7.83-52900). Plasma MIF did not correlate with GFR or ACR (Spearman's r=-0.50, p=1.0, Spearman's

r=0.5, p=1.0, respectively). There was no correlation between the healthy urinary MIF/Cr ratio levels and that of plasma MIF levels (p=0.90, Spearman's r=0.04).

Below is a comparison of the baseline plasma MIF levels in healthy, Non-DM and DM patients. A significant difference is seen between the plasma MIF in Non-DM and DM groups, Kruskal-Wallis test shows the medians of all the populations are significantly different (p<0.01, see above for medians and range). Dunn's comparison test shows this difference continues to be significant between Non-DM and DM groups (p<0.05), see Fig 3.1.1.2.

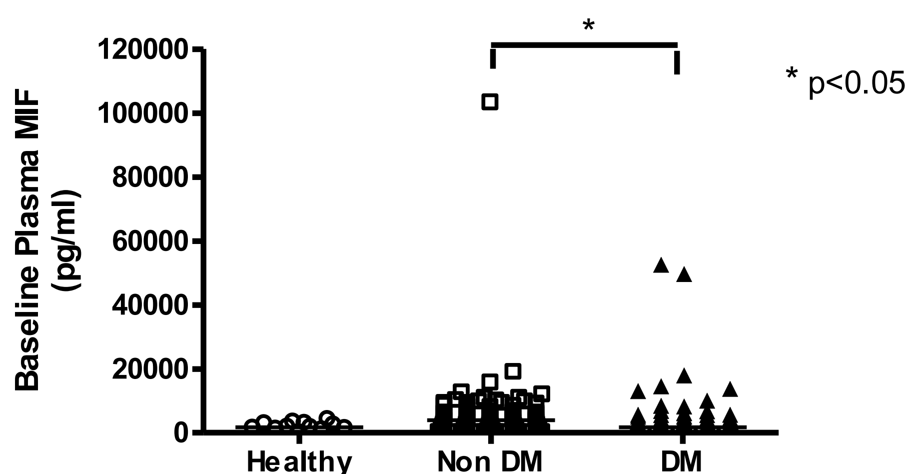


Figure 3.1.1.2 Baseline plasma MIF of healthy, Non-DM and DM groups.

Summary

Baseline urinary MIF/Cr ratio in healthy volunteers, are much lower than those found in proteinuric renal disease. Baseline urinary MIF/Cr ratio levels in Non-DM were significantly higher than those in healthy and DM groups. DM plasma MIF levels were significantly lower than plasma levels in Non-DM (Kruskal-Wallis p<0.05).

3.1.2 Baseline Urinary MIF/Cr ratio

In DM patients, there was no correlation between baseline urinary MIF/Cr ratio and baseline GFR (p=0.55, Spearman's r=-0.06) in DM, see Fig 3.1.2.0. Spearman's correlation showed no relationship between baseline urinary MIF/Cr ratio and age, gender, HbA1C, systolic BP, BMI, smoking, or MAP in DM and Non-DM groups. Cholesterol did not significantly correlate with baseline urinary MIF/Cr ratio in DM but was significant in Non-DM patients. There was no significant correlation with UPCR in Non-DM whereas urinary MIF/Cr ratio correlated significantly

with ACR in DM. Urinary MIF/Cr ratio correlated with baseline GFR in Non-DM, see table 3.4 for details.

Table 3.4 Comparison of DM and Non-DM baseline urinary MIF/Cr ratio

Clinical Risk Factor	**Baseline Urinary MIF in DM (n=102)** **Median (range)**	**P value** **Statistical test**	**Baseline Urinary MIF in Non-DM (n=106) Median (range)**	**P value** **Statistical test**
Age	65 (28-83)	p=0.12 ns Spearman's r=-0.16	54 (21-93)	p=0.62 ns Spearman's r=0.06
Gender	M 78.5(0.1-5100) F 86.1(0.1-2140)	Mann-Whitney U 1110 p=0.94 ns	M 312(11.2-9370) F 214(0.3-11200)	Mann-Whitney U 1270 p=0.51 ns
HbA1C	8.0 (5.2-12.7)	p=0.91 ns Spearman's r=-0.01	5.6 (4.7-9.2)	p=0.72 ns Spearman's r=-0.06
Systolic BP	140 (104-200)	p=0.34 ns Spearman's r=0.10	131 (100-220)	p=0.38 ns Spearman's r=-0.09
Cholesterol	4.0 (2.2-10.4)	p=0.68 ns Spearman's r=-0.05	4.6 (2.8-8.6)	p=0.04* Spearman's r=-0.24
Smoking	Non 86.2(0.1-2710) Smoker 67.9(0.1-5100)	Mann-Whitney U 1180 p=0.43 ns	Non 85.1(0.3 -11200) Smoker 122(12-2900)	Mann-Whitney U 321 p=0.27 ns
BMI	30 (21-55)	p=0.07 Spearman's r=-0.18	27 (18-45)	p=0.76 ns Spearman's r=-0.07
MAP	87.5 (18-220)	p=0.64 ns Spearman's r=0.05	65.1(24.7-175)	p=0.81 ns Spearman's r=-0.02
ACR	20 (0.1-624)	p=0.005** Spearman's r=0.28	n/a	n/a
UPCR	n/a	n/a	31 (0.1-1016)	p=0.48 ns Spearman's r=0.08
Baseline GFR	40.4 (11.1-117)	p=0.55 ns Spearman's r=-0.06	50 (6.1-99.5)	p=0.03* Spearman's r=-0.22

ns= non-significant, * p<0.05, ** p<0.01, *** p<0.001

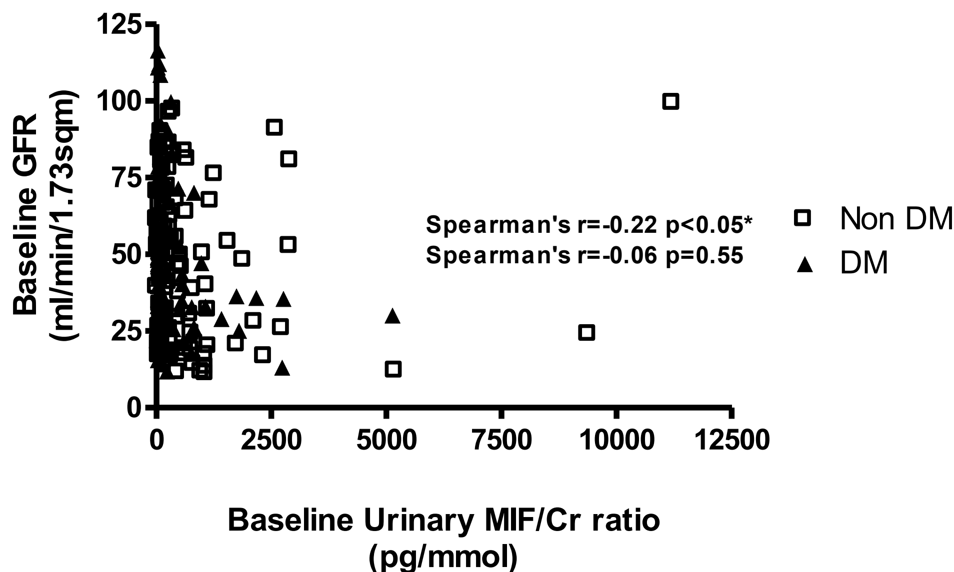
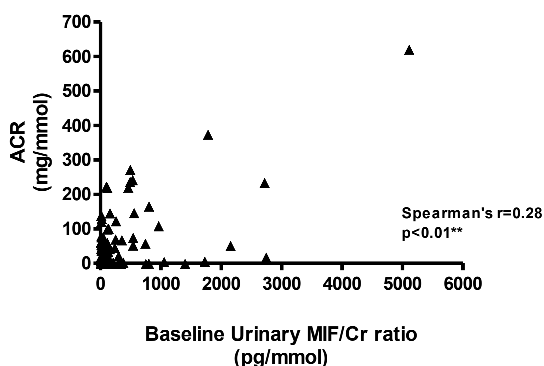


Figure 3.1.2.0 Baseline urinary MIF/Cr ratio and GFR in Non-DM and DM.

Baseline urinary MIF/Cr ratio levels in Non-DM patients significantly correlated with baseline GFR ($p=0.03$, Spearman's $r=-0.22$) see Fig 3.1.2.0. The decline in GFR in Non-DM group results in an increasing baseline UMIF/Cr ratio.



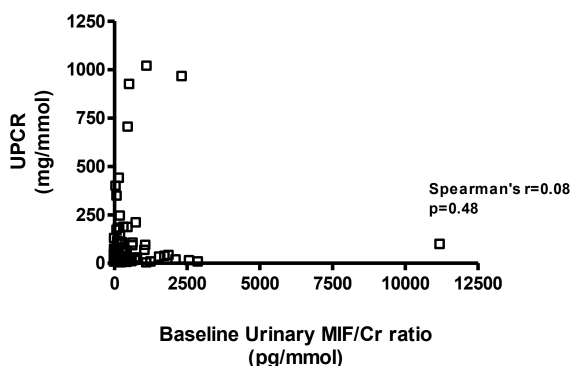


Figure 3.1.2.1 Correlation of baseline urinary MIF/Cr ratio and ACR/UPCR in DM and Non-DM, respectively.

In DM group, Spearman's correlation was significant between baseline urinary MIF/Cr ratio and ACR ($p<0.01$, $r=0.28$) see Fig 3.1.2.1. An increasing urinary MIF/Cr ratio correlated with a raised

ACR. There was no correlation between baseline urinary MIF/Cr ratio and UPCR in Non-DM patients (p=0.48, Spearman's r=0.08).

White and Asian groups had higher levels of baseline urinary MIF/Cr ratio regardless of whether DM or Non-DM, however, this was not significant and may reflect the small sample size. There was no significant difference in baseline urinary MIF/Cr ratio levels between gender or smokers and non-smokers in DM or Non-DM groups.

Summary

A raised baseline urinary MIF/Cr ratio in DM patients significantly correlates with an increase in ACR. In Non-DM patients a raised baseline urinary MIF/Cr ratio was seen with a decreasing GFR and lower cholesterol levels.

3.1.3 Baseline Plasma MIF

There was no correlation between baseline plasma MIF and age, gender, HbA1C, systolic BP, cholesterol, MAP or BMI for either group. Non-DM, non-smokers had significantly higher plasma MIF levels compared with smokers within the same group (p<0.0001 Mann-Whitney U 1110, see Table 3.5 for median and range).

Table 3.5 Comparison of DM and Non-DM baseline plasma MIF

Clinical Risk Factor	**Baseline Plasma MIF in DM (n=94)** **Median (range)**	**P value** **Statistical test**	**Baseline Plasma MIF in Non-DM (n=93)** **Median (range)**	**P value** **Statistical test**
Age	65 (28-83)	p=0.38 ns Spearman's r=0.09	54 (21-93)	p=0.76 ns Spearman's r=-0.04
Gender	M 1510(7.8-52900) F 1980(9.0-50000)	Mann-Whitney U 865 p=0.44 ns	M 3540(13.9-103000) F 4260(14.9-15500)	Mann-Whitney U 957 p=0.47 ns
HbA1C	8.0 (5.2-12.7)	p=0.71 ns r=-0.04	5.6 (4.7-9.2)	p=0.63 ns Spearman's r=0.08
Systolic BP	140 (104-200)	p=0.47 ns Spearman's r=-0.07	131 (100-220)	p=0.26 ns Spearman's r=0.13
Cholesterol	4.0 (2.2-10.4)	p=0.82 ns Spearman's r=0.03	4.6 (2.8-8.6)	p=0.34 ns Spearman's r=0.12
Smoking	Non 1810(7.9-52900) Smoker	Mann-Whitney U 1080 p=0.89 ns	Non 4180 (13.9-18900) Smoker	Mann-Whitney U 1110 p<0.0001****

	1660(7.8-15000)		350(12.1-9370)	
BMI	30 (21-55)	p=0.12 ns Spearman's r=0.17	27 (18-45)	p=0.17 ns Spearman's r=0.31
MAP	87.5 (18-220)	p=0.14 ns Spearman's r=0.15	65.1(24.7-175)	p=0.68 ns Spearman's r=0.05
ACR	19.4 (0.1-621)	p=0.66 ns Spearman's r=0.05	n/a	n/a
UPCR	n/a	n/a	31 (0.1-1016)	p=0.06 ns Spearman's r=0.23
Baseline GFR	40.4 (11.1-117)	p<0.001*** Spearman's r=-0.35	50 (6.1-99.5)	p<0.05* Spearman's r=-0.21

ns= non-significant, * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

No difference was seen between plasma MIF levels and smoking status in DM group. Spearman's correlation was significant in DM patients with high plasma MIF correlating with a low baseline GFR (p<0.001, r=-0.345) see Fig 3.1.3.0. White and Asian groups had higher levels of plasma MIF independent of DM or Non-DM grouping, however, there was no significant difference that may reflect the small sample size.

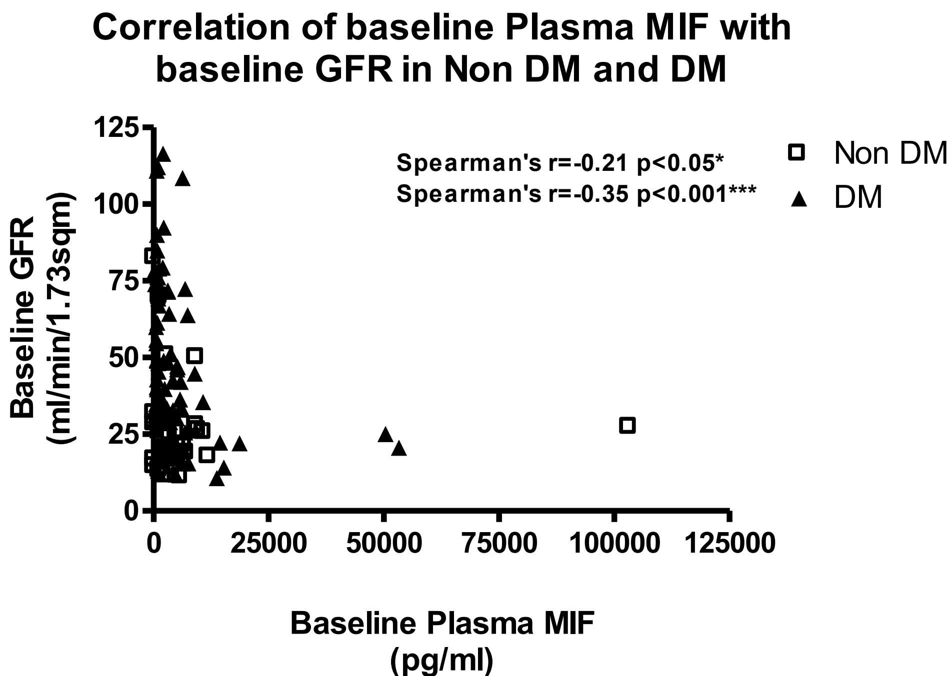


Figure 3.1.3.0 Baseline plasma MIF and GFR in DM and Non-DM

Baseline GFR significantly correlates with baseline plasma MIF in DM (Spearman's p<0.001, r=-0.35) and Non-DM patients (Spearman's correlation p<0.05, r=-0.21).

Baseline Plasma MIF significantly increases with a decreasing GFR in Non-DM patients. There is no correlation between baseline plasma MIF; and UPCR in Non-DM (Spearman's r=0.23, p=0.06), and ACR in DM patients (Spearman's r=0.05, p=0.66).

Summary

High baseline plasma MIF in DM significantly correlates with a decrease in GFR that is also seen in Non-DM, however, the latter is not as significantly correlated. There are no correlations in either group for baseline plasma MIF and ACR or UPCR. High plasma MIF levels were seen in Non-DM non-smokers.

<u>Summary</u>

White and Asian groups appeared to trend towards high levels of baseline urinary and plasma MIF however this did not reach significance. There was no significant difference between cytokine production and age or gender in this cohort. Higher baseline urinary MIF/Cr ratio levels trended in those with a high BMI in DM however this did not reach significance. Higher baseline urinary levels of MIF/Cr ratio correlated with a lower GFR and lower cholesterol levels in Non-DM patients. Baseline urinary MIF/Cr ratio levels were high in DM patients with high baseline ACR.

Baseline plasma MIF levels significantly correlate with baseline GFR in DM and Non-DM with a low GFR associated with high plasma MIF levels. The correlation was less significant in the Non-DM group. There was no correlation between plasma MIF levels and ACR or UPCR. Non-smokers in Non-DM had significantly higher plasma MIF levels compared to smokers in the same group. This difference was not seen in the DM group.

3.2 MCP-1

3.2.1 Baseline Urinary MCP-1

Baseline urinary MCP-1/Cr ratio had a significant inverse correlation with baseline GFR in DM (Spearman's p=0.006, r=-0.31) see Figure 3.2.1.0. An increasing urinary MCP-1/Cr ratio occurs with a decreasing GFR in DM. This relationship was previously presented by my predecessor Dr Qureshi, whereby the subgroup of macroalbuminurics correlated strongly with baseline urinary MCP-1/Cr ratio. The above results represent all DM and I have not subdivided these into different levels of albuminuria in view of the limitations of statistically analysing small sized samples that occur with subdividing levels of albuminuria. I present this data combined with Non-DM to allow

direct comparison between the groups, see Fig 3.2.1.0. The original cohort analysed suggested the normal range for urinary MCP-1 in healthy volunteers is 0-11.5ng/mmol with a median of 1.6ng/mmol (Qureshi MD 2012).

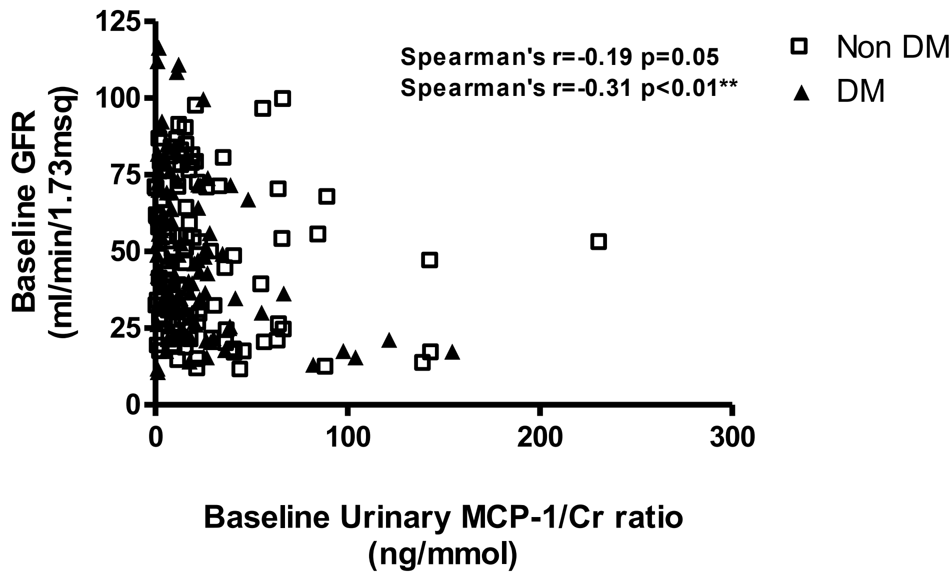


Figure 3.2.1.0 Baseline urinary MCP-1/Cr ratio and GFR in DM and Non-DM

There is a significant correlation between baseline urinary MCP-1/Cr ratio and baseline ACR in DM (Spearman's correlation p=0.001, r=0.34) see Fig 3.2.1.1. A high level of albuminuria is seen in DM with high levels of urinary MCP-1.

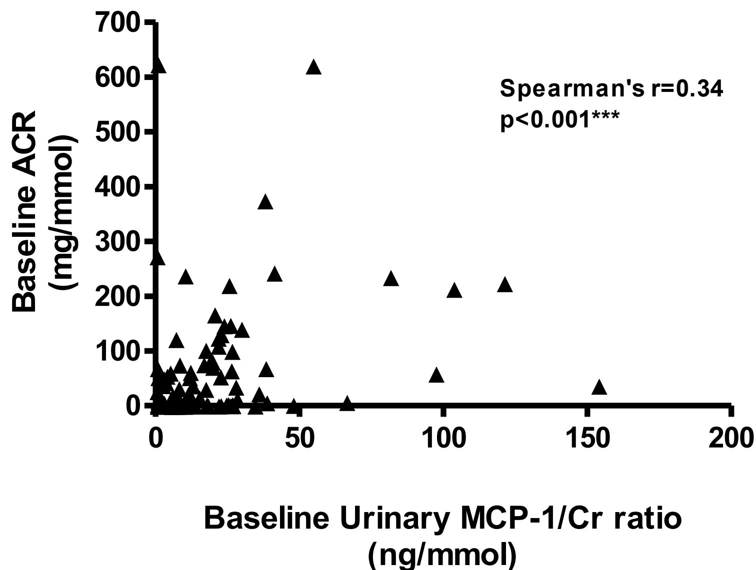


Figure 3.2.1.1 Baseline urinary MCP-1/Cr ratio and ACR in DM

Baseline urinary MCP-1/Cr ratio does not correlate with GFR (p=0.05, r=-0.19) or baseline UPCR in Non-DM (Spearman's p=0.053, r=0.218).

3.2.2 Baseline Plasma MCP-1

There is no significant correlation between baseline plasma MCP-1 and baseline GFR in DM and Non-DM (Spearman's correlation p=0.61, r=-0.06, p=0.62, r=-0.05, respectively). There is no correlation between baseline plasma MCP-1 and UPCR (p=0.48, r=-0.08) in Non-DM. The original cohort analysed suggested the normal range for plasma MCP-1 in healthy volunteers is 0.06-0.66ng/ml with a median of 0.18ng/ml (Qureshi MD 2012).

<u>Summary</u>

A high baseline urinary MCP-1/Cr ratio correlates with a low GFR and a high ACR in DM patients. There is no correlation between urinary MCP-1/Cr ratio levels and GFR or UPCR in Non-DM group. There are no correlations between plasma MCP-1 and GFR, ACR or UPCR in either group.

3.3 CCL18

3.3.1 Baseline Urinary CCL18

The following findings for baseline urinary CCL18/Cr ratio have previously been reported by my predecessor Dr Qureshi and illustrated according to the level of albuminuria. I have analysed this data and presented the effects of baseline urinary CCL18/Cr ratio correlating with baseline GFR in both DM and Non-DM to allow for direct comparison. Baseline urinary CCL18/Cr ratio is significantly correlated to baseline GFR with Spearman's correlation ($p<0.05$, $r=-0.36$) in DM and in Non-DM patients ($p<0.01$, $r=-0.32$) see Fig 3.3.1.0. This suggests that a low GFR is associated with higher urinary CCL18/Cr ratio in both groups.

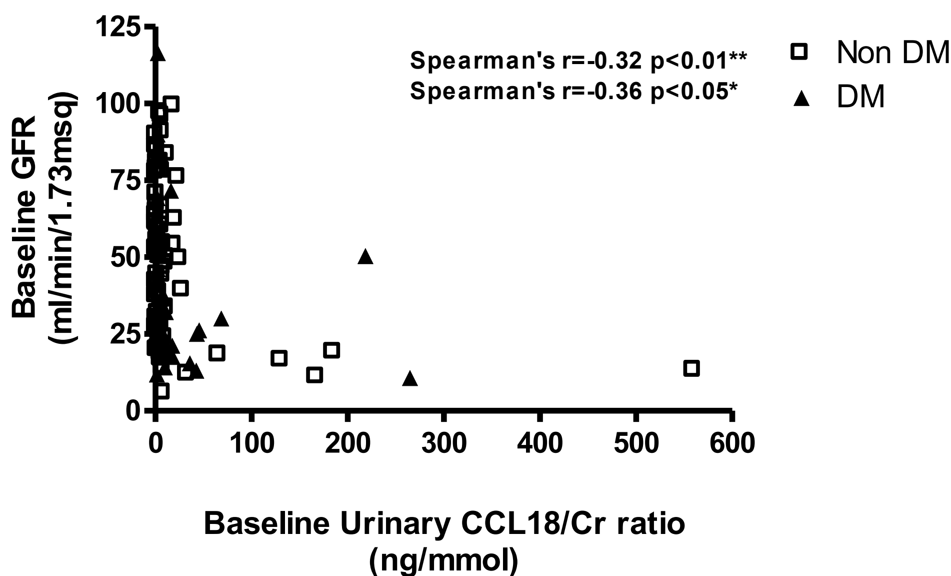


Figure 3.3.1.0 Baseline urinary CCL18/Cr ratio and GFR in DM and Non-DM

Baseline urinary CCL18/Cr ratio and baseline ACR significantly correlated in DM patients (Spearman's correlation $p<0.0001$, $r=0.74$) see Fig 3.3.1.1. This relationship was previously presented by my predecessor Dr Qureshi whereby macroalbuminurics correlated strongly with baseline urinary CCL18/Cr ratio. The above results are representative of all DM patients and have not been subdivided into different levels of albuminuria in view of the limitations of small sized samples. Baseline urinary CCL18/Cr ratio does not correlate with UPCR in Non-DM group

(Spearman's p=0.99, r=-0.001). The original cohort analysed suggested the normal range for urinary CCL18 in healthy volunteers is 0-4.05ng/mmol with a median of 0.67ng/mmol (Qureshi MD 2012).

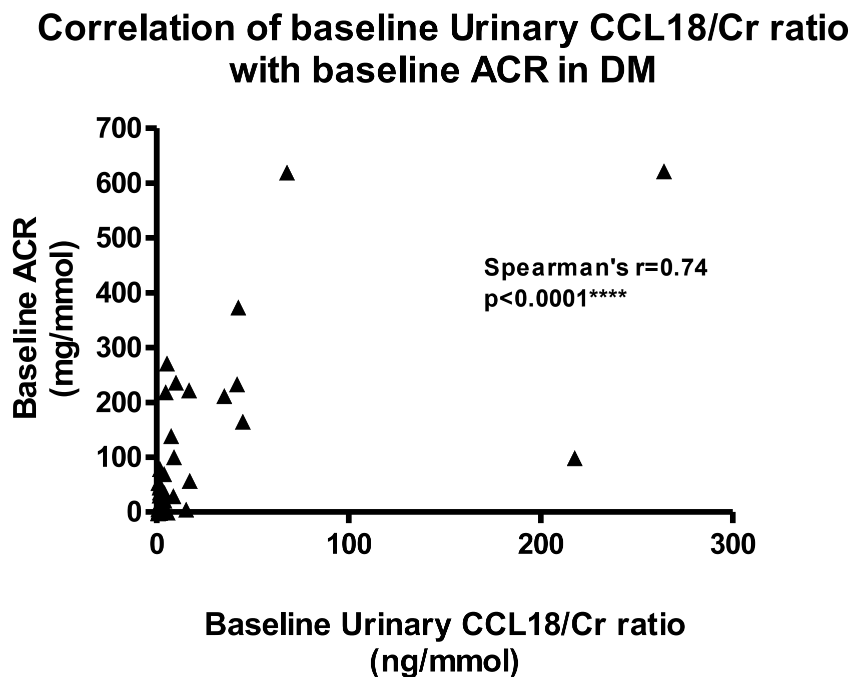


Figure 3.3.1.1 Baseline urinary CCL18/Cr ratio and ACR in DM

3.3.2 Baseline Plasma CCL18

There is no significant correlation between baseline plasma CCL18 and baseline GFR in DM and Non-DM (Spearman's correlation p=0.25, r=-0.132, p=0.23, r=-0.131, respectively). There is no significant correlation between baseline plasma CCL18 and baseline ACR in DM (Spearman's p=0.98, r=-0.002) or UPCR in Non-DM (Spearman's p=0.97, r=-0.004). The original cohort analysed suggested the normal range for plasma CCL18 in healthy volunteers is 76.6-357.9ng/ml with a median of 163.5ng/ml (Qureshi MD 2012).

<u>Summary</u>

In Non-DM and DM patients, high levels of baseline urinary CCL18/Cr ratio are found in those with a low GFR. High urinary CCL18/Cr ratio in DM is seen in those with high levels of albuminuria. There is no correlation between urinary CCL18 levels and UPCR at baseline in Non-DM. There are no correlations between the baseline plasma CCL18 and GFR, ACR or UPCR of DM and Non-DM groups.

3.4 Clinical correlation of urinary and plasma cytokines with GFR, ACR and UPCR at >3 years.

The correlation between cytokines and GFR, ACR, UPCR in DM and Non-DM groups, respectively were compared at the >3 year time point to those seen at baseline. Spearman's correlation was used for this analysis as the sample size was smaller than the original cohort. At >3 years a raised urinary MIF/Cr ratio is associated with a decrease in GFR in Non-DM patients ($p<0.05$). There is no relationship between urinary MIF/Cr ratio and ACR, UPCR or GFR in DM. Plasma MIF at >3 years has no relationship with GFR, ACR or UPCR in DM and Non-DM groups.

Raised urinary MCP-1/Cr ratio levels at >3 years is associated with a decrease in GFR in Non-DM ($p<0.01$) that had not been previously present at baseline. The relationship between urinary MCP-1/Cr ratio and a raised ACR seen at baseline continued at >3years ($p=0.046$), with no effect seen with UPCR. Plasma MCP-1 levels in contrast had no relationship with GFR, ACR or UPCR in DM or Non-DM.

A raised urinary CCL18/Cr ratio continued to correlate with a high ACR ($p=0.032$) at >3 years, the same as the baseline relationship (see Table 3.6). The cytokine profile in these groups changes over time. The correlation between urinary MIF/Cr ratio and GFR continues in Non-DM. A new correlation between urinary MCP-1/Cr ratio with GFR is seen in Non-DM >3 years follow up that was not present at baseline.

Table 3.6 Comparison of urinary cytokines in DM and Non-DM >3 years later with GFR, ACR and UPCR

Comparisons at 3 years with 3 years urinary cytokines levels	**DM**		**Non-DM**	
	Spearman's r	**p value**	**Spearman's r**	**p value**
GFR vs UMIF	-0.33	0.09	-0.39	p<0.05*
ACR vs UMIF	0.29	0.10	n/a	n/a
UPCR vs UMIF	n/a	n/a	0.24	0.18
GFR vs UMCP-1	-0.20	0.31	-0.48	p<0.01**
ACR vs UMCP-1	0.38	p<0.05*	n/a	n/a
UPCR vs UMCP-1	n/a	n/a	0.22	0.23

GFR vs UCCL18	-0.13	0.53	0.09	0.60
ACR vs UCCL18	0.38	p<0.05*	n/a	n/a
UPCR vs UCCL18	n/a	n/a	0.30	0.09

There were no significant relationships between plasma cytokines and GFR, ACR or UPCR at >3 years (see Table 3.7). Plasma MIF originally correlated with GFR in DM and Non-DM groups, however, this is not maintained at >3 years.

Table 3.7 Comparison of plasma cytokines in DM and Non-DM >3 years later with GFR, ACR and UPCR

Comparisons at 3 years with 3 years Plasma cytokines levels	**DM**		**Non-DM**	
	Spearman's r	**p value**	**Spearman's r**	**p value**
GFR vs PMIF	-0.1	0.61	0.21	0.24
ACR vs PMIF	-0.32	0.05	n/a	n/a
UPCR vs PMIF	n/a	n/a	0.08	0.67
GFR vs PMCP-1	-0.13	0.51	-0.27	0.12
ACR vs PMCP-1	0.24	0.15	n/a	n/a
UPCR vs PMCP-1	n/a	n/a	-0.27	0.14
GFR vs PCCL18	-0.05	0.80	0.15	0.39
ACR vs PCCL18	-0.07	0.69	n/a	n/a
UPCR vs PCCL18	n/a	n/a	0.24	0.19

Summary

High urinary MIF/Cr ratio detected at >3 years correlated with a decrease in GFR in Non-DM, a relationship that was observed at baseline in the DM group alone. At >3 years a raised urinary MCP-1 and CCL18/Cr ratio, continued to correlate with a raised ACR previously seen at baseline. In contrast, a high urinary MCP-1/Cr ratio at >3 years, correlated with a low GFR in Non-DM patients. There were no correlations between any plasma cytokines and GFR, ACR or UPCR at >3 years in either DM or Non-DM groups.

3.5 Baseline versus prospective urinary and plasma cytokines.

Wilcoxon matched pairs signed rank was used to determine the degree of change in the cytokines over time from the baseline as the sample size analysed was small.

3.5.1 Urinary and Plasma MIF comparison of baseline and prospective cytokines

3.5.1.0 Urinary MIF

DM **Non-DM**

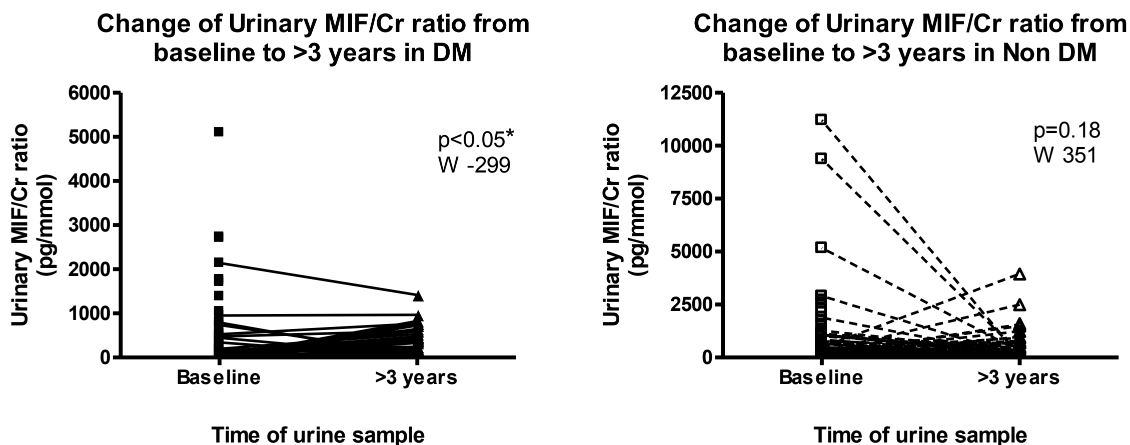


Figure 3.5.1.0 Change of urinary MIF/Cr ratio at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Urinary MIF/Cr ratio significantly increase over time in DM patients (Wilcoxon matched pairs signed rank W -299, $p<0.05$). Baseline urinary MIF/Cr ratio median was 84pg/mmol, with >3 years median of 244pg/mmol.

Urinary MIF/Cr ratio did not significantly increase over time in Non-DM group (Wilcoxon matched pairs signed rank W 351, $p=0.18$). Baseline urinary MIF/Cr ratio median was 275pg/mmol, with >3 years median of 218pg/mmol.

Summary

Urinary MIF/Cr ratio levels increased significantly following >3 years in DM group. These levels were measured in the same person over time and the finding was not reciprocated in the Non-DM group.

3.5.1.1 Plasma MIF

DM **Non-DM**

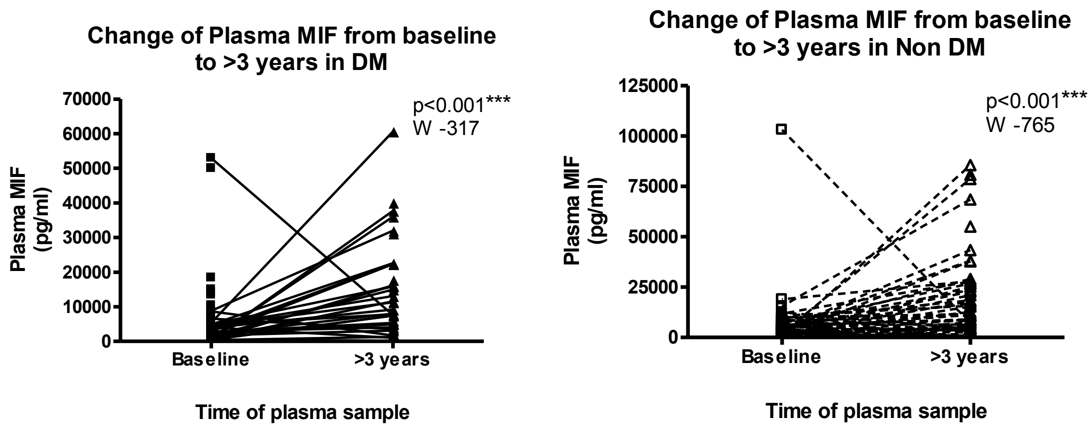


Figure 3.5.1.1 Change of plasma MIF at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Plasma MIF significantly increases over time in DM group (Wilcoxon matched pairs signed rank W -317, p<0.001). Baseline plasma MIF median was 558pg/ml, with >3 years median of 2250pg/ml. There was a single case where there was a decrease in plasma levels of MIF in both the DM and Non-DM group. The rise seen in plasma MIF over time in the DM group is not as high as those seen in the Non-DM group.

Plasma MIF significantly increases over time in Non-DM patients (Wilcoxon matched pairs signed rank W -765, p<0.001). Baseline plasma MIF median was 3900pg/ml, with >3 years median of 8500pg/ml.

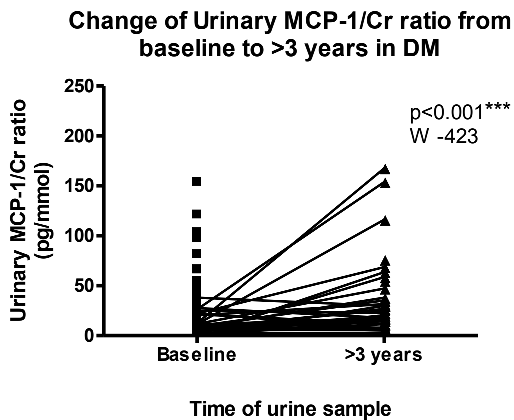
Summary of results

Urinary MIF/Cr ratio significantly increases over time in DM with little change in Non-DM group. Plasma MIF significantly increases in DM and Non-DM patients at >3 years. Higher baseline plasma MIF is seen in Non-DM compared to DM group.

3.5.2 Urinary and Plasma MCP-1 comparison of baseline and prospective cytokines

3.5.2.0 Urinary MCP-1

DM **Non-DM**



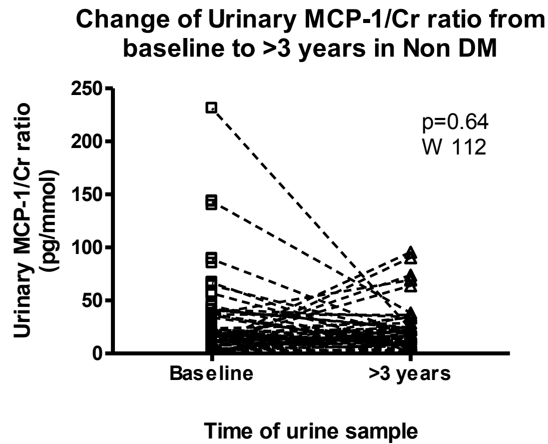


Figure 3.5.2.0 Change of urinary MCP-1/Cr ratio at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Urinary MCP-1/Cr ratio significantly increases over time in DM (Wilcoxon matched pairs signed rank W -423, p<0.001). Baseline urinary MCP-1/Cr ratio median was 10.4pg/mmol, with >3 years median of 19.9pg/mmol. Urinary MCP-1/Cr ratio is variable over time in Non-DM patients (Wilcoxon matched pairs signed rank W 112, p=0.64), this may reflect the diversity of the Non-DM group. Baseline urinary MCP-1/Cr ratio median was 16.3pg/mmol, with >3 years median of 15.8pg/mmol.

Summary

Urinary MCP-1/Cr ratio significantly increases over time in DM whereas there is no change in Non-DM patients.

3.5.2.1 Plasma MCP-1

DM **Non-DM**

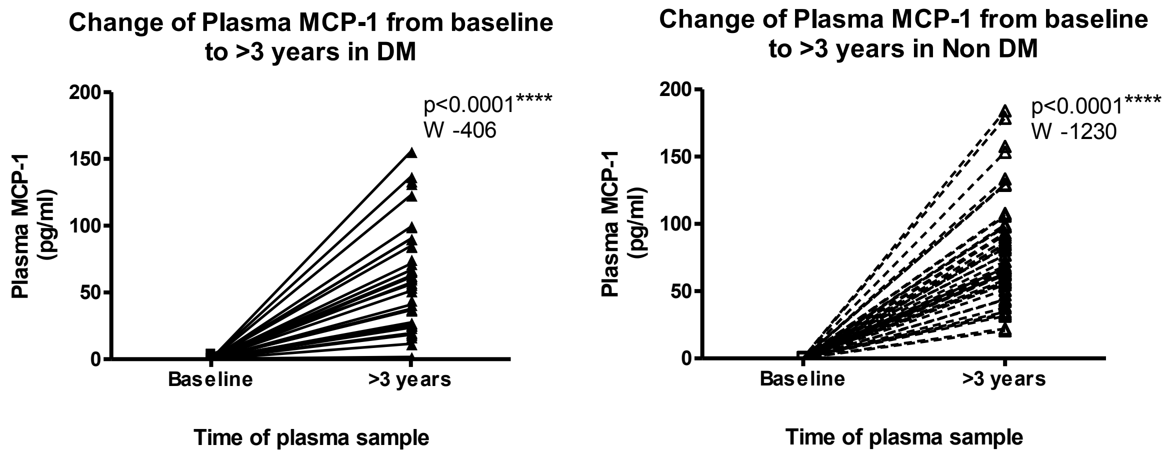


Figure 3.5.2.1 Change of plasma MCP-1 at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Plasma MCP-1 significantly increases over time in DM and Non-DM groups (Wilcoxon matched pairs signed rank W -406, p<0.0001, W -1230, p<0.0001, respectively). DM patients baseline plasma MCP-1 had median of 0pg/ml, with >3 years median of 58.8pg/ml. Non-DM patients baseline plasma MCP-1 median was 0pg/ml, with >3 years median of 67.4pg/ml.

<u>Summary of results</u>

Urinary MCP-1/Cr ratio increases significantly over time in DM with no difference in Non-DM groups. Plasma MCP-1 significantly increases in DM and Non-DM patients over time.

3.5.3 Urinary and Plasma CCL18 comparison of baseline and prospective cytokines

3.5.3.0 Urinary CCL18

DM **Non-DM**

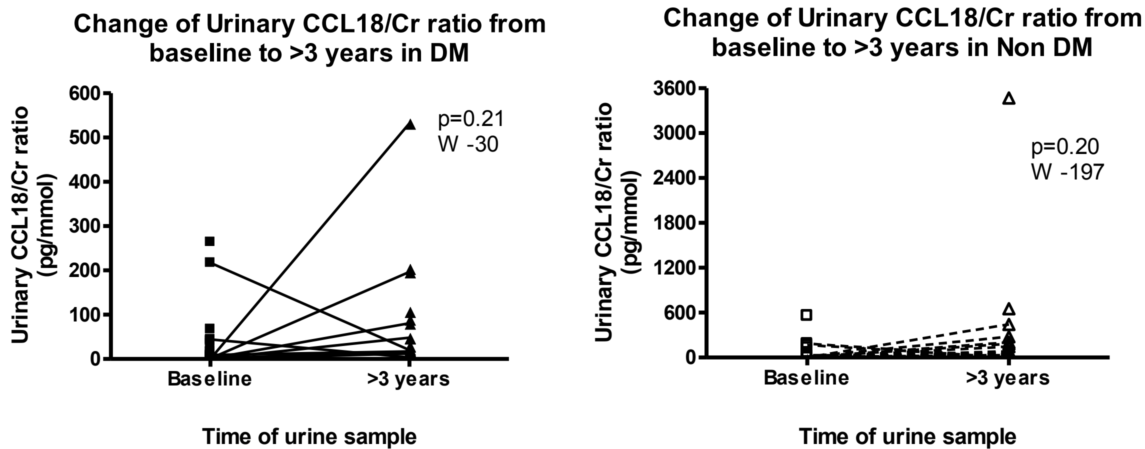


Figure 3.5.3.0 Change of urinary CCL18/Cr ratio at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Urinary CCL18/Cr ratio trend to increase over time in DM and Non-DM groups but does not reach significance (Wilcoxon matched pairs signed rank W -30, p=0.21, W -197, p=0.20, respectively). DM baseline urinary CCL18/Cr ratio has median of 0pg/mmol, with >3 years median of 12.2pg/mmol. Non-DM baseline urinary CCL18/Cr ratio has median of 0pg/mmol, with >3 years median of 7.89pg/mmol.

Summary

Urinary CCL18/Cr ratio trend to increase over time in DM and Non-DM patients but did not reach statistical significance.

3.5.3.1 Plasma CCL18

DM **Non-DM**

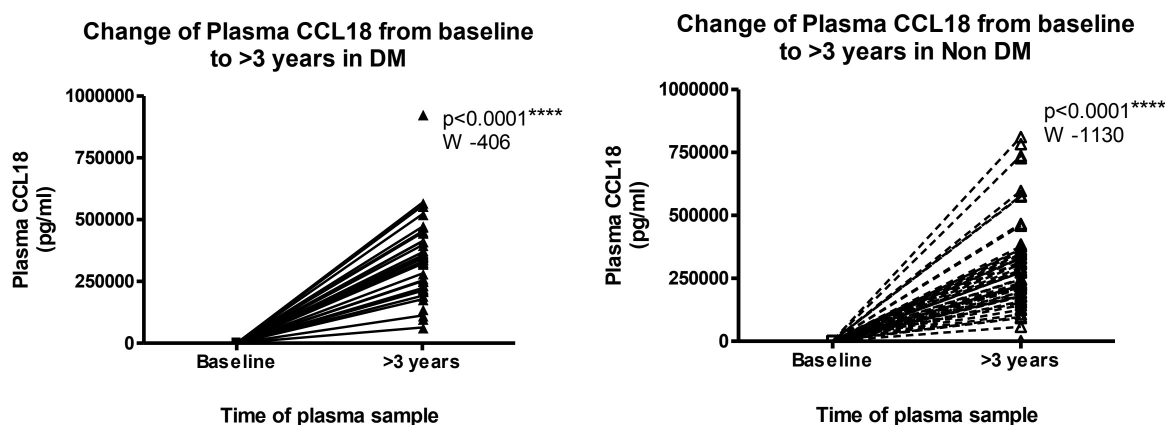


Figure 3.5.3.1 Change of plasma CCL18 at baseline and >3 years in DM and Non-DM. Key; W = sum of signed ranks

Plasma CCL18 significantly increases over time in DM patients (Wilcoxon matched pairs signed rank W -406, p<0.0001). Baseline plasma CCL18 has median of 134pg/ml, with >3 years median of 331000pg/ml. Plasma CCL18 significantly increases over time in Non-DM group (Wilcoxon matched pairs signed rank W -1130, p<0.0001). Baseline plasma CCL18 has median of 137pg/ml, with >3 years median of 247000pg/ml.

Summary of results

There are no significant changes in urinary CCL18/Cr ratio over time in DM or Non-DM groups. Plasma CCL18 significantly increases over time in both groups.

Summary for Baseline versus prospective Urinary and plasma cytokines

All urinary MIF, MCP-1 and CCL18 levels were raised in DM at >3 years and this was significant in MCP-1 and MIF. There was no significant change in Non-DM urinary MIF, MCP-1 or CCL18 levels. Plasma MIF, MCP-1 and CCL18 levels were significantly increased over time in DM. This was also seen in Non-DM at >3 years.

3.6 Correlation of urinary and plasma cytokines

Baseline urinary and plasma samples were compared to determine whether there was any relationship between urinary and plasma levels of the same cytokine. A systemic production of the

cytokine rather than a localised production would be suggested by correlation between urinary and plasma levels. There is no correlation between urinary and plasma MIF, MCP-1 or CCL18 in DM or Non-DM patients at baseline. These results suggest the quantities of cytokines detected in the urine or plasma, are independent of each other at baseline, see Table 3.8.

Table 3.8 Shows the relationship between urinary and plasma levels of the same cytokine at baseline and at >3 years. U prefix = Urinary, P prefix = Plasma, B prefix = Baseline, 3 prefix = >3 years

Comparisons of urinary and plasma cytokines	**DM**		**Non-DM**	
	Spearman's r	**p value**	**Spearman's r**	**p value**
BUMIF vs BPMIF	-0.02	0.83	-0.1	0.37
BUMCP-1 vs BPMCP-1	0.14	0.23	0.19	0.08
BUCCL18 vs BPCCL18	-0.08	0.69	0.19	0.13
3UMIF vs 3PMIF	-0.21	0.21	0.15	0.25
3UMCP-1 vs 3PMCP-1	0.11	0.48	0.14	0.29
3UCCL18 vs 3PCCL18	0.28	0.09	0.34	p<0.01**

Urinary and plasma MIF and MCP-1 at >3 years in DM and Non-DM group show no correlation. Urinary and plasma CCL18 levels in Non-DM at >3 years however, significantly correlate with each other (see Table 3.8). A rising urinary CCL18/Cr ratio correlates with a rise in plasma CCL18 see Figure 3.6. This correlation was not seen in DM or at baseline in Non-DM group.

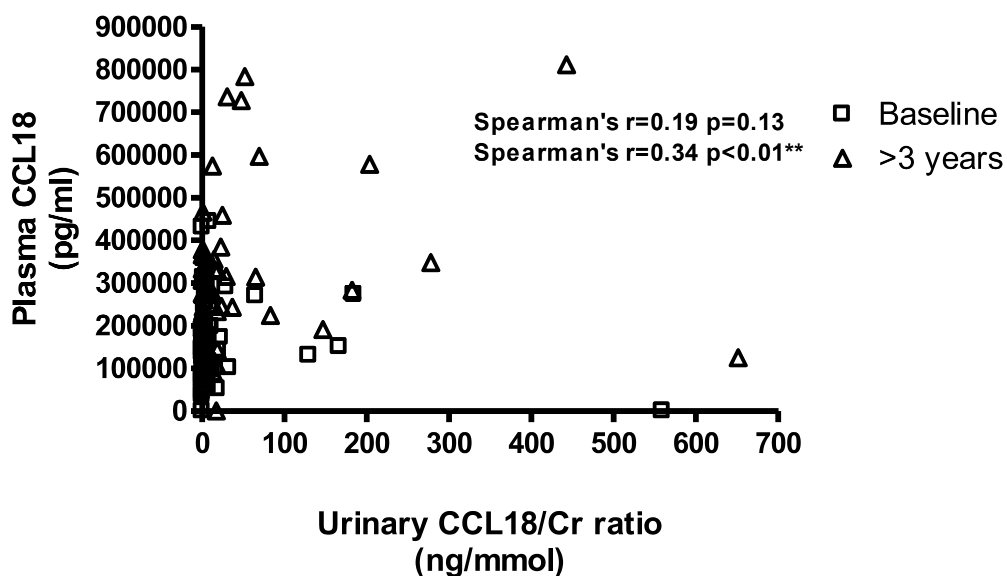


Figure 3.6 Correlation between urinary and plasma CCL18 levels over time in Non-DM

Summary

There was no correlation between baseline urinary MIF, MCP-1 and CCL18 and plasma cytokines at baseline in DM and Non-DM groups. At >3 years this continued to be the same except for urinary and plasma CCL18 levels that positively correlated in the Non-DM group.

3.7 Distribution of cytokines at baseline and >3 years in DM and Non-DM

The distribution of cytokines at baseline and >3 years is illustrated below to determine whether the profile of the combination of the three cytokines changes over time in DM or Non-DM patients.

3.7.1 Distribution of Urinary cytokines at baseline and >3 years

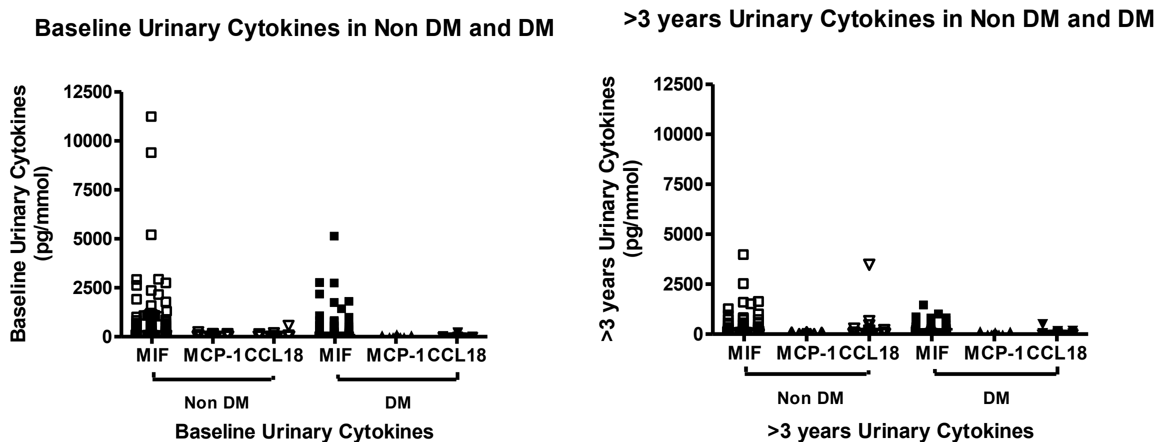


Figure 3.7.1 Distribution of urinary cytokines at baseline and >3 years

Urinary MIF is produced in higher quantities compared with MCP-1 and CCL18 that is maintained over >3 years. The paired cytokine analysis, however, showed urinary MIF and MCP-1 significantly increase over time with urinary CCL18 levels trending to increase in DM group (see section 3.5). There was no increase in Non-DM in all three cytokines. The paired sample size was small and larger numbers may show that urinary CCL18 levels also significantly increase over time.

3.7.2 Distribution of Plasma cytokines at baseline and >3 years

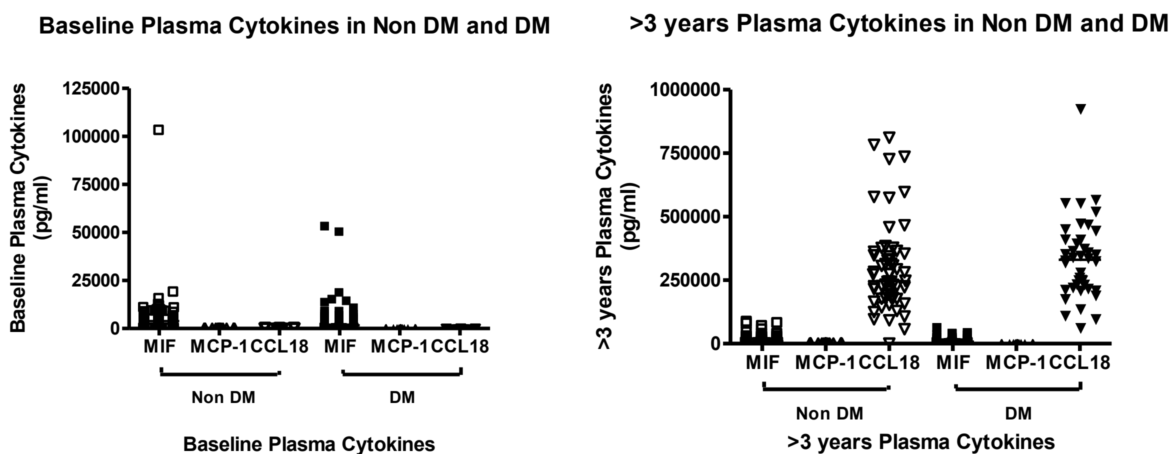


Figure 3.7.2 Distribution of plasma cytokines at baseline and >3 years

Plasma MIF levels were highest at baseline compared with MCP-1 and CCL18. The y axis at >3 years show MIF to have increased over time though less than plasma CCL18 at >3 years. Plasma

CCL18 levels dramatically increased over time in both Non-DM and DM groups. Plasma MCP-1 remained constant over time.

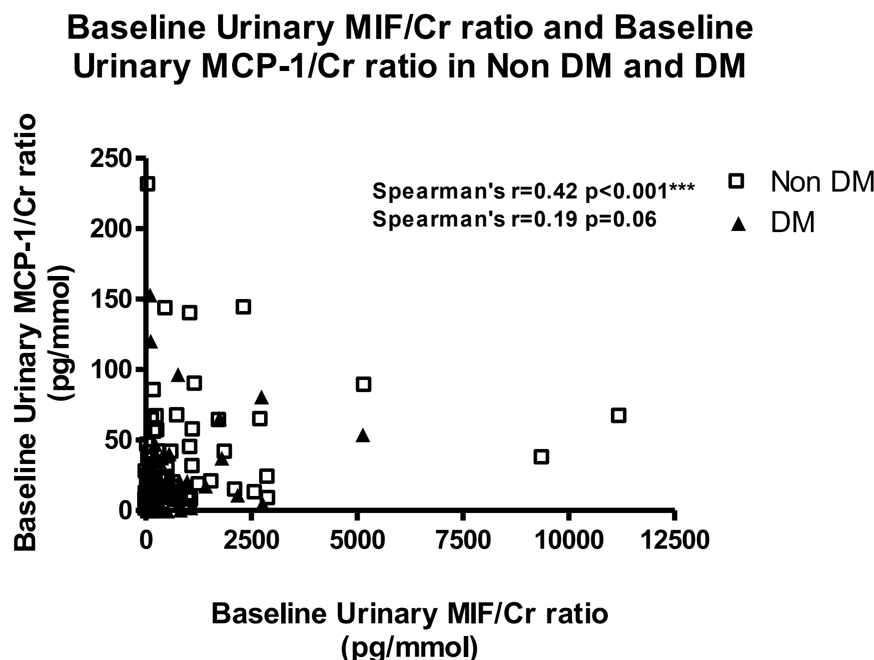
Summary

Urinary and plasma cytokine profile for DM and Non-DM groups differs over time. There were no significant differences seen in prospective cytokine production between DM and Non-DM patients. Both groups produced larger amounts of CCL18 in plasma prospectively compared to baseline production of plasma CCL18. Plasma MIF levels appear to be higher in the Non-DM group, however, this did not reach significance. There were higher levels of cytokines found in prospective samples. The clinical findings presented thus far suggest there may be communication between the cytokines. Section 3.8 determines whether any correlations exist between the different cytokines within the same clinical samples and if this is maintained over time.

3.8 Correlation of urinary cytokines

At baseline, urinary MIF, MCP-1 and CCL18 all positively correlate in Non-DM. In DM, urinary CCL18 positively correlates with high levels of MIF and MCP-1. Urinary MIF and MCP-1 levels are unrelated, that continues to be the same at >3 years in DM, however, the only correlation that is maintained over time in Non-DM is that of urinary MCP-1 and CCL18. The baseline results are illustrated in Fig 3.8.1-3.8.3 with the results at the two time points summarised in Table 3.9-3.10.

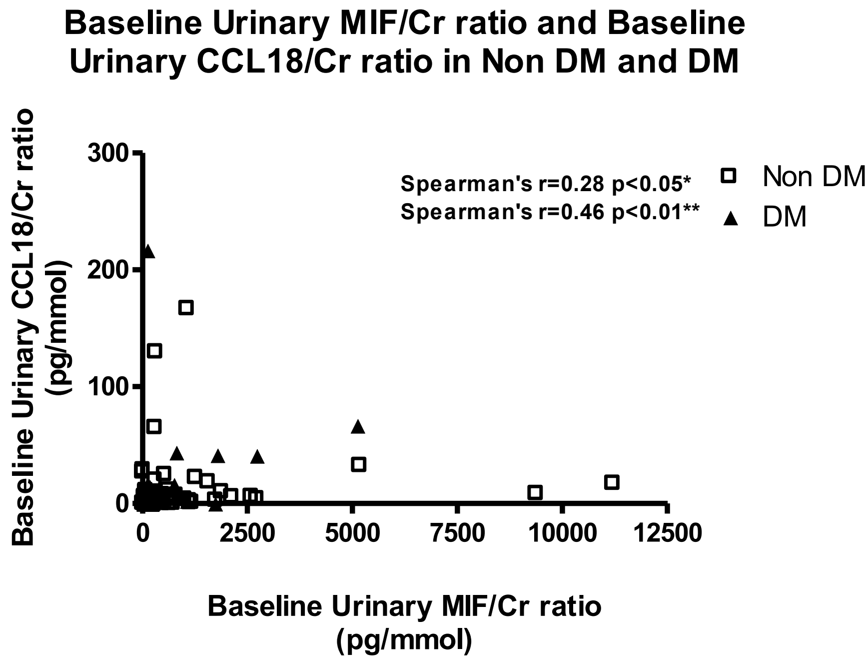
3.8.1 Urinary MIF and MCP-1



A significant correlation between urinary MIF and MCP-1 is seen in Non-DM (Spearman's r=0.42, p<0.001). An increasing urinary MIF is associated with higher levels of urinary MCP-1 that was not seen in DM (Spearman's r=0.19, p=0.06).

Figure 3.8.1 Correlation between baseline urinary MIF and MCP-1 in DM and Non-DM

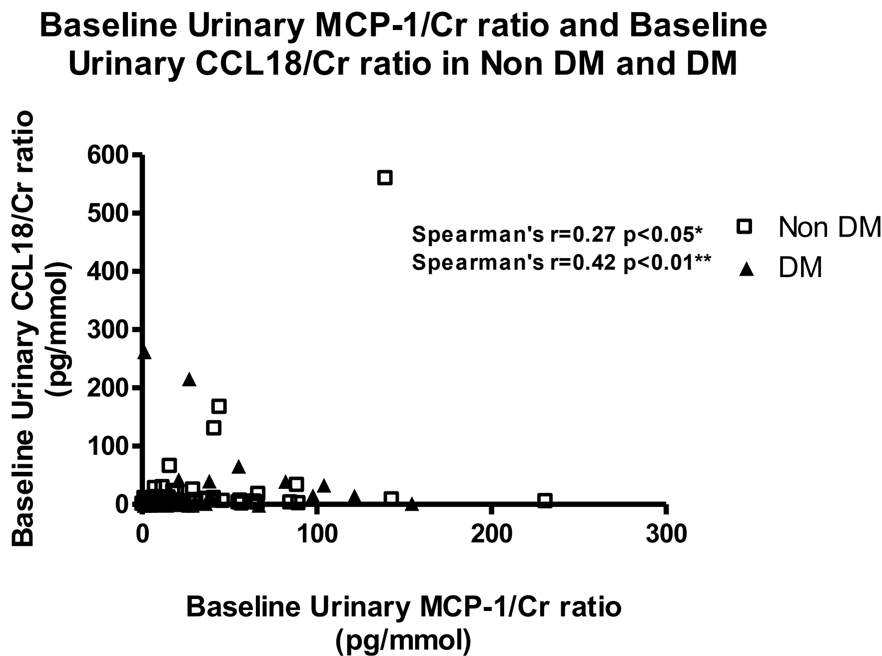
3.8.2 Urinary MIF and CCL18



Baseline urinary MIF and CCL18 were found to significantly correlate in both Non-DM and more significantly in DM patients ($p<0.05$, $p<0.01$, respectively).

Figure 3.8.2 Correlation between baseline urinary MIF and CCL18 in DM and Non-DM

3.8.3 Urinary MCP-1 and CCL18



In Non-DM and DM patients a high urinary MCP-1 significantly correlates with a high urinary CCL18 level (Spearman's $r=0.27$, $p<0.05$, Spearman's $r=0.42$, $p<0.01$).

Figure 3.8.3 Correlation between baseline urinary MCP-1 and CCL18 in DM and Non-DM

Table 3.9 Showing the comparison between different urinary cytokines at baseline and >3 years.

Comparisons at urinary cytokines	DM		Non-DM	
	Spearman's r	**P value**	**Spearman's r**	**p value**
BUMIF vs BUMCP-1	0.19	0.06	0.42	p<0.001***
BUMIF vs BUCCL18	0.46	p<0.01**	0.28	p<0.05*
BUMCP-1 vs BUCCL18	0.42	p<0.01**	0.27	p<0.05*
3UMIF vs 3UMCP-1	0.27	0.10	0.24	0.06
3UMIF vs 3UCCL18	0.52	p<0.01**	0.22	0.08
3UMCP-1 vs 3UCCL18	0.40	p<0.05*	0.31	p<0.05*

<u>Summary</u>

Urinary MIF and CCL18, and urinary CCL18 and MCP-1 significantly correlate in DM patients at baseline and at >3 years. There was no correlation between urinary MIF and MCP-1 in DM group. In contrast, baseline urinary MIF, MCP-1 and CCL18 all significantly correlated in Non-DM group. At >3 years the relationship between urinary MCP-1 and CCL18 was the only one to be maintained in Non-DM group.

3.9 Correlation of plasma cytokines

There was no correlation between baseline plasma levels CCL18, MCP-1 and MIF with each other in either DM or Non-DM groups, see Table 3.10. Plasma MIF and MCP-1 correlated at >3 years in DM patients.

Table 3.10 Comparison between different plasma cytokines at baseline and >3 years.

Comparisons at plasma cytokines	DM		Non-DM	
	Spearman's r	**P value**	**Spearman's r**	**p value**
BPMIF vs BPMCP-1	0.06	0.61	-0.18	0.11
BPMIF vs BPCCL18	0.08	0.51	0.13	0.30
BPMCP-1 vs BPCCL18	0.14	0.26	0.005	0.97

3PMIF vs 3PMCP-1	-0.31	p<0.05*	-0.08	0.55
3PMIF vs 3PCCL18	-0.09	0.57	0.13	0.31
3PMCP-1 vs 3PCCL18	0.14	0.39	0.09	0.49

Summary

There were no correlations between plasma cytokines in DM and Non-DM patients at baseline. Plasma MIF and MCP-1 correlated at>3 years in DM group.

3.10 Univariant analysis to determine the Predictive value of Baseline urinary and plasma MIF, MCP-1 and CCL18 in DM and Non-DM

The calculations showing how the rate of change for GFR, ACR and UPCR were calculated are below. The >18 months value is subsequently changed for the >3 years value using the time point when the sample was collected from. This allows the actual rate of change of the outcome measures; GFR, ACR and UPCR to be used, when correlated with baseline urinary and plasma cytokines. Those that began RRT were excluded from the analysis.

Calculation for dGFR= (>18 months GFR-baseline GFR)/time interval in months)

Calculation for dACR= (>18 months ACR-baseline ACR)/time interval in months)

Calculation for dUPCR= (>18 months UPCR-baseline UPCR)/time interval in months)

3.10.1 Predictive value of Baseline urinary MIF, MCP-1 and CCL18 in DM and Non-DM at >18 months

Baseline urinary cytokines did not predict changes in GFR, ACR or UPCR in DM and Non-DM, respectively (see Table 3.11).

Table 3.11 Predictability of urinary cytokines in DM and Non-DM >18 months with GFR, ACR and UPCR Prefix d= rate of change, n/a= not applicable to the analysis.

Predictability of urinary cytokines at >18months	DM		Non-DM	
	Spearman's r	p value	Spearman's r	p value
dGFR vs UMIF	-0.08	0.59	0.01	0.91
dACR vs UMIF	-0.14	0.42	n/a	n/a

dUPCR vs UMIF	n/a	n/a	-0.11	0.54
dGFR vs UMCP-1	-0.10	0.50	0.06	0.62
dACR vs UMCP-1	-0.13	0.45	n/a	n/a
dUPCR vs UMCP-1	n/a	n/a	-0.12	0.50
dGFR vs UCCL18	-0.16	0.45	-0.02	0.90
dACR vs UCCL18	-0.42	0.17	n/a	n/a
dUPCR vs UCCL18	n/a	n/a	0.06	0.77

3.10.2 Predictive value of Baseline plasma MIF, MCP-1 and CCL18 in DM and Non-DM at >18 months

Plasma cytokines did not predict outcome measures of GFR, ACR or UPCR, see table 3.12.

Table 3.12 Predictability of plasma cytokines in DM and Non-DM >18months with GFR, ACR and UPCR Prefix d= rate of change, n/a= not applicable to the analysis.

Predictability of plasma cytokines at >18months	DM		Non-DM	
	Spearman's r	p value	Spearman's r	p value
dGFR vs PMIF	-0.12	0.44	-0.19	0.10
dACR vs PMIF	-0.08	0.67	n/a	n/a
dUPCR vs PMIF	n/a	n/a	-0.06	0.77
dGFR vs PMCP-1	-0.13	0.45	0.04	0.75
dACR vs PMCP-1	0.36	0.12	n/a	n/a
dUPCR vs PMCP-1	n/a	n/a	0.08	0.65
dGFR vs PCCL18	-0.15	0.33	-0.02	0.83
dACR vs PCCL18	0.04	0.83	n/a	n/a
dUPCR vs PCCL18	n/a	n/a	0.16	0.41

3.10.3 Predictive value of Baseline urinary MIF, MCP-1 and CCL18 in DM and Non-DM at >3 years

Baseline urinary cytokines did not correlate with GFR, ACR or UPCR at >3 years, see Table 3.13.

Table 3.13 Predictability of urinary cytokines in DM and Non-DM >3 years with GFR, ACR and UPCR dGFR= rate of change of GFR.

Predictability of urinary cytokines at >3 years	DM		Non-DM	
	Spearman's r	p value	Spearman's r	p value
dGFR vs UMIF	-0.03	0.83	0.03	0.81
dACR vs UMIF	-0.23	0.17	n/a	n/a
dUPCR vs UMIF	n/a	n/a	0.21	0.12
dGFR vs UMCP-1	-0.02	0.91	0.05	0.68
dACR vs UMCP-1	0.17	0.31	n/a	n/a
dUPCR vs UMCP-1	n/a	n/a	-0.08	0.57
dGFR vs UCCL18	-0.20	0.38	-0.004	0.97
dACR vs UCCL18	-0.16	0.57	n/a	n/a
dUPCR vs UCCL18	n/a	n/a	0.30	0.06

3.10.4 Predictive value of Baseline plasma MIF, MCP-1 and CCL18 in DM and Non-DM at >3 years

There were no correlations between baseline plasma MIF and GFR, ACR or UPCR at >3 years, see Table 3.14. Baseline plasma MCP-1 levels did not correlate with GFR in DM or Non-DM nor UPCR. There were no correlations between baseline plasma CCL18 and ACR or UPCR.

*Table 3.14 Predictability of plasma cytokines in DM and Non-DM >3 years with GFR, ACR and UPCR dGFR= rate of change of GFR, *p<0.05, **p<0.01.*

Predictability of plasma cytokines at >3 years	DM		Non-DM	
	Spearman's r	p value	Spearman's r	p value
dGFR vs PMIF	-0.07	0.67	-0.14	0.29
dACR vs PMIF	0.02	0.90	n/a	n/a
dUPCR vs PMIF	n/a	n/a	0.04	0.80
dGFR vs PMCP-1	0.13	0.42	0.11	0.38
dACR vs PMCP-1	0.44	0.02*	n/a	n/a

dUPCR vs PMCP-1	n/a	n/a	0.23	0.12
dGFR vs PCCL18	0.44	0.004**	-0.06	0.66
dACR vs PCCL18	0.30	0.09	n/a	n/a
dUPCR vs PCCL18	n/a	n/a	0.05	0.76

At >3 years a slower decline in GFR was seen in DM patients with high baseline plasma CCL18 (Spearman's r=0.44, p<0.01), see below.

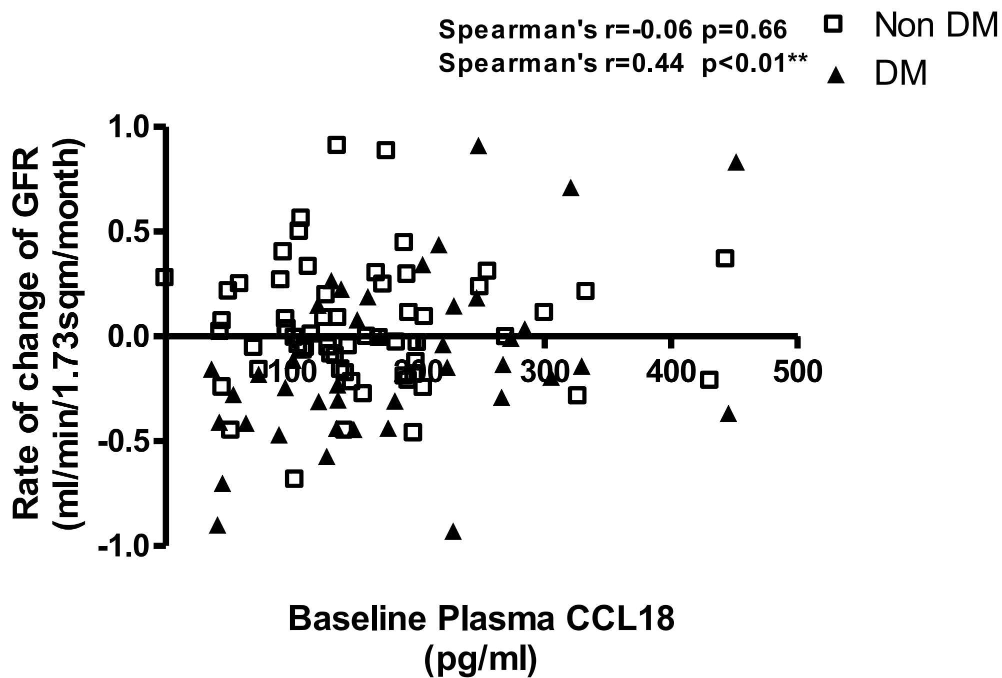


Figure 3.10.4.0 Correlation of baseline plasma CCL18 with >3 year GFR in DM and Non-DM

Urinary ACR was seen to worsen, i.e. increase with high levels of baseline plasma MCP-1 in DM (Spearman's r=0.44, p<0.05).

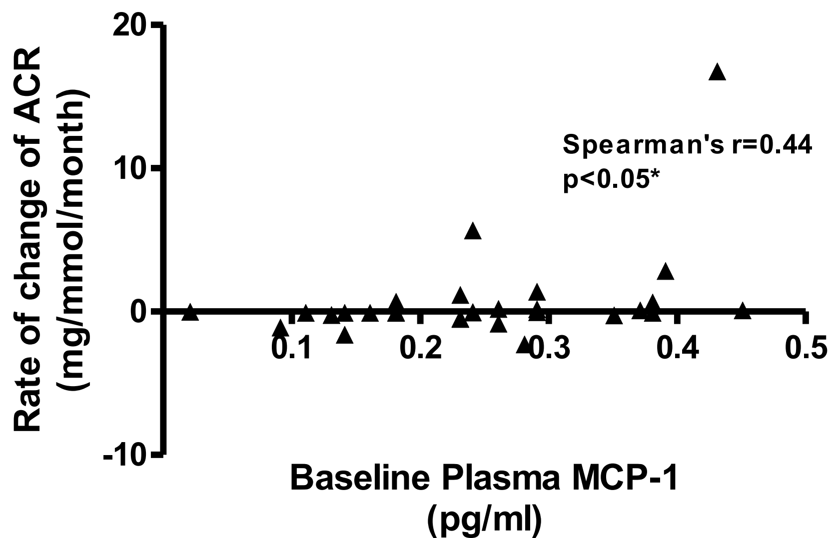


Figure 3.10.4.1 Correlation of baseline plasma MCP-1 with >3 year GFR in DM and Non-DM

Summary

There were no correlations between baseline urinary or plasma cytokines with GFR, ACR or UPCR at >18 months. The analysis showed that at >3 years when using rate of change in GFR in months, high levels of baseline plasma CCL18 seem to predict an improvement in GFR in DM patients. Raised baseline plasma MCP-1 significantly correlated with increasing ACR at >3 years. This difference may reflect the use of plasma samples to predict GFR over a longer period of time. These results differ from my predecessors' work where urinary MCP-1 significantly correlated with ACR. This may arise from the more intensive use of ACEi/ARB that may decrease urinary levels of MCP-1. Urinary MCP-1 has been reported to increase over time in DM (Tam, Riser et al. 2009). The median follow up time from Dr Qureshi was 1.89 years that showed urinary MCP-1 did not correlate with deteriorating GFR. My findings are consistent with this at >3 years. This cohort is different to that of the initial observational cohort study with a 6 years follow up by my supervisor. He showed that a raised urinary MCP-1 level correlated with a decreasing GFR in DM (Tam, Riser et al. 2009). Nowadays RAS inhibitors are more commonly used and may explain the difference seen. (Ogawa, Kobori et al. 2009) reported a decrease in urinary MCP-1 and albuminuria with ACEi/ARB.

3.11 Mixed models for predictive value of Baseline urinary and plasma MIF, MCP-1 and CCL18 in DM and Non-DM

To determine the predictive value of baseline MIF, MCP-1 and CCL18 with GFR, ACR and UPCR a mixed model was developed by Imperial College Statistician Dr Fabiana Gordon. The mixed model applies to longitudinal data and is the most appropriate methodology for analysis of this data. The model takes into account the correlated observations over time in months. In this study the effect of time on observations cannot be assumed to be uncorrelated and hence application of this model. The model takes account of gender, age, immunosuppression, MAP, DM together with urinary and plasma cytokines measured at baseline. These factors were used to determine whether they had an effect on the outcome measures of; death, RRT, GFR, ACR and UPCR in DM and Non-DM groups, respectively. Dr Qureshi found urinary CCL18 to correlate with decreasing GFR, however, this longer prospective follow up no longer showed this. No correlation was previously reported between urinary and serum samples of MCP-1 (Banba, Nakamura et al. 2000; Wada, Furuichi et al. 2000). No correlation has previously been reported between MCP-1 levels and serum or ACR (Tam, Sanders et al. 2004), though a decrease in urinary MCP-1 has been reported in patients given immunosuppression for vasculitis and lupus.

The model excluded patients with any missing data and excluded variables that were not significant. The final cohort had less numbers for analysis following the exclusions identified by the model. The final model contains only the significant effects that are presented herein.

3.11.1 Outcome measures

3.11.1.0 Survival

The mixed model showed that those who had DM had a significantly lower GFR at the time of death ($p<0.03$) compared with Non-DM that had a higher GFR. This suggests that death in the DM group may be due to the complications of DM, including DN. From the deaths recorded the majority of those with DM died from sepsis, cancer and MI and those with Non-DM died from sepsis and cancer.

3.11.1.1 GFR Outcome

GFR was seen to be lower in the DM compared with Non-DM. A significant interaction was seen in the GFR of those on immunosuppression who were male compared to females ($p=0.03$). Immunosuppressed males had a higher GFR compared with females in this group

(69ml/min/1.73msq versus 46ml/min/1.73msq, respectively), suggesting there is an added benefit on GFR if male and on immunosuppression. This effect was not seen in those without immunosuppression i.e. the DM group. The GFRs in the non-immunosuppressed group that were predominantly DM were males 32ml/min/1.73msq and females 39ml/min/1.73msq.

3.11.1.2 ACR Outcome

No significant differences were seen with baseline cytokines on ACR over time in either group.

3.11.1.3 UPCR Outcome

Baseline cytokines did not predict an effect on UPCR over time in either group.

3.11.2 Predictors

3.11.2.0 Baseline Plasma MIF

The effect of baseline plasma MIF on GFR changed over time in the DM group whilst no effect was seen in the Non-DM. Baseline plasma MIF predicted a lower GFR in DM at >18 months (p=0.03), that stopped at >3 years. Fig 3.11.2.0 illustrates the change in baseline plasma MIF in DM and Non-DM over time.

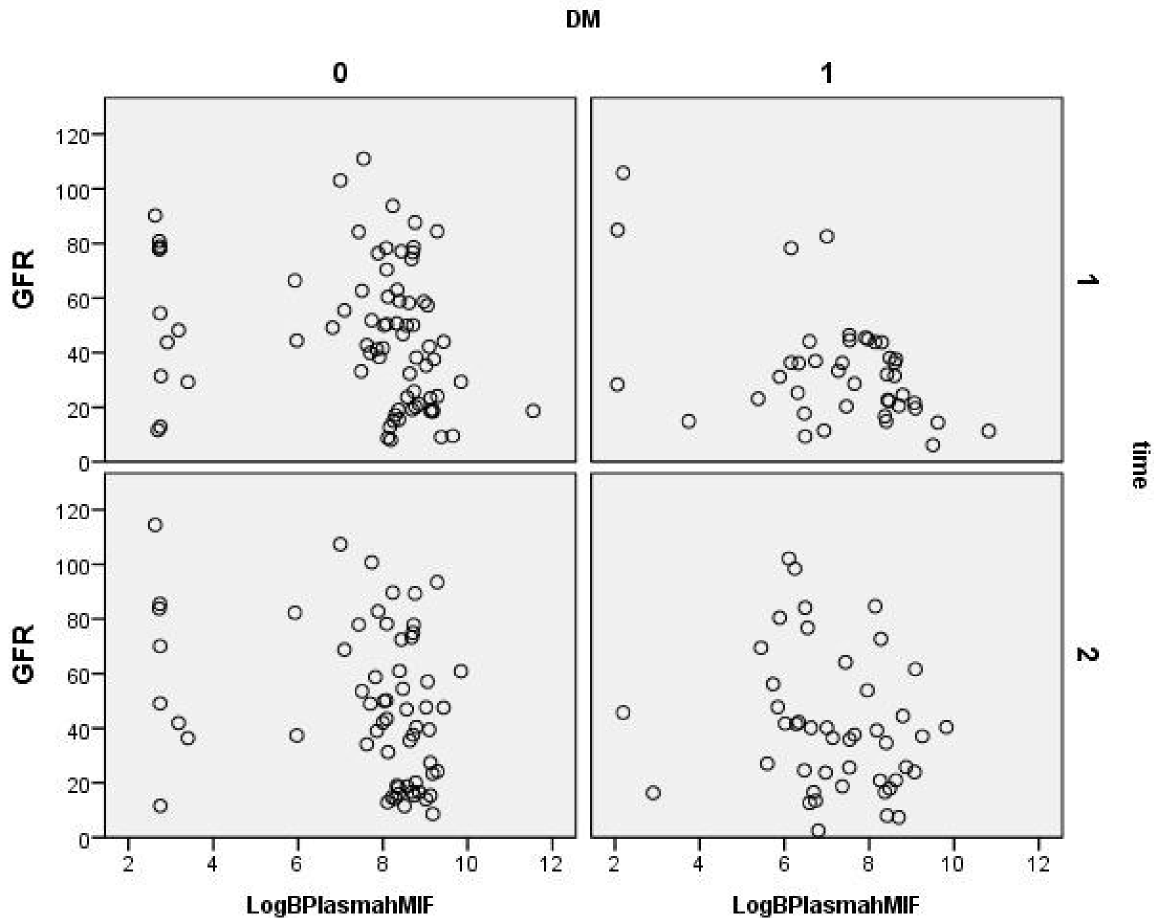


Figure 3.11.2.0 Predictive effect of baseline plasma MIF over time. Key: DM=1 Non-DM=0, Time 1= 18 months, Time 2= >3 years, LogBPlasmahMIF = baseline plasma MIF using logarithmic scale.

3.11.2.1 Baseline Plasma CCL18

The effect of baseline plasma CCL18 on GFR changed over time in the DM group whilst no effect was seen in the Non-DM. Baseline plasma CCL18 predicted a lower GFR in DM at >18 months (p=0.03), that stopped at >3 years (p=0.07). Figure 3.11.2.1 suggests that with an increased sample size the predictive significant effect on GFR may continue in DM at a longer time point. Further larger studies would be needed to confirm these findings. This was different from the effect seen in plasma CCL18 on GFR in univariant analysis. The model accounts for other variables and hence likely to have resulted in the difference arising between the results.

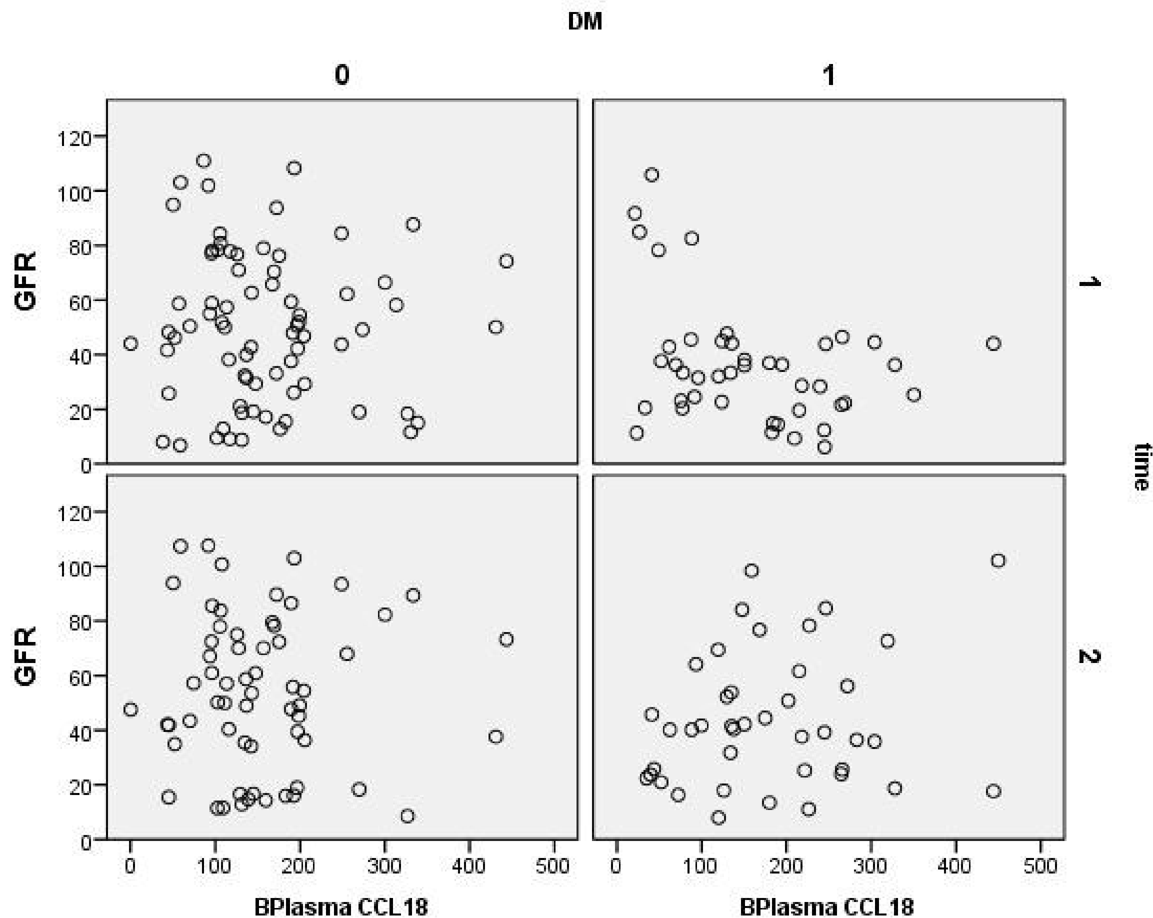


Figure 3.11.2.1 Predictive effect of baseline plasma CCL18 over time. Key: DM=1 Non-DM=0, Time 1= 18 months, Time 2= >3 years, BPlasma CCL18 = baseline plasma CCL18.

<u>Summary</u>

The predictive model shows mortality is higher in DM with low GFR. Immunosuppression preserves GFR in males and in turn this may contribute to the difference seen in cytokines. Specifically in this model, baseline plasma CCL18 and baseline plasma LogMIF were predictive of a lower GFR in DM at more than 18 months. The significance declines with time; however, the analysis suggests that baseline plasma CCL18 may continue to have its predictive effect at >3 years. Larger numbers specifically looking at this factor could help clarify this; however, it is known that DM have a higher morbidity and thus the study would need to be adequately powered. Baseline cytokines are not predictive of ACR or UPCR outcomes though the analysis is limited by the small number analysed over the follow up period.

3.12 Clinical correlation of serum and plasma cytokines

Literature exists that suggest the collection of cytokines is affected by the coagulation cascade and hence collecting serum samples may be more reflective of circulating cytokines compared with collection of plasma samples (Johnson, Aarden et al. 1996). My predecessor had collected plasma samples and stored these. I subsequently collected plasma samples in the prospective cohort to allow comparisons to be made. To ensure the circulating cytokines did not alter with the EDTA tubes, I simultaneously collected serum samples. Serum tubes are normally used to measure renal function thus if cytokine levels could be measured in the same sample collected by the patient to assess their renal function, this would be more practical. Spearman's correlation was used to establish whether there are differences between the serum and plasma levels of an individual and whether these alter with cytokine analysed. The results are presented below and summarised in Table 3.16.

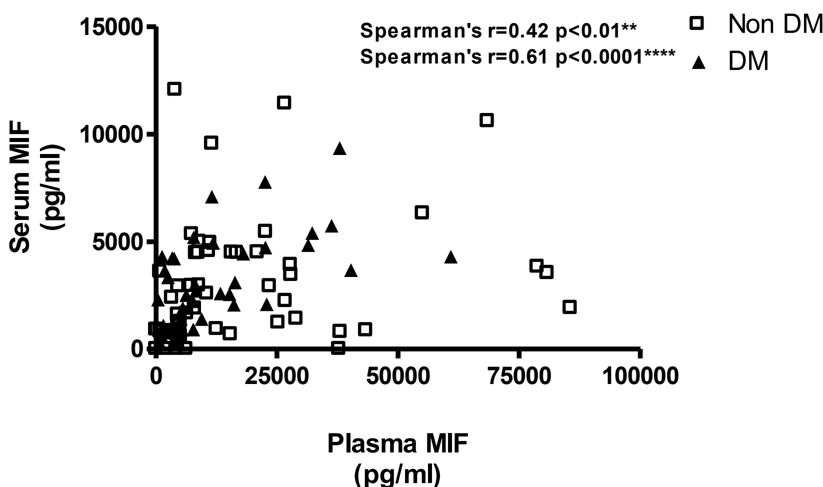


Figure 3.12.1 MIF Serum and plasma levels

Serum and plasma MIF levels correlate significantly in both Non-DM and DM patients, with DM having a higher level of significance, see Figure 3.12.1. The serum levels detected were lower than cytokine in plasma samples.

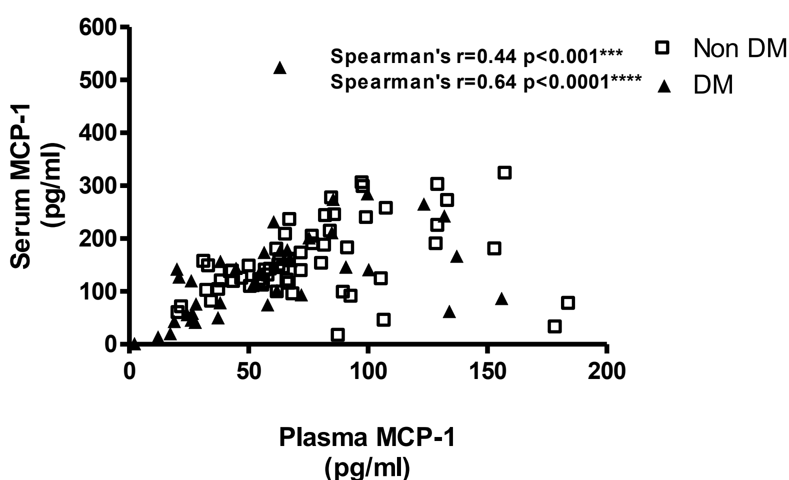
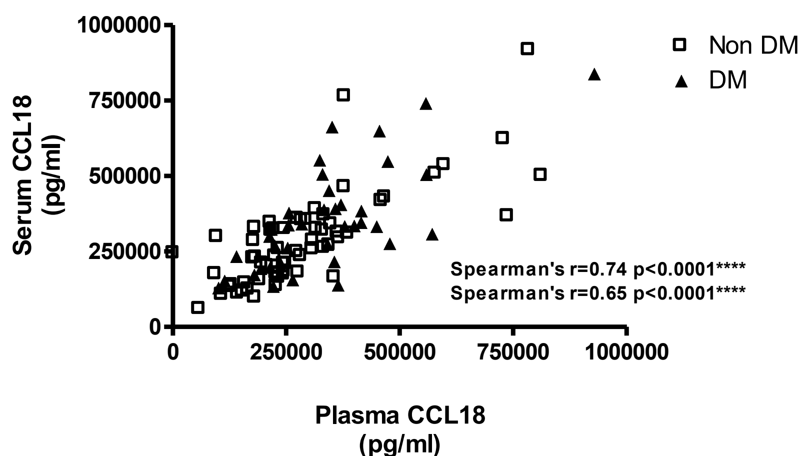


Figure 3.12.2 MCP-1 serum and plasma levels

Serum levels of MCP-1 were higher than those detected in plasma samples see Figure 3.12.2. Both were correlated significantly with the correlation being highly significant in the DM group.



Serum and plasma CCL18 levels were detected in similar quantities and correlated significantly in both Non-DM and DM, see Figure 3.12.3.

Figure 3.12.3 CCL18 serum and plasma levels

Table 3.15 Comparisons of plasma and serum cytokines at >3 years. Key: P prefix = plasma, S prefix = serum

Comparisons at >3 years plasma and serum cytokines	DM		Non-DM	
	Spearman's r	p value	Spearman's r	p value
PMIF vs SMIF	0.61	p<0.0001	0.42	p<0.05
PMCP-1 vs SMCP-1	0.64	p<0.0001	0.44	p<0.001
PCCL18 vs SCCL18	0.65	p<0.0001	0.74	p<0.0001

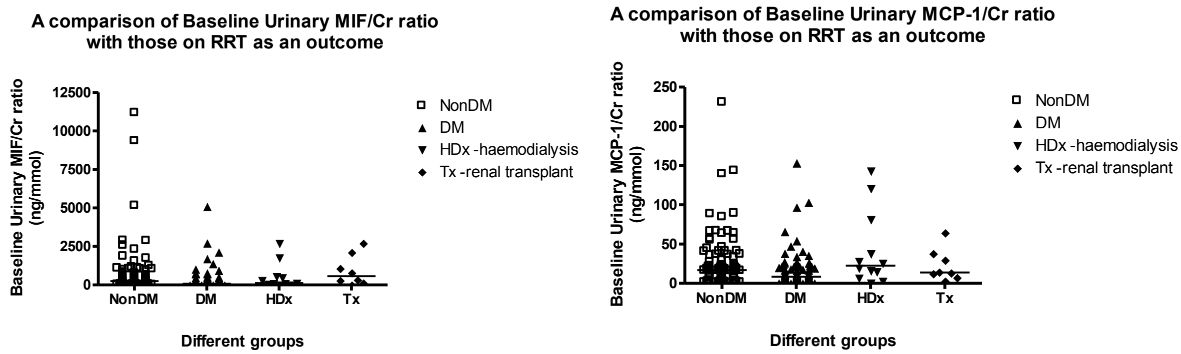
Summary

Serum and plasma levels correlate significantly in all cytokines in both DM and Non-DM groups. There is a higher level of significance with serum and plasma CCL18 in both groups. MIF and MCP-1 serum and plasma levels are slightly less significant in Non-DM patients. Serum levels of MIF were lower than plasma levels.

3.13 Cytokine profile in RRT, DM and Non-DM

The amount of cytokines measured in HD and RT patients were small. Those that went onto HDx and Tx within the follow up period had their baseline cytokines compared with DM and Non-DM group to determine whether a different cytokine profile was seen. The results are presented below

3.13.1 Urinary cytokines



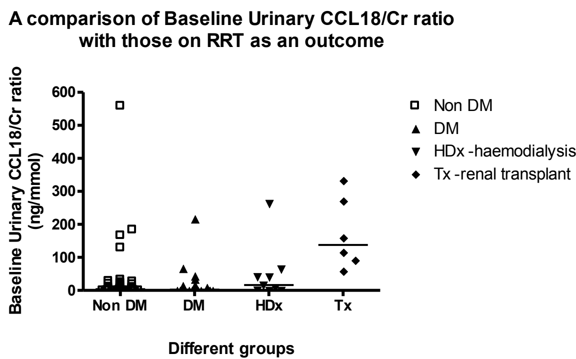


Figure 3.13.1 Urinary cytokines in RRT

Comparable levels of urinary MIF and MCP-1 were seen at baseline in all groups with little difference in those that went on to require RRT. The numbers in this group size were small but those leading to Tx had much higher urinary CCL18 levels compared with the other groups.

3.13.2 Plasma cytokines

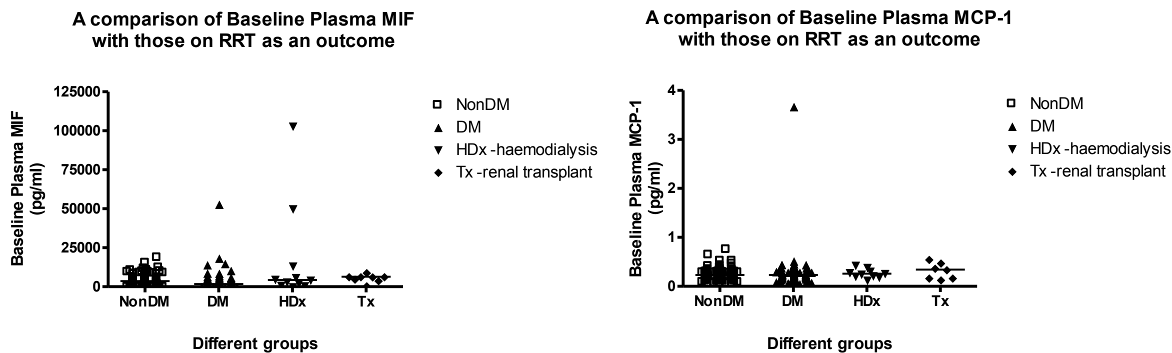


Figure 3.13.2 Plasma cytokines in RRT

A comparison of Baseline Plasma CCL18 with those on RRT as an outcome

Baseline Plasma CCL18 (pg/ml)

500 400 300 200 100 0

NonDM DM HDx Tx

Different groups

NonDM ▲ DM ▼ HDx -haemodialysis ◆ Tx -renal transplant

The DM and Non-DM groups appeared to have higher levels of baseline plasma MIF compared to those that went on to start RRT. There are comparable levels of plasma MCP-1 at baseline in all groups. There was a large spread of baseline plasma CCL18 levels between the groups. A larger sample size looking at the profile of those leading to RRT may illustrate a difference. In addition,

measuring the levels of cytokines in those on RRT may be informative although cytokines in RRT is a complex field with many variables affecting cytokines.

<u>Summary</u>

Higher baseline urinary CCL18/Cr ratio seemed to be more prevalent in patients needing RRT. There did not appear to be any trends in the remaining cytokines in baseline urine or plasma levels. A larger group size would be needed to confirm this finding as the effects of RRT on cytokines are multifactorial and complex, that is beyond the scope of this project.

3.14 Discussion

Despite modern medical therapy for the treatment of diabetes it is clearly seen in this cohort that the natural history of diabetes still continues to result in significant mortality. This prospective cohort study has a number of limitations that will be discussed in Chapter 5.0. The main limitation of this prospective cohort study is the sample size, whereby analysis of cytokines to determine the long-term outcome is affected by those who have died, have left the study by starting on RRT or have been lost to follow up by either returning to primary care or moving out of the area. This was not a matched controlled prospective cohort study and one of the strengths of the findings is the recruitment did not have a large exclusion criteria. Those excluded on the analysis were those with a urinary tract infection that could affect the interpretation of cytokine analysis. The findings of this study may be applicable to diabetic patients seen in clinic though larger multicentre studies would be required to ensure reproducibility of this novel data. Power calculations may be constructed with this data to adequately power future studies.

The prospective study shows there is a need to understand the factors leading to the progression of DN as 31.3% died by >3 years follow up compared with the proteinuric renal disease Non-DM group that had 8.6% deaths at >3 years. There are specific changes that occur in patients with DN compared with those with proteinuric renal disease leading to higher mortality. The mortality depicted is related to the lower GFR seen in this group, whereas the GFR is not significantly different in the Non-DM group over time.

The cytokine profile differs in DM and Non-DM groups. There is also a difference between cytokine measured and when the sample is taken in the disease process. There are good correlations between plasma and serum samples across all the cytokines, however, these do not correlate with the urinary levels. This reflects the complexity of the human body that allows cytokines not only to be systemically produced but to be consumed or produced locally in organs, e.g. the kidney.

Urinary cytokines

A difference is seen between the relationship of baseline urinary MIF, MCP-1 and CCL18 in Non-DM and DM patients when correlating these to GFR, ACR and UPCR. A raised baseline urinary MIF level is related to macroalbuminuria. Macroalbuminuria is related to high urinary MCP-1 and CCL18 levels. This observation may be causal, from the effects of the cytokines or an association, as increased albuminuria may reflect poor glomerular structure and as a consequence of this there maybe cytokine leakage. Interestingly, the findings with albuminuria change at >3 years, with high urinary MCP-1 and CCL18 levels correlated with macroalbuminuria, but urinary MIF no longer correlated with albuminuria. The latter would suggest that urinary cytokines identified in the urine at different time points are more likely to arise from synthesis rather than a problem with filtration.

High baseline urinary MIF levels in Non-DM are seen to correlate with a decreasing GFR that continues at >3 years. This was established using samples taken at these time points and correlating them with the urinary cytokine taken at the same time point. This indicates that some relationships between the cytokines and GFR do not change over time. In contrast the decrease in GFR seen at baseline with baseline plasma MIF in both DM and Non-DM is no longer seen in the >3 year samples.

High baseline urinary MCP-1 and CCL18 levels correlated with a lower GFR in DM patients. High urinary CCL18 continued to correlate with lower GFR in Non-DM group. At >3 years this was no longer seen, though high urinary MCP-1 levels at this longer time point correlated with a decrease GFR in Non-DM group. The clinical cohort shows that at >3 years DM had lower GFRs compared with Non-DM and this may reflect the loss of correlation between MCP-1 with declining GFR.

Urinary cytokines correlate with GFR, ACR and UPCR in DM and Non-DM groups. Changes in these correlations are seen over time. Increasing levels of urinary MIF and MCP-1 are seen over time in DM with no change in urinary CCL18 levels. In contrast, there were no changes in the urinary levels of cytokines detected in Non-DM patients. This may reflect a change in disease state that increases or decreases cytokine production and may help determine a cytokine profile for disease progression. Understanding why and how these changes occur may provide further insight into the progression of different disease states, in particular DN. New therapeutic targets may be identified with further understanding.

Urinary cytokine interactions

Urinary MIF rises with increasing urinary MCP-1 and CCL18 in Non-DM patients. In contrast, urinary CCL18 rises with increasing urinary MIF and MCP-1 in DM patients. These relationships were more significant in the baseline urinary findings in DM than Non-DM group. There was no correlation between urinary MIF and MCP-1 in DM. Cytokines have different relationships with GFR, ACR and UPCR in DM compared with Non-DM group. In addition, the observation that the cytokines do not all interact with each other suggests that cytokines have different functions that may protect or cause further damage or inflammation resulting in disease progression. This data suggests that cytokines may alter or regulate progression of disease as they up or down regulate at different points during the disease. The difference observed in levels of cytokines may support more than a biomarker role and may reflect involvement in the pathogenesis of disease. Chapter 4.0 looks to unravel the possible mechanism behind these observations on a cellular level.

Plasma cytokines

Baseline plasma cytokines and those at >3 years did not correlate with GFR, ACR or UPCR in DM or Non-DM. No correlations were seen, however, over time the levels of systemic cytokines altered. In both DM and Non-DM groups there was an increase in plasma levels of MIF, MCP-1 and CCL18. The increase seen in systemic levels did not result in a correlation with urinary levels except for plasma CCL18 levels that correlated with increasing urinary CCL18 levels in Non-DM at >3 years. This was not observed in the DM group despite comparable high plasma CCL18 levels and lower GFR. This supports there is a difference in the behaviour of the cytokines between DM and Non-DM patients. Interestingly, this also suggests that immunosuppression may not affect all cytokine actions as some Non-DM patients were on immunosuppression.

Plasma cytokine interactions

Plasma MIF and MCP-1 are seen to correlate at >3 years in DM, a relationship that was not seen at baseline. No other plasma cytokines were related. This prospective cohort illustrates how differently MIF, MCP-1 and CCL18 behave in DM and Non-DM despite the small group size. In addition, these interactions change with urinary and plasma levels. Plasma and serum levels correlate very strongly. The lack of interaction between the plasma and urinary levels suggest that the kidney may be able to produce these independently to the systemic circulation. The levels may be specific to disease occurring within the kidney itself.

Predictive value of cytokines

Univariant analysis

Univariant analysis was used to look at whether baseline urinary and plasma cytokines could predict a change in GFR, ACR or UPCR. No correlations were seen at >18 months. In >3 years there was a suggestion that high baseline plasma CCL18 levels correlated with less decline in GFR in DM. Urinary ACR was seen to worsen, i.e. increased ACR with high levels of baseline plasma MCP-1 in DM at >3 years that was consistent with baseline analysis. The limitation of this analysis is the inability to account for different variables, such as increasing age, change of BMI over time.

Mixed models analysis

The model used within this chapter used baseline cytokine measurements to predict outcome in DM and Non-DM. The prelude to this model illustrates how the cytokines behave at baseline and over time and how they interact between systemic circulating levels and those measured in the urine. I have explored how the cytokines interact with each other and how this changes over time. The mixed model is limited due to the number of patients lost to follow up, those who have died or commenced RRT. Despite these limitations the model shows that high baseline plasma CCL18 and plasma LogMIF levels in DM correlate strongly with a decreasing GFR. Baseline plasma CCL18 correlation weakens over time. Baseline plasma Log MIF correlates with decreasing GFR at >18 months but this relationship is lost by >3 years. Identifying high plasma CCL18 and Log MIF could allow identification of patients that would progress more rapidly and benefit from closer follow up. This could help unravel what mechanisms occur to cause disease progression compared with those that have DM with no or slower progression rate. The model supports a role for cytokine measurements in the clinical environment whilst understanding how and why the changes occur over time becomes the challenge to determine the mechanisms driving the disease. There are limitations within this model as it is unable to differentiate the speed with which an individual within a group may deteriorate and hence in order to address this, a larger multicentre trial would need to be undertaken. The novel findings of this cohort can be used to establish a power calculation that would allow a sufficiently large number of patients to be followed up and for a number of cytokines to be analysed prospectively. The number of patients lost throughout the trial would be able to be approximated on the basis of this cohort study thereby allowing findings to be robustly tested.

There are limitations to both univariant and mixed model analysis used for determining predictability of baseline cytokines with outcomes. The major limitation is that of sample size and in the model

the inability to differentiate who within the group has a faster declining GFR. Both analyses suggest that it may be possible to predict some outcome from a set of baseline cytokines.

Cytokine Profile

There is a suggestion from looking at the baseline cytokines in those that went on to require RRT that there were higher urinary CCL18/Cr ratio levels although a larger study would be needed to determine whether this finding is predictive.

Proposed pathways of observed associations of cytokines on; GFR, ACR, UPCR and cytokines.

The cytokines have been seen to interact with each other and; GFR, ACR or UPCR in different ways. I have used the correlations seen within this cohort to propose the associations seen on decreasing GFR and increasing ACR in DM patients and on decreasing GFR and increasing UPCR in Non-DM patients. I have illustrated these in Fig 3.14.0 and 3.14.1, respectively.

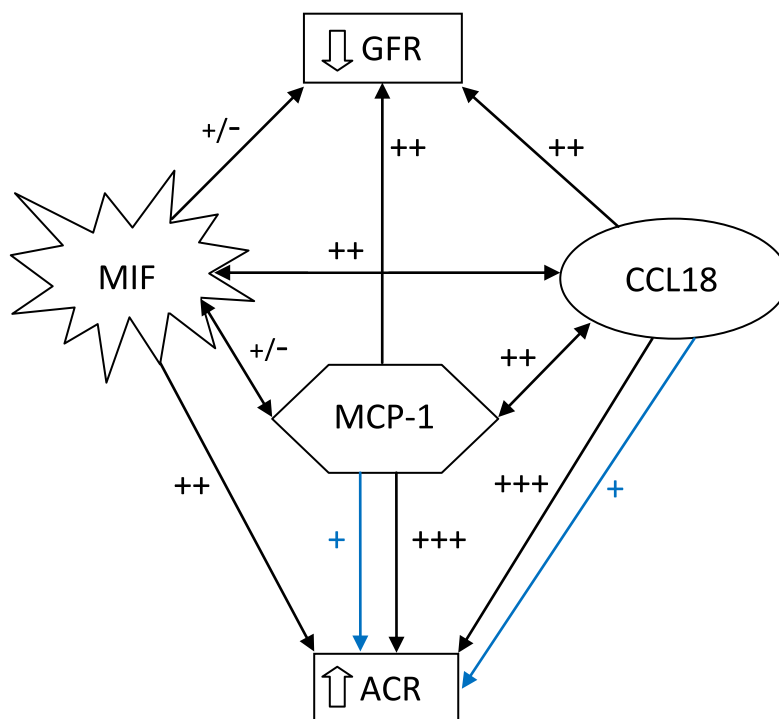


Figure 3.14.0 Interactions of urinary cytokines between each other and their effects on GFR and ACR in DM. The black arrows are correlations seen at baseline with blue arrows illustrating those seen at >3 years. The figure shows how higher levels of urinary CCL18 and MCP-1 are seen in DM and occur with a decrease in GFR compared with Non-DM (Fig 3.14.1). Large amounts of urinary CCL18, MCP-1 and MIF are seen in DM with increase ACR, in contrast to proteinuric Non-DM

renal diseases that have slightly raised urinary MIF, therefore the cytokines distribution is specific to the diabetic environment.

These differ from those seen in Non-DM seen below.

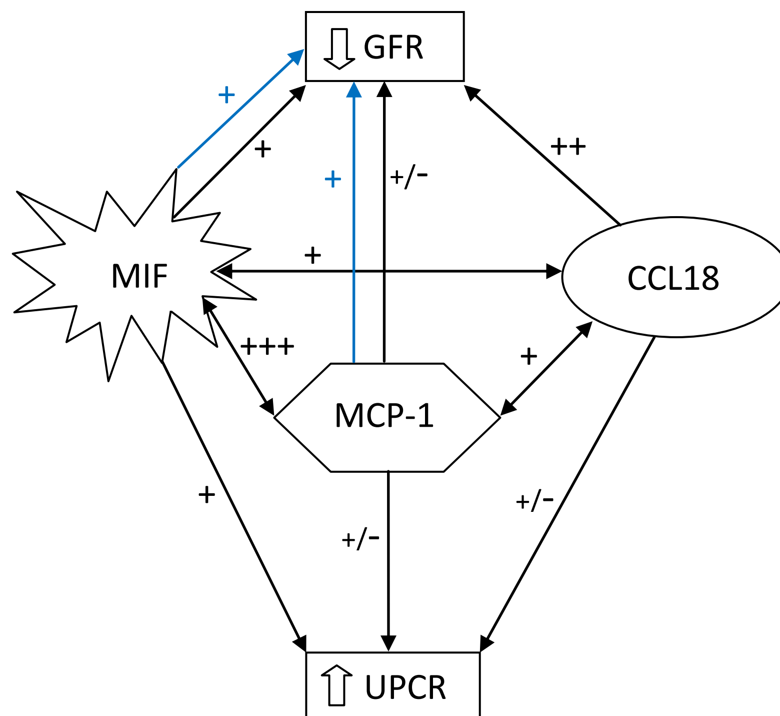


Figure 3.14.1 Interactions of urinary cytokines between each other and their effects on GFR and UPCR in Non-DM. The black arrows are correlations seen at baseline with blue arrows illustrating those seen at >3 years. Urinary MIF and CCL18 at baseline are seen to contribute to a decline in GFR in proteinuric Non-DM renal disease in contrast to that seen in DM (Fig 3.14.0), Increase UPCR is seen with increased urinary MIF thus illustrating a different balance of urinary cytokines seen in DM with raised ACR.

A decreasing GFR in DM patients is associated with an increase in urinary MCP-1 and CCL18, in contrast to Non-DM group where urinary MIF and CCL18 are seen to have the greater effect in decreasing GFR. All urinary cytokines have an effect on rising ACR. In contrast, an increase in urinary MIF is associated with a high UPCR. Urinary CCL18 correlates with MIF and MCP-1 in DM, however, urinary MIF seems to be related strongly with MCP-1 and less with CCL18 in Non-DM patients. There were no correlations with baseline plasma cytokines in DM or Non-DM groups and these have not been illustrated. Plasma MIF and MCP-1 were seen to correlate at >3 years in DM with no other correlations or effects. These findings suggest there may be local mechanisms in

the kidney that are contributing to the production of these cytokines. In addition it is possible these observations suggest a communication between the cytokines that is explored further in Chapter 4.0.

Cytokines differ in DM and Non-DM groups and a larger sample size would allow further analysis within the different levels of albuminuria in DM patients to determine whether this is more predictive of the associations seen in Fig 3.14.0. Ideally the cohort would have large enough numbers to have an immunosuppressed and non-immunosuppressed arm to draw comparisons between the effects of immunosuppression on cytokine levels. Unfortunately, the sample size did not allow for the separate effects of immunosuppression itself on GFR to be established. An interaction was found between the GFR of those immunosuppressed and gender. This may reflect a difference in gender or be a reflection of the disparate numbers of male/females in each group. A future larger study would require a more uniform Non-DM group to be recruited and followed up prospectively.

The following chapter aims to explain some of the clinical findings herein and the results of both of these chapters will be discussed in Chapter 5.0.

CHAPTER 4.0 – RESULTS – SCIENTIFIC BASIS FOR CLINICAL FINDINGS

This chapter aims to explain the mechanism underlying the production of cytokines MIF, MCP-1 and CCL18 found in the urine of DN patients. In addition, I shall investigate which renal cells produce these cytokines and whether there is any communication or feedback mechanism allowing the levels of these cytokines to be enhanced or reduced depending on the local environment using *in vitro* experiments. Mesangial cells (tHMC), tubuloepithelial cells (HK2) and podocytes, as human cell lines, are used to help discover the mechanisms behind the clinical findings. Following pilot cell culture data, further cell culture experiments were carried out in HK2 cells and podocytes to determine whether the original findings were reproducible and to allow statistical evaluation of the findings. Each chosen experiment was repeated three times on different days to ensure the results were reproducible. Each condition was repeated 6 times (n=6). In total all these experiments would have the same condition 18 times. The results were not pooled but analysed as per each experiment (n=6) so statistically the results can be compared with regard to reproducibility.

MTT assays were done on the HK2 cells and podocytes experiments to help determine the effect of cell viability on interpretation of the changes in cytokines. Cell signalling and activation of apoptotic pathways are briefly looked at to ensure the findings are not secondary to activation of cell death pathways. A discussion at the end of this chapter will establish the laboratory findings and potential pathways of communication between the different cytokines. Chapter 5 – the final discussion will combine the clinical findings of Chapter 3 with the scientific findings of Chapter 4 to determine whether the scientific findings are translational.

MIF and MCP-1 have been previously seen with IHC in the glomerulus of patients with DN. CCL18 has been stained in gastric tissue but never in renal biopsy tissue. In order to determine whether CCL18 is found in renal biopsy tissue and where it is localised IHC was undertaken. It is unknown whether CCL18 is found within intrinsic renal cells or cells infiltrating the renal interstitium. This would help to explain whether the urinary CCL18 found in DN patients is localised to a particular area or cell type within the kidney.

The production of cytokines in intrinsic renal cells was determined with *in vitro* experiments of different purified renal cells: tHMC, HK2 cells and podocytes. The following experiments determine whether these cell types are able to produce the said cytokines, whether this alters with the basal conditions and whether levels of the cytokines produced alter with recombinant stimulation of

the cytokine in a particular environment. This allows us to determine what cytokines cells produce, the influence of environment on this production, together with determining any communication or feedback systems that occur between the cytokines in the different environments i.e. is there natural inhibition or self-induction of cytokines from the intrinsic renal cells in high glucose or glycated albumin.

A 48 hour time point was taken following pilot data not presented herein. tHMC and podocytes were stimulated by a colleague with high glucose over a period spanning 4 weeks. The results presented in my mid-MD report showed little difference between the production of cytokines over the weeks and hence a 48 hour time point was chosen for the pilot data presented herein for all the intrinsic cell types. tHMC stimulated with normal glucose, mannitol and high glucose produced low levels of CCL18 that remained constant for the 4 weeks, with little variation between stimuli. I also analysed the supernatant of these samples in podocytes that showed slightly higher levels of CCL18 after 1 week of stimulation with normal glucose, mannitol and high glucose that again remained stable over the 4 weeks.

Fibronectin was measured in the supernatants from the HK2 cells and podocyte experiments using ELISA. The effects of cytokines on fibronectin production, was determined with statistical analysis. This aimed to support whether production of cytokines involved in inflammation contributes to fibrosis. CCL18 has previously been described to have an independent TGF-β pathway to fibrosis that may support the finding of higher urinary CCL18 levels in DN patients seen in this cohort.

Cell signalling involving active phospho p38 MAPK and phospho p44/42 MAPK were looked at with Western blot, as these are known to be activated by MCP-1 and MIF respectively. It is unknown whether these cell signalling pathways are activated or used by CCL18 to induce its effects. Caspases were also looked at with Western blot to ensure the findings were not a phenomena arising from cell death. MTT assay in combination with the Western blots suggest interactions between the cytokines is not secondary to cell death.

4.1 Immunohistochemistry (IHC)

Based on the published literature I optimised the conditions for IHC of CCL18 in human histopathological samples using gastric cancer tissue (Leung, Yuen et al. 2004). Gastric cancer tissue was stained for CCL18 using the protocol described in Chapter 2 section 2.2. Six gastric cancer tissue samples were stained to ensure reproducible results. This positive control located CCL18 in cells near the margin of tumour invasion (Fig 4.1A).

Native renal biopsy tissue from 6 patients with DN, was stained for CCL18. The antibody was reconstituted to a concentration of 100µg/ml and the following dilutions were used; 1:50, 1:100, 1:200, 1:500 initially in gastric cancer tissue. The 1:100 dilution of CCL18 was subsequently used for renal biopsy staining. CCL18 Ab used was a rabbit polyclonal antibody and rabbit serum was used as a control, with 0.01% rabbit serum concentration allowing distinction of CCL18 staining from the background (Fig 4.1B). PBS/Tween (Fig 4.1C) was another control used in both gastric cancer tissue and subsequently renal biopsy tissue. CCL18 was not found in renal DN biopsy tissue (Fig 4.1D). Six sections from native renal biopsies with tubulointerstitial nephritis were also stained for CCL18 as this condition also had patients producing large amounts of urinary CCL18 (Qureshi et al, unpublished results). CCL18 was not present in this tissue (Fig 4.1E). Different microwave times were used to determine whether this affected CCL18 staining in renal biopsy tissue together with longer Ab incubation; there was still no localisation of CCL18 in renal biopsy tissue.

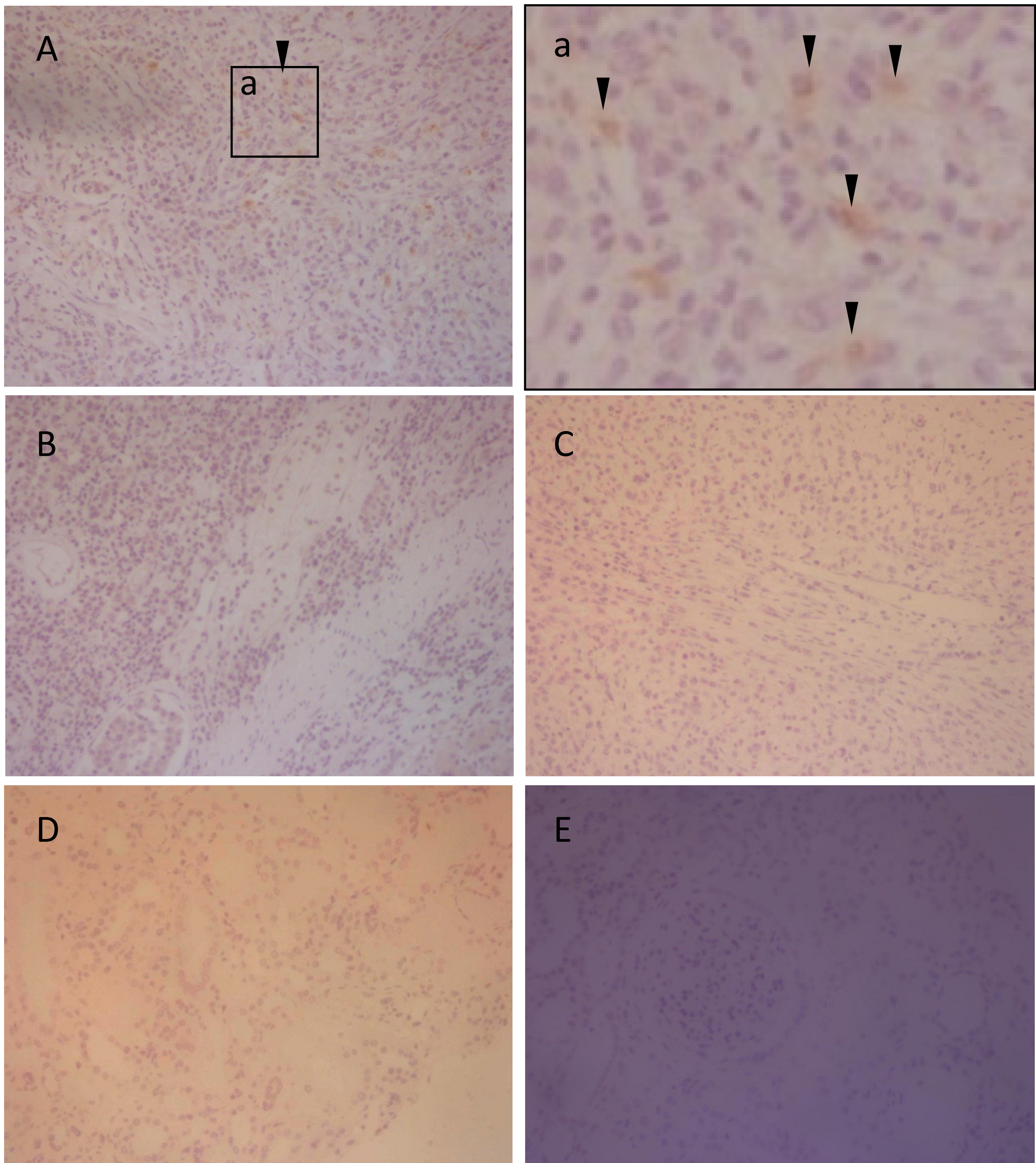


Figure 4.1 CCL18 IHC in gastric tissue with controls and in DN and TIN renal biopsy A=gastric tumour tissue with CCL18 shown with arrows, a=expanded view of gastric tumour tissue showing CCL18+ cells at the tumour invasion front shown with black arrows. Macrophage morphology of CCL18+ cells light brown staining extracellular. B=gastric tumour tissue with rabbit serum background control, C=gastric tumour tissue PBS/Tween background control, D=CCL18 in DN renal biopsy, E=CCL18 in TIN renal biopsy.

4.2 Cell culture in diabetic milieu; Pilot study data

This pilot study was done to determine whether the intrinsic renal cells of the kidney would produce MIF, MCP-1 or CCL18 in diabetic conditions or their controls. The pilot study went on to determine if the levels of these cytokines changed when stimulated with extracellular or external stimulus with a particular cytokine, i.e. stimulation of MIF may increase MIF but increase or decrease MCP-1 or CCL18. The effect of this stimulus may change with the DM environment and with the dose. A dose response was done in these pilot studies to determine the amount of external cytokine needed to have an effect. The outcomes of the pilot studies were used to decide what conditions and dose of cytokine should be used to stimulate the cells in subsequent experiments. This design also allows the interactions between the different cytokines to be explored.

In this pilot study all cell culture experiments were incubated in; normal glucose concentration (4mM D-glucose), mannitol (26mM+4mM D-glucose), high glucose concentration (30mM D-glucose) or glycated albumin (100µg/ml+4mM D-glucose (A1), 500µg/ml+4mM D-glucose (A2)) for 24 hours. Supernatant was collected; however, time limitations did not allow these to be analysed. Diabetic stimuli were applied for a further 48 hours with the addition of recombinant cytokine at doses of 0ng/ml, 2ng/ml, 10ng/ml and 20ng/ml to determine a dose response, (see Fig 4.2.0). Each condition was in duplicate (n=2), to determine the optimum condition and cytokine concentration. The supernatant and cell lysates were collected following 48 hours of stimulation. Supernatant was analysed for MIF, MCP-1 and CCL18 with ELISAs. Cell lysates were collected for DNA analysis and stored in trysol at -80ºC.

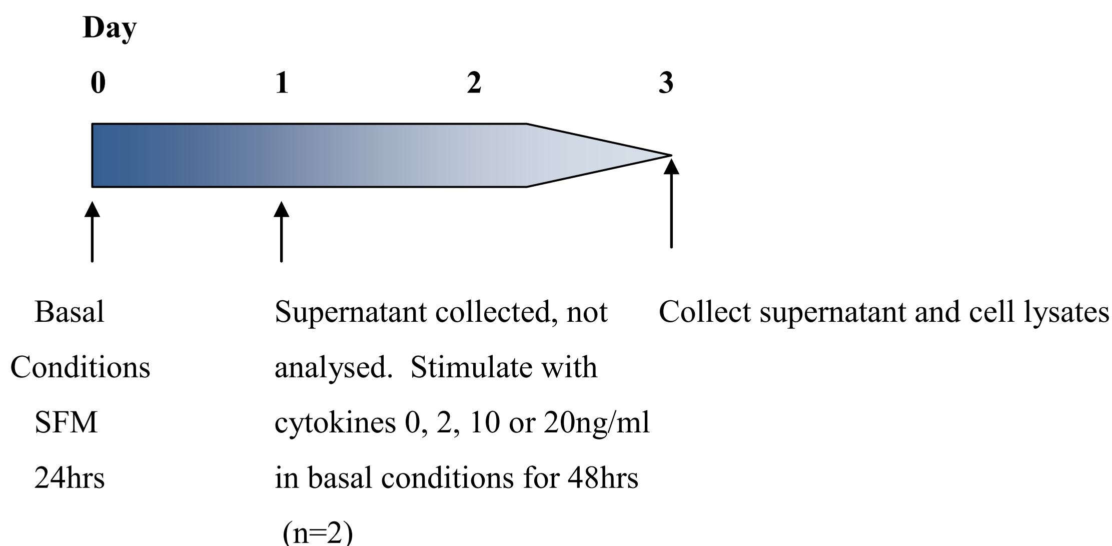


Figure 4.2.0 Timeline for pilot experiment stimulation (Key: SFM= serum free media, n= number of duplicates per condition)

4.2.1 Transformed human mesangial cells (tHMC) stimulated with diabetic milieu over 48 hours

Basal levels of MIF, MCP-1 and CCL18 are determined by ELISA in tHMC with n=2 as the pilot study.

Basal MIF levels in tHMC

More MIF was produced basally in mannitol in tHMC stimulated for 48 hours see Fig 4.2.1.0. The median detectable concentrations of MIF in normal glucose were 8650pg/ml, glycated albumin 1 were 6150pg/ml, glycated albumin 2 were 8870pg/ml, mannitol were 12300pg/ml and high glucose were 9120pg/ml. There was no significant difference between the groups. MIF is detected in tHMC in the different basal conditions.

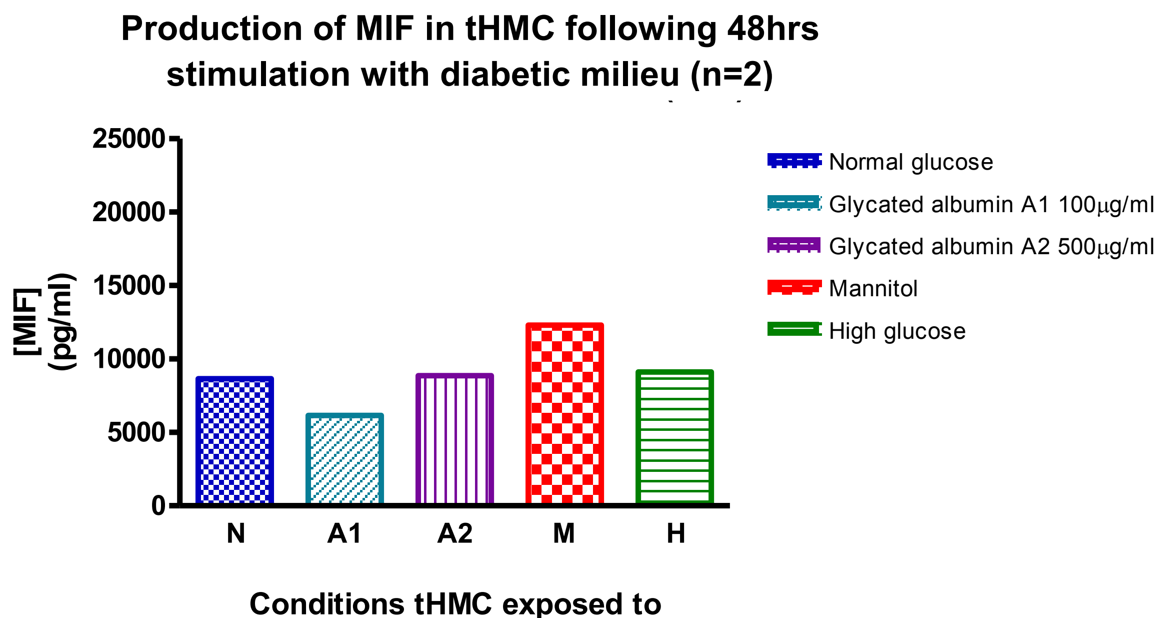


Figure 4.2.1.0 MIF production after 48 hours of basal condition stimulation

Basal MCP-1 levels in tHMC

There was no production of MCP-1 in tHMC as the levels were below the sensitivity of the standard curve for the MCP-1 ELISA (n=2), however in one experiment the basal levels of MCP-1 in tHMC were raised. See Fig 4.2.1.0.1. The median MCP-1 concentrations for normal glucose were 190pg/ml, glycated albumin 1 were 285pg/ml, glycated albumin 2 were 226pg/ml, mannitol were 146pg/ml and high glucose were 248pg/ml. There was no significant difference between the groups.

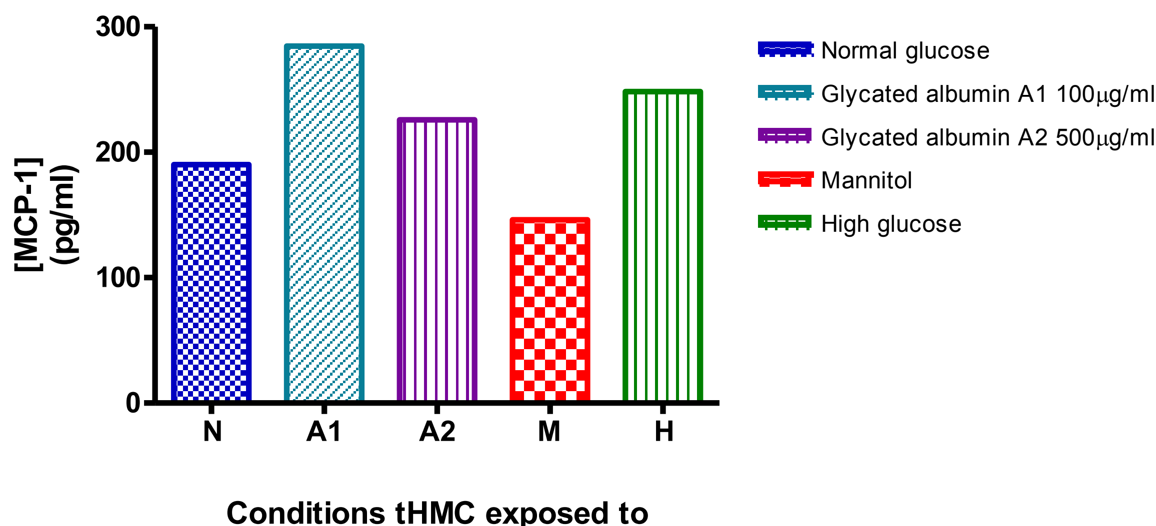


Figure 4.2.1.0.1 MCP-1 production after 48 hours of basal condition stimulation

Basal CCL18 levels in tHMC

There was no CCL18 in the supernatant of tHMC at 48 hours as the levels were below the sensitivity of the standard curve for the CCL18 ELISA (n=2).

4.2.1.1 tHMC stimulated with MIF over 48 hours

All the following experiments have (n=2) per condition and dose. In view of the large quantities of MIF detected the y axis has been presented as a log scale. Increasing dose of recombinant MIF (rMIF) were added to tHMC and collected following 48 hours.

MIF in tHMC stimulated with rMIF

The basal levels of MIF in tHMC appear to decrease with stimulation of its recombinant form in ng/ml irrespective of the background diabetic stimulus. Increasing dose of rMIF reduces measurable amounts of MIF in the supernatant see Fig 4.2.1.1.0. The higher levels seen at basal conditions need to be repeated as (n=2) and this pilot may reflect true inhibition or higher basal MIF levels due to cell proliferation or death. Morphologically the cells appeared viable.

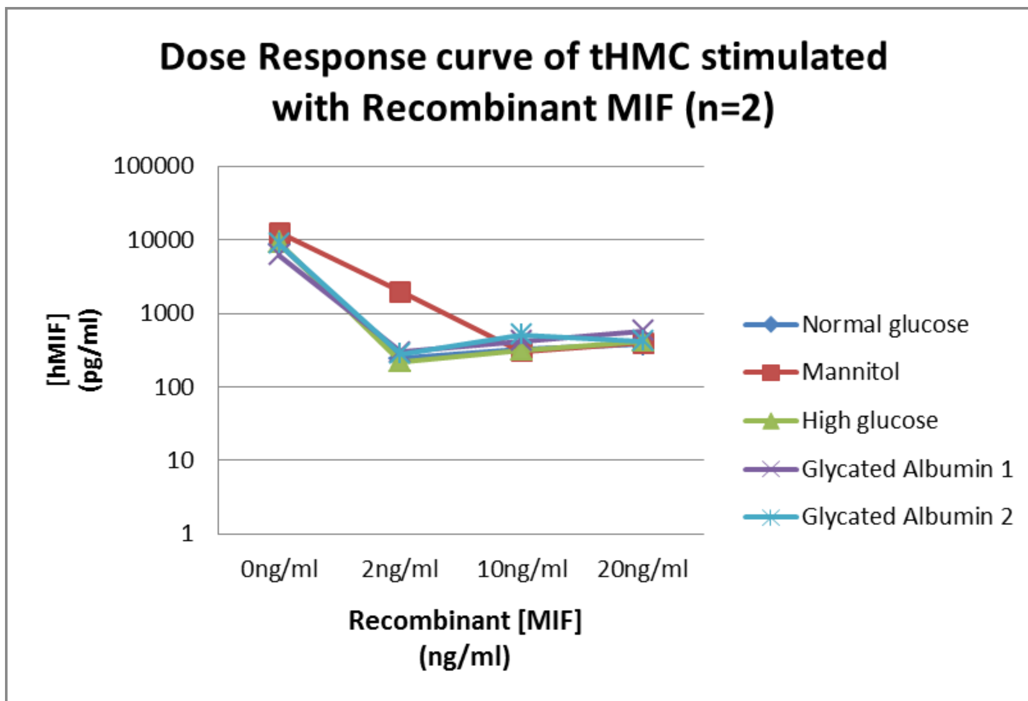


Figure 4.2.1.1.0 Dose response curve of rMIF in tHMC

MCP-1 in tHMC stimulated with rMIF

There is a decline in the levels of MCP-1 following stimulation with rMIF, suggesting a dose response change. There appears to be an inhibition of MCP-1 with high rMIF stimulation in tHMC that is non-specific to the diabetic conditions, see Fig 4.2.1.1.1. In other experiments the MCP-1 basal levels have been beneath the sensitivity of the MCP-1 standard curve thus it is unclear whether there is a true reduction in MCP-1 with rMIF stimulation.

This pilot experiment would need to be repeated to establish the actual basal production of MCP-1 in tHMC. Morphologically there was no difference between the basally stimulated cells and those stimulated with rMIF.

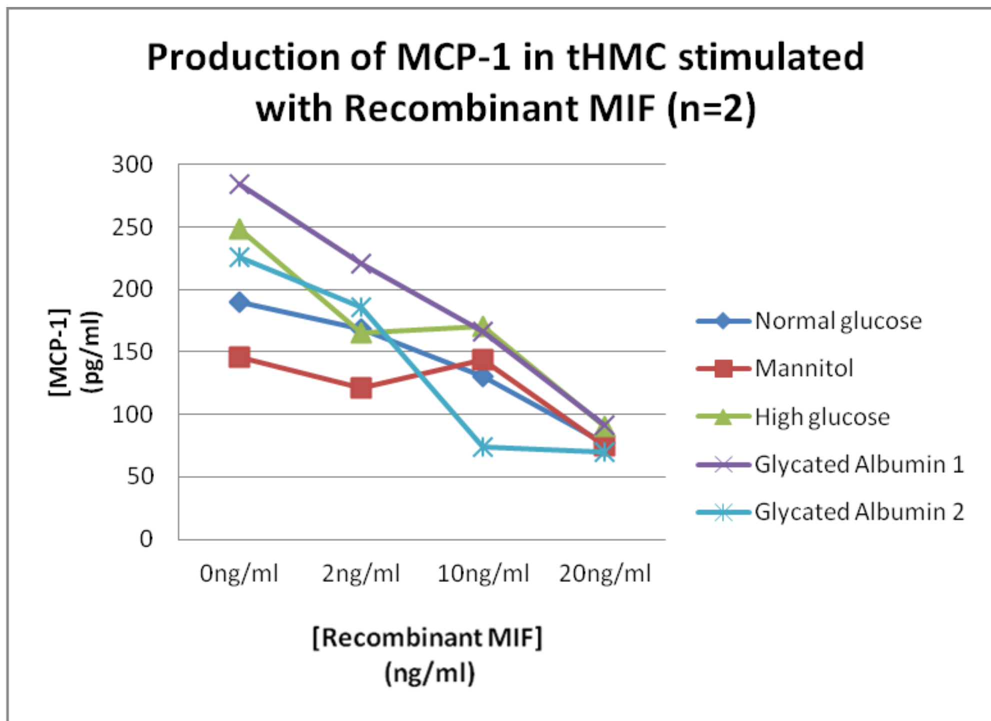


Figure 4.2.1.1.1 MCP-1 production after 48 hours of rMIF stimulation

<u>*CCL18 in tHMC stimulated with rMIF*</u>

There were no detectable levels of CCL18 following increasing stimulation of tHMC with rMIF in any of the conditions.

<u>***Summary of results***</u>

tHMC stimulated with increasing dose of rMIF results in a decrease in MIF levels and a sharp decline in the production of MCP-1 in all conditions. No CCL18 was detected in tHMC following 48 hours of stimulation in the basal conditions or following rMIF in these conditions. These experiments need to be repeated to ensure these findings are robust as n=2 was used for this pilot.

4.2.1.2 tHMC stimulated with MCP-1 over 48 hours

All the following experiments were (n=2) per condition and dose. Increasing doses of recombinant MCP-1 (rMCP-1) were added to tHMC and collected following 48 hours.

<u>*MCP-1 in tHMC stimulated with Recombinant MCP-1*</u>

Stimulation of tHMC with increasing doses of rMCP-1 in ng/ml results in a rise of MCP-1 detected by ELISA in all the background conditions. Higher doses of rMCP-1 appeared to trend higher levels of MCP-1 in diabetic conditions, see Fig 4.2.1.2.0.

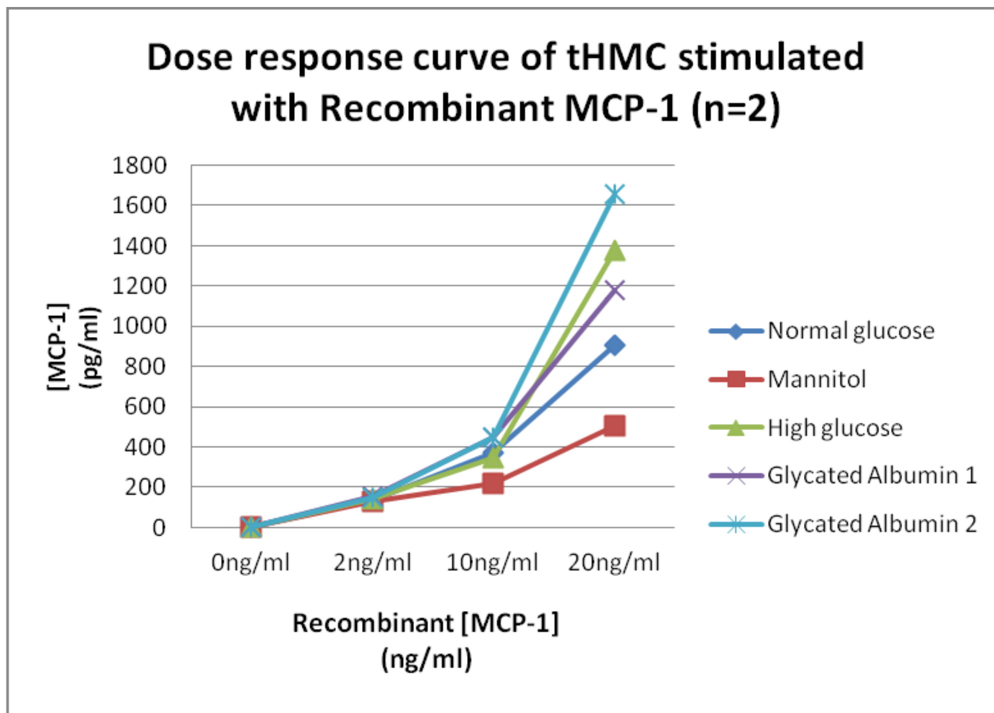


Figure 4.2.1.2.0 Dose response curve of MCP-1 in tHMC

MIF in tHMC stimulated with rMCP-1

Stimulation with 2ng/ml of rMCP-1 appears to increase the levels of MIF in tHMC, however, this trend changes with a higher dose of rMCP-1 where MIF levels return to their basal production, see Fig 4.2.1.2.1. High glucose conditions appear to raise MIF levels slightly above basal levels when stimulated with 20ng/ml of rMCP-1; however, the numbers in this pilot study are small and would need to be repeated to ensure this finding is reproducible.

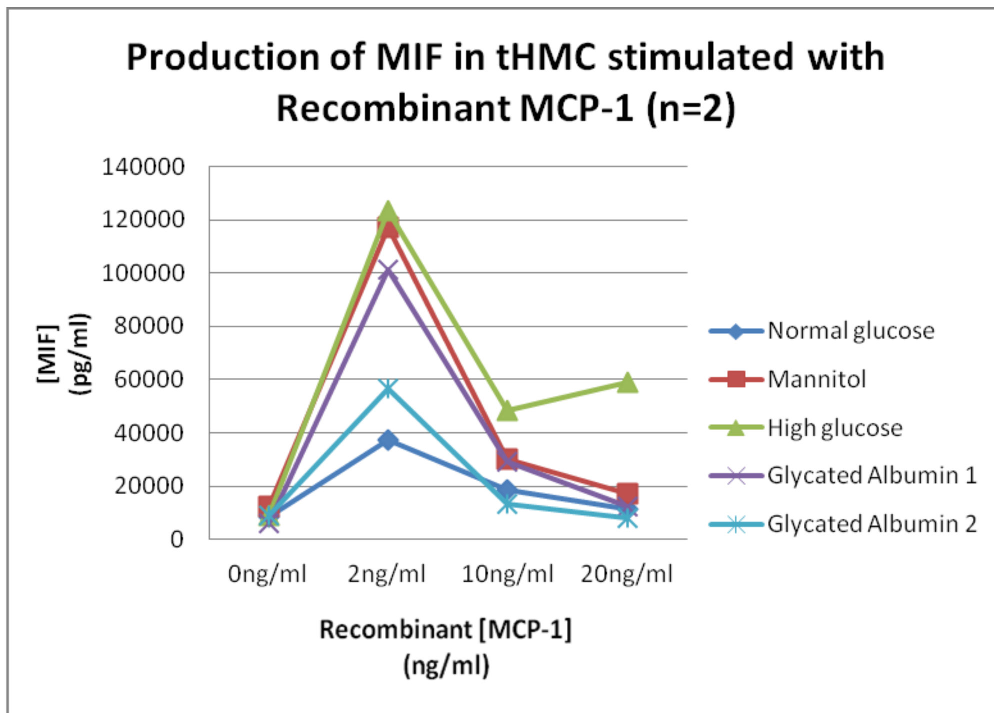


Figure 4.2.1.2.1 MIF production after 48 hours of stimulation with rMCP-1

<u>*CCL18 in tHMC stimulated with rMCP-1*</u>

There were no detectable levels of CCL18 following increasing stimulation of tHMC with rMCP-1 in any of the conditions.

<u>***Summary of results***</u>

tHMC stimulated with rMCP-1 results in a dose response curve that has higher levels of MCP-1 in diabetic conditions. Increasing rMCP-1 in tHMC results in levels of MIF peaking at a lower 2ng/ml dose of rMCP-1 that decreases to basal levels with high dose rMCP-1. High glucose seems to induce more MIF than basal conditions when stimulated with 20ng/ml of rMCP-1. There is no detection of CCL18 with the stimulation of rMCP-1.

4.2.1.3 tHMC stimulated with CCL18 over 48 hours

All the following experiments were (n=2) per condition and dose. Increasing doses of recombinant CCL18 (rCCL18) were added to tHMC and collected following 48 hours.

CCL18 in tHMC stimulated with rCCL18

Stimulation of tHMC with increasing doses of rCCL18 results in a steady rise of CCL18 detected by ELISA in all basal conditions, see Fig 4.2.1.3.0. The effect appears to be more prominent in high glucose and glycated albumin conditions.

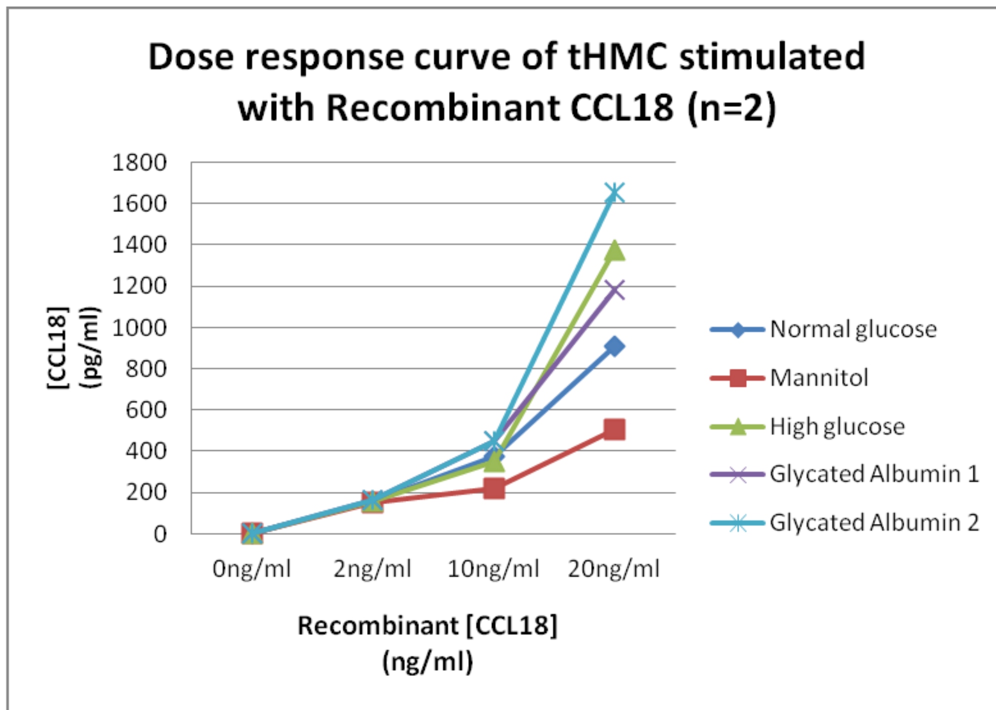


Figure 4.2.1.3.0 Dose response curve of rCCL18 in tHMC

MIF in tHMC stimulated with rCCL18

MIF levels appear to increase in tHMC following stimulation with high dose 20ng/ml of rCCL18 in all conditions, see Fig 4.2.1.3.1.

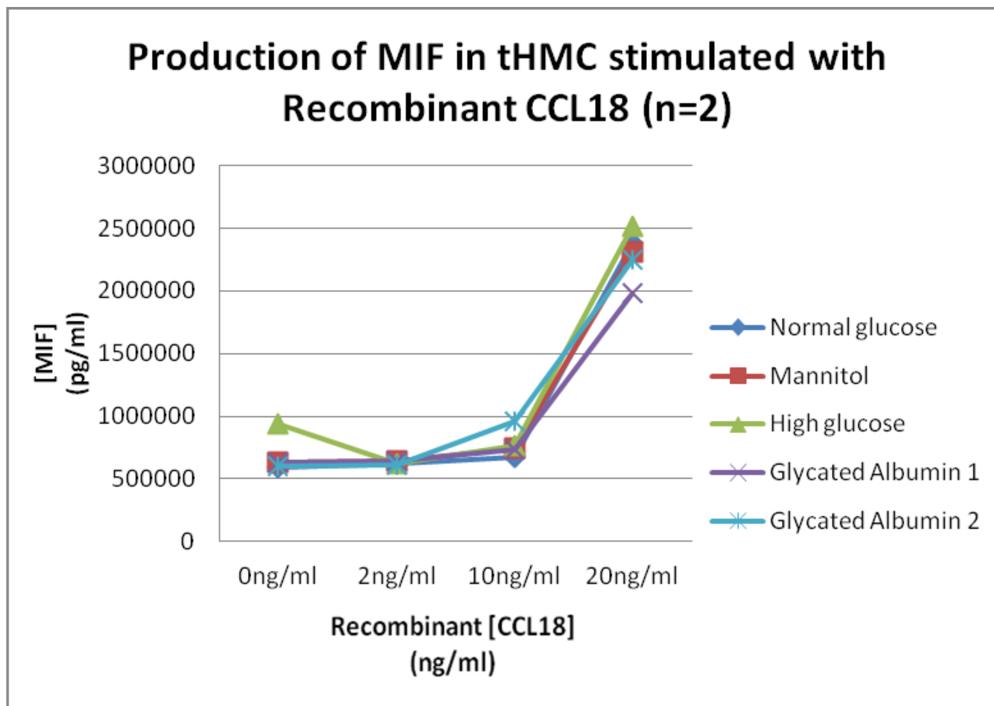


Figure 4.2.1.3.1 MIF production after 48 hours of rCCL18 stimulation

MCP-1 in tHMC cells stimulated with rCCL18

There are no detectable levels of MCP-1 following increasing stimulation of tHMC with rCCL18 in any of the conditions. The amounts fall below the sensitivity of the MCP-1 standard curve.

Summary of results

tHMC stimulated with rCCL18 results in a dose response curve with higher levels of CCL18 detected in diabetic condtions. MIF levels increase following stimulation with high dose rCCL18 in all conditions. MCP-1 is not detected following stimulation with rCCL18.

Summary of results in tHMC

tHMC in all basal conditions are able to produce MIF and MCP-1 cytokines in vitro. There is no basal production of CCL18 in tHMC at 48 hours. Stimulation with high dose rMIF results in a decline in MCP-1. Stimulation with low dose rMCP-1 results in a rise in MIF that is attenuated with increasing doses of rMCP-1. These effects on MIF are more pronounced in high glucose conditions. There is no effect on CCL18 production with rMIF or rMCP-1 stimulation regardless of the basal conditions. Stimulation with rCCL18 however, results in a dramatic increase in MIF with high dose rCCL18 stimulation. tHMC are able to produce or release MIF and MCP-1 in certain conditions and respond to CCL18. CCL18 may need stimulation in basal conditions for

longer as tHMC has previously been seen to have CCL18 at one week post stimulation. No further experiments done on tHMC in this thesis.

4.2.2 HK-2 cells in diabetic milieu over 48 hours

HK2 cell pilot data is shown below for MIF levels at basal conditions and for HK2 cells stimulated with rMIF over 48 hours. The pilot experiments showing basal MCP-1 and CCL18 production and following stimulation with MCP-1 and CCL18 are seen in Appendix 4.0. These initial results have not been shown herein as these HK2 experiments were repeated three times with n=6 each time. These are presented in section 4.3 with reference to whether these findings are consistent with the pilot data. HK2 cells in various basal conditions produced varying levels of cytokines at 48 hours, see figures below.

<u>*Basal MIF levels in HK2 cells*</u>

There are variable amounts of MIF produced in the different conditions following 48 hours of stimulation with basal conditions. The median MIF concentrations in normal glucose were 24200pg/ml, glycated albumin 1 were 12200pg/ml, glycated albumin 2 were 15400pg/ml, mannitol were 9910pg/ml and high glucose were 10900pg/ml. Glycated albumin levels of basal MIF are lower than its control normal glucose.

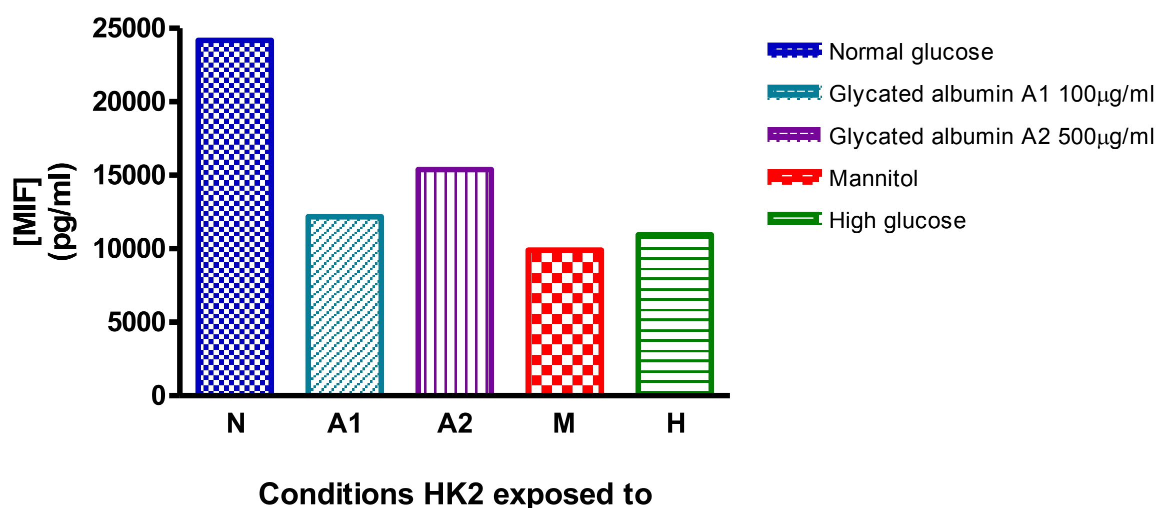


Figure 4.2.2.0 MIF production after 48 hours of basal condition stimulation

4.2.2.1 HK-2 cells stimulated with rMIF over 48 hours

All the following experiments were (n=2) per condition and dose.

MIF in HK2 cells stimulated with rMIF

There is a sharp rise in MIF with stimulation of HK-2 cells with 10ng/ml of rMIF, see Fig 4.2.2.1.0. This occurs in all diabetic milieu, however, at 20ng/ml only glycated albumin 1 and 2 (100µg/ml, 500µg/ml, respectively) continue to maintain higher MIF levels seen at 10ng/ml.

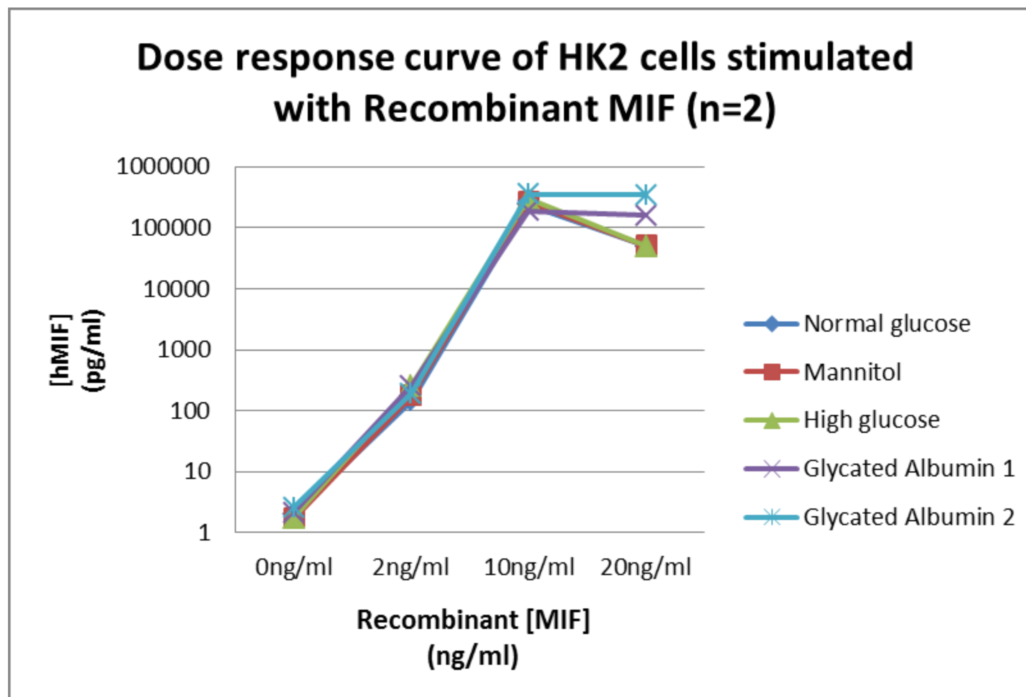


Figure 4.2.2.1.0 Dose response curve for rMIF in HK2 cells

MCP-1 in HK2 stimulated with rMIF

There were no detectable levels of MCP-1 following increasing stimulation of HK2 cells with rMIF in any of the conditions.

CCL18 in HK2 cells stimulated with rMIF

Detectable levels of CCL18 were only found in HK2 cells stimulated for 48 hours in a single well of normal glucose and mannitol stimulated with 20ng/ml of rMIF (20.2pg/ml and 13.1pg/ml respectively). CCL18 was otherwise not detected in HK2 cells following stimulation with rMIF.

Summary of results

HK2 cells stimulated with rMIF result in higher levels of MIF in all conditions. rMIF had no discernible effect on the detection of MCP-1 or CCL18. There is a suggestion that CCL18 may be found in mannitol and normal glucose following high doses of rMIF stimulation.

Summary of results in HK2 cells

All basal conditions are able to produce MIF in vitro in HK2 cells. Stimulation with rMIF seems to induce a larger difference in glycated albumin conditions. In the pilot data seen in Appendix 4.0 showed rMCP-1 stimulation slightly increased detectable levels of MIF in HK2 cells. MIF production in HK2 cells was attenuated with high dose stimulation with rCCL18 in all conditions. The decrease in MIF was more evident in glycated albumin. The effects of rMCP-1 and rCCL18 will be further determined in the repeat experiments in section 4.3 together with the basal production of these cytokines in HK2 cells.

4.2.3 Human Podocytes stimulated with diabetic milieu over 48 hours

Podocytes stimulated with diabetic milieu are able to produce MIF and MCP-1 *in vitro*. In particular, MIF is seen in high concentrations in this cell type. The pilot data for rMIF and rMCP-1 stimulation of podocytes is shown in Appendix 4.0 with formal repeated rMIF and rMCP-1 podocyte experiments presented herein. The effects of rCCL18 are presented below.

Basal CCL18 levels in Podocytes

There was no production of CCL18 in Podocytes as the levels were below the sensitivity of the standard curve for the CCL18 ELISA (n=2).

Summary

The level of MIF measured in all podocyte conditions decreased following stimulation with increasing rMIF in diabetic conditions. MCP-1 production increases with 2 and 10ng/ml doses of rMIF and then decreases with high dose rMIF stimulation. See Appendix 4.0 for details. Formal experiments repeated section 4.3. Stimulation with high dose 20ng/ml rMIF results in a rise in CCL18 production.

4.2.3.1 Human Podocytes stimulated with rCCL18 over 48 hours

All the following experiments were (n=2) per condition and dose.

<u>*CCL18 in Podocytes stimulated with rCCL18*</u>

There is a dose related rise in the level of CCL18 in podocytes following stimulation with rCCL18 regardless of the basal conditions, see Fig 4.2.3.1.0.

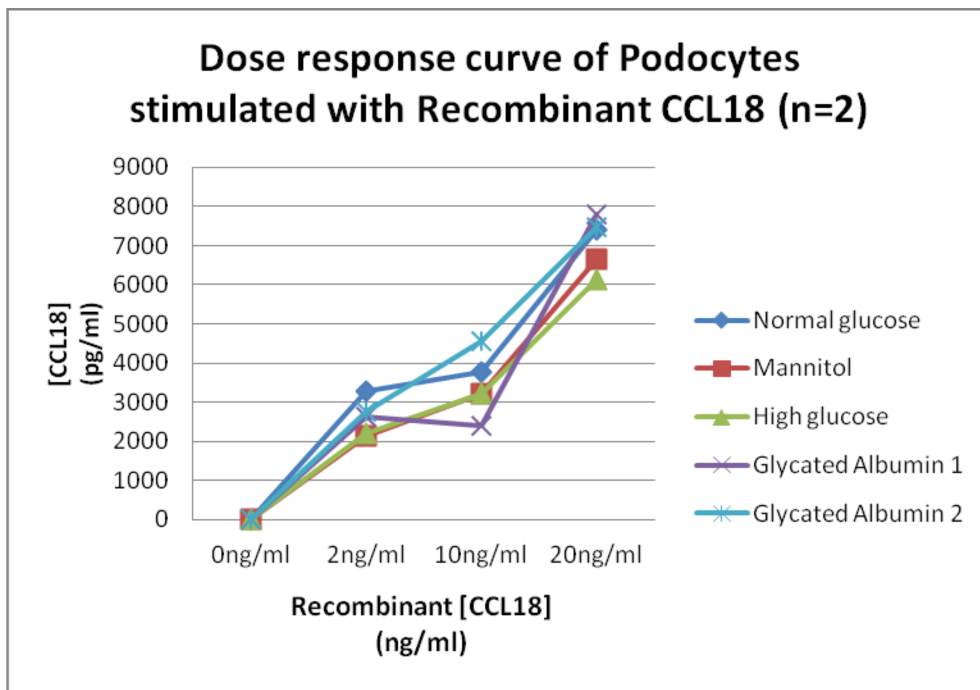


Figure 4.2.3.1.0 Dose response curve for rCCL18 in Podocytes

<u>*MIF in Podocytes stimulated with rCCL18*</u>

There is a dramatic inhibition of MIF production with stimulation with rCCL18 in podocytes stimulated with diabetic conditions. There is little change seen in basal MIF levels of the control conditions, see Fig 4.2.3.1.1. The experiments would need to be repeated as n=2.

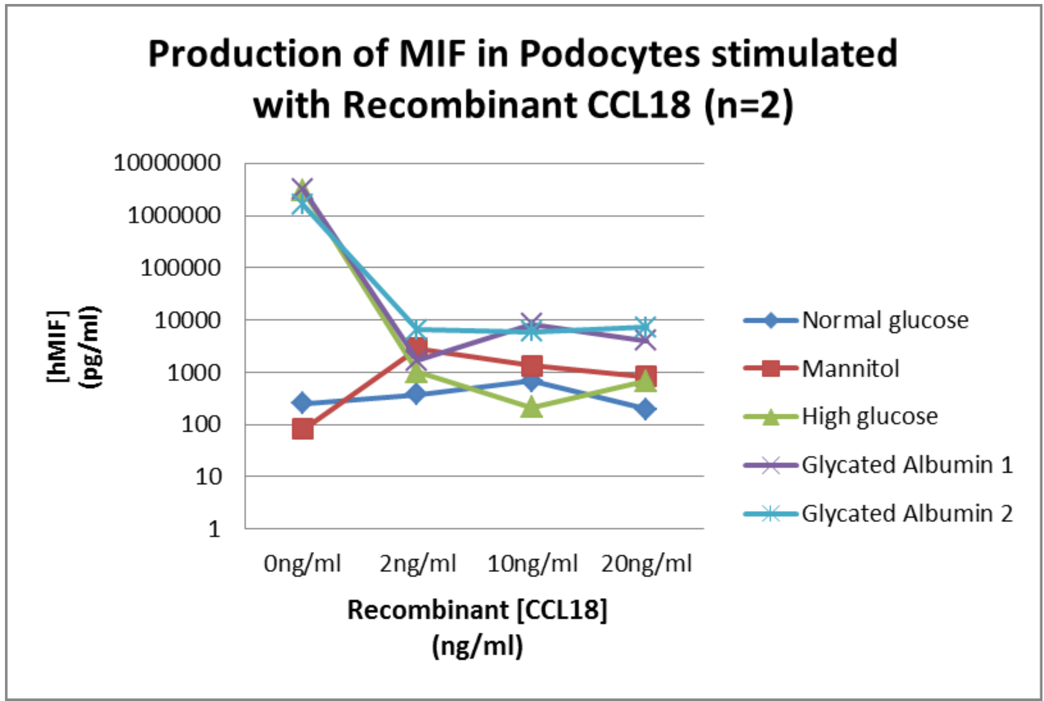


Figure 4.2.3.1.1 Production of MIF in Podocytes following rCCL18

MCP-1 in Podocytes stimulated with rCCL18

The production of MCP-1 is decreased in podocytes when stimulated with rCCL18, see Fig 4.2.3.1.2.

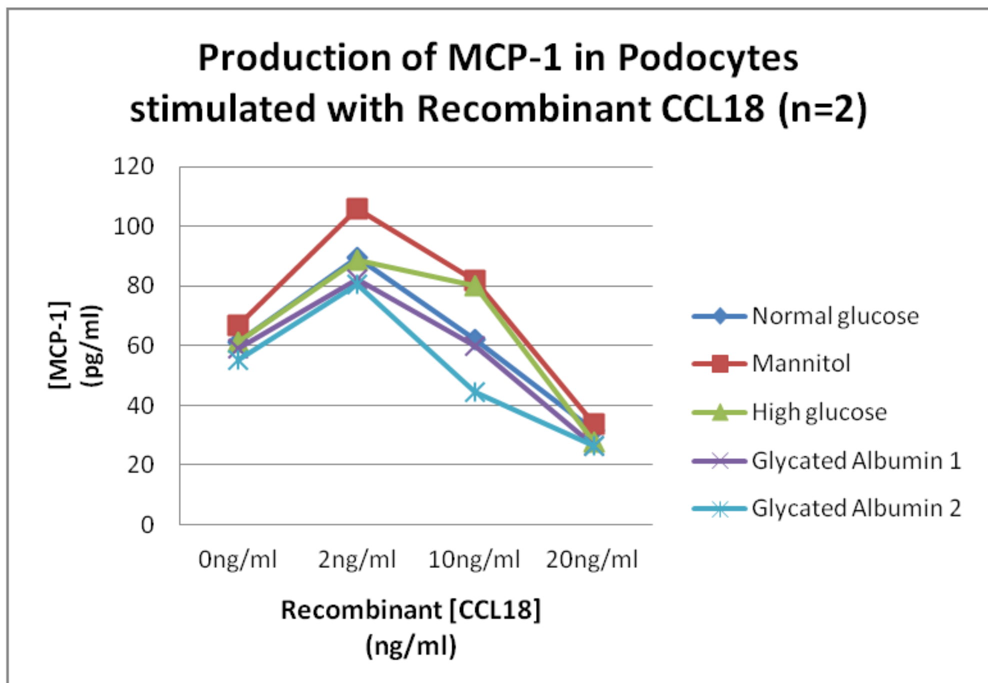


Figure 4.2.3.1.2 Production of MCP-1 in Podocytes stimulated with rCCL18

Summary of results

The production of MIF in diabetic conditions is effectively attenuated with rCCL18 in podocytes. MCP-1 production is also decreased in podocytes exposed to increasing levels of rCCL18. These changes occur irrespective of the basal conditions, however, they need to be repeated in view of the small numbers (n=2) used in the pilot study.

Summary of results in Podocytes

MIF and MCP-1 are produced in podocytes stimulated under all conditions. The levels of cytokines are affected by stimulation with recombinant cytokine and basal conditions also potentiate the effect of the stimulus. Low doses of rMIF stimulate the production of MCP-1 whereas high doses stimulate the production of CCL18, see Appendix 4.0 pilot data for further details. rMCP-1 stimulation in Podocytes results in a bimodal rise in MIF levels in diabetic conditions. Stimulation of Podocytes with rCCL18 results in a decrease in the production of MIF in diabetic conditions and overall decrease in MCP-1 in all basal conditions. There are no detectable levels of CCL18 following rMCP-1 stimulation. rMIF and rMCP stimulation of Podocytes were repeated to ensure reproducibility, see section 4.4

MIF is detected in large quantities in podocytes exposed to diabetic conditions following 2ng/ml and 20ng/ml rMCP-1, refer to Appendix 4.0. This experiment would need to be repeated to determine whether this bimodal distribution continues to be present. There are no detectable levels of CCL18 following rMCP-1 stimulation.

4.2.4 Summary of Pilot cell culture data

The effect of cytokine interactions seen in all pilot conditions are shown below in Table 4.0. These initial findings need to be repeated to see whether they are reproducible and statistically significant.

Table 4.0 Summary of Pilot cell culture data

Cell Type	***Effect***
tHMC	*MIF and MCP-1 are produced following basal stimulation* *rMIF inhibits MCP-1* *rMCP-1 stimulates MIF at low dose* *High dose rCCL18 stimulates MIF production*

HK-2 cells	*MIF produced in all basal conditions* *rMIF does not stimulate the production of CCL18 or MCP-1* *rMCP-1 slightly increases MIF levels* *rCCL18 inhibits MIF*
Podocytes	*MIF and MCP-1 are produced following basal stimulation* *Increasing MIF inhibits itself production in diabetic conditions* *rMIF increases then decreases levels of MCP-1* *rMIF increases CCL18* *rMCP-1 has a bimodal effect on MIF that is dose dependent* *rCCL18 inhibits MIF in diabetic conditions* *rCCL18 inhibits MCP-1 in all conditions*

HK2 cells stimulated with rCCL18 and rMCP-1 were repeated to determine whether the above interactions were reproducible. Podocytes stimulated with rMIF and rMCP-1 were also repeated to ensure the above findings continued to be seen. Cell cultures were stimulated with basal conditions and cytokine stimulation with 10ng/ml and 20ng/ml. In view of time limitations 0ng/ml and 20ng/ml were analysed and are herein presented. Each experiment had the same condition in n=6 with the whole experiment repeated 3 times on different days to ensure reproducibility of findings. The experiments will be referred to as Experiment 1, 2 or 3 and compared with each other. The details of the other experiments are presented in Appendix 4.0 and may be referred to.

4.3 Cell culture HK-2 cells

Following the pilot data (see Appendix 4.0 for details) HK2 cells were chosen to be repeated with the following conditions and with stimulation with rCCL18 or rMCP-1 at 0, 10 and 20ng/ml. ELISA analysis was undertaken at the 0 and 20ng/ml dose of cytokine stimulation. The 10ng/ml supernatants and cell lysates are stored in the -80ºC freezer, together with the supernatant collected at 24 hours after the initial serum free media period. HK2 cells were stimulated with normal glucose concentration (4mM D-glucose) the control for higher dose glycated albumin (500µg/ml + 4mM normal D-glucose), Mannitol (26mM + 4mM normal D-glucose) the osmotic control for High glucose (30mM D-glucose). See Figure 4.3.0 for experimental timeline.

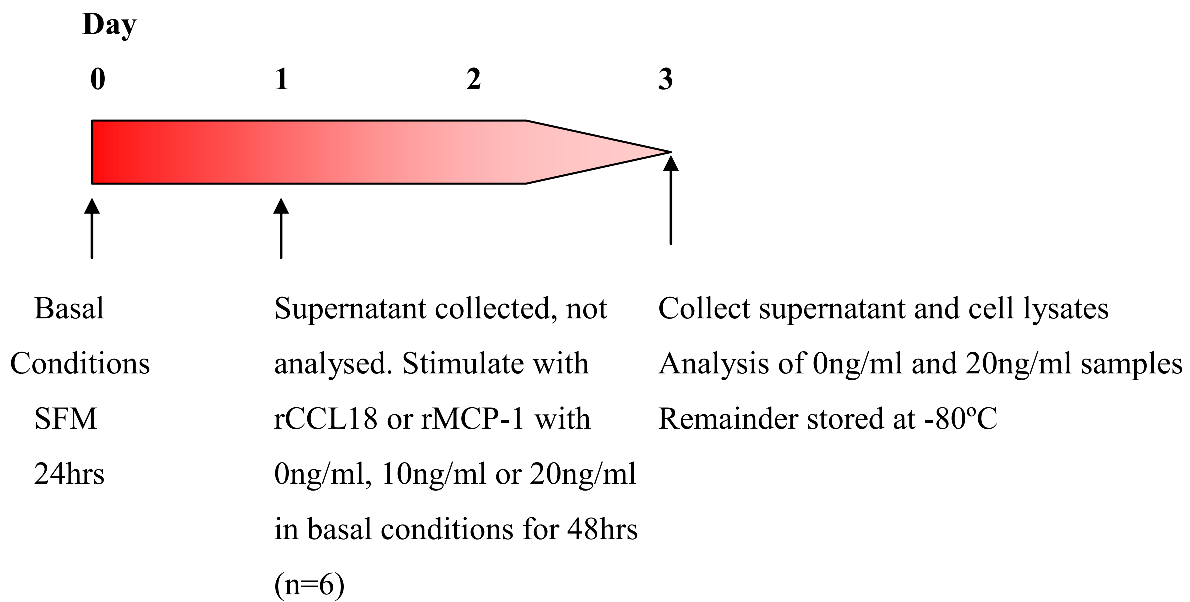


Figure 4.3.0 Timeline for HK2 experiment stimulation (Key: SFM= serum free media, n= number of duplicates per condition).

4.3.1 HK2 cells stimulated with rCCL18 - MTT Assay

The MTT assay has been presented first in order to determine whether the effects seen by the cytokines are related to cell viability or to stimulation with the recombinant cytokine. Each experiment was repeated three times with 6 wells per condition. Three of these wells were used for MTT assay, with the cell lysates for the remaining three collected and stored for Western blot analysis. The graphical presentation of the MTT assay is representative of the experiment illustrated throughout this section, see Figure 4.3.1.0. The results of the other two experiments have been shown in the tables below to determine whether the findings were consistent.

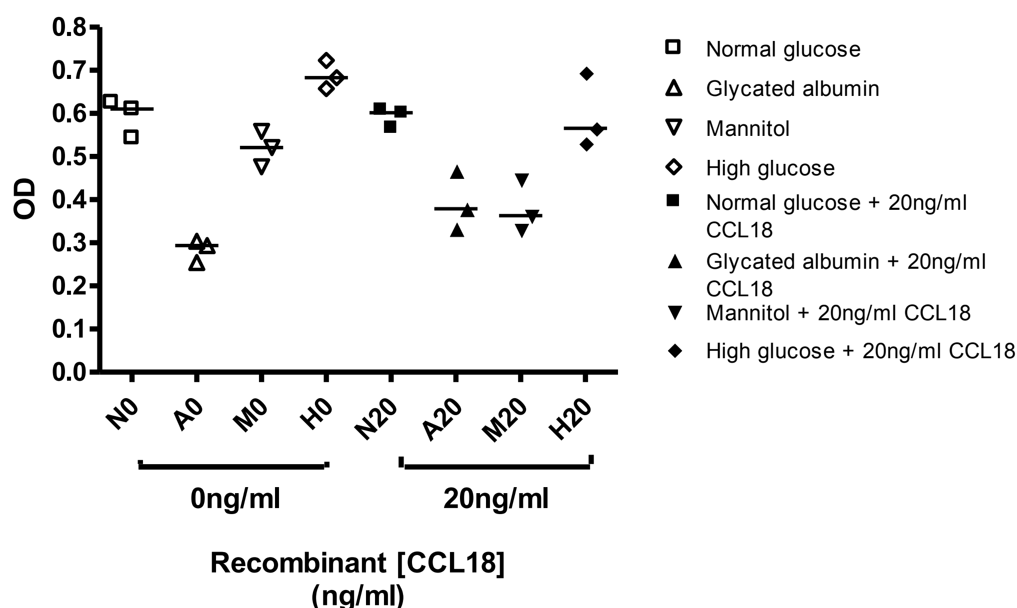


Figure 4.3.1.0 MTT assay for HK2 cells stimulated with rCCL18 under different conditions

There was a decrease in the number of surviving cells following 48 hours stimulation with glycated albumin compared with its control normal glucose. In contrast there seem to be an increase in the number of cells at 48 hours in high glucose compared with its control mannitol. This relationship was similar in the conditions stimulated with rCCL18. There appear to be more surviving cells in glycated albumin following treatment with rCCL18 compared with basal glycated albumin. Experiments 2 and 3 exhibited the same pattern of MTT assay with less cell viability in glycated albumin stimulated HK2 cells, see Table 4.1. Cell viability was improved following stimulation with rCCL18. Experiment 3 however showed lower cell viability in high glucose conditions stimulated with rCCL18 compared to basal conditions and to the other experiments.

Table 4.1 MTT Assay HK2 cell Experiment 2 and 3 0ng/ml or 20ng/ml with rCCL18

Condition with 0ng/ml or 20ng/ml Recombinant CCL18	**MTT Assay Expt 2 Median (range) OD**	**MTT Assay Expt 3 Median (range) OD**
N0	0.67 (0.64-0.68)	0.61 (0.60-0.64)
A0	0.33 (0.29-0.37)	0.33 (0.33-0.34)
M0	0.67 (0.63-0.77)	0.55 (0.55-0.55)
H0	0.87 (0.70-0.96)	0.80 (0.62-0.82)
N20	0.67 (0.54-0.70)	0.67 (0.66-0.68)
A20	0.37 (0.31-0.38)	0.47 (0.47-0.51)
M20	0.62 (0.47-0.69)	0.67 (0.44-0.69)
H20	0.75 (0.70-0.82)	0.53 (0.48-0.55)

Summary

Less HK2 cells survived in glycated albumin conditions, however more survived with simultaneous stimulation with rCCL18. Mannitol showed a decrease in cell number following rCCL18 stimulation with little change in normal glucose or high glucose conditions.

4.3.1.1 Effects of rCCL18 on CCL18 in HK2 cells

The results for HK2 cells stimulated with 0 and 20ng/ml of rCCL18 in their diabetic conditions are seen in Figure 4.3.1.1. The experiments of n=6 per condition were repeated in total three separate times (referred to as Experiment 1, 2 or 3) to ensure the findings were reproducible. For simplicity of understanding the findings of a single experiment (Experiment 1) have been illustrated below with tables to summarise the findings.

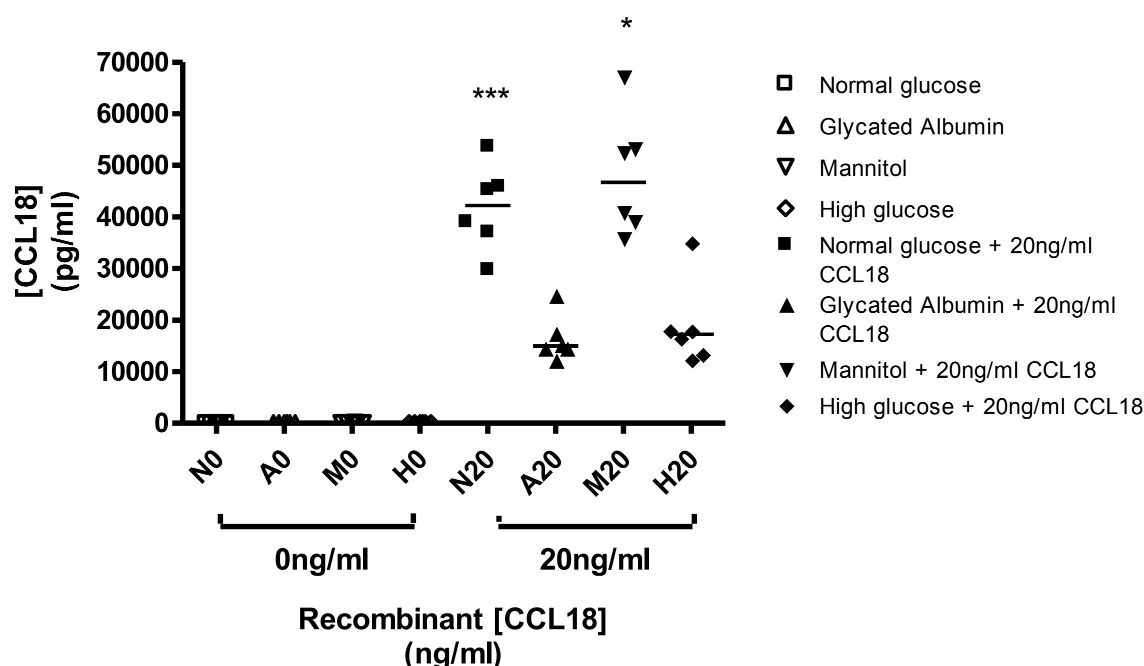


Figure 4.3.1.1 Production of CCL18 in HK2 cells under basal conditions and following rCCL18 stimulation. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of CCL18 stimulation, 20=20ng/ml of CCL18 stimulation.
**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of CCL18 (N0) compared to Normal glucose with 20ng/ml of CCL18 (N20). *p<0.05, **p<0.01, ***p<0.001.*

The graph shows that CCL18 was consistently detected in HK2 cells after 48 hours of stimulation with mannitol, see Table 4.2 for median. Low levels of CCL18 were detected inconsistently in glycated albumin and high glucose with no detectable levels in normal glucose conditions. The amounts of CCL18 in HK2 cells increase following stimulation with rCCL18. When comparing stimulation with 20ng/ml of rCCL18 within the same condition, normal glucose significantly increased supernatant levels of CCL18 ($p<0.001$), mannitol also had increased CCL18 ($p<0.05$).

Glycated albumin and high glucose did not have significantly higher levels of CCL18 following stimulation with rCCL18 when comparing their wells without recombinant stimulation, thereby suggesting a difference within the diabetic milieu to rCCL18 stimulation. The diabetic environment i.e. glycated albumin or high glucose, affects CCL18 production or consumption in HK2 cells

following 48 hours of stimulation with rCCL18. The changes observed are not a reflection of cell viability as glycated albumin had a lower cell viability compared with that of high glucose. Glycated albumin had an improved survival with rCCL18 stimulation though lower survival than its control normal glucose. Mannitol had lower cell viability compared with its control high glucose whose cell survival was slightly lower than its unstimulated form.

Both diabetic conditions have lower levels of CCL18 detected following stimulation that is not related to the variation in cell survival. The levels of CCL18 detected are not related to cell death but related to the condition the HK2 cells are in.

Table 4.2 The amount of CCL18 found in HK2 cells without and with rCCL18. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant CCL18	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 (0) vs 0 (0-26.4)	ns
0ng/ml	M vs H	56.1(13.7-129) vs 0 (0-39.5)	ns
20ng/ml	N vs A	42200 (29800-53700) vs 14900 (12300-24800)	ns
20ng/ml	M vs H	46700 (35900-67100) vs 17300 (12300-35100)	ns
CCL18 stimulation	N20 vs N0	42200 (29800-53700) vs 0 (0)	p<0.001
CCL18 stimulation	A20 vs A0	14900 (12300-24800) vs 0 (0-26.4)	ns
CCL18 stimulation	M20 vs M0	46700 (35800-67000) vs 56.1(13.7-129)	p<0.05
CCL18 stimulation	H20 vs H0	17300 (12300-35100) vs 0 (0-39.5)	ns

Similar to experiment 1, experiments 2 and 3 showed glycated albumin and mannitol were able to produce CCL18 with no CCL18 detected in the other basal conditions. CCL18 had lower levels in high glucose stimulated with rCCL18 compared with its control mannitol in experiments 1 and 2, see Table 4.3. Experiment 3 however, had higher levels of CCL18 in high glucose with rCCL18. These differences may be explained by a change of cell viability. In experiment 3 there was less cell survival compared with mannitol, see Table 4.4. High glucose conditions with rCCL18 resulted in more cell survival in experiments 1 and 2 compared with its control mannitol. Experiment 3 showed

an increase of CCL18 in high glucose compared with mannitol. There was less cell survival with high levels of CCL18 in high glucose.

Table 4.3 CCL18 following rCCL18 in HK2 Experiment 2

Recombinant CCL18	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 (0-18) vs 9.32 (0-163)	ns
0ng/ml	M vs H	0 (0-19) vs 0 (0)	ns
20ng/ml	N vs A	46500 (30100-70700) vs 56800 (40700-76300)	ns
20ng/ml	M vs H	46100 (35700-74300) vs 20500 (13800-26100)	ns
CCL18 stimulation	N20 vs N0	56800 (40700-76300) vs 0 (0-18)	p<0.05
CCL18 stimulation	A20 vs A0	56800 (40700-76300) vs 9.32 (0-163)	p<0.01
CCL18 stimulation	M20 vs M0	46100 (35700-74300) vs 0 (0-19)	p<0.01
CCL18 stimulation	H20 vs H0	20500 (13800-26100) vs 0 (0)	ns

Table 4.4 CCL18 following rCCL18 in HK2 Experiment 3

Recombinant CCL18	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 (0) vs 38.3 (0-138)	ns
0ng/ml	M vs H	0 (0) vs 0 (0)	ns
20ng/ml	N vs A	50500 (47700-57300) vs 87600 (51500-91600)	ns
20ng/ml	M vs H	20100 (17400-25300) vs 37200 (31300-64200)	ns
CCL18 stimulation	N20 vs N0	50500 (47700-57300) vs 0 (0)	p<0.01
CCL18 stimulation	A20 vs A0	87600 (51500-91600) vs 38.3 (0-138)	p<0.05

CCL18 stimulation	M20 vs M0	20100 (17400-25300) vs 0 (0)	ns
CCL18 stimulation	H20 vs H0	37200 (31300-64200) vs 0 (0)	p<0.01

Summary

CCL18 is produced inconsistently in HK2 cells following basal conditions for 48 hours as in the pilot. Following rCCL18 there is a rise in CCL18 in all conditions that is significant in normal glucose and mannitol. The rise in diabetic milieu is less prominent.

4.3.1.2 Effects of rCCL18 on MIF in HK2 cells

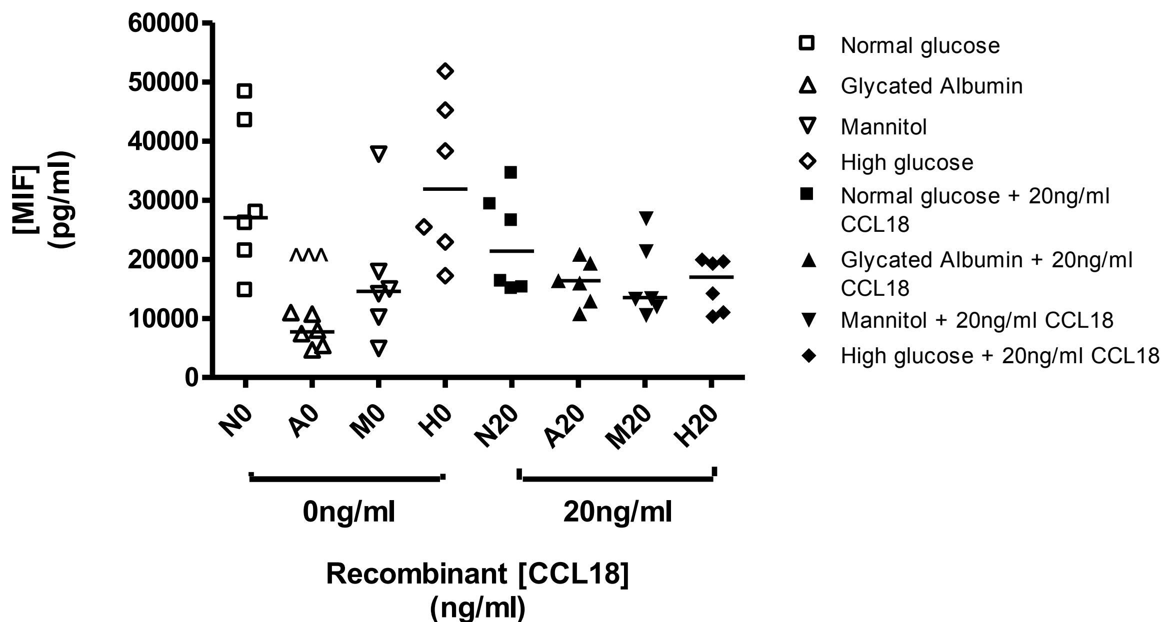


Figure 4.3.1.2 Production of MIF in HK2 cells under basal conditions and following rCCL18 stimulation. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of CCL18 stimulation, 20=20ng/ml of CCL18 stimulation.

^ depicts the level of significance between basal conditions and their controls ie. Normal glucose concentration (N0) compared to glycated albumin (A0). The osmotic control Mannitol (M0) compared to high glucose (H0). ^ p<0.05, ^^ p<0.01, ^^^ p<0.001.

MIF is produced by HK2 cells regardless of the basal condition stimulus at 48 hours, see Fig 4.3.1.2. There is significantly less MIF produced from HK2 cells following 48 hours of glycated albumin compared to its control; normal glucose ($p<0.001$). There appears to be higher levels of MIF in high glucose; however, when comparing this to its control this does not reach statistical significance. There is little change in the level of MIF production following stimulation with rCCL18 in HK2 cells. High glucose had a decrease in MIF levels following stimulation with rCCL18, however, glycated albumin had an increase in MIF levels following stimulation with rCCL18; these were not sigificant using Kruskal-Wallis test with Dunn's multiple comparison, see Table 4.5.

Table 4.5 The amount of MIF found in HK2 cells without and with rCCL18. Key: N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant CCL18	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	27000 (14700-48300) vs 7710 (4650-11000)	p<0.001
0ng/ml	M vs H	14600 (4910-37800) vs 31900 (17200-51800)	ns
20ng/ml	N vs A	21400 (15000-34500) vs 16400 (11000-21000)	ns
20ng/ml	M vs H	13500 (10700-27100) vs 17000 (10500-20100)	ns
CCL18 stimulation	N20 vs N0	21400 (15000-34500) - 27000 (14700-48300)	ns
CCL18 stimulation	A20 vs A0	16400 (11000-21000)- 7710 (4650-11000)	ns
CCL18 stimulation	M20 vs M0	13500 (10700-27100)- 14600 (4910-37800)	ns
CCL18 stimulation	H20 vs H0	17000 (10500-20100) -31900 (17200-51800)	ns

Experiment 2 showed no significant difference between MIF levels in basal conditions and their controls (see Appendix 4.0). There was a trend for lower levels of MIF in basal mannitol and high glucose compared to normal glucose and glycated albumin. Glycated albumin stimulated with

rCCL18 had significantly lower levels of MIF ($p<0.01$), similar to experiment 1. In experiment 3 the the levels of MIF trended to be higher in glycated albumin compared to normal glucose. Experiments 2 and 3 trended to have higher levels of MIF in high glucose stimulated with rCCL18 compared with its basal condition alone, although this did not reach significance. This was different to Experiment 1. Experiment 3 may differ as the cell viability in high glucose with rCCL18 was lower compared with experiments 1 and 2.

Summary

MIF was found in HK2 cells stimulated with all the different cell culture conditions reproducible to the pilot study. There was little change in the levels of MIF production following stimulation with rCCL18 in HK2 cells. There is a downward trend of MIF production in all conditions with rCCL18. High glucose had a decrease in MIF levels following stimulation with rCCL18, however, glycated albumin had an increase in MIF levels following stimulation with rCCL18; neither reached significance. In some experiments, stimulation with glycated albumin was associated with reduction of MIF in the cell culture supernatant. This may be partly related to a reduction in cell viability compared with the other conditions, as shown by MTT assay.

4.3.1.3 Effects of rCCL18 on MCP-1 in HK2 cells

No MCP-1 was produced in HK2 cells following 48 hours of stimulation in normal glucose, glycated albumin, mannitol and high glucose, as per the pilot study. MCP-1 was produced by HK2 cells following stimulation with rCCL18 in mannitol ($p=0.36$). These findings are summarised in Figure A4F12, Table A4T4-T6 in Appendix 4.0. Repeated experiments confirmed the same observation.

Summary

MCP-1 was inconsistently produced in basal conditions and MCP-1 levels did not change with rCCL18 stimulation.

4.3.1.4 Effects of rCCL18 on Fibronectin in HK2 cells

Fibronectin is produced in all basal conditions with higher levels in glycated albumin however, this was not significant when compared with its control. See Fig 4.3.1.4 and Table 4.6.

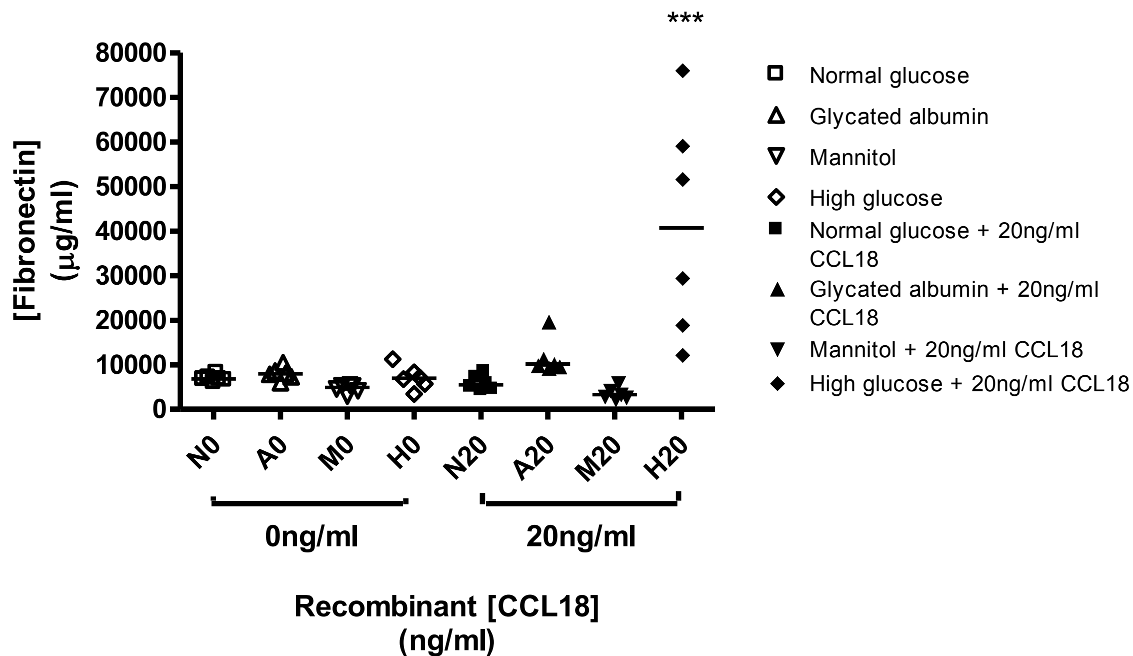


Figure 4.3.1.4 Production of Fibronectin in HK2 cells under basal conditions and following rCCL18 stimulation. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of CCL18 stimulation, 20=20ng/ml of CCL18 stimulation.

**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of CCL18 (N0) compared to Normal glucose with 20ng/ml of CCL18 (N20). *p<0.05, **p<0.01, ***p<0.001.*

There was a significant rise in the production of fibronectin following stimulation with rCCL18 in high glucose conditions compared to high glucose alone (Kruskal-Wallis post Dunns comparison $p<0.001$), see Table 4.6. The median fibronectin level in high glucose following stimulation with rCCL18 was higher than its control mannitol for experiment 2, median 7010 (range 773-15500) vs 3780 (3350-5830) µg/ml and experiment 3, median 20200 (range 3350-39000) vs 2790 (1970-4790) µg/ml, however, these did not reach significance (see Appendix 4.0 for more details of experiments 2 and 3). The median fibronectin levels in conditions stimulated with glycated albumin and rCCL18, were higher than normal glucose though this was not significantly different (see Table A4T7-T8 Appendix 4.0).

Table 4.6 The amount of Fibronectin found in HK2 cells without and with rCCL18. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant CCL18	**Basal condition**	**Median (range) (µg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	6810 (6110-8210) vs 7980 (6000-10400)	ns
0ng/ml	M vs H	4880 (2970-5400) vs 6950 (3410-11200)	ns
20ng/ml	N vs A	5470 (4360-8470) vs 10200 (9460-19800)	ns
20ng/ml	M vs H	3270 (2390-6010) vs 40700 (12400-76200)	p<0.001
CCL18 stimulation	N20 vs N0	5470 (4360-8470) - 6810 (6110-8210)	ns
CCL18 stimulation	A20 vs A0	10200 (9460-19800) - 7980 (6000-10400)	ns
CCL18 stimulation	M20 vs M0	3270 (2390-6010) - 4880 (2970-5400)	ns
CCL18 stimulation	H20 vs H0	40700 (12400-76200)- 6950 (3410-11200)	ns

Summary

Fibronectin was produced by all basal conditions at 48 hours. There was a significant rise in the production of fibronectin following stimulation with rCCL18 in high glucose conditions but little change in fibronectin basal levels in the other conditions.

<u>Summary of results</u>

Glycated albumin or high glucose affects CCL18 production or consumption in HK2 cells following 48 hours of stimulation with rCCL18, as these levels were lower than their respective controls. MIF was produced in HK2 cells stimulated for 48 hours in all conditions. MIF levels were found to be lower in glycated albumin with higher levels found in high glucose conditions. Following stimulation with rCCL18, MIF levels were significantly higher in glycated albumin. There was a trend to decrease MIF levels in high glucose conditions stimulated with rCCL18 compared to its control mannitol; with median MIF levels being higher than median high glucose condition alone. This latter finding was similar to the pilot study where there was a suggestion

that rCCL18 could decrease MIF levels in diabetic conditions. This experiment shows this effect to be more specific to high glucose conditions.

MCP-1 was not produced following stimulation with the basal conditions or with rCCL18. Fibronectin is produced by all conditions following exposure to these conditions for 48 hours. Fibronectin is significantly increased in high glucose following stimulation with CCL18. There is a trend for higher fibronectin levels in glycated albumin regardless of CCL18 stimulation. MTT assay showed an overall decrease in cell survival in glycated albumin and more cells overall in high glucose compared to their controls.

4.3.2 HK2 cells stimulated with rMCP-1 - MTT Assay

HK2 cells were stimulated with 0, 10 and 20ng/ml of rMCP-1 in their basal conditions. In view of project time constraints ELISA's were performed for 0ng/ml and 20ng/ml samples for all of the three separately repeated experiments (n=6 per experiment).

There were a similar number of cells in all basal conditions stimulated with rMCP-1 after 48 hours except for glycated albumin that had less than its control; normal glucose. High glucose had more cells compared with mannitol. Cell viability appeared to improve following rMCP-1 stimulation in glycated albumin but with less viability in high glucose conditions compared to their basal levels. The same distribution of cell viability was seen post rMCP-1 stimulation as that seen in basal conditions with respect to the diabetic conditions and their controls, see Fig 4.3.2.

MTT Assay for HK2 cells stimulated under diabetic milieu with different concentrations of Recombinant MCP-1

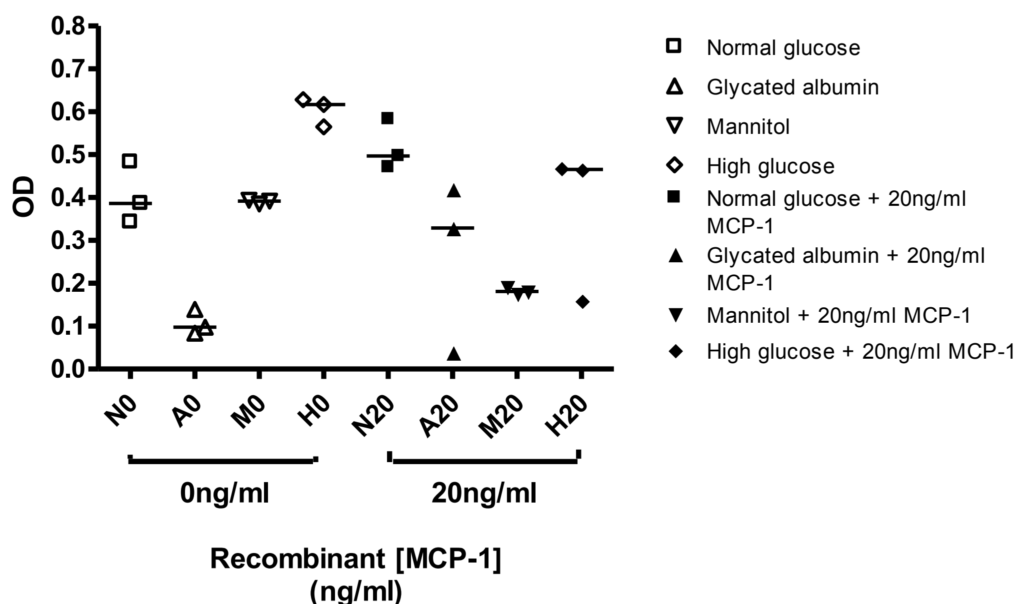


Figure 4.3.2 MTT assay for HK2 cells of Experiment 1

Experiments 2 and 3 were concordant with the results of experiment 1 (see Appendix 4.0).

Summary

Glycated albumin stimulus for 48 hours consistently reduced HK2 cell viability. This improved following 48 hours of rMCP-1 stimulation in this condition. High glucose conditions had a higher cell viability compared to its control mannitol that remained constant following rMCP-1 stimulation. rMCP-1 decreased HK2 overall cell number when compared to basal conditions alone.

4.3.2.1 Effects of rMCP-1 on MCP-1 in HK2 cells

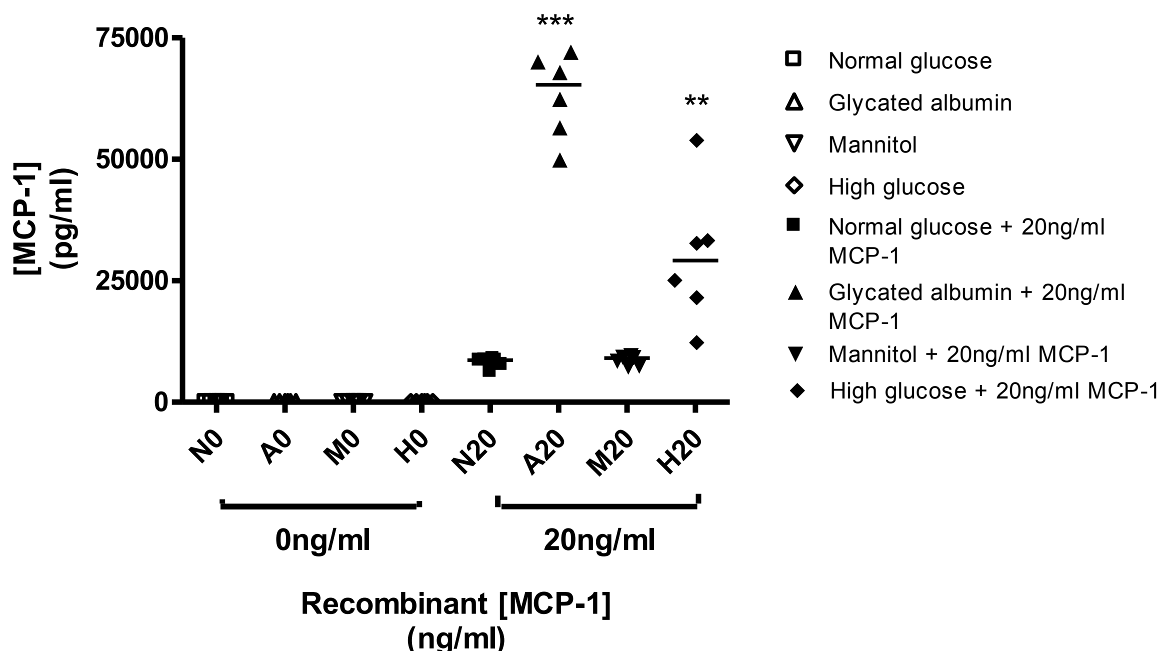


Figure 4.3.2.1 Production of MCP-1 in HK2 cells under basal conditions and following rMCP-1 stimulation. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MCP-1 (N0) compared to Normal glucose with 20ng/ml of MCP-1 (N20). *p<0.05, **p<0.01, ***p<0.001.*

No MCP-1 was produced by HK2 cells following 48 hours of basal conditions stimulation, consistent with previous findings. Following stimulation of HK2 cells with rMCP-1, MCP-1 was detected in the supernatant with higher levels in the diabetic milieu, see Fig 4.3.2.1. MCP-1 significantly increased in glycated albumin ($p<0.001$) and high glucose ($p<0.01$) stimulated with rMCP-1 compared to the baseline production of MCP-1 (see Table 4.7). These levels were higher than their controls however, this did not reach significance and may be a reflection of the number of groups compared for statistical analysis.

Table 4.7 The amount of MCP-1 found in HK2 cells without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	8660 (6340-8990) vs 65400 (50100-72300)	ns
20ng/ml	M vs H	9110 (7550-9860) vs 29200 (12500-54100)	ns
MCP-1 stimulation	N20 vs N0	8660 (6340-8990) - 0	ns
MCP-1 stimulation	A20 vs A0	65400 (50100-72300) – 0	p<0.001
MCP-1 stimulation	M20 vs M0	9110 (7550-9860) - 0	ns
MCP-1 stimulation	H20 vs H0	29200 (12500-54100) – 0	p<0.01

Experiment 2 reflects the same trends of rMCP-1 stimulation resulting in an increased amount of MCP-1 detected in glycated albumin compared with normal glucose, and increased levels seen in high glucose compared with mannitol. None or minimal inconsistent amounts of MCP-1 were detected following basal stimulus in both experiments 2 and 3 (see Appendix 4.0). Experiment 3 showed a slight decrease in MCP-1 detected in high glucose compared to its control that was different from experiments 1 and 2 although the MCP-1 levels detected in these conditions were very similar. This finding is not explained by changes in the MTT assay.

Summary

MCP-1 was not detected following stimulation with basal conditions for 48 hours in HK2 cells. rMCP-1 stimulation resulted in high levels of MCP-1 being detected in glycated albumin and high glucose conditions. These findings were not dependent on MTT assay and were consistent in all three experiments.

4.3.2.2 Effects of rMCP-1 on MIF in HK2 cells

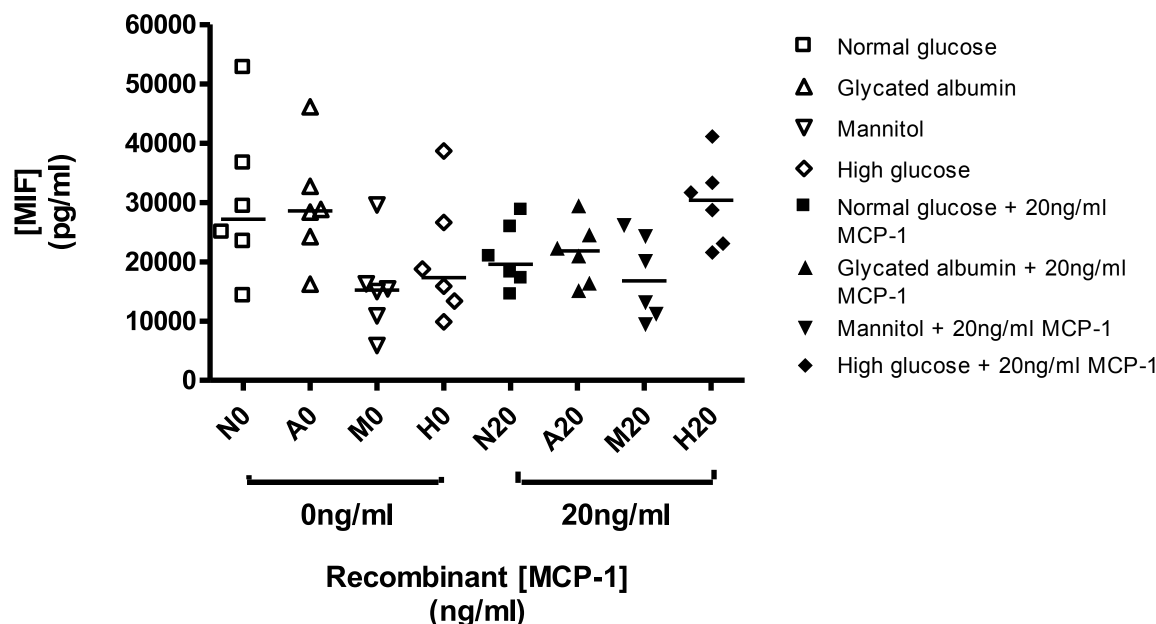


Figure 4.3.2.2 Production of MIF in HK2 cells under basal conditons and following rMCP-1 stimulation. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

There was a mixture of effects on the production of MIF following stimulation with rMCP-1, see Fig 4.3.2.2. None of these were significant. The diabetic milieu had similar MIF levels compared to its controls. The findings in experiment 1 were slightly different to the previous HK2 experiments where lower levels of MIF were seen in glycated albumin compared with normal glucose. Experiment 1 showed MIF levels in high glucose were similar to mannitol following 48 hours stimulation. In contrast experiments 2 and 3 were the same as the previous findings where lower levels of MIF were found following stimulation with glycated albumin. This difference is not explained by the cell viability as the MTT assay for all these experiments consistently shows a decrease in cell viability in glycated albumin conditions. Basal levels of MIF were similar in high glucose conditions and mannitol in all three experiments.

MIF levels were not significantly raised following stimulation with rMCP-1 in any of the conditions in experiments 2 and 3. There was a tendency for MIF to be reduced in glycated albumin conditions, however, this did not reach significance when compared to normal glucose. There appeared to be an increase in MIF with rMCP-1 in high glucose that did not reach significance when compared to its control mannitol. See Table A4T12-T14 Appendix 4.0 for details.

Summary

Stimulation with rMCP-1 did not increase MIF in glycated albumin conditions There was little change from basal MIF levels. MIF appeared in two out of three experiments to be lower in glycated albumin that is consistent with cell viability, however there were increased MIF levels detected despite lower cell viability in experiment 1 with higher levels found with rMCP-1 in experiment 2 and 3 with an improved cell survival.

4.3.2.3 Effects of rMCP-1 on CCL18 in HK2 cells

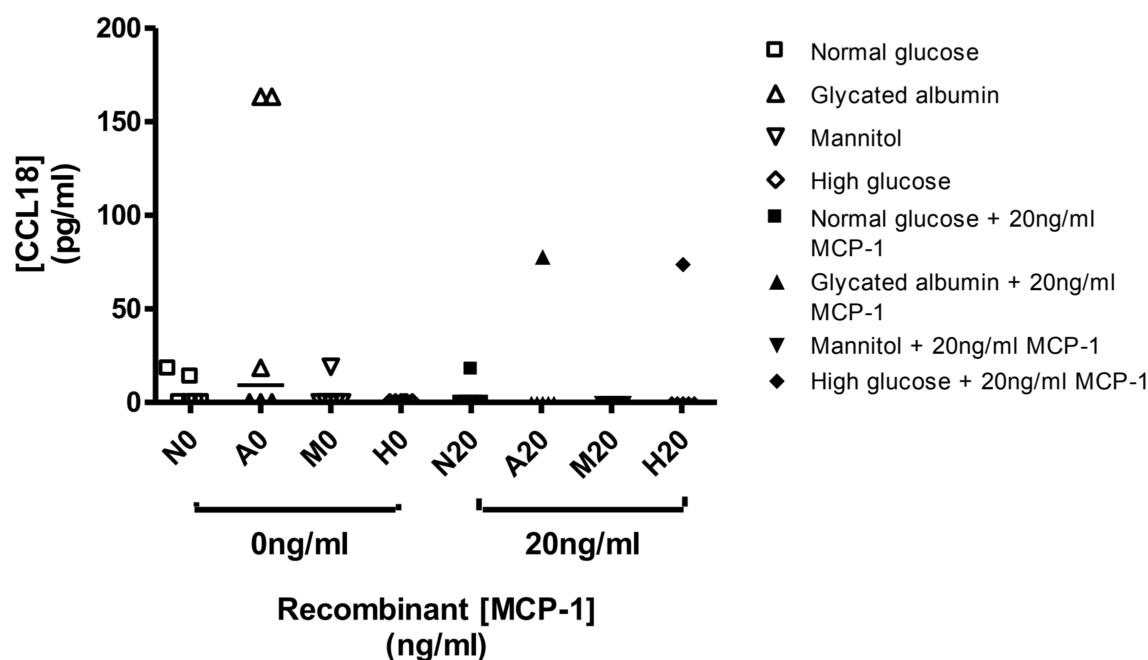


Figure 4.3.2.3 Production of CCL18 in HK2 cells in basal conditions stimulated with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

CCL18 was produced in small amounts after 48 hours of basal conditions stimulation of HK2 cells, see Fig 4.3.2.3. There was no significant difference in CCL18 in basal conditions stimulated with rMCP-1.

Summary

CCL18 was not consistently affected by rMCP-1 stimulation in any basal conditions.

4.3.2.4 Effects of rMCP-1 on Fibronectin in HK2 cells

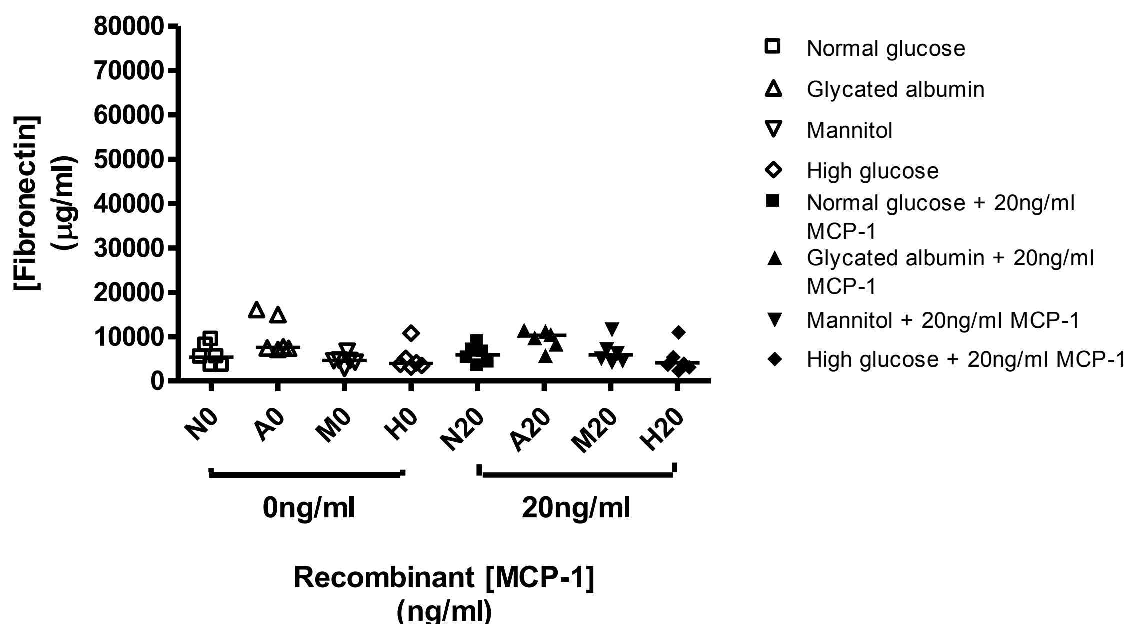


Figure 4.3.2.4 Production of Fibronectin in HK2 cells in basal conditions stimulated with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

There was no significant difference between fibronectin levels in basal conditions and those stimulated with rMCP-1, see Fig 4.3.2.4. There was a trend for higher levels of fibronectin in glycated albumin with rMCP-1, however, experiment 3 had the opposite effect with a decrease in

fibronectin levels (see Appendix 4.0). No effect on fibronectin levels was seen in high glucose with rMCP-1 except for experiment 3 that suggested a possible rise in fibronectin in this environment; this was not significant.

Summary

Fibronectin was higher in glycated albumin with rMCP-1 compared with normal glucose. Experiment 3 suggested a higher fibronectin level in high glucose with rMCP-1 that was not seen in the two previous experiments. The production of fibronectin seen in basal normal glucose and mannitol conditions did not change with rMCP-1. These findings were not significant.

Summary of results

MCP-1 was not detected following stimulation with basal conditions for 48 hours. MCP-1 levels were significantly higher in glycated albumin and high glucose conditions following rMCP-1 compared with their controls. MIF decreased in glycated albumin and normal glucose but increased in high glucose following rMCP-1 this was not significant. Low levels of CCL18 were detected in basal conditions with rMCP-1 that were not significant. An overall decrease was seen in fibronectin production following rMCP-1 stimulation that was not significant. An increase in cell survival was seen following rMCP-1 stimulation in glycated albumin with increased cell death in mannitol and high glucose. Overall, there were more surviving cells in high glucose conditions. There were slightly higher levels of MIF seen in high glucose with rMCP-1 where MTT assay reflects lower cell viability, but no changes were seen in mannitol, however this was an inconsistent finding. Some of the trends illustrated, presented and seen in Appendix 4.0 are striking, however, they do not reach statistical significance. In part this may reflect the multiple comparisons made within each group. Smaller experiments comparing 2 conditions would need to be repeated to determine whether the trends seen are significant.

4.3.3 Summary of HK2 cell culture data

HK2 cells results are summarised below in Table 4.8

Table 4.8 Summary of HK2 cell culture data

HK2 cells	***Effects***
Basal conditions	*Less MIF in glycated albumin*

	More MIF in high glucose *No MCP-1 at baseline* *CCL18 in variable small amounts in all conditions* *Fibronectin in all conditions with more in glycated albumin*
CCL18 stimulation	*Less CCL18 detected in glycated albumin and high glucose with rCCL18* *rCCL18 increases MIF in glycated albumin* *rCCL18 results in a decrease in MIF in high glucose* *rCCL18 no effect on MCP-1* *rCCL18 increases fibronectin in high glucose with decreased cell survival* *rCCL18 increased cell survival in glycated albumin*
MCP-1 stimulation	*rMCP-1 stimulation produces significantly more MCP-1 in glycated albumin and high glucose* *Trend for rMCP-1 to increase MIF in high glucose but decrease MIF in glycated albumin compared with basal levels* *rMCP-1 no effect on CCL18 in all conditions* *rMCP-1 trend to decrease fibronectin in all conditions* *rMCP-1 stimulated cells increased cell survival in glycated albumin*

4.4 Cell culture Human Podocytes

Following the pilot data podocyte experiments were chosen to be repeated with stimulation for 48 hours in basal conditions without and with stimulation with rMIF or rMCP-1 at 0, 10 and 20ng/ml. Each of these experiments was repeated three times with experiment 1 graphically presented and reference made to the reproducibility of the results from experiments 2 and 3 (see Appendix 4.0). ELISA analysis was to be undertaken at the 0 and 20ng/ml dose of cytokine stimulation. The 10ng/ml supernatants and cell lysates were stored in the -80ºC freezer, together with the supernatant collected at 24 hours after the initial period of podocytes in media with the conditions. Podocytes were not able to be serum free as this resulted in cell death, thus they were placed in the basal conditions with 5% FCS for 24 hours prior to the start of the experiment. Podocytes were stimulated with normal glucose concentration (4mM D-glucose) the control for higher dose glycated albumin (500µg/ml + 4mM normal glucose). Podocytes were also stimulated with mannitol (26mM + 4mM

normal glucose), the osmotic control for high glucose (30mM D-glucose), each condition maintained 5% FCS. The experimental timeline following 2 weeks of podocyte differentiation incubated at 37^0C is shown in Figure 4.4.0.

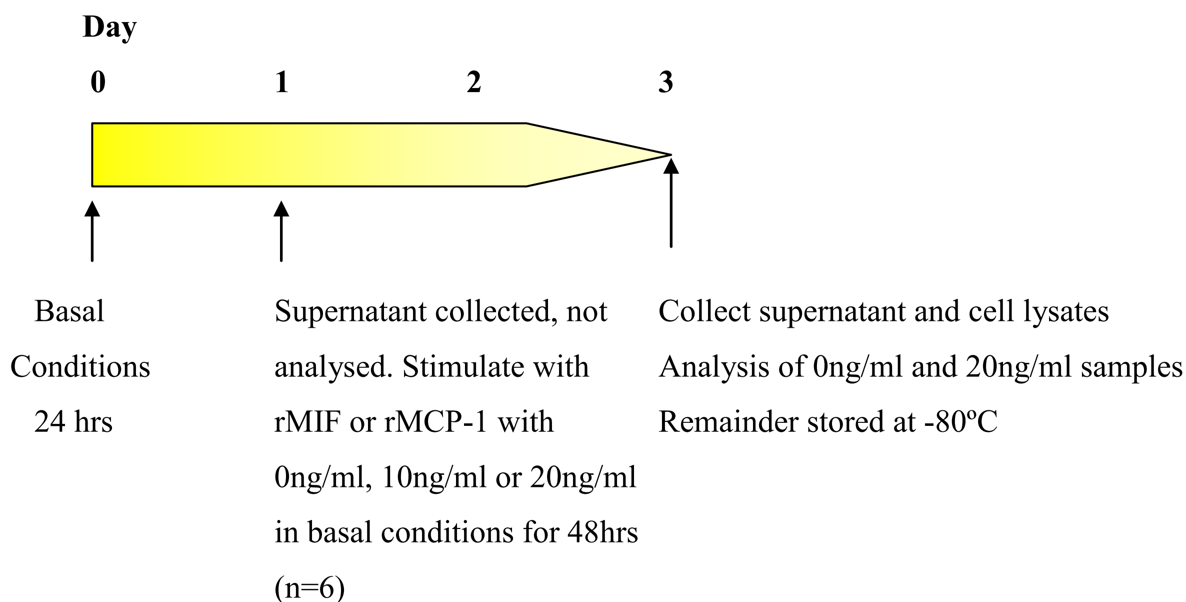


Figure 4.4.0 Timeline for Podocyte experiment stimulation (Key: n= number of duplicates per condition)

4.4.1 Podocytes stimulated with rMIF- MTT Assay

The MTT assay showed there were a similar number of cells in all conditions following 48 hours stimulation with basal conditions, see Fig 4.4.1. There was a slight rise in cell survival following stimulation with rMIF particularly in glycated albumin and high glucose conditions.

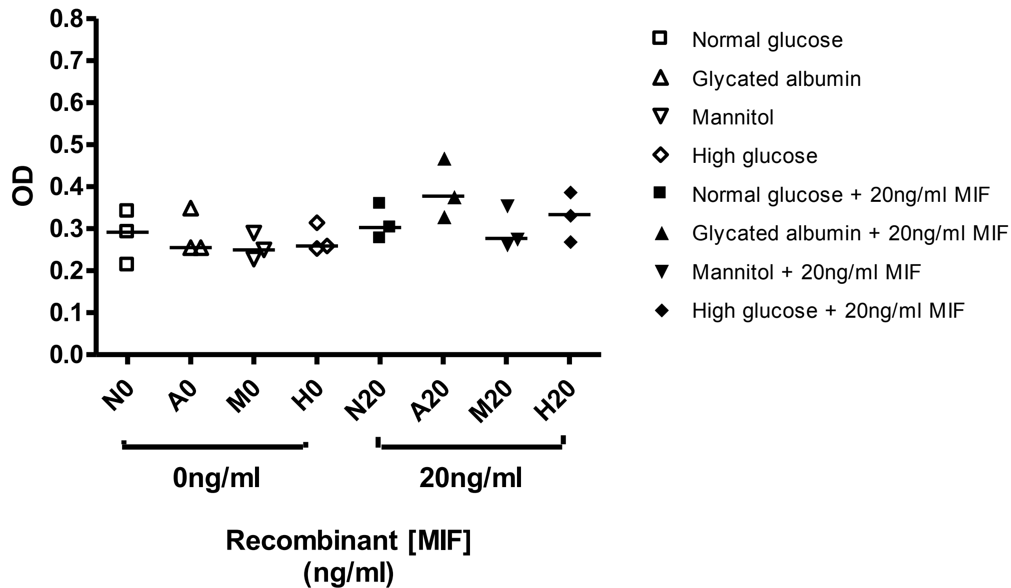


Figure 4.4.1 MTT assay for podocytes stimulated with rMIF

Experiments 2 & 3 showed cell viability improved in high glucose with rMIF compared with its control and to basal levels (see Appendix 4.0).

4.4.1.1 Effects of diabetic milieu and rMIF on Podocytes

MIF was detected in podocyte supernatant in all basal conditions following 48 hours stimulation as seen in the pilot study. The basal levels of MIF found in podocytes were similar to those detected in HK2 cells. Lower levels of MIF were detected in mannitol and high glucose. There was slightly more MIF in glycated albumin and high glucose compared to their respective controls; that was not significant. Experiment 2 showed a similar trend in higher MIF levels seen in glycated albumin. In contrast, experiment 3 showed slightly lower levels of MIF in high glucose during basal stimulation compared to their controls (see Appendix 4.0).

A variable amount of MIF was detected in the supernatant of podocytes stimulated with rMIF. The largest amount of MIF detected was in glycated albumin, mannitol and high glucose with little change in levels detected in normal glucose conditions, see Figure 4.4.1.1 below and Table A4T22-T24, Appendix 4.0. Mannitol stimulated with rMIF had significantly more MIF ($p<0.01$), compared

to mannitol stimulation alone. There were no other significant rises in MIF detected post rMIF stimulation in the other conditions.

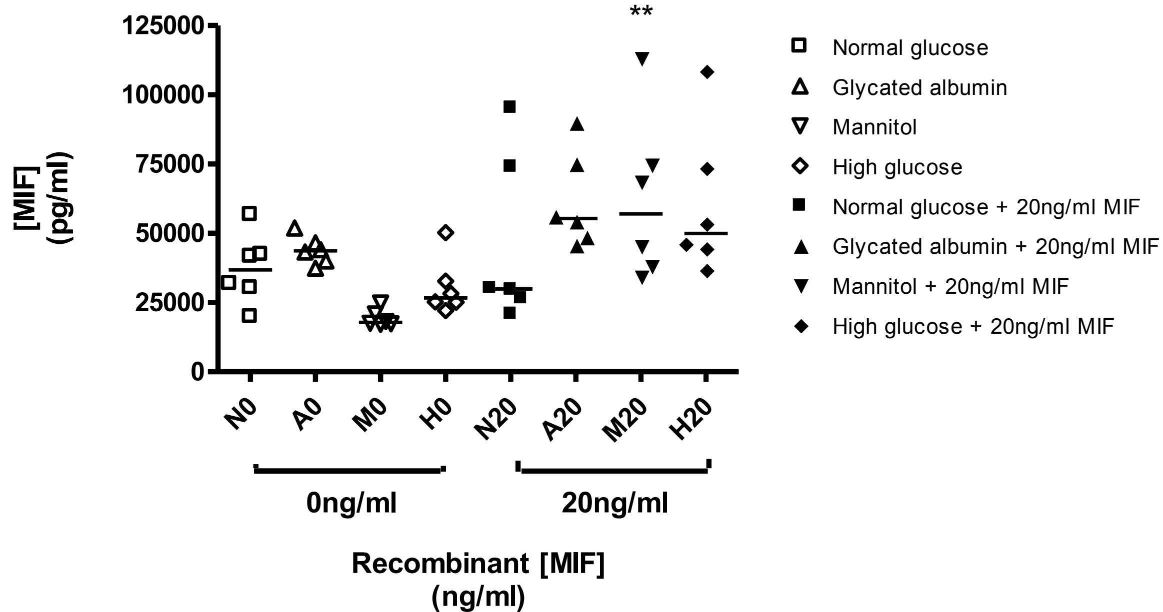


Figure 4.4.1.1 Production of MIF in Podocytes in DM milieu following stimulation with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MIF stimulation, 20=20ng/ml of MIF stimulation.

**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MIF (N0) compared to Normal glucose with 20ng/ml of MIF (N20). *p<0.05, **p<0.01, ***p<0.001.*

All three experiments showed a trend for higher levels of MIF in glycated conditions without and with rMIF, that were not significant. Experiment 3 showed a significant rise in MIF levels in high glucose conditions stimulated with rMIF (p<0.001) that was not reproduced in the other experiments. There was a downward trend for lower MIF levels in high glucose conditions with rMIF compared with its control mannitol (see Appendix 4.0).

Summary

Podocytes are able to produce MIF following stimulation with basal conditions for 48 hours. These levels increase further with rMIF. There were higher detectable level of MIF in glycated albumin that was not significant. In contrast there lower MIF levels in high glucose with rMIF when compared to its control. MIF mRNA could be measured in future to determine whether there is synthesis of MIF in podocytes following rMIF stimulation in the different conditions or whether the increase in MIF is due to the addition of rMIF itself.

4.4.1.2 Effects of rMIF on MCP-1 in Podocytes

MCP-1 was detected in podocytes stimulated with basal conditions and those stimulated with rMIF at 48 hours. There was significantly more MCP-1 produced in podocytes in high glucose with rMIF compared to those in high glucose only ($p<0.01$), see 4.4.1.2. MCP-1 was significantly increased in normal glucose following stimulation with rMIF ($p<0.05$), see Appendix 4.0.

MCP-1 detection seems to be increased in podocytes in diabetic milieu with rMIF stimulation compared with the condition stimulus alone.

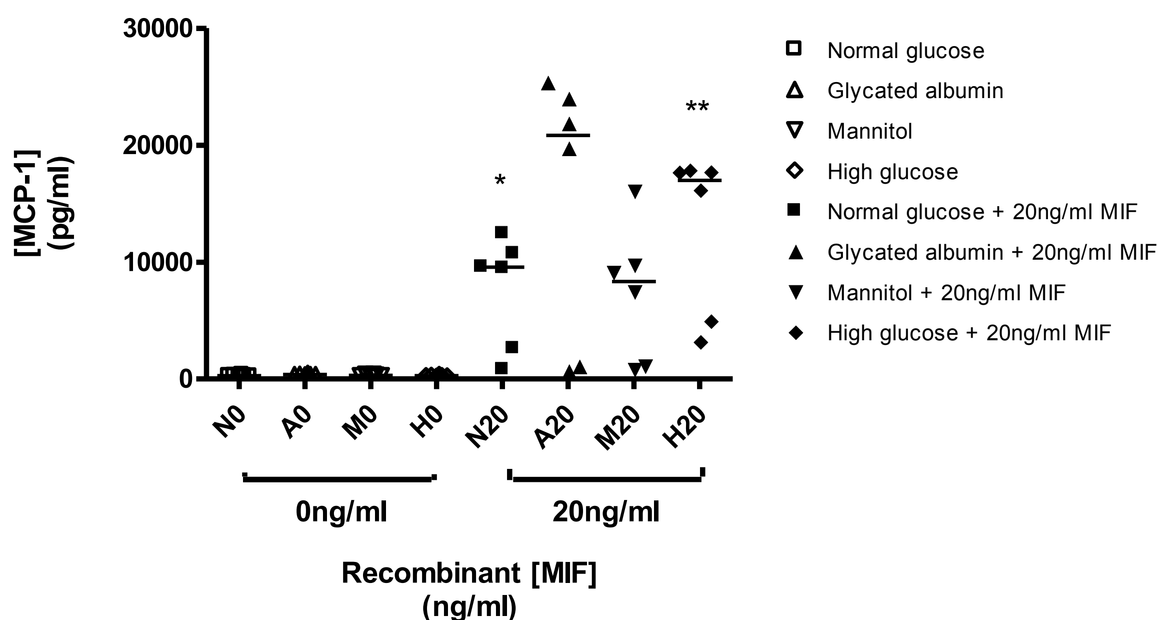


Figure 4.4.1.2 Production of MCP-1 in Podocytes in DM milieu following stimulation with rMIF

Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MIF stimulation, 20=20ng/ml of MIF stimulation.

**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MIF (N0) compared to Normal glucose with 20ng/ml of MIF (N20). *p<0.05, **p<0.01, ***p<0.001.*

There was an increase MCP-1 in glycated albumin with rMIF in experiments 1 and 2 that was not seen in experiment 3, see Table 4.9. Cell viability in glycated albumin with rMIF improved in these two experiments although this was similar to its controls thus this would not explain the trend to increase MCP-1 in this environment.

Table 4.9 The amount of MCP-1 found in podocytes without and with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MIF	**Basal condition**	**Median (range) (pg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	294 (213-377) vs 379 (356-476)	ns
0ng/ml	M vs H	316 (203-416) vs 284 (250-384)	ns
20ng/ml	N vs A	9560 (841-12500) vs 20800 (732-25400)	ns
20ng/ml	M vs H	8350 (873-16100) vs 17000 (3240-17900)	ns
MIF stimulation	N20 vs N0	9560 (841-12500)-294 (213-377)	p<0.05
MIF stimulation	A20 vs A0	20800 (732-25400)-379 (356-476)	ns
MIF stimulation	M20 vs M0	8350 (873-16100)-316 (203-416)	ns
MIF stimulation	H20 vs H0	17000 (3240-17900)-284 (250-384)	p<0.01

Summary

Following 48 hours of stimulation, MCP-1 was detected in podocytes in all basal conditions. There was a significant increase in MCP-1 in high glucose with rMIF stimulation. Increasing MCP-1 was seen in glycated albumin with rMIF stimulation that did not reach significance. These changes were

unrelated to cell viability. MCP-1 in podocytes was affected by the diabetic milieu more than control conditions with rMIF.

4.4.1.3 Effects of rMIF on CCL18 in Podocytes

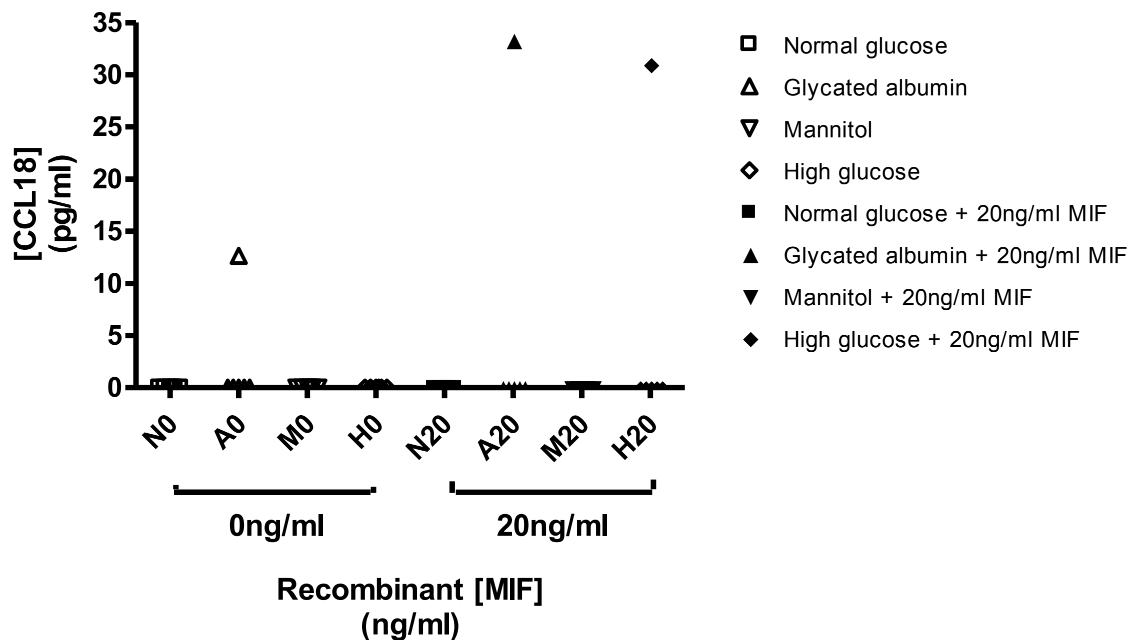


Figure 4.4.1.3 Production of CCL18 in Podocytes in DM milieu when following stimulation with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MIF stimulation, 20=20ng/ml of MIF stimulation.

CCL18 was inconsistently detected in glycated albumin conditions in podocytes following 48 hours stimulation, see Fig 4.4.1.3. Other basal conditions did not stimulate the production of CCL18 in podocytes. CCL18 was detected in some podocytes following stimulation with rMIF in high glucose and glycated albumin compared with controls. There was no significant difference in the levels of CCL18 detected in podocytes following stimulation with rMIF irrespective of the basal conditions. This was consistent in all the experiments.

Summary

Low levels of CCL18 were inconsistently detected in podocytes in all three experiments regardless of the stimulus or presence of rMIF with the conditions.

4.4.1.4 Effects of rMIF on Fibronectin in Podocytes

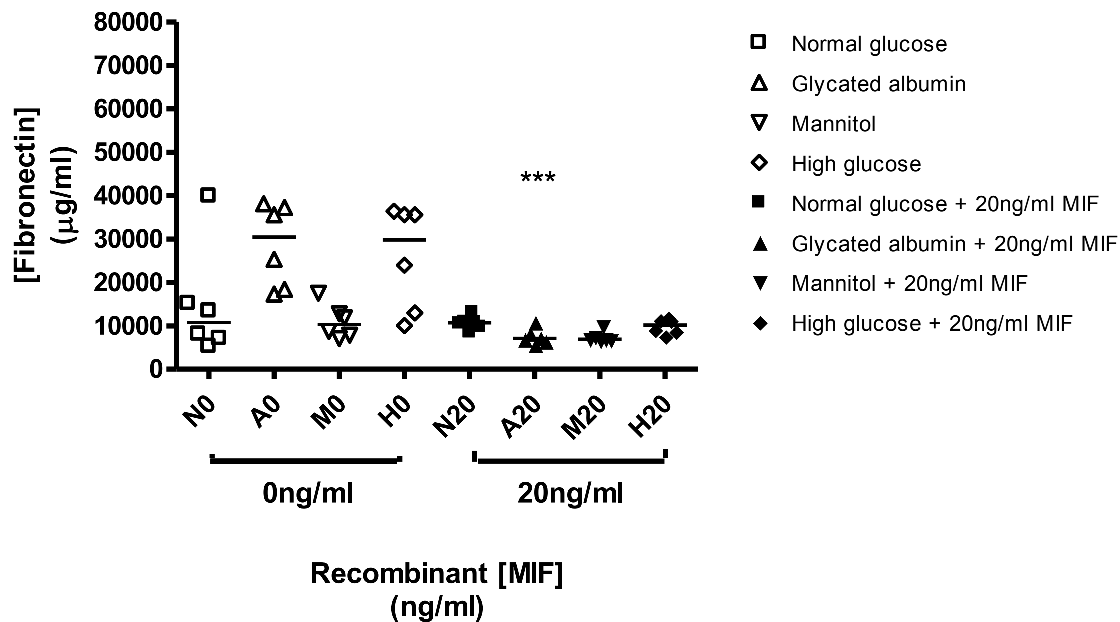


Figure 4.4.1.4 Production of Fibronectin in Podocytes in DM milieu following stimulation with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MIF stimulation, 20=20ng/ml of MIF stimulation.
**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MIF (N0) compared to Normal glucose with 20ng/ml of MIF (N20). *p<0.05, **p<0.01, ***p<0.001.*

High levels of fibronectin were detected in podocytes following 48 hours stimulation with glycated albumin and high glucose compared to their controls, see Fig 4.4.1.4. This difference did not reach significance. The exception was experiment 3 where fibronectin was significantly higher in basal glycated albumin compared with normal glucose ($p<0.001$). I Increased levels of fibronectin were seen in high glucose conditions in all the experiments thatwas not significant.

There was an overall decrease in the production of fibronectin in podocytes stimulated with rMIF that was evident in experiments 1 and 2. The cells in experiment 3 had a lower cell viability compared with experiments 2 and 3. There were elevated levels of fibronectin in glycated albumin and high glucose with rMIF compared with these basal conditions alone in experiment 3 (see Appendix 4.0). Where there were fewer surviving podocytes MIF stimulation resulted in increased fibronectin.

Summary

Fibronectin was detected in podocytes following stimulation with basal conditions for 48 hours. There were higher basal fibronectin levels in the diabetic milieu. Fibronectin levels detected were reduced following rMIF stimulation with the basal conditions. Poor cell viability resulted in an increase in fibronectin in glycated albumin and high glucose conditions with rMIF.

Summary of results

MIF and MCP-1 were detected in all basal conditions at 48 hours in podocytes. CCL18 was detected inconsistently in podocytes regardless of the stimulus used. MIF was raised in normal glucose and glycated albumin conditions however, this is not maintained with rMIF stimulation where MIF levels are higher in glycated albumin levels than normal glucose. Stimulation with rMIF results in an increase in MCP-1 that is significant in normal and high glucose conditions. There was an increase in MCP-1 in glycated albumin with rMIF however, this is not significant. CCL18 is not significantly different following rMIF with basal conditions. Stimulation with rMIF decreases fibronectin production in all conditions and is significantly decreased in glycated albumin. The MTT assay suggests an increased cell survival in glycated albumin following rMIF.

4.4.2 Podocytes stimulated with rMCP-1 - MTT Assay

Cell viability was higher in podocytes in high glucose for 48 hours that decreased following rMCP-1 stimulation, see Fig 4.4.2. Stimulation with rMCP-1 in glycated albumin resulted in an increase in cell viability compared with glycated albumin alone or its control, normal glucose. This was seen in both experiments 1 and 2.

MTT Assay for Podocytes stimulated under diabetic milieu with different concentrations of Recombinant MCP-1

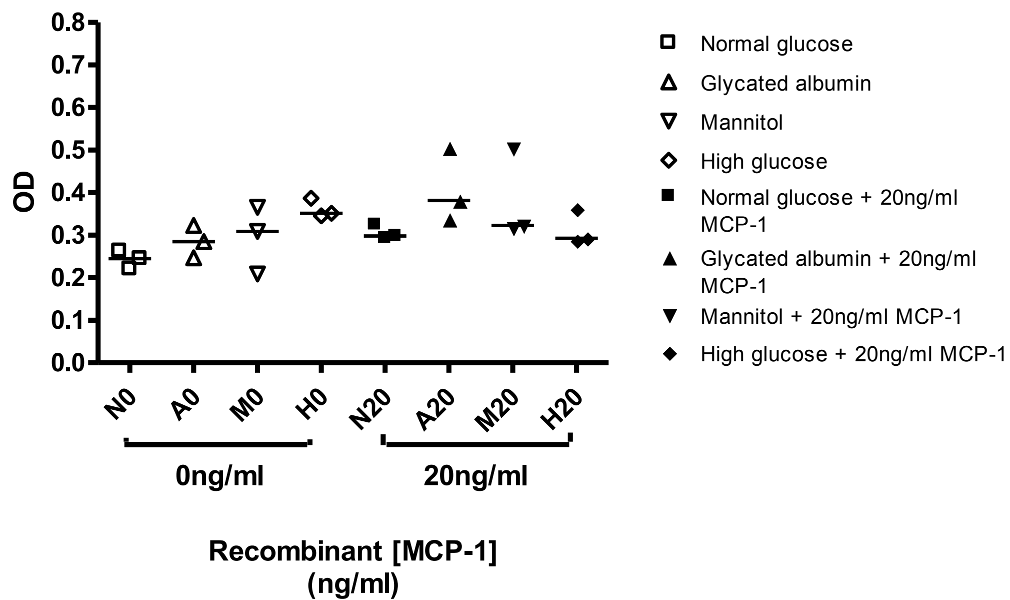


Fig 4.4.2 MTT assay for podocytes in diabetic milieu stimulated with rMCP-1.

Experiment 3 had similar findings despite lower cell viability. This was consistent with the cell viability seen in experiment 3 of podocytes stimulated with rMIF.

MCP-1 was detected by podocytes stimulated in basal conditions for 48 hours.

4.4.2.1 Effects of rMCP-1 on MCP-1 in Podocytes

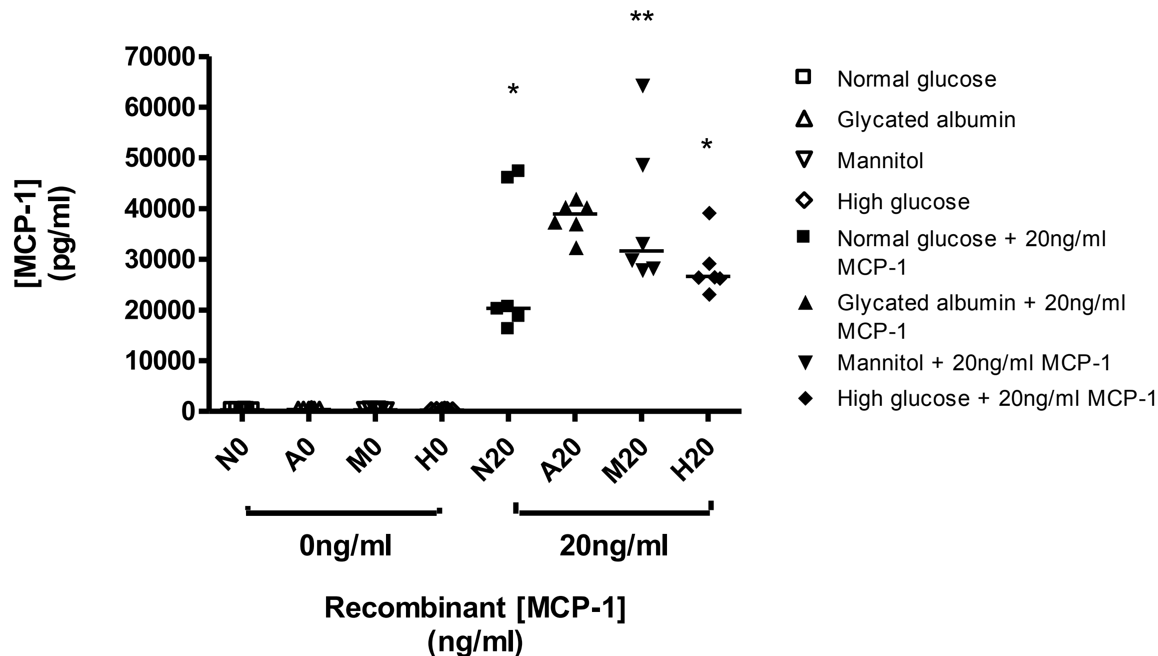


Fig 4.4.2.1 Production of MCP-1 in diabetic milieu and following stimulation with rMCP-1.
Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.
**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MCP-1 (N0) compared to Normal glucose with 20ng/ml of MCP-1 (N20). *p<0.05, **p<0.01, ***p<0.001.*

There was a significant rise in MCP-1 in podocytes following stimulation with rMCP-1 in normal glucose ($p<0.05$), mannitol ($p<0.01$) and high glucose ($p<0.05$), see Fig 4.4.2.1. There was no significant change between the conditions and their controls following stimulation with rMCP-1. Part of the increased amount of MCP-1 would be due to the addition of rMCP-1 (20ng/ml) used in the stimulation. In some of the wells, however, the amount of MCP-1 detected was much higher than 20 ng/ml, that may suggest localised production.

Experiments 2 and 3 show a significant rise in MCP-1 in podocytes stimulated with glycated albumin (p<0.05, p<0.001, respectively). All three experiments consistently show a significant rise in MCP-1 in high glucose with rMCP-1.

Summary

MCP-1 is produced by podocytes with basal stimulation. This increases significantly when in glycated albumin with rMCP-1 or high glucose with rMCP-1. These changes did not reflect differences in cell viability.

4.4.2.2 Effects of rMCP-1 on MIF in Podocytes

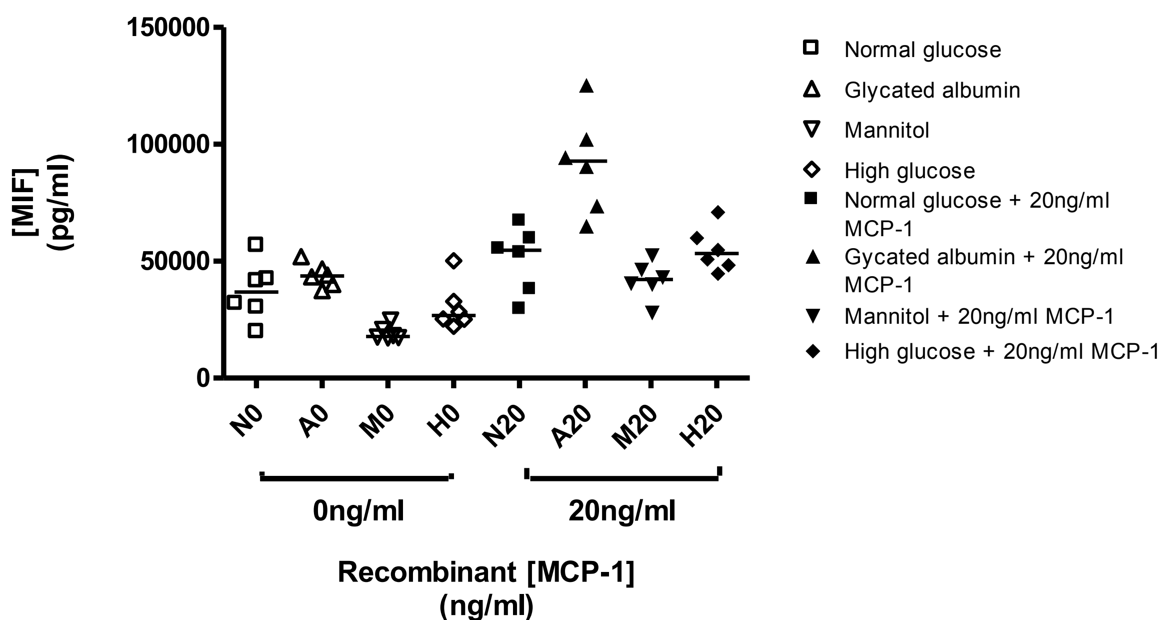


Fig 4.4.2.2 Detection of MIF in podocytes in diabetic milieu and following stimulation with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

MIF was detected in podocytes after 48 hours of basal condition stimulation as per previous findings. There was a rise in MIF in all conditions following stimulation with rMCP-1 that was not significant, see Fig 4.4.2.2. All three experiments had an increase in MIF in glycated albumin with rMCP-1. Experiments 1 and 2 showed an increase in MIF found in high glucose with rMCP-1. In contrast,

experiment 3 showed MIF levels to be decreased in high glucose with rMCP-1. This may reflect the low cell viability seen in the MTT assay. Experiment 2 showed significantly more MIF compared to basal conditions without rMCP-1 stimulus in normal glucose, glycated albumin and high glucose (all $p<0.05$). These findings were also seen in the other experiments, however, they did not reach significance.

Summary

MIF was produced in all conditions at baseline in podocytes following 48 hours stimulation. There was an increase in MIF in all conditions following rMCP-1. There were higher levels in glycated albumin and high glucose conditions with rMCP-1. Experiment 3 had a decrease in podocyte MIF levels with high glucose and rMCP-1 with low cell viability.

4.4.2.3 Effects of rMCP-1 on CCL18 in Podocytes

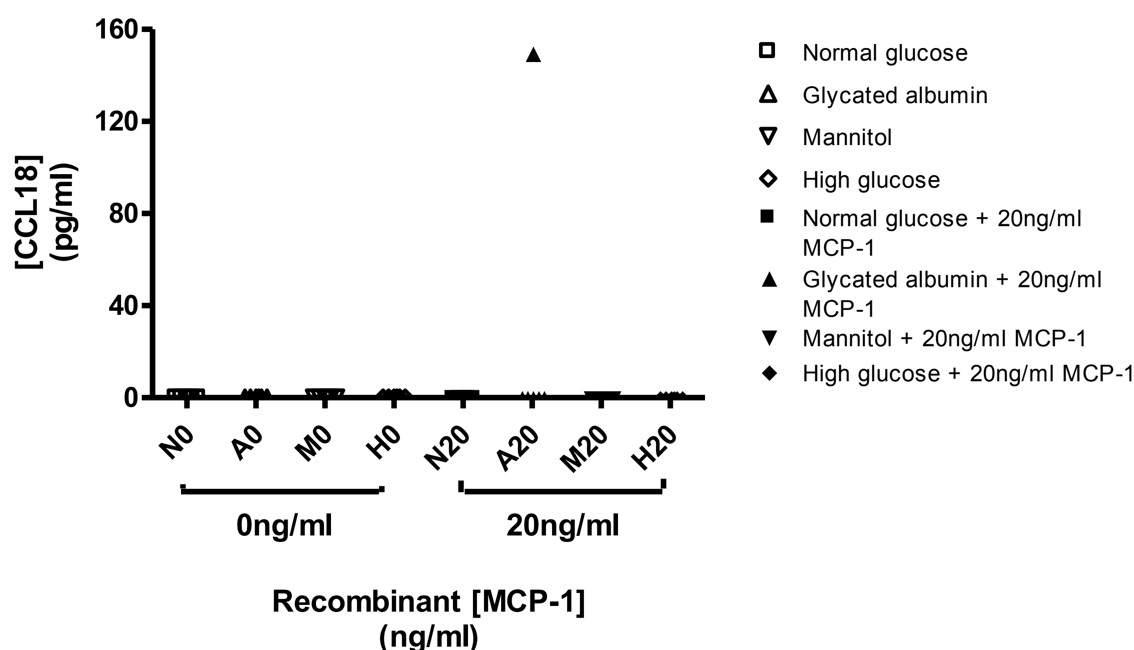


Figure 4.4.2.3 Production of CCL18 in Podocytes in diabetic milieu stimulated with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

CCL18 was not detected in podocytes stimulated with basal conditions after 48 hours, see Fig 4.4.2.3. Podocytes produced minimal amounts of CCL18 after stimulation with rMCP-1, however, this was an inconsistent finding and was not specific to a condition.

Summary

There is inconsistent detection of CCL18 in podocytes in different basal conditions with or without rMCP-1.

4.4.2.4 Effects of rMCP-1 on Fibronectin in Podocytes

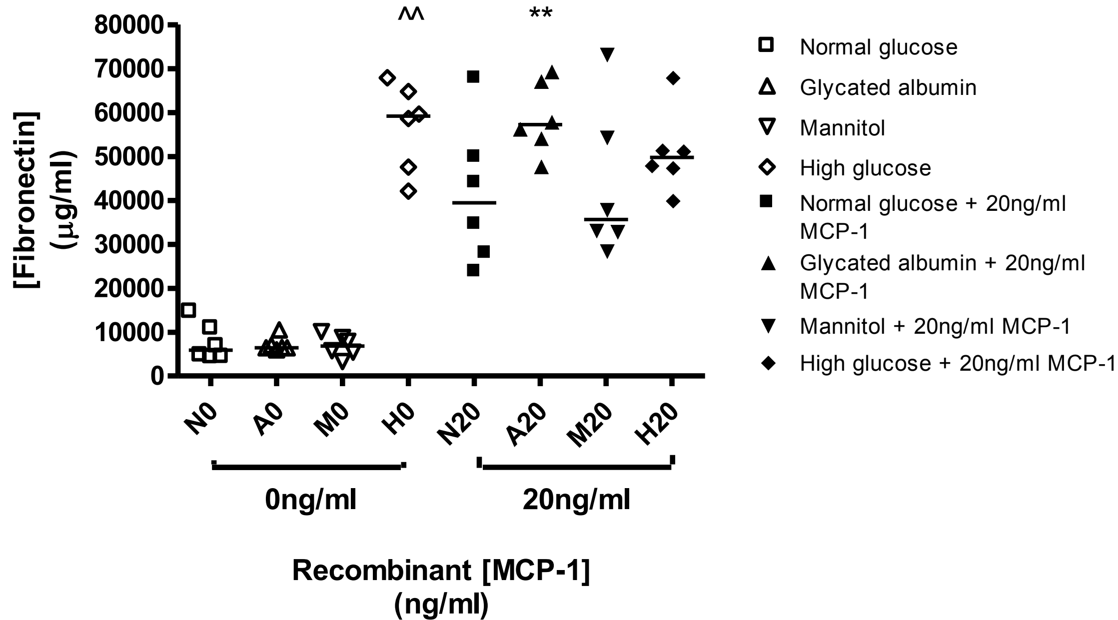


Figure 4.4.2.4 Production of Fibronectin in podocytes in diabetic milieu stimulated with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of MCP-1 stimulation, 20=20ng/ml of MCP-1 stimulation.

^ depicts the level of significance between basal conditions and their controls ie. Normal glucose concentration (N0) compared to glycated albumin (A0). The osmotic control Mannitol (M0) compared to high glucose (H0). ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$.

*depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of MCP-1 (N0) compared to Normal glucose with 20ng/ml of MCP-1 (N20). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Fibronectin is consistently produced by podocytes after 48 hours stimulation of basal conditions as seen in previous experiments. There was significantly more fibronectin produced in podocytes stimulated with high glucose compared with mannitol ($p<0.01$), see Fig 4.4.2.4. This was not seen in experiments 2 or 3 although there were higher levels detected in high glucose; the median was 29800µg/ml with a range of 10000-36400µg/ml. Experiment 3 median for high glucose was 3220µg/ml with a range of 1030-8310µg/ml. The median for mannitol was 10300µg/ml with a range of 7050-17500µg/ml in experiment 2 and a median of 2190µg/ml with a range of 336-3960µg/ml in experiment 3. Experiment 3 detected significantly higher levels of fibronectin in basal glycated albumin ($p<0.01$), a finding also seen in experiments 1 and 2 (see Appendix 4.0).

There was a significant rise in fibronectin following stimulation with rMCP-1 in glycated albumin compared to basal levels ($p<0.01$), that was duplicated in experiment 2 ($p<0.001$). There was an increase in fibronectin levels seen in glycated albumin and high glucose with rMCP-1 compared with their controls, that was not significant. Interestingly, this finding was reversed in experiment 2 where the levels of fibronectin were lower in the diabetic milieu compared to their controls. The MTT assay for experiment 2 showed the cell viability in glycated albumin with rMCP-1 to have less of a survival advantage compared to normal glucose. Comparing this to the other 2 experiments may account for the difference seen. The table for results of experiments 1-3 are included in the Appendix 4.0.

Summary

Fibronectin is increased in podocytes in glycated albumin or high glucose for 48 hours compared to their controls. Fibronectin levels increase in glycated albumin and high glucose conditions with rMCP-1 compared to their respective controls. This was not seen in one experiment where the cell viability was slightly lower in the glycated albumin with rMCP-1 stimulus compared to the other experiments.

Summary of results

MCP-1 is produced by podocytes in all conditions and rises further with stimulation with its recombinant form in all conditions. MIF is produced by podocytes in all conditions and is increased with stimulation of rMCP-1. There is a significant rise in MIF levels in podocytes exposed to glycated albumin. Podocytes did not produce CCL18 in the basal conditions or following stimulation with rMCP-1. Fibronectin is detected in podocytes after 48 hours stimulation with basal conditions. Fibronectin levels in podocytes increased following stimulation with rMCP-1. There were slightly more surviving cells in high glucose compared with other conditions. Cell survival seemed to increase in glycated albumin with rMCP-1 stimulation except for one experiment. rMCP-1 further increases MIF levels in podocytes in glycated albumin with rMCP-1 and in high glucose conditions with rMCP-1.

4.4.3 Summary of Podocyte cell culture data

Podocytes are able to produce MIF and MCP-1 in all conditions. CCL18 may be produced invariably. The results are summarised below in Table 4.10

Table 4.10 Summary of Podocyte cell culture data

Podocytes	***Effects***
Basal conditions	*MIF is detected in all conditions in podocytes* *MCP-1 is detected in all conditions in podocytes* *CCL18 is inconsistently detected in all conditions* *Fibronectin was higher in high glucose and glycated albumin*
MIF stimulation	*rMIF increases MIF in all conditions* *rMIF increases MCP-1 in normal and high glucose with an increase in glycated albumin* *rMIF has little or no effect on CCL18* *rMIF significantly decreases fibronectin in glycated albumin with a decrease fibronectin in high glucose* *rMIF stimulation increases cell survival in glycated albumin*
MCP-1 stimulation	*rMCP-1 increases in all conditions* *rMCP-1 significantly increases MIF in glycated albumin*

	rMCP-1 has no effect on CCL18 *rMCP-1 significantly increases fibronectin in all conditions* *rMCP-1 increases cell survival in glycated albumin*

4.5 Signalling pathways activated with cytokine stimulation

The active forms of the cell signalling pathways of p38 MAPK and p44/42 MAPK were measured with Western blot (phospho p38 MAPK and phospho p44/42 MAPK, respectively). Phospho p38 MAPK and Phospho p44/42 MAPK protein levels were determined by band densitometry following Western blotting. Actin levels were consistent allowing comparison of band density between different stimuli. In view of time limitations Actin was tested initially on the Western blot to establish the reproducibility of the BCA. Actin was not tested on every sample presented as the total protein for p38 MAPK and p44/42 MAPK was to be measured to quantify the degree of activation by comparing this with the level of phospho protein.

The cleaved caspases were to be re-blotted with the total caspase blots to determine the degree of caspase activity. Unfortunately, this aspect of the project was not done, thus the phospho active forms of p38 MAPK and p44/42 MAPK together with the total caspases 3, 7 and 9 are presented herein. The protein lysates were collected in conjunction with the supernatant and are at a 48 hours time point. Shorter time point experiments would need to be undertaken to determine whether these pathways are activated and influenced following cytokine stimulation. Those seen at 48 hours are likely to be the established pathways that continue with persistent cytokine stimuli. Caspase activation in combination with the MTT assay will help determine whether the findings are secondary to activation of cellular death. The Western blot analysis was done for cell lysates of Experiment 1.

4.5.1 HK-2 cells stimulated with CCL18 or MCP-1

The protein lysates from the cells stimulated with basal conditions and those stimulated with rCCL18 and rMCP-1 were analysed. The effect of the cytokine stimulation in a condition was compared to those stimulated with the condition alone.

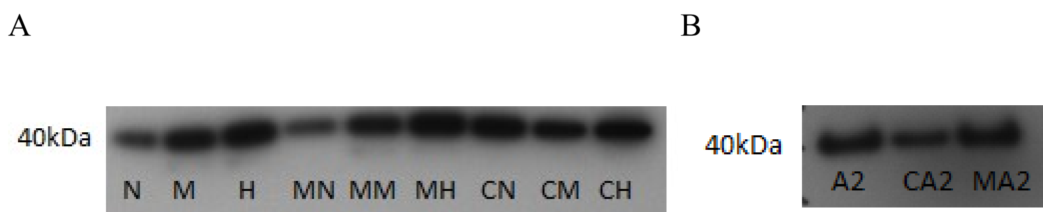
4.5.1.1 Phospho p38 MAPK

Phospho p38 MAPK is found in HK2 cells stimulated with all the different conditions. More is seen in the diabetic conditions compared to their controls. MCP-1 is known to use phospho p38 MAPK

as a cell signalling mechanism (Sheryanna, Bhangal et al. 2007) and hence activation of phospho p38 MAPK in conditions with rMCP-1 may be a true reflection of the persistent stimulation with rMCP-1. Stimulation of HK-2 cells with rCCL18 and rMCP-1 in normal glucose, mannitol and high glucose appear to have little effect on the production of phospho p38 MAPK compared to these conditions alone. Normal glucose with rCCL18 appears to have higher levels of phospho p38 MAPK with little change in those stimulated with rMCP1. Alternatively there may be an increased use of this signalling pathway leading to less active protein. Shorter time point experiments will help determine this.

Phospho p38 MAPK is decreased in HK2 cells in glycated albumin with rCCL18 or rMCP-1 compared to glycated albumin alone, see Fig 4.5.1.1. Glycated albumin stimulated with rMCP-1 appears to have higher phospho p38 MAPK than those stimulated with rCCL18, suggesting there is an upregulation of this protein in HK-2 cells that may mediate or maintain the effect of rMCP-1. The activated form would ideally be compared with the total p38 MAPK protein to ensure there is a difference in the activation of this protein, however the effect of cytokine stimulation is compared to that of stimulation with the basal condition. At 48 hours timepoint it is unclear whether the subsequent activation and stimulation of other pathways may lead to the increase seen in phospho p38 MAPK production. A time course experiment looking at cell signalling would be able to determine this and was beyond the scope of this project. MTT assay showed a survival advantage of HK2 cells in glycated albumin with rMCP-1 at 48 hours.

A B



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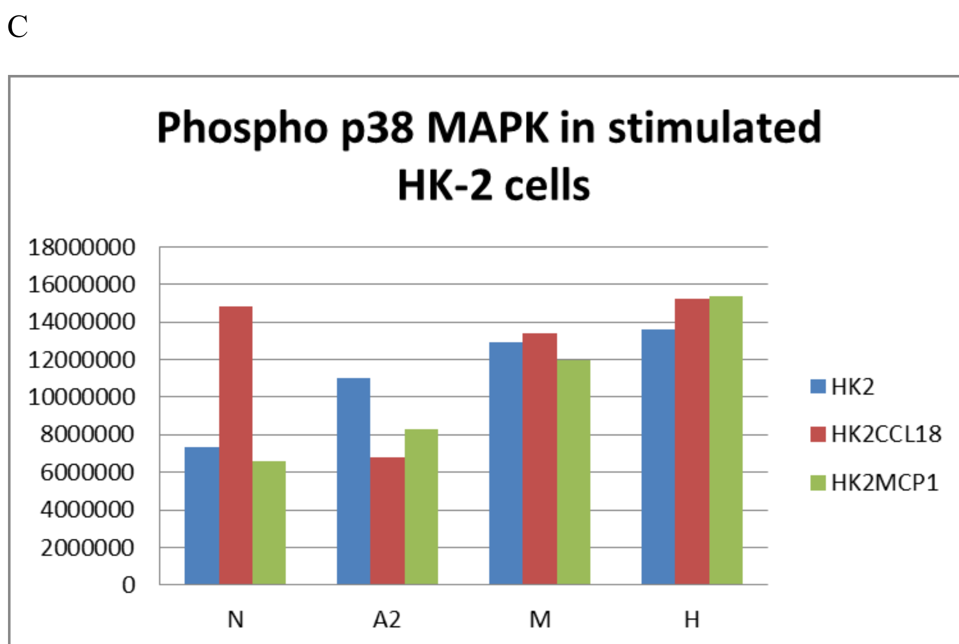


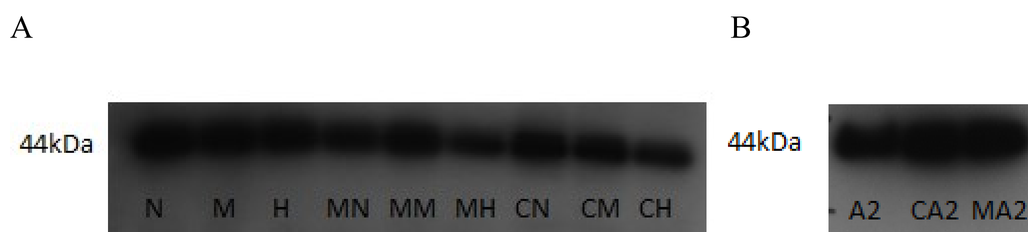
Figure 4.5.1.1 A: p38 MAPK (40kDa) from HK2 cells stimulated with normal glucose, mannitol and high glucose. B: p38 MAPK(40kDa) from

HK2 cells stimulated with glycated albumin. C: Phospho p38 MAPK in stimulated HK2 cells. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and C – rCCL18 (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are HK2 cells stimulated with 20ng/ml rCCL18, the green bars are HK2 cells stimulated with 20ng/ml rMCP-1.

4.5.1.2 Phospho p44/42 MAPK (ERK1/2)

Phospho p44/42 MAPK was found in all conditions with or without recombinant stimulation. Similar amounts of phospho p44/42 MAPK were seen in normal glucose and mannitol that were stimulated with or without recombinant cytokines. There was a decrease in phospho p44/42 MAPK in high glucose conditions stimulated with rCCL18 or rMCP-1 compared to high glucose alone, see Fig 4.5.1.2. Higher levels of phospho p44/42 MAPK were seen in glycated albumin compared to normal glucose. A further rise was seen in this activated protein in HK2 cells in glycated albumin with rCCL18 and rMCP-1. An improved cell viability was seen in glycated albumin stimulated with rCCL18 and rMCP-1 that may partly contribute to the increased protein expression. It is unclear whether the increased protein level found is a reflection of the cytokine stimulus or whether downstream effectors from the initial cell signalling are producing this protein following 48 hours of stimulation. When comparing glycated albumin without and with cytokine stimulation the latter show an increase in phospho p44/42 MAPK levels.

A B



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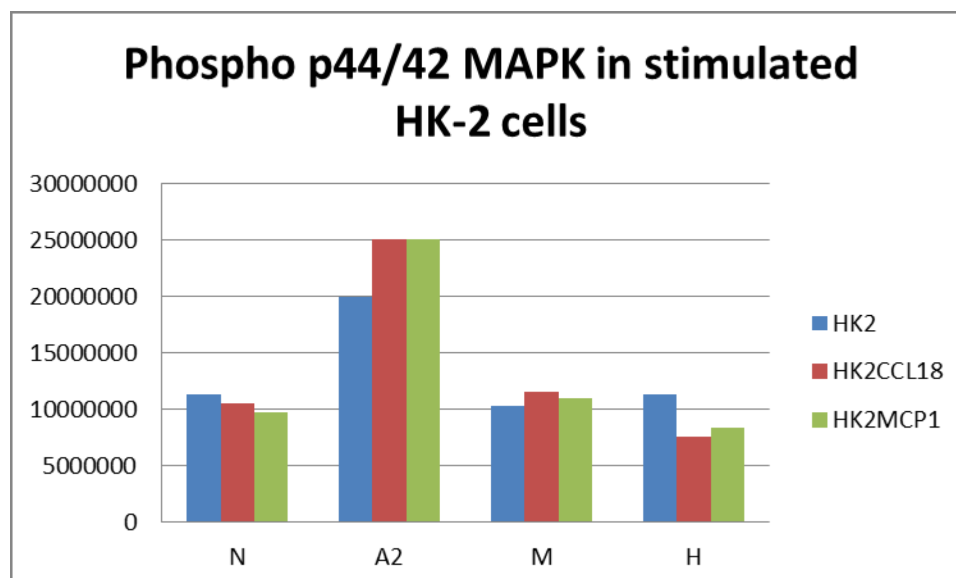


Figure 4.5.1.2 A: p44/42 MAPK (42, 44kDa) from HK2 cells stimulated with normal glucose, mannitol and high glucose. B: p44/42 MAPK (42, 44kDa)

from HK2 cells stimulated with glycated albumin. C: Phospho p44/42 MAPK in stimulated HK2 cells. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and C – rCCL18 (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are HK2 cells stimulated with 20ng/ml rCCL18, the green bars are HK2 cells stimulated with 20ng/ml rMCP-1.

<u>Summary of HK2 cell signalling</u>

After 48 hours of stimulation phospho p38 MAPK is evident in all basal conditions with higher levels in mannitol and high glucose. There is a decrease in these protein levels in glycated albumin with rMCP-1 and rCCL18. High glucose conditions with rCCL18 or rMCP-1 have more phospho p38 MAPK activation. In comparison phospho p44/42 MAPK has higher activity in HK2 cells in glycated albumin. This increases further with rCCL18 or rMCP-1, the mechanism by how this occurs needs further investigation.

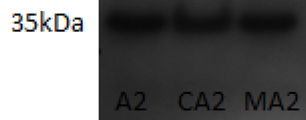
Glycated albumin activates both phospho p38 MAPK and phospho p44/42 MAPK regardless of cytokine stimulation. Phospho p44/42 MAPK is higher with cytokine stimulation. HK2 cells stimulated with high glucose with rCCL18 or rMCP-1 stimulation produce more phospho p38 MAPK than HK2 cells solely exposed to high glucose. The MTT assay of this experiment shows that basal glycated albumin had the lowest cell survival and despite this there was greater phospho p44/42 MAPK activation with cytokine stimulation. The levels of phospho p38 MAPK were lower with cytokine stimulation in glycated albumin compared to glycated albumin alone. Cytokine stimulation at 48 hours affected levels of activated p38 and p44/42 MAPK compared to basal conditions alone, irrespective of cell viability.

4.5.1.3 Caspase 3

Caspase 3 is an effector caspase that once activated cleaves cytoskeletal and nuclear proteins to induce apoptosis. Caspase 3 Ab detects 3 bands 17, 19, 35kDa with Cleaved caspase 3 detecting the two proteins at 17, and 19kDa if present. HK-2 stimulated cells produced a single 35kDa band in all conditions though this was markedly reduced in normal glucose, mannitol, and high glucose conditions stimulated with rMCP-1, see Fig 4.5.1.3. Higher levels of caspase 3 were seen in glycated albumin however stimulation with rCCL18 or rMCP-1 in glycated albumin had lower levels with improved cell survival on MTT assay.

A

B



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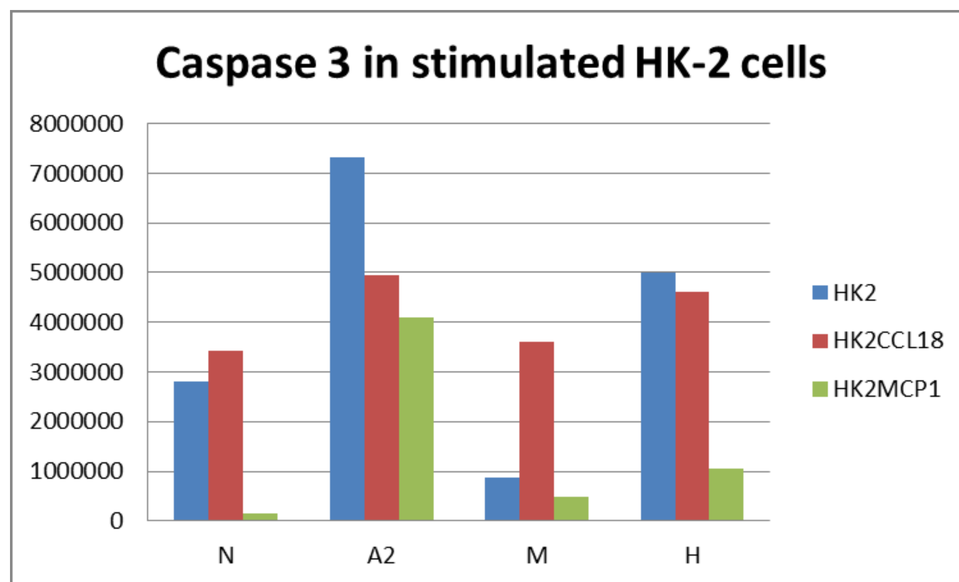
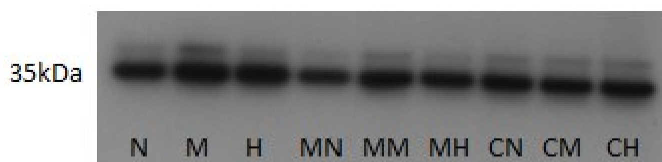


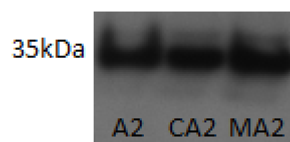
Figure 4.5.1.3 A: Caspase 3 (17,19,35kDa) from HK2 cells stimulated with normal glucose, mannitol and high glucose. B: Caspase 3 (17,19,35kDa) from HK2 cells stimulated with glycated albumin. C: Caspase 3 in stimulated HK2 cells. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and C – rCCL18 (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are HK2 cells stimulated with 20ng/ml rCCL18, the green bars are HK2 cells stimulated with 20ng/ml rMCP-1.

4.5.1.4 Caspase 7

Caspase 7 is also an effector caspase that once activated may induce apoptosis. Caspase 7 detects 2 bands 20, and 35kDa. Cleaved caspase 7 has its product at 20kDa. The 20kDa band was not detected in any of the conditions or following cytokine stimulation. Cleaved caspase 7 would need to be repeated in Western blot to determine the activity of the protein, however, the protein is unlikely to be seen as the band was not detected in the total form. HK2 cells are able to produce caspase 7 with higher levels seen in glycated albumin conditions stimulated with rCCL18 or rMCP-1, see Fig 4.5.1.4. Those in normal glucose had higher levels following cytokine stimulation though less when compared to glycated albumin. High glucose conditions had less caspase 7 compared to its control mannitol, with slightly higher levels in HK2 cells in high glucose with rMCP-1.

A B





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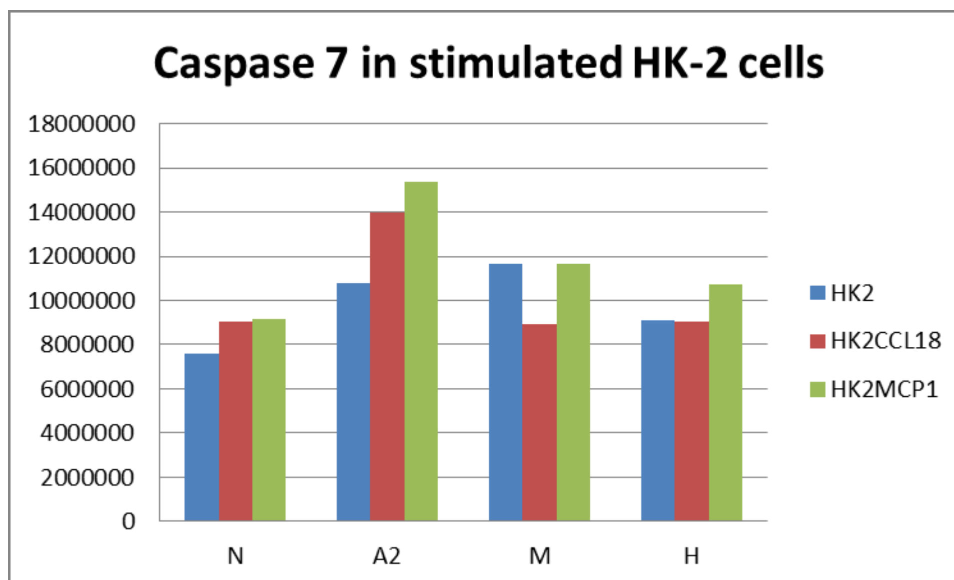


Figure 4.5.1.4 A: Caspase 7 (20, 35kDa) from HK2 cells stimulated with normal glucose, mannitol and high glucose. B: Caspase 3 (17,19,35kDa) from HK2 cells stimulated with glycated albumin. C: Caspase 7 in stimulated HK2 cells. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and C – rCCL18 (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are HK2 cells stimulated with 20ng/ml rCCL18, the green bars are HK2 cells stimulated with 20ng/ml rMCP-1.

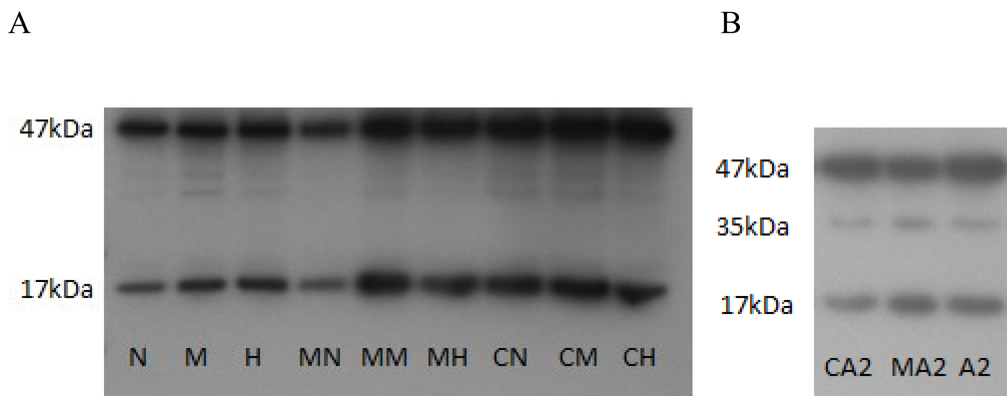
There were less viable cells in glycated albumin conditions in HK2 cells however, Caspase 7 levels were further increased with cytokine stimulation where good cell viability was seen. The raised Caspase 7 activity did not correlate with increased cell death.

4.5.1.5 Caspase 9

Caspase 9 has 4 protein bands at 17, 35, 37, and 47kDa with cleaved caspase 9 having 2 protein bands at 17, and 37kDa, bands 1 and 3 respectively. This initiator caspase shows activation in all bands in glycated albumin conditions in HK2 cells. The other conditions show that HK2 cells at 48 hours have the ability to produce caspase 9 protein bands 1 and 4, see Fig 4.5.1.5. Glycated albumin had lower levels of caspase 9 following rCCL18 or rMCP-1 stimulation that may act to decrease the

initiation of apoptosis in these cells. The cleaved caspase 9 form would need to be re-blotted to determine the activity of this caspase. rCCL18 and rMCP-1 stimulation increased the levels of caspase 9 in mannitol and high glucose in a similar manner.

A B



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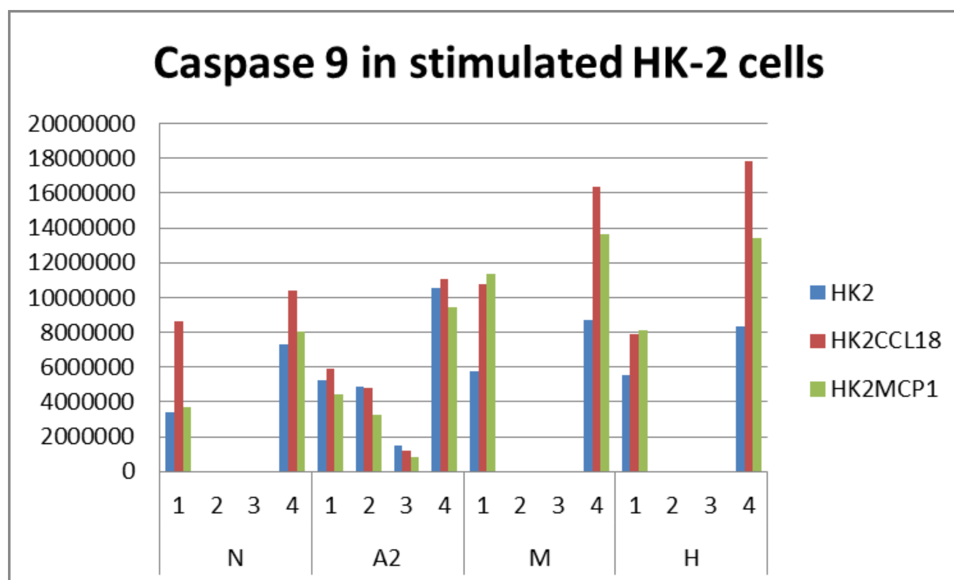


Figure 4.5.1.5 A: Caspase 9 (17,35, 37, 47kDa) from HK2 cells stimulated with normal glucose, mannitol and high glucose. B: Caspase 9 (17,35, 37, 47kDa) from HK2 cells stimulated with glycated albumin. C: Caspase 9 in stimulated HK2 cells. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and C – rCCL18 (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are HK2 cells stimulated with 20ng/ml rCCL18, the green bars are HK2 cells stimulated with 20ng/ml rMCP-1. The four bands seen in total caspase 9 are at 17, 35, 37, and 47kDa presented as 1 to 4 per condition. The cleaved forms are 17, and 37kDa, bands 1 and 3 respectively.

Summary of results of Caspases in HK2 cells

Glycated albumin had higher levels of caspase 3 and 7, the effector caspases. Caspase 3 production was abrogated with rMCP-1 stimulation although an increase in cleaved caspase 7 product was seen in glycated albumin irrespective of cytokine stimulation. Western blot for cleaved caspases would help inform the degree of activity within these cells at the 48 hour time point. Repeating the experiments with collection of cell lysates at different time points would be more informative of the time frame whereby the environment activates certain pathways.

There was more caspase 9 in glycated albumin. Overall there was more caspase 9 activity with rCCL18 stimulation in all conditions that were independent of cell viability as compared to the MTT assay. High glucose with rCCL18 or rMCP-1 stimulation, had higher levels of caspase 9. Cleaved caspases would determine the level of activity of this protein within the cells. Glycated albumin MTT assay was lower compared to other conditions regardless of cytokine stimulation. The MTT assay showed that cytokine stimulation in glycated albumin conditions conferred an increase in cell survival, the mechanisms by how this is achieved are unknown. The time point whereby glycated albumin cell survival differs from other conditions would need to be determined together with analysis of cell signalling and caspases at earlier time points as 48 hours may already establish a fixed generation of protein caspases.

4.5.2 Human Podocytes stimulated with MIF or MCP-1

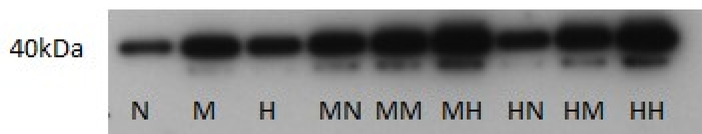
Protein lysates from the cells stimulated with basal conditions and those stimulated with rMIF and rMCP-1 were analysed.

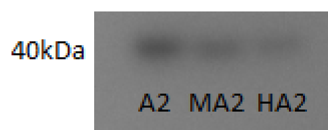
4.5.2.1 Phospho p38 MAPK

Phospho p38 MAPK is found in podocytes in all conditions. There are low levels of phospho p38 MAPK produced by podocytes in glycated albumin, compared to cytokine stimulation. There was no effect on cell viability as the glycated albumin podocytes had the same number of cells with or without cytokine stimulus. Stimulation of the other basal conditions with rMIF showed a slight reduction in phospho p38 MAPK compared to rMCP-1 stimulated conditions, see Fig 4.5.2.1. Phospho p38 MAPK levels were higher in podocytes with conditions with rMIF compared to unstimulated conditions. MCP-1 is known to act through phospho p38 MAPK however whether the degree of protein production of this activated form is maintained at 48hours in podocytes is unknown.

A

B





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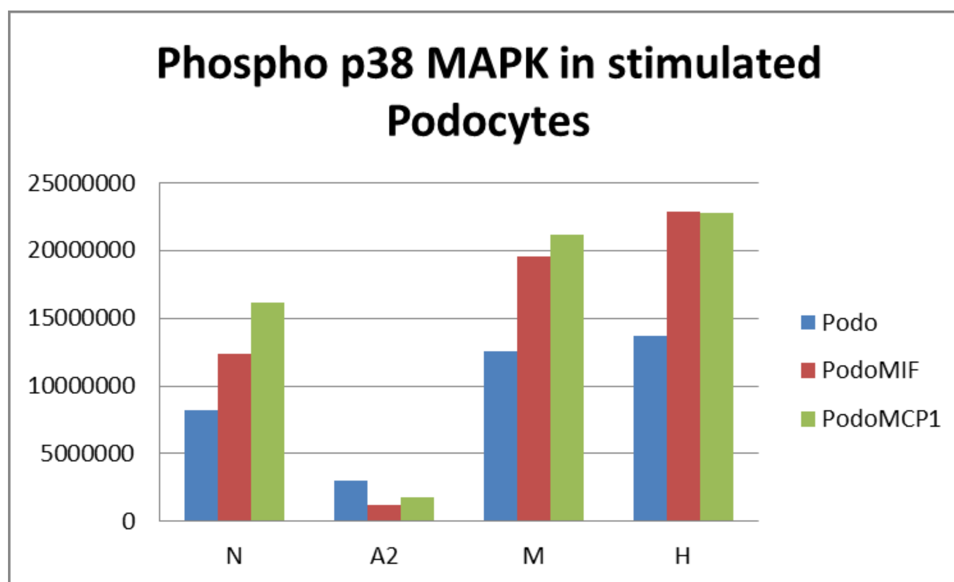


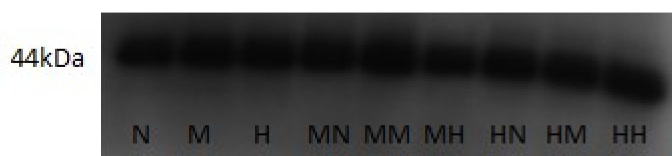
Figure 4.5.2.1 A: p38 MAPK (40kDa) from podocytes stimulated with normal glucose, mannitol and high glucose. B: p38 MAPK(40kDa) from podocytes stimulated with glycated albumin. C: Phospho p38 MAPK in stimulated Podocytes. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and H – rMIF (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are podocytes stimulated with 20ng/ml rMIF, the green bars are podocytes stimulated with 20ng/ml rMCP-1.

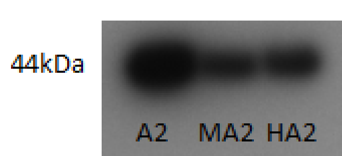
4.5.2.2 Phospho p44/42 MAPK (ERK1/2)

There was a higher level of phospho p44/42 MAPK in podocytes stimulated for 48 hours in glycated albumin, see Fig 4.5.2.2. This rise was abrogated with stimulation with rMIF or rMCP-1 in this environment. Other conditions and stimuli had lower levels of this activated form of p44/42 MAPK. MIF is known to use p44/42 MAPK pathways in cell signalling however, whether other mechanisms are employed to continue its effects are unknown. The activity would need to be compared with the total protein to determine whether the lower levels seen of phospho p44/42 MAPK in other conditions are still significantly higher than would be expected.

A

B





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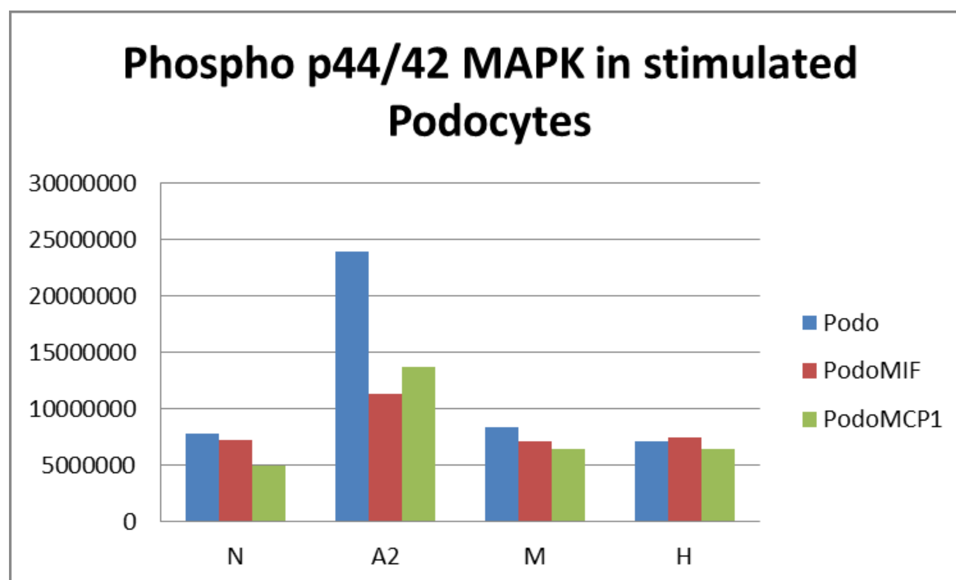

Figure 4.5.2.2 A: p44/42 MAPK (42, 44kDa) from podocytes stimulated with normal glucose, mannitol and high glucose. B: p44/42 MAPK (42, 44kDa) from podocytes stimulated with glycated albumin. C: Phospho p44/42 MAPK in stimulated Podocytes. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and H – rMIF (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are podocytes stimulated with 20ng/ml rMIF, the green bars are podocytes stimulated with 20ng/ml rMCP-1.

Summary of Podocyte cell signalling

There is a marked reduction of activated phospho p38 MAPK in glycated albumin regardless of cytokine stimulation. rMIF and rMCP-1 stimulated conditions appear to increase the levels of phospho p38 MAPK at 48 hours in podocytes in normal glucose, mannitol and high glucose. rMIF and rMCP-1 may therefore use p38 MAPK in these conditions to induce their effects. These pathways however, may have already been switched on following the effects induced by the cytokines and other pathways may have been employed at 48 hours. Short time interval signalling experiments would need to be done to determine whether p38 MAPK is the main cell signalling pathway used by rMIF and rMCP-1 in normal glucose, mannitol and high glucose. Phospho p44/42 MAPK was higher in glycated albumin conditions with less activity seen in podocytes

stimulated with rMIF and glycated albumin. There was no significant change in cell viability to explain these different findings as per the MTT assay.

4.5.2.3 Caspase 3

The findings of caspase 3 suggest glycated albumin has higher levels of cleaved caspase (17, and 19kDa), however, the membrane would need to be reblotted to determine this. Other conditions tended to have a single band with little change of the level of protein found following cytokine stimulation in the same condition. The exception is that of normal glucose where cytokine stimulation appeared to have lower levels of caspase 3 than in normal glucose alone. Mannitol had no lower detectable bands suggesting no cleaved caspase 3, see Fig 4.5.2.3. The MTT assay shows similar cell survival suggesting this change did not affect cell survival.

A B

C

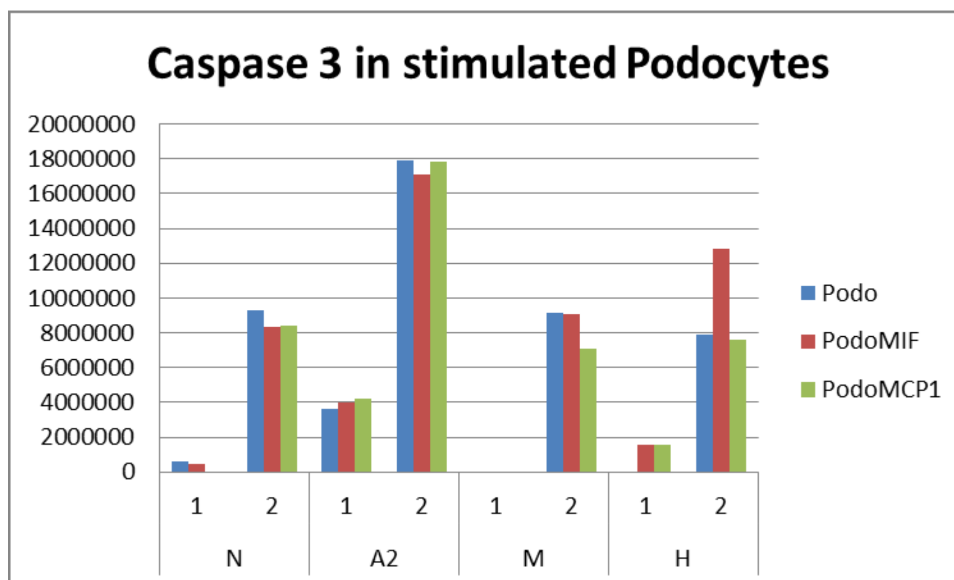

Figure 4.5.2.3 A: Caspase 3 (17,19,35kDa) from podocytes stimulated with normal glucose, mannitol and high glucose. B: Caspase 3 (17,19,35kDa) from podocytes stimulated with glycated albumin. C: Caspase 3 in stimulated Podocytes. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin; Prefix's M – rMCP-1 (20ng/ml) and H – rMIF (20ng/ml). The blue bars indicate the

0ng/ml basal conditions at 48 hours, the red bars are podocytes stimulated with 20ng/ml rMIF, the green bars are podocytes stimulated with 20ng/ml rMCP-1.

4.5.2.4 Caspase 7

Unfortunately there was insufficient cell lysate for Caspase 7 to be analysed in glycated albumin. Time limitations did not allow the entire analysis to be repeated in all the experiments to ensure no sample bias. All these results need to be confirmed with further analysis, however, there is a suggestion of lower levels of caspase 7 in normal glucose, mannitol and high glucose following rMIF stimulation, see Fig 4.5.2.4. There was equivalent cell viability in this experiment between conditions. The MTT assay in podocytes stimulated with rMIF in mannitol and high glucose showed improved cell survival with little difference following rMCP-1 stimulation. 20kDa caspase 7 bands were not identified. The levels of caspase 7 were less in podocytes with these stimuli than those produced by HK2 cells.

A

B

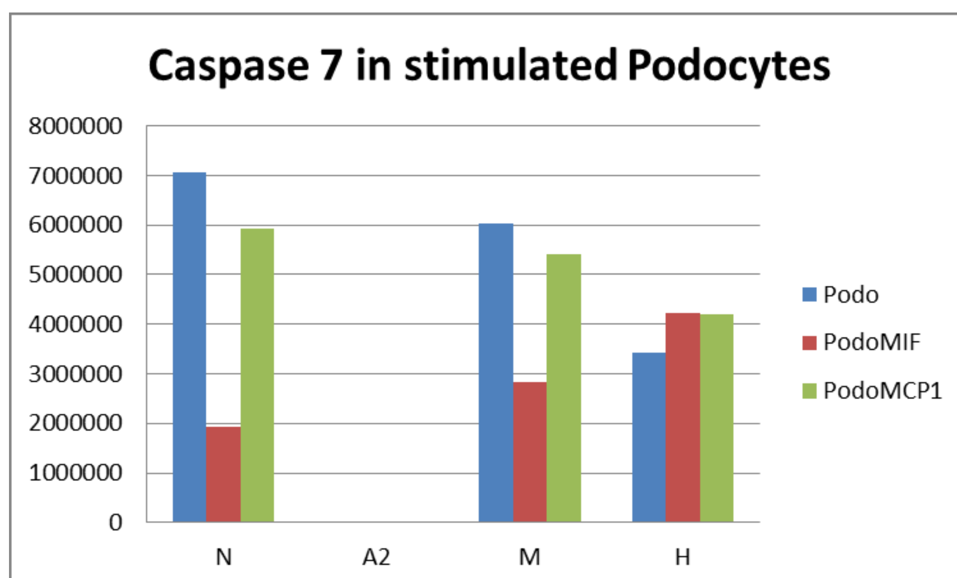

Figure 4.5.2.4 A: Caspase 7 (20, 35kDa) from podocytes stimulated with normal glucose, mannitol and high glucose. B: Caspase 7 in stimulated Podocytes. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated

albumin; Prefix's M – rMCP-1 (20ng/ml) and H – rMIF (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are podocytes stimulated with 20ng/ml rMIF, the green bars are podocytes stimulated with 20ng/ml rMCP-1.

4.5.2.5 Caspase 9

An increase in 3 of the total 4 bands of caspase 9 in podocytes with glycated albumin, was detected that remained constant following rMIF or rMCP-1. Cleaved caspase 9 are found at 17 and 37kDa. Bands 1 and 4 only are present in other stimulated conditions with bands at 1 and 3 suggesting the presence of cleaved caspase 9. The membrane would need to be reblotted with cleaved caspase 9 to determine its activation. High glucose resulted in higher protein levels of caspase 9 in podocytes stimulated with rMIF that did not affect cell survival, see Fig 4.5.2.5.

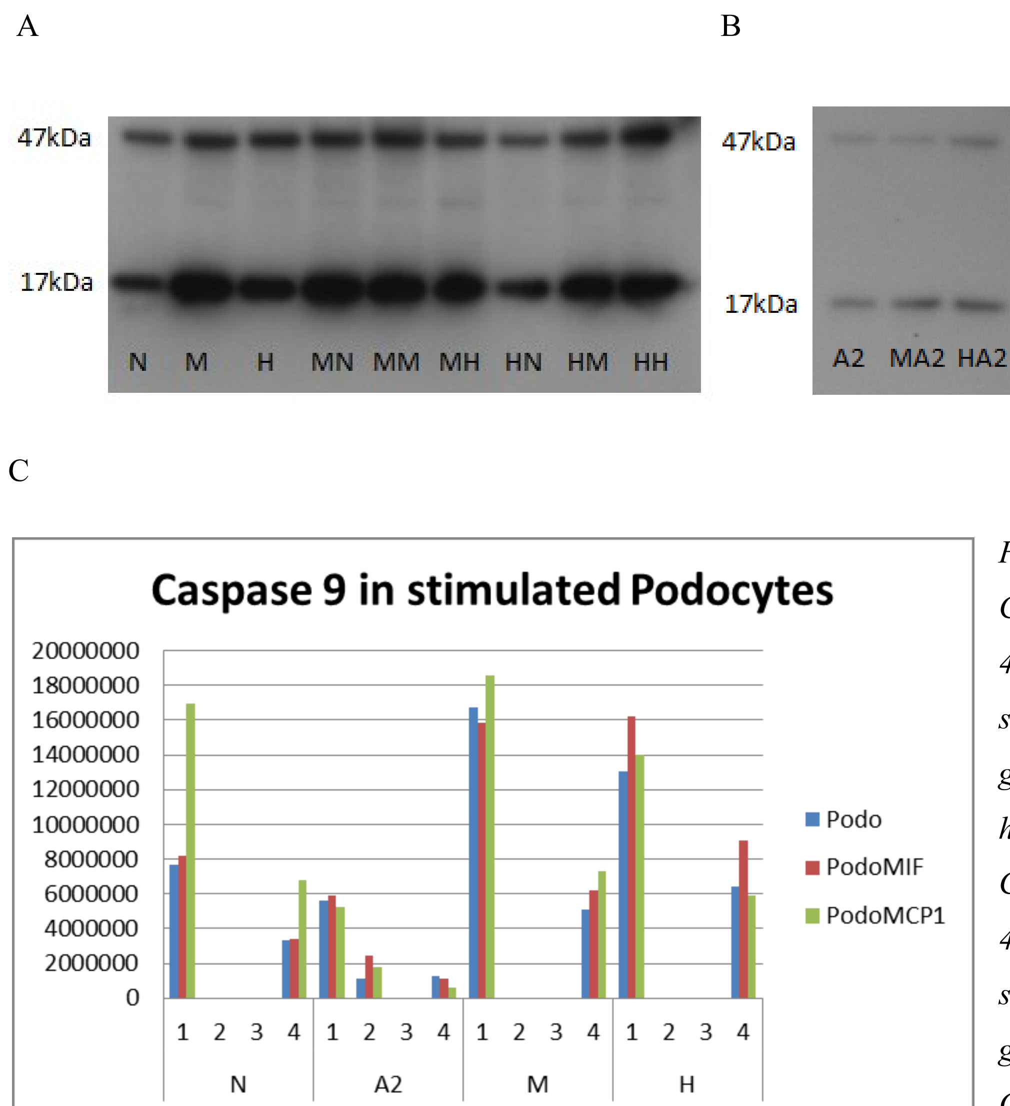

Figure 4.5.2.5 A: Caspase 9 (17,35, 37, 47kDa) from podocytes stimulated with normal glucose, mannitol and high glucose. B: Caspase 9 (17,35, 37, 47kDa) from podocytes stimulated with glycated albumin. C: Caspase 9 in stimulated Podocytes. Key; N – normal glucose, M – mannitol, H – high glucose, A2 – glycated albumin;

Prefix's M – rMCP-1 (20ng/ml) and H – rMIF (20ng/ml). The blue bars indicate the 0ng/ml basal conditions at 48 hours, the red bars are podocytes stimulated with 20ng/ml rMIF, the green bars are podocytes stimulated with 20ng/ml rMCP-1. The four bands seen in total caspase 9 are at 17, 35, 37, and 47kDa presented as 1 to 4 per condition. The cleaved forms are 17, and 37kDa, bands 1 and 3 respectively.

<u>Summary of results of Caspases in Podocytes</u>

Caspase 3 is increased in podocytes in glycated albumin and in high glucose with rMIF. There was insufficient sample for the analysis of caspase 7 in glycated albumin conditions of podocytes. The high glucose and mannitol conditions had lower levels of caspase 7 in podocytes following rMIF or rMCP-1 stimulation at 48 hours; this correlated with an increased cell survival on MTT assay. Three bands of caspase 9 were present in glycated albumin conditions compared to its control. Lower cell viability was seen in glycated albumin podocytes, however, cell viability improved following cytokine stimulation. rMIF appeared to increase caspase 9 activity. This data suggests cell survival is influenced by conditions and cytokine stimulation.

4.6 Discussion of Results – Scientific basis for Clinical findings

This chapter aimed to determine where MIF, MCP-1 or CCL18 are found, the cells producing them and whether there is any communication amongst the different cytokines. The impact of these communications on fibrosis was later determined by looking at the effect on fibronectin using ELISA in the repeated experiments in HK2 cells and podocytes. Time limitations did not allow for all the interesting findings in the pilot data to be repeated. The effects of the cytokines in the HK2 cells and podocytes were interpreted alongside the MTT assay determining the effect on cell viability. Each experiment was repeated three times to ensure reproducibility of findings. This allows statistical findings to be robust. The statistical difference between certain conditions is weakened to trends in some of the analysis as there are multiple comparators within the same group when using Dunn's multiple comparisons. It was unclear what would be found in these novel experiments and hence the multiple conditions and stimulations were done to investigate the concept of cytokine communication and whether this changes with the environment and recombinant cytokine stimulation. These experiments can allow smaller focused experiments to be carried out to ensure the initial findings are robust.

Western blot looking at caspase induction was also used to determine activation of apoptotic pathways that may contribute to the findings. Western blot looked at cell signalling at the 48 hours

and these findings would need to be confirmed with shorter time course cell signalling experiments. This could determine the initiating cell signalling mechanism that leads to the sustained signalling response seen and was beyond the scope of this project.

4.6.1 IHC

Previous studies have localised MIF and MCP-1 histologically to DN glomeruli (Duque, Gomez-Guerrero et al. 1997; Sanchez-Nino, Sanz et al. 2009). CCL18 has not previously been reported in renal tissue. IHC showed the gastric tumour tissue control to stain for CCL18, however despite optimising the conditions for IHC, CCL18 was not found in DN or TIN renal biopsy tissue. Both conditions had high levels of urinary CCL18/Cr ratio. This suggests that CCL18 may be released directly into the urine in a cytosolic mechanism similar to MIF or that CCL18 may be lost due to the formalin fixative used in renal biopsy tissue. In contrast gastric tumour tissue maintains CCL18 that is found within tumour associated macrophages in paraformaldehyde fixed tissue.

The next step would be to determine whether the recently found receptor for CCL18, PITPNM3 is found in renal biopsy tissue (Bonecchi, Locati et al. 2011). It is unclear whether CCL18 has other receptors that may induce its actions or whether its effects are mediated in a paracrine or autocrine manner. Alternatively PCR could be used to determine whether CCL18 is produced locally in the kidney using mRNA. This was beyond the scope of this project.

4.6.2 Pilot study

The pilot study was designed in order to determine whether MIF, MCP-1 and CCL18 could be produced by intrinsic renal cells *in vitro* as they are found in human urine in certain disease conditions. In addition, different environments were used in order to determine whether this has an effect on cytokine production, as the clinical studies suggest a variation in the urinary findings according to disease. A dose response was conducted in the pilot study to determine the effects of different cytokine concentration in the different conditions and whether this affected the levels of other cytokines to either increase or decrease their levels *in vitro*. This guided the design of a more detailed study by determining the cell type of interest with the environment and cytokine stimulus that appears to communicate with other cytokines produced. The time point was chosen on the basis of the stability of the production of cytokines in the initial 4 week study in tHMC and podocytes where CCL18 levels were unchanged regardless of the stimulus or the length of time the stimulus continued. The pilot study therefore conducted in the three different intrinsic renal cell types showed the cytokines behaved in a different manner dependent on the basal conditions they were stimulated

with. In addition, an effect of increasing dose of recombinant cytokine stimulation within the conditions was seen to influence the levels of different cytokines detected.

tHMC could produce MIF and MCP-1 following 48 hours stimulation with basal conditions with higher levels of MIF detected in high glucose conditions. rMIF resulted in a dose response decrease in MIF and MCP-1 irrespective of the basal condition. These results suggest there is cross talk amongst the cytokines and natural feedback mechanisms exist to either increase or decrease the cytokine in question. In tHMC it appears that high doses of rMIF induced a negative feedback to switch off further production of MIF. In contrast, higher doses of rMIF caused a rise in MCP-1. Both of these cytokines are proinflammatory and have chemoattractant properties that would induce monocyte and T-cell infiltration. Interestingly rMCP-1 seemed to release MIF or stimulate its production from tHMC in high glucose conditions. This supports an important role of the environment affecting how these cytokines interact.

When tHMCs were stimulated with rCCL18 the levels of detectable CCL18 were subsequently higher in diabetic conditions suggesting that although CCL18 is not detected following basal stimulation of these cells it is possible to synthesise CCL18 following recombinant stimulation. Under the right conditions therefore, it is possible for tHMC to produce CCL18 or initiate the autoinduction of CCL18 specifically in glycated albumin and high glucose conditions. High dose rCCL18 also resulted in higher levels of MIF. It is unclear whether this is due to increased synthesis of MIF or whether this is due to cellular release of MIF stores. Shorter and longer time point experiments are needed to determine this.

The pilot study in HK2 cells showed that MIF was detected in all basal conditions and this was reciprocated in the subsequent more detailed experiments. The pilot also showed higher levels of MIF in glycated albumin. No MCP-1 or CCL18 were detected following 48 hours basal stimulation. rMIF increased MIF in a dose dependent manner with no effect on MCP-1 or CCL18 and was found to be higher in glycated albumin. An increase in rCCL18 resulted in a decrease in MIF with no changes in MCP-1. This decrease in MIF was seen as a trend in the subsequent experiments and specifically seen in high glucose conditions, however, this did not reach significance. rMCP-1 had a dose dependent rise in MCP-1 with a slight increase in MIF and no effect on CCL18.

MIF and MCP-1 were detected in the basal conditions in the pilot podocyte experiments that were subsequently reproduced. rMIF decreased MIF in diabetic conditions compared with its controls suggesting a negative feedback mechanism within the diabetic environment. MCP-1 initially increased then decreased following increasing rMIF stimulation in diabetic conditions. High dose

rMIF resulted in detectable levels of CCL18 in all conditions. In contrast rMCP-1 had no effect on CCL18, although rMCP-1 increased MIF levels in a dose dependent bimodal distribution with higher detectable levels following 2 and 20ng/ml of rMCP-1 in diabetic conditions. rCCL18 resulted in lower detectable levels of MIF in diabetic conditions with overall lower levels of MCP-1, again showing interactions between the cytokines within the different environments.

These preliminary findings of n=2 would need to be repeated and in view of the timescale for the project two cell types were chosen. Interestingly this preliminary data showed communication amongst the different cytokines that altered with the different cell type and in the basal conditions. This part of the project aimed to determine the effects of cytokines with regard to disease progression and the role this may have between the induction of inflammation and fibrosis. HK2 cells were chosen as the effect on the tubulointerstitium in DN is less well described with regard to disease progression and cytokine production in the cells upstream may influence these cells. The pilot study suggested crosstalk between rCCL18 and MIF and rMCP-1 and MIF hence these were the stimuli chosen for the repeated experiments in HK2 cells. rMIF and rMCP-1 seemed to have greater crosstalk in podocytes and hence these stimuli were chosen. The higher dose of glycated albumin was chosen in view of its more pronounced effect and 0, 10 and 20ng/ml dosing of recombinant stimuli were used. Time limitations allowed 0 and 20ng/ml dosing to be analysed.

4.6.3 HK2 cells

The effects seen in these larger experiments of n=6, repeated three times showed the importance of stimulation with basal conditions in these cell types. It is apparent as illustrated in the proposed pathways for cytokine communication section 4.6.4, that the basal conditions affect the levels of cytokines detected *in vitro* and the amount of fibronectin detected in these cells. In addition, stimulation with recombinant cytokine results in increasing, decreasing or having no effect on the other cytokines or fibronectin. In the proposed pathways the effects of rMIF have not been repeated and hence the trends seen in the pilot data have been used and illustrated in blue, however, these pathways would need to be confirmed with further experiments. The pilot data has been used to illustrate the effects on the other cytokines in view that similar findings were reproduced in the more extensive experiments and thus may hold true. The effects of MIF on fibronectin in HK2 have not been looked at in this project.

4.6.3.1 Basal effects of normal glucose compared with glycated albumin

Following 48 hours of normal glucose stimulation high levels of MIF were detected in HK2 cells with small amounts of fibronectin and inconsistent amounts of CCL18. MCP-1 was not detected in

HK2 cells stimulated with normal glucose. In contrast, when HK2 cells were stimulated with glycated albumin, small levels of MIF were detected with high levels of fibronectin and again inconsistent amounts of CCL18 with no MCP-1 following this 48 hours stimulus. The lower levels of MIF may arise from the reduced cell number seen in the MTT assay compared with normal glucose conditions however, cell viability may not be sufficient to explain the difference as recombinant cytokine stimulation affects MIF levels and cell viability. AGEPs are known to be profibrotic in humans interacting with the renin angiotensin system, cell signalling and interacting with RAGE all disrupting the cellular matrix (Ban and Twigg 2008). It is likely other mechanisms that have not been measured have been employed to disrupt the cellular matrix. The increase in fibronectin may be a reflection of attempts for cellular repair, where cell death is occurring via these unidentified mechanisms. Other experiments looking at RAGE, TGF-β, CTGF with other fibrotic markers and cell signalling may be used to determine why and how this difference in cell viability is occurring.

4.6.3.2 Basal effects of mannitol compared with high glucose

Mannitol conditions produce the same basal cytokine profile as normal glucose conditions in HK2 cells despite having a different osmolarity. High levels of MIF with small amounts of fibronectin and inconsistent amounts of CCL18 were seen in HK2 cells with mannitol. MCP-1 was not detected after 48 hours. In contrast high glucose conditions result in much higher levels of MIF and raised levels of fibronectin. High glucose has inconsistent amounts of CCL18 and no MCP-1 in HK2 stimulated cells. The consistent findings of basal stimulation with the different conditions, shows CCL18 is produced inconsistently with no detectable levels of MCP-1.

The cell viability was slightly higher in high glucose conditions compared with mannitol. This may reflect an increase in cell proliferation in high glucose rather than an improvement in cell survival.

4.6.3.3 rCCL18 stimulation on HK2 cells in normal glucose or glycated albumin

Stimulation with rCCL18 in normal glucose results in a rise in CCL18 with an inhibition of basal MIF levels in HK2 cells. Interestingly, this chemoattractant cytokine with pro-fibrotic characteristics does not affect fibronectin levels in normal glucose and has inconsistent effects on MCP-1 see Figure 4.6.4.1. There was a trend for a decrease in cell viability in normal glucose with rCCL18 but this effect was not pronounced. In HK2 cells with glycated albumin the rCCL18 stimulus results in increasing MIF and further increasing fibronectin levels. There are again inconsistent effects on the levels of MCP-1. The environment has reversed the effect of CCL18 on MIF indicating there is a

dynamic communication and reaction to the stimulus. In contrast, glycated albumin HK2 cells without rCCL18 had improved cell viability compared with those with rCCL18. This is interesting as increasing MIF could arise from release of cellular stores however the 48 hour time point would be at the point where cells would need to synthesis more MIF in order to maintain its levels. Fibronectin is increased and this may be a reflection of increased fibrosis or, in view of the increase in cell survival seen, this may be as a consequence of cell proliferation within this environment associated with increasing the extracellular matrix of more cells.

The increase in MIF in a systemic model may result in more damage as there would be an increase in infiltrating cells with this stimulus. It is unclear as to what type of cells would infiltrate and the cytokine profile they would release to influence whether this is an anti-inflammatory or pro-fibrotic response. CCL18 has previously been described to influence T lymphocytes to induce a TGF-β dependent fibrotic pathway whilst also being described to affect resident fibroblasts to induce fibrosis through a TGF-β independent pathway (Luzina, Papadimitriou et al. 2006). More experiments would need to be done to determine whether stimulation with CCL18 induces more fibrotic markers or whether there is a protective effect of this stimulus in this environment by improving cell viability.

4.6.3.4 rMCP-1 stimulation on HK2 cells in normal glucose or glycated albumin

rMCP-1 in normal glucose results in an increase in MCP-1 levels and further stimulation of fibronectin. rMCP-1 also inhibits MIF in normal glucose conditions and has an improved cell survival following stimulation in this condition compared with normal glucose conditions alone see Fig 4.6.4.1. There is little or no effect on CCL18 following rMCP-1. CCL18 is known to inhibit MIF in normal glucose as per the previous experiments. It is possible the improved survival seen in normal glucose following rMCP-1 arises from the dual inhibition of MIF from MCP-1 and CCL18 as this may counteract the basal MIF levels produced from normal glucose conditions in HK2 cells alone.

The increased cell viability seen in glycated albumin HK2 cells with rMCP-1 is similar in effect to that of rCCL18 and differs from normal glucose conditions predominantly where the effect on MIF is reversed. rMCP-1 levels increase more in glycated albumin compared with normal glucose and stimulate production of CCL18 that in turn increases MIF and increases fibronectin. rMCP-1 increases MIF and fibronectin in glycated albumin and may provide an increase in cell survival by allowing extracellular matrix to be strengthened with the higher levels of fibronectin detected in these cells.

Glycated albumin in HK2 cells improves cell viability following rCCL18 and rMCP-1 *in vitro*. It is unclear whether this would still occur with other cell types or infiltrating cells and thus these experiments illustrate how HK2 cells are able to respond to different environments and alter their production of cytokines in accordance to external stimuli.

4.6.3.5 Cell signalling and caspases in rCCL18, rMCP-1 stimulation on HK2 cells in normal glucose or glycated albumin

Recombinant stimuli result in a decrease in phospho p38 MAPK and increase in phospho p44/42 MAPK at 48 hours in glycated albumin. Lower levels of p38 MAPK have previously been described with cell survival as raised levels are used to help maintain the cell cytoskeleton, regulate proinflammatory cytokines and help initiate apoptosis (Dai, Yang et al. 2003). MIF is known to induce cell proliferation via p44/42 MAPK and p38 pathways whilst causing synthesis of prostaglandin E in leukocytes and fibroblasts. In this closed system the improved cell survival seen in glycated albumin may arise following increased activation of MIF thereby inducing synthesis of phospho p44/42 MAPK seen on Western blot at this time point. Signalling time course experiments would need to be done to determine what cytokine is inducing these effects or whether a combination of these cytokines, use the pathways to induce their effects. Normal glucose had a similar signalling profile with both recombinant cytokine stimulation despite rCCL18 inducing little effect on cell survival and MCP-1 improving cell survival. In contrast, glycated albumin phospho p38 MAPK levels were higher with little change in phospho p44/42 MAPK following recombinant stimulation. This may reflect the difference in normal glucose conditions inhibiting MIF with recombinant stimulation whilst glycated albumin with recombinant stimulation promotes MIF production.

The total caspase profile of these experiments show no total caspase 3 in normal glucose with an increase in caspase 7 and 9 with rCCL18 and only a rise in caspase 7 with rMCP-1. Glycated albumin conditions with rCCL18 and rMCP-1 had a decrease in total caspase 3 with an increase in caspase 7 and 9 compared with the basal form. This would suggest that apoptosis has not been instigated.

4.6.3.6 rCCL18 stimulation on HK2 cells in mannitol or high glucose

rCCL18 increases CCL18 in mannitol whilst having little effect on MIF, MCP-1 or fibronectin after 48 hours stimulation. In contrast, rCCL18 in high glucose results in a slight increase in CCL18 that further inhibits MIF and increases fibronectin production. There is an inconsistent effect on MCP-1. There was a decrease in cell viability in mannitol following recombinant stimulation of cytokines

that was also seen with high glucose when compared with their respective basal levels. The decreased cell viability is not a reflection of the osmotic effect of mannitol or high glucose on HK2 cells as the cell viability without recombinant stimulation illustrated a higher cell viability in high glucose that may reflect an increase in cell proliferation. The decrease in cell viability affects both mannitol and high glucose with recombinant stimulation. It is possible this may reflect a decrease in cell proliferation or alternatively an increase such that this leads to cell death due to a decrease in nutrients within the *in vitro* system. Alternatively the recombinant cytokine stimulus may result in cell growth arrest and hence cell viability is reduced. A time course experiment looking at cell proliferation or cell cycle growth arrest may provide insight to these findings.

4.6.3.7 rMCP-1 stimulation on HK2 cells in mannitol or high glucose

rMCP-1 results in high levels of MCP-1 in high glucose conditions compared with mannitol. rMCP-1 with mannitol has little effect on MIF, CCL18 and fibronectin. High glucose with rMCP-1 results in an increase in MIF levels with little effect on fibronectin and CCL18. Despite these differences there is poor cell viability in both conditions when given rMCP-1 or rCCL18 stimulation. More cells survived in high glucose compared with mannitol though rMCP-1 stimulation resulted in a decrease in cell survival compared with their basal levels. The difference seen between high glucose cell viability and mannitol continues despite overall reduction in cell viability with recombinant cytokine stimulation. This may reflect higher MIF levels despite inhibition with CCL18 as this will affect cell proliferation. Fibronectin levels increased in 2 of the 3 experiments with one showing decreased levels with rMCP-1. This may influence cell viability if fibronectin is required for the increased cell turnover enhanced by high glucose conditions and high MIF levels. The effect of MIF in HK2 cells needs to be studied to determine the effect of this on fibronectin.

4.6.3.8 Cell signalling and caspases in rCCL18, rMCP-1 stimulation on HK2 cells in mannitol or high glucose

There was no change in phospho p38 MAPK or phospho p44/42 MAPK levels in recombinant stimulated mannitol conditions compared to mannitol alone. In contrast there were slightly lower levels of phospho p44/42 MAPK following rCCL18/rMCP-1 stimulation in high glucose conditions compared to high glucose alone with no change in phospho p38 MAPK levels. This difference however did not improve cell viability in HK2 cells.

Caspase 3 levels were higher in rCCL18 mannitol conditions with no change in caspase 7 from basal condition and a rise in caspase 9 levels with recombinant cytokine stimulation. These findings were

the same for high glucose conditions stimulated with recombinant cytokines with the exception that caspase 3 was lower in rMCP-1 high glucose conditions. These differences do not suggest activation of apoptotic pathways to explain the reduced cell viability, however Western blot would be needed to determine the activity of cleaved caspases within the total caspases looked at.

4.6.4 Proposed pathways for cytokine interaction in diabetic milieu in HK2 cells

It is clear from the data that the environment affects the way that HK2 cells respond to cytokines. In view of this I propose the following pathways for interaction of cytokines according to the environment the HK2 cells are exposed to. I have included the control followed by the diabetic condition to see how this differs. The results show that there are also differences in the way HK2 cells respond in glycated albumin compared to that of high glucose. The combination of both these diabetic environments may further change the proposed interactions between the cytokines. The data herein supports the idea that cytokines do interact with each other and may autoregulate themselves or have other cytokines that promote or inhibit their production, thereby having a feedback loop that has been described in other systems of the human body. Further studies would be required to test these proposed pathways.

Figure 4.6.4.1 Normal glucose in HK2 cells. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data. Tubuloepithelial cells produce MIF, fibronectin and intermittently CCL18 under normal

physiological conditions. In contrast, HK2 cells in glycated albumin seen in DM, produce more fibronectin with lower amounts of MIF and variable amounts of CCL18 see Fig 4.6.4.2. CCL18 and MCP-1 increase fibronectin production further in glycated albumin conditions, emphasising how diabetic conditions influence cytokine production.

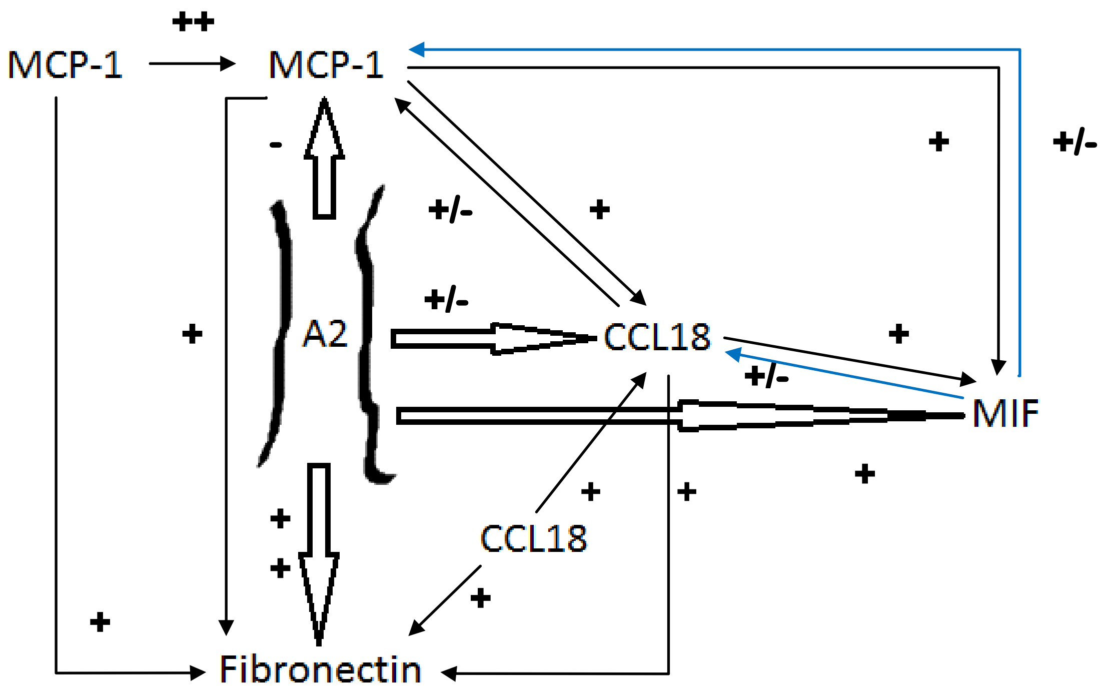

Figure 4.6.4.2 Glycated albumin in HK2 cells. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

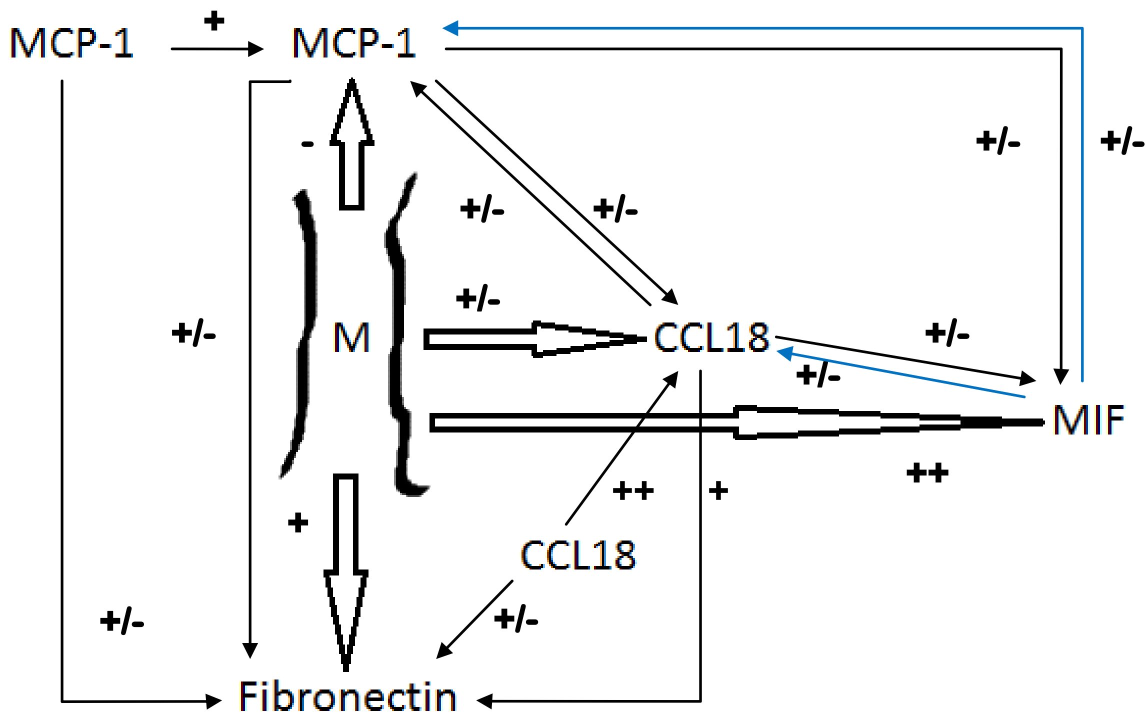

Figure 4.6.4.3 Mannitol in HK2 cells. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data. Mannitol, the osmotic control for high glucose is seen to produce MIF, fibronectin and intermittently CCL18 in HK2 cells. In the high glucose environment (Fig 4.6.4.4), there is a much higher production of fibronectin and MIF. CCL18 in high glucose inhibits MIF production and increases fibronectin production further with MCP-1 in contrast, inhibiting fibronectin production. These differences illustrate how a diabetic environment affects these cell types cytokine production.

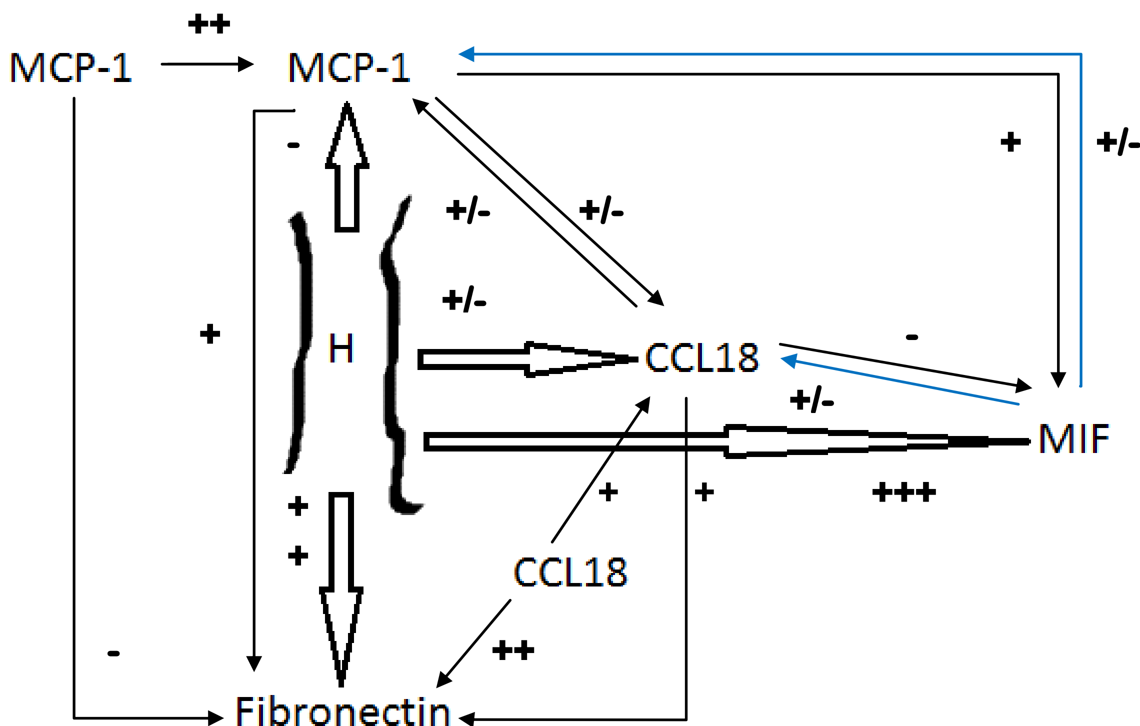

Figure 4.6.4.4 High glucose in HK2 cells. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

The main difference between diabetic conditions and their controls on the proposed pathways for HK2 cells appears to be the further stimulation of MIF with rMCP-1. Interestingly normal glucose and high glucose result in CCL18 inhibiting MIF, with glycated albumin conditions stimulating the production of MIF with MCP-1. In addition, there is a consistent rise in fibronectin with stimulation of CCL18 that is enhanced specifically in the diabetic conditions. This may reflect direct stimulation of CCL18 on fibronectin or disruption of the cellular matrix resulting in the release of fibronectin. HK2 cells are more responsive to CCL18 compared with podocytes.

MCP-1 also increases fibronectin production in all conditions once there are detectable levels however, HK2 cells do not produce MCP-1 over 48 hours irrespective of the environment. This is interesting as the urinary MCP-1 detected may illustrate the production of this prominent chemoattractant from mesangial cells, podocytes, glomerular epithelial cells or alternatively infiltrating cells. MCP-1 however, when present does affect MIF, CCL18 and fibronectin according to the environment. There were much higher levels of MCP-1 detected in diabetic conditions stimulated with rMCP-1 in HK2 cells. MIF levels were higher in diabetic conditions with rMCP-1.

MCP-1 has previously been described to correlate with tubulointerstitial injury and found in the urine of those with proteinuria. CTGF decreases with increased MCP-1 and was not measured in these experiments, but it would be interesting to see how this is influenced with the environment in this cell type and its interactions with the cytokines. An increase in MCP-1 will result in monocytes chemoattractant actions suggesting an acute injury may be occurring. MCP-1 would be raised in an acute injury in order to recruit anti-inflammatory cells to prevent further injury and remove damaging debris.

4.6.5 Podocytes

These experiments of n=6 repeated three times, looked at the effects of the diabetic environment on podocytes and their ability to produce MIF, MCP-1, CCL18 and fibronectin. In addition, the effects of rMIF and rMCP-1 on the cytokines and fibronectin in the different environments were also compared. The results were interpreted in the context of cell viability and are discussed below. The proposed pathways in section 4.6.6 illustrate the findings set out below.

4.6.5.1 Basal effects of normal glucose compared with glycated albumin

Podocytes produced high amounts of MIF with lower levels of MCP-1 and fibronectin 48 hours after stimulation with normal glucose see Fig 4.6.6.1. CCL18 was inconsistently produced in this environment. In contrast lower levels of MIF were produced in glycated albumin with similar levels of MCP-1 see Fig 4.6.6.2. More fibronectin was found in podocytes in glycated albumin with CCL18 inconsistently found in podocytes. There were similar levels of cell viability in both conditions.

4.6.5.2 Basal effects of mannitol compared with high glucose

Low levels of MIF, MCP-1 and fibronectin were detected after 48 hours in podocytes in mannitol see Fig 4.6.6.3. Inconsistent amounts of CCL18 were detected in the supernatant. Podocytes in high glucose (see Fig 4.6.6.4) have similar levels of MIF, MCP-1 and CCL18 after 48 hours, however, high levels of fibronectin are detected compared to those seen in mannitol. Podocyte cell viability remained constant for all the conditions irrespective of the basal condition.

4.6.5.3 rMIF stimulation on podocytes in normal glucose or glycated albumin

rMIF slightly increases MIF in podocytes under normal glucose conditions, and increases MCP-1 while inhibiting CCL18. There is an inconsistent effect of MIF on fibronectin in normal glucose. In contrast rMIF in glycated albumin has higher levels of MIF with inhibition of fibronectin. There are

inconsistent effects on CCL18 and MCP-1. Cell viability does not change in podocytes in normal glucose with rMIF, however, cell viability improves in glycated albumin and may be a consequence of increasing MIF that inhibits fibronectin production in this environment. Alternatively this may occur following the increase in MCP-1 seen in rMIF normal glucose podocytes that does not occur in rMIF glycated albumin podocytes. MCP-1 has been reported to induce apoptosis together with TGF-β induction in podocytes that may lead to cell death (Nam, Paeng et al. 2012). This has been described in high glucose environments but not in glycated albumin.

4.6.5.4 rMCP-1 stimulation on podocytes in normal glucose or glycated albumin

High levels of MCP-1 are detected following rMCP-1 in both normal glucose and glycated albumin podocytes. Low levels of fibronectin are produced with high levels of MIF and inconsistent levels of CCL18 in rMCP-1 normal glucose podocytes. In contrast there are higher levels of fibronectin detected following rMCP-1 in glycated albumin podocytes with lower levels of MIF and inconsistent levels of CCL18. Cell viability in both of these conditions increased compared to basal stimulus alone.

4.6.5.5 Cell signalling and caspases in rMIF, rMCP-1 stimulation on podocytes in normal glucose or glycated albumin

There was an increase in phospho p38 MAPK following stimulation of normal glucose podocytes with recombinant cytokines with no change in phospho p44/42 MAPK. In contrast glycated albumin podocytes with or without recombinant cytokine stimulus have lower levels of phospho p38 MAPK. rMIF/rMCP-1 stimulated glycated albumin podocytes have lower levels of phospho p44/42 MAPK levels with higher cell viability. It is unclear whether the protein levels have been downregulated by 48 hours and other signalling pathways have been activated to maintain the milieu. It has previously been established MIF uses phospho p44/42 MAPK to induce some of its effects whilst also using phospho p38 MAPK that is predominantly used as a cell signalling pathway for MCP-1 to induce its effects. Short time course signalling experiments would need to be done to determine when these mechanisms are employed. There is however a difference seen between the use of these pathways according to the environment the podocytes are in.

Caspase 3 is constantly found in normal glucose podocytes irrespective of the stimulus. rMIF has lower caspase 7 levels and rMCP-1 has higher caspase 9 levels in normal glucose. In contrast there is a rise in caspase 3 and 9 in glycated albumin. The levels of caspase 7 are unknown. The cleaved caspases would need to be determined to ensure there is no activation of the apoptotic pathways.

There was increased cell viability in glycated albumin under recombinant cytokine stimulation compared to its basal stimulus and to its control. It does not appear that the downregulation in phospho p38 MAPK and phospho p44/42 MAPK is due to increasing cell death. It is possible that these pathways were used in cell growth and with increased fibronectin production.

4.6.5.6 rMIF stimulation on podocytes in mannitol or high glucose

rMIF mannitol podocytes produce high levels of MIF and induce MCP-1 while decreasing basal fibronectin levels. CCL18 is inconsistently produced in mannitol and high glucose podocytes with rMIF. High glucose rMIF podocytes have much higher levels of MCP-1 and fibronectin levels are reduced albeit the basal production of fibronectin in high glucose alone, is much higher than other conditions. Cell viability did not change in podocytes stimulated in mannitol with rMIF however there was increased cell viability in those stimulated with rMIF in high glucose. It is unclear why podocyte survival is better in the rMIF high glucose group after 48 hours. The pilot data suggest that CCL18 acts to inhibit both MIF and MCP-1 and this could aid cell survival except, the levels of CCL18 in podocytes were inconsistent and it is likely other mechanisms are employed. The improved cell viability is likely to reflect increased cellular activity as MIF and MCP-1 aid the cellular growth and proliferation. The inhibition of fibronectin in high glucose by MIF is novel and has not previously been described in the literature.

4.6.5.7 rMCP-1 stimulation on podocytes in mannitol or high glucose

Podocytes stimulated with rMCP-1 in mannitol results in much higher levels of MCP-1 that increases MIF levels with no discernible effect on CCL18. There is increased production of fibronectin in mannitol. rMCP-1 in high glucose podocytes results in high levels of MCP-1 and higher MIF levels than its control. Again there was no effect on CCL18. Interestingly rMCP-1 inhibits fibronectin in high glucose with rMCP-1 after 48 hours. In high glucose the basal stimulation of fibronectin is inhibited with increasing MIF and MCP-1 levels in these podocytes. There is a decrease in cell viability with rMCP-1 in high glucose podocytes compared with basal conditions alone, however, cell survival does not change. Previous studies, whereby stimulation of cultured podocytes with 10ng/ml MCP-1 in high glucose, have reported induction of apoptosis with an increase in p38 MAPK pathways (Nam, Paeng et al. 2012). The dose used in this experiment was 20ng/ml where there was a detectable increase in MCP-1 compared to that produced in high glucose conditions alone. The decreased cell viability in this case would be consistent with apoptosis though there was only a slight decrease in cell viability compared with basal conditions and compared with mannitol. It is possible that at 48 hours apoptotic pathway have been initiated. Further time course

experiments in these conditions would be able to clarify the cause of the change in cell viability with a short time course experiment to determine cell signalling pathways involved.

4.6.5.8 Cell signalling and caspases in rMIF, rMCP-1 stimulation on podocytes in mannitol or high glucose

There is an increase in phospho p38 MAPK following recombinant cytokine stimulation in podocytes in mannitol and high glucose with unchanged levels of phospho p44/42 MAPK when compared to basal conditions alone.

Total caspases 3, 7 and 9 are all raised in high glucose conditions with recombinant cytokine stimulation. This occurs when there is improved cell viability with rMIF in high glucose. In contrast there is a decrease in cell viability in the presence of raised total caspases 3, 7 and 9 with rMCP-1. Cleaved caspases in this case will help differentiate between activation of apoptotic pathways that may contribute to the differences seen in cell viability. Mannitol stimulated podocytes have equal caspase 3 levels regardless of cytokine stimulus whereas caspase 7 activity decreases with rMIF and caspase 9 increases with rMCP-1. This is similar to that seen in normal glucose conditions with rMIF/rMCP-1 podocytes that have similar cell viability. The significance of these findings are unclear and further short time experiments would help understand these findings.

4.6.6 Proposed pathways for cytokine interaction in diabetic milieu in Podocytes

The data in podocytes also show the environment affects the way cytokines interact and how their levels alter with the condition and with recombinant stimulus. I propose the following pathways for how MIF, MCP-1 and CCL18 react to each other while illustrating their effect on fibronectin in podocytes. I have included the control followed by the diabetic condition to see how this differs. The results show that there are also differences in the way podocytes respond in glycated albumin compared to that of high glucose, similar principles to those seen in HK2 cells. The combination of both these diabetic environments may further change the proposed interactions between the cytokines however further experiments need to be undertaken to establish any changes. Future studies would be required to test these proposed pathways.

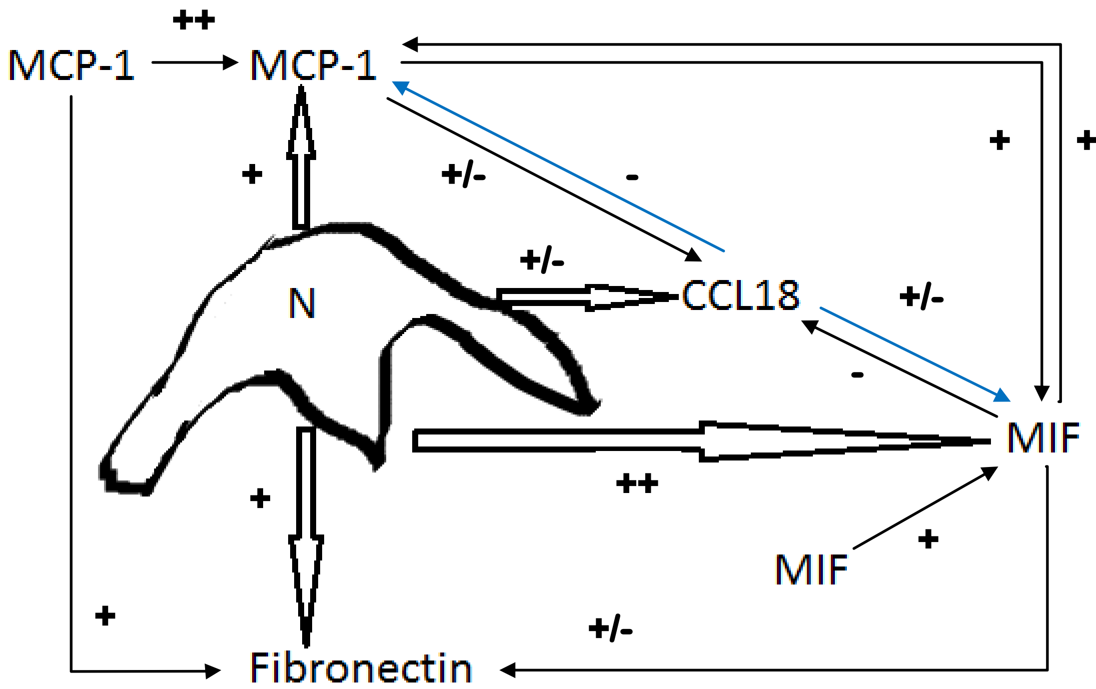

Figure 4.6.6.1 Normal glucose in Podocytes. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

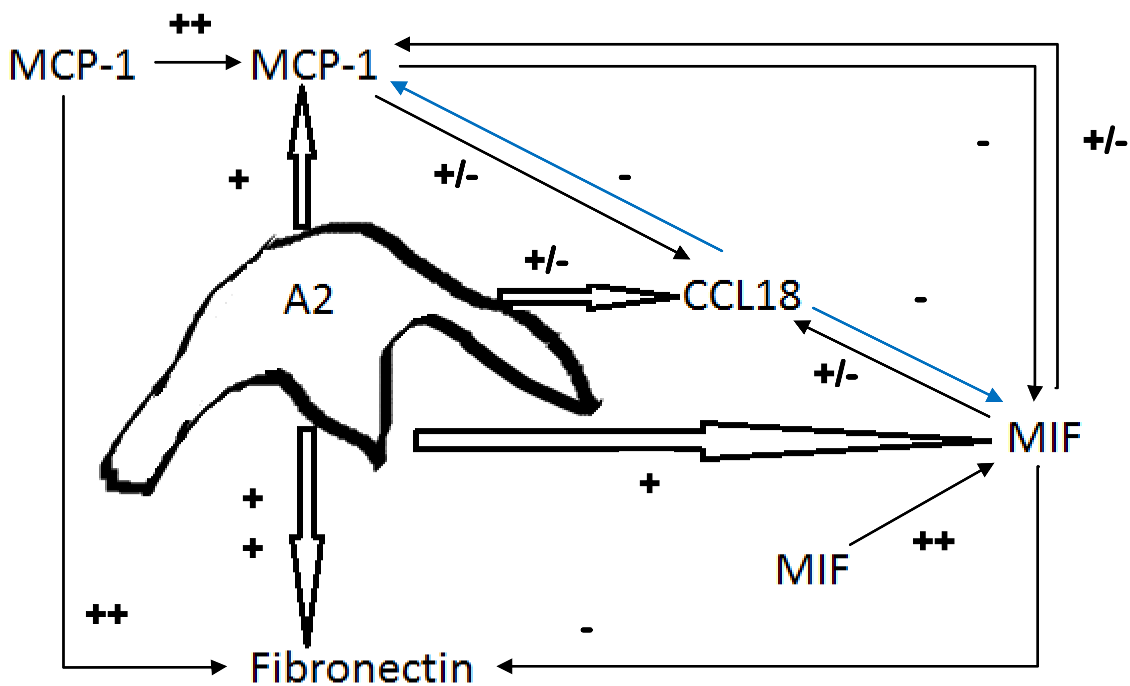

Figure 4.6.6.2 Glycated albumin in Podocytes. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

Figure 4.6.6.3 Mannitol in Podocytes. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

Figure 4.6.6.4 High glucose in Podocytes. Key Large arrows – effects of condition, black thin arrows – effects from statistical repeated experiments, blue thin arrows – effects from pilot data.

The main difference between diabetic conditions and their controls on the proposed pathways for podocytes is further stimulation of MIF and increase in fibronectin production. MCP-1 inhibits MIF in glycated albumin but increases this in high glucose. High levels of MIF will inhibit fibronectin allowing increase cell proliferation, recruitment and cell growth in high glucose conditions MCP-1 also inhibits fibronectin and may contribute to this finding. The pilot study suggests that CCL18 in high glucose conditions would inhibit MCP-1 and MIF in podocytes and this would be interesting to repeat to see whether there is a natural inhibition of these cytokines in this environment in podocytes. In contrast, glycated albumin has a greater need to increase extracellular matrix and have higher levels of fibronectin following stimulation with MCP-1. CCL18 and MCP-1 act to inhibit MIF in podocytes in glycated albumin whilst increasing fibronectin levels.

MCP-1 may increase to induce further recruitment of circulating monocytes to infiltrate and phagocytose debris or alternatively induce apoptotic pathways previously described to be induced by MCP-1 in podocytes in high glucose. The latter mechanism reports induction of its effects through TGF-β dependent and independent pathways (Nam, Paeng et al. 2012). The effect of MIF inducing MCP-1 is lessened in podocytes in glycated albumin and it is possible that changing the balance where MCP-1 becomes the dominant cytokine acts as a natural feedback mechanism for the cell to stop producing MIF. These pathways are a constant reflection of the cell types' ability to react to the environment and change according to the different stimuli affecting it. A number of chemokine receptors have been reported to be on podocytes and so further experiments to determine which receptors are activated may help differentiate the predominant stimulus (Huber, Reinhardt et al. 2002). There may be downregulation of the MIF receptor CD74 (Sanchez-Nino, Sanz et al. 2009) on podocytes in glycated albumin environments with upregulation of other cytokine receptors hence determining the local cytokine milieu and thereby regulating its effects.

Summary

CCL18 is not found in renal biopsy tissue with IHC. tHMC, HK2 cells and podocytes are able to produce different cytokines following stimulation with diabetic and Non-DM conditions after 48 hours. Stimulation with recombinant cytokines alters the levels of other cytokines in these cells and the responses vary according to the basal condition.

HK2 cells and podocytes produce different cytokines and have different communication pathways between cytokines and fibronectin that alters with diabetic conditions compared with their controls. These changes are not dependent on cell viability but may affect cell viability. More experiments with shorter time points will help determine the cell signalling pathways

involved and detection of cleaved caspases will aid the differentiation of activation of apoptotic pathways. Longer time points will be useful to determine whether the interactions between the cytokines changes. MIF, MCP-1, CCL18 and fibronectin were measured, however, there will be other factors that are influencing the outcome and these would require further investigation.

The proposed pathways are based on the *in vitro* findings that enable us to understand on a cellular basis what the effects are of certain stimuli. The main limitation is this *in vitro* work is unable to illustrate the effects of how a full system involving all cell types interacts. Chapter 5 Discussion will discuss the limitations of this work together with translational aspects of Chapter 3 and 4 and future work.

CHAPTER 5.0 – DISCUSSION

This thesis aimed to improve the understanding of cytokines in DN. The clinical study with DN and proteinuric Non-DM renal disease as a control determined whether there were specific changes seen in DN. The laboratory study was conducted to attempt to understand and explain the mechanisms behind the urine cytokine findings as there seemed to be local independent production of cytokines in the urine that did not correlate with the systemic circulating levels of the same cytokines. The hypothesis was that; MIF, MCP-1 and CCL18 are causative factors in the development of renal inflammation and fibrosis in DN and are useful biomarkers of disease progression. This novel data suggests that these cytokines, that are found in DM patients may act as biomarkers, however larger studies are needed to determine whether a cytokine profile exists and could be used as a marker of progression. The laboratory work shows that these cytokines react differently in diabetic conditions compared with their controls and may increase fibronectin as a marker of fibrosis, in addition to further potentiating inflammation by increasing levels of chemoattractant cytokines. This would allow infiltration of other cell types further exacerbating inflammation and fibrosis within the kidney.

There were a number of limitations in trying to determine whether this hypothesis was true. These limitations need to be taken into account when interpreting the results and potential pathways that arise from analysis of the data derived from the clinical and laboratory findings. These limitations are discussed below, followed by proposing a new theory of interaction of these cytokines contributing to disease progression by combining the clinical and laboratory findings. Finally there is a section on future work that would help see whether these findings are true.

5.1 Limitations

5.1.0 Clinical Study

The clinical prospective cohort study was not a matched control or a randomised control trial. This would be difficult in view of the numbers needed and the follow up period would need to be more extensive. In lengthening the follow up period there would be higher numbers of patients being lost to follow up. Power calculations may be derived using this pilot study to determine the number of patients that would be needed to look at a number of cytokines, whilst accounting for those lost to follow up, RRT or death.

The study would need to be a multicentre trial in order to allow for the larger number of patients to be recruited, together with a longer follow up period with shorter time intervals for sample collection. The true pattern of disease could then be determined. It would be possible to look into the cytokine profile produced from patients that progressed more rapidly leading to RRT and those who died as a consequence of diabetic complications. The difficulty with this study would be the need to involve primary care, in view of the number of patients with DN that are discharged from hospital clinics back to the community. A cytokine profile may help determine those who should remain under hospital follow up whilst allowing further understanding of what production of these cytokines in the urine or plasma/serum mean.

The control group was limited due to the heterogeneity of recruitment and should ideally have larger numbers of patients with or without immunosuppression as part of their treatment. By doing this it would be possible to further differentiate the effect of immunosuppression on cytokines. The group would need to have a more uniform diagnosis and concordant treatment, either all on immunosuppression or not. Alternatively, patients with proteinuric renal diseases that are known to progress over time, who are not on immunosuppression would provide a better comparison and when recruiting for a future study strict entry and exclusion criteria need to be defined to limit the degree of variability. It is unclear whether the differences seen between the DM and Non-DM group are a reflection of immunosuppression, and if so, this may suggest a role for immunosuppression in the treatment of DN. This, however, is not proven and larger, more detailed studies addressing this question in particular would need to be conducted.

Despite the limitations of this prospective cohort study a difference was found between the amounts of MIF, MCP-1 and CCL18 in the urine and blood of DM and Non-DM patients. Relationships were seen with decreasing GFR and increasing ACR or UPCR with urinary cytokines in DM and Non-DM groups, respectively and occurred despite the heterogeneity of the Non-DM group. This could suggest a role for the development of a urinary cytokine profile to detect the different levels of cytokines, however, more numbers are needed to determine whether within the DM group the urinary or plasma levels can profile the severity or progression of disease. Ideally this would be correlated with renal biopsy tissue rather than ACR as it is clear from the literature that DN is not a stepwise progression of ACR and people may progress with varying levels of albuminuria (See Chapter 1 section 1.4).

The observations from this study suggest the different cytokine levels seen in the urine correlate with an increase or decrease in other cytokines that differ between DM and Non-DM patients. This study suggests that the diabetic environment may be influencing cytokine levels as proteinuric Non-DM patients have different relationships between the cytokines. This was seen in urinary cytokine levels rather than plasma/serum levels. The study also showed that serum and plasma levels significantly correlated in both groups, however, this correlation may decrease depending on the cytokine, albeit the significance continues. Laboratory testing could therefore be done on the same sample tubes taken for routine clinic visits.

The statistical mixed models showed there was a significant correlation in DM patients with high baseline plasma CCL18 levels and progression of DN characterised by a worsening GFR. This seemed to be maintained at >3 years however, significance was not reached. In addition, it appeared that high baseline plasma LogMIF predicted a worse GFR at >18 months though this was not maintained. There are limitations with the model that are dependent on the number of variables looked at i.e. the cytokines and their outcome. Over time less data was available for analysis due to the exclusion of those on RRT or lack of data due to death or loss to follow up. The model, despite the limitations suggests that baseline plasma CCL18 and MIF samples in DM patients can predict worsening GFR at >18 months and <3 years. A larger study would be needed to confirm this finding that could help differentiate patients in clinic that need closer follow up. Further limitation in this model arises from the inability to differentiate how quickly the decline in GFR occurs in an individual however, a larger sample size may clarify this. These findings were not seen in the Non-DM group.

5.1.1 Laboratory Study

A number of interesting novel findings were seen in stimulating the different intrinsic renal cells with cytokines (MIF, MCP-1 and CCL18). Some of the interesting findings were chosen to be repeated in the HK2 cells and podocytes and within these experiments there is further suggestion of trends that may be significant. For example, CCL18 increased fibronectin in HK2 cells in high glucose but did not reach significance. Experiments would be required to determine whether a single condition and its control stimulated with CCL18 significantly increases fibronectin production under these conditions. Repeating the experiment will ensure the trends seen, are reproducible. The HK2 cells and podocyte experiments would ideally have been done for all of the cytokine stimulations in the different environments as per the pilot study however, time was a limiting factor.

There are some consistent findings seen throughout this data. This in part may be a reflection of the large amount of comparisons made on small numbers that would be lost statistically. The experiment should be repeated with a large number of duplicates consisting of only one condition and control thereby minimising the potential error and allowing for direct comparison. The conducted experiments have shown a number of areas that need to be explored in this novel area. The effects of MIF, MCP-1 and CCL18 looked in combination has not previously been described. Neither have the effects of diabetic conditions on the combination of these or the effects of recombinant cytokine stimulation within tHMC, HK2 cells and podocytes. A number of more focused detailed experiments can be conducted with this knowledge to further understand the interactions between the cytokines, the ability of the cells to produce the cytokines and their effects on potentiating fibrosis.

There were also a number of interesting findings in tHMC culture that suggest further cross talk amongst the cytokines and how these alter between different environments. I conducted these experiments in single intrinsic renal cells and not a biological system that allows correlation of plasma and urine levels to be determined whilst stimulating with different cytokines, hence the interpretation of these results is limited. However, *in vitro* cell culture work is a reasonable manner to determine the effects seen within a single system and how a diabetic environment (high glucose, glycated albumin) influences this. These experiments have shown how different renal cells react however it would be important to see whether these effects continue in an *in vivo* model. When each cell type is taken individually, they have the ability to produce and/or react to MIF, MCP-1 and CCL18 that can subsequently affect the levels of the other cytokines or fibrotic markers such as fibronectin. The major limitation with cell culture work is the lack of a systemic system. Human cells may behave differently when placed on a plastic coated plate compared with 'in vivo'. An 'in vivo' system may provide further understanding to these pathways, however, animal 'in vivo' work may not determine what occurs in a human, thus both have limitations. Human 'in vitro' cell culture work in combination with 'in vivo' studies will allow further comparison as to the validity of these results.

With the discovery of a CCL18 receptor PITNMP3 IHC experiments, could be repeated to detect this. Alternatively, laser capture techniques could be used on renal biopsy tissue to isolate RNA to determine whether CCL18 is located to a specific area in the human kidney. *In vitro* work suggests

that there is variable intrinsic production of CCL18 in the renal intrinsic cells and looking at CCL18 through real time PCR may help to determine whether there is upregulation of CCL18 within a specific area in DN. Infiltrating cells attracted by local production of MIF and MCP-1 may be the source of CCL18 production and hence may induce fibrotic changes that subsequently, in different diabetic environments induce the cells to react to varying levels of cytokines. Further work is needed in this area and may show that the concept of redundancy in actions of cytokines is not true and the presence of specific cytokines to induce actions is necessary. This study has shown that although MIF, MCP-1 and CCL18 may be similar in certain aspects, the actions of these cytokines are individual and their production, actions and interactions with each other alters with cell type and environment. The recombinant cytokine stimulation may represent exposure of intrinsic renal cells to cytokine production upstream or from other cell types.

This study was limited to look at the interactions of MIF, MCP-1 and CCL18, however, some of the effects seen may use other cytokines or signalling pathways that were not measured. Further time and funding would allow for the detection of other cytokines known to be found in DN e.g. TNF-α, and determine how they interact with other cytokines and cell types. Cell signalling experiments could be conducted in these environments to determine the initial pathways used to induce these effects and whether these differ between the cytokines. In addition, cell signalling experiments will enable differentiation of initial activation of pathways and identify what pathways continue to be activated in order to maintain a response within a cell type or environment. It would be interesting to understand the established pathways because if specific to certain environments this may allow targeted inhibition. This could prevent the continuous production or stimulus leading to the production of fibrotic markers involved in disease progression.

5.2 Clinical findings - Can a single test predict worse outcome in DN?

The clinical cohort analysis, with its limitations suggests that high plasma CCL18 and MIF levels may help to determine who with DN will progress in >18 months but less than 3 years. These tests correlate with a worse GFR and would be beneficial as these people could be looked at more closely to understand why they progress.

Subsequent measurements of plasma or urine cytokines may allow further understanding of how and when progression occurs as the relationship between cytokines, GFR and ACR change over time.

This test would be more sensitive than GFR as it would allow prediction of function within a defined period, however, a larger study would be required to confirm these findings. It is not clear whether multiple tests looking for these cytokines will aid more than the initial baseline test taken on the person's first visit or if low levels are found how often these levels would need to be measured, hence the benefit of a further study.

Urinary cytokines were detected at different levels with varying effects on decreasing GFR and increasing ACR at baseline that would traditionally be reflective of disease progression, albeit the latter is seen with greater variability. The clinical study showed raised urinary CCL18 with increasing urinary MCP-1 levels that both resulted in a declining GFR. CCL18 was also seen with increasing urinary MIF (refer to Chapter 3 Fig 3.13.1). These findings do not reflect a loss of systemic cytokines as the urinary and plasma levels did not correlate. Urinary MIF was not associated with a declining GFR although urinary MIF correlated with increased urinary CCL18 levels. These urinary cytokine findings were different in Non-DM group, where urinary MCP-1 was not associated with a loss of GFR but was seen with high levels of urinary MIF and CCL18. ACR was increased with raised urinary CCL18, MCP-1 and MIF levels at baseline, although these findings change over time within the same person and is not specific to a level of albuminuria.

In the future the urinary profile of a diabetic may be more sensitive than serial blood tests. A study with a larger number of patients and a urinary cytokine profile for DN could be developed to include different markers that could determine the different stages of disease. It is clear from this work that more studies are required to understand DN progression, however, cytokine action may not be a biomarker alone but play a role in the pathogenesis of DN progression.

5.3 Laboratory findings – Can the science explain the clinical findings?

Previous studies have shown mesangial cells produce fibronectin and TGF-β following stimulus with MCP-1 (Huber, Reinhardt et al. 2002). This group also reported the CCR2 – MCP-1 receptor to be on podocytes and mesangial cells and showed that TGF-β also increased MCP-1. High levels of urinary MCP-1 have previously been reported in DN (Tam, Riser et al. 2009). The *in vitro* work shows and supports that the diabetic environment is important not only to what intrinsic renal cells produce but how this is influenced with recombinant stimulation of cytokines. This may represent the production of cytokine by cells upstream from these cells e.g. mesangial cell production of

cytokines in a particular cellular environment may differ from that of podocytes and tubuloepithelial cells. Subsequently podocytes and tubuloepithelial cells will induce certain cytokines within that environment and be stimulated by a higher level of cytokine that may produce or inhibit further production of cytokines or fibrotic factors that influence the disease state.

The production of cytokines and fibronectin detected in high glucose and glycated albumin conditions were higher when compared to their controls. The effects also differ between these two diabetic conditions. This may be used to determine progression disease of disease as hyperglycaemia usually occurs before AGEPs. Further studies specifically looking into these environments may improve our understanding of DN.

5.4 Proposal of new theory – Linking the clinical with the scientific findings

The limitations of the study need to be taken into account however, this study shows that cytokines are influenced by DN and these changes differ from other proteinuric Non-DM renal diseases. Previous literature has shown that cytokines have a role in DN (Navarro-Gonzalez, Mora-Fernandez et al. 2011), though if this precedes or occurs following hyperglycaemic and haemodynamic changes remain unclear. The *in vitro* work presented herein supports that a diabetic environment is important not only in affecting intrinsic renal cells, previously supported by other studies using high glucose conditions, but MIF, MCP-1 and CCL18 behave differently on their interactions together but also with recombinant stimulation. Recombinant stimulation may represent the production of cytokines further upstream or that from other cell types within the local area. The effects of this also differ with diabetic environment and support that these cytokines have a role in DN.

Cytokines have previously been reported to interact with Ang2 in diabetic conditions and it is unclear whether this begins the process. Interestingly, despite ACEi/ARB for blood pressure control and good glycaemic control there continues to be DN progression. From combining the clinical findings with the *in vitro* work I propose that high MIF levels are seen in high glucose conditions intrinsically produced by tubuloepithelial cells and in the first instance this may be seen in those with high ACR as albumin is toxic to the tubules (Dixon and Brunskill 1999) and may induce further production of MIF. As more AGEPs are formed the MIF production decreases and over time MIF no longer correlates with high ACR. Fibronectin is produced by tubuloepithelial cells and podocytes that are in high glucose or glycated albumin conditions and thus this in itself will induce

inflammation and change the extracellular matrix. It is unclear whether this mechanism is to repair damage in the first instance or if in high glucose conditions this mechanism is switched off by MCP-1. The progression to glycated albumin may cause MCP-1 to stimulate fibronectin production in tubuloepithelial cells and podocytes. Remarkably there is consistency of the effect of MCP-1 in the same environment in different cell types with regard to its effects on fibronectin.

In contrast high MIF levels inhibit fibronectin production in high glucose and glycated albumin conditions. MCP-1 is increased by MIF in high glucose conditions and this may trigger infiltration of other cells whilst also affecting mesangial cell cytokine production. MCP-1 in diabetic patients is seen to decrease GFR. This may result from a decrease of fibronectin production in podocytes whereby the podocytes are unable to repair themselves thus leading to decreasing GFR. There are multiple mechanisms that may also trigger haemodynamic changes to affect GFR. This data would suggest that inflammatory changes may affect the natural repair system that would occur on a cellular basis and it would be beneficial to look at the effects of the diabetic environment on tHMC and fibronectin. With disease progression there is formation of AGEPs, and podocytes in glycated albumin results in MCP-1 increasing fibronectin production. This may be a reflection of further disruption of normal ECM and an impairment of the natural healing mechanisms. The effects of CCL18 on fibronectin in podocytes, is unknown.

The pilot tHMC data suggested an inhibition of MIF with CCL18 that may indicate disease progression or production of CCL18 from infiltrating macrophages. CCL18 further increases fibronectin production in tubuloepithelial cells and is seen to be raised in baseline urinary levels of DM patients with decreasing GFR and increasing ACR. CCL18 also increases with increasing urinary MCP-1 levels. Interestingly, as AGEPs are formed i.e. glycated albumin conditions in tubuloepithelial cells MCP-1 further increases fibronectin and CCL18, that further increases fibronectin. It is therefore plausible with the clinical and laboratory findings to postulate that MIF plays an early role in DN with MCP-1 changing its effects on cells, other cytokines and fibronectin according to disease progression. The formation of AGEPs or the production of CCL18 from either mesangial cells or infiltrating cells further causes imbalance and increases fibronectin and possibly other fibrotic markers. These would occur later in the disease or indicate disruption of renal architecture and herald ongoing localised inflammatory damage that will lead on to disease progression.

This study would need to be repeated to look at other cytokines reported to be affected in DN so that interactions can be further determined. This work would allow for urinary profiling to indicate disease progression in a non-invasive manner and may be able to tailor treatment for different time points of progression. By understanding these interactions further then specific therapies can be tailored and perhaps the imbalance that occurs with the development of the different environments stopped. Further studies are essential, however, the *in vitro* studies could possibly explain the clinical findings and may be translational though these are only initial findings. This project looks at a few cytokines, though it is possible the effects seen may arise from other factors being stimulated or inhibited.

5.5 Future work

There are a number of studies that would need to be done to determine the mechanisms that interplay for DN to progress. Some of these have been described in the sections above. A larger clinical study adequately powered would enable more cytokines to be looked at. Establishing those found in the urine or plasma would enable further *in vitro* and subsequently *in vivo* work to be done. Further biostatistical methods and models for data analysis of this work would be useful, to determine any further interaction of these cytokines in combination.

To further determine the cell signalling mechanisms involved in the experiments inhibitors of established pathways should be added and the supernatant and cells collected to determine whether the markers have altered compared to the original experiments. A time course experiment would be useful to determine how quickly the pathways are activated. Identification of the receptors would also be helpful as specific blockade of pathways may lead to new, more specific treatments with less potential side effects if the main signalling cascades are not targeted. Determining the time line of events for signalling cascades and those that are activated specifically in DN with certain cytokines or fibrotic markers may be amenable to inhibition.

The studies show a difference occurs in inflammatory and fibrotic mechanisms induced by the diabetic environment compared with controls. It is therefore plausible that there will be a way to use natural agonists or antagonists to switch these signals on and off. Alternatively synthetic drugs could be designed to target more specific interactions that may halt disease progression. This novel data

needs to be confirmed, though it is promising and supportive that there are other mechanisms that contribute to disease progression than those traditionally described.

Using an *in vivo* model of DN to determine whether these markers may inhibit or induce progressive changes either in the glomerulus or tubulointerstitial compartment would be of benefit. The limitation with this is currently there is a lack of a good DN murine model.

5.6 Final remarks

Despite the limitations of this study it further supports the role of an inflammatory component in leading to progression of DN that has not been addressed with the treatments currently available. Understanding the inflammatory component in more depth may lead to effective therapeutic interventions and delay or stop the progression of DN. This could ultimately lead to improved quality and longevity of life for those with DN.

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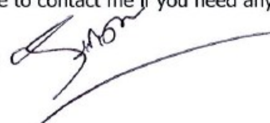
APPENDIX 1.0 – PERMISSION LETTER FROM DR. QURESHI

To whom it may concern

This is to confirm, i am happy that Dr Montero can use the data previously collected for cytokine analysis CCL18, MCP-1 that i did for my MD thesis.

Please do not hesitate to contact me if you need any further details



Regards
Dr Ashfaq Qureshi
14.07.2013

APPENDIX 2.0 – SCIENTIFIC PROTOCOLS

2.1.0 Immunohistochemistry (IHC) protocol

Label slides with date and;

Rabbit serum 0.01% (1µl in 10mls)

CCL18 (Peprotech, EC) Rabbit polyclonal antibody 1:100 (2µl in 200µl)

PBS/Tween 200µl

Put slides into a slide rack

De-wax slides: Xylene – Ethanol leaving slides in each trough for 3 minutes

The slides are then put in the water bath and then into distilled water

Citrate buffer is made with Tri-Sodium Citrate 1.5g in 500mls distilled water

Warm citrate buffer and distilled water in microwave for 5 minutes

Transfer the Tri-Sodium Citrate to cover the slide rack and heat in microwave 3 times 5 minutes ensuring the slides are covered by adding warm distilled water

Take slides out of microwave

Pour Tri-Sodium Citrate away replacing this with PBS

Dip each slide into PBS/Tween and with wax pen draw around tissue

Add 2 drops of rabbit peroxidase block and leave for 10 minutes

Dip slides in PBS/Tween

Add to respective labelled slides; rabbit serum, CCL18 1:100, PBS/Tween - 200µl volume to each slide

Incubate for 2 hours

Wash slides in PBS/Tween for 2 minutes and repeat this after changing PBS/Tween wash

Add 2 drops of polymer and incubate for 30 minutes

Wash slides in PBS/Tween for 2 minutes and repeat this after changing PBS/Tween wash

Make 1.6ml of DAB

Add 200µl of DAB per slide and leave for 5 minutes

Put slides in PBS/Tween and then wash in waterbath to stop reaction

Filter the haematoxylin and then put slide rack into haematoxylin for 30 seconds

Place back into waterbath then dip rack into Acetic Acid

Remove and then put into Ethanol through to Xylene leaving slides for 3 minutes each

Fix slides, dry and look under microscope

2.2.0 MCP-1 ELISA protocol

A 96 well plate is covered with 100µl of MCP-1 Capture antibody (MAB679) per well.

Plate covered overnight in the fridge

The plate is washed three times with washing solution (PBS/Tween). A total volume of 3 times 360µl/w

300µl of blocking solution is added to each well and the plate is covered for 1 hour.

Standards are prepared. MCP-1 Standard (279-MC).

Preparation of standards

5µl of Stock to 5000µl of Diluent concentration 10000pg/ml (label as Standard)

11 tubes are labelled with the following concentrations; 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, 19.53, 9.76, 0

500µl of Diluent is added to each tube (solution S for serum samples)

500µl from each tube labelled Standard is added to tube labelled 5000 and then to tube 2500 continuing serial dilution except for tube 0 where none is added.

Tubes labelled 2500 to 0 are used

Prepare samples

Wash plate 3 times 360µl/well

Add 100µl Standards to the standard part of the plate

Add 100µl of samples to the remaining empty wells of the plate

Cover the plate for 2 hours rotating on a slow shaker

Take out the MCP-1 Detection antibody (BAF279) and prepare 22µl in 11mls of Diluent

Wash the plate 3 times 360µl/well

Add 100µl of Detection Antibody to each well.

Cover the plate for 2 hours

Take out the substrate from the fridge

Wash plate 3 times 360µl/well

Prepare Streptavidin 2µl in 20mls PBS

Add Streptavidin to the plate 100µl/well and cover for 20 minutes

Prepare Substrate 5 minutes before use (before washing with Streptavidin). Add 5.5ml of A to 5.5ml B

Wash the Streptavidine 3 times 360µl/well

Add 100µl Substrate to each well of the plate

Cover and protect from direct sunlight for 5-30 minutes until the 2500pg/ml Standard becomes deep blue.

Prepare Stop Solution (10mls H_2SO4 into 90mls of water)

Set up the ELISA plate reader for 450nm wavelength

Add 50µl Stop Solution to each well of the plate and mix gently – samples and standards will turn yellow in colour

Read the plate in the ELISA plate reader

2.2.1 Human MIF duoset (DY289) ELISA protocol

A 96 well plate is covered with 100µl of MIF Capture antibody (Part 840489) per well.

Plate covered overnight in the fridge

The plate is washed three times with washing solution (PBS/Tween). A total volume of 3 times 360µl/w

300µl of Reagent Diluent is used as the blocking solution and added to each well. The plate is covered for 1 hour.

Standards are prepared. MIF Standard (Part 840491).

Preparation of standards

40µl of Stock to 560µl of Diluent concentration
2000pg/ml (label as Standard)
8 tubes are labelled with the following concentrations;
2000, 1000, 500, 250, 125, 62.5, 31.25, 0
300µl of Reagent Diluent is added to each tube
(solution S for serum samples)
300µl from each tube labelled Standard is added to tube
labelled 2000 and then to tube 1000 continuing serial
dilution except for tube 0 where none is added.
Tubes labelled 2000 to 0 are used

Prepare samples

Wash plate 3 times 360µl/well

Add 100µl Standards to the standard part of the plate

Add 100µl of samples to the remaining empty wells of the plate

Cover the plate for 2 hours rotating on a slow shaker

Take out the MIF Detection antibody (Part 840490) and prepare 38.9µl in 7mls of Diluent

Wash the plate 3 times 360µl/well

Add 100µl of Detection Antibody to each well.

Cover the plate for 2 hours

Take out the substrate from the fridge

Wash plate 3 times 360µl/well

Prepare Streptavidin HRP 100µl in 20mls Reagent Diluent

Add Streptavidin HRP to the plate 100µl/well and cover for 20 minutes

Prepare Substrate 5 minutes before use (before washing with Streptavidin HRP). Add 5.5ml of A to 5.5ml B

Wash the Streptavidin HRP 3 times 360µl/well

Add 100µl Substrate to each well of the plate

Cover and protect from direct sunlight for 5-30 minutes until the 2500pg/ml Standard becomes deep blue.

Prepare Stop Solution (10mls H_2SO4 into 90mls of water)

Set up the ELISA plate reader for 450nm wavelength

Add 50µl Stop Solution to each well of the plate and mix gently – samples and standards will turn yellow in colour

Read the plate in the ELISA plate reader

2.2.2 CCL18/PARC ELISA protocol

A 96 well plate is covered with 100µl of CCL18 Capture antibody (MAB394) per well. 22µl Capture antibody in 11ml in PBS.

Plate covered overnight in the fridge

The plate is washed three times with washing solution (PBS/Tween). A total volume of 3 times 360µl/w

300µl of blocking solution is added to each well. The plate is covered for 1 hour.

Standards are prepared. CCL18 Standard 5µl in 12.5ml of Diluent (394-PA).

Preparation of standards 5µl of Stock to 5000µl of Diluent concentration
10000pg/ml (label as Standard)
11 tubes are labelled with the following concentrations;
5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06,
19.53, 9.5, 0
500µl of Diluent is added to each tube
(solution S for serum samples)
500µl from each tube labelled Standard is added to tube

labelled 5000 and then to tube 2500 continuing serial dilution except for tube 0 where none is added.

Tubes labelled 5000 to 0 are used

Prepare samples

Wash plate 3 times 360µl/well

Add 100µl Standards to the standard part of the plate

Add 100µl of samples to the remaining empty wells of the plate

Cover the plate for 2 hours rotating on a slow shaker

Take out the CCL18 Detection antibody (BAF394) and prepare 22µl in 11mls of Diluent

Wash the plate 3 times 360µl/well

Add 100µl of Detection Antibody to each well.

Cover the plate for 2 hours

Take out the substrate from the fridge

Wash plate 3 times 360µl/well

Prepare Streptavidin 2µl in 20mls PBS

Add Streptavidin to the plate 100µl/well and cover for 20 minutes

Prepare Substrate 5 minutes before use (before washing with Streptavidin). Add 5.5ml of A to 5.5ml B

Wash the Streptavidin 3 times 360µl/well

Add 100µl Substrate to each well of the plate

Cover and protect from direct sunlight for 5-30 minutes until the 5000pg/ml Standard becomes deep blue.

Prepare Stop Solution (10mls H_2SO4 into 90mls of water)

Set up the ELISA plate reader for 450nm wavelength

Add 50µl Stop Solution to each well of the plate and mix gently – samples and standards will turn yellow in colour

Read the plate in the ELISA plate reader

2.2.3 Fibronectin ELISA protocol

This protocol was initially established in the Renal Research Laboratory, University of Leicester.

A 96 well plate is covered with 100µl of Fibronectin Capture antibody (F3648) per well. 10µl Capture antibody in 10mls in coating buffer.

Plate covered overnight in the 4ºC fridge

The plate is washed three times with washing solution (PBS/Tween). A total volume of 3 times 360µl/w

100µl of blocking solution (2%BSA/PBS) is added to each well. The plate is covered for 1 hour.

Standards are prepared. Fibronectin Standard 1mg/ml stock concentration (F0895).

Preparation of standards 10µl of Stock to 5mls of PBS concentration
2000pg/ml (label as Standard)
11 tubes are labelled with the following concentrations;
2000, 1000, 500, 250, 125, 62.5, 31.25, 15.6,
7.8, 3.9, 1.95, 0
200µl of Diluent is added to each tube
(solution S for serum samples)
100µl from each tube labelled Standard is added to tube
labelled 2000 and then to tube 1000 continuing serial
dilution except for tube 0 where none is added.
Tubes labelled 2000 to 0 are used

Prepare samples

Wash plate 3 times 360µl/well

Add 50µl Standards to the standard part of the plate

Add 50µl of samples to the remaining empty wells of the plate

Cover the plate overnight in the 4ºC fridge

Take out the Fibronectin Detection antibody (F7387) and prepare 10µl in 5mls of Diluent (1:500 dilution)

Wash the plate 3 times 360µl/well

Add 50µl of Detection Antibody to each well.

Cover the plate for 1 hour

Take out the substrate from the fridge

Wash plate 3 times 360µl/well

Prepare Streptavidin 1µl in 10mls PBS

Add Streptavidin to the plate 50µl/well and cover for 1 hour

Prepare Substrate immediately before use (after washing with Streptavidin).

Wash the Streptavidin 3 times 360µl/well

Add 50µl Substrate to each well of the plate

Cover and protect from direct sunlight for 5-30 minutes until the 2000pg/ml Standard becomes deep brown.

Prepare Stop Solution (10mls H_2SO4 into 90mls of water)

Set up the ELISA plate reader for 492nm wavelength

Add 75µl Stop Solution to each well of the plate and mix gently – samples and standards will turn orange in colour

Read the plate in the ELISA plate reader

2.3.0 MTT Assay protocol

Using a six well plate

Remove supernatant from the wells

Add 1ml of fresh complete media + 10µl of MTT solution

Incubate at 37ºC overnight

Remove media and discard

Add 1ml of PBS +100µl of 10% SDS per well

Place plate into the 37ºC incubator overnight

Transfer 200µl of the purple liquid into a 96 plate well

Read at 550nm on ELISA plate reader (spectrophotometer)

2.4.0 Transformed Human Mesangial Cells

Use T75 flasks, 15mls medium per flask and are grown at 37ºC incubator

Split 1:3 in 5% FCS, 5mls trypsin, FCS 1ml

Maintain in; RPMI 1640 (with glutamine with no glucose) 500mls with:
5% FCS
PSF (penicillin, streptomycin) (5mls)
4mM glucose (5ml 400nM)

2.5.0 HK-2 cells

T75 flasks were used to grow cells in 37ºC incubator.

Maintenance Medium: Keratinocyte media supplemented with bovine pituitary extract and epidermal growth factors
5% FCS
PSF (penicillin, streptomycin) (5mls)

2.6.0 Human Podocytes

T75 flasks were used to grow in 33ºC incubator.

Once the required cell number has been reached the cells are seeded and transferred to 37ºC for cell differentiation. They are maintained here for 2 weeks prior to experimental use.

Medium; RPMI 1640
PSF (penicillin, streptomycin) (5mls)
4mM glucose
5% FCS

2.7.0 Cell Culture Cells and Supernatant Collection

Collection of supernatant

Aspirate the supernatant into 1.5ml eppendorf tubes

Centrifuge 14000RPM 4^{0}C for 10 minutes (10958g)

Remove the supernatant into fresh labelled tubes and store -80^{0}C freezer

Discard pellet

Collection of cells

The pellet left in the eppendorf with removal of supernatant is used

1ml of Hanks solution put into tube

Centrifuge 1500RPM 4^{0}C for 5 minutes (128g)

Discard supernatant and add further 1ml Hanks solution to wash cells

Centrifuge 1500RPM 4^{0}C for 5 minutes (128g)

Discard supernatant

Add 1ml Trysol to pellet and store in -80^{0}C freezer

2.7.1 Collection of cells for Western blotting

Cell lysis buffer was added 100µl per well and then a cell scraper was used to scrape the cells from the well. These were placed in an eppendorf on ice for 30 minutes with vortex every 10 minutes. The tubes were centrifuged for 10 minutes 1500rpm 4ºC (128g) eppendorf centrifuge 5417R and the supernatant collected in a fresh tube. The BCA assay was later done to determine the amount of protein per sample.

Cell lysis buffer

For total of 12mls;

1.2mls of Protease Inhibitor

40µl if PMSF in DMSO

10760µl of Invitrogen buffer to be kept on ice

2.7.2 BCA Protein Assay

Mix protein assay reagent A and B (working reagent)

Protein assay reagent A:Protein assay reagent B= 50:1

200µl per well

Dilute Standard BSA (Albumin Standard Ampules 2mg/ml) with water in a 96 well plate (see table below)

Table A2T1 BCA protein assay

[Standard] (mg/ml)	dH_2O (µl)	BSA (µl)	Lysis buffer (µl)
0	8 x10 = 80	0	2
0.1	7.5 x10 = 75	0.5 x10 = 5	2
0.2	7 x10 = 70	1 x10 = 5	2
0.4	6 x10 = 60	2 x10 = 20	2
0.6	5 x10 = 50	3 x10 = 30	2
0.8	4 x10 = 40	4 x10 = 40	2
1	3 x10 = 30	5 x10 = 50	2
1.2	2 x10 = 20	6 x10 = 60	2
1.4	1 x10 = 10	7 x10 = 70	2

Dilute each sample in 96 well plate (2µl sample in 8µl if dH_2O)

2µlx1.5=3µl in dH_2O, 8µlx1.5=12µl in dH_2O

Add 200µl working reagent to each well and 5µl samples and series of standards

Gently mix on plate shaker for 30 seconds

Cover plate and incubate at 37ºC for 30 minutes

Cool plate to room temperature and read on ELISA plate reader at 562nm

2.7.3 Freeze cells

To freeze: Trypsinise, stop and spin down at 500rpm for 5 minutes (28g)

Discard medium and add 1ml 10% DMSO in complete medium and mix well

Freeze in cryotube at -80ºC

Next day transfer to liquid nitrogen

2.8.0 Western blotting protocol

40mls 20x NuPage running buffer was placed in 800mls dH_2O

The running buffer is determined according to the molecular weight of the protein the western blot is to detect

Select the NuPage gel according to the molecular weight proteins to be identified

Add 500µl of Invitrogen Antioxidant to the central compartment of the holder

Heat the block to 95ºC for 5 minutes

Centrifuge the defrosted cell lysate samples and the BioRAD ladder

Comb removed from NuPage gel from Invitrogen and place into gel holder

Wash with running buffer and flush each well with buffer

To avoid protein degradation 10µl of 5x sample buffer is added to the cell lysate and centrifuged 9000rpm for a few seconds

The samples are then placed in heating block 100ºC for 10 minutes

The samples are loaded according to the pre-determined protein content ascertained by BCA assay. Approximately 40µl per well

Once loaded then cover and run the voltage at 100v for 90 minutes ensuring the samples run down the gel

Using the thermo-semi wet transfer (iBlot invitrogen) on PVDF membranes

TBST is used to wet the blotting paper

The gel is opened with a spatula and subsequently cut to size according to band width

The gel is placed on the transfer with the wet blotting paper over this and sandwiched by the machine for 7 minutes

The protein bands have transferred to the membrane that is cut and placed into a tray with 20mls of chemiluminescent blocking for 1 hour on a shaker

This is washed x1 with TBST and the primary Ab of choice is then put onto the membrane and incubated overnight at 4ºC (cold room)

Primary Ab is made in 5% BSA with TBST and re-used

The following day the Primary Ab is collected and the membrane washed with TBST x 3 every 10 minutes

Secondary Ab is made in the appropriate dilution with milk and TBST

This added and left to incubate on a shaker for 1 hour

Further x 3 washes with TBST 10 minutes each

ECL primer is used for development of the membrane and maintained in an x-ray case until developed

Radiographic film is placed over the membrane for varying exposure time and developed in the dark room

Membranes are stored at 4ºC to allow for re-blotting of proteins

2.8.1 Western blotting re-blot protocol

The membrane was placed in 20mls of stripping buffer for 30mins on a plate shaker

The membrane was removed and washed in TBST for 10mins

The membrane was then placed in 20mls of chemiluminescent blocking for 1 hour on the plate shaker

The membrane was removed and washed in TBST for 10mins

Actin Ab was made in dilution of 1:200 in 20mls of TBST with 5%BSA that is later collected for repeated use

The membrane is incubated at room temperature on the plate shaker for 1 hour

The membrane washed with TBST x 3 every 10 minutes

ECL primer is used for development of the membrane and maintained in an x-ray case until developed

Radiographic film is placed over the membrane for varying exposure time and developed in the dark room

Membranes are stored at 4ºC

APPENDIX 3.0 – RESULTS – PROSPECTIVE CLINICAL COHORT

3.1.0 Prospective study of CCL18 in serum and urine – 3yr follow up

Date

Name | Gender M/F

Hospital number | Date of Birth

Ethnicity | Laboratory number

Medication list

Smoking status | pack years

BP | Weight (kg) | Height (m)

Urine dipstick

Leu ☐ Nit ☐ Pro ☐ pH ☐ Bld ☐ SG ☐
Ket ☐

Samples to be collected for research lab

1 x Urine universal sample ☐ (white top universal urine 20mls container)

1 x Serum sample ☐ (EDTA 4mls light purple top)

1 x Serum sample ☐ (Clotted serum 6mls red top)

Bloods to be sent

HbA1c ☐ U+Es ☐ eGFR ☐ FBC ☐

Bone profile ☐ Lipids ☐ Vit D ☐ (25 hydroxy)

Urine to be sent

ACR ☐ MC+S ☐ UPCR ☐

Diabetic related Complications

Retinopathy	Background	☐
	Pre-proliferative	☐
	Proliferative	☐
	Maculopathy	☐
Neuropathy	Peripheral	☐
	Autonomic	☐
CVA		☐
IHD	MI	☐
	Angina	☐
	CABG	☐
	Angioplasty	☐
PVD		☐

Renal Biopsy or other renal diagnosis

Date

Results

3.2.0 Consent form

Imperial College London

Imperial College Healthcare NHS

NHS Trust

Centre Number:
Study Number: SALA1004 Date: 1 October 2009 Version: 3
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Investigation of lymphocyte responses in renal diseases, including analysis of **any** biomarkers

Name of Researcher: Dr Alan D. Salama and Dr. F.W.K. Tam

Please initial box

1. I confirm that I have read and understand the information sheet dated 1 October 2009 (version 5) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [company name] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

3.3.0 Patient information sheet DM

Date: 1 October 2009 Version 5

Patient information Sheet-for patients with renal disease other than glomerulonephritis

Investigation of white blood cell responses in renal diseases, including analysis of biomarkers which may be useful markers of diseases

You are being invited to take part in a research study. This information sheet will help you understand why we are undertaking the research and what we would like you to do. Please read the sheet carefully and feel free to discuss it with others if you wish. Please ask us if there is anything that is not clear to you or if you would like more information. Take time to decide whether you wish to take part or not.

We will be happy to let you have a copy of the leaflet entitled 'Medical Research and You' published by Consumers for Ethics in Research (CERES). This leaflet gives more information about medical research and looks at some questions you may want to ask.

Thank you for reading this.

What is the purpose of the study?

White blood cells normally work to fight infections. However, in many forms of kidney disease (called glomerulonephritis) they start to attack the kidney(called autoimmunity), leading to kidney failure. It is not clear why this happens to certain individuals at particular times and not to others.
We believe that by understanding what has gone wrong in patients with glomerulonephritis we may be able to better treat them and return their immune system to the normal, healthy state, where the white blood cells are unable to cause any damage, but continue their normal job of fighting infections. We intend to do this by studying the responses of white blood cells from patients with glomerulonephritis and comparing them with patients who have other forms of kidney disease. We can analyse cells or the chemicals they make, in the blood, urine, on or kidney biopsies. We can study the genes that control these responses from the same blood samples.

What does the research involve for me?

There are 4 types of samples that we may ask your permission to use in the laboratory: 1) samples of blood; 2) sample of your DNA (taken from your blood), 3) urine samples and 4) samples from kidney biopsies that you have had done only if you need a biopsy for clinical reasons. Details of each of these samples are given below.

1) Blood samples. To look at the function of T cells from different people we need blood samples from which we can purify the cells. We will need about 50-100mls from you each time – that's less than ¼ of what you would donate

if you were giving blood. We are undertaking the research over 2 years and may need to take 4 to 6 samples from you during this time. In virtually all cases we would take the blood at the same time as you were having your routine clinic bloods done so no extra needle pricks will normally be involved.

2) Sample of your DNA. DNA will be extracted from the same blood sample and stored, using an anonymous code, in a freezer. All the data is completely confidential and any unused DNA will be discarded at the end of the study.

3) Samples of your urine. Urine contains cells and chemicals produced in the kidney and we have already shown that certain chemicals are found in much higher amounts in patients with glomerulonephritis. We will use the urine samples to see if we can detect patterns of chemicals that mirror disease activity. Every time you come to clinic you normally give a urine sample. We will then store part of this sample in a freezer and analyse the chemicals in the urine at a later date.

4) Samples from kidney biopsies. If you have a kidney biopsy we would like to look at any spare tissue that remains. We will not take any extra samples at the time of biopsy and we do not want to perform another biopsy for research purposes. We would simply use any spare material left after all that has been needed for your clinical diagnosis has been used.

At no time will we be giving you any extra medications or giving back the cells we have taken from you for this research. We simply need samples from you that we keep in the laboratory. Some of the samples may be frozen for some months as we may need to repeat some of the laboratory tests.

No one in the laboratory who is working with your cells will know who you are. All the samples we take will be coded and only the principal investigators will be able to relate your clinical condition to the results of the laboratory research.
The samples you give are purely for research and the results of the research will have no direct benefit for you. However, we hope that the information we obtain from this study will give a better understanding of why and how glomerulonephritis occurs and how we may be able to better treat it.

Why have I been chosen?

You have been chosen because you have a form of kidney disease which is not glomerulonephritis.

Do I have to take part?

NO. It is entirely up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and we will ask you to sign a consent form. If you do decide to take part you are still free to withdraw at any time and without giving a reason. A decision not to take part or to withdraw at any time, will in no way affect the standard of clinical care you receive.

Will taking part have any negative effects on my health?

No. We will only take blood from you when we have a recent blood count available. If your blood count is low (i.e. if you are anaemic), we won't take blood. Taking a larger amount of blood is no more painful than taking the samples you have done routinely for your clinic appointments.

There are risks associated with having a kidney biopsy done but this will only be done for clinical reasons and the risks will be explained by the doctor doing the biopsy. Using the tissue left over from the clinical analysis involves no added risk whatsoever as no extra tissue will be taken from you for this research.

Do I have to alter my lifestyle to take part?

No.

Will my taking part in this study be kept confidential?

Yes. All information collected about you during the research will be kept strictly confidential. Any information about you, which leaves the hospital or is published, will be anonymous so that no one can identify you from the available information. Your name and address, hospital number and date of birth will not be published or passed on in any way. Urine samples and anonymous information collected about you may be sent outside of the European Union, for example to the United States of America and Canada. This information may be stored and electronically processed for the purpose of scientific evaluation.

What will happen to the results of the study?

The results will be used as a basis for further research. If we find things out that are deemed interesting by researchers from other hospitals and universities, the results may be published in a scientific journal. If this happens, no one will be able to identify you as having taken part in the research, as all the information about patients will be anonymised.

Who is organising and funding the research?

The research is being organised by Dr Salama and Dr. Tam from the Renal department, in collaboration with Professor Pusey, Professor Maxwell, Dr Levy, Dr Lightstone and Dr Gaskin. No doctor taking part in this study is getting paid extra for including you in the study. Funding for the scientists undertaking the research comes from the Department of Health, the Medical Research Council, the National Kidney Research Fund, The Wellcome Trust, Hammersmith Hospitals Trust Research Committee, Biogen Idec Inc. and from Imperial College.

Is there any payment for taking part?

No, we are not paying patients or volunteers for providing blood samples.

Who has reviewed this study?

The study has been reviewed by the Research Ethics Committee of the Hammersmith, Queen Charlotte's and Chelsea and Acton Hospitals.

You can keep this information sheet and a copy of the consent form. If you wish to have further information about the study, please contact Dr Salama or Dr. Tam via, Anjili Jagpal, on 020 8383 3152

3.4.0 Patient information sheet Non-DM

Date: 1 October 2009 **Version: 5**

Patient information Sheet-for patients with Glomerulonephritis

Investigation of white blood cell responses in renal diseases, including analysis of biomarkers which may be useful markers of diseases

You are being invited to take part in a research study. This information sheet will help you understand why we are undertaking the research and what we would like you to do. Please read the sheet carefully and feel free to discuss it with others if you wish. Please ask us if there is anything that is not clear to you or if you would like more information. Take time to decide whether you wish to take part or not.

We will be happy to let you have a copy of the leaflet entitled 'Medical Research and You' published by Consumers for Ethics in Research (CERES). This leaflet gives more information about medical research and looks at some questions you may want to ask.

Thank you for reading this.

What is the purpose of the study?

White blood cells normally work to fight infections. However, in many forms of kidney disease (called glomerulonephritis) they start to attack the kidney, leading to kidney failure. It is not clear why this happens to certain individuals at particular times and not to others.
We believe that by understanding what has gone wrong in patients with glomerulonephritis we may be able to better treat them and return their immune system to the normal, healthy state, where the white blood cells are unable to cause any damage, but continue their normal job of fighting infections. We intend to do this by studying the responses of white blood cells from patients with glomerulonephritis and comparing them with healthy controls. We can analyse cells or the chemicals they make, in the blood, urine, on or kidney biopsies. We can study the genes that control these responses from the same blood samples.

What does the research involve for me?

There are 4 types of samples that we may ask your permission to use in the laboratory: 1) samples of blood; 2) sample of your DNA (taken from your blood), 3) urine samples and 4) samples from kidney biopsies that you have had done only if you need a biopsy for clinical reasons. Details of each of these samples are given below.

1) Blood samples. To look at the function of cells from different people we need blood samples from which we can purify the cells. We will need about 50-100mls from you each time – that's less than ¼ of what you would donate if you were giving blood. We are undertaking the research over 2 years and

may need to take 3 to 4 samples from you during this time. In virtually all cases we would take the blood at the same time as you were having your routine clinic bloods done so no extra needle pricks will normally be involved.

2) Sample of your DNA/RNA. DNA/RNA will be extracted from the same blood sample and stored, using an anonymous code, in a freezer. All the data is completely confidential and any unused DNA/RNA will be discarded at the end of the study.

3) Samples of your urine. Urine contains cells and chemicals produced in the kidney and we have already shown that certain chemicals are found in much higher amounts in patients with glomerulonephritis. We will use the urine samples to see if we can detect patterns of chemicals that mirror disease activity. Every time you come to clinic you normally give a urine sample. We will then store part of this sample in a freezer and analyse the chemicals in the urine at a later date.

4) Samples from kidney biopsies. If you have a kidney biopsy we would like to look at any spare tissue that remains. We will not take any extra samples at the time of biopsy and we do not want to perform another biopsy for research purposes. We would simply use any spare material left after all that has been needed for your clinical diagnosis has been used.

At no time will we be giving you any extra medications or giving back the cells we have taken from you for this research. We simply need samples from you that we keep in the laboratory. Some of the samples may be frozen for some months as we may need to repeat some of the laboratory tests.

No one in the laboratory who is working with your cells will know who you are. All the samples we take will be coded and only the principal investigators will be able to relate your clinical condition to the results of the laboratory research.
The samples you give are purely for research and the results of the research will have no direct benefit for you. However, we hope that the information we obtain from this study will give a better understanding of why and how glomerulonephritis occurs and how we may be able to better treat it.

Why have I been chosen?

You have been chosen because you have, or have had in the past a form of glomerulonephritis.

Do I have to take part?

NO. It is entirely up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and we will ask you to sign a consent form. If you do decide to take part you are still free to withdraw at any time and without giving a reason. A decision not to take part or to withdraw at any time, will in no way affect the standard of clinical care you receive.

Will taking part have any negative effects on my health?

No. We will only take blood from you when we have a recent blood count available. If your blood count is low (i.e. if you are anaemic), we won't take blood. Taking a larger amount of blood is no more painful than taking the samples you have done routinely for your clinic appointments.

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Do I have to alter my lifestyle to take part?

No.

Will my taking part in this study be kept confidential?

Yes. All information collected about you during the research will be kept strictly confidential. Any information about you, which leaves the hospital or is published, will be anonymous so that no one can identify you from the available information. Your name and address, hospital number and date of birth will not be published or passed on in any way. Urine samples and anonymous information collected about you may be sent outside of the European Union, for example to the United States of America and Canada. This information may be stored and electronically processed for the purpose of scientific evaluation.

What will happen to the results of the study?

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Who is organising and funding the research?

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APPENDIX 4.0 – RESULTS – SCIENTIFIC BASIS FOR CLINICAL FINDINGS

4.1.0 Pilot study HK-2 tubuloepithelial cells stimulated in diabetic milieu for 48 hours

Basal MCP-1 levels in HK2 cells

There was no production of MCP-1 in HK2 cells as the levels were below the sensitivity of the standard curve for the MCP-1 ELISA (n=2).

Basal CCL18 levels in HK2 cells

There was no production of CCL18 in HK2 cells as the levels were below the sensitivity of the standard curve for the CCL18 ELISA (n=2).

4.1.1 HK-2 tubuloepithelial cells stimulated with rMCP-1 over 48 hours

All the following experiments (n=2) per condition and dose.

MCP-1 in HK2 stimulated with rMCP-1

A rise is seen in MCP-1 production that is dose dependent in HK2 cells. A sharp rise is seen in the conditions with glycated albumin and 20ng/ml stimulation of rMCP-1.

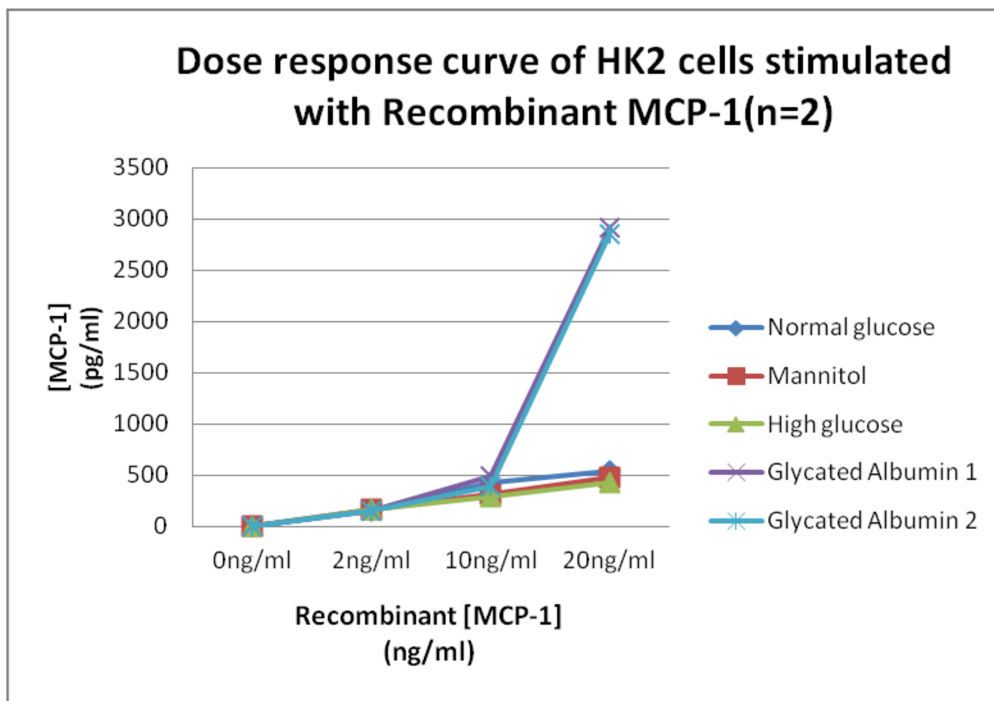

Figure A4F1 Dose response curve of rMCP-1 in HK2 cells

MIF in HK2 stimulated with rMCP-1

MIF levels appear to increase from baseline with stimulation with higher dose of rMCP-1 in HK2 cells.

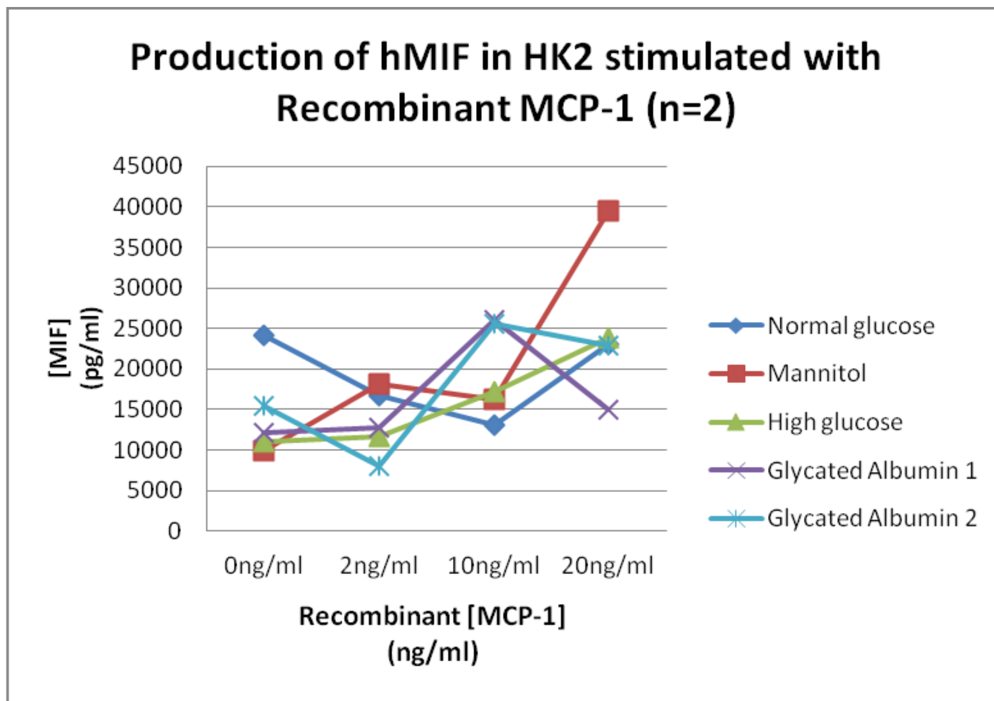

Figure A4F2 Production of MIF following stimulation with rMCP-1 in HK2 cells

CCL18 in HK2 stimulated with rMCP-1

There are no detectable levels of CCL18 following increasing stimulation of HK2 cells with rMCP-1 in any of the conditions.

Summary of pilot results

Stimulation with 20ng/ml rMCP-1 resulted in higher amounts of MCP-1 found in glycated albumin conditions. There was an inconsistent rise in the levels of MIF following stimulation with increasing dose of rMCP-1. There were no detectable levels of CCL18 following stimulation with rMCP-1.

4.1.2 HK-2 tubuloepithelial cells stimulated with rCCL18 over 48 hours

All the following experiments (n=2) per condition and dose.

CCL18 in HK2 stimulated with rCCL18

There is a dose dependent rise in CCL18 with increasing rCCL18 in HK2 cells. This effect appears more linear in the glycated albumin basal conditions compared to other conditions.

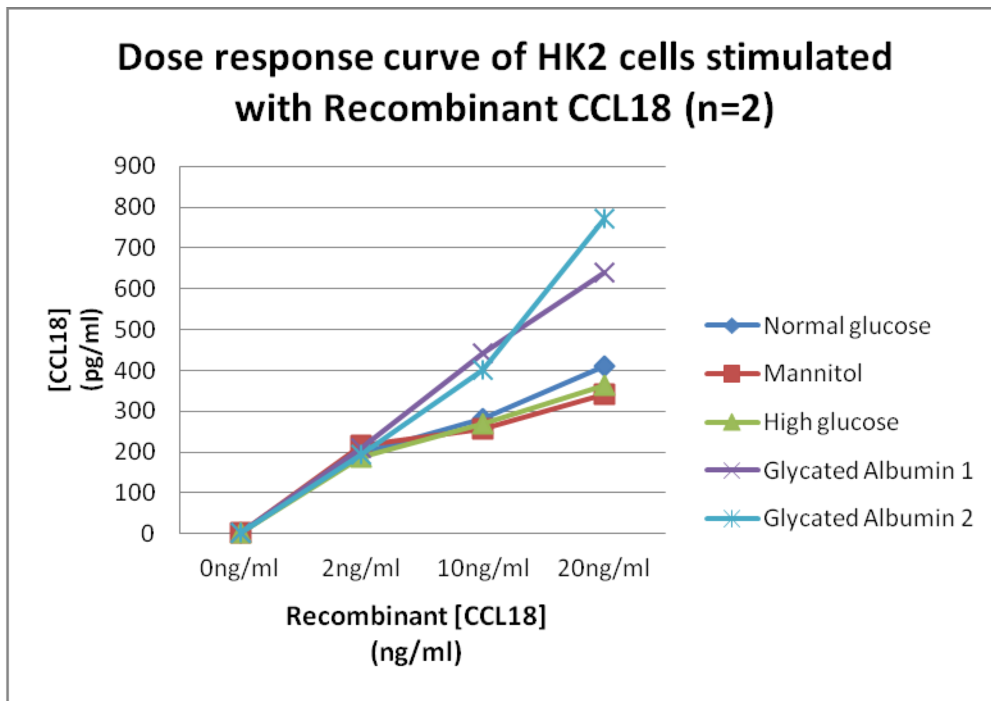

Figure A4F3 Dose response curve of rCCL18 in HK2 cells

<u>*MIF in HK2 stimulated with rCCL18*</u>

There is a decrease in the basal levels of MIF in HK-2 cells following increasing dose of rCCL18. This appears to be more profound in glycated albumin 2 and is illustrated on the Log10 y axis used due to the large amounts of MIF detected.

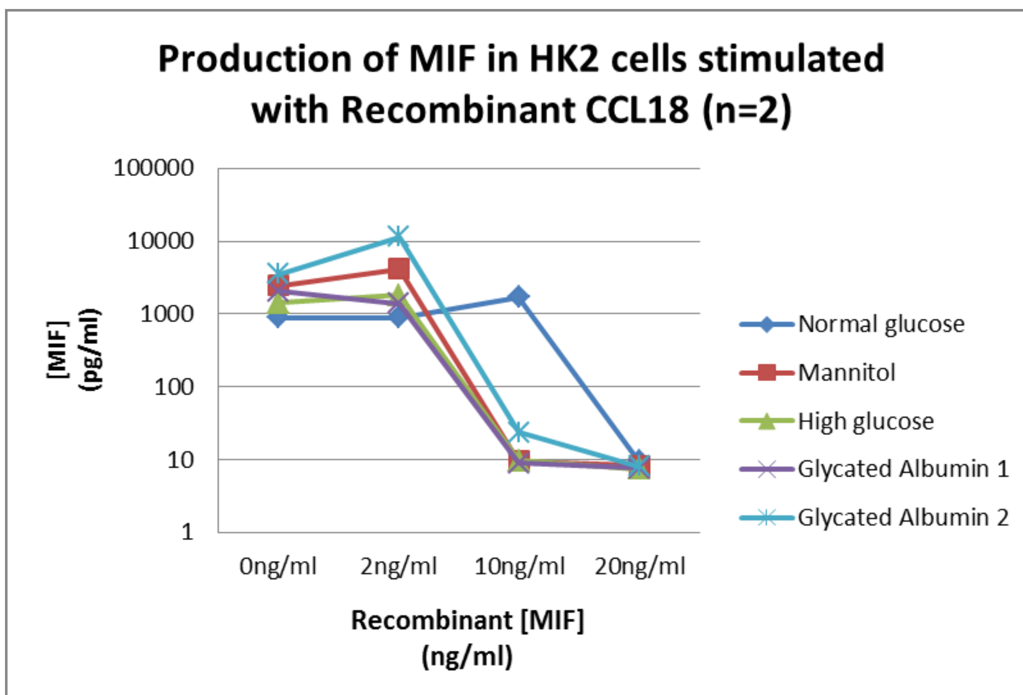

Figure A4F4 Production of MIF following stimulation with rCCL18 in HK2 cells

MCP-1 in HK2 stimulated with rCCL18

There are no detectable levels of MCP-1 following increasing stimulation of HK2 cells with rCCL18 in any of the conditions.

Summary of results

Increasing dose of rCCL18 resulted in a more linear increase in CCL18 detected in glycated albumin. MIF production appeared to increase in glycated albumin with 2ng/ml stimulation of rCCL18 with attenuation in the production of MIF in all conditions with higher doses of rCCL18. rCCL18 had no effect on MCP-1.

Summary of pilot results in HK2 cells

All basal conditions are able to produce MIF in vitro in HK2 cells. Stimulation with recombinant cytokines seems to induce a larger difference in glycated albumin conditions. rMCP-1 stimulation slightly increased detectable levels of MIF in HK2 cells. MIF production in HK2 cells was attenuated with high dose stimulation with rCCL18 in all conditions, the effect was more evident in glycated albumin.

4.2.0 Pilot study Human Podocytes stimulated in diabetic milieu for 48 hours

Podocytes stimulated with diabetic milieu are able to produce MIF and MCP-1 in vitro. In particular MIF is seen in high concentrations in this cell type.

Basal MIF levels in Podocytes

Large amounts of MIF are detected in podocytes following 48 hours stimulation with basal conditions. Median numbers for normal glucose (3.8 $x10^{14}$pg/ml), glycated albumin 1 (3.23 $x10^{14}$pg/ml), glycated albumin 2 (3.01$x10^{6}$pg/ml), mannitol (3.15 $x10^{6}$pg/ml) and high glucose (1.58 $x10^{6}$pg/ml).

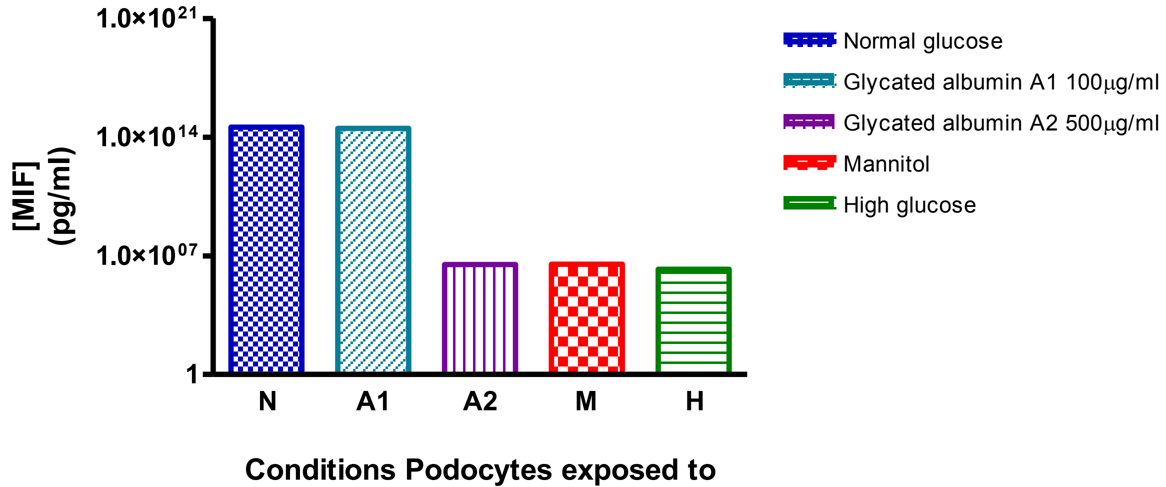

Figure A4F5 Production of MIF following stimulation with diabetic milieu

Basal MCP-1 levels in Podocytes

Median numbers for normal glucose (61pg/ml), glycated albumin 1 (59.2pg/ml), glycated albumin 2 (55.1pg/ml), mannitol (66.7pg/ml) and high glucose (61.2pg/ml).

Figure 4.1.1 MCP-1

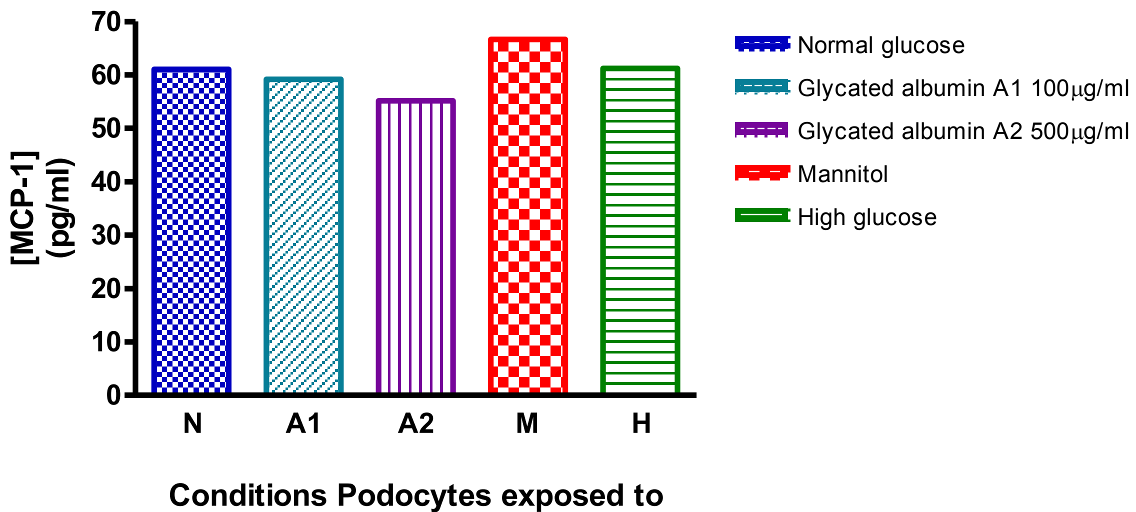

Figure A4F6 Production of MCP-1 following stimulation with diabetic milieu

4.2.1 Human Podocytes stimulated with rMIF over 48 hours

All the following experiments (n=2) per condition and dose.

MIF in Podocytes stimulated with rMIF

The dose response curve for MIF following rMIF stimulation has used a Log10 y axis in view of the large quantities detected in the ELISA. High levels of MIF are seen in podocytes stimulated with diabetic milieu. High glucose and the lower dose of glycated albumin produce higher levels of MIF than their controls in the supernatant collected by stimulated podocytes. The addition of rMIF to podocytes in these conditions in a dose increment, results in a decrease in production. There is a rise in MIF with stimulation with recombinant form in the control conditions; normal glucose and mannitol.

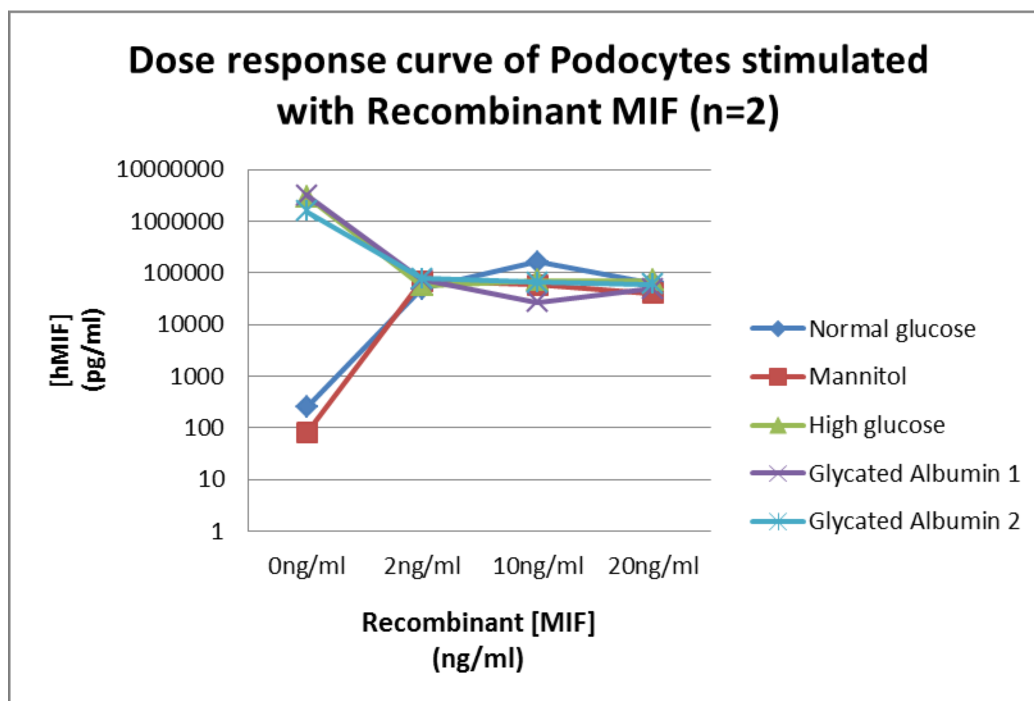

Figure A4F7 Dose response of rMIF in Podocytes

MCP-1 in Podocytes stimulated with rMIF

There appears to be an increase followed by a decrease in MCP-1 with increasing stimulation of rMIF in all conditions in podocytes.

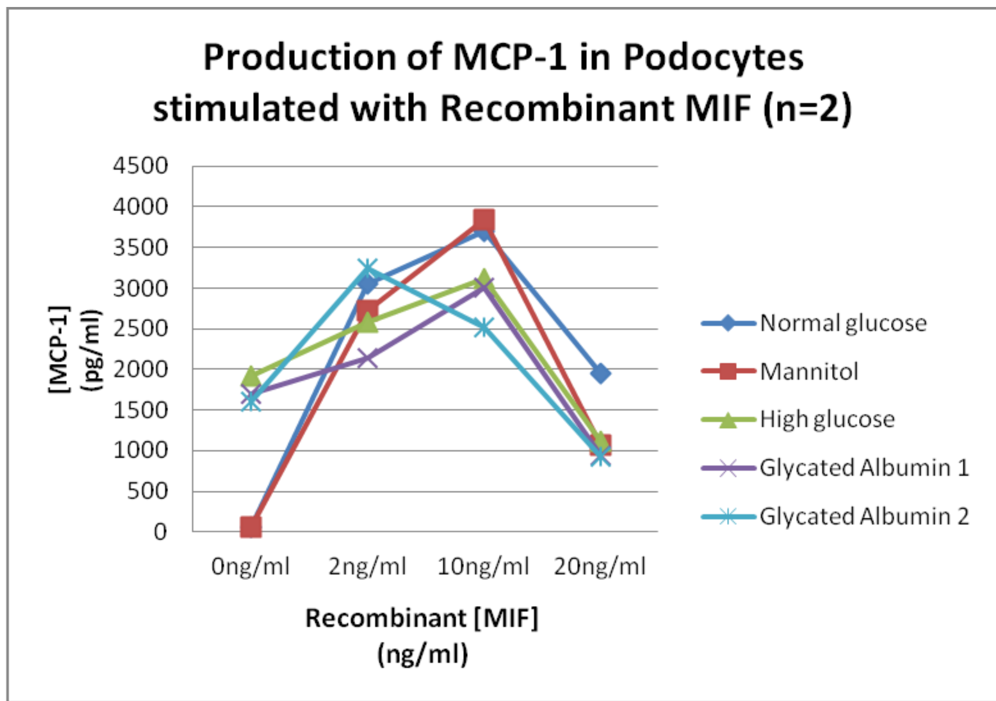

Figure A4F8 Production of MCP-1 following stimulation with rMIF

<u>*CCL18 in Podocytes stimulated with rMIF*</u>

A high dose of rMIF results in an increase in CCL18 production in all the conditions except normal glucose. These findings would need to be repeated to ensure they are reproducible.

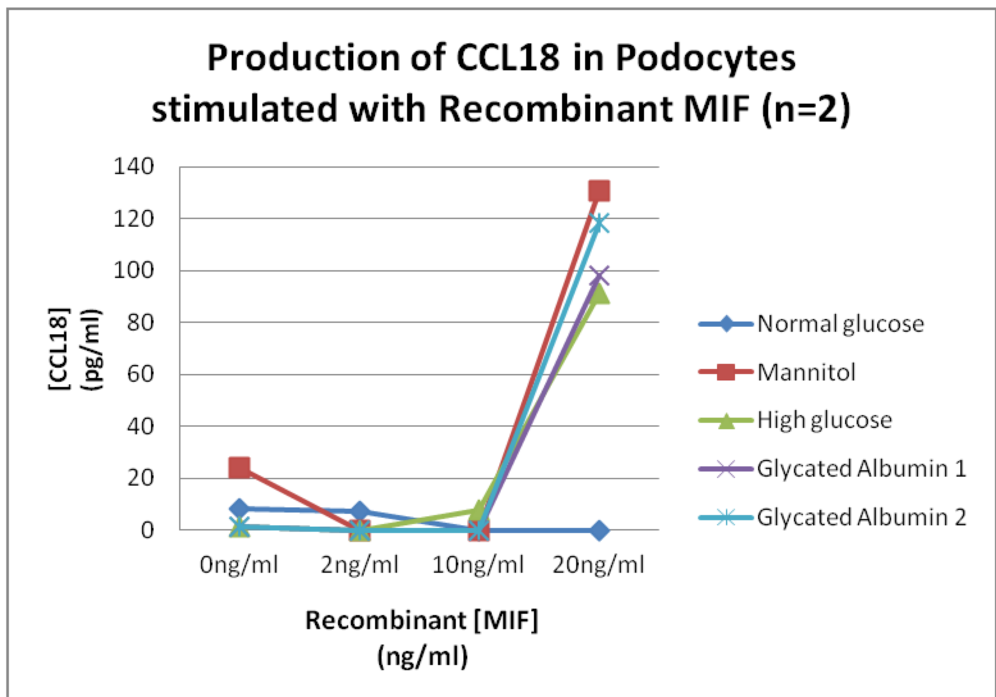

Figure A4F9 Production of CCL18 following stimulation with rMIF

Summary

The level of MIF measured in all podocyte conditions decreases following stimulation with increasing rMIF in diabetic conditions. MCP-1 production increases with 2 and 10ng/ml doses of rMIF and then decreases with high dose rMIF stimulation. Stimulation with high dose 20ng/ml rMIF results in a rise in CCL18 production.

4.2.2 Human Podocytes stimulated with rMCP-1 over 48 hours

All the following experiments (n=2) per condition and dose.

MCP-1 in Podocytes stimulated with rMCP-1

There is a sharp rise in the levels of MCP-1 following stimulation with increasing dosing of rMCP-1 on podocytes.

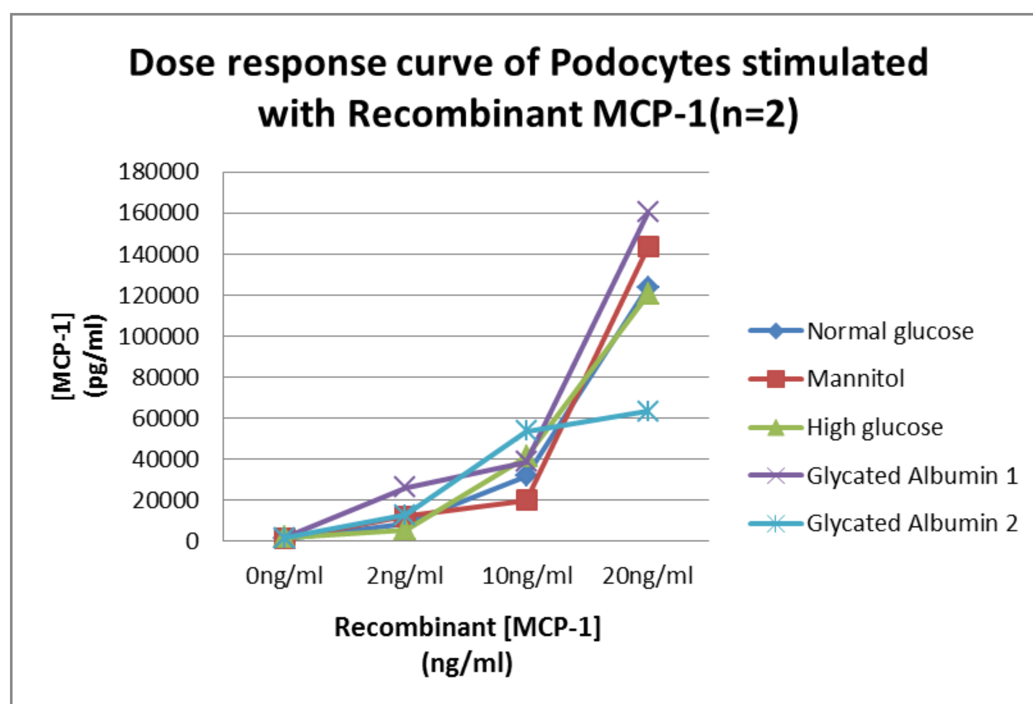

Figure A4F10 Dose response curve of rMCP-1 in Podocytes pilot data

MIF in Podocytes stimulated with rMCP-1

Stimulation of Podocytes with rMCP-1 causes a bimodel rise in MIF production with increasing dose following stimulation with diabetic conditions. The basal production of MIF in podocytes is lower in diabetic conditions that increases MIF with the higher dose of rMCP-1. Normal glucose maintains basal levels of MIF irrespective of increasing rMCP-1 dosing.

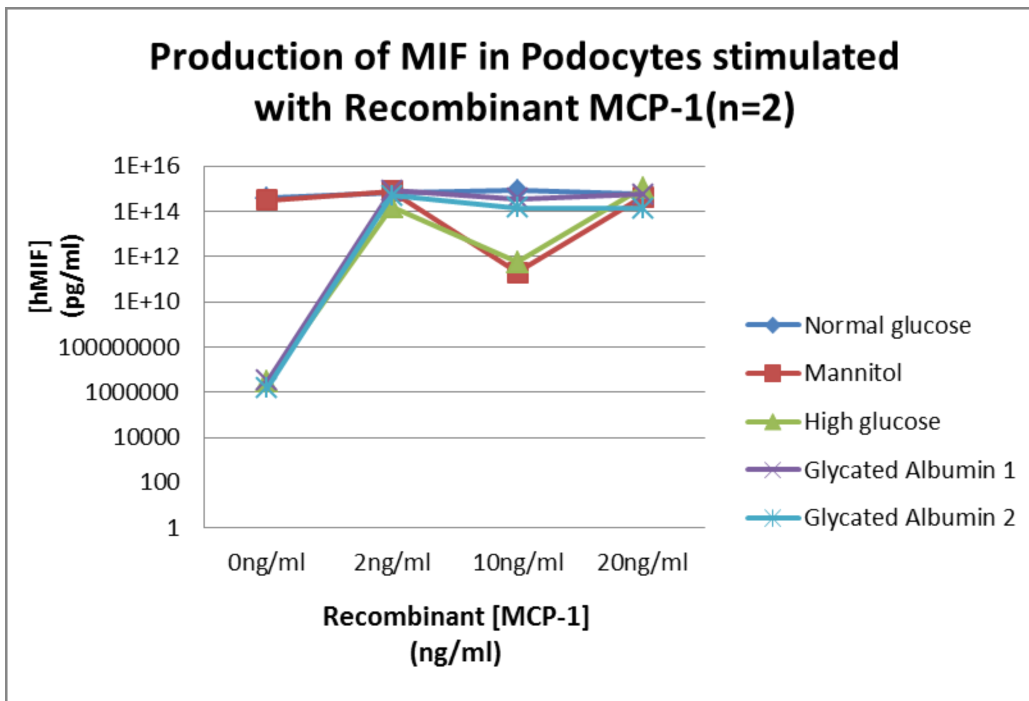

Figure A4F11 Production of MIF following stimulation with rMCP-1 in Podocytes pilot data

<u>*CCL18 in Podocytes stimulated with rMCP-1*</u>

There was no production of CCL18 in Podocytes stimulated with rMCP-1 as the levels were below the sensitivity of the standard curve for the CCL18 ELISA.

Summary

MIF is detected in large quantities in podocytes exposed to diabetic conditions following 2ng/ml and 20ng/ml rMCP-1. This experiment would need to be repeated to determine whether this bimodal distribution continues to be present. There are no detectable levels of CCL18 following rMCP-1 stimulation.

4.3.0 Summary of Pilot cell culture data

The effects of cytokine interactions seen in all conditions are shown below in Table A4T1. These are initial findings that would need to be repeated to see whether these findings are statistically significant.

Table A4T1 Summary of pilot cell culture data

Cell Type	***Effect***
tHMC	*MIF and MCP-1 are produced following basal stimulation* *MIF inhibits MCP-1* *MCP-1 stimulates MIF at low dose* *High dose CCL18 stimulates MIF production*
HK-2 cells	*MIF produced in all basal conditions* *MIF does not stimulate the production of CCL18 or MCP-1* *MCP-1 slightly increases MIF levels* *CCL18 inhibits MIF*
Podocytes	*MIF and MCP-1 are produced following basal stimulation* *Increasing MIF inhibits itself production in diabetic conditions* *Increasing MIF increases then decreases levels of MCP-1* *MIF increases CCL18* *MCP-1 has a bimodal effect on MIF that is dose dependent* *CCL18 inhibits MIF in diabetic conditions* *CCL18 inhibits MCP-1 in all conditions*

HK2 cells stimulated with CCL18 and MCP-1 were repeated to determine whether the above interactions were reproducible. Podocytes stimulated with MIF and MCP-1 were also repeated to ensure the above findings continued to be seen. Cell cultures were stimulated with basal conditions and cytokine stimulation with 10ng/ml and 20ng/ml. In view of time limitations 0ng/ml and 20ng/ml were analysed and are herein presented.

4.4.0 Formal HK2 cell culture experiments - Effects of rCCL18 on MIF

The tables below are similar findings to that of Experiment 1, presented in the thesis

Table A4T2 MIF following rCCL18 in HK2 Experiment 2

Recombinant CCL18	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	27200 (14300-52700) vs 28600 (16200-46100)	ns
0ng/ml	M vs H	15200 (5860-29600) vs 17300 (9880-	ns

		38700)	
20ng/ml	N vs A	57900 (27600-76900) vs 12600 (2130-29300)	p<0.01
20ng/ml	M vs H	22600 (17200-34800) vs 23300 (16900-32000)	ns
CCL18 stimulation	N20 vs N0	57900 (27600-76900) vs 27200 (14300-52700)	ns
CCL18 stimulation	A20 vs A0	12600 (2130-29300) vs 28600 (16200-46100)	ns
CCL18 stimulation	M20 vs M0	22600 (17200-34800) vs 15200 (5860-29600)	ns
CCL18 stimulation	H20 vs H0	23300 (16900-32000) vs 17300 (9880-38700)	ns

Table A4T3 MIF following rCCL18 in HK2 Experiment 3

Recombinant CCL18	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	22600 (15300-32600) vs 15300 (12900-20300)	ns
0ng/ml	M vs H	16600 (13000-25700) vs 11300 (3380-20300)	ns
20ng/ml	N vs A	26100 (12200-27800) vs 24400 (21000-32100)	ns
20ng/ml	M vs H	16400 (8700-29000) vs 13800 (12000-20700)	ns
CCL18 stimulation	N20 vs N0	26100 (12200-27800) vs 22600 (15300-32600)	ns
CCL18 stimulation	A20 vs A0	24400 (21000-32100) vs 15300 (12900-20300)	ns
CCL18 stimulation	M20 vs M0	16400 (8700-29000) vs 16600 (13000-25700)	ns
CCL18 stimulation	H20 vs H0	13800 (12000-20700) vs 11300 (3380-20300)	ns

4.4.1 Effects of rCCL18 on MCP-1 in HK2 cells

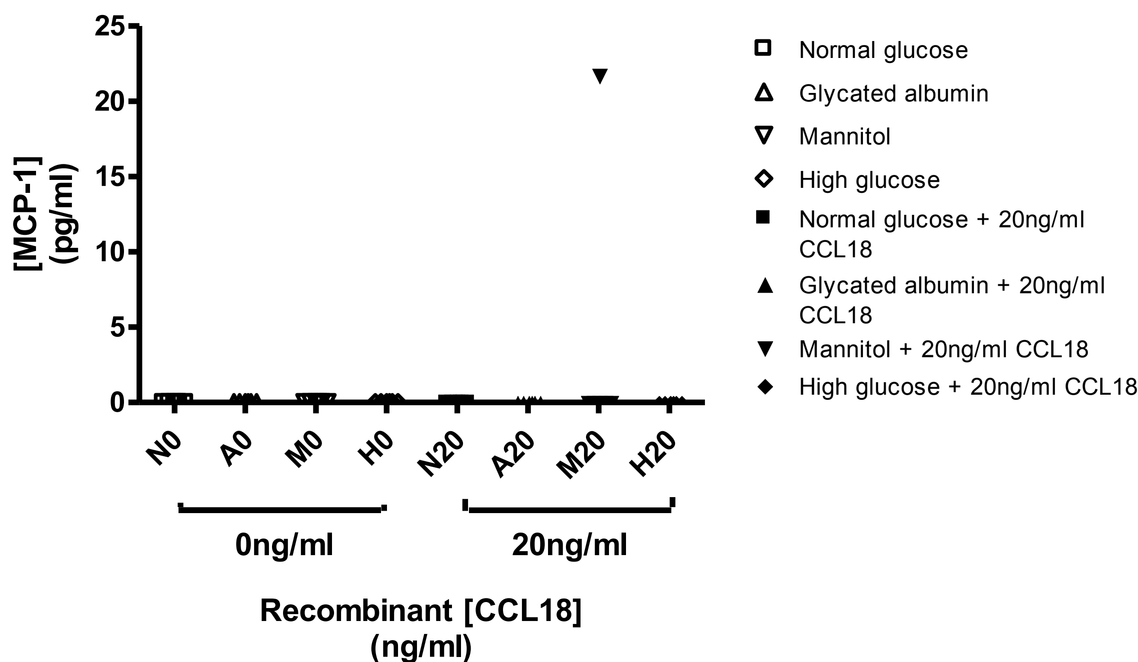

Figure A4F12 Production of MCP-1 following stimulation with rCCL18 in HK2 cells
Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose, 0=0ng/ml of CCL18 stimulation, 20=20ng/ml of CCL18 stimulation.
^ depicts the level of significance between basal conditions and their controls ie. Normal glucose concentration (N0) compared with glycated albumin (A0). The osmotic control Mannitol (M0) compared with high glucose (H0). ^ p<0.05, ^^ p<0.01, ^^^ p<0.001
**depicts the level of significance between between the same basal condition with or without cytokine stimulation ie. Normal glucose with 0ng/ml of CCL18 (N0) with Normal glucose with 20ng/ml of CCL18 (N20). *p<0.05, **p<0.01, ***p<0.001*
#depicts the level of significance between CCL18 stimulated conditions and their controls ie. Normal glucose with 20ng/ml of CCL18 (N20) compared with glycated albumin with 20ng/ml of CCL18 (A20). #p<0.05, ##p<0.01, ###p<0.001

Table A4T4 The amount of MCP-1 found in HK2 cells without and with rCCL18. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant CCL18	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns

20ng/ml	N vs A	0 vs 0	ns
20ng/ml	M vs H	0 (0-21.74) vs 0	ns
CCL18 stimulation	N20 vs N0	0 vs 0	ns
CCL18 stimulation	A20 vs A0	0 vs 0	ns
CCL18 stimulation	M20 vs M0	0 (0-21.74) vs 0	ns
CCL18 stimulation	H20 vs H0	0 vs 0	ns

Table A4T5 MCP-1 following rCCL18 in HK2 Experiment 2

Recombinant CCL18	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	0 (0-13.1) vs 0	p<0.05
20ng/ml	M vs H	0 vs 0	ns
CCL18 stimulation	N20 vs N0	0 (0-13.1) vs 0	p<0.05
CCL18 stimulation	A20 vs A0	0 vs 0	ns
CCL18 stimulation	M20 vs M0	0 vs 0	ns
CCL18 stimulation	H20 vs H0	0 vs 0	ns

Table A4T6 MCP-1 following rCCL18 in HK2 Experiment 3

Recombinant CCL18	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 (0-30.5) vs 0	ns
0ng/ml	M vs H	0 (0-26) vs 0 (0-58.2)	ns
20ng/ml	N vs A	0 vs 0	ns
20ng/ml	M vs H	0 vs 0	ns
CCL18 stimulation	N20 vs N0	0 vs 0 (0-30.5)	ns
CCL18 stimulation	A20 vs A0	0 vs 0	ns

CCL18 stimulation	M20 vs M0	0 vs 0 (0-26)	ns
CCL18 stimulation	H20 vs H0	0 vs 0 (0-58.2)	ns

4.4.2 Effects of rCCL18 on Fibronectin in HK2 cells

The repeated experiments are shown below

Table A4T7 Fibronectin following rCCL18 in HK2 Experiment 2

Recombinant CCL18	**Basal condition**	**Median (range) (µg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	5380 (3570-9380) vs 7560 (7120-16100)	ns
0ng/ml	M vs H	4610 (2830-6690) vs 3940 (3150-10800)	ns
20ng/ml	N vs A	7460 (3130-15200) vs 10200 (8690-21100)	ns
20ng/ml	M vs H	3780 (3350-5830) vs 7010 (773-15500)	ns
CCL18 stimulation	N20 vs N0	7460 (3130-15200) vs 5380 (3570-9380)	ns
CCL18 stimulation	A20 vs A0	10200 (8690-21100) vs 7560 (7120-16100)	ns
CCL18 stimulation	M20 vs M0	3780 (3350-5830) vs 4610 (2830-6690)	ns
CCL18 stimulation	H20 vs H0	7010 (773-15500) vs 3940 (3150-10800)	ns

Table A4T8 Fibronectin following rCCL18 in HK2 Experiment 3

Recombinant CCL18	**Basal condition**	**Median (range) (µg/ml)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	10700 (9010-15700) vs 9310 (8580-12300)	ns
0ng/ml	M vs H	8830 (7050-10700) vs 14500 (8780-22800)	ns
20ng/ml	N vs A	3860 (2380-38100) vs 3200 (2780-39900)	ns
20ng/ml	M vs H	2790 (1970-4790) vs 20200 (3350-39000)	ns

CCL18 stimulation	N20 vs N0	3860 (2380-38100) vs 10700 (9010-15700)	ns
CCL18 stimulation	A20 vs A0	3200 (2780-39900) vs 9310 (8580-12300)	ns
CCL18 stimulation	M20 vs M0	2790 (1970-4790) vs 8830 (7050-10700)	ns
CCL18 stimulation	H20 vs H0	20200 (3350-39000) vs 14500 (8780-22800)	ns

4.5.0 Stimulated with rMCP-1 - MTT Assay

Experiment 1 is present in thesis

Table A4T9 MTT Assay HK2 cell Experiment 2 and 3, stimulated with 0ng/ml or 20ng/ml with rMCP-1

Condition with 0ng/ml or 20ng/ml Recombinant MCP-1	**MTT Assay HK2 Expt 2 Median (range) OD**	**MTT Assay HK2 Expt 3 Median (range) OD**
N0	0.59 (0.54-0.60)	0.49 (0.47-0.61)
A0	0.27 (0.24-0.28)	0.16 (0.15-0.19)
M0	0.50 (0.45-0.51)	0.53 (0.37-0.59)
H0	0.63 (0.58-0.67)	0.63 (0.57-0.68)
N20	0.42 (0.42-0.44)	0.45 (0.40-0.58)
A20	0.33 (0.32-0.41)	0.33 (0.31-0.35)
M20	0.39 (0.34-0.39)	0.38 (0.37-0.44)
H20	0.66 (0.55-0.77)	0.60 (0.56-0.78)

4.5.1 Effects of rMCP-1 on MCP-1 in HK2 cells

Table A4T10 MCP-1 following rMCP-1 in HK2 Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	1190 (10300-14200) vs 51900 (29400-70800)	ns
20ng/ml	M vs H	14300 (9810-14900) vs 32400 (21800-96400)	ns

MCP-1 stimulation	N20 vs N0	1190 (10300-14200) vs 0	ns
MCP-1 stimulation	A20 vs A0	51900 (29400-70800) vs 0	p<0.001
MCP-1 stimulation	M20 vs M0	14300 (9810-14900) vs 0	ns
MCP-1 stimulation	H20 vs H0	32400 (21800-96400) vs 0	p<0.01

Table A4T11 MCP-1 following rMCP-1 in HK2 Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 (0-30.5) vs 0	ns
0ng/ml	M vs H	0 (0-26) vs 0 (0-58.2)	ns
20ng/ml	N vs A	17600 (15400-19900) vs 13400 (11500-16300)	ns
20ng/ml	M vs H	24800 (18200-25400) vs 20600 (16200-24700)	ns
MCP-1 stimulation	N20 vs N0	17600 (15400-19900) vs 0 (0-30.5)	ns
MCP-1 stimulation	A20 vs A0	13400 (11500-16300) vs 0	ns
MCP-1 stimulation	M20 vs M0	24800 (18200-25400) vs 0 (0-26)	p<0.01
MCP-1 stimulation	H20 vs H0	20600 (16200-24700) vs 0 (0-58.2)	p<0.01

4.5.2 Effects of rMCP-1 on MIF in HK2 cells

Table A4T12 The amount of MIF found in HK2 cells without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	27200 (14300-52700) vs 28600 (16200-46100)	ns
0ng/ml	M vs H	15200 (5860-29600) vs 17300 (9880-38700)	ns
20ng/ml	N vs A	19600 (14500-28700) vs 21800 (15300-29600)	ns

20ng/ml	M vs H	16800 (9600-26300) vs 30400 (21800-41300)	ns
MCP-1 stimulation	N20 vs N0	19600 (14500-28700)- 27200 (14300-52700)	ns
MCP-1 stimulation	A20 vs A0	21800 (15300-29600)- 28600 (16200-46100)	ns
MCP-1 stimulation	M20 vs M0	16800 (9600-26300)- 15200 (5860-29600)	ns
MCP-1 stimulation	H20 vs H0	30400 (21800-41300)- 17300 (9880-38700)	ns

Table A4T13 MIF following rMCP-1 in HK2 Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	27000 (14700-48300) vs 7710 (4650-11000)	p<0.01
0ng/ml	M vs H	14600 (4910-37800) vs 31900 (17200-51800)	ns
20ng/ml	N vs A	17700 (8880-25400) vs 25000 (17700-36700)	ns
20ng/ml	M vs H	14500 (10500-18300) vs 21000 (11800-46200)	ns
MCP-1 stimulation	N20 vs N0	17700 (8880-25400) vs 27000 (14700-48300)	ns
MCP-1 stimulation	A20 vs A0	25000 (17700-36700) vs 7710 (4650-11000)	p<0.01
MCP-1 stimulation	M20 vs M0	14500 (10500-18300) vs 14600 (4910-37800)	ns
MCP-1 stimulation	H20 vs H0	21000 (11800-46200) vs 31900 (17200-51800)	ns

Table A4T14 MIF following rMCP-1 in HK2 Experiment 3

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	22600 (15300-32600) vs 15300 (12900-20300)	ns
0ng/ml	M vs H	16600 (13000-25700) vs 11300 (3380-20300)	ns
20ng/ml	N vs A	18000 (6030-43200) vs 23900 (21000-43500)	ns
20ng/ml	M vs H	15200 (9280-32100) vs 17700 (9820-40200)	ns
MCP-1 stimulation	N20 vs N0	18000 (6030-43200) vs 22600 (15300-32600)	ns
MCP-1 stimulation	A20 vs A0	23900 (21000-43500) vs 15300 (12900-20300)	ns
MCP-1 stimulation	M20 vs M0	15200 (9280-32100) vs 16600 (13000-25700)	ns
MCP-1 stimulation	H20 vs H0	17700 (9820-40200) vs 11300 (3380-20300)	ns

4.5.3 Effects of rMCP-1 on CCL18 in HK2 cells

Table A4T15 The amount of CCL18 found in HK2 cells without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 (0-18) vs 9.32 (0-163)	ns
0ng/ml	M vs H	0 (0-19) vs 0	ns
20ng/ml	N vs A	0 (0-17.7) vs 0 (0-78.5)	ns
20ng/ml	M vs H	0 vs 0 (0-74.4)	ns
MCP-1 stimulation	N20 vs N0	0 (0-17.7) - 0 (0-18)	ns
MCP-1 stimulation	A20 vs A0	0 (0-78.5) - 9.32 (0-163)	ns
MCP-1 stimulation	M20 vs M0	0 - 0 (0-19)	ns
MCP-1 stimulation	H20 vs H0	0 (0-74.4) – 0	ns

Table A4T16 CCL18 following rMCP-1 in HK2 Experiment 2

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 vs 0 (0-26.4)	ns
0ng/ml	M vs H	56.1 (13.7-129) vs 0 (0-39.5)	p<0.01
20ng/ml	N vs A	0 vs 0 (0-16.5)	ns
20ng/ml	M vs H	0 vs 0	ns
MCP-1 stimulation	N20 vs N0	0 vs 0	ns
MCP-1 stimulation	A20 vs A0	0 (0-16.5) vs 0 (0-26.4)	ns
MCP-1 stimulation	M20 vs M0	0 vs 56.1 (13.7-129)	p<0.001
MCP-1 stimulation	H20 vs H0	0 vs 0	ns

Table A4T17 CCL18 following rMCP-1 in HK2 Experiment 3

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 vs 38.3 (0-138)	p<0.01
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	6.98 (0-46.8) vs 22.9 (0-233)	ns
20ng/ml	M vs H	0 (0-16.2) vs 0 (0-10)	ns
MCP-1 stimulation	N20 vs N0	6.98 (0-46.8) vs 0	ns
MCP-1 stimulation	A20 vs A0	22.9 (0-233) vs 38.3 (0-138)	ns
MCP-1 stimulation	M20 vs M0	0 (0-16.2) vs 0	ns
MCP-1 stimulation	H20 vs H0	0 (0-10) vs 0	ns

4.5.4 Effects of rMCP-1 on Fibronectin production in HK2 cells

Table A4T18 The amount of Fibronectin found in HK2 cells without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	5380 (3570-9380) vs 7560 (7120-16100)	ns
0ng/ml	M vs H	4610 (2830-6690) vs 3940 (3150-10800)	ns
20ng/ml	N vs A	5880 (3410-8870) vs 10300 (5940-11700)	ns
20ng/ml	M vs H	5900 (4440-11900) vs 4090 (2570-11200)	ns

MCP-1 stimulation	N20 vs N0	5880 (3410-8870)-5380 (3570-9380)	ns
MCP-1 stimulation	A20 vs A0	10300 (5940-11700)-7560 (7120-16100)	ns
MCP-1 stimulation	M20 vs M0	5900 (4440-11900)-4610 (2830-6690)	ns
MCP-1 stimulation	H20 vs H0	4090 (2570-11200)-3940 (3150-10800)	ns

Table A4T19 Fibronectin following rMCP-1 in HK2 Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	6810 (6110-8210) vs 7980 (6000-10400)	ns
0ng/ml	M vs H	4880 (2970-5400) vs 6950 (3410-11200)	ns
20ng/ml	N vs A	7600 (4600-16800) vs 11600 (8560-18200)	ns
20ng/ml	M vs H	3430 (2770-5740) vs 4650 (3060-9200)	ns
MCP-1 stimulation	N20 vs N0	7600 (4600-16800) vs 6810 (6110-8210)	ns
MCP-1 stimulation	A20 vs A0	11600 (8560-18200) vs 7980 (6000-10400)	ns
MCP-1 stimulation	M20 vs M0	3430 (2770-5740) vs 4880 (2970-5400)	ns
MCP-1 stimulation	H20 vs H0	4650 (3060-9200) vs 6950 (3410-11200)	ns

Table A4T20 Fibronectin following rMCP-1 in HK2 Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	10700 (9010-15700) vs 9310 (8580-12300)	ns
0ng/ml	M vs H	8830 (7050-10700) vs 14500 (8780-22800)	ns
20ng/ml	N vs A	18700 (7920-33700) vs 3450 (2580-4890)	$p<0.01$
20ng/ml	M vs H	3220 (2490-3490) vs 10200 (6080-11400)	ns

MCP-1 stimulation	N20 vs N0	18700 (7920-33700) vs 10700 (9010-15700)	ns
MCP-1 stimulation	A20 vs A0	3450 (2580-4890) vs 9310 (8580-12300)	ns
MCP-1 stimulation	M20 vs M0	3220 (2490-3490) vs 8830 (7050-10700)	ns
MCP-1 stimulation	H20 vs H0	10200 (6080-11400) vs 14500 (8780-22800)	ns

4.6.0 Formal human Podocyte cell culture experiments - Stimulated with rMIF- MTT Assay

Table A4T21 MTT Assay Podocyte Experiment 2 and 3 0ng/ml or 20ng/ml with rMIF

Condition with 0ng/ml or 20ng/ml Recombinant MIF	**MTT Assay Podocyte Expt 2 Median (range) OD**	**MTT Assay Podocyte Expt 3 Median (range) OD**
N0	0.23 (0.15-0.29)	0.08 (0.08-0.09)
A0	0.33 (0.28-0.34)	0.12 (0.12-0.14)
M0	0.22 (0.17-0.24)	0.11 (0.11-0.19)
H0	0.29 (0.19-0.29)	0.11 (0.10-0.12)
N20	0.32 (0.25-0.34)	0.12 (0.10-0.20)
A20	0.33 (0.30-0.36)	0.11 (0.10-0.17)
M20	0.27 (0.27-0.35)	0.10 (0.10-0.16)
H20	0.44 (0.27-0.88)	0.14 (0.12-0.24)

4.6.1 Effects of diabetic milieu and rMIF on podocytes

Table A4T22 The amount of MIF found in podocytes without and with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	36800 (20000-56800) vs 43700 (37400-51900)	ns
0ng/ml	M vs H	17900 (17100-24800) vs 26800 (22200-50200)	ns
20ng/ml	N vs A	30000 (21000-	ns

		95300) vs 55300 (45800-90000)	
20ng/ml	M vs H	57100 (34400-113000) vs 49900 (36800-109000)	ns
MIF stimulation	N20 vs N0	30000 (21000-95300) - 36800 (20000-56800)	ns
MIF stimulation	A20 vs A0	55300 (45800-90000)- 43700 (37400-51900)	ns
MIF stimulation	M20 vs M0	57100 (34400-113000)- 17900 (17100-24800)	p<0.01
MIF stimulation	H20 vs H0	49900 (36800-109000) -26800 (22200-50200)	ns

Table A4T23 MIF following rMIF in Podocytes Experiment 2

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	12800 (7270-23400) vs 31100 (27900-41800)	ns
0ng/ml	M vs H	15200 (6960-36400) vs 21400 (13800-38100)	ns
20ng/ml	N vs A	45300 (33100-79900) vs 55400 (46800-64600)	ns
20ng/ml	M vs H	34500 (23100-43900) vs 24900 (15300-28000)	ns
MIF stimulation	N20 vs N0	45300 (33100-79900) vs 12800 (7270-23400)	p<0.001
MIF stimulation	A20 vs A0	55400 (46800-64600) vs 31100 (27900-41800)	ns
MIF stimulation	M20 vs M0	34500 (23100-43900) vs 15200 (6960-36400)	ns
MIF stimulation	H20 vs H0	24900 (15300-28000) vs 21400 (13800-38100)	ns

Table A4T24 MIF following rMIF in Podocytes Experiment 3

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	28600 (4750-52900) vs 32400 (24600-57300)	ns
0ng/ml	M vs H	32400 (21200-45900) vs 17200 (12200-22100)	ns
20ng/ml	N vs A	50700 (24200-162000) vs 65800 (40200-148000)	ns
20ng/ml	M vs H	41700 (19300-97500) vs 79400 (30400-172000)	ns
MIF stimulation	N20 vs N0	50700 (24200-162000) vs 28600 (4750-52900)	ns
MIF stimulation	A20 vs A0	65800 (40200-148000) vs 32400 (24600-57300)	ns
MIF stimulation	M20 vs M0	41700 (19300-97500) vs 32400 (21200-45900)	ns
MIF stimulation	H20 vs H0	79400 (30400-172000) vs 17200 (12200-22100)	p<0.001

4.6.2 Effects of rMIF on MCP-1 in Podocytes

Table A4T25 MCP-1 following rMIF in Podocytes Experiment 2

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	357 (328-1150) vs 334 (222-644)	ns
0ng/ml	M vs H	470 (357-595) vs 321 (204-449)	ns
20ng/ml	N vs A	1170 (1100-1540) vs 868 (794-942)	ns
20ng/ml	M vs H	1270 (842-1580) vs 1840 (1480-2100)	ns
MIF stimulation	N20 vs N0	1170 (1100-1540) vs 357 (328-1150)	ns
MIF stimulation	A20 vs A0	868 (794-942) vs 334 (222-644)	ns

MIF stimulation	M20 vs M0	1270 (842-1580) vs 470 (357-595)	ns
MIF stimulation	H20 vs H0	1840 (1480-2100) vs 321 (204-449)	p<0.001

Table A4T26 MCP-1 following rMIF in Podocytes Experiment 3

Recombinant MIF	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	493 (346-4990) vs 529 (410-661)	ns
0ng/ml	M vs H	757 (545-4050) vs 471 (426-534)	ns
20ng/ml	N vs A	319 (246-421) vs 434 (352-580)	ns
20ng/ml	M vs H	329 (264-407) vs 371 (314-436)	ns
MIF stimulation	N20 vs N0	319 (246-421) vs 493 (346-4990)	p<0.05
MIF stimulation	A20 vs A0	434 (352-580) vs 529 (410-661)	ns
MIF stimulation	M20 vs M0	329 (264-407) vs 757 (545-4050)	p<0.001
MIF stimulation	H20 vs H0	371 (314-436) vs 471 (426-534)	ns

4.6.3 Effects of rMIF on CCL18 in Podocytes

Table A4T27 The amount of CCL18 found in podocytes without and with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MIF	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 vs 0 (0-12.65)	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	0 vs 0 (0-33.28)	ns
20ng/ml	M vs H	0 vs 0 (0-31)	ns
MIF stimulation	N20 vs N0	0 vs 0	ns
MIF stimulation	A20 vs A0	0 (0-33.28) vs 0 (0-12.65)	ns
MIF stimulation	M20 vs M0	0 vs 0	ns
MIF stimulation	H20 vs H0	0 (0-31) vs 0	ns

Table A4T28 CCL18 following rMIF in Podocytes Experiment 2

Recombinant MIF	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	0 vs 0 (0-150)	ns
20ng/ml	M vs H	0 vs 0	ns
MIF stimulation	N20 vs N0	0 vs 0	ns
MIF stimulation	A20 vs A0	0 (0-150) vs 0	ns
MIF stimulation	M20 vs M0	0 vs 0	ns
MIF stimulation	H20 vs H0	0 vs 0	ns

Table A4T29 CCL18 following rMIF in Podocytes Experiment 3

Recombinant MIF	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	0 (0-49.1) vs 0	ns
0ng/ml	M vs H	0 (0-19.2) vs 0	ns
20ng/ml	N vs A	0 vs 0	ns
20ng/ml	M vs H	0 vs 0	ns
MIF stimulation	N20 vs N0	0 vs 0 (0-49.1)	ns
MIF stimulation	A20 vs A0	0 vs 0	ns
MIF stimulation	M20 vs M0	0 vs 0 (0-19.2)	ns
MIF stimulation	H20 vs H0	0 vs 0	ns

4.6.4 Effects of rMIF on Fibronectin in Podocytes

Table A4T30 The amount of Fibronectin found in podocytes without and with rMIF. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MIF	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	10800 (5310-39900) vs 30400 (17300-38100)	ns
0ng/ml	M vs H	10300 (7050-17500) vs 29800 (10000-36400)	ns
20ng/ml	N vs A	10700 (8520-13200) vs 7070 (5620-10900)	ns
20ng/ml	M vs H	6900 (6560-9930) vs 10200 (7540-11900)	ns
MIF stimulation	N20 vs N0	10700 (8520-13200)-10800 (5310-39900)	ns

MIF stimulation	A20 vs A0	7070 (5620-10900)-30400 (17300-38100)	p<0.001
MIF stimulation	M20 vs M0	6900 (6560-9930)-10300 (7050-17500)	ns
MIF stimulation	H20 vs H0	10200 (7540-11900)-29800 (10000-36400)	ns

Table A4T31 Fibronectin following rMIF in Podocytes Experiment 2

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	5880 (4440-14800) vs 6460 (5980-10500)	ns
0ng/ml	M vs H	6820 (3280-10100) vs 59200 (42100-67900)	ns
20ng/ml	N vs A	712 (457-1360) vs 960 (507-1310)	ns
20ng/ml	M vs H	639 (393-1980) vs 754 (551-803)	ns
MIF stimulation	N20 vs N0	712 (457-1360) vs 5880 (4440-14800)	ns
MIF stimulation	A20 vs A0	960 (507-1310) vs 6460 (5980-10500)	ns
MIF stimulation	M20 vs M0	639 (393-1980) vs 6820 (3280-10100)	ns
MIF stimulation	H20 vs H0	754 (551-803) vs 59200 (42100-67900)	p<0.001

Table A4T32 Fibronectin following rMIF in Podocytes Experiment 3

Recombinant MIF	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	1520 (906-1940) vs 8450 (7340-11200)	p<0.001
0ng/ml	M vs H	2190 (336-3960) vs 3220 (1030-8310)	ns
20ng/ml	N vs A	4140 (1910-5550) vs 10100 (6950-17400)	ns
20ng/ml	M vs H	2860 (1960-3860) vs 7160 (2790-10400)	ns
MIF stimulation	N20 vs N0	4140 (1910-5550) vs 1520 (906-1940)	ns
MIF stimulation	A20 vs A0	10100 (6950-17400)	ns

		vs 8450 (7340-11200)	
MIF stimulation	M20 vs M0	2860 (1960-3860) vs 2190 (336-3960)	ns
MIF stimulation	H20 vs H0	7160 (2790-10400) vs 3220 (1030-8310)	ns

4.7.0 Stimulated of Podocytes with rMCP-1 - MTT Assay

Table A4T33 MTT Assay Podocyte Experiment 2 and 3 0ng/ml or 20ng/ml with rMCP-1

Condition with 0ng/ml or 20ng/ml Recombinant MCP-1	MTT Assay Podocyte Expt 2 Median (range) OD	MTT Assay Podocyte Expt 3 Median (range) OD
N0	0.24 (0.17-0.40)	0.10 (0.09-0.13)
A0	0.25 (0.16-0.27)	0.13 (0.12-0.15)
M0	0.29 (0.23-0.41)	0.13 (0.12-0.13)
H0	0.23 (0.22-0.27)	0.12 (0.10-0.13)
N20	0.36 (0.35-0.44)	0.11 (0.10-0.17)
A20	0.28 (0.25-0.41)	0.15 (0.15-0.18)
M20	0.32 (0.26-0.38)	0.21 (0.18-0.21)
H20	0.31 (0.30-0.35)	0.13 (0.13-0.16)

4.7.1 Effects of rMCP-1 on MCP-1 in Podocytes

Table A4T34 The amount of MCP-1 found in podocytes without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	Basal condition	Median (range)	Dunn's Multiple comparison p value
0ng/ml	N vs A	294 (213-377) vs 379 (356-476)	ns
0ng/ml	M vs H	316 (203-416) vs 284 (250-384)	ns
20ng/ml	N vs A	20400 (16200-47300) vs 39000 (32500-42100)	ns
20ng/ml	M vs H	31600 (28100-64400) vs 26600 (23300-39400)	ns
MCP-1 stimulation	N20 vs N0	20400 (16200-47300)- 294 (213-377)	p<0.05

MCP-1 stimulation	A20 vs A0	39000 (32500-42100)- 379 (356-476)	ns
MCP-1 stimulation	M20 vs M0	31600 (28100-64400)- 316 (203-416)	p<0.01
MCP-1 stimulation	H20 vs H0	26600 (23300-39400)- 284 (250-384)	p<0.05

Table A4T35 MCP-1 following rMCP-1 in Podocytes Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	357 (328-1150) vs 334 (222-644)	ns
0ng/ml	M vs H	470 (357-595) vs 321 (204-449)	ns
20ng/ml	N vs A	24100 (17200-26400) vs 23800 (22200-28200)	ns
20ng/ml	M vs H	35200 (22200-42700) vs 31100 (28600-35600)	ns
MCP-1 stimulation	N20 vs N0	24100 (17200-26400) vs 357 (328-1150)	ns
MCP-1 stimulation	A20 vs A0	23800 (22200-28200) vs 334 (222-644)	p<0.05
MCP-1 stimulation	M20 vs M0	35200 (22200-42700) vs 470 (357-595)	p<0.05
MCP-1 stimulation	H20 vs H0	31100 (28600-35600) vs 321 (204-449)	p<0.001

Table A4T36 MCP-1 following rMCP-1 in Podocytes Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	493 (346-4990) vs 529 (410-661)	ns
0ng/ml	M vs H	757 (545-4050) vs 471 (426-534)	ns
20ng/ml	N vs A	11100 (9040-15200) vs 18900 (16300-2300)	ns

20ng/ml	M vs H	15700 (12600-18600) vs 13200 (7860-18800)	ns
MCP-1 stimulation	N20 vs N0	11100 (9040-15200) vs 493 (346-4990)	ns
MCP-1 stimulation	A20 vs A0	18900 (16300-2300) vs 529 (410-661)	p<0.001
MCP-1 stimulation	M20 vs M0	15700 (12600-18600) vs 757 (545-4050)	ns
MCP-1 stimulation	H20 vs H0	13200 (7860-18800) vs 471 (426-534)	p<0.01

4.7.2 Effects of rMCP-1 on MIF in Podocytes

Table A4T37 The amount of MIF found in podocytes without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	36800 (20000-56800) vs 43700 (37400-51900)	ns
0ng/ml	M vs H	17900 (17100-24800) vs 26800 (22200-50200)	ns
20ng/ml	N vs A	54600 (29700-67400) vs 92900 (65400-126000)	ns
20ng/ml	M vs H	42200 (28400-52900) vs 53300 (45200-71400)	ns
MCP-1 stimulation	N20 vs N0	54600 (29700-67400)- 36800 (20000-56800)	ns
MCP-1 stimulation	A20 vs A0	92900 (65400-126000)- 43700 (37400-51900)	ns
MCP-1 stimulation	M20 vs M0	42200 (28400-52900)- 17900 (17100-24800)	ns
MCP-1 stimulation	H20 vs H0	53300 (45200-71400)- 26800 (22200-50200)	ns

Table A4T38 MIF following rMCP-1 in Podocytes Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	12800 (7270-23400) vs 31100 (27900-41800)	ns
0ng/ml	M vs H	15200 (6960-36400) vs 21400 (13800-38100)	ns
20ng/ml	N vs A	35500 (32300-44600) vs 89400 (78800-112000)	ns
20ng/ml	M vs H	34500 (28200-43600) vs 68900 (36600-85500)	ns
MCP-1 stimulation	N20 vs N0	35500 (32300-44600) vs 12800 (7270-23400)	p<0.05
MCP-1 stimulation	A20 vs A0	89400 (78800-112000) vs 31100 (27900-41800)	p<0.05
MCP-1 stimulation	M20 vs M0	34500 (28200-43600) vs 15200 (6960-36400)	ns
MCP-1 stimulation	H20 vs H0	68900 (36600-85500) vs 21400 (13800-38100)	p<0.05

Table A4T39 MIF following rMCP-1 in Podocytes Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	28600 (4750-52900) vs 32400 (24600-57300)	ns
0ng/ml	M vs H	32400 (21200-45900) vs 17200 (12200-22100)	ns
20ng/ml	N vs A	63100 (40700-87200) vs 75600 (42900-133000)	ns
20ng/ml	M vs H	54900 (38800-91200) vs 39600 (27800-67500)	ns
MCP-1 stimulation	N20 vs N0	63100 (40700-87200) vs 28600	ns

		(4750-52900)	
MCP-1 stimulation	A20 vs A0	75600 (42900-133000) vs 32400 (24600-57300)	ns
MCP-1 stimulation	M20 vs M0	54900 (38800-91200) vs 32400 (21200-45900)	ns
MCP-1 stimulation	H20 vs H0	39600 (27800-67500) vs 17200 (12200-22100)	ns

4.7.3 Effects of rMCP-1 on CCL18 in Podocytes

Table A4T40 The amount of CCL18 found in podocytes without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	0 vs 0 (0-149.7)	ns
20ng/ml	M vs H	0 vs 0	ns
MCP-1 stimulation	N20 vs N0	0 – 0	ns
MCP-1 stimulation	A20 vs A0	0 (0-149.7) – 0	ns
MCP-1 stimulation	M20 vs M0	0 – 0	ns
MCP-1 stimulation	H20 vs H0	0 – 0	ns

Table A4T41 CCL18 following rMCP-1 in Podocytes Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	0 vs 0	ns
0ng/ml	M vs H	0 vs 0	ns
20ng/ml	N vs A	0 vs 0	ns
20ng/ml	M vs H	0 vs 0	ns
MCP-1 stimulation	N20 vs N0	0 vs 0	ns
MCP-1 stimulation	A20 vs A0	0 vs 0	ns
MCP-1 stimulation	M20 vs M0	0 vs 0	ns
MCP-1 stimulation	H20 vs H0	0 vs 0	ns

Table A4T42 CCL18 following rMCP-1 in Podocytes Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**

0ng/ml	N vs A	0 (0-49.1) vs 0	ns
0ng/ml	M vs H	0 vs 0 (0-19.2)	ns
20ng/ml	N vs A	0 vs 0 (0-35.1)	ns
20ng/ml	M vs H	0 (0-32.5) vs 0 (0-99.2)	ns
MCP-1 stimulation	N20 vs N0	0 vs 0 (0-49.1)	ns
MCP-1 stimulation	A20 vs A0	0 (0-35.1) vs 0	ns
MCP-1 stimulation	M20 vs M0	0 (0-32.5) vs 0	ns
MCP-1 stimulation	H20 vs H0	0 (0-99.2) vs 0 (0-19.2)	ns

4.7.4 Effects of rMCP-1 on Fibronectin in Podocytes

Table A4T43 The amount of Fibronectin found in podocytes without and with rMCP-1. Key; N=Normal glucose, A=glycated albumin, M=mannitol, H=High glucose

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	5880 (4440-14800) vs 6460 (5980-10500)	ns
0ng/ml	M vs H	6820 (3280-10100) vs 59200 (42100-67900)	p<0.01
20ng/ml	N vs A	39400 (23900-67900) vs 57200 (47900-69500)	ns
20ng/ml	M vs H	35700 (28700-73400) vs 49700 (40100-68100)	ns
MCP-1 stimulation	N20 vs N0	39400 (23900-67900)- 5880 (4440-14800)	ns
MCP-1 stimulation	A20 vs A0	57200 (47900-69500)- 6460 (5980-10500)	p<0.01
MCP-1 stimulation	M20 vs M0	35700 (28700-73400)- 6820 (3280-10100)	ns
MCP-1 stimulation	H20 vs H0	49700 (40100-68100)- 59200 (42100-67900)	ns

Table A4T44 Fibronectin following rMCP-1 in Podocytes Experiment 2

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**

0ng/ml	N vs A	10800 (5310-39900) vs 30400 (17300-38100)	ns
0ng/ml	M vs H	10300 (7050-17500) vs 29800 (10000-36400)	ns
20ng/ml	N vs A	12200 (8040-15600) vs 6540 (5280-9000)	ns
20ng/ml	M vs H	12600 (9350-15600) vs 10300 (6720-13900)	ns
MCP-1 stimulation	N20 vs N0	12200 (8040-15600) vs 10800 (5310-39900)	ns
MCP-1 stimulation	A20 vs A0	6540 (5280-9000) vs 30400 (17300-38100)	p<0.001
MCP-1 stimulation	M20 vs M0	12600 (9350-15600) vs 10300 (7050-17500)	ns
MCP-1 stimulation	H20 vs H0	10300 (6720-13900) vs 29800 (10000-36400)	ns

Table A4T45 Fibronectin following rMCP-1 in Podocytes Experiment 3

Recombinant MCP-1	**Basal condition**	**Median (range)**	**Dunn's Multiple comparison p value**
0ng/ml	N vs A	1520 (906-1940) vs 8450 (7340-11200)	p<0.01
0ng/ml	M vs H	2190 (336-3960) vs 3220 (1030-8310)	ns
20ng/ml	N vs A	5470 (2180-11700) vs 29300 (13500-63400)	ns
20ng/ml	M vs H	4550 (3910-6180) vs 8900 (4830-19200)	ns
MCP-1 stimulation	N20 vs N0	5470 (2180-11700) vs 1520 (906-1940)	ns
MCP-1 stimulation	A20 vs A0	29300 (13500-63400) vs 8450 (7340-11200)	ns
MCP-1 stimulation	M20 vs M0	4550 (3910-6180) vs 2190 (336-3960)	ns
MCP-1 stimulation	H20 vs H0	8900 (4830-19200) vs 3220 (1030-8310)	ns