The Role of Melanocortin 1 Receptor in Kidney Disease

Annika Lindskog Jonsson

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2012

Cover illustration: Melanocortin 1 receptor co-localizing with synaptopodin in a human glomerulus. Courtesy of Dr Kerstin Ebefors

The Role of Melanocortin 1 Receptor in Kidney Disease

annika.lindskogjonsson@wlab.gu.se

ISBN 978-91-628-8554-0
Abstract and summary sections of this thesis are available online:
http://hdl.handle.net/2077/30263

Printed in Gothenburg, Sweden 2012
Ale Tryckteam AB

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kunskapen om mekanismerna bakom kroniska njursjukdomar är bristfällig. Patienter behandlas med läkemedel som har ospecifika anti-inflammatoriska effekter vilket medför kraftiga biverkningar. I slutet av 1990-talet behandlades patienter med olika typer av kroniska njursjukdomar, där njuren läcker stora mängder protein till urinen, med adrenokortikotropt hormon (ACTH) i syfte att undersöka ACTHs eventuella blodfettssänkande effekter. Behandlingen visades även - helt oväntat - resultera i goda effekter på njursjukdomen med en dramatisk minskning av proteinläckage i urinen samt en förbättrad njurfunktion. Denna goda effekt hos njursjuka patienter har sedan dess kunnat upprepas och bekräftas i andra studier.

Målet med den här avhandlingen har varit att ta reda på mekanismerna bakom ACTHs goda behandlingseffekter mot nefrotiska sjukdomar. Hypotesen är att ACTH utövar sin effekt via en specifik receptor i njuren. Genuttrycket av alla ACTH receptorer, melanokortin receptorer (MCR) 1-5, undersöktes därför i njurvävnad. Genuttryck av MC1R, men inte andra MCR, påvisades i njure samt i cellerna som finns i njurens filtrationsbarriär mellan blod och urin (endotelceller, mesangiala celler och podocyter). MC1R-proteinet återfanns på samma ställe som en podocytmarkör och slutsatsen är därför att MC1R huvudsakligen sitter på podocyterna. Genuttrycket av MC1R uppreglerades starkt när cellerna utsattes för ämnen som framkallar nefrotiska syndrom.

Vidare fann vi att MCR-agonister hade god effekt i en experimentell modell för kronisk njursjukdom, som efterliknar den humana sjukdomen membranös nefropati. Behandling med selektiva MC1R-agonister minskade proteinläckaget i urin, förbättrade njurens morfologi samt minskade skada orsakad av oxidativ stress. Samma behandling var verkningslös i en annan experimentell modell, som efterliknar fokal segmentell glomeruloskleros hos människa. Detta tyder på att olika mekanismer ligger bakom olika njursjukdomar. När podocyter stimulerades med en selektiv MC1R-agonist, aktiverades flera kända signalvägar, vilket tyder på ett ökat försvar mot skador i cellen.

Sammanfattningsvis så har både gen-och proteinuttryck av en ACTH-receptor, MC1R, påvisats i njure. Selektiva agonister hade god effekt i modellen för membranös nefropati och kan vara ett framtida behandlingsalternativ för patienter med nefrotisk sjukdom, främst de med membranös nefropati.

ABSTRACT

Nephrotic syndrome is a term describing a group of poorly understood glomerular diseases that are responsible for a steadily increasing number of patients requiring active uremic care. Characteristic symptoms of nephrotic syndrome are proteinuria, hypoalbuminemia, hyperlipidemia and peripheral edema, and treatment of these symptoms, rather than their cause, is currently the only option available to the clinician. While the mechanisms underlying these diseases remain elusive, a number of studies have lately revisited adrenocorticotropic hormone (ACTH) as a potential treatment option since it has been shown to reduce proteinuria and improve glomerular function. Thus, the aim of this thesis has been to elucidate the mechanisms behind this treatment strategy.

The hypothesis is that ACTH mediates its effect by a kidney specific receptor. The gene expression of all ACTH receptors, melanocortin receptors (MCR) 1-5, was therefore investigated. MC1R gene expression was detected in kidney tissue, including cells specific for the glomerular filtration barrier (endothelial cells, podocytes and mesangial cells). MC1R protein was also detected and found to be co-localized with synaptopodin, a podocyte specific marker. In order to assess the relevance of MC1R in disease, selective agonists were used in experimental nephrotic models. MC1R agonists ameliorated the disease in a rat model resembling membranous nephropathy, and reduced proteinuria, improved morphology and reduced oxidative stress. MC1R agonists did not reduce proteinuria in a model resembling focal segmental glomerulosclerosis, suggesting different mechanistic pathways. Signaling pathways were investigated by stimulating podocytes with a selective MC1R agonist. Several known intracellular pathways were activated, including cAMP, phosphorylation of ERK1/2 and activation of catalase, an anti-oxidative enzyme. MC1R stimulation may also have a protective effect in nephrotoxin-induced rearrangement of the actin cytoskeleton.

In conclusion, this thesis has provided new data on the mechanisms behind the beneficial effects of ACTH treatment in nephrotic patients. MC1R, expressed in podocytes, likely mediates these effects. The results presented herein will pave the way for new, more specific and possibly curative treatment options, without severe side effects, for nephrotic patients.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Melanocortin 1 receptor agonists reduce proteinuria

Lindskog A, Ebefors K, Johansson ME, Stéfansson B, Granqvist A, Arnadottir M, Berg AL, Nyström J, Haraldsson B

J Am Soc Nephrol. 2010, 21: 1290-1298

II. Effects of melanocortin 1 receptor agonists in experimental nephropathies

Lindskog Jonsson A, Granqvist A, Elvin J, Haraldsson B, Nyström J

Manuscript

III. Melanocortin 1 receptor function and signaling in podocytes

Elvin J, Lindskog Jonsson A, Buvall L, Granqvist A, Nyström J, Haraldsson B

Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

AC	adenylyl cyclase
ACE-I	angiotensin-converting enzyme inhibitor
ACTH	adrenocorticotropic hormone
AGRP	agouti-related protein
ARB	angiotensin receptor blocker
ASIP	agouti signaling protein
β-LPH	β-lipotrophin
cAMP	cyclic adenosine monophosphate
CKD5	chronic kidney disease stage 5
CPE	carboxypeptidase
CRE	cAMP responsive element
CREB	cAMP responsive element-binding protein
C_T	threshold cycle
DN	diabetic nephropathy
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ESL	endothelial cell surface layer
ESRD	end stage renal disease = CKD5
FSGS	focal segmental glomerulosclerosis
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GPCR	G-protein-coupled receptor
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
JNK	c-Jun N-terminal kinase
MAC	membrane attack complex
MAP	mitogen-activated protein
MCR	melanocortin receptor
MN	membranous nephropathy
MRAP	melanocortin receptor accessory protein
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
N-AT	*n*-acetyltransferase
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B

	cells
PAM	α-amidating monooxygenase
PAN	puromycin aminonucleoside
PC	prohormone converting enzyme
PCR	polymerase chain reaction
PHN	passive Heymann nephritis
PKA	protein kinase A
PLA_2R	phospholipase A_2 receptor
POMC	pro-opiomelanocortin
PVDF	polyvinylidene difluoride
RAS	renin-angiotensin system
ROS	reactive oxygen species
suPAR	soluble urokinase receptor
TBARS	thiobarbituric acid-reactive substances
TGF-β	transforming growth factor β
ULEX	ulex europeaus agglutinin
VEGF-A	vascular endothelial growth factor A
WT-1	Wilms' tumor

1 INTRODUCTION

Patients with end-stage renal disease (ESRD), or rather chronic kidney disease stage 5 (CKD5), have a severe handicap with life-long dependence on dialysis and/or transplantation reducing their quality of life. To this date, there are around 8500 patients in active uremic care in Sweden and the numbers are constantly growing.[1] Glomerulonephritis is the most common diagnosis within the CKD5 group. However diabetic nephropathy is the most common diagnosis in patients starting treatment today.[1] There is an unmet need for a remedial treatment in these patients with glomerular disorders. Hence, the aim of the work compiled in this thesis has been to elucidate the mechanisms behind a rediscovered and potentially curative therapeutic strategy: adrenocorticotropic hormone (ACTH).

1.1 The Kidney

The kidney has several important functions including regulation of body fluid volume and composition, maintenance of acid-base balance and production of hormones that regulate calcium balance, blood pressure and production of red blood cells. One major function is to filter blood, thus removing waste products and excess fluid via the urine. This process takes place in a functional unit called the nephron, which consists of a glomerular capillary network surrounded by Bowman's capsule attached to a tubular part. Each kidney has about one million nephrons, and 180 L of fluid is filtered across the glomerular capillary walls every day. Most of the fluid and solutes are reabsorbed in the tubular system, so the final daily urine volume is only around 1.5 L. Under normal conditions, larger proteins and blood cells are retained in the blood. Accumulation of proteins in the urine, proteinuria, is evidence of a malfunctioning filtration barrier and constitutes a defining characteristic of renal glomerular disease.

1.1.1 The Glomerular Filtration Barrier

The glomerular filtration barrier is a highly specialized structure consisting of four different layers (Figure 1). From the capillary lumen to the urinary space they are arranged in the following manner: endothelial cell surface layer (ESL), endothelial cells, glomerular basement membrane (GBM) and podocytes.[2] This complex barrier filters blood based on size, shape and charge. Importantly, water is freely filtered whereas passage of larger and negatively charged molecules, such as albumin, is restricted to various degrees.[3-6] Mesangial cells are also a part of the glomerulus, but they serve as

structural support between the capillaries and will not be further discussed in this thesis. Past research has focused on the barrier's individual components and it has been shown that damage to any of the layers results in proteinuria.[7,8] Depending on diagnosis, one specific layer can be involved, but it has also been shown that cross-talk between cells of different layers can be of importance.[2,9]

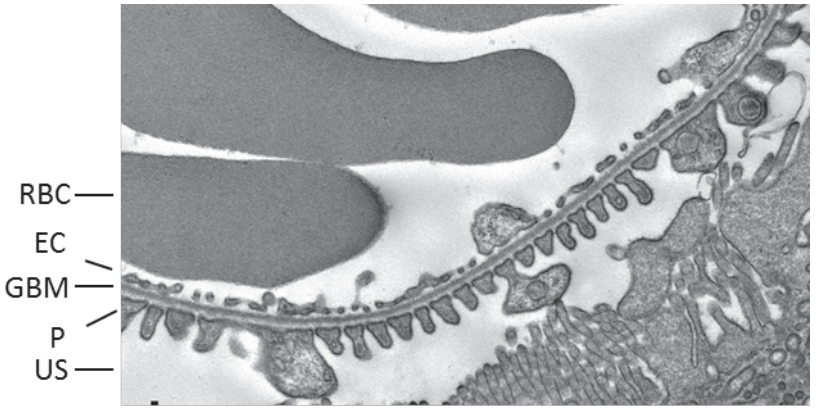

Figure 1. Transmission electron microscopy of a glomerular capillary cross section containing red blood cells (RBC) and showing fenestrated endothelium (EC) lining the lumen, the basement membrane (GBM) and the podocyte (P) with its foot processes facing the urinary space (US). Courtesy of Dr A Granqvist.

Endothelial Cell Surface Layer

The first level of the barrier is the ESL, a gel-like layer, composed of a membrane bound glycocalyx and a more loosely attached endothelial cell coat.[2] The content of negatively charged glycoproteins, glycosaminoglycans and proteoglycans contributes to the permselective properties of this layer. Visualization of the ESL using lipid droplets has indicated a thickness of 100-400 nm.[10,11] Modification of the ESL by enzymatic treatment with hyaluronidase, heparinase or chondroitinase, decreased the distance between the droplets and the capillary wall.[11] Similar effects were obtained by treatment with hypertonic sodium chloride.[12] Both these treatments were accompanied by an increased fractional clearance of albumin, suggesting an important role for the ESL in barrier function. In addition, studies in immortalized glomerular endothelial cells showed that treatment with neuraminidase and heparinase, increased albumin flux.[13] Moreover, mice with adriamycin-induced nephropathy displayed a reduced thickness of ESL compared with control mice.[14] This effect was accompanied by foot process effacement and a decreased glomerular filtration rate. Another recently published study showed that treatment with neuraminidase resulted in loss of

endothelial glycocalyx accompanied by albuminuria, thus confirming the importance of ESL for normal glomerular function.[15]

Endothelial Cells

The property that distinguishes glomerular from other endothelial cells is that they are heavily fenestrated with up to 50% of the surface area covered by fenestrae.[16] A filamentous glycocalyx plug that covers the fenestrae, approximately 60-80 nm in size, has been detected[17] suggesting that albumin with a size of about 3.6 nm do encounter some resistance at this level of the filtration barrier. The importance of the endothelial cells has been shown in preeclampsia, a pregnant-related disease, which is characterized by glomerular endotheliosis, loss of glomerular endothelial pores and proteinuria.[18] Eremina et al. showed that mice heterozygous for podocyte-specific vascular endothelial growth factor (VEGF)-A display preeclampsia-like characteristics, thus highlighting the importance of communication between the glomerular cell types.[9]

Basement Membrane

The GBM is a thick, acellular extracellular matrix layer composed of type IV collagens, laminins, nidogen/entactin and proteoglycans.[19] In addition to its structural supportive role for adjacent cells, the GBM restricts fluid flux across the barrier[20,21] and serves as a stimulus for cell polarization, migration and differentiation[19]. GBM-related genes have been shown to be associated with pathological conditions. For example, Alport's syndrome, hereditary glomerulonephritis, is caused by mutations in collagen chains which results in disruption of the membrane.[22-24] Other diseases, including membranous nephropathy (MN) and diabetic nephropathy (DN), are characterized by an altered thickness of the basement membrane.[25,26]

Podocytes

The podocytes are attached via $\alpha 3\beta 1$ integrins to collagen, fibronectin and laminin in the GBM.[27] In addition, cell surface-expressed proteoglycans are believed to be critical for podocyte - matrix interaction.[28] Dystroglycans have also been described as a link, but are probably not critical for kidney development and function as recently shown by Jarad et al.[29]

Podocytes are highly differentiated and specialized epithelial cells surrounding the glomerular capillaries. They consist of three segments: a cell body, major processes and foot processes that are long extensions arranged in an extremely organized manner resembling a zipper. The content of the slit diaphragms, which are the structures that bridge the foot processes from one podocyte to another, have been revealed during recent years, and specific slit

proteins have been shown to be crucial for normal glomerular function.[30] For example, a mutation in *NPHS1*, the gene encoding nephrin, causes congenital nephrotic syndrome of the Finnish type.[31] Also, *NPHS1* knock-out mice are nephrotic and fail to develop foot processes.[32] *NPHS2*, and its gene product podocin, accounts for both familial and sporadic forms of nephrotic syndrome[33,34], and conditional knockout mice develop focal segmental glomerulosclerosis (FSGS)[35].

The slit diaphragm protein complexes are further connected to well-organized filament bundles of the actin cytoskeleton and synaptopodin is partly responsible for this association.[36] Synaptopodin, expressed in brain and differentiated but not undifferentiated podocytes[37], induces actin stress fibers via regulation of RhoA[38]. Generally, disruption and rearrangement of the actin cytoskeleton leads to loss of foot processes, a state called foot process effacement.[39,40] In summary, an integral actin cytoskeleton is important in maintaining podocyte structure and function, thus upholding the permselective properties of the barrier (Figure 2). Given that foot process effacement is often accompanied by proteinuria[40,41], many nephrotic diseases are considered to be podocytopathies.

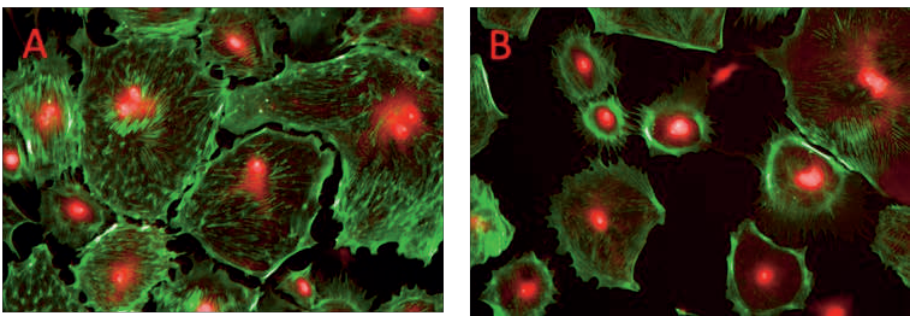

Figure 2. Actin cytoskeleton staining (green) in cultured podocytes. A) Untreated podocytes with normal actin filament bundles. B) Treatment with puromycin aminonucleoside (PAN) results in rearrangement of the actin cytoskeleton. Courtesy of Dr V. Gupta.

1.2 Nephrotic Syndrome

Nephrotic syndrome is characterized by proteinuria, hypoalbuminemia, hyperlipidemia and peripheral edema. Normally, the protein loss in urine is less than 30 mg/day while levels of 30-300 mg reflect microalbuminuria. Nephrotic syndrome is characterized by even higher levels, over 3.5 g/day. Albumin excretion can be estimated either as an amount per day or in relation to creatinine, which corrects for differences in urine dilution. Both methods

are commonly used in experimental models to estimate renal disease. Regarding the edema seen in nephrotic syndrome, recent findings suggest that it is caused by specific activation of tubular sodium transporters by increased urine concentrations of plasmin.[42] The symptoms of nephrotic syndrome can have primary causes, such as in glomerulonephritis, or they can be secondary due to other diseases such as DN or malignancy. As previously mentioned, there are also genetic explanations as seen in congenital nephrotic syndrome of Finnish type[31] and Alport's syndrome[22-24]. Diagnosis is based on evaluating morphological features in kidney biopsies.

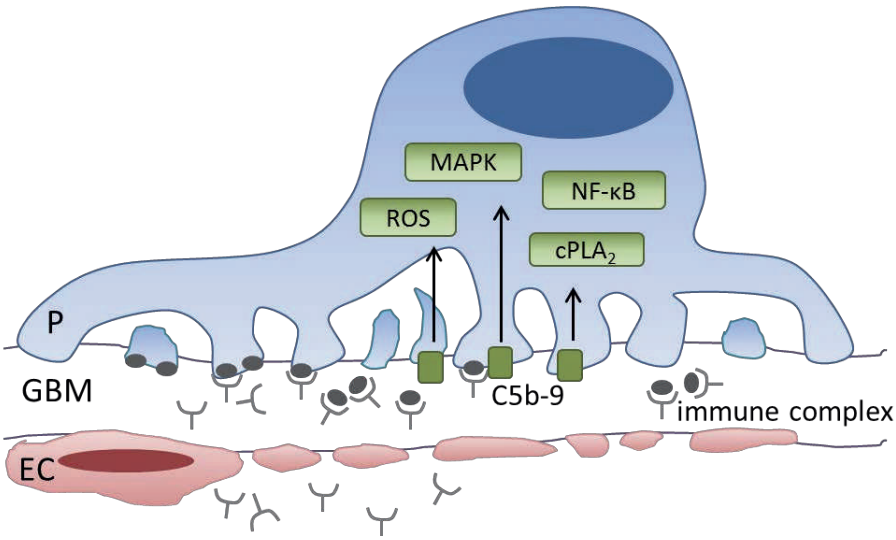

Figure 3. Schematic figure of immune complex formation in membranous nephropathy. Circulating antibodies pass through the endothelial cell (EC) barrier and bind to antigens on the podocyte (P) surface. Formed immune complexes shed into the glomerular basement membrane (GBM) and stimulate insertion of C5b-9 into the podocyte cell membrane. MAPK = mitogen-activated protein kinase, ROS = reactive oxygen species, NF-κB = nuclear factor κB, $cPLA_2$ = cytosolic phospholipase A_2.

1.2.1 Membranous Nephropathy

MN is one of the most common causes of nephrotic syndrome in adults and can be either primary idiopathic (focus of this thesis) or secondary due to cancer, infections or drugs and toxic agents.[43] Diagnosis is based on kidney biopsy revealing capillary wall thickening, subepithelial deposits in electron microscope and IgG along the capillary wall on immunofluorescence.[44] Other characteristics include foot process effacement and increased GBM thickness.[25] About one third of the patients spontaneously go into remission while the remainder divides into two groups: one with an unchanged disease

state and one that will progress into CKD5.[45,46] Heymann nephritis, a rat model resembling MN, was described for the first time in 1959[47] and during the past decades much effort has been put into understanding the underlying mechanisms of this disease (Figure 3). In situ immune complexes are formed by circulating antibodies that bind to a specific antigen (megalin in rat) present on the podocyte surface.[48-52] The immune complexes detach from the cell surface and shed into the GBM and, in addition, cause insertion of the C5b-9 membrane attack complex (MAC) into the podocyte cell membrane.[53,54] Sublytic concentrations of MAC results in an intracellular signaling cascade with multiple effects including production of reactive oxygen species (ROS)[55,56], alteration of slit diaphragm proteins[57] and actin cytoskeleton[58], activation of nuclear factor kappa B (NF-κB)[59], and effects on mitogen-activated protein (MAP) kinase pathways including p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)[59-61]. All these processes will ultimately lead to foot process effacement and proteinuria. A recent study showed that the human megalin equivalent might be the M-type phospholipase A_2 receptor (PLA_2R), present on the podocyte surface in 70% of idiopathic MN patients[62], and for many patients antibody titer correlated with disease progression and degree of proteinuria[63,64].

1.2.2 Focal Segmental Glomerulosclerosis

FSGS has become one of the most frequent causes of CKD5 in Western countries, especially among the African-American and US Hispanic populations.[44,65] As indicated by its name, FSGS is characterized by focal glomerulosclerosis, segmental hyalinosis and scarring. Similar to MN, FSGS can be either primary idiopathic, or secondary and as previously mentioned there are also genetic causes, reviewed in Gbadgesin et al.[66] FSGS has been considered as a podocytopathic disease, and mutations in many podocyte-related genes are indeed linked to the disease: *ACTN4*[67], *CD2AP*[68] and S*YNPO*[69]. In 2011, soluble urokinase receptor (suPAR) was identified as a factor that may be involved in the pathogenesis of FSGS with increased serum levels in FSGS patients.[70]

1.3 Treatment of Nephrotic Syndrome

The mechanisms behind most of the diseases causing nephrotic syndrome remain unclear and thus treatment options are only symptomatic and not curative. In addition, there is an absence of good randomized controlled trials that compare old and new treatment options. Patients with a renal function, not requiring active uremic care, are commonly treated with

immunosuppressive drugs that do not cure the disease, but simply delay symptoms. In addition such drugs are associated with severe side effects, and therefore the physician and patient must always balance risk against benefit. Unfortunately, today's treatment is focused on control rather than cure, and the main treatment objective is to reduce proteinuria. Nephrotic patients often present with cardiovascular symptoms, and proteinuria is associated with an increased risk for cardiovascular events.[71,72] Consequently, a secondary treatment objective is to reduce hypertension, edema and hyperlipidemia. Below follows a brief account of both recommended and new potential treatment options for nephrotic diseases, based on the 2012 KDIGO Clinical Practice Guideline for Glomerulonephritis.[44]

1.3.1 Immunosuppression

Immunosuppressive therapy, that is alkylating agents and corticosteroids, is often the first choice therapy in nephrotic diseases. For MN patients, first reported in 1984 by Ponticelli[73], the strategy has been to use a 6-month regimen of monthly alternating oral chlorambucil or cyclophosphamide and corticosteroids. In FSGS, the typical initial treatment is corticosteroids alone, given as prednisolone or prednisone. Although many patients seem to benefit from these drugs, there is an association with severe short- and long-term side effects including myelosuppression, infertility, cancer, gastrointestinal problems (peptic ulcers), nausea, anorexia and liver dysfunction.[74,75] Usually, patients are left untreated for about 6 months to see if there is a spontaneous remission, followed by 6 months of corticosteroid treatment. If the patient does not respond to treatment, another agent is tried, for example Rituximab (an anti-B cell antibody). There is also a possibility that the patient suffers from a steroid-resistant or genetic variety of the disease and then no corticosteroid therapy will ameliorate the disease.

1.3.2 Basal and Symptomatic Therapy

The renin-angiotensin system (RAS) contributes to the control of blood pressure by regulating sodium balance and hence, plasma volume. RAS blockade is commonly used when treating nephrotic patients. The aim is to reduce proteinuria by lowering the glomerular capillary hydrostatic pressure. In some patients hypertension is present, and then RAS blockade reduces the risk for cardiovascular events, by controlling blood pressure. The goal is to keep blood pressure at or below 130/80 mmHg. Angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARB) are first-choice therapy. These agents also have an antiproteinuric effect which can be additive when they are used in combination.[76] Nephrotic edema is normally treated by restriction of dietary sodium in combination with oral loop

diuretics. The latter inhibit electrolyte transporters in the thick ascending loop of Henle with ensuing increased urinary output. Hyperlipidemia is controlled with statins (3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors). Prophylactic anticoagulation, in Sweden Fragmin or Warfarin, is given when the level of serum albumin falls below 25 g/L and the risk of thrombotic events increases.

In summary, there is no curative treatment for nephrotic syndrome. Many of the different agents used when treating the symptoms can cause severe side effects, alone or in combination. Therefore the patient must be carefully monitored and risk must be weighed against benefit and the patient's quality of life must be evaluated. In the best of cases, a nephrotic patient spontaneously goes into remission. The next best is that short-term treatment with corticosteroids is successful. In the worst case scenario treatment does not have any effect on the degree of proteinuria and symptoms are only treated to reduce the risk of later cardiovascular events.

1.3.3 Adrenocorticotropic Hormone

ACTH is an endogenous hormone that was used already in the 1950s and 60s as treatment for patients with nephrotic syndrome.[77-79] When oral corticosteroids, prednisone and prednisolone, became available, and also due to ACTH's immunosuppressive-like side effects, physicians discontinued subscription of the drug. In 1999, Berg et al. treated nephrotic patients with ACTH with the aim of studying a potential lipid lowering effect.[80] By chance, it was (re-) discovered that ACTH also improved glomerular function and reduced proteinuria. These observations were repeated in other studies[81,82] including a trial comparing ACTH with the earlier described Ponticelli treatment regimen[83]. In these European reports, a synthetic analogue of ACTH was typically given parenterally at an approximate dose of 1 mg/kg twice a week for up to one year. In USA, lack of availability has instead resulted in using a gel formulation that seems to confirm the beneficial effects in nephrotic patients, primarily in MN patients.[84]

The administered dose has been very low but the amount of cortisol release from the adrenal gland upon ACTH stimulation is still high enough to cause Cushingoid like side effects, including edema, increased blood pressure and osteoporosis. However, the cortisol concentration is much lower compared with the doses used when treating patients with renal disease, suggesting an alternative treatment effect. Furthermore, corticosteroids alone in low or moderate doses do not result in beneficial effects.[85,86] Due to the normal physiological response, ACTH treatment results in side effects comparable to

those seen for the corticosteroids and it would be preferable with a drug that minimizes these. There is a lack of well executed, randomized controlled trials comparing ACTH to other treatments. Such trials, in combination with mechanistic studies, would be of great interest since they could reveal new drug targets, allowing more specific and possibly curative treatment of nephrotic disease without severe side effects.

1.4 The Melanocortin System

1.4.1 Melanocortin Receptor Ligands

ACTH and other melanocortins are small peptide hormones derived by posttranslational processing of the protein pro-opiomelanocortin (POMC), (Figure 4).[87] Prohormone converting (PC) enzymes 1 and 2 are important for the production of melanocortins.[88] Thus, proteolytic cleavage of POMC by PC1 results in the products β-lipotrophin (LPH) and pro-ACTH, which is then further cleaved by the same enzyme to generate ACTH. A second proteolytic cleavage by PC2 generates the melanocortins γ-, and β-melanocyte-stimulating hormones (MSH). Further cleavage of ACTH by PC2 and carboxypeptidase followed by amidation and acetylation, yields a peptide hormone that constitutes the C-terminal 13 amino acids, namely α-MSH. POMC, as well as the different melanocortins, have been detected in both brain and peripheral tissues.[89]

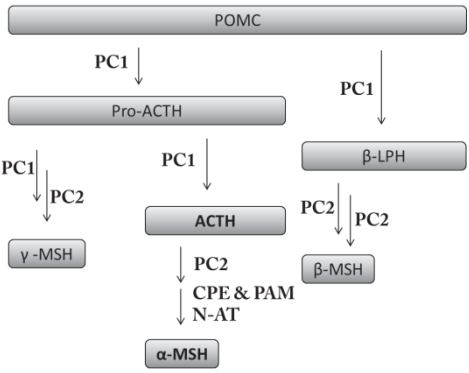

Figure 4. Posttranslational processing of POMC protein involving prohormones converting enzymes 1 and 2 (PC1 and 2) as well as carboxypeptidase (CPE), α-amidating monooxygenase (PAM) and n-*acetyltransferase (N-AT). Figure adapted from Getting et al.*[90]

There is a high degree of sequence homology between the melanocortin receptors (MCRs), both across species and within the receptor family[91], and one conserved amino acid sequence, His-Phe-Arg-Trp, (HFRW) is required for receptor binding and activation[92]. Since the melanocortins stimulate the MCRs in an unspecific manner, several selective and potent agonists have been synthesized during the past decades in order to identify the (patho)

physiological role of a specific receptor. Nle4,DPhe7-α-MSH (NDP-MSH) is a more stable and potent analog of α-MSH[93], MS05 has strong selective agonistic activity for MC1R[94] and BMS-470539 is a synthetic and highly potent MC1R agonist[95]. These and many other agonists have facilitated the work in elucidating the role of each MCR. In addition, there are two endogenous antagonists, the widely distributed agouti signaling protein (ASIP) and agouti-related protein (AGRP) where expression is concentrated to the central nervous system.[89,92]

1.4.2 Melanocortin Receptors and Signaling

To date, five intronless subtypes of the melanocortin receptors have been cloned, MC1-5R (Figure 5). They belong to a family of G-protein-coupled 7 transmembrane receptors (GPCR). Melanocortin signaling involving adenylyl cyclase (AC) and intracellular production of cyclic adenosine monophosphate (cAMP) was first reported in 1965 based on pigmentation experiments in frogs.[96] The increased level of cAMP causes activation of protein kinase A (PKA) which will lead to binding of cAMP responsive element-binding protein (CREB) to cAMP response elements (CRE) in the DNA.[97-99] In addition, several other intracellular signaling pathways have been associated with MCR signal transduction. For example, ligand affinity and signaling are enhanced at physiological Ca^{2+} concentrations compared with Ca^{2+}-free conditions.[100] Further downstream signaling pathways and effects of MCR stimulation include phosphorylation and activation of the MAP kinases p38[101] and ERK1/2[102] where phosphorylation and activity are associated with melanogenesis and proliferation. In addition, stimulation with α-MSH inhibits the activation of NF-κB, which is particularly important for inflammatory processes.[103,104]

MC1R, the first melanocortin receptor to be cloned in 1992[105,106], is known to be expressed in melanocytes and regulate skin pigmentation. Mutations may be associated with an increased risk for melanoma.[107] MC1R stimulation is also involved in the defense against UV-induced oxidative stress through activation of catalase, an antioxidant enzyme that converts hydrogen peroxide to water and oxygen.[108] In addition, the MC1R is also known for its anti-inflammatory properties, and it is expressed in several different immune cells.[95,103,109-111] The natural ligands for MC1R are, in order of potency, α-MSH ≥ ACTH > β-MSH >> γ-MSH.[105,106]

ACTH is the only melanocortin that binds and activates MC2R, which is therefore also known as the ACTH-receptor. As reviewed by Veo et al. this selectivity requires an additional amino acid motif, limited to ACTH, and

possibly also interaction with melanocortin receptor accessory protein (MRAP) 1.[112] The normal physiological response to stimuli is production and release of steroids from the adrenal gland.[113,114]

All the melanocortins (α, β- and γ) and ACTH stimulates and are equipotent for MC3R.[115] The receptor distribution is somewhat different compared to MC1R and MC2R, as it is mainly found in brain[115] associated with energy homeostasis[116]. In addition, expression has been detected in placenta[115], in heart[117] in correlation with protective cardiovascular function in ischemia-reperfusion injury[118], and in macrophages suggesting a role in some inflammatory diseases[119-122].

MC4R is expressed primarily in the brain[123], no expression has been found in 20 different peripheral tissues[117]. Studies in mice revealed a function in feeding control and homeostasis, possibly an agouti-mediated antagonizing effect.[124] Of the melanocortins, ACTH and α-MSH are equipotent, while β-MSH is a less potent receptor activator.

The final member of the MCR gene family to be cloned was MC5R[125] and similar to MC1R and MC4R, this receptor is equally activated by ACTH and α-MSH, while γ-MSH has no activity.[89,125] The receptor is expressed in several peripheral tissues including leukocytes, suggesting a role in inflammation.[117,125] It is also expressed in exocrine glands and disruption of the MC5R gene results in mice with impaired water repulsion and thermoregulation.[126]

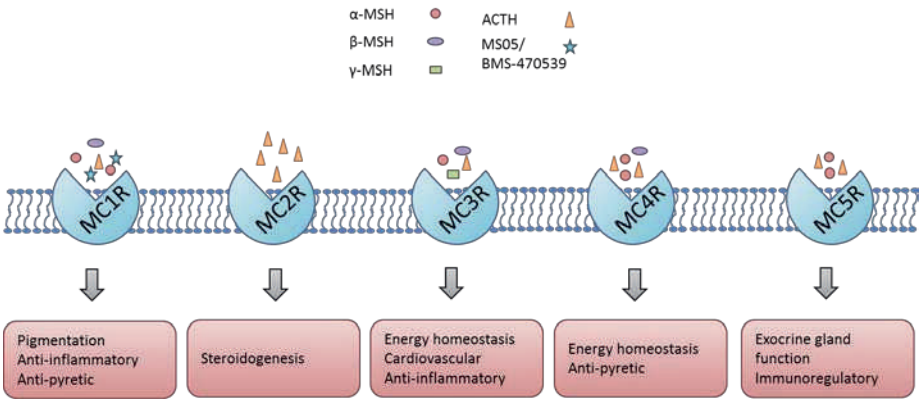

Figure 5. The melanocortin ligands and receptors with main functions.

2 ORIGIN AND AIMS

This thesis originates from the finding that ACTH improves clinical parameters in patients with nephrotic syndrome. The mechanisms behind many nephrotic diseases are unclear and treatments are unspecific. Therefore, it is of great importance to elucidate the mechanisms so that drug targets can be defined and new treatment options can become available.

The overall aim of this thesis was to explain the mechanisms behind the positive effect of ACTH in nephrotic patients.

The specific aims were to:

1. Determine which ACTH receptor(s) is/are expressed in the kidney.
2. Evaluate specific MC1R agonists as a treatment option in different nephrotic diseases.
3. Identify key signaling pathways involved in MC1R signaling in podocytes.

3 METHODOLOGICAL CONSIDERATIONS

The following is a brief overview of the methods used in this thesis, with special emphasis on why they were chosen. More detailed descriptions of materials and methods are found in each paper.

3.1 Patients

All patients were diagnosed with biopsy-proven idiopathic MN. After an observation period of at least 15 months, they were treated with synthetic ACTH (Synachten Depot, Novartis, Switzerland) at the University Hospitals of Lund and Sahlgrenska in Sweden, according to previously published protocols.[80,81] Proteinuria was followed during and after treatment.

3.2 Experimental Nephrotic Models

There are several experimental models for nephrotic syndrome. In order to gain knowledge of the mechanisms behind the ACTH effect, the two most well-known and characterized *in vivo* models have been used in this thesis: Passive Heymann Nephritis (PHN), resembling human MN, and adriamycin-induced nephropathy, resembling FSGS. The studies were approved by Gothenburg Ethical Board for Animal Experiments. The ethical permits for the studies with PHN rats are numbered 213-2007 and 237-2009 for paper I and II, respectively. The ethical permit number for the study with adriamycin-induced nephropathy in mice in paper II is 124-2012.

All animals had free access to food and water and were housed in a 12-hour dark-light cycle. Surgical procedures were performed during anesthesia that was induced and maintained by inhalation of isoflurane (2-3% v/v, Schering-Plough, Stockholm, Sweden) mixed with air (~1 L/min) in an isoflurane vaporizer (Ohmeda Isotec 5, Simtec engineering, Askim, Sweden). Temgesic® (0.1 ml/100 g body weight, Schering-Plough, Stockholm, Sweden) was given as a post-operative pain reliever.

3.2.1 Experimental MN

PHN is a well-studied and characterized experimental model resembling human MN and was therefore chosen for investigating the effect of ACTH *in vivo*. This model was initially described in 1959 and over the years it has been an important tool in the unraveling of many questions and mechanisms related to human MN.[47] PHN is induced by injection of an antibody against

the tubular brush border fraction, anti-Fx1A. The disease is characterized by subepithelial immune deposits that are formed *in situ* in the glomerulus, triggered by antigen expression on the podocyte surface, mainly megalin.[127] Subepithelial electron-dense deposits are visible already 3-5 days after disease induction and proteinuria reaches a peak level after two weeks. New immune deposit formation decreases with time, but proteinuria persists throughout life.[128]

PHN experiments were performed on male Sprague Dawley rats with an initial body weight of 125-165 g. To induce PHN, anti-Fx1A, 30 mg/ml (Probetex Inc., San Antonio, TX), was intravenously injected. A dose-response study was performed in order to assess the ideal dose. The optimal dose regimen was found to be 1.5 ml at day 0, followed by a booster dose of 0.5 ml at day 7, leading to a high and stable level of proteinuria 28 days after the first injection. Consequently this dose scheme was used in the subsequent experiments. Agonist treatment started two weeks after the first injection, when proteinuria reached a high and stable level, and one of the following MC1R agonists was used: $ACTH_{1-24}$ (Novartis, Switzerland); α-MSH (Sigma-Aldrich, St Louis, MO); or MS05 (custom-made peptide from Sigma-Aldrich in paper I, St Louis, MO, or Agrisera in paper II, Vännäs, Sweden). The dose was 10 μg/day in Paper I and 100 μg/day in Paper II. Drugs were administered through osmotic pumps (Alzet® Osmotic Pumps, Cupertino, CA) in paper I and II and via subcutaneous injections in paper II.

3.2.2 Experimental FSGS

The adriamycin model in mice resembles FSGS in patients. Injection of adriamycin generally leads to proteinuria, and morphological characteristics include glomerulosclerosis, tubulointerstitial inflammation and fibrosis.[129] There is also glomerular infiltration by macrophages and, at later stages, interstitial infiltration by $CD4^+$ and $CD8^+$ T cells.[129]

In paper II, experiments were performed on male BALB/c mice with an initial body weight of 22-26 g. Adriamycin (doxorubicin hydrochloride, Sigma-Aldrich, St Louis, MO) was injected via the tail vein, and controls received an equal volume of saline. Treatment, either 0.14 M BMS-470539 (synthesized by Enamine, Ukraine), or 0.15 mM α-MSH (Sigma-Aldrich, St Louis, MO) diluted in 1:1 PEG400 and water, was administered through osmotic pumps (Alzet® Osmotic Pumps, Cupertino, CA) and started one day before adriamycin injection. Healthy controls and adriamycin-treated controls received vehicle. To prevent weight loss, a glucose-electrolyte solution was given intraperitoneally on day 1 to 11.

3.3 Podocyte Cell Culture

Cultured podocytes were used as a complement to the *in vivo* studies, for further understanding of MC1R signaling pathways. Isolating and working with primary glomerular cells is both difficult and time-consuming. Much effort has therefore been put into creating cell-lines as a substitute for primary glomerular cells *in vitro*. One of these, a conditionally immortalized mouse podocyte cell-line, was used in paper III. These cells have been transformed with a temperature sensitive mutant of SV-40 T antigen. Podocytes grown under the permissive temperature at 33°C proliferate rapidly. After thermo switch to the non-permissive temperature of 37°C, the cells stop to proliferate and differentiate to express the podocyte markers Wilms' tumor (WT-1) and synaptopodin.[130] The cells were allowed to differentiate for at least 9 days before starting experiments.[130]

Since MC1R has a very low basal expression level in mouse podocytes, they were transfected with human MC1R. Differentiated podocytes were exposed to lentivirus containing a vector for expression of MC1R-EGFP or a control vector for expression of EGFP. The cells were used 72 hours after lentivirus addition. Expression of human MC1R mRNA was confirmed indicating successful transfection. To study MC1R signaling pathways, podocytes were stimulated with the synthetic MC1R agonist BMS-470539 at different times and concentrations. Protein and mRNA was harvested and further analyzed with quantitative real-time PCR, western blot and functional assays to detect intracellular levels of cAMP and catalase activity. Nephrosis-inducing agents, such as puromycin aminonucleoside (PAN) and the previously mentioned adriamycin, are known to cause rearrangement of the actin cytoskeleton and therefore serve as suitable tools for studying foot process effacement.[131-133]

3.4 Gene Expression Analysis

Quantitative real-time PCR was performed on kidney tissue and cells of both human, rat and mouse origin. RNA quality was verified with a 2100 Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany) or with Experion™ (Bio-Rad, Hercules, CA). Pre-designed primers and probes verified by ABI were used, see Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a stably expressed endogenous control, unaffected by any of the treatments used in the different studies. Expression of MCRs in paper I was analyzed in terms of the threshold cycle (C_T) level. A C_T level above 35 is generally considered as 0-1 copy numbers of the gene. The comparative $\Delta\Delta C_T$ method was used in paper II and III for estimation of any change in mRNA levels compared to control samples.

Table 1. Information about primer/probes used in the different papers.

Primer/Probe	Species	Number	Paper
MC1R	human	Hs00267167_s1	I, II and III
MC2R	human	Hs00265039_s1	I
MC3R	human	Hs00252036_s1	I
MC4R	human	Hs00271877_s1	I
MC5R	human	Hs00271882_s1	I
GAPDH	human	Hs99999905_m1	I and II
MC1R	rat	custom made GenBankID AB306978.1	I and II
GAPDH	rat	Rn01775763_g1	I and II
MC1R	mouse	Mm00434851_s1	II and III
GAPDH	mouse	Mm99999915_g1	II and III

3.5 Protein Analysis

3.5.1 Spot Urine Analysis

Proteinuria is a hallmark of kidney disease. Normally, there is little or no protein in the urine, reflecting the high selectivity of the normal glomerular barrier and tubular reabsorption of the small amount of albumin that is present in primary urine. The tubular uptake of albumin is dependent on intact megalin-cubilin complexes.[134] However, with an impaired glomerular function, the amount of protein in the urine is correspondingly increased. In this thesis, albuminuria is used in both paper I and II as a primary outcome and reflection of glomerular disease. Spot urine samples were collected twice a week (PHN) or daily (adriamycin) and albumin was analyzed with an enzyme-linked immunosorbent assay (ELISA). To correct for diluted urine

samples, creatinine was also measured with the Jaffé reaction using a creatinine standard solution.

3.5.2 Western Blot

Western blot was used for detection of proteins and confirmation of gene expression findings (paper I). This method was also used for studying protein phosphorylation (paper III), which is a common step in activating various cell signaling pathways. Protein lysates were separated on NuPAGE 4-12% BisTris gels (Novex, San Diego, CA), transferred to polyvinylidene difluoride (PVDF) membranes and blocked. Primary antibodies, described in Table 2, were used for detection and immunoreactive bands were visualized with a chemiluminescent kit and a CCD camera (Bio-Rad Laboratories Inc., Hercules, CA; Fujifilm, Tokyo, Japan). GAPDH was used as a loading control to ensure equal loading. Science Lab Image Gauge Version 4.0 was used to compare the phosphorylated proportion to total protein.

3.5.3 Immunohistochemistry

Immunohistochemistry is a standard method to detect and visualize protein expression and location in a cell or tissue. Cryosections from kidney tissue, from both healthy individuals and MN patients, were used to analyze the expression of MC1R in paper I. Co-localization studies were performed with both endothelial- (ulex europeaus agglutinin, ULEX) and podocyte (synaptopodin) specific markers. Rhodamine-Phalloidin, which binds to F-actin, was used for detection of the actin cytoskeleton in podocytes in paper III. The antibodies are described in Table 2.

3.5.4 Activity assays

In order to examine the functional response after MC1R stimulation in podocytes, two different activity assays were used: intracellular cAMP levels (measured with the cAMP Direct Biotrak^{TK} EIA kit, GE Healthcare, Sweden) and catalase activity (measured with the Amplex® Red Catalase Assay Kit from LIFE technologies, Sweden). As previously described, both of these have been related to MC1R stimulation in other cell types.

3.6 Morphological Analysis

Transmission electron microscopy was used to examine the intrinsic structure of glomeruli in the experimental models of nephrotic syndrome in paper I and II. This is an excellent technique for inspection of the different layers in the filtration barrier, allowing visualization of, for example, foot process

effacement and changes in GBM thickness. At the end of the experiments, the renal artery and vein were clamped and the kidney was fixed by subcapsular injection of Karnovsky's fixative. The kidneys were processed according to standard procedures as previously described.[135] In paper I, sections were analyzed in a blinded fashion by a pathologist. The number of foot processes per 10 μm GBM was calculated and compared between the groups.

Table 2. Information about the primary antibodies used in Western Blot (WB) and Immunohistochemistry (IHC).

Primary Antibody	**Paper**	**Application**	**Company**
Anti-MC1R	I	WB and IHC	Alomone Labs, Israel
Anti-synaptopodin	I	IHC	Abcam Ltd, UK
ULEX	I	IHC	Vector Laboratories, CA
Anti C5b-9	I	IHC	Santa Cruz Biotechnology, Santa Cruz, CA
Rhodamine-Phalloidin	III	IHC	Sigma-Aldrich, Sweden
Anti-GFP	III	WB	Abcam Ltd, UK
Anti Phospho-p44/42 MAPK (ERK1/2)	III	WB	Cellsignaling, Beverly, MA
Anti-p44/42 MAPK (ERK1/2)	III	WB	Cellsignaling, Beverly, MA
Anti-GAPDH	III	WB	Abcam Ltd, UK

4 REVIEW OF RESULTS

This thesis is based on three papers. The focus of paper I and II was to identify a possible target / receptor for ACTH in kidney and to further elucidate the mechanisms *in vivo*. In paper III, the signaling pathways of MC1R stimulation were further studied.

4.1 ACTH treatment in MN patients (Paper I)

In paper I, we confirmed previous observations that ACTH treatment ameliorates nephrotic disease (Figure 6). The nephrotic condition in five MN patients with biopsy-proven diagnosis, treated with ACTH for at least 7 months, was dramatically and significantly improved. Proteinuria was reduced by 86 ± 6% and consequently serum albumin increased with 88 ± 7% to reach normal levels ($P < 0.001$ for both parameters). The effect was sustained in all patients with a follow-up time from 1 to 15 months.

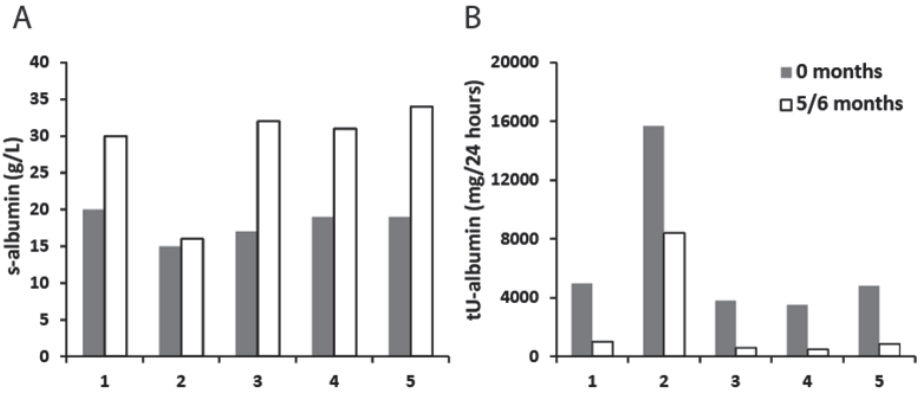

Figure 6. A) Serum albumin (s-albumin) and B) total urine albumin (tU-albumin) are improved in five MN patients treated with ACTH for 5 or 6 months.

4.2 Expression of MC1R in kidney (Paper I and II)

The hypothesis was that ACTH signaling is mediated through a kidney-specific receptor and therefore, expression in renal tissue of all MCRs 1-5 was investigated. Only MC1R mRNA was clearly detected in kidney tissue, namely in glomerular cells of human origin, with a C_T value below 30, see Table 3. MC1R protein was confirmed with western blot in podocytes and to a lesser extent in endothelial cells. Co-localization studies with a glomerular

cell-specific marker showed that MC1R is present in podocytes where it co-localizes with synaptopodin.

Since two experimental models of nephrotic disease were used, MC1R mRNA was also examined in rodents. There was a weak expression in both mouse and rat glomeruli, with no change upon disease induction (PHN in rats and adriamycin in mice).

Table 3. Gene expression of MC1R in kidney from different species.

Species	Tissue / Cell type	n	MC1R C_T level	GAPDH C_T level
Human	Kidney	1	28.9	21.1
Human	Podocyte cell-line	8	28.1 ± 0.19	19.7 ± 0.28
Rat	Glomeruli	4	34.3 ± 0.50	19.8 ± 0.53
Mouse	Glomeruli	10	33.5 ± 0.37	16.6 ± 0.08
Mouse	Podocyte cell-line	5	32.4 ± 0.13	15.2 ± 0.10

4.3 MC1R agonists in experimental nephrotic syndrome

After confirming expression of the ACTH receptor MC1R in kidney tissue, the effect of receptor agonists was evaluated in two different nephrotic models: PHN in rats, resembling human MN, and adriamycin in mice, resembling FSGS.

4.3.1 Experimental MN (Paper I and II)

In paper I, treatment with different MCR agonists, including the specific MC1R agonist MS05, ameliorated the disease by reducing the level of proteinuria, improving morphology and reducing oxidative stress. After four weeks of treatment, MS05 and α-MSH reduced albuminuria by 60% ($P < 0.01$) and 52% ($P < 0.05$), respectively, compared with untreated PHN. In PHN rats, glomerular morphology was disrupted as in MN, displaying subepithelial deposits, foot process effacement and a thickened GBM (Figure 7). In an attempt to quantify the damage, the number of foot processes per 10 µm GBM was calculated. MCR agonist treatment increased this parameter to

13.4 ± 0.57, compared with 10.7 ± 0.71 for untreated PHN ($P < 0.01$). As a further indication of the positive effect of MC1R agonism, oxidative stress was reduced. Thus, thiobarbituric acid-reactive substances (TBARS) were decreased in MS05-treated versus untreated PHN rats, 34.3 ± 3.2 and 76.8 ± 19.4 nmol TBARS/mg creatinine, respectively ($P < 0.01$).

Additional findings in paper II, suggested that the effect remained after treatment withdrawal, comparable with what is seen in patients. Four weeks of treatment with MS05 gradually reduced the level of proteinuria compared with untreated PHN. One week after treatment withdrawal, the effect was sustained and the degree of albuminuria was 56% compared with untreated PHN ($P < 0.05$).

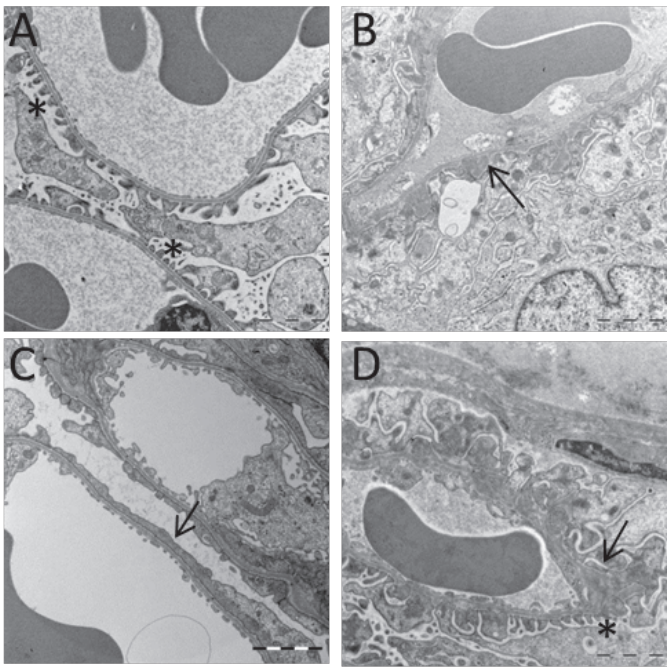

*Figure 7. Transmission electron microscopy of ultrastructural morphology in (A) a control rat, (B) untreated PHN, (C) adriamycin-treated mouse and (D) an MC1R agonist-treated PHN rat. The control displays a normal structure while the untreated PHN rat and adriamycin-treated mouse show podocyte foot process effacement. The MC1R agonist treated PHN rat has a restored structure. *Normal foot process, → disrupted glomerular barrier structure and loss of foot processes. Scale bar2 µm.*

4.3.2 Experimental FSGS (Paper II)

Most of the clinical findings suggest ACTH to be beneficial in treating MN-induced nephrotic syndrome, however less is known for other diagnoses of kidney disease. Therefore, MC1R agonism was assessed in a second *in vivo* model for nephrotic syndrome, specifically adriamycin-induced nephropathy in mice, resembling FSGS. This model is more acute compared with PHN and modest albuminuria was indeed apparent within 5 days after adriamycin injection. When analyzing albuminuria as a primary outcome, no difference was observed at day 12, the end of the study, in MC1R agonist treated compared with untreated adriamycin mice. This model is characterized by severe loss of body weight, which we managed to keep at an acceptable level, approximately 5%, due to daily intraperitoneal injections with a glucose-salt solution. Thus, there was no significant difference in weight between the different treatment groups.

4.4 MC1R signaling pathways (Paper III)

Expression of MC1R mRNA was confirmed in wild-type mouse podocytes with a C_T level of 33 (Table 3). In MC1R-transfected podocytes, gene expression of human MC1R was 84 times greater compared with native murine MC1R mRNA. Over-expression was also confirmed by western blot where two distinct bands were detected from podocytes transfected with MC1R-EGFP, corresponding to the molecular weight of EGFP (29 kDa) and EGFP + MC1R (64 kDa), whereas in control cells only the 29 kDa band, corresponding to EGFP, was detected.

MC1R signaling pathways have been extensively studied in other cell types including melanocytes, but in podocytes the effects of MC1R stimulation remain to be elucidated. Podocytes (both transfected and untransfected) were treated with the synthetic MC1R agonist BMS-470539 in an attempt to reveal the signaling pathways activated by stimulation of this receptor. Firstly, intracellular cAMP levels were increased 4-fold to 44% of maximum response ($P < 0.001$). Secondly, MC1R stimulation induced phosphorylation of ERK1/2 proteins after 4 hours. Phosphorylation was observed in both control and MC1R over-expressed podocytes. Thirdly, MC1R stimulation for 24, 48 and 72 hours increased the activity of catalase. Again, the effect was notable in both control and MC1R over-expressed cells: 14% and 37% compared with control at 48 and 72 hours, respectively ($P < 0.05$).

Rearrangement of the actin cytoskeleton is coupled to foot process effacement *in vivo.* In cell culture, PAN has been extensively used in order to

study these effects.[131,132] The gene expression of MC1R was up-regulated in wild-type podocytes stimulated with two different nephrosis-inducing agents: PAN and adriamycin. Stimulation with PAN resulted in an almost 17-fold increase and adriamycin in a 7-fold increase at 48 and 24 hours respectively, compared with control. When visualizing the actin cytoskeleton in immunohistochemical experiments, 15% of wild-type podocytes subjected to PAN was shown to be unaffected, whereas this number was 24% in the cells stimulated with both PAN and BMS. These results suggest that MC1R agonism can have a protective effect and restore foot process effacement through rearrangement of the actin cytoskeleton.

5 DISCUSSION

The mechanisms behind nephrotic diseases are poorly understood and consequently there are no effective or curative treatment options. Some patients spontaneously go into remission, but the majority will need some kind of treatment. Unfortunately, many patients progress to CKD5 where the only options for survival are dialysis or kidney transplantation.

Previous reports suggest that ACTH has beneficial effects in nephrotic patients with improvement of clinical parameters such as proteinuria and glomerular filtration rate (GFR), although the underlying mechanisms are completely unknown. However, ACTH has Cushingoid side effects due to its cortisol-releasing effect, and a more selective substance would be of great interest. Therefore, the aim of this thesis has been to elucidate the mechanisms behind the beneficial ACTH effect on nephrotic disease and, at least at an experimental level, to point towards a new treatment strategy. In a nutshell: to retain the beneficial ACTH effects while eliminating unwanted side effects.

Initially, we searched for an ACTH receptor, MCR, expressed specifically in the kidney (paper I). When expression of MC1R was confirmed, we proceeded to *in vivo* studies and treated nephrotic rodents with different MCR agonists, including specific MC1R agonists (paper I and II). Finally, we started to unravel the signaling pathways upon MC1R stimulation in podocytes, an important glomerular cell type involved in many nephrotic conditions.

5.1 ACTH ameliorates nephrotic disease in MN patients

The interest in using ACTH as a treatment option for nephrotic patients has risen considerably after the pioneering work of Berg and Arnadottir.[80] Evidence for a beneficial effect has been documented mostly in MN patients where approximately 90% of patients went into complete or partial remission.[81,83,84] Although other patients with other nephrotic diagnoses have also benefitted from ACTH treatment, the effects have been inconclusive and more patients need to be included before definite conclusions can be drawn. In this study, five MN nephrotic patients were treated with ACTH and all went into remission, partial or complete. Since patients were persistently nephrotic for at least 15 months before treatment start, the improvement seen

in these case reports is unlikely to be due to spontaneous remission, which is otherwise the natural outcome for one third in this group. Another important finding was that the effect was sustained with a follow-up of up to 15 months after treatment withdrawal, suggesting that ACTH has a curative effect. All of these findings are consistent with previously reported effects.[82,83,136]

How does ACTH exert its beneficial action on the kidneys? One hypothesis is that it acts through the specific ACTH receptor, MC2R, expressed in the adrenal glands.[113,114] However, there are two main objectives pointing towards another mechanism. Firstly, the cortisol release upon ACTH stimulation is almost one order of magnitude lower than the conventional corticosteroid doses used. Secondly, according to meta analyses, corticosteroids alone are not effective in MN.[85,86] Together, these facts point towards a different mechanism, paving the way for the hypothesis that ACTH exerts its effect through a kidney specific melanocortin receptor.

After the initiation of this project and search for an ACTH receptor in the kidney, an exciting finding was published concerning the antigen forming immune complexes in human MN. The antigen corresponding to megalin in rat was described as the PLA_2R and serum samples contained autoantibodies in 70% of MN patients.[62] ACTH has a curative effect even after initiation of the disease, when the immune complexes have already formed and initiated pathological signaling events in the cells. It seems that ACTH affects one of the already ongoing intracellular signaling processes started by the MAC C5b-9. Therefore, it would be interesting to compare the immune complex formation and disease progression in relation to treatment with ACTH in MN nephrotic patients. These kinds of studies have already been done for other promising treatment strategies, including the anti-B cell antibody Rituximab, where the level of anti-PLA_2R antibodies was shown to correlate with the state of the disease. Those patients who experienced beneficial effects of Rituximab also had lower levels of antibodies.[64] Although Rituximab has many advantages, including no risk for hypertension or nephrotoxic effects, there are still severe side effects that can be potentially fatal.[74] As with ACTH, more randomized controlled trials are needed to conclude its effect in patients.

5.2 Expression of MC1R in kidney

Despite the diverse effects and expression of MCRs, previous knowledge of MCR expression in kidney is sparse. It has been demonstrated that α-MSH inhibits ischemic acute renal failure in mice and rats, probably acting directly on renal tubules through an MCR.[137] While some have demonstrated kidney-

specific expression of MC3R[138,139], others have shown MC4R[138], or MC5R[117,138,140] to be present. There is one previous report on MC1R expression in kidney[139].

In this thesis, the renal expression of all five MCRs was investigated by quantitative real-time PCR (paper I). In contrast to many others, we found glomerular expression of MC1R but failed to detect other MCRs. These differences could partly be explained by the analysis methods and the quality of the analyzed material. We have used RNA of excellent quality and determined the gene expression with a more sensitive method. We thoroughly investigated MCR expression in all glomerular cell types of human, rat and mouse origin. An interesting finding was that the gene expression of MC1R was up-regulated in mouse podocytes stimulated with adriamycin or PAN. This might reflect a response to harmful stimuli or an anti-inflammatory response by the podocyte. MC1R gene expression was not up-regulated in adriamycin-treated mice 12 days after disease induction. However, an early response and up-regulation of the gene cannot be excluded, and there could be a time-dependent regulation that we observed *in vitro* but failed to detect *in vivo*.

We also demonstrated that the MC1R protein, a transmembrane receptor, co-localized with synaptopodin in podocytes. It would be very interesting to further investigate the exact location in podocytes: Is it connected to proteins related to the actin cytoskeleton? Is it expressed in the slit diaphragm? The position could give more clues to its role in a potentially protective mechanism/pathway in nephrotic diseases.

5.3 MC1R agonists ameliorate nephrotic disease in experimental MN

Based on the finding that MC1R is present in kidney, more specifically in glomeruli and podocytes, the next step was to evaluate MC1R agonists as a treatment option in experimental nephrotic models (paper I and II). The best evidence that ACTH ameliorates nephrotic disease comes from MN patients, but there is also a very good experimental model resembling MN: the passive Heymann nephritis (PHN) model in rats. Nephrotic rats were treated with different MCR agonists including: ACTH, an agonist for all MCRs; α-MSH, an agonist for all MCRs except MC2R; and MS05, a specific MC1R agonist. All rats were nephrotic before treatment was started, resembling the natural course in patients. There was a tendency that ACTH reduced proteinuria although it did not reach statistical significance. Treatment with both α-MSH

and MS05 resulted in a 50-60% reduction in proteinuria, the latter suggesting a specific effect mediated by MC1R. These two agonists also improved glomerular morphology, including reestablishing the podocyte foot processes, and reduced oxidative stress measured as levels of lipid peroxidation products. In similarity with clinical observations, the effect remained after treatment withdrawal.

A pilot study was performed in order to establish an optimal treatment dose and it was clear that higher doses of ACTH caused side effects. Therefore, a low dose was chosen, and the other MCR agonists were administered in equimolar doses. The fact that ACTH did not result in a better effect than observed is therefore not surprising. What is noteworthy is that the other two agonists had such remarkable effects in equimolar doses. These results suggest that patients would benefit from a specific MC1R agonist, considering the severe side-effects caused by effective doses of ACTH.

It seems unlikely that the major beneficial effects of ACTH would be mediated through MC1R expressed on immune cells, since reports indicate that there is no involvement of inflammatory cells in MN[141]. Oxidative stress is probably involved, and indeed we showed that TBARS were decreased in MC1R agonist treated PHN compared with untreated PHN rats. In line with our results, it has previously been shown in experimental MN that C5b-9 MAC formation results in formation of ROS associated with lipid peroxidation.[55,56] Another potential mechanism includes an anti-inflammatory response. In immune cells, it has been shown that α-MSH inhibits NF-κB translocation to the nucleus, a cAMP mediated process.[103] We showed that MC1R is mainly expressed in podocytes and it is possible that MC1R stimulation has similar anti-inflammatory effects in these cells. It has already been suggested that nephrin deficiency leads to activation of NF-κB.[142] Down-regulation of nephrin, a slit diaphragm protein associated with the actin cytoskeleton, is seen in experimental MN[57,143], and this potential mechanism would be interesting to investigate further. Possibly, MC1R stimulation in different cell types contributes to the beneficial effects. Hence both antibody production by B cells and antigen presentation on the podocyte surface could be affected in MN.[144]

5.4 Lack of effect by MC1R agonists in experimental FSGS

ACTH has been shown to be beneficial primarily in MN patients, whereas the effects in other nephrotic diagnoses have been less studied.[84] We therefore

thought it would be interesting to evaluate the effects in another experimental model besides MN and treated mice with adriamycin-induced nephropathy, FSGS-resembling, with MCR agonists (paper II). Neither the unspecific MCR agonist α-MSH, nor the specific MC1R agonist BMS-470539, resulted in a decreased level of proteinuria. No morphological improvement was seen in treated versus untreated mice. There are a few possible explanations to this finding, and they will be further discussed below.

The first, and perhaps most intriguing explanation, is that there are distinct mechanisms underlying the diseases resulting in MN and FSGS, respectively. As previously mentioned, more information on the pathological mechanisms underlying MN has been become available in the last decades, showing that immune complex formation and assembly of the C5b-9 MAC are key signaling events. Inflammatory cells are not present in experimental MN[141] whereas there is an infiltration in experimental FSGS[129]. Although the podocyte is of great importance in both diseases, a different nature of the cell injury could explain the different treatment effects. In both diseases there is foot process effacement due to actin reorganization. In addition, FSGS is associated with a prominent podocyte depletion[145], probably a consequence of the TGF-β up-regulation, seen in both patients[146] and experimental models[147,148]. The podocyte injury in MN is perhaps coupled primarily to structural rearrangements of the actin cytoskeleton while loss of podocytes due to detachment and apoptosis are the major effects in FSGS.

Another explanation of the different effects of MCR agonists in the two experimental models reflects the models themselves. PHN is well characterized and the molecular mechanisms seem to fit well with clinical MN. Adriamycin, on the other hand, is originally an anticancer drug that induces nephropathy. It is a more crude model using a nephrotoxin with severe side effects including body weight loss. As reviewed by Lee et al., the therapeutic window is very narrow and strain-dependent (9.8 – 10.4 mg/kg in BALB/c mice) and batch-to-batch effects have large impact on the disease severity.[149] The level of proteinuria in our adriamycin model was very high, and we are considering that the effect is too severe to be able to treat. Reflecting the possibility of a structure-based – as opposed to apoptotic - MC1R agonist effect, it could well be that we are unable to detect any improvement in kidney function because of too high doses in our experimental set up. Furthermore, FSGS is not easily diagnosed in the clinic. Consequently, some patients with minimal change disease that do not respond to corticosteroids have later been diagnosed with FSGS.

A final argument for the lack of effect in the FSGS model lies in the sequence homology of MC1R between species; the sequence identity is only 76% between mouse and man[106]. As discussed below there is also a difference in the response to receptor stimulation in mouse compared with man.

5.5 MC1R signaling pathways in podocytes

MC1R has been extensively studied in melanocytes and immune cells. Our finding that MC1R is expressed in podocytes led us to explore the possible signaling pathways in this cell type (paper III, Figure 8). It has been shown that mouse MC1R may not respond in the same way to stimuli as the human MC1R.[150] Therefore, we used a system to over-express the receptor in immortalized mouse podocytes, which are cells that well resemble the human podocytes[151]. In this way, we were able to investigate specific signaling pathways in the podocyte after MC1R stimulation. Overall, we found that MC1R stimulation resulted in distinct intracellular responses that seem to be coupled to the actin cytoskeleton. The question is: how do these signaling pathways affect the healthy versus diseased podocyte?

One of the first events after MC1R stimulation was increased levels of intracellular cAMP. This pathway has been described in podocytes and following MC1R stimulation in other cell types, but never in this unique combination. The family of MAPKs including ERK1/2, JNK and p38, are important for cell proliferation and apoptosis and also for cytoskeletal stability.[152-154] We found that MC1R stimulation resulted in a time-dependent phosphorylation of ERK1/2. It has been shown that podocytes stimulated with PAN and adriamycin also increased the levels of phosphorylated ERK1/2 and that this effect is coupled to podocyte injury in PAN-treated animals.[152,153] However, yet another report suggests that phosphorylation protects against PAN-induced apoptosis in mouse podocytes.[155] In conclusion, these observations indicate that the outcome of ERK1/2 phosphorylation is context-dependent.

Nephrotic syndrome is associated with oxidative stress, and anti-oxidative enzymes have been shown to be down-regulated in glomeruli from both human nephrotic patients and PAN-treated rats.[156] Catalase is expressed in many tissues, including kidney, and an increased catalase activity strengthens the defense against oxygen radical formation.[157] It has previously been shown that MC1R stimulation leads to an increased catalase activity in melanoma cells[158] and in UV exposed melanocytes[108]. In this thesis, we showed a similar response in podocytes; catalase activity was up-regulated up to 72 hours after MC1R stimulation. Together with the finding that MC1R agonists reduce the

level of TBARS in PHN rats, these results suggest that MC1R stimulation mediates oxidative stress-related protective effects.

Nephrotic diseases and podocytopathies are often coupled to rearrangement of the actin cytoskeleton. A crucial question is therefore if MC1R stimulation has any impact at this level? So far, we have only briefly investigated the effects of MC1R stimulation, but we have seen a tendency towards protective effects against PAN-induced actin cytoskeleton rearrangement. Further experiments need to be performed in order to conclude the role of MC1R signaling in this aspect. As mentioned previously, it would be important and interesting to determine the exact location of MC1R in podocytes, Does MC1R interact with slit diaphragm proteins such as nephrin or podocin? Is there a connection with the actin cytoskeleton or cytoskeleton associated proteins?

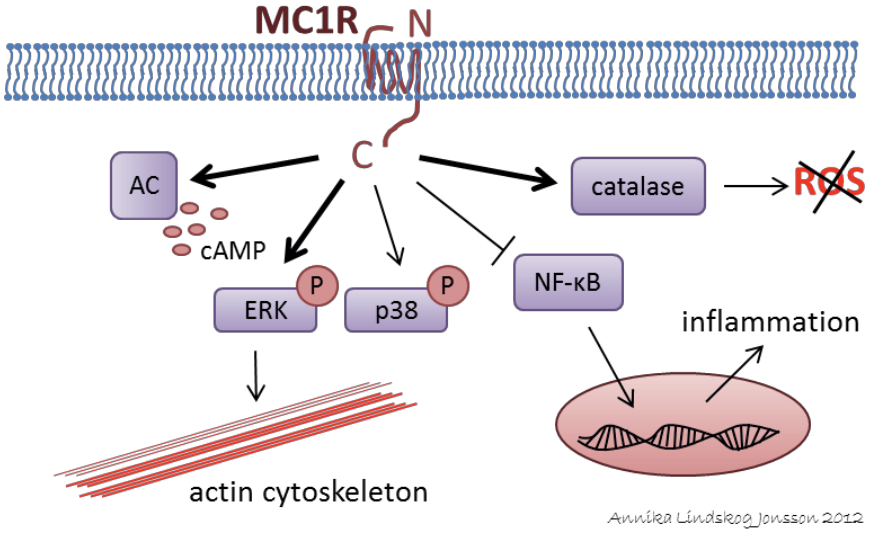

Figure 8. Schematic figure of MC1R signaling pathways in the podocyte. Thin arrows are previously known and described pathways in other cell types, thick arrows demonstrate the activated pathways shown in this thesis. AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, ERK = extracellular signal-regulated kinase, NF-κB = nuclear factor κB, ROS = reactive oxygen species.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The major finding in this thesis is that MC1R agonists reduce proteinuria in an experimental model of nephrotic syndrome. This effect is probably podocyte-mediated and involves rearrangement of the actin cytoskeleton and reduction of oxidative stress. Stimulation of MC1R in podocytes was found to activate similar signaling pathways, both anti-inflammatory and anti-oxidative, as previously reported in other cell types.

The first aim of this thesis was to define the renal expression of the ACTH receptors, MCRs. To our knowledge, we are the first to demonstrate expression of MC1R in glomeruli, specifically in podocytes. Further studies will reveal the exact location, probably cell membrane specific, and if there is an association with other slit diaphragm proteins, such as nephrin and podocin, or connection with other actin cytoskeleton related proteins.

The second aim was to evaluate specific MC1R agonists as a treatment option in different nephrotic diseases. As mentioned above, MC1R agonists had beneficial effects in experimental MN and the effect remained after treatment withdrawal. Further optimization studies are needed to determine the ideal dose, way of administration and treatment time. The next step would be to treat patients with this more selective agonist type, which should have milder side effects, such as increased pigmentation, compared with ACTH. Today, there is no selective agonist available as treatment option. However, α-MSH, the endogenous hormone, could serve as a starting point since it stimulates MC1R but not MC2R, hence the beneficial effects on the kidney would be kept, but unwanted MC2R related side effects would be lost. MC1R agonists were also tested in an experimental model resembling FSGS, but did not seem beneficial in this disease which could be explained by the different disease mechanisms.

The last aim was to identify key signaling pathways involved in MC1R signaling, specifically in podocytes. Our data so far suggests that MC1R signaling protect the actin cytoskeleton, which is of great importance for an intact and functional podocyte. The mechanisms behind this connection remain to be defined. Future studies will reveal if the signaling pathways involved are unique to podocytes and how the activated pathways are related to inflammation and/or oxidative stress. Since cross-talk between the

glomerular cell types seem to be of importance, such studies require both *in vitro* as well as *in vivo* models.

In the end, the most important question is what kind of clinical implications the results from this thesis will have. How can patients benefit from these findings? The results suggest that selective MC1R agonists are a future treatment option for nephrotic patients, who will not suffer from the severe ACTH Cushingoid-like side effects but will still benefit from the kidney effects. To conclude, the results from this thesis indicate that selective MC1R agonists do have beneficial effects in nephrotic disease.

ACKNOWLEDGEMENTS

Till sist skulle jag vilja tacka alla kollegor, familj och vänner som hjälpt mig under de här åren. Ett särskilt tack till:

Mina handledare, **Börje Haraldsson** för din entusiasm och ständigt positiva tänkande, **Jenny Nyström** för all stöttning och peppning samt goda råd. Tack för ert engagemang och all kunskap ni delat med er av!

Anna Granqvist, min bihandledare, det sista året hade aldrig gått utan dig! Tack för gott samarbete och stöd.

Mina medförfattare **Bergur Stéfansson**, **Margret Arnadottir**, **Anna-Lena Berg** och **Martin Johansson** för kliniska perspektiv och bra samarbete.

Annika Åstrand, min mentor, för goda råd och trevliga luncher.

Njurgruppen: **Heidi** för alla goda fikor och bus, **Kerstin** för galna och roliga upptåg, **Johannes** för gott samarbete, **Ulf** för värdefull skrivhjälp, **Madeleine** för all hjälp på labbet när jag var ny, **Vincent** för trevligt sällskap vid HPLCn**, Peidi** och **Deman** mina western kompisar, **Lisa** och **Jennie** för trevligt sällskap, **Marie** för ELISA-support, **Emelie**, **Katarina** och **Paula** för all hjälp med djurförsöken, **Christel** för all administrativ hjälp.

Alla på **Wallenberglab** för en inspirerande arbetsmiljö. Särskilt tack till **Magnus**, **Merja** och **Christina** för all hjälp med det praktiska och **Svenne** för IT-support. Kollegor och rumskamrater på **Fysiologen** för nya kunskaper på labb och vid undervisning.

Annika, Anna-Karin, **Karin**, **Frida O** och **Frida L** för att ni hjälpte mig med en bra start i forskningskarriären och alla våra mysiga after works.

Alla mina vänner för perspektiv på tillvaron.

Min extrafamilj **Carin** och **Björn** samt **Thomas** och **Jenny**, tack för all hjälp och trevligt sällskap de senaste åren.

Mamma och **Pappa**, tack för allt! Mina syskon **Elisabeth** och **Henrik med familjer** som jag alltid har så roligt med!

Min fina familj: **Maja**, min lilla solstråle; **Mathias**, min klippa och trygghet, tack för att du alltid stöttar mig och finns vid min sida!

REFERENCES

1. Aktiv uremivård i Sverige 1991-2010. *Swedish Renal Registry* (2011).
2. Haraldsson, B., Nystrom, J. & Deen, W.M. Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol Rev* **88**, 451-487 (2008).
3. Chang, R.L.*, et al.* Permselectivity of the glomerular capillary wall to macromolecules. II. Experimental studies in rats using neutral dextran. *Biophys J* **15**, 887-906 (1975).
4. Chang, R.L., Deen, W.M., Robertson, C.R. & Brenner, B.M. Permselectivity of the glomerular capillary wall: III. Restricted transport of polyanions. *Kidney international* **8**, 212-218 (1975).
5. Sorensson, J., Ohlson, M., Lindstrom, K. & Haraldsson, B. Glomerular charge selectivity for horseradish peroxidase and albumin at low and normal ionic strengths. *Acta Physiol Scand* **163**, 83-91 (1998).
6. Ohlson, M.*, et al.* Effects of filtration rate on the glomerular barrier and clearance of four differently shaped molecules. *Am J Physiol Renal Physiol* **281**, F103-113 (2001).
7. Kalluri, R. Proteinuria with and without renal glomerular podocyte effacement. *J Am Soc Nephrol* **17**, 2383-2389 (2006).
8. Jarad, G. & Miner, J.H. Update on the glomerular filtration barrier. *Current opinion in nephrology and hypertension* **18**, 226-232 (2009).
9. Eremina, V.*, et al.* Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *The Journal of clinical investigation* **111**, 707-716 (2003).
10. Hjalmarsson, C., Johansson, B.R. & Haraldsson, B. Electron microscopic evaluation of the endothelial surface layer of glomerular capillaries. *Microvasc Res* **67**, 9-17 (2004).
11. Jeansson, M. & Haraldsson, B. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *American journal of physiology. Renal physiology* **290**, F111-116 (2006).
12. Friden, V.*, et al.* The glomerular endothelial cell coat is essential for glomerular filtration. *Kidney international* **79**, 1322-1330 (2011).
13. Singh, A.*, et al.* Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. *J Am Soc Nephrol* **18**, 2885-2893 (2007).
14. Jeansson, M., Bjorck, K., Tenstad, O. & Haraldsson, B. Adriamycin alters glomerular endothelium to induce proteinuria. *J Am Soc Nephrol* **20**, 114-122 (2009).
15. Salmon, A.H.*, et al.* Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. *Journal of the American Society of Nephrology : JASN* **23**, 1339-1350 (2012).
16. Bulger, R.E., Eknoyan, G., Purcell, D.J., 2nd & Dobyan, D.C. Endothelial characteristics of glomerular capillaries in normal, mercuric chloride-induced, and gentamicin-induced acute renal failure in the rat. *The Journal of clinical investigation* **72**, 128-141 (1983).

17. Rostgaard, J. & Qvortrup, K. Sieve plugs in fenestrae of glomerular capillaries--site of the filtration barrier? *Cells, tissues, organs* **170**, 132-138 (2002).
18. Maynard, S.E., Venkatesha, S., Thadhani, R. & Karumanchi, S.A. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. *Pediatr Res* **57**, 1R-7R (2005).
19. Weber, M. Basement membrane proteins. *Kidney international* **41**, 620-628 (1992).
20. Daniels, B.S., Hauser, E.B., Deen, W.M. & Hostetter, T.H. Glomerular basement membrane: in vitro studies of water and protein permeability. *The American journal of physiology* **262**, F919-926 (1992).
21. Deen, W.M., Lazzara, M.J. & Myers, B.D. Structural determinants of glomerular permeability. *American journal of physiology. Renal physiology* **281**, F579-596 (2001).
22. Barker, D.F.*, et al.* Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science* **248**, 1224-1227 (1990).
23. Lemmink, H.H.*, et al.* Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome. *Human molecular genetics* **3**, 1269-1273 (1994).
24. Mochizuki, T.*, et al.* Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome. *Nat Genet* **8**, 77-81 (1994).
25. Wasserstein, A.G. Membranous glomerulonephritis. *J Am Soc Nephrol* **8**, 664-674 (1997).
26. Adler, S. Diabetic nephropathy: Linking histology, cell biology, and genetics. *Kidney international* **66**, 2095-2106 (2004).
27. Adler, S. Characterization of glomerular epithelial cell matrix receptors. *The American journal of pathology* **141**, 571-578 (1992).
28. Chen, S.*, et al.* Podocytes require the engagement of cell surface heparan sulfate proteoglycans for adhesion to extracellular matrices. *Kidney international* **78**, 1088-1099 (2010).
29. Jarad, G., Pippin, J.W., Shankland, S.J., Kreidberg, J.A. & Miner, J.H. Dystroglycan does not contribute significantly to kidney development or function, in health or after injury. *American journal of physiology. Renal physiology* **300**, F811-820 (2011).
30. Mundel, P. & Kriz, W. Structure and function of podocytes: an update. *Anat Embryol (Berl)* **192**, 385-397 (1995).
31. Kestila, M.*, et al.* Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell* **1**, 575-582 (1998).
32. Putaala, H., Soininen, R., Kilpelainen, P., Wartiovaara, J. & Tryggvason, K. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Human molecular genetics* **10**, 1-8 (2001).
33. Machuca, E.*, et al.* Clinical and epidemiological assessment of steroid-resistant nephrotic syndrome associated with the NPHS2 R229Q variant. *Kidney international* **75**, 727-735 (2009).

34. McKenzie, L.M., *et al.* NPHS2 variation in sporadic focal segmental glomerulosclerosis. *Journal of the American Society of Nephrology : JASN* **18**, 2987-2995 (2007).
35. Mollet, G., *et al.* Podocin inactivation in mature kidneys causes focal segmental glomerulosclerosis and nephrotic syndrome. *Journal of the American Society of Nephrology : JASN* **20**, 2181-2189 (2009).
36. Mundel, P., *et al.* Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J Cell Biol* **139**, 193-204 (1997).
37. Mundel, P., Gilbert, P. & Kriz, W. Podocytes in glomerulus of rat kidney express a characteristic 44 KD protein. *J Histochem Cytochem* **39**, 1047-1056 (1991).
38. Asanuma, K., *et al.* Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat Cell Biol* **8**, 485-491 (2006).
39. Whiteside, C.I., Cameron, R., Munk, S. & Levy, J. Podocytic cytoskeletal disaggregation and basement-membrane detachment in puromycin aminonucleoside nephrosis. *Am J Pathol* **142**, 1641-1653 (1993).
40. Greka, A. & Mundel, P. Cell biology and pathology of podocytes. *Annu Rev Physiol* **74**, 299-323 (2012).
41. Ryan, G.B. & Karnovsky, M.J. An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. *Kidney international* **8**, 219-232 (1975).
42. Svenningsen, P., *et al.* Plasmin in nephrotic urine activates the epithelial sodium channel. *J Am Soc Nephrol* **20**, 299-310 (2009).
43. Ponticelli, C. Membranous nephropathy. *J Nephrol* **20**, 268-287 (2007).
44. KDIGO. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. KDIGO Clinical Practice Guideline for Glomerulonephritis. *Kidney international* **Suppl. 2**, 139–274 (2012).
45. Schieppati, A., *et al.* Prognosis of untreated patients with idiopathic membranous nephropathy. *The New England journal of medicine* **329**, 85-89 (1993).
46. Polanco, N., *et al.* Spontaneous remission of nephrotic syndrome in idiopathic membranous nephropathy. *Journal of the American Society of Nephrology : JASN* **21**, 697-704 (2010).
47. Heymann, W., Hackel, D.B., Harwood, S., Wilson, S.G. & Hunter, J.L. Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. *Proc Soc Exp Biol Med* **100**, 660-664 (1959).
48. Kerjaschki, D. & Farquhar, M.G. The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 5557-5561 (1982).
49. Kerjaschki, D., Miettinen, A. & Farquhar, M.G. Initial events in the formation of immune deposits in passive Heymann nephritis. gp330-anti-gp330 immune complexes form in epithelial coated pits and rapidly become attached to the glomerular basement membrane. *J Exp Med* **166**, 109-128 (1987).
50. Saito, A., Pietromonaco, S., Loo, A.K. & Farquhar, M.G. Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9725-9729 (1994).

51. Couser, W.G., Steinmuller, D.R., Stilmant, M.M., Salant, D.J. & Lowenstein, L.M. Experimental glomerulonephritis in the isolated perfused rat kidney. *The Journal of clinical investigation* **62**, 1275-1287 (1978).
52. Van Damme, B.J., Fleuren, G.J., Bakker, W.W., Vernier, R.L. & Hoedemaeker, P.J. Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab Invest* **38**, 502-510 (1978).
53. Cybulsky, A.V., Quigg, R.J. & Salant, D.J. The membrane attack complex in complement-mediated glomerular epithelial cell injury: formation and stability of C5b-9 and C5b-7 in rat membranous nephropathy. *Journal of immunology* **137**, 1511-1516 (1986).
54. Cybulsky, A.V., Rennke, H.G., Feintzeig, I.D. & Salant, D.J. Complement-induced glomerular epithelial cell injury. Role of the membrane attack complex in rat membranous nephropathy. *The Journal of clinical investigation* **77**, 1096-1107 (1986).
55. Neale, T.J.*, et al.* Reactive oxygen species and neutrophil respiratory burst cytochrome b558 are produced by kidney glomerular cells in passive Heymann nephritis. *Proc Natl Acad Sci U S A* **90**, 3645-3649 (1993).
56. Neale, T.J.*, et al.* Proteinuria in passive Heymann nephritis is associated with lipid peroxidation and formation of adducts on type IV collagen. *J Clin Invest* **94**, 1577-1584 (1994).
57. Nakatsue, T.*, et al.* Nephrin and podocin dissociate at the onset of proteinuria in experimental membranous nephropathy. *Kidney international* **67**, 2239-2253 (2005).
58. Topham, P.S., Haydar, S.A., Kuphal, R., Lightfoot, J.D. & Salant, D.J. Complement-mediated injury reversibly disrupts glomerular epithelial cell actin microfilaments and focal adhesions. *Kidney international* **55**, 1763-1775 (1999).
59. Takano, T., Cybulsky, A.V., Yang, X. & Aoudjit, L. Complement C5b-9 induces cyclooxygenase-2 gene transcription in glomerular epithelial cells. *American journal of physiology. Renal physiology* **281**, F841-850 (2001).
60. Aoudjit, L., Stanciu, M., Li, H., Lemay, S. & Takano, T. p38 mitogen-activated protein kinase protects glomerular epithelial cells from complement-mediated cell injury. *Am J Physiol Renal Physiol* **285**, F765-774 (2003).
61. Cybulsky, A.V., Papillon, J. & McTavish, A.J. Complement activates phospholipases and protein kinases in glomerular epithelial cells. *Kidney international* **54**, 360-372 (1998).
62. Beck, L.H., Jr.*, et al.* M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *The New England journal of medicine* **361**, 11-21 (2009).
63. Hofstra, J.M., Beck, L.H., Jr., Beck, D.M., Wetzels, J.F. & Salant, D.J. Anti-phospholipase A(2) receptor antibodies correlate with clinical status in idiopathic membranous nephropathy. *Clin J Am Soc Nephrol* **6**, 1286-1291 (2011).
64. Beck, L.H., Jr.*, et al.* Rituximab-induced depletion of anti-PLA2R autoantibodies predicts response in membranous nephropathy. *Journal of the American Society of Nephrology : JASN* **22**, 1543-1550 (2011).

65. Braden, G.L., *et al.* Changing incidence of glomerular diseases in adults. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **35**, 878-883 (2000).
66. Gbadegesin, R., Lavin, P., Foreman, J. & Winn, M. Pathogenesis and therapy of focal segmental glomerulosclerosis: an update. *Pediatric nephrology* **26**, 1001-1015 (2011).
67. Kaplan, J.M., *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* **24**, 251-256 (2000).
68. Kim, J.M., *et al.* CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* **300**, 1298-1300 (2003).
69. Dai, S., *et al.* Functional analysis of promoter mutations in the ACTN4 and SYNPO genes in focal segmental glomerulosclerosis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **25**, 824-835 (2010).
70. Wei, C., *et al.* Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat Med* **17**, 952-960 (2011).
71. Brantsma, A.H., *et al.* Urinary albumin excretion and its relation with C-reactive protein and the metabolic syndrome in the prediction of type 2 diabetes. *Diabetes Care* **28**, 2525-2530 (2005).
72. Halbesma, N., *et al.* Macroalbuminuria is a better risk marker than low estimated GFR to identify individuals at risk for accelerated GFR loss in population screening. *Journal of the American Society of Nephrology : JASN* **17**, 2582-2590 (2006).
73. Ponticelli, C., *et al.* Controlled trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. *The New England journal of medicine* **310**, 946-950 (1984).
74. Waldman, M. & Austin, H.A., 3rd. Treatment of Idiopathic Membranous Nephropathy. *Journal of the American Society of Nephrology : JASN* (2012).
75. Schacke, H., Docke, W.D. & Asadullah, K. Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & therapeutics* **96**, 23-43 (2002).
76. Cravedi, P., Ruggenenti, P. & Remuzzi, G. Intensified inhibition of renin-angiotensin system: a way to improve renal protection? *Current hypertension reports* **11**, 118-124 (2009).
77. Charlton, D., *et al.* The nephrotic syndrome; observations of the effects of A.C.T.H. in 40 patients. *Acta Med Scand* **161**, 33-56 (1958).
78. Cameron, J.S. Immunosuppressant agents in the treatment of glomerulonephritis. 1. Corticosteroid drugs. *J R Coll Physicians Lond* **5**, 282-296 (1971).
79. Pachioli, R. & Genova, R. Long-term steroid-immunosuppressive treatment of the childhood nephrotic syndrome. *Pediatrics* **47**, 731-736 (1971).
80. Berg, A.L., Nilsson-Ehle, P. & Arnadottir, M. Beneficial effects of ACTH on the serum lipoprotein profile and glomerular function in patients with membranous nephropathy. *Kidney international* **56**, 1534-1543 (1999).
81. Berg, A.L. & Arnadottir, M. ACTH-induced improvement in the nephrotic syndrome in patients with a variety of diagnoses. *Nephrol Dial Transplant* **19**, 1305-1307 (2004).

82. Picardi, L.*, et al.* ACTH therapy in nephrotic syndrome induced by idiopathic membranous nephropathy. *Clinical nephrology* **62**, 403-404 (2004).
83. Ponticelli, C.*, et al.* A randomized pilot trial comparing methylprednisolone plus a cytotoxic agent versus synthetic adrenocorticotropic hormone in idiopathic membranous nephropathy. *Am J Kidney Dis* **47**, 233-240 (2006).
84. Bomback, A.S.*, et al.* Treatment of nephrotic syndrome with adrenocorticotropic hormone (ACTH) gel. *Drug Des Devel Ther* **5**, 147-153 (2011).
85. Hogan, S.L., Muller, K.E., Jennette, J.C. & Falk, R.J. A review of therapeutic studies of idiopathic membranous glomerulopathy. *Am J Kidney Dis* **25**, 862-875 (1995).
86. Perna, A.*, et al.* Immunosuppressive treatment for idiopathic membranous nephropathy: a systematic review. *Am J Kidney Dis* **44**, 385-401 (2004).
87. Eberle, A. *The Melanotropins*, (Karger, Basel, 1988).
88. Benjannet, S., Rondeau, N., Day, R., Chretien, M. & Seidah, N.G. PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 3564-3568 (1991).
89. Catania, A., Gatti, S., Colombo, G. & Lipton, J.M. Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacological reviews* **56**, 1-29 (2004).
90. Getting, S.J. Targeting melanocortin receptors as potential novel therapeutics. *Pharmacology & therapeutics* **111**, 1-15 (2006).
91. Schioth, H.B.*, et al.* Remarkable synteny conservation of melanocortin receptors in chicken, human, and other vertebrates. *Genomics* **81**, 504-509 (2003).
92. Wikberg, J.E.*, et al.* New aspects on the melanocortins and their receptors. *Pharmacol Res* **42**, 393-420 (2000).
93. Sawyer, T.K.*, et al.* 4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 5754-5758 (1980).
94. Szardenings, M., Muceniece, R., Mutule, I., Mutulis, F. & Wikberg, J.E. New highly specific agonistic peptides for human melanocortin MC(1) receptor. *Peptides* **21**, 239-243 (2000).
95. Herpin, T.F.*, et al.* Discovery of tyrosine-based potent and selective melanocortin-1 receptor small-molecule agonists with anti-inflammatory properties. *Journal of medicinal chemistry* **46**, 1123-1126 (2003).
96. Bitensky, M.W. & Burstein, S.R. Effects of cyclic adenosine monophosphate and melanocyte-stimulating hormone on frog skin in vitro. *Nature* **208**, 1282-1284 (1965).
97. Busca, R. & Ballotti, R. Cyclic AMP a key messenger in the regulation of skin pigmentation. *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society* **13**, 60-69 (2000).
98. Harris, M.*, et al.* Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. *The Journal of clinical investigation* **107**, 111-120 (2001).

99. Sarkar, S., Legradi, G. & Lechan, R.M. Intracerebroventricular administration of alpha-melanocyte stimulating hormone increases phosphorylation of CREB in TRH- and CRH-producing neurons of the hypothalamic paraventricular nucleus. *Brain research* **945**, 50-59 (2002).
100. Gerst, J.E., Sole, J. & Salomon, Y. Dual regulation of beta-melanotropin receptor function and adenylate cyclase by calcium and guanosine nucleotides in the M2R melanoma cell line. *Mol Pharmacol* **31**, 81-88 (1987).
101. Smalley, K. & Eisen, T. The involvement of p38 mitogen-activated protein kinase in the alpha-melanocyte stimulating hormone (alpha-MSH)-induced melanogenic and anti-proliferative effects in B16 murine melanoma cells. *FEBS Lett* **476**, 198-202 (2000).
102. Englaro, W.*, et al.* Mitogen-activated protein kinase pathway and AP-1 are activated during cAMP-induced melanogenesis in B-16 melanoma cells. *The Journal of biological chemistry* **270**, 24315-24320 (1995).
103. Manna, S.K. & Aggarwal, B.B. Alpha-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-kappa B activation induced by various inflammatory agents. *Journal of immunology* **161**, 2873-2880 (1998).
104. Haycock, J.W.*, et al.* Alpha-melanocyte-stimulating hormone inhibits NF-kappaB activation in human melanocytes and melanoma cells. *The Journal of investigative dermatology* **113**, 560-566 (1999).
105. Chhajlani, V. & Wikberg, J.E. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett* **309**, 417-420 (1992).
106. Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. & Cone, R.D. The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248-1251 (1992).
107. Palmer, J.S.*, et al.* Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? *Am J Hum Genet* **66**, 176-186 (2000).
108. Song, X.*, et al.* alpha-MSH activates immediate defense responses to UV-induced oxidative stress in human melanocytes. *Pigment Cell Melanoma Res* **22**, 809-818 (2009).
109. Kang, L.*, et al.* A selective small molecule agonist of the melanocortin-1 receptor inhibits lipopolysaccharide-induced cytokine accumulation and leukocyte infiltration in mice. *Journal of leukocyte biology* **80**, 897-904 (2006).
110. Leoni, G.*, et al.* The melanocortin MC(1) receptor agonist BMS-470539 inhibits leucocyte trafficking in the inflamed vasculature. *British journal of pharmacology* **160**, 171-180 (2010).
111. Neumann Andersen, G.*, et al.* MC(1) receptors are constitutively expressed on leucocyte subpopulations with antigen presenting and cytotoxic functions. *Clin Exp Immunol* **126**, 441-446 (2001).
112. Veo, K.*, et al.* Observations on the ligand selectivity of the melanocortin 2 receptor. *General and comparative endocrinology* **172**, 3-9 (2011).
113. Lefkowitz, R.J., Roth, J., Pricer, W. & Pastan, I. ACTH receptors in the adrenal: specific binding of ACTH-125I and its relation to adenyl cyclase. *Proc Natl Acad Sci U S A* **65**, 745-752 (1970).

114. Buckley, D.I. & Ramachandran, J. Characterization of corticotropin receptors on adrenocortical cells. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 7431-7435 (1981).
115. Gantz, I.*, et al.* Molecular cloning of a novel melanocortin receptor. *The Journal of biological chemistry* **268**, 8246-8250 (1993).
116. Butler, A.A.*, et al.* A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* **141**, 3518-3521 (2000).
117. Chhajlani, V. Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochemistry and molecular biology international* **38**, 73-80 (1996).
118. Guarini, S.*, et al.* MC(3) receptors are involved in the protective effect of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias. *Naunyn Schmiedebergs Arch Pharmacol* **366**, 177-182 (2002).
119. Getting, S.J., Gibbs, L., Clark, A.J., Flower, R.J. & Perretti, M. POMC gene-derived peptides activate melanocortin type 3 receptor on murine macrophages, suppress cytokine release, and inhibit neutrophil migration in acute experimental inflammation. *J Immunol* **162**, 7446-7453 (1999).
120. Getting, S.J., Allcock, G.H., Flower, R. & Perretti, M. Natural and synthetic agonists of the melanocortin receptor type 3 possess anti-inflammatory properties. *Journal of leukocyte biology* **69**, 98-104 (2001).
121. Getting, S.J., Christian, H.C., Flower, R.J. & Perretti, M. Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. *Arthritis and rheumatism* **46**, 2765-2775 (2002).
122. Getting, S.J.*, et al.* Redundancy of a functional melanocortin 1 receptor in the anti-inflammatory actions of melanocortin peptides: studies in the recessive yellow (e/e) mouse suggest an important role for melanocortin 3 receptor. *J Immunol* **170**, 3323-3330 (2003).
123. Gantz, I.*, et al.* Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *The Journal of biological chemistry* **268**, 15174-15179 (1993).
124. Huszar, D.*, et al.* Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* **88**, 131-141 (1997).
125. Gantz, I.*, et al.* Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochemical and biophysical research communications* **200**, 1214-1220 (1994).
126. Chen, W.*, et al.* Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* **91**, 789-798 (1997).
127. Farquhar, M.G., Saito, A., Kerjaschki, D. & Orlando, R.A. The Heymann nephritis antigenic complex: megalin (gp330) and RAP. *J Am Soc Nephrol* **6**, 35-47 (1995).
128. Salant, D.J. & Cybulsky, A.V. Experimental glomerulonephritis. *Methods Enzymol* **162**, 421-461 (1988).
129. Wang, Y., Wang, Y.P., Tay, Y.C. & Harris, D.C. Progressive adriamycin nephropathy in mice: sequence of histologic and immunohistochemical events. *Kidney international* **58**, 1797-1804 (2000).

130. Mundel, P*., et al.* Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp Cell Res* **236**, 248-258 (1997).
131. Ransom, R.F., Lam, N.G., Hallett, M.A., Atkinson, S.J. & Smoyer, W.E. Glucocorticoids protect and enhance recovery of cultured murine podocytes via actin filament stabilization. *Kidney international* **68**, 2473-2483 (2005).
132. Saleem, M.A*., et al.* Co-localization of nephrin, podocin, and the actin cytoskeleton: evidence for a role in podocyte foot process formation. *Am J Pathol* **161**, 1459-1466 (2002).
133. Liu, H*., et al.* alpha-Actinin-4 is involved in the process by which dexamethasone protects actin cytoskeleton stabilization from adriamycin-induced podocyte injury. *Nephrology (Carlton, Vic* (2012).
134. Birn, H*., et al.* Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. *J Clin Invest* **105**, 1353-1361 (2000).
135. Jeansson, M. & Haraldsson, B. Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. *J Am Soc Nephrol* **14**, 1756-1765 (2003).
136. Berg, A., Stefánsson, B. & Arnadottir, M. A randomized, controlled study on treatment with adrenocorticotropic hormone in idiopathic membranous nephropathy. *ASN* **F-PO1112**(2006).
137. Kohda, Y., Chiao, H. & Star, R.A. alpha-Melanocyte-stimulating hormone and acute renal failure. *Current opinion in nephrology and hypertension* **7**, 413-417 (1998).
138. Ni, X.P., Bhargava, A., Pearce, D. & Humphreys, M.H. Modulation by dietary sodium intake of melanocortin 3 receptor mRNA and protein abundance in the rat kidney. *American journal of physiology* **290**, R560-567 (2006).
139. Lee, Y.S., Park, J.J. & Chung, K.Y. Change of melanocortin receptor expression in rat kidney ischemia-reperfusion injury. *Transplant Proc* **40**, 2142-2144 (2008).
140. Fathi, Z., Iben, L.G. & Parker, E.M. Cloning, expression, and tissue distribution of a fifth melanocortin receptor subtype. *Neurochem Res* **20**, 107-113 (1995).
141. Salant, D.J., Belok, S., Madaio, M.P. & Couser, W.G. A new role for complement in experimental membranous nephropathy in rats. *The Journal of clinical investigation* **66**, 1339-1350 (1980).
142. Hussain, S*., et al.* Nephrin deficiency activates NF-kappaB and promotes glomerular injury. *J Am Soc Nephrol* **20**, 1733-1743 (2009).
143. Yuan, H*., et al.* Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathy. *Journal of the American Society of Nephrology : JASN* **13**, 946-956 (2002).
144. Bomback, A.S. & Radhakrishnan, J. Treatment of nephrotic syndrome with adrenocorticotropic hormone (ACTH). *Discovery medicine* **12**, 91-96 (2011).
145. Wharram, B.L*., et al.* Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *Journal of the American Society of Nephrology : JASN* **16**, 2941-2952 (2005).

146. Kim, J.H., Kim, B.K., Moon, K.C., Hong, H.K. & Lee, H.S. Activation of the TGF-beta/Smad signaling pathway in focal segmental glomerulosclerosis. *Kidney international* **64**, 1715-1721 (2003).
147. Sadlier, D.M.*, et al.* Sequential extracellular matrix-focused and baited-global cluster analysis of serial transcriptomic profiles identifies candidate modulators of renal tubulointerstitial fibrosis in murine adriamycin-induced nephropathy. *The Journal of biological chemistry* **279**, 29670-29680 (2004).
148. Pereira, R.L.*, et al.* Bradykinin receptor 1 activation exacerbates experimental focal and segmental glomerulosclerosis. *Kidney international* **79**, 1217-1227 (2011).
149. Lee, V.W. & Harris, D.C. Adriamycin nephropathy: a model of focal segmental glomerulosclerosis. *Nephrology* **16**, 30-38 (2011).
150. Jackson, I.J., Budd, P.S., Keighren, M. & McKie, L. Humanized MC1R transgenic mice reveal human specific receptor function. *Human molecular genetics* **16**, 2341-2348 (2007).
151. Chittiprol, S., Chen, P., Petrovic-Djergovic, D., Eichler, T. & Ransom, R.F. Marker expression, behaviors, and responses vary in different lines of conditionally immortalized cultured podocytes. *Am J Physiol Renal Physiol* **301**, F660-671 (2011).
152. Koshikawa, M.*, et al.* Role of p38 mitogen-activated protein kinase activation in podocyte injury and proteinuria in experimental nephrotic syndrome. *J Am Soc Nephrol* **16**, 2690-2701 (2005).
153. Liu, S., Ding, J., Fan, Q. & Zhang, H. The activation of extracellular signal-regulated kinase is responsible for podocyte injury. *Mol Biol Rep* **37**, 2477-2484 (2010).
154. Cargnello, M. & Roux, P.P. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* **75**, 50-83 (2011).
155. Wada, T., Pippin, J.W., Nangaku, M. & Shankland, S.J. Dexamethasone's prosurvival benefits in podocytes require extracellular signal-regulated kinase phosphorylation. *Nephron. Experimental nephrology* **109**, e8-19 (2008).
156. Granqvist, A., Nilsson, U.A., Ebefors, K., Haraldsson, B. & Nystrom, J. Impaired glomerular and tubular antioxidative defense mechanisms in nephrotic syndrome. *American journal of physiology. Renal physiology* **299**, F898-904 (2010).
157. Goyal, M.M. & Basak, A. Human catalase: looking for complete identity. *Protein & cell* **1**, 888-897 (2010).
158. Maresca, V.*, et al.* MC1R stimulation by alpha-MSH induces catalase and promotes its re-distribution to the cell periphery and dendrites. *Pigment Cell Melanoma Res* **23**, 263-275 (2010).