Physiological Genomics of the Rat Renal Failure QTLs *Rf-1* through *Rf-5*

Fysiologisch Genoom Onderzoek van de Rat Nierschade QTLs *Rf-1* tot en met *Rf-5*

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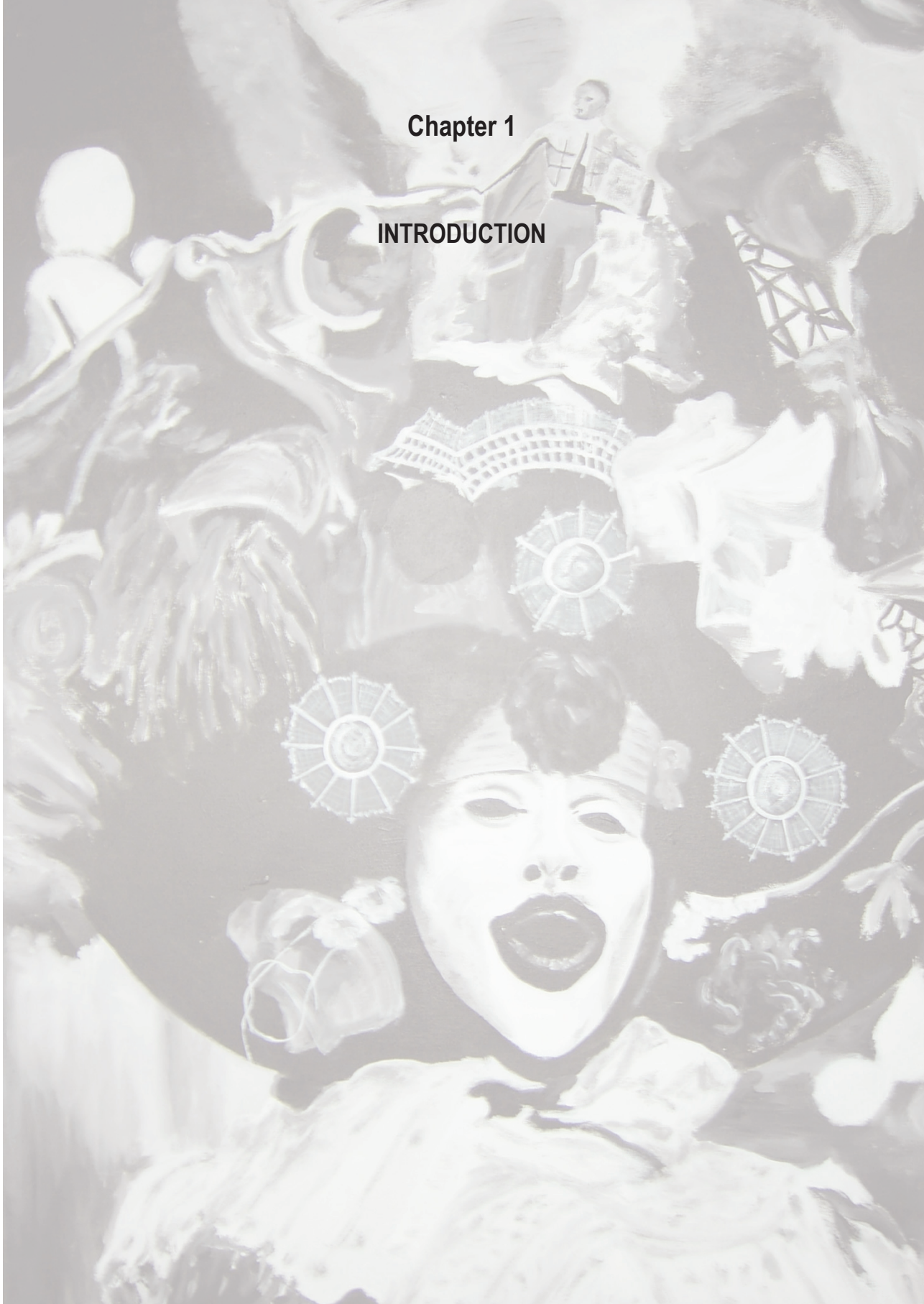
Bloed, zweet en tranen

Contents

List of abbreviations:

ACI	August x Copenhagen Irish
AT1	Angiotensin II receptor 1
BN	Brown Norway
BUF	Buffalo
CKD	Chronic Kidney Disease
cM	centiMorgan
CVD	Cardiovascular Disease
DOCA	Deoxycorticosterone Acetate
ENU	N-ethyl-N-nitroso-urea
ESRD	End-Stage Renal Disease
ESRF	End-Stage Renal Failure
F1	first offspring
FGS	Focal glomerulosclerosis
FSGS	Focal segmental glomerulosclerosis
FHH	Fawn-Hooded Hypertensive
GFR	Glomerular Filtration Rate
Htn-assoc.	Hypertension-associated
IgA	Immunoglobulin A
K/DOQI	Kidney Disease Outcome Quality Initiative
LEW	Lewis
LH	Lyon Hypertensive
LN	Lyon Normotensive
L-NAME	N-nitro-L-arginine methyl ester
LOD	Logarithm of the Odds
Mb	Megabases
MIF	Migration Inhibitory Factor
MWF	Munich Wistar Fromter
NKF	National Kidney Foundation (USA)
NO	Nitric oxide
PGA	Program for Genomic Applications
P_{GC}	Intraglomerular pressure
QTL	Quantitative Trait Locus
Rf-	Renal Failure-
RGD	Rat Genome Database
RI	Recombinant Inbred
RKM	Remnant Kidney Model
SBH/y	Sabra Hypertensive/Yagil
SBN/y	Sabra Normotensive/Yagil

SHR	Spontaneous Hypertensive Rat
SHRSP	Spontaneous Hypertensive Rat Stroke-Prone
SLE	Systemic Lupus Erythematosus
SS	Dahl Salt-Sensitive
T1D	Type I Diabetes
T2D	Type II Diabetes
UAV	Albuminuria
UNX	Unilateral Nephrectomy
UPV	Proteinuria
USRDS	United States Renal Data System
WKY	Wistar Kyoto



Chapter 1

INTRODUCTION

1.1 Chronic Kidney Disease

1.1.1 Introduction

There is a growing awareness that chronic kidney disease (CKD) poses a major health problem worldwide. Large screening programs indicate that 5-10% of the adult population show mild to moderate kidney damage, with or without concomitant renal function loss. It is clear that CKD is a risk factor for the development of end-stage renal failure (ESRF), but more importantly for the development of cardiovascular disease (CVD) and overall mortality. Interestingly, the majority of CKD cases are not associated with primary renal disease, but with systemic conditions like diabetes and hypertension. Only a minority of patients with diabetes and/or hypertension develops CKD, indicating that other aspects also determine susceptibility to develop CKD. Familial clustering and ethnic differences in the prevalence of CKD point to the importance of genetic and/or socio-economic factors influencing the development of CKD.

Completion of sequencing of the human, mouse and rat genomes enables the rapid expansion of comparative genomic studies that may be advantageous to identify genes involved in human complex diseases. Rodent models of CKD will eventually lead to positional cloning of genes that influence the susceptibility to renal damage in various conditions, such as hypertension, diabetes, or reduced renal mass. The genes identified can then be examined in humans. Furthermore, they may help to resolve pathophysiological mechanisms, and possibly lead to new targets for treatment or prevention of CKD.

1.1.2 Definition and staging of chronic kidney disease.

Kidneys are regulatory organs important in helping to preserve the constancy of the internal environment necessary for cells and organ systems to function normally. This is achieved by excretion of toxic waste products produced during several metabolic pathways, and by selectively conserving and excreting water and electrolytes. Furthermore, kidneys secrete hormones that participate in the regulation of systemic and renal haemodynamics, red cell production, and mineral metabolism. The glomerular filtration rate (GFR) is considered to be a good indicator of renal function.[211,264]

In 2002 the National Kidney Foundation in the USA published guidelines for the definition and staging of CKD.[142,177] In the NKF report CKD was divided into five severity stages (Table 1). The staging system is based on the GFR calculated from serum creatinine levels[42,141], and kidney damage ascertained by either biopsy or markers of kidney damage, such as proteinuria or albuminuria.

Table 1. Stages and prevalence of chronic kidney disease

Stage	Description	GFR	Related Terms	US prevalence
.1.	Kidney damage with normal or ↑ GFR	≥90	Albuminuria, proteinuria, hematuria	3.3%
.2.	Kidney damage with mild ↓ GFR	60-89	Albuminuria, proteinuria, hematuria	3.0%
.3.	Moderate ↓ GFR	30-59	Chronic renal insufficiency, early renal insufficiency	4.3%
.4.	Severe ↓ GFR	15-29	Chronic renal insufficiency, renal failure, pre-ESRD	0.2%
.5.	Kidney failure	< 15 (or dialysis)	Renal failure, uremia, ESRD: Renal replacement therapy	0.1%

GFR in ml/min per 1.73 m^2. Related terms for CKD stages 1 and 2 depend on the marker of kidney damage. Albuminuria, proteinuria, and hematuria have been studied most. Related terms for CKD stages 3 to 5 do hot have specific definitions, except for ESRD.

1.1.3 Epidemiology of CKD, and CKD as risk factor for kidney failure, cardiovascular disease and mortality

The prevalence of all stages of CKD was about 11-12% of the US population, surveyed in the early and late 1990s CKD (Table 1).[43,44] Screening programs in Australia, Japan, the Netherlands, and Thailand identified between 6-11% of the adult population having some degree of CKD.[34,58,104,112] In the US study, a total of up to 6.6% have signs of kidney damage, but the calculated GFR is still ≥60 ml/min per 1.73 m^2, i.e. ≥50% of normal GFR. Another 4.3% has a GFR between 25 and 50% of normal. Despite a high prevalence, CKD awareness is low.[44]

Prevalence rates of CKD increase with age and are strongly associated with the presence or absence of two major risk factors – diabetes and hypertension.[263] Depending on age prevalence rates of CKD in patients with diabetes and hypertension are 10-18 times higher compared to those without diabetes and/or hypertension. Prevalence CKD rates in patients with either diabetes or hypertension are 4-7 times higher than those without both risk factors. The prevalence of CKD further increases to 50-60% when high-risk individuals are screened.[26]

It is not clear how this high prevalence of CKD relates to number of patients affected by ESRF or treated by dialysis or kidney transplantation. As indicated in Table 1 the prevalence of kidney failure in the USA is about 0.1%. This equals only 1% of those defined as having CKD. Apparently only a fraction of those with CKD eventually progress to kidney failure and renal replacement therapy. A study of the natural history of stage 2-4 CKD patients indicated that during a 5-year observation period 3.1% progressed to kidney failure (dialysis or transplantation), whereas 24.9% died. The rate of renal replacement therapy was 1.1%, 1.3%, and 19.9%, respectively for the stages 2, 3, and 4, while mortality rates were 19.5%, 24.3%, and 45.7%, respectively. Therefore, death, mainly from CVD, was far more common than dialysis at these stages of CKD.[123] Consequently, patients at all stages CKD should be considered in the “highest risk group” for the development of CVD.[161,217]

1.1.4 Epidemiology of End-Stage Renal Disease (ESRD)

The terms ESRD and kidney failure are not synonymous.[143] ESRD is an administrative term based on conditions for payment for health care by the Medicare ESRD Program for patients treated by dialysis and transplantation. Every year the US Renal Data System documents the incidence, prevalence, age, and primary cause of renal failure in ESRD patients. The most recent one reports data from 2002, the 20th year of coverage.[263] More than 1.5 million people have now been treated through this program, and in 2002 over 100.000 entered for therapy (an adjusted incident rate of 333 new cases per million population), while the total program expenditures reached over $25 billion in 2002. Of the reported ESRD patients 50% was aged 65 or older, while over 70% had diabetes and hypertension as primary diagnosis. Primary renal diseases like glomerulonephritis, interstitial nephritis, polycystic kidneys and renal neoplasms, etc. accounted for less than one third of the ESRD patients.

The distribution of primary diagnosis in the pediatric ESRD population differs significantly from that in adults. Diabetes and hypertension are virtually absent. Hypoplasia, dysplasia and hereditary disease have been the main causes of ESRD in the 0-4 year-old, while glomerulonephritis and pyelonephritis predominate in the older child.[265] The incidence rates in the pediatric age group are low. In the USA, the incidence rate between 0-19 years amounts to only 1% of that age 65 or older, 15 vs. 1500 patients per-million-population.[263] Because of the uniqueness of the pediatric ESRD population, a special CKD classification system for CKD in children and adolescents has been established.[106]

1.1.5 Risk factors for CKD and concept of CKD susceptibility

A risk factor is defined as “an attribute that is associated with increased risk of an outcome”. Four types of risk factors for adverse outcome in CKD have been distinguished (Table 2).[177] In principle, risk factors for the early development of CKD would include susceptibility factors and initiation factors. The difficulty of detecting the onset of CKD makes it hard to determine whether identified risk factors relate more to susceptibility, initiation, or progression.

Table 2. Types and examples of risk factors for adverse outcomes of chronic kidney disease

Type	Definition	Examples
Susceptibility factors	Increased susceptibility to kidney damage	Old age, ethnicity, family history
Initiation factors	Directly initiate kidney damage	Diabetes, High blood pressure
Progression factors	Cause worsening of kidney damage and faster decline in kidney function after initiation	Poor glycemic control, Higher blood pressure level Higher proteinuria, Smoking
End-stage factors	Cause complications in patients with kidney failure	Inadequate dialysis, Anemia

A partial list of clinical and sociodemographic factors that have been implicated as susceptibility or initiation factors is presented in Table 3. Hypertension, diabetes, and reduced renal mass are established risk factors in the initiation and progression of CKD. However, whether these factors are susceptibility, initiation, or progression risk factors for CKD is less clear.[14,67,71,127] For instance, three possibilities exist regarding the role of hypertension in CKD: (1) hypertension is necessary and sufficient to produce CKD; (2) hypertension is necessary but not sufficient to produce CKD, other risk factors are also needed; (3) hypertension is neither necessary nor sufficient to produce CKD, but increases the risk in individuals who are otherwise predisposed or increases the rate of progression of CKD. In the first situation, CKD is hypertension-induced, while in the latter two situations CKD will be hypertension-associated.[25]

Table 3. Potential risk factors for susceptibility to and initiation of chronic kidney disease

Clinical factors	Socio-demographic factors
Diabetes Hypertension	Family history of CKD
Reduction in kidney mass Autoimmune disease Systemic infections	Ethnic status: African American, Native American, Australian Aboriginal, Maori, Pacific Islander
Urinary tract infections Urinary stones	Older-age
Urinary tract obstruction Low birth weight	Low income/education

In the absence of primary renal disease, the development of CKD associated with hypertension and/or diabetes appears to be limited to susceptible individuals. Essential hypertension causes renal injury, but renal susceptibility genes may determine whether hypertension-induced renal damage occurs and how severe it is.[262] Multiple lines of evidence suggest that susceptibility to develop CKD or ESRF has a significant genetic component.[19] A genetic or familial predisposition to develop CKD is implicated from the observation that CKD commonly clusters within families.[11,79,121,139,220,227] A genetic predisposition is also implicated by the large ethnic differences in CKD prevalence rate. African and Native Americans in the USA, Afro-Caribbeans in the UK, and indigenous peoples in Australia and New Zealand all show a high prevalence of CKD related to diabetes, hypertension, or both.[27,201,229,247,263] Although it is attractive to imply genetic factors to explain the familial clustering and ethnic differences of CKD, other socio-economic explanations are also possible as extensively discussed by Cass et al.[33]

Genetic studies in humans have not yet been very successful in defining candidate genes influencing susceptibility to develop renal damage. Studies in congenital and familial forms of nephrotic syndrome has led to the identification of genes and podocyte-specific proteins that are essential to the maintenance of the normal structure of glomerular filtration barrier.[122,125,149,179,194,295] Mutations in these genes result in proteinuria and glomerulosclerosis, but none of these mutations has yet been definitively linked to the occurrence of CKD in the general population.

Linking CKD and various polymorphisms of candidate genes encoding putative mediators of kidney damage has provided various results.[151,164] Most studies aimed at delineating a possible role of genetic variability in the renin-angiotensin-aldosterone system in nephropathy. An association between angiotensin-

converting enzyme genotype and diabetic nephropathy was first described in 1994.[60,155] Numerous studies in diabetic and other forms of nephropathy followed this finding with inconsistent results, as did studies investigating a possible role of polymorphisms of the angiotensinogen gene, the angiotensin-type1 receptor gene, and the aldosterone synthase gene.[163] A new approach is to look simultaneously at genetic variants and whether they act synergistically to increase the risk or confer protection to develop renal failure.[70]

Another approach to identify genes that predispose for kidney failure is to carry out a whole genome scan in multiple families. A number of studies have resulted in the detection of linkage between several chromosomal regions .[18,37,39,74,76,77,78,80,86,111,114,166,184,202,203,259,277,294] These regions, summarized in Table 4, may be considered as "priority regions for further study" (Freedman et al 2004).[77]

Table 4. Linkage obtained in genome scans in human nephropathy

Chr. region	LOD-score	ESRD-type	Race/Ethnicity	Reference
7q 3q, 9, 20p	2.7 1.1 – 1.9	T2DM	Pima Indian	111
3q	3.1	T1DM	Caucasian	166
11q21-q22	9.9	FSGS	Caucasian	295
6q22-q23	5.6	IgAN	Caucasian	86
1q25-31	4.0	FSGS	Brazilian	259
10q	3.4	DN and non-DN ESRD	African American	78
2q34-q35 10q22.3 11p15.6	 3.2 3.3	SLE nephritis	European American and African American	202,203
18q22.3-q23	6.1	T2DM	Turkish	277
9q31-q32	5.4	Htn-assoc ESRD	African American	39
10p	n.a.	T2DM	Caucasian and African American	114
1q31-q41	n.a.	IgAN	Japanese	184
3q, 7p, 18q	4.6, 3.6, 3.7	T2DM	African American	18
3q23-q24	n.a.	T1DM	Russian	37
1q25.1, 2q32 9p21.3, 13q33	1.6, 3.9 2.0, 1.0	Htn-assoc ESRD	African American	77
1q25.1, 4p15.32 9q34.3, 13q33.3	 1.0, 1.1 1.2, 1.7	All cause ESRD	African American	76

LOD is logarithm of odds; ESRD is end-stage renal disease; T2DM and T1DM are type 2 and type 1 diabetes mellitus, respectively; FSGS is focal segmental glomerulosclerosis; IgAN is IgA Nephropathy; DN and non-DN are diabetic- and non-diabetic nephropathy; SLE is systemic lupus erythematosus; Htn-assoc is hypertension associated.

1.2 How to investigate the genetics of susceptibility to CKD

1.2.1 Comparative genomics (human, mouse, rat)

Apparently CKD in humans has all characteristics of a complex trait. There is genetic heterogeneity, complicated by gene-gene and gene-environment interactions affecting the susceptibility of the kidney to be damaged. This complexity will markedly hinder the direct discovery of susceptibility genes in humans. Studies in animal models may help to reveal genes influencing genetic susceptibility to develop renal damage. With the completion of the sequencing of the human, mouse and rat genome, genomic comparisons between these three species are greatly facilitated.[87,135,278,287] Comparative genomics combined with physiological information will facilitate the discovery of mammalian genes that underlie physiological pathways that are involved in disease.[261] In addition, it should lead to the development of better pre-clinical models of human disease, which will aid in the discovery of new therapeutic targets.[23,116,250]

1.2.2 Mouse and rat model systems

The mouse and rat model systems have several advantages over humans for the investigation of mammalian biology. In both species there are genetically well-defined lines that differ in phenotypic characteristics. Coupled with their modest costs maintenance and short generation times has resulted in an explosion of studies in genetic mapping and development of genetic modification tools. Consequently, both mice and rats are used in experimental studies aiming to detect genes involved in complex multi-factorial diseases such as diabetes, obesity, hypertension, cardiovascular disease, asthma and chronic kidney damage.[48]

1.2.3 How to assess renal susceptibility in rodent models?

Assessment of renal damage in rodent models is usually done by determining the urinary excretion of albumin and/or total protein or by determining the incidence and/or severity of structural renal damage. Structural renal damage can then be separated in glomerular or tubulo-interstitial damage. Inbred rodent models are ideally suited to detect genetic components of renal susceptibility. Not only are they genetically identical, all genes are homozygous present. When renal susceptibility is compared between inbred strains under identical circumstances, ideally differences will only be due to genetic divergence.

Differences in renal susceptibility are present between strains of aging animals with two intact kidneys[10], and between strains with different forms of genetic hypertension.[20,66,95,99,215,238,245,283] However, differences in renal susceptibility between strains of rats and mice are mostly tested following surgical or pharmacological intervention, or by the induction of various forms of nephritic renal disease. Surgical intervention consists of unilateral nephrectomy (UNX)[239,291] or more extensive forms of renal ablation.[16,97,130] Pharmacological intervention upon the kidney may be indirect by the induction of a systemic disease, such as hypertension[274,275] or diabetes[119], or directly by injection of glomerular toxins, such as puromycin[98,138] or adriamycin.[183] Combinations of surgical and pharmacological interventions have also been applied. In addition, it has been well established that dietary protein intake (total amount as well origin – animal or vegetable) influences the development of renal damage.

Employing various models has revealed marked differences in renal susceptibility between inbred rodent strains.[25,84,175,195,224,225,234,235] This opens the opportunity to carry out genetic linkage studies aiming to

detect genes (chromosomal loci) influencing renal susceptibility. As the pathophysiological mechanisms may differ between strains and the various test models, it is highly likely that renal susceptibility genes may also differ. Thus, it is to be expected that eventually a substantial number of renal susceptibility genes will emerge from rodent studies investigating multiple model systems. Once identified, the rodent susceptibility genes can then be examined in humans to ascertain a possible role in explaining differences in renal susceptibility in humans.

1.2.4 Mouse studies

Mouse strains have been shown to differ in susceptibility with respect to spontaneous renal lesions[109,126,185], to renal damage induced by hyperglycemia[307], after renal mass reduction[69,130,152], and following DOCA-salt-treatment.[100] The ICGN mouse strain has spontaneous glomerular lesions and albuminuria.[185,186] A single gene (named *nep*) was thought to be responsible, which was localized by linkage mapping on the distal part of mouse chromosome 15.[188] The FGS/Nga mouse strain is a model for spontaneous glomerulosclerosis, and is considered to have two pairs of autosomal recessive genes associated with FGS.[109,302] Linkage analysis in a HIV-1 transgenic mouse strain identified a locus on mouse chromosome 3 linked with nephropathy.[85] A genome-wide scan in a murine IgA-nephropathy model identified a susceptibility locus on mouse chromosome 10, in a region syntenic to human IGAN1 on chromosome 6q22-23.[253] The importance of the genetic background has been shown in a mouse model (ROP-OS/+ strain) of glomerulosclerosis due to a 50% reduction in nephron number.[102] Glomerulosclerosis in ROP-OS/+ mice appears inherited to be in a recessive fashion involving 8-10 loci.[140] Recently, a study in KK/Ta mice has detected a region on chromosome 2 of the mouse that is linked to albuminuria.[233] This region is homologous to the *Rf-3* region of the FHH rat.

Studies in congenic mice have been crucial to unravel the complex genetics of lupus nephritis.[171,49,297] Several mouse models of diabetic nephropathy have been characterized and are potentially useful for genetic studies.[4,119,230] Molecular profiling of diabetic mouse kidneys has revealed several novel genes that might be involved in glomerular damage.[252] Furthermore, a role of leptin has been suggested to play a role in diabetic nephropathy in a mouse model of lipoatrophic diabetes.[251]

The technology for disrupting gene expression (knockout) or produce over-expression (transgenic) is well established in the mouse. Various mouse models have been successfully employed to investigate the function of genes and their possible connection or disconnection with kidney disease.[254] As new models constantly emerge, only a few examples will be presented. Transgenic Alb/TGF-β mice that are over-expressing transforming growth factor-β1 develop progressive renal disease.[128] Glomerulosclerosis was found in mice transgenic for human insulin-like growth factor-binding protein-1[61], while Wt1+/R394W transgenic mice display glomerulo-sclerosis and early-onset renal failure characteristic of human Denys-Drash syndrome.[82] Mice transgenic for the macrophage migration inhibitory factor (MIF) develop podocyte injury and progressive mesangial sclerosis.[218]

No aggravation of renal injury was seen in apolipoprotein E knockout mice after subtotal nephrectomy.[29] Knockout mice have also been employed to delineate the role of angiotensin-converting enzyme in renal function and blood pressure control[13], and the function of several podocyte-specific molecules like Neph-1 and nephrin[59,147,212,296], CD2-adaptor protein[296], and podocin.[212]

Recently, Rathkolb et al. reported on a large-scale albuminuria screen for nephropathy models in N-ethyl-N-nitrosourea (ENU) induced mouse mutants. Proteinuria was used as a parameter for identifying nephropathy phenotypes in ENU-mutagenized mice. This is the first study describing ENU-mutagenesis especially used for detecting nephropathy models in mice.[209]

Taken together, studies in mice concentrate on transgenic and knockout model, rather than linkage studies in established models of spontaneous renal damage. Overexpression or loss of different single genes does influence the development of renal damage. The few linkage studies indicate that spontaneous renal damage appears to be inherited from one, two or multiple genes.

1.2.5 Rat studies

Like mice, inbred rat strains vary widely in their susceptibility to develop renal damage. In contrast to mice, some of the strains are well characterized and have already been used in linkage analyses to detect chromosomal regions, i.e. quantitative trait loci (QTLs) influencing the development of renal damage.

The FHH (Fawn-Hooded Hypertensive) rat is one of the best-characterized models of hypertension-associated renal damage. The FHH rat is prone to develop mild hypertension and marked proteinuria (UPV), albuminuria (UAV), and focal and segmental glomerulosclerosis (FGS) at relatively young age. Numerous studies have been performed in the FHH rat to characterize the (patho)physiology[51,52,53,54,189,198,210,238,239,272,273,279,288,] and histopathology.[131,132,133] Furthermore, several studies have established the efficacy of inhibiting the renin-angiotensin system to prevent or slow down the progression of renal damage in the FHH rat.[279,282,308]

Altogether, these studies indicated that in FHH the moderately elevated systemic blood pressure is transmitted into the glomerulus due to an impaired myogenic renal autoregulation. The resulting increased intraglomerular pressure initiates the development of vascular-pole-associated glomerulosclerosis, eventually progressing to total nephron degeneration and interstitial fibrosis through misdirected filtration and peritubular spreading of the filtrate. There appears to be a general up-regulation of systems important in the control of intra-renal haemodynamics, like preglomerular renin activity, and NO-synthase and cyclo-oxygenase-2 activities in the macula densa. However, no primary defect initiating these abnormalities has yet been identified. It is surmised that genetic studies will eventually lead to the detection of the responsible gene(s), the molecular events, and physiological pathways.

Other susceptible inbred strains have also been studied to detect the genetics of renal damage susceptibility. They include BUF (Buffalo), MWF (Munich Wistar Fromter), SHRSP (Spontaneously Hypertensive Rat-Stroke Prone), and SS (Dahl Salt Sensitive). However, in contrast to FHH, they are all deficient in pathophysiological characterization. The availability of large sets of genetic markers has opened the possibility of detailed linkage analyses employing the renal susceptible strains and crossing them with contrasting renal resistant strains, such as ACI (August Copenhagen Irish), WKY (Wistar Kyoto), LEW (Lewis), SHR (Spontaneously Hypertensive Rat), and BN (Brown Norway).[25,84,175,195,224,225,234,235]

1.3 Finding genes influencing renal susceptibility

1.3.1 Linkage analysis to detect 'renal susceptibility QTLs'

Linkage analysis offers the possibility to detect quantitative trait loci (QTLs) influencing parameters of functional renal damage, like proteinuria and albuminuria, or structural renal damage, like the incidence or severity of glomerular or tubular damage. About 90 QTLs have been identified linking chromosomal markers to renal damage traits, summarized in Table 5 and Figure 1.

The first linkage analyses involving crosses between FHH and the renal resistant ACI rat revealed the presence of five QTLs linked to UPV and other parameters of renal damage. These QTLs were named *Renal-failure-1* (*Rf-1*) to *Rf-5*.[25,234] It is surmised that each of these QTLs contains gene(s) that play a role in the initiation and/or progression of renal damage in the FHH rat. The *Rf-1*-QTL has by far the highest LOD-score and is considered to play a major role. In addition, the linkage analysis suggested complex interactions between the five QTLs. The *Rf-2*, *Rf-3*, *Rf-4*, and *Rf-5* QTL, being homozygous for FHH, by itself showed little effect on UPV. However, a marked increase in UPV level was noted when one of these QTLs was combined with *Rf-1*.[234]

As indicated in Table 5 and Figure 1, QTLs linked to parameters of renal damage are spread all over the rat genome. With the exception of chromosomes 20, all chromosomes carry one or more QTLs. Some of the renal QTLs appear to cluster, i.e. on chromosomes 1, 2, 3, 6, 8, 9, 10, 11, and 17. Taken together about 15-20 QTL clusters can be distinguished. Such a sizeable number of QTLs indicates that the renal susceptibility is indeed a complex trait. Although some strains may have QTLs in common, it seems that different, strain specific, sets of genes underlie renal susceptibility in the various rat models.

Table 5: Localization of rat kidney damage QTLs

cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
Bn/SsNHsd x SS/JrHsdMcwi	Rf-6	3.1	1	4.3-12.0	D1Rat167	4.4	D1Rat167, D1Rat4	Renal Function QTL 6	salt loaded renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf28	4.7	1	?-78.2	D1Mit10	~43	D1Mgh3, DMgh5	Renal Function QTL 28	potassium excretion rate	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf27	4.6	1	29.7-78.2	D1Mgh3	?	D1Rat8, D1Mgh5	Renal Function QTL 27	urine sodium concentration	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf29	4.6	1	56.7-78.2	D1Mit10	~43	D1Mit1, D1Mgh5	Renal Function QTL 29	urine potassium concentration	235
(SHR x SHRSP) F2 intercross	Rds2	?	1	19.7-54.8	D1Rat127	47	D1Rat6, D1Rat201	Renal Damage Susceptibility QTL2	renal lesions	88
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Uae1	3.8	1	90.3-186.2	D1Rat38	133.6	D1Rat27, D1Rat216	Urinary albumin excretion QTL 1	urine albumin level	224
(LH/Mav x LN/Mav) F2 intercross	Rf46	3.8	1	103.2-154.2	D1Rat43	139.0	D1Rat29, D1Rat140	Renal Function QTL 46	serum creatinine concentration	17
(ACI x FHH) x FHH backcross	**Rf-2**	**3.7**	**1**	**135.0-147.2**	**D1Mit3**	**147**	**D1Mit2, D1Mit3**	**Renal failure 2**	**UPV, FGS**	**25, 234**
SHR.BN-D1Mit3/Igf2	Rds1	?	1	147.0-203.1	?	?	D1Mit3, Igf2	Renal Damage Susceptibility QTL1	renal lesions	243
Bn/SsNHsd x SS/JrHsdMcwi	Rf-7	3.0	1	190.0-227.3	D1Rat295	213.7	D1Rat130, D1Rat74	Renal Function QTL 7	salt loaded urine creatinine concentration	172
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae20	3.5	1	227.1-267.4	D1Rat75	235.3	D1Rat74, D1Rat190	Urinary albumin excretion QTL 20	urine albumin level	225
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae5	4.0	1	153.6-259.5	D1Mco35	238.5	D1Rat158, D1Rat86	Urinary albumin excretion QTL 5	urine albumin level	84
WKY.SHR-D1Mit7	Rhf1	5.3	1	224.7-263.8	D1Mit7	241.2	D1Mgh14, D1Mit18	Renal hemodynamic functions QTL1	renal blood flow	113
(ACI x FHH) x FHH backcross	**Rf-1**	**16.9**	**1**	**217.4-247.4**	**D1Mgh12**	**247.4**	**D1Mit6, D1Mgh12**	**Renal failure 1**	**UPV, FGS**	**25, 2334**
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Glom2	3.3	1	236.6-266.6	D1Rat151	251.7	?	Glomerulus QTL 2	number of surface glomeruli (SG)	224
Bn/SsNHsd x SS/JrHsdMcwi	Rf-8	3.8	1	234.7-267.4	D1Rat90	267.3	D1Rat301, D1Rat90	Renal Function QTL 8	salt loaded renal vascular resistance	172
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Glom4	2.4	2	?	D2Rat14	42.4	?	Glomerulus QTL 4	number of superficial glomeruli in renal cortex	225

cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
(LH/Mav x LN/Mav) F2 intercross	Rf50	3.5	2	53.6-75.7	D2Mit35	131.5	DRat270, D2Rat21	Renal Function QTL 50	urine volume	17
(LH/Mav x LN/Mav) F2 intercross	Rf48	2.9	2	75.7-215.0	D2Rat221	145.7	D2Rat21, D2Rat118	Renal Function QTL 48	serum creatinine concentration	17
Bn/SsNHsd x SS/JrHsdMcwi	Rf-9	3.3	2	138.6-210.7	D2Rat40	163.1	D2Rat147, D2Mgh12	Renal Function QTL 9	hypertension related glomerular damage	172
(SS/Fub x SHR/Fub) F2 intercross	Uae13	4.4	2	~160-218.5	D2Rat126	189.8	D2Rat36, D2Rat57	Urinary albumin excretion QTL 13	urine albumin level	195
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae6	10.0	2	79.6-227.2	D2Rat50	200.4	D2Mco18, D2Rat61	Urinary albumin excretion QTL 6	urine albumin level	84
Bn/SsNHsd x SS/JrHsdMcwi	Rf30	3.7	3	36.5-87.9	D3Mgh6	50.4	D3Mgh7, D3Rat27	Renal Function QTL 30	urine potassium concentration	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf-12	3.8	3	58.6-121.6	D3Rat169	?	D3Rat180, D3Rat17	Renal Function QTL 12	salt loaded renal blood flow	172
(ACI x FHH) x FHH backcross	**Rf-3**	**6.5**	**3**	**116.0-145.6**	**D3Mit4**	**131.1**	**?**	**Renal failure 3**	**UPV, FGS**	**25, 234**
Bn/SsNHsd x SS/JrHsdMcwi	Rf-11	3.4	3	121.4-148.0	D3Rat11	132.2	D3Rat17, D3Rat6	Renal Function QTL 11	hypertension related renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf-10	3.6	3	163.1-166.7	D3Rat1	169.9	D3Rat137, D3Rat77	Renal Function QTL 10	hypertension related renal vascular resistance	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf31	3.0	4	36.7-74.0	D4Mit2	55.6	D4Mgh2, D4Mgh24	Renal Function QTL 31	urine potassium concentration	235
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae21	2.4	4	116.3-146.3	D4Rat95	131.4	?	Urinary albumin excretion QTL 21	urine albumin level	225
Bn/SsNHsd x SS/JrHsdMcwi	Rf-13	3.9	4	161.2-185.2	D4Rat204	182.6	D4Rat67, D4Rat70	Renal Function QTL 13	salt loaded renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf34	4.2	5	~0-95.4	D5Mgh5	45.5	D5Mgh1, D5Mit4	Renal Function QTL 34	renal blood flow	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf35	4.4	5	~0-90.6	D5Mgh5	45.5	D5Mgh1, D5Rat19	Renal Function QTL 35	renal blood flow	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf36	3.3	5	3.5-101.5	D5Mit4	95.4	D5Rat131, D5Rat149	Renal Function QTL 36	renal blood flow	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf33	4.1	5	45.5-135.9	D5Mgh23	115.4	D5Mgh5, D5Rjr1	Renal Function QTL 33	renal vascular resistance	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf32	2.8	5	135.9-149.8	D5Mgh8	149.8	D5Rjr1, D5Mgh8	Renal Function QTL 32	renal vascular resistance	235

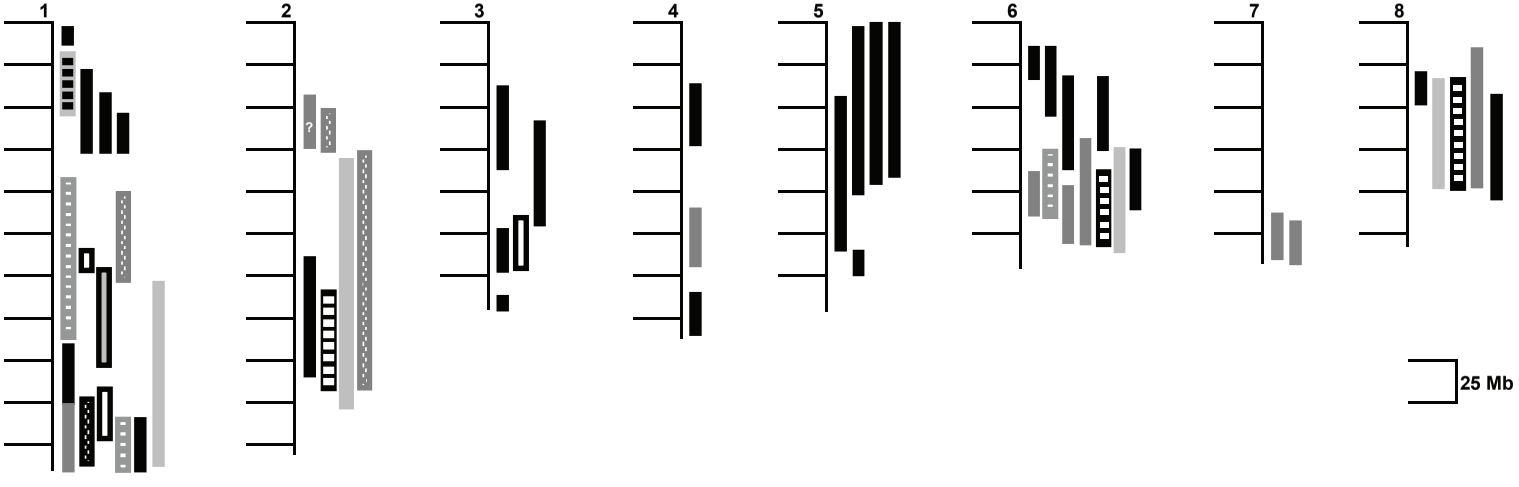
cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
Bn/SsNHsd x SS/JrHsdMcwi	Rf-16	3.9	6	13.2-35.3	D6Rat62	5.5	D6Rat46, D6Rat35	Renal Function QTL 16	salt loaded renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf-15	?	6	13.2-56.8	D6Rat42	19.9	D6Rat42, D6Rat26	Renal Function QTL 15	salt loaded renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf38	2.9	6	~34-75.1	D6Mgh11	~41	D6Mgh8, D6Mit3	Renal Function QTL 38	renal vascular resistance	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf-14	2.9	6	35.1-80.2	D6Rat26	56.7	D6Rat35, D6Rat126	Renal Function QTL 14	salt loaded renal blood flow	172
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Glom7	5.6	6	82.5-112.5	D6Mit8	97.6	?	Glomerulus QTL 7	number of superficial glomeruli in renal cortex	225
Bn/SsNHsd x SS/JrHsdMcwi	Rf37	3.2	6	75.1-~111	D6Mit8	97.6	D6Mit3, D6Rat163	Renal Function QTL 37	renal vascular resistance	235
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Uae2	2.7	6	76.5-113.9	D6Rat71	110.5	D6Rat12, D6Mgh5	Urinary albumin excretion QTL 2	urine albumin level	224
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Glom8	7.0	6	98.9-128.9	D6Rat12	114	?	Glomerulus QTL 8	glomerulus number	225
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae22	10	6	68.0-~130	D6Rat12	114	D6Rat106, D6Rat6	Urinary albumin excretion QTL 22	urine albumin level	225
(SS/Fub x SHR/Fub) F2 intercross	Uae14	6.5	6	88.7-~130	D6Rat12	114	D6Rat6, D6Rat104	Urinary albumin excretion QTL 14	urine albumin level	195
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae7	3.5	6	75.0-136.6	D6Uia5	114	D6Mit3, D6Wox13	Urinary albumin excretion QTL 7	urine albumin level	84
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Glom5	2.5	7	111.3-141.3	D7Rat7	126.4	?	Glomerulus QTL 5	number of superficial glomeruli in renal cortex	225
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae23	2.4	7	119.6-143.1	D7Rat4	134.7	?	Urinary albumin excretion QTL 23	urine albumin level	225
(SS/Fub x SHR/Fub) F2 intercross	Uae15	2.9	8	30.8-74.3	D8Rat46	43.3	D8Rat162, D8Rat30	Urinary albumin excretion QTL 15	urine albumin level	195
Bn/SsNHsd x SS/JrHsdMcwi	Rf39	3.9	8	44.5-103.7	D8Mit16	61.9	D8Mit4, D8Mgh15	Renal Function QTL 39	urine volume	235
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae24	6.4	8	19.7-98.6	D8Rat35	64.6	D8Rat53, D8Rat19	Urinary albumin excretion QTL 24	urine albumin level	225
Bn/SsNHsd x SS/JrHsdMcwi	Rf-17	2.9	8	29.6-~47	D8Rat49	?	D8Rat51, D8Rat159	Renal Function QTL 17	salt loaded renal blood flow	172
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae8	5.0	8	32.1-99.1	D8Rat62	70	D8Mit5, D8Rat130	Urinary albumin excretion QTL 8	urine albumin level	84

cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae25	3.5	9	22.0-100.1	D9Rat10	68.6	D9Rat29, D9Rat3	Urinary albumin excretion QTL 25	urine albumin level	225
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae9	4.5	9	21.6-105.9	D9Uia6	77.4	D9Rat70, D9Mco6	Urinary albumin excretion QTL 9	urine albumin level	84
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Glom6	2.8	9	70.3-100.3	D9Rat5	85.4	?	Glomerulus QTL 6	number of superficial glomeruli in renal cortex	225
(SS/Fub x SHR/Fub) F2 intercross	Uae16	8.0	9	55.7-99.9	D9Rat5	85.4	D9Rat3, D9Mit3	Urinary albumin excretion QTL 16	urine albumin level	195
Bn/SsNHsd x SS/JrHsdMcwi	Rf40	3.9	10	30.0-65.4	D10Mgh11	30.0	D10Mgh11, D10Mgh14	Renal Function QTL 40	renal vascular resistance	235
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Pur2	5.5	10	22.8-95.2	D10Mco57	?	D10Uia2, D10Rat17	Proteinuria QTL 2	proteinuria	84
(SS/Fub x SHR/Fub) F2 intercross	Uae17	3.6	10	23.4-81.9	D10Rat30	63.8	D10Rat43, D10Rat93	Urinary albumin excretion QTL 17	urine albumin level	195
Bn/SsNHsd x SS/JrHsdMcwi	Rf-18	3.4	10	82.4-103.0	D10Rat17	95.1	D10Rat86, D10Rat201	Renal Function QTL 18	salt loaded urine volume	172
(SHR x SHRSP) F2 intercross	Rds3	?	10	83.4-89.3	Igfbp4	?	D10Rat21, D10Rat55	Renal Damage Susceptibility QTL3	renal lesions	88
(SS/Fub x SHR/Fub) F2 intercross	Uae18	3.7	11	16.6-45.4	D11Mit2	30.9	D11Rat20, D11Rat6	Urinary albumin excretion QTL 18	urine albumin level	195
Bn/SsNHsd x SS/JrHsdMcwi	Rf-20	4.4	11	29.6-62.2	D11Rat71	41.8	D11Rat15, D11Rat38	Renal Function QTL 20	hypertension reloated glomerular damage	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf-19	3.4	11	41.7-84.7	D11Rat6	45.3	D11Rat71, D11Mgh1	Renal Function QTL 19	salt loaded creatinine clearance	172
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae10	6.0	11	28.1-85.0	D11Rat67	45.9	D11Rat17, D11Rat50	Urinary albumin excretion QTL 10	urine albumin level	84
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Uae3	4.9	12	0.0-21.0	D12Rat37	10.8	D12Rat59, D12Rat7	Urinary albumin excretion QTL 3	urine albumin level	224
Bn/SsNHsd x SS/JrHsdMcwi	Rf-21	4.4	12	9.6-33.2	D12Mgh5	29	D12Rat2, D12Rat36	Renal Function QTL 21	salt loaded renal blood flow	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf41	3.0	12	29.0-41.3	D12Mgh9	41.3	D12Mgh9, D12Mgh5	Renal Function QTL 41	renal blood flow	235
(BUF/Mna x WKY/NCrj)F1 x BUF/Mna	Pur1	18	13	9.2-46.5	D13Mgh4	38.5	D13Mgh2, D13Rat25	Proteinuria QTL 1	proteinuria	175
(LH/Mav x LN/Mav) F2 intercross	Rf47	3.7	13	19.0-106.0	D13Rat26	56.8	D13Rat53, D13Rat163	Renal Function QTL 47	kidney renin concentration	17

cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Glom3	2.9	13	45.6-75.6	D13Rat62	60.7	?	Glomerulus QTL 3	number of surface glomeruli (SG)	224
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae11	5.7	13	46.2-80.6	D13Rat58	71.1	D13Wox5, D13Rat63	Urinary albumin excretion QTL 11	urine albumin level	84
Bn/SsNHsd x SS/JrHsdMcwi	Rf-22	3.9	14	44.8-101.8	D14Rat90	73.9	D14Rat16, D14Rat95	Renal Function QTL 22	salt loaded renal vascular resistance	172
(ACI x FHH) x FHH backcross	**Rf-4**	**4.1**	**14**	**?**	**D14Mgh7**	**11.6**	**?**	**Renal failure 4**	**UPV, FGS**	**25, 234**
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Pur3	2.3	15	6.8-36.8	D15Rat66	21.9	?	Proteinuria QTL 3	proteinuria	225
Bn/SsNHsd x SS/JrHsdMcwi	Rf42	3.1	15	20.1-80.4	D15Mgh11	50.1	D15Mit3, D15Mgh8	Renal Function QTL 42	renal blood flow	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf-23	3.6	15	45.8-106.3	D15Mgh9	89.6	D15Rat14, D15Rat106	Renal Function QTL 23	salt loaded renal vascular resistance	172
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae26	2.4	15	82.6-109.8	D15Rat102	97.7	?	Urinary albumin excretion QTL 26	urine albumin level	225
Bn/SsNHsd x SS/JrHsdMcwi	Rf-24	3.6	16	3.4-23.1	D16Rat87	3.5	D16Rat87, D16Rat76	Renal Function QTL 24	salt loaded creatinine clearance	172
(SHR x SHRSP) F2 intercross	Rds4	?	16	?	D16Mit2	?	?	Renal Damage Susceptibility QTL4	renal lesions	88
(LH/Mav x LN/Mav) F2 intercross	Rf49	2.9	17	?-81.1	D17Rat102	27.1	D17Rat94, D17Rat58	Renal Function QTL 49	serum creatinine concentration	17
Bn/SsNHsd x SS/JrHsdMcwi	Rf43	2.9	17	21.3-28.9	D17Rat59	27.7	D17Mgh2, D17Ra9	Renal Function QTL 43	renal vascular resistance	235
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Uae4	4.9	17	42.3-92.0	D17Rat58	81.5	D17Rat17, D17Rat133	Urinary albumin excretion QTL 4	urine albumin level	224
(ACI x FHH) x FHH backcross	***Rf-5***	**3.0**	**17**	**47.7-77.7**	**D17Mit12**	***?***	**?**	**Renal failure 5**	**UPV, FGS**	**25, 234**
Bn/SsNHsd x SS/JrHsdMcwi	Rf-25	3.0	17	82.2-92.7	D17Rat47	85.1	D17Rat45, D17Rat52	Renal Function QTL 25	salt loaded creatinine clearance	172
Bn/SsNHsd x SS/JrHsdMcwi	Rf44	3.0	18	15.9-32.5	D18Mgh9	25.6	D18Mgh8, D18Mit5	Renal Function QTL 44	urine protein level	235
Bn/SsNHsd x SS/JrHsdMcwi	Rf-26	3	18	55.0-69.0	D18Rat91	61.3	D18Mgh3, D18Rat55	Renal Function QTL 26	salt loaded potassium excretion	172
(SS/JrMco x SHR/NHsd)F1 x SS/JrMco	Uae12	5.0	19	22.6-?	D19Rat29	31.2	D19Rat34, D19Uia1	Urinary albumin excretion QTL 12	urine albumin level	84

cross	QTL	LOD	Chr	region (Mb)	Peak	pos.	Flank markers	Name	Trait	Ref
(SS/Fub x SHR/Fub) F2 intercross	Uae19	5.5	19	7.9-45.0	D19Rat11	31.6	D19Mit7, D19Rat19	Urinary albumin excretion QTL 19	urine albumin level	195
Bn/SsNHsd x SS/JrHsdMcwi	Rf45	2.8	19	16.8-?	D19Mit10	32.9	D19Mit4, D19Mit14	Renal Function QTL 45	renal blood flow	235
(MWF/Fub x SHR/Fub)F1 x MWF/Fub	Uae27	2.7	X	26.3-56.3	DXRat8	41.4	?	Urinary albumin excretion QTL 27	urine albumin level	225
(MWF/Fub x LEW/Rkb)F1 x MWF/Fub	Glom1	2.8	X	?	DXRat96	122.1	?	Glomerulus QTL 1	number of superficial glomeruli	224

Bold indicates our own renal failure QTLs; QTL: quantitative trait locus; LOD: Logarithm of the odds; Chr.: Chromosome; Pos.: position; Ref. Reference.[17,25,84,88,172,175,195,224,225,234,235,249]



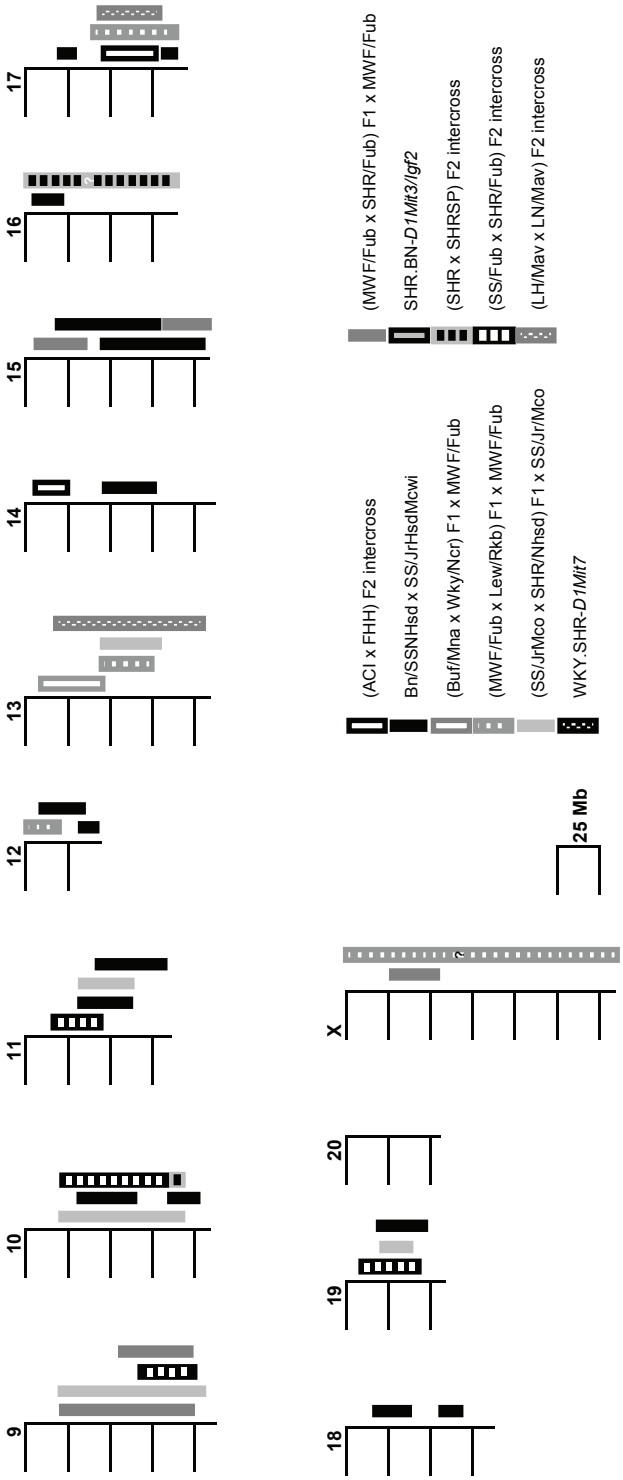


Figure 1: Localization of rat kidney damage QTLs

1.3.2 From linkage to susceptibility genes

Localizing a QTL is only the beginning of a long road to gene identification via a positional cloning strategy.[90,117] Once a set of QTLs is identified, the next step is to generate a series of congenic strains in which each chromosomal region carrying a QTL is introgressed from the renal susceptible strain to the genome of the renal resistant strain or vice versa. These congenic rat strains may then be tested for changes in renal susceptibility. This approach is currently widely tested in other complex traits like hypertension, diabetes, arthritis, and obesity. For instance, over 25 congenic rat strains and substrains, carrying various blood pressure QTLs, have been generated and studied.[56,159,207]

The generation and testing of a panel of congenic strains carrying the various *Rf*-QTLs from the FHH strain onto the genomic background of the ACI strain will be described in this thesis. The first congenic strain being tested carried a 20cM (24.2 Mb) region including the *Rf-1* QTL from FHH upon the ACI genomic background. These ACI.FHH-*Rf1* rats showed an increased susceptibility to renal damage when compared with the ACI parental strain. However, an increased renal susceptibility in ACI.FHH-*Rf1* was only noted in the presence of an increased haemodynamic stress upon the kidney, i.e. following unilateral nephrectomy (UNX) or UNX combined with L-NAME-induced hypertension.[199]

The degree of renal damage observed in strains congenic for only a single *Rf*-QTL remains far below that of the FHH parental strain. This is in accordance with the assumption that renal damage in FHH is a polygenic trait. Consequently, significant gene-gene interactions can be expected. Such interactions can be investigated in double or multiple congenic rats.

Apart from our experimental work in ACI.FHH congenics no other studies have yet been reported employing congenic rat strains that have been generated based on previous linkage studies. However, an effect on renal susceptibility has been reported in a congenic strain generated for hypertension research. An increased renal susceptibility was reported after transgressing a chromosomal region (*D1Mit3-Igf*) from BN into the SHR genome.[243] This region coincides with the *Rf-2* region found in the FHH.[25,234]

1.3.3 Consomic rats

A consomic rat strain is one in which an entire chromosome is introgressed into the isogenic background of another inbred strain. Consomic rats are yet another tool to help identifying genes influencing complex traits. Whereas congenic rats are developed based on QTL regions linked to a specific trait (blood pressure, proteinuria, etc), a panel of consomic rats is generated by replacing the chromosome from a disease model one by one by the chromosome from a healthy control strain.[45,46] In the PhysGen project (http://pga.mcw.edu; part of the NIH Program for Genomic Applications (PGA) initiative) two panels of consomic rats will be generated that are of major interest to study susceptibility to renal damage. By replacing the individual chromosomes from the Dahl Salt-sensitive (SS) or the FHH (two models of progressive renal damage) by those from the Brown Norway (BN) rat, a total of 44 consomic strains will be generated and phenotyped for various renal and blood pressure related traits. When a chromosome is thought to harbor a QTL, consomic rats enable the rapid development of congenic strains over a narrow region in just two generations. Furthermore, they enable one to perform F2 linkage studies to positionally locate QTLs on a single chromosome with a fixed genetic background.

The situation as of June 24, 2005 (Release 17) regarding UPV, UAV and mean arterial blood pressure (MAP) is summarized in Table 6. In the SS strain several chromosomes appear to influence the development of UPV and/or UAV (i.e. chromosomes 5, 6, 7, 8, 11, 13, and 18) and blood pressure (i.e. chromosomes 5, 7, 8, 13, and 18). Five consomic strains (i.e. chromosome 5, 7, 8, 13, and 18) show a simultaneous reduction of UPV, UAV and MAP when compared with SS rats, suggesting that attenuation of renal damage is achieved through a reduction in blood pressure. Studies with congenic rats derived from these consomics should further elucidate these suppositions.

In the FHH, the replacement of chromosome 1, 14, 16 and 20 significantly attenuates the development of UPV, while only the replacement of chromosome 1 significantly attenuated the development of UAV. The MAP was attenuated only by replacement of chromosome 20. These findings are in line with the presence of the *Rf-1* and *Rf-2* QTLs on chromosome 1, and *Rf-4* on chromosome 14 influencing UPV and UAV. In contrast, a role of the *Rf-5* QTL on chromosome 17 is not confirmed in the FHH-17^{BN} consomic rats. The FHH chromosomes 16 and 20 could contain QTLs affecting UPV that were not found in the linkage analysis using FHH and ACI rats.[25,234] An effect on MAP was only noted by replacing chromosome 20. No QTL had been found on this chromosome, while the previously reported QTLs for SBP on chromosomes 1 and 17 were not confirmed in the consomic strains.

Further studies dealing with the FHH-1^{BN} consomic rats, confirmed the significance of FHH chromosome 1 in renal susceptibility[158], emphasizing the important role of the *Rf-1* and *Rf-2* QTLs both located on FHH chromosome 1. Studies in FHH-1^{BN} subcongenics resulted in the positional cloning of a strong candidate gene for *Rf-2* and the bleeding disorder present in FHH rats.[204] Additional publications involving consomic rat strains keep emerging[47,63,65,72,157], indicating the potential strength of using the consomic rats in functional genomics.

Consomic strains were also used to investigate the genetic basis of the difference in renal susceptibility between two strains of Sabra rats. The Sabra SBH/y rat strain is more susceptible to develop proteinuria than the Sabra SBN/y strain. Consomic rats were constructed by introgressing chromosome 1 (which harbors *Rf-1* and *Rf-2*) or chromosome 17 (which harbors *Rf-5*) from SBH/y onto the SBN/y genomic background. Following UNX, the consomic strains developed more proteinuria than SBN/y, suggesting a functional role of gene systems located on chromosomes 1 and 17 in inducing proteinuria in the SBH/y strain.[299]

Table 6: Proteinuria (UPV), albuminuria (UAV) and mean arterial pressure in male BN.FHH and BN.SS consomics and in male BN, FHH, and SS parental strain rats (Situation after Release 17, June 24, 2005)

Strain	UPV	UAV	MAP	Strain	UPV	UAV	MAP
(8% NaCl)	(mg/day)	(mg/day)	(mm Hg)	(8% NaCl + L-NAME)	(mg/day)	(mg/day)	(mm Hg)
Parental strains				**Parental strains**			
SS	211±16 (51)	80±8 (51)	177±3 (50)	FHH	453±34 (41)	170±15 (41)	180±3 (41)
BN	**73±12 (10)**	**4±1 (10)**	**111±1 (9)**	BN	**32±5 (8)**	**5±1 (8)**	**121±3 (8)**
Consomic strains				**Consomic strains**			
SS-1B				FH-1B	**189±37 (10)**	**69±17 (10)**	167±5 (9)
SS-2B	174±23 (10)	74±13 (10)	175±2 (9)	FH-2B	*393±90 (5)*	*118±39 (5)*	*182±15 (5)*
SS-3B				FH-3B			
SS-4B	176±30 (7)	54±9 (7)	181±6 (7)	FH-4B	*575±172 (3)*	*146±71 (3)*	*194±3 (3)*
SS-5B	**46±7 (10)**	28±5 (10)	**130±3 (7)**	FH-5B	435±69 (7)	201±42 (7)	174±9 (7)
SS-6B	**90±15 (9)**	**26±6 (9)**	176±5 (10)	FH-6B	409±69 (11)	210±31 (11)	177±5 (9)
SS-7B	**73±8 (11)**	**21±3 (11)**	**146±3 (10)**	FH-7B	422±104 (8)	165±54 (8)	*195±5 (6)*
SS-8B[1]	**100±23 (21)**	**21±7 (21)**	**156±7 (20)**	FH-8B	*401±155 (4)*	*145±57 (4)*	*160±10 (3)*
SS-9B	170±23 (10)	65±11 (10)	161±3 (9)	FH-9B			
SS-10B	207±53 (11)	85±22 (11)	171±7 (10)	FH-10B	*556±52 (6)*	*264±29 (6)*	*187±7 (5)*
SS-11B	121±21 (16)	**35±10 (16)**	164±3 (11)	FH-11B	*670±56 (3)*	*175±55 (3)*	*190±7 (3)*
SS-12B[2]	303±23 (12)	87±11 (12)	191±5 (10)	FH-12B	368±46 (11)	112±15 (11)	190±5 (12)
SS-13B	**75±8 (16)**	**11±2 (16)**	**143±1 (18)**	FH-13B	447±65 (10)	171±37 (10)	189±5 (10)
SS-14B	151±30 (7)	52±8 (7)	176±8 (8)	FH-14B	**261±72 (8)**	139±34 (8)	165±8 (8)

Consomic strains				Consomic strains			
SS-15B	139±36 (7)	58±12 (7)	160±5 (7)	FH-15B			
SS-16B	145±23 (10)	50±11 (10)	155±3 (9)	FH-16B	**239±83 (8)**	82±26 (8)	165±13 (8)
SS-17B	466±47 (9)	176±16 (9)	184±9 (9)	FH-17B	538±64 (10)	152±22 (10)	187±10 (8)
SS-18B	**52±8 (9)**	**18±3 (9)**	**135±2 (8)**	FH-18B	425±56 (9)	79±29 (9)	182±4 (9)
SS-19B	*62±17 (6)*	*27±7 (6)*	*151±13 (5)*	FH-19B	453±76 (7)	227±45 (7)	166±8 (7)
SS-20B	134±19 (9)	44±9 (9)	147±8 (7)	FH-20B	**282±60 (8)**	138±33 (8)	**149±5 (7)**
SS-XB				FH-XB	582±69 (7)	205±19 (7)	*186±7 (6)*
SS-YB	153±16 (17)	43±6 (17)	163±4 (15)	FH-YB	*343±121 (5)*	*157±66 (5)*	*165±11 (4)*

BN, Brown Norway rat strain; SS, Dahl salt-sensitive, SS-nBN, SS consomic for BN chromosome n; FHH, fawn hooded hypertensive, FH-nBN, FHH consomic for BN chromosome n; [1)] Not consomic, but congenic between markers D8rat163 and D8rat81; [2)] Not consomic, but congenic between markers D12arb13 and D12rat79; All data are mean±SEM, (n) number of rats, **Bold figures** P<0.05 vs SS or FHH parental rats, ANOVA + Dunnett's test for pairwise comparison. *Italic figures* not included in ANOVA as n ≤ 6. Blank, no data available.

1.3.4 Gene expression studies

After identifying a QTL and the subsequent confirmation of an effect on the trait under investigation in a congenic strain, a next step on the path from QTL to gene may be the application of gene expression studies.[196] To narrow the number of candidate genes in a QTL region, one may focus on genes that show differential expression between the parental strains as well as the congenic and the recipient strain. This approach has been used to identify *Cd36* as a gene underlying cellular defects in glucose and fat metabolism in the spontaneously hypertensive rat (SHR).[1,2] However, successful studies using microarray expression profiling to identify genes in complex traits are scarce.

A new concept called 'genetical genomics' or 'expression genetics' applies gene expression assays from an experimental cross, treating the transcript abundance as a quantitative trait, to identify QTLs that influence the expression of genes.[24] Regulation of gene expression was studied in tissues in a panel of rat recombinant inbred strains derived from the SHR and BN strains. By combining the gene expression QTLs with previously mapped blood pressure QTLs a data set of 73 candidate genes for hypertension was generated that merit further testing.[108] A similar approach for identifying candidate genes influencing renal susceptibility has not yet been reported.

1.3.5 Bioinformatics to integrate human, mouse and rat

In the current era, several websites are providing data for researchers on various species, for instance Rat Genome Database (RGD, rgd.mcw.edu), Human Phenome Database (HPD, hpd.mcw.edu), and comparative maps of human, rat and mouse (http://www.ncbi.nlm.nih.gov/projects/Homology). Integrating rat physiology with mouse genetics and clinical results from human by using the respective genomes provides a novel route to capitalize on comparative genomics and the strengths of model organism biology (Figure 2).[116,260,261] In the end, it will hopefully lead to a better understanding of the pathogenesis and pathophysiology of a disease and ultimately prevention of a disease.[145,150]

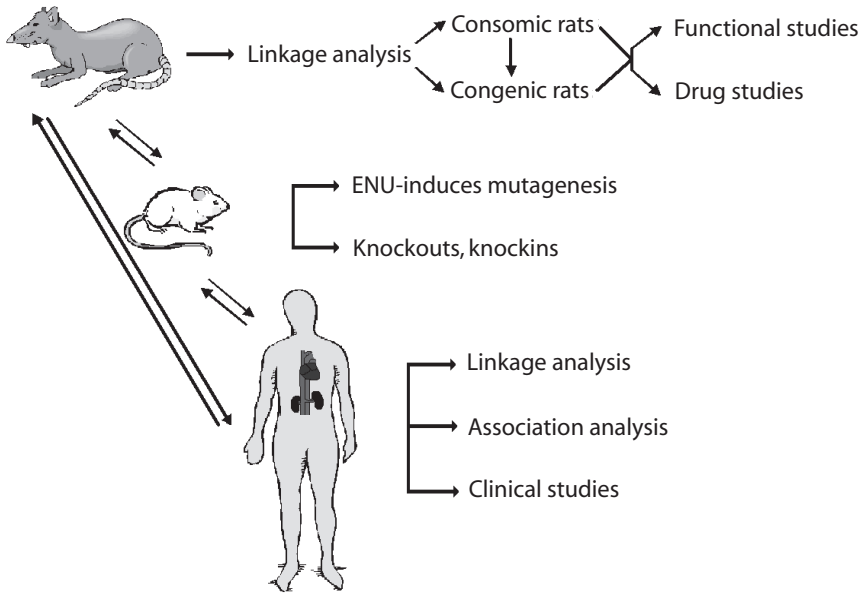


Figure 2: Using data from rat and mouse studies for studies of human complex diseases (adapted from Jacob, Kwitek, 2002)[116]

1.4 Outline of this thesis

Linkage analyses of involving crosses between FHH and ACI rats revealed the presence of five QTLs linked to parameters of renal damage (*Rf-1* through *Rf-5*), and two QTLs linked to SBP. The first aim of this thesis is to directly assess the role of each of these QTLs in determining the susceptibility to renal damage. These studies are performed in single congenic rats, carrying one of the *Rf*-QTLs from the FHH strain on the genomic background of the ACI strain. The linkage analyses also suggested interactions between *Rf-1* and other *Rf*-QTLs. Therefore the second aim is to directly assess the possible interaction between *Rf-1* and the other four *Rf*-QTLs in enhancing renal damage susceptibility. These studies are performed in double congenic rats, carrying both *Rf-1* and another *Rf*-QTL from FHH on the genomic background of the ACI strain. The third aim is to establish whether the supposed interactions between the QTLs are affected by the treatments employed to induce chronic renal damage. It has been shown that an impaired renal autoregulation is involved in the high susceptibility to renal damage in the FHH rat. Thus, the fourth aim is to establish the role of the *Rf*-QTLs in the autoregulation of the renal blood flow.

The outline of this thesis is as follows. **Chapter 2** describes the generation and genetic lay-out of the ACI.FHH-*Rf* single and double congenic rats. The congenic strains were generated using a speed congenic strategy with marker-assisted breeder selection. Each of the congenic rats has been characterized by dense genotyping.

Chapter 3 describes the renal damage susceptibility and renal autoregulation in single congenics rats carrying either the *Rf-1B* or *Rf-5* region of the FHH rat onto an ACI genomic background. The susceptibility to renal damage was assessed using four models. First the control situation where no interventions were made, and the rats remained with two kidneys and were normotensive. In the second model, an UNX is performed to reduce the functional renal mass. The third model consisted of rats receiving L-NAME to induce a level of hypertension, which is normally present in the FHH rat. The fourth model was a combination of UNX and L-NAME induced hypertension.

Chapter 4 describes the renal damage susceptibility and renal autoregulation in congenic rats carrying either the *Rf-1A* or *Rf-4* regions alone as well as the combination of *Rf-1A* and *Rf-4*. Renal damage susceptibility is again tested using the four treatment models.

Chapter 5 describes the role of the *Rf-3* region alone and combined with the *Rf-1A* region in the development of renal damage.

Chapter 6 describes the susceptibility to renal damage of the combination of the *Rf-5* region with *Rf-1B* and the comparison with a similar double congenic carrying the *Rf-1B* and *Rf-4* region.

Chapter 7 describes the renal damage susceptibility and renal autoregulation in double congenic rats carrying the *Rf-1A* and *Rf-2* regions in comparison with FHH and FHL rats. Due to an increased susceptibility to L-NAME, renal damage susceptibility in these strains is only tested in the 2K and UNX-situation.

Chapter 8 describes the results of employing the remnant kidney model (RKM) to assess differences in renal susceptibility. In the RKM about 75% of the renal mass is removed inducing hypertension and chronic renal failure.

Chapter 9 contains the general discussion and presents the conclusions drawn from the various studies described in this thesis. It also presents perspectives for future studies.

Chapter 10 ends the thesis with an English and a Dutch summary.

Chapter 2

A panel of congenic rat strains derived from ACI and FHH rats to study the genetics of progressive renal damage

SABINE J. VAN DIJK, PATRICIA A.C. SPECHT, ABRAHAM P. PROVOOST

To be submitted

Introduction

Chronic kidney disease (CKD) is a major health problem all over the world.[160,244,263,285] The presence of CKD is not only a risk factor for the development of end-stage renal failure (ESRF), but also for cardiovascular disease (CVD).[217] Hypertension and diabetes are the underlying causes in most patients.[181] However, hypertension and diabetes do not always result in ESRF, but are rather risk factors influencing the susceptibility to renal damage.[71,75,127] Familial clustering and ethnic differences in the prevalence of ESRF are well documented.[11,201,220,222,226,247,248,263] Therefore, genetic factors are thought to play an important role in the initiation and rate of progression of ESRF. Genetic linkage studies in patients with various forms of nephropathy indicate that multiple chromosomal loci appear to be involved.[18,37,39,74,77,78,86,111,114,166,184,202,203,259,277,294] In addition, there may be predisposing environmental risk factors, and gene-gene and gene-environment interactions.[38,169,219] Animal studies may be helpful to unravel the complex genetics of ESRF.

Renal damage susceptibility in rats

Inbred rat strains also vary widely in their susceptibility to develop renal damage. Strains such as the Fawn-Hooded Hypertensive (FHH), the Buffalo (BUF), the Munich Wistar Fromter (MWF), and the Dahl Salt Sensitive (SS) develop some degree of spontaneous proteinuria (UPV) and albuminuria (UAV). Linkage analyses have identified about 70 quantitative trait loci (QTL) linking chromosomal markers to renal damage traits, such as UPV, UAV, structural renal damage, etc.[25,84,88,113,172,175,224,225,234,235] Our studies involve the FHH rat, prone to develop mild hypertension and marked renal damage at a young age, and are well characterized by numerous physiological and histological studies.[133,198,238,239,272,273,279] Crosses between FHH and the renal damage resistant ACI rat revealed the presence of five QTLs linked to UPV and other parameters of renal damage. These QTLs were named *Rf-1* to *Rf-5*.[25,234] The LOD-scores and the position of these QTLs are presented in Table 1. The *Rf-1*, *Rf-2*, and *Rf-3* QTLs were significantly linked to UPV, whereas the *Rf-4* and *Rf-5* QTLs were suggestive for linkage. It is surmised that each of these QTLs contain one or more genes that play a role in the development of progressive renal damage in the FHH rat. The high LOD-score peak for UPV indicates that *Rf-1* must contain one or more genes that play an important role in defining the high susceptibility to renal failure in the FHH rat. However, the presence of four additional QTLs that influence UPV underscores the complexity of the genetics of renal failure.[234]

Localization of the chromosomal regions that contain susceptibility genes is only the beginning of a long road that should lead to the identification of the individual genes. Gene identification is essential for further understanding of the function of genes and the pathway by which the mutated genes may result in an increased susceptibility to renal damage. The first step to reach these goals is to generate a panel of congenic rat strains.[89,300]

Table 1: Location of the rat renal failure QTLs and their human and mouse homologs.

QTL	LOD-score	Rat 95% C.I.		Human Homolog		Mouse Homolog	
		Chr.	Mb	Chr.	Mb	Chr.	Mb
Rf-1	16.7 (1)	1	243-257	10	95-109	19	37-50
Rf-2	5.4 (1)	1	138-173	15 11 11	81-78 88-71 3-15	7	68-102
Rf-3	6.1 (1)	3	117-146	20 20 20	1-25 0-1 29-33	2	126-155
Rf-4	4.1 (2)	14	5-20	1 4	90-89 89-73	5	103-88
Rf-5	3.0 (2)	17	55-68	7 1 10 1	39-42 0.2-0.2 32-29 0.2-0.2	13 18 13	17-13 7-3 13-12

QTL = quantitative trait locus; LOD = logarithm of the odds; *Rf* = congenic rat strain; Chr. = chromosome; C.I. = confidence interval; Mb = Megabase; (1): significant linkage for UPV; (2): suggestive linkage for UPV.

Congenic strains in general

Congenic strains are animal strains in which the native genomic background of the recipient is maintained unchanged except for a specific genomic region of interest, which incorporates the QTL that is transferred from a donor strain.[207,236] Congenic strains are useful for studying the effects of specific genes or genomic regions against a common inbred background. The use of congenic strains has evolved during the past decade as the major working algorithm used by researchers in their attempts to narrow down the span of QTLs.[136,300]

Single congenics provide direct evidence for an effect of the QTL, i.e. on the disease trait or a physiological parameter of a disease trait. Interactions between a gene and the environment can also be revealed. Double congenics are used because they provide direct evidence for gene-gene interactions that can either be additive or epistatic and for gene-environment interactions. When more QTLs are involved, triple or multiple congenics can also be generated and studied.

Breeding of congenic rats

Several crossing methods have been developed for generating congenic rat strains[207,154,236], depending on whether the characteristic that has to be dealt with is either recessive, co-dominant or dominant. The method shown in Figure 1 is a widely used method to generate congenic rat strains. After eight to twelve backcrosses, according to Mendelian laws, more than 99% of the genetic background is that of the recipient strain. On completion of the backcrossing, the final step of brother x sister mating fixes the strain so that the desired chromosomal region is homologous for the donor's alleles in one quarter of the offspring. The generation of congenic rat strains can be accelerated by using marker-assisted breeding selection or "speed" congenic method. This allows for the selection of a 'best male' at each stage of backcrossing which has the least amount of donor alleles in the genetic background whilst still maintaining heterozygosity at

the chromosomal region of interest. By selecting for the 'best male' the number of backcross generations needed to establish congenic strains in the rat is reduced to approximately five instead of the traditional eight to twelve. This speed congenic strategy can save nearly two years of backcrossing at the price of genotyping hundreds of markers.[120,154,159,207]

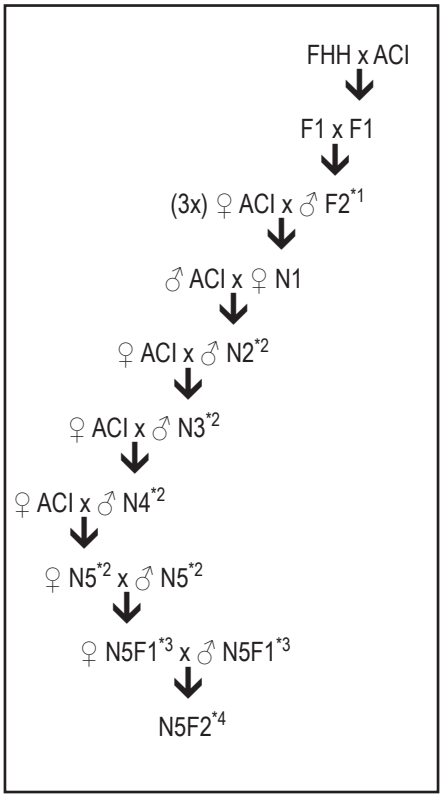


Figure 1: Scheme of producing an *Rf*-congenic rat

*1 selected on development of renal damage; *2 genotyped for heterozygosity of *Rf* and with the most ACI alleles; *3 selected for homozygosity of *Rf*; *4 can be used to set up a congenic line; N = generation; F = offspring

Two types of congenic strains can be generated. In the first type, a QTL region that is linked to a trait in the disease in the recipient strain can be substituted by the region of a non-diseased donor. These congenics are then tested to determine if the trait is absent, or less severe. In other words, research is focused on (partly) rescuing the phenotype by the congenic substitution. In the second type, congenic rats are generated by introgressing a QTL region of a diseased donor strain into the genomic background of the non-diseased recipient. This type of congenics is then tested to determine if the disease trait does (partly) occur in the congenic strain because of the introgressed QTL region. For our studies the second type of congenic strains were generated.

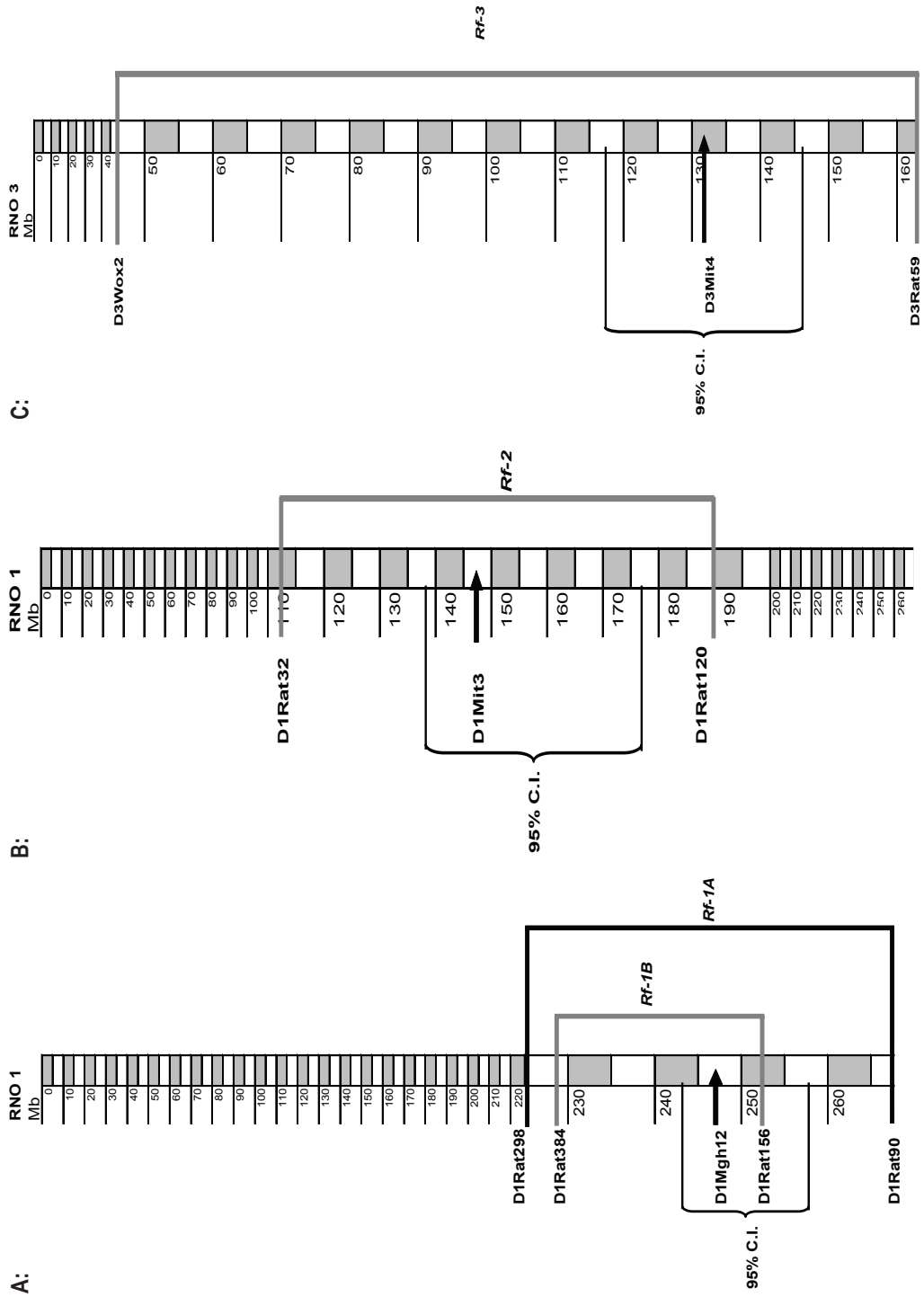
The advantage of congenic rats is that it is possible to establish alleles of a small and well-defined set of loci on a common genetic background. The major disadvantages are the loss of genetic variability and the extreme effort that is necessary to develop and maintain congenic rats.[89]

So far, several congenic strains have been generated to discover genes influencing the development of hypertension.[56,64,159,207] Congenic strains for diseases such as diabetes, arthritis and obesity are also widely used.[3,22,107,170,241] However, the use of congenic strains for assessing susceptibility to renal damage is still marginal and mainly linked to hypertension research. Congenic rats carrying *Rf*-regions will expand the amount of congenic strains available for assessing susceptibility to the development of renal damage.

Congenic rat strains to study renal susceptibility

Over the last decade we have been generating congenic rat strains carrying *Rf*-QTLs of the FHH rat on the background of the ACI rat strain. Here we present an overview of our progress in generating congenic strains carrying one or more *Rf*-regions.

Congenic lines were generated by using the speed congenic method.[154,199,267,270] Since the *Rf-1* QTL gave a LOD-score of around 16, our main interest is the single *Rf-1* congenics and congenics carrying *Rf-1* in combination with other *Rf*-QTLs. At this moment we have two *Rf-1* single congenic rat strains, a single *Rf-2*, a single *Rf-3*, a single *Rf-4* and a single *Rf-5* congenic. The *Rf* single congenic rats carry 25-117Mb of the FHH rat onto an ACI genomic background, which means that the rats are 96-99% ACI with the exception of the 1-4% *Rf*-region of the FHH rat. Figure 2 visualizes the *Rf* single congenics by depicting which *Rf*-regions of the FHH are introgressed into the ACI genomic background. Table 2 shows the total size of the introgressed regions of the FHH, where the regions are located and on which chromosome.


A:
RNO 1
Mb
D1Rat298
D1Rat384
D1Mgh12
D1Rat156
D1Rat90
95% C.I.
Rf-1A
Rf-1B
B:
RNO 1
Mb
D1Rat32
D1Mit3
D1Rat120
95% C.I.
Rf-2
C:
RNO 3
Mb
D3Wox2
D3Mit4
D3Rat59
95% C.I.
Rf-3

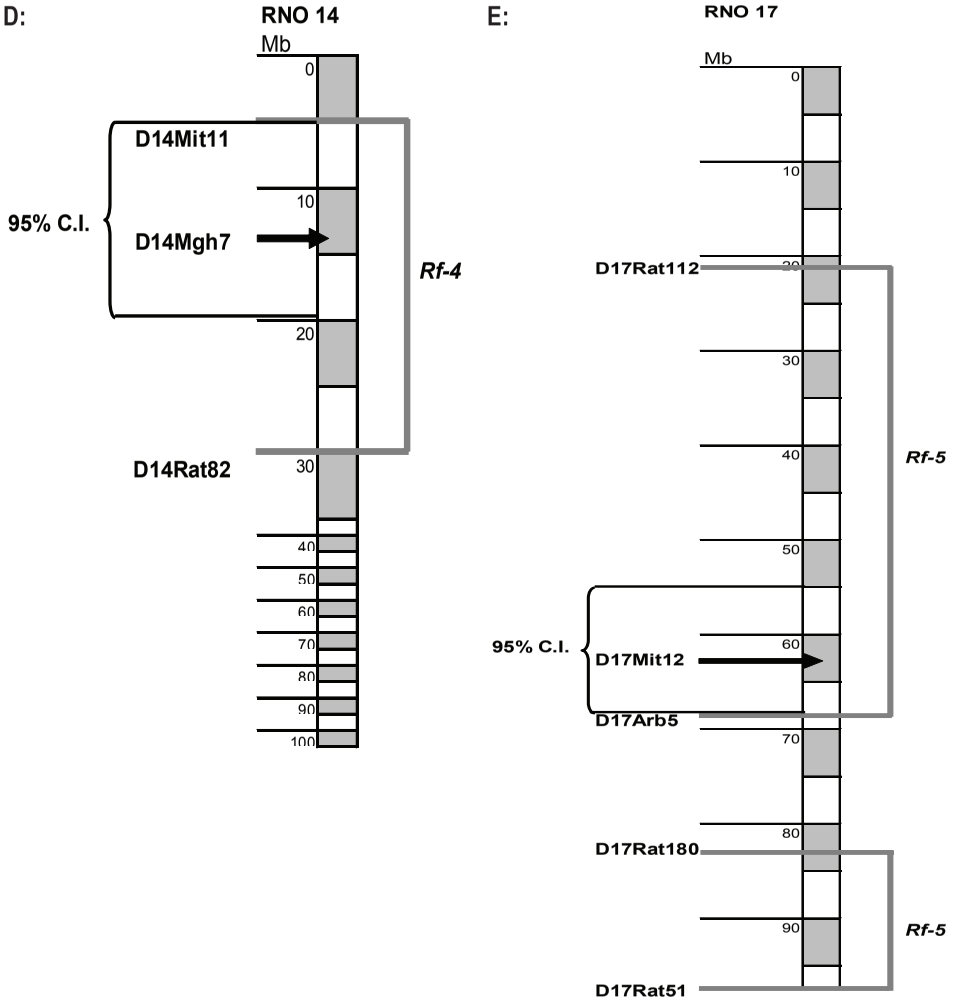


Figure 2: Genetic composition of our ACI.FHH single congenic rat strains.

A: *Rf-1* single congenic rats; **B**: *Rf-2* single congenic rat; **C**: *Rf-3* single congenic rat; **D**: *Rf-4* single congenic rat; **E**: *Rf-5* single congenic rat. The whole genomic background of these congenic rats is ACI, except for the areas shown at the right hand side above with the different markers. These areas are homozygous for FHH, and contain the QTL peak. The arrows at the left hand side indicate the locations of the QTL peaks plus 95% C.I. found in previous studies.[25,234]

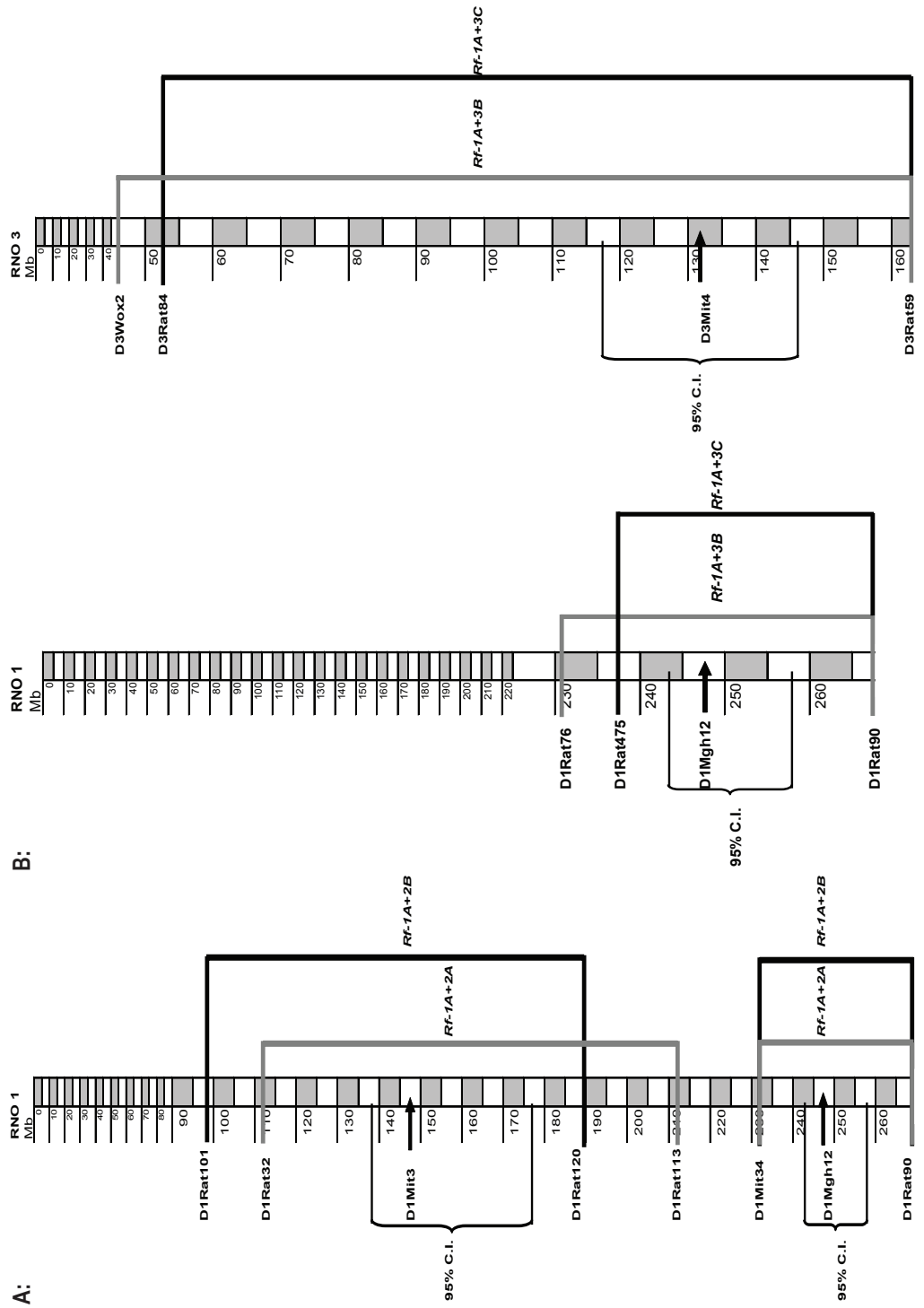
Table 2: *Rf*-regions introgressed in the *Rf* single congenics

Congenic	RNO	region	Total (Mb)
Rf-1A	1	225-267	42
Rf-1B	1	229-254	25
Rf-2	1	112-188	76
Rf-3	3	46-163	117
Rf-4	14	5-30	25
Rf-5	17	21-69 / 82-97	48 / 15

Rf = renal failure; RNO = rat chromosome; Mb = Megabase

Double congenics are available in the combinations of *Rf-1* and *Rf-2*, *Rf-1* and *Rf-3*, *Rf-1* and *Rf-4*, and *Rf-1* and *Rf-5*. The *Rf* double congenic rats carry 74-154Mb of the FHH rat onto an ACI genomic background, which means that the rats are 95-98% ACI with the exception of the 2-5% *Rf*-region of the FHH rat. Figure 3 visualizes the *Rf* double congenics by depicting which *Rf*-regions of the FHH are introgressed into the ACI genomic background. Table 3 shows the total size of the introgressed regions of the FHH, where the regions are located and on which chromosome.

Complex gene-gene interactions should be revealed in double or multiple congenics, as well as the interaction of genes with environmental factors known to influence the progression of renal failure, such as hypertension, renal mass reduction, protein intake, and so forth. The ultimate goal will be to generate a congenic rat carrying all five *Rf*-QTLs of the FHH rat onto an ACI genomic background.


A:
RNO 1
Mb
D1Rat101
D1Rat32
D1Mit3
D1Rat120
D1Rat113
D1Mit34
D1Mgh12
D1Rat90
95% C.I.
95% C.I.
Rf-1A+2A
Rf-1A+2B
Rf-1A+2A
Rf-1A+2B
B:
RNO 1
Mb
D1Rat76
D1Rat475
D1Mgh12
D1Rat90
95% C.I.
Rf-1A+3B
Rf-1A+3C
RNO 3
Mb
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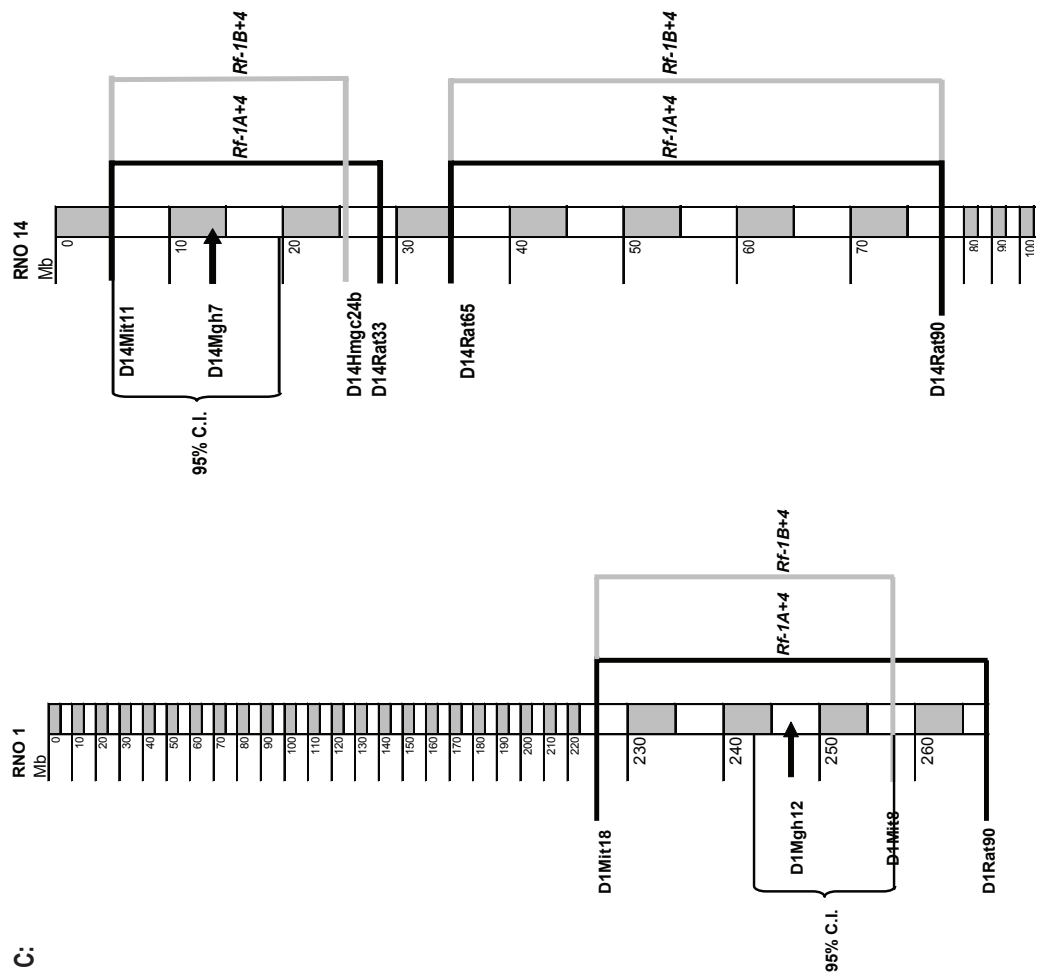
D3Rat84
D3Mit4
D3Rat59
95% C.I.
Rf-1A+3B
Rf-1A+3C


RNO 14
Mb
0
10
20
30
40
50
60
70
80
90
100
Rf-1B+4
Rf-1A+4
Rf-1B+4
Rf-1A+4
D14Mit11
D14Mgh7
95% C.I.
D14Hmgc24b
D14Rat33
D14Rat65
D14Rat90
C:
RNO 1
Mb
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
Rf-1B+4
Rf-1A+4
D1Mit18
D1Mgh12
95% C.I.
D1Mit8
D1Rat90

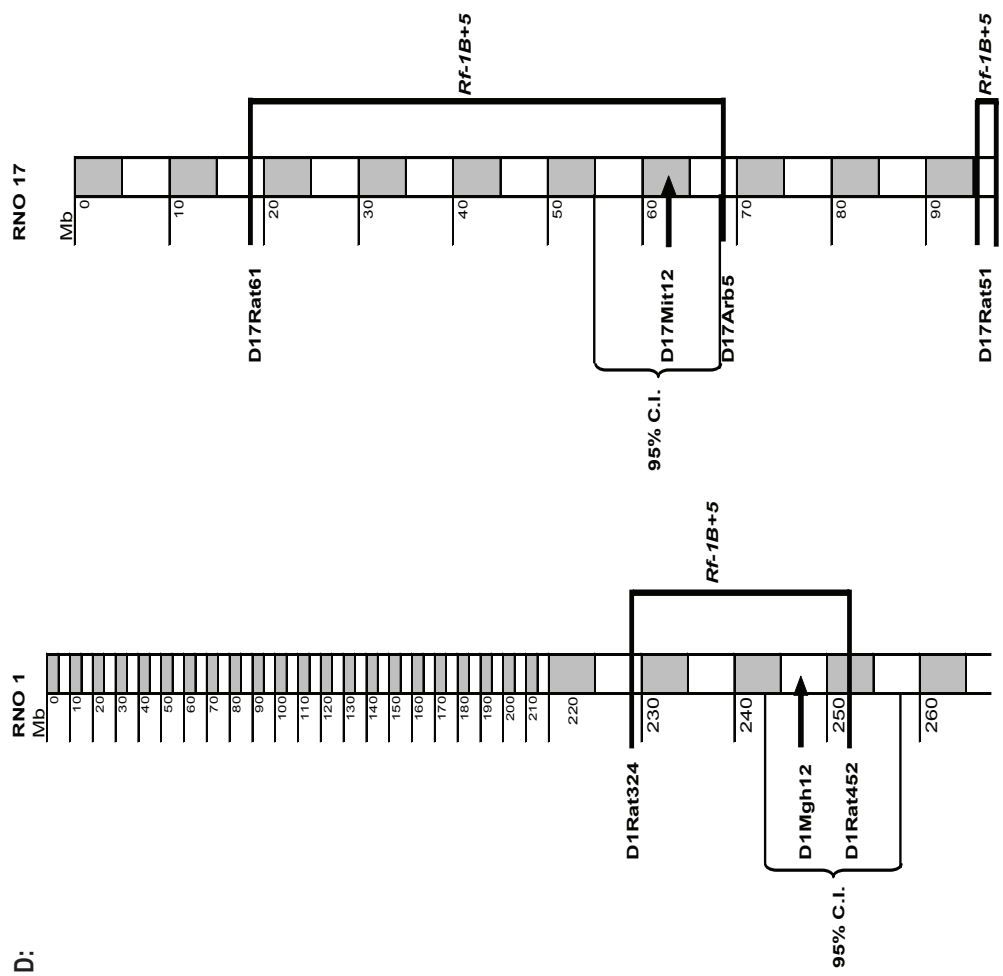


Figure 3: Genetic composition of our ACI.FHH double congenic rat strains.
A: *Rf-1+2* double congenic rats; **B**: *Rf-1+3* double congenic rats; **C**: *Rf-1+4* double congenic rats; **D**: *Rf-1+5* double congenic rat. The whole genomic background of these congenic rats is ACI, except for the areas shown at the right hand side above with the different markers. These areas are homozygous for FHH, and contain the QTL peak. The arrows at the left hand side indicate the locations of the QTL peaks plus 95% C.I. found in previous studies.[25,234]

Table 3: *Rf*-regions introgressed in the *Rf* double congenics

Congenic	RNO	region	Total (Mb)
Rf-1A+2A	1	230-267	37
	1	112-212	100
Rf-1A+2B	1	230-267	37
	1	97-188	91
Rf-1A+3B	1	230-267	37
	3	46-163	117
Rf-1A+3C	1	238-267	29
	3	53-163	110
Rf-1A+4	1	224-267	43
	14	5-29 / 36-74	24 / 38
Rf-1B+4	1	224-258	34
	14	5-26 / 36-74	21 / 38
Rf-1B+5	1	229-253	24
	17	20-69 / 96-97	49 / 1

Rf = renal failure; RNO = rat chromosome; Mb = Megabase

Combining physiology with genetics

Physiology must be combined with genetics in order to find out which genes in the *Rf*-regions are responsible for susceptibility, initiation or progression of renal damage. The *Rf*-congenic rat strains that carry either a single *Rf*-QTL or the combination of *Rf-1* and another *Rf*-QTL can be helpful to unravel the mechanisms of the development of severe renal damage.

Unravelling the mechanisms behind the development of renal damage can be done using various studies. First, renal susceptibility should be assessed. When renal susceptibility is present, studies looking into renal physiology should be performed. Gene expression studies can be done to find out if there are differences in gene activity between two rat strains. When finding renal susceptibility genes in one species, comparative genomics can help to find where these renal susceptibility genes are present in other species.

Various methods are available to assess susceptibility to develop renal damage. Several studies have shown that reduced renal mass, either congenital or acquired, increases the risk of developing renal damage.[169,256,289] Renal mass can be reduced by performing unilateral nephrectomy (UNX) or 5/6 subtotal nephrectomy.[97,274] Another risk factor for the development of renal damage is hypertension. Hypertension can be spontaneously present[117] or induced in several ways: deoxycorticosterone acetate-salt-induced hypertension[100,144], cadmium-induced hypertension[8], angiotensin II-induced hypertension[221], ouabain-induced hypertension[213], or L-NAME-induced hypertension.[274,275,276] For our studies we tested four experimental situations to compare the susceptibility to develop chronic kidney damage. First, we have a control situation, where no interventions are made. This model looks at a situation where renal mass and systolic blood pressure (SBP) are normal. The second model looks at the effect of reduced renal mass following UNX. The third model is a two-kidney model with L-NAME induced hypertension. The fourth model

combines UNX and L-NAME induced hypertension. With these models, differences in development of renal damage, dependent on the presence of renal susceptibility genes, can be detected.[199,267,274,275,276]

In addition, studying the renal physiology of the congenic strains can unravel potential pathways that may explain an enhanced susceptibility and may thus be helpful in the gene identification studies. A possible pathway could be an impaired renal autoregulation. Previous studies indicated that FHH rats have an impaired myogenic response of the autoregulation system, however, the tubular glomerular feedback (TGF) is intact.[272,273] Renal autoregulation system in general can be determined by assessing renal blood flow when increasing renal perfusion pressure. Whether myogenic response or TGF is impaired can be assessed by performing micropuncture studies.[273] Another possible pathway is an increased glomerular permeability. Normally, the glomerular membrane is impermeable for proteins. When this glomerular membrane is damaged, leakage of proteins into the urine can occur. The permeability of the glomerular membrane can be assessed by the albumin permeability assay.[232]

Gene expression studies create an opportunity to reveal differences in gene activity between different kinds of rats. There are many techniques that help us identify and isolate transcripts that are differentially expressed, e.g. microarray or suppressive subtraction hybridisation (SSH).[57,83,223] Microarrays are commercially available (Affymetrix) but can also be custom made. Microarrays are used to assess which genes are upregulated or downregulated. The SSH can be performed with the help of commercially available kit. It is a powerful technique that makes it possible to compare two populations of mRNA and obtain clones of genes that are expressed in one rat strain but has a reduced expression or is not expressed at all in the other rat strain. The main difference between microarrays and SSH is that the SSH method leaves out the genes that are expressed equally in both subjects. The SSH method is particularly well suited for the identification of target cDNAs that correspond to rare transcripts, which are typically the most difficult to obtain in microarrays.[57,83,223]

Gene expression studies can reveal differences between FHH and ACI. More detailed differences can be obtained comparing ACI and congenic strains, especially when differences are present between genes located within the congenic regions of the FHH. These studies will hopefully lead to identification of genes involved in the development of renal damage.

Rat, mouse and human homology

With the completion of the sequencing of the human, mouse and rat genome, genomic comparisons between these three species are greatly facilitated.[87,135,278,287] Comparative genomics combined with physiological information will facilitate the discovery of mammalian genes that underlie physiological pathways that are involved in disease.[261] In addition, it should lead to the development of better pre-clinical models of human disease, which will aid in the discovery of new therapeutic targets.[23,116,250] Chromosomal regions of human and mouse homologous to the *Rf*-regions in the rat are depicted in Table 1 (http://www.ncbi.nlm.nih.gov/projects/Homology).

Conclusion

The mapping of many physiological traits to the genome should help to accelerate our understanding of the biology of disease by guiding and facilitating the identification and functional understanding of disease genes.[116] Etiology and pathology of the susceptibility to the development of chronic renal insufficiency can be unravelled in congenic animal models. Characterization of genes and gene-gene interactions in rats can lead to identification of homologous genes in humans. Finally, this can lead to a change in prediction, diagnosing, treatment and prevention of progressive renal damage in humans.

Acknowledgements

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Chapter 3

Renal damage susceptibility and autoregulation in *Rf-1* and *Rf-5* congenic rats

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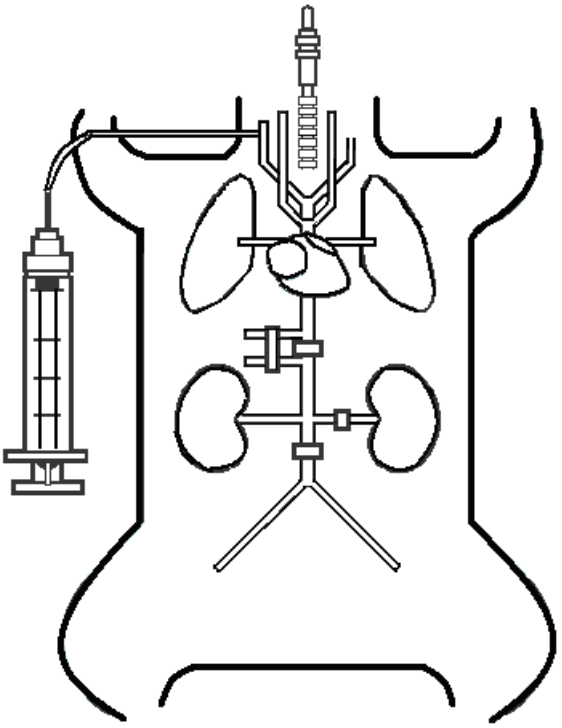

Abstract

Background: Linkage analyses of crosses of the susceptible to renal damage FHH (fawn-hooded hypertensive) and the resistant to kidney damage ACI (August x Copenhagen Irish) rats indicated that five quantitative trait loci (QTLs), *Rf-1* to *Rf-5*, influence proteinuria (UPV), albuminuria (UAV) and focal glomerulosclerosis (FGS). Here we present data obtained in congenic rats to directly assess the role of the *Rf-1* and *Rf-5* QTLs.

Methods: Renal damage (UPV, UAV, and FGS) was assessed in ACI, ACI.FHH-(*D1Rat324-D1Rat156*) (*Rf-1B*), and ACI.FHH-(*D17Rat112-D17Arb5)(D17Rat180-D17Rat51*) (*Rf-5*) congenic rats in the two-kidney (2K) control situation, and following L-NAME-induced hypertension, unilateral nephrectomy (UNX), and UNX combined with L-NAME. In addition we investigated renal blood flow (RBF) autoregulation in 2K congenic and parental ACI and FHH rats.

Results: Compared to ACI, *Rf-1B* congenic rats after all three treatments showed a significant increase in susceptibility to renal damage. The increase was most pronounced after UNX with L-NAME. In contrast, the degree of renal damage in *Rf-5* congenic rats was not different from the ACI. Like FHH, *Rf-1B* rats had an impaired renal autoregulation. In contrast, RBF autoregulation of *Rf-5* rats does not differ from ACI. *Conclusion:* The *Rf-5* QTL does not show any direct effect. The *Rf-1* QTL carries one or more genes impairing renal autoregulation and influencing renal damage susceptibility. Whether these are the same genes remains to be established.

Introduction

The incidence and progression of kidney damage, eventually resulting in end-stage renal failure (ESRF) is an increasing international health care problem.[263] Systemic hypertension is often seen in chronic kidney disease, but not every patient with hypertension develops ESRF. This indicates that systemic hypertension may be a risk factor, but is in itself not sufficient to cause ESRF.[14,71,127] Over the last 8 years, several studies in human and animal models have demonstrated that genetic components play a critical role in hypertension-associated ESRF.[12,198,290]

The FHH (Fawn-Hooded hypertensive) rat is known to develop hypertension that is followed by renal damage at a young age.[238] Our previous studies with crosses of the FHH and the ACI (August x Copenhagen Irish), which is resistant to renal failure, revealed the presence of 5 quantitative trait loci (QTLs) linked to the development of renal damage. These QTLs were named *Renal failure-1* (*Rf-1*) to *Rf-5*.[25,234] It is surmised that each of these QTLs contains one or more genes that play a role in the development of progressive renal damage in the FHH rat. Although the nature of the genes has yet to be identified, the separate role of each QTL in the initiation and progression of renal damage can be studied in single congenic rat strains carrying one *Rf*-region of the FHH rat transgressed into the genomic background of the resistant ACI rat. The ability to use these congenic rats, carrying about 30-60 Mb (i.e. 1-2%) of the FHH genome offers unparalleled power to investigate the mechanisms underlying the development of chronic renal damage. In a later stage, interaction between the QTLs can be studied in double, multiple congenic strains, or consomic strains.[116]

As the ACI is normotensive, and the renal failure requires an interaction with blood pressure, there is a need to increase blood pressure in the congenic animals. Previously, we reported about the development of renal damage after unilateral nephrectomy (UNX), and UNX combined with L-NAME induced hypertension in the congenic ACI.FHH-(*D1Rat324-D1Rat156*) rat, *Rf-1B* for short.[199] The *Rf-1B* rats developed significantly more proteinuria (UPV) and albuminuria (UAV) than the ACI control strain during a 24-week study. As expected this difference was most pronounced when UNX was combined with L-NAME-induced hypertension. These findings indicated that the *Rf-1B* region of the FHH rat contains one or more genes affecting the susceptibility to progressive renal damage.

Studies in the parental FHH strain have indicated an impaired autoregulation of the renal blood flow (RBF) and intraglomerular pressure (P_{GC}) as an important mechanism in the early development of renal damage.[272] Due to an impaired myogenic response, FHH rats are unable to maintain a constant P_{GC} when faced with a change in systemic blood pressure.[273] We hypothesized that autoregulation would be impaired in one or more of the congenic animals carrying a single renal failure locus.

Methods

Animals (general)

Male *Rf-1B*, and ACI.FHH-(*D17Rat112-D17Arb5*)(*D17Rat180-D17Rat51*) (*Rf-5* for short) congenic rats and control ACI/EUR and FHH/EUR rats were used. All breeding was performed at the Animal Research Centre at Erasmus MC, Rotterdam, the Netherlands. Animals were housed in individually-ventilated cages under SPF-conditions.[180] Lights were on from 7:00 a.m. to 7:00 p.m. Standard commercial rat chow containing 44% carbohydrates, 29% digestible protein, 7% fat, 4% fibre, and 8% minerals (SRM-A; Hope Farms, Woerden, the Netherlands) and drinking fluid (tap water, acidified to pH 2.4-2.8) were provided *ad libitum*. The protocols were approved by the animal ethical committee (DEC) at Erasmus MC, Rotterdam.

The *Rf-5* congenic strain was generated using a speed congenic strategy as described for the *Rf-1B* strain by Provoost et al.[119] The congenic (homozygous FHH) region of the *Rf-1B* strain is on chromosome 1 between markers *D1Rat384* (229.4 Mb) and *D1Rat156* (253.7 Mb), spanning 24.3 Mb, about 9% of the chromosome. The *Rf-5* congenic region on chromosome 17 is split in two parts. One between markers *D17Rat112* (21.0 Mb) and *D17Arb5* (69.3 Mb), spanning 48.3 Mb, the other between markers *D17Rat180* (82.8 Mb) and *D17Rat51* (96.6 Mb), spanning 13.8 Mb. The two regions cover about 64% of chromosome 17. A whole genome scan with about 150 genetic markers distributed across the genome in each of these two congenic rats showed that they did not contain genetic contamination of FHH on other chromosomes.[119]

Renal damage susceptibility

Animals

Experiments for assessment of renal damage susceptibility were performed on 101 animals (35 ACI, 31 *Rf-1B*, and 35 *Rf-5*) divided across the control and three treatment groups starting from the age of 6-7 weeks, with a body weight (BW) of 100-120g. The control group had both kidneys and received no treatment (2K). Group two had both kidneys and was chronically treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) (2K+L-NAME) to induce systemic hypertension. The third group was unilaterally nephrectomized (UNX). The fourth group had both an UNX and received L-NAME (UNX+L-NAME). The number of rats per strain for each treatment is presented in Table 1.

Unilateral nephrectomy and induction of hypertension

UNX was performed as previously described.[274] L-NAME was dissolved in the acidified drinking water at a concentration of 150 mg/L.[274] Treatment with L-NAME was started 5 days after surgery. Control animals were provided with acidified drinking water. Actual L-NAME intake was in the order of 8-13 mg/day/kg BW in the 2K+L-NAME situation, and 11-15 mg/day/kg BW in the UNX+L-NAME situation. No significant differences in L-NAME intake were present between the four strains at all time points following 2K+L-NAME treatment, nor at the first and second evaluation with UNX+L-NAME treatment. However, at the final evaluation, the *Rf-1B* rats showed a significantly higher intake of L-NAME compared to ACI (data not shown).

Urine collection and blood pressure monitory

Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period.

Following the urine collection, SBP was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained rats, as described previously.[199]

Autopsy

An autopsy was carried out shortly after the last series of urine collections and SBP measurements. The rats were anaesthetized and a 20-gauge needle (Braun, Melsungen, Germany) was introduced into the aorta at the bifurcation of the left and right iliac artery and a blood sample was taken. Kidneys were collected and weighed and the left kidney was used to determine extent of glomerular damage, i.e. the incidence of focal glomerulosclerosis (%FGS). Briefly, kidneys were longitudinally dissected, and were then fixed by immersion in 3% Formalin (Fresenius Kabi, 's-Hertogenbosch, the Netherlands). After fixation, the kidneys were embedded in paraffin, sectioned and prepared for light microscopy. The %FGS was determined in 1-µm sections stained with periodic acid-Schiff (PAS) reagent, by examining 50 glomeruli.[275]

Renal blood flow autoregulation

Experiments to assess RBF autoregulation were performed on 63 animals (15 ACI, 15 FHH, 18 *Rf-1B*, and 15 *Rf-5* rats) with an average age of 14 weeks. Animals were anaesthetized with a mixture of 3% Isoflurane®, 30% N_2O, and 60% O_2 and surgically prepared for autoregulation studies.[272] After surgery and a 10-min equilibration period, the relationship between the left renal artery blood flow and the renal perfusion pressure (RPP) was determined. In each animal RPP was increased by ligating the celiac and mesenteric arteries together. RBF was recorded using a 1.5 mm flow probe and a flow meter (Transonic Systems Inc, Ithaca, USA) as the RPP measured at the femoral artery, using a Datex-Ohmeda S/5 Light Monitor (Datex-Ohmeda, Hoevelaken, the Netherlands) was lowered from 150 to 80 mm Hg in 10 mm Hg steps by tightening the clamp around the aorta above the renal arteries, followed by a 3-min equilibration period. When the RPP did not reach 150 mm Hg, we put a clamp around the aorta beneath the renal arteries and measured the RPP at the carotid artery.

The degree of renal damage was also determined. Before the autoregulation experiment rats were put in the metabolic cages for one day, and a 24-hr urine sample was collected to measure UPV and UAV. After the evaluation, the left kidney was used to determine %FGS.

Calculations

To normalize the outcome of the individual rats, the RBF at a RPP of 100 mm Hg (RBF_{100}) was considered to be 100%. At each level of RPP ($_x$) the percentage RBF (%RBF) was calculated using the following formula:

$$\% RBF_x = (RBF_x/RBF_{100}) * 100$$

Renal autoregulatory indexes (RAIs) over the range of pressures from 80 to 150 mm Hg were calculated by the method of Semple and de Wardener[228] using the following formula:

$$RAI = [(RBF_2 - RBF_1)/(RBF_1)]/[(RPP_2 – RPP_1)/RPP_1)]$$

The RAIs were calculated assuming that RPP was reduced in a single step from a high pressure (RPP_2) to a lower pressure (RPP_1). A RAI of 0 indicates perfect autoregulation of RBF, and a RAI of 1 indicates that there is no autoregulation present due to a fixed renal vascular resistance.

Analytical procedures and statistics

Plasma and urinary samples were analysed with an ELAN system (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Total urinary protein was determined colorimetrically with pyrogallol red-molybdate complex.[286] Urine and plasma albumin (Palb) was determined with bromocresol green.[62] Plasma creatinine (Pcreat) was determined with the Jaffe method without de-proteinisation.[240]

Data are presented as mean ± SEM. Statistical differences in mean values between groups were compared using one way analysis of variance (ANOVA). The ANOVA test was followed by the Bonferroni t-test to determine which of the congenic strains were significantly different from each other. In all tests, a p-value <0.05 was considered statistically significant. All tests were performed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996).

Results

Assessment of renal damage susceptibility

Proteinuria, albuminuria, and systolic blood pressure

The UPV and UAV levels during the various treatments in the ACI and congenic rat strains are presented in Figure 1A-1D. To correct for small differences in BW between the strains and treatments, UPV and UAV values are presented per 100g BW.

In the 2K-situation no significant differences in UPV and UAV were observed between the three strains (Figure 1A). A significant increase in UPV and UAV was present in *Rf-1B* rats following 12 and 18 weeks of 2K+L-NAME treatment (Figure 1B). In *Rf-1B* congenics an elevation of UPV and UAV was present at 18 weeks after UNX (Figure 1C). Following UNX+L-NAME treatment, UPV and UAV were significantly increased in the *Rf-1B* rats at all three time-points (Figure 1D). No statistically significant differences in UPV or UAV were found in the *Rf-5* rats when compared to ACI, regardless of treatment and time (Figure 1A-1D).

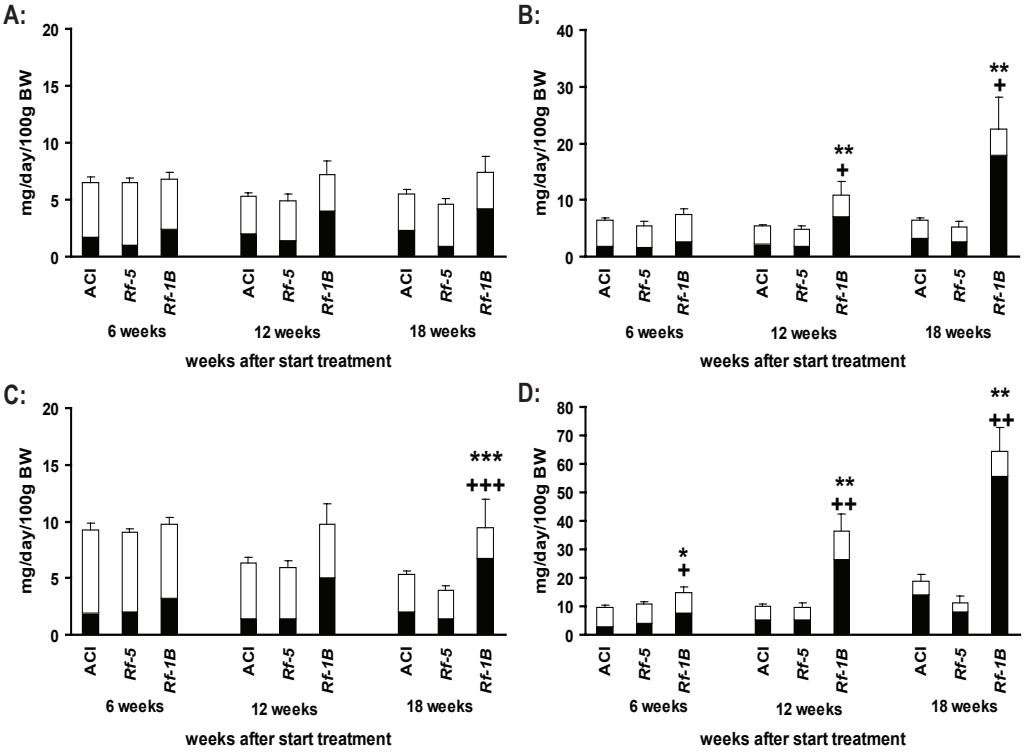

Figure 1: Proteinuria (total bar) and albuminuria (black part of the bar) in ACI, *Rf-1B*, and *Rf-5* rats after 6, 12 and 18 weeks of follow-up. **A**: 2K, 2-kidneys; **B**: 2K+L-NAME; **C**: UNX, unilateral nephrectomy; **D**: UNX+L-NAME. Values are given as mean ± SEM; Note the differences in scaling of the Y-axis. *: $p < 0,05$ versus ACI for UPV; **: $p < 0.05$ versus ACI and *Rf-5* for UPV; ***: $p < 0.05$ versus *Rf-5* for UPV; $^{+}$: $p < 0.05$ versus ACI for UAV; $^{++}$: $p < 0.05$ versus ACI and *Rf-5* for UAV; $^{+++}$: $p < 0.05$ versus *Rf-5* for UAV.

The SBP levels during the 18 week follow-up are presented in Figure 2A-2D. In the 2K- and UNX-situation, SBP in all strains remained normotensive, with no significant differences present (Figure 2A and 2C). As anticipated, chronic L-NAME treatment raised SBP in all strains. Significant differences were occasionally noted. At 12 weeks, SBP after 2K+L-NAME treatment in *Rf-1B* and *Rf-5* was significantly increased compared to ACI, while after UNX+L-NAME treatment, *Rf-5* had a significantly higher SBP compared to ACI rats. At 18 weeks, SBP after 2K+L-NAME in *Rf-1B* was significantly increased compared to ACI. No significant differences were present between 2K+L-NAME or UNX+L-NAME treated *Rf-1B* and *Rf-5* congenic rats, indicating that blood pressure alone cannot account for the differences in proteinuria between the two congenic strains.

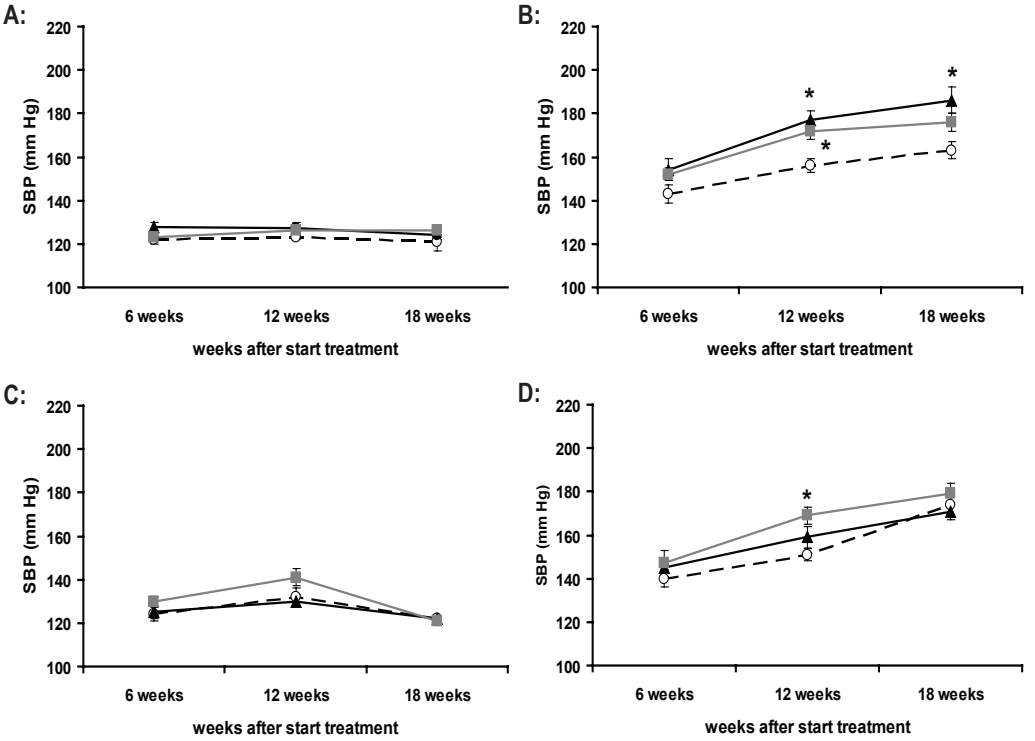

Figure 2. Systolic blood pressure in ACI (○), *Rf-1B* (▲), and *Rf-5* (■) rats after 6, 12 and 18 weeks of follow-up. **A**: 2K, 2-kidneys; **B**: 2K+L-NAME; **C**: UNX, unilateral nephrectomy; **D**: UNX+L-NAME. Values are given as mean ± SEM (error bars); *: $p < 0.05$ versus ACI.

Autopsy findings are summarized in Table 1. The % FGS was significantly higher in *Rf-1B* rats compared to ACI following UNX+L-NAME treatment. In comparison to ACI, the Pcreat level was significantly higher in *Rf-1B* rats following UNX+L-NAME treatment, while the Palb level was significantly decreased.

Table 1: Autopsy findings in the chronic experiment.

	n	BW (gram)	FGS (% glom.)	Pcreat (µmol/l)	Palb (g/l)
2K					
ACI	8	315 ± 6	8 ± 2	49 ± 3	29.2 ± 0.2
Rf-5	7	283 ± 8*	2 ± 1	49 ± 3	29.7 ± 0.5
Rf-1B	8	309 ± 3	15 ± 3°	48 ± 2	28.5 ± 0.5
ANOVA		P = 0.022	P = 0.006	P = 0.945	P = 0.167
2K+L-NAME					
ACI	8	312 ± 2	6 ± 2	51 ± 2	29.0 ± 0.4
Rf-5	9	296 ± 8	5 ± 6	48 ± 1	29.9 ± 0.4
Rf-1B	7	293 ± 6	16 ± 5°	59 ± 5	27.4 ± 1.3
ANOVA		P = 0.159	P =0.035	P = 0.090	P = 0.090
UNX					
ACI	8	308 ± 5	5 ± 1	54 ± 2	29.4 ± 0.5
Rf-5	10	301 ± 4	2 ± 1	51 ± 2	28.3 ± 0.6
Rf-1B	8	284 ± 5*	9 ± 3°	52 ± 1	27.3 ± 0.9
ANOVA		P = 0.008	P = 0.037	P = 0.568	P = 0.176
UNX+L-NAME					
ACI	11	292 ± 9	13 ± 2	68 ± 4	27.9 ± 0.3
Rf-5	9	284 ± 6	8 ± 2	65 ± 2	28.7 ± 0.6
Rf-1B	8	235 ± 9*°	41 ± 9*°	118 ± 21*°	24.0 ± 1.0*°
ANOVA		P < 0.001	P < 0.001	P = 0.010	P < 0.001

Values are given as means ± SEM. 2K, 2-kidneys; UNX, unilateral nephrectomy; BW, body weight; FGS, incidence of glomerulosclerosis; Pcreat, plasma creatinine level; Palb, plasma albumin level. *: p <0.05 versus ACI; °: p< 0.05 versus *Rf-5*.

Assessment of renal blood flow autoregulation

Autoregulation curves for the relative RBF (%RBF) are presented in Figure 3. There are significant differences in %RBF between different strains as RPP is increased between 110 to 150 mm Hg. The relative increase in RBF at RPP at 150 mm Hg, compared to RBF at 100 mm Hg, was significantly higher in FHH (35±3%) and *Rf-1B* (35±2%) as compared to ACI (12±1%) and *Rf-5* (9±1%). The nearly 1 to 1 correlation in %RBF with each change in RPP in the FHH was as expected.[272] Interestingly, only the *Rf-1B* congenic had an alteration in autoregulation, and appeared to completely recapitulate the FHH phenotype, while only carrying at most 1% of the FHH genome. These data suggests the gene(s) responsible for autoregulation is within the *Rf-1* interval.

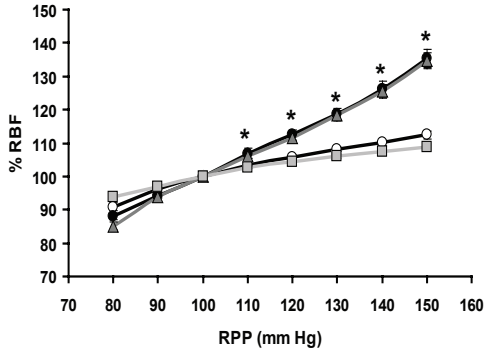

Figure 3: Relationship between percentage of renal blood flow (%RBF) and renal perfusion pressure (RPP) in ACI (○, n=15), FHH (●, n=15), *Rf-1B* (▲, n=18), and *Rf-5* (■, n=15) rats. Values (as % RBF at RPP of 100 mm Hg) are given as means ± SEM. *: $p < 0.05$ FHH and *Rf-1B* versus ACI and *Rf-5*.

Figure 4 shows the renal autoregulation index (RAI), another way to express the efficacy of the autoregulation. Over the pressure ranges from 90-110, 110-130, and 130-150 mm Hg, the RAIs were in the order of 0.2-0.3 in ACI as well as *Rf-5* rats, indicating normal renal autoregulation. In contrast, RAI values were significantly increased to levels of about 0.7-0.9 in FHH and *Rf-1B* rats. At no pressure interval was there any significant difference in RAI between ACI and *Rf-5* rats, nor between FHH and *Rf-1B* rats.

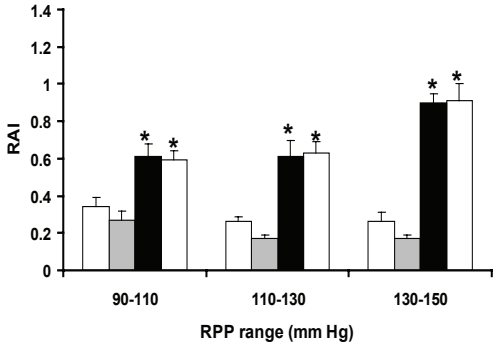

Figure 4: Renal blood flow autoregulatory index (RAI) for several renal perfusion pressures (RPP) ranges in ACI, FHH, *Rf-1B*, and *Rf-5* rats. Values are given as mean ± SEM. ACI (white bar, n=15); *Rf-5* (light grey bar, n=15); FHH (black bar, n=15); *Rf-1B* (dark gray bar, n=18). *: $p < 0.05$ versus ACI and *Rf-5*.

Assessment of the degree of renal damage in the rats used this acute experiment indicated that UPV and UAV were significantly higher in FHH rats compared to all other strains. No significant differences were seen between ACI, and *Rf-1B*, and *Rf-5* congenic rats (Table 2). Relatively small but statistically significant increases in %FGS were noted in FHH and *Rf-1B* when compared to ACI.

Table 2: Indices of renal damage in the animals used for the autoregulation study.

	n	BW (gram)	UPV (mg/d/100 gBW)	UAV (mg/d/100 gBW)	FGS (% glomeruli)
ACI	15	269 ± 4	6.3 ± 0.3	1.8 ± 0.2	6 ± 1
Rf-5	15	248 ± 6	3.7 ± 0.2	1.3 ± 0.2	5 ± 1
FHH	15	308 ± 4*	39.0 ± 2.9*	28.7 ± 2.6*	13 ± 1*
Rf-1B	18	254 ± 4	7.5 ± 0.4	3.0 ± 0.2	10 ± 1*

Values are given as mean ± SEM. BW, body weight; UPV, proteinuria; UAV, albuminuria; FGS, incidence of focal glomerulosclerosis. *: $P < 0.05$ versus ACI.

Discussion

The chronic *in vivo* studies reconfirmed that the *Rf-1B* congenic rat is more susceptible to the development of renal damage than the ACI rat. In contrast, *Rf-5* congenic rats do not show an increased susceptibility to renal damage, despite equivalent increases in blood pressure. As our hypothesis was that the inability to autoregulate renal blood flow is a contributing mechanism to renal failure in the FHH, we tested autoregulation in the two different congenic strains. If autoregulation was linked to both congenics, then it was the result of more then one gene and may even have been secondary to the renal disease.

This study showed that all the congenics in the control group were normotensive. Moreover, these data validate that *Rf-1* and *Rf-5* loci did not carry genes responsible for hypertension in this cross.[25,234] As there is a clear interaction between blood pressure and renal disease there was a need to increase blood pressure, as the congenics we used are on the normotensive genome background of the ACI. We increased blood pressure by inhibiting nitric oxide using L-NAME. Hypertension is considered to be a key feature in the development of renal damage, and is one leading causes of ESRF all over the world.[14,71,127] Loss of functional renal mass is another characteristic of progressive renal damage.[21] Therefore, we also tested if the renal disease was simply the result of a decrease in renal mass, mimicked by UNX. It should be noted that the ACI was almost completely protected from significant changes in UPV, UAV, Pcreat, and Palb, despite changes in renal mass and the presence of hypertension. Therefore, any change in renal indices found in the congenics must be the result of FHH genomic regions introgressed into the ACI genome.

In none of the four conditions was there significant difference in the degree of renal damage between *Rf-5* congenics and ACI rats. Neither hypertension nor renal mass reduction led to more severe kidney damage in *Rf-5* congenic rats. In clear contrast, a significant increase in renal susceptibility of *Rf-1B* rats, compared to ACI, was present in all three conditions. Importantly, even in the 2K-situation, slight increase in UPV and UAV, albeit not statistically significant, were present in the final evaluation at 25 weeks of age. It is likely that with a longer follow-up the difference in UPV between *Rf-1B* and ACI would have become significant. Elevating the SBP in the 2K-situation to levels similar to that of FHH rats[134] enhances the differences in renal damage in the *Rf-1B* congenics, but not in the ACI rats or *Rf-5* congenics. A similar outcome was found by reducing renal mass. As expected, the most profound differences in renal damage between *Rf-1B* and ACI rats were present following UNX+L-NAME treatment.[199]

In concordance with an increased UPV and UAV in *Rf-1B* rats following UNX+L-NAME treatment, the incidence of FGS is also higher compared to other rat strains subjected to the same treatment. Similarly, Pcreat levels in the *Rf-1B* rats following UNX+L-NAME treatment were almost twice as high as obtained in

the other strains. Thus, there appears to be a good association between the degrees of structural glomerular damage, i.e. FGS, and functional damage, i.e. UPV, UAV, and Pcreat. A final indication for an impaired renal function was the finding that in *Rf-1B* rats after UNX+L-NAME, the increase in UAV was paralleled by a decrease in Palb.

Next to 2K-control rats, three experimental situations were studied to detect differences in renal susceptibility. Reproducibility is by far the best in the UNX-model. Following UNX alone, *Rf-1B* rats already show increased levels of UAV and UPV compared to ACI and *Rf-5* congenics, albeit statistically significant compared to *Rf-5* congenics only. However, in contrast to the parental FHH strain the congenic rats remain normotensive. Consequently, we have to raise blood pressure to detect changes in renal susceptibility resulting from the introgression of the *Rf*-QTLs on the ACI background. Based on our earlier experience we used chronic L-NAME treatment to raise systemic blood pressure.[274,275] As indicated in our previous studies, using chronic L-NAME treatment has some disadvantages. It is difficult, if not impossible, to match SBP between the various strains at a level normally present in the FHH rat. Chronic L-NAME treatment may directly affect the vascular structure in the kidney, independent of its blood pressure effects.[305] Furthermore, reducing endothelial NO-synthase activity by L-NAME may have a negative effect on the protective action of NO in organs that are targets of hypertensive injury.[101] Thus, differences in renal damage between *Rf-1B* congenics and ACI may be partly due to an increased susceptibility to L-NAME.

Normally, adequate renal autoregulation is able to protect the glomerular capillary structures from injury due to systemic hypertension. Impaired autoregulation increases susceptibility for renal damage in various rat models including FHH.[15,94,272] Previous studies were not able to localize impaired autoregulation to particular chromosomal region. Here we clearly demonstrate that the *Rf-1* region of rat chromosome 1 contains one or more gene(s) responsible for impairing autoregulation before the development of severe renal damage. Whether it is the same gene responsible for both a lack of autoregulation and renal disease remains to be determined. In contrast to the findings in *Rf-1B*, the *Rf-5* congenics were shown to have a normal autoregulation, comparable to that of ACI rats. Consequently, the *Rf-5* region contains no genes that influence renal autoregulation.

The results from this study also shows that the severity of the renal damage in the *Rf-1B* congenic is significantly less than the FHH, as would be anticipated if more than one region of the FHH were required to recapitulate the disease in the ACI background. Our previous linkage analyses indicated that renal damage in FHH is polygenic, and that the presence of *Rf-1* would only have a small effect.[25,234] The low LOD score (2.99 for UPV) of the *Rf-5* QTL compared to *Rf-1* (16.74 for UPV) would predict an even smaller effect on renal damage than *Rf-1*, a prediction confirmed in our present study. It is expected that combinations of two or three QTLs, one of them being *Rf-1* are needed to markedly increase renal susceptibility, although this may still not be sufficient to induce the same severity of renal damage seen in the FHH rat. Consequently, although *Rf-5* single congenics do not develop renal damage and do have a normal renal autoregulation, it is still possible that the *Rf-5* QTL does influence development of renal damage, but only when interacting with other loci. It will require additional studies using double or multiple congenics carrying *Rf-1* plus other loci. Given that impaired renal autoregulation alone does not account for the full spectrum of renal damages seen in FHH, it will also require physiological studies to explore other mechanisms that contribute to renal failure.

Finally, it is worth noting that comparative genomics can be used to infer gene function between species most notably human, once a biological trait is assigned to a genomic location. Here we conclusively show that a gene or gene(s) influencing renal autoregulation reside within a 24.2Mb (229.5-253.7Mb) stretch of rat chromosome 1. The human homologous region is located on chromosome 10q24. In recent years, several studies have looked into a role of this region in human ESRF and found that it may contribute to renal disease in humans.[78,114,303] This linkage in humans and our present data, suggests that lack of RBF autoregulation may contribute to renal disease in humans. Obviously, this question will require additional study.

In summary, the present studies indicate that transferring the *Rf-1* region from the FHH to the ACI rat impairs renal autoregulation and increases susceptibility to renal damage, suggesting either a direct relationship or that the gene(s) responsible for autoregulation and the gene(s) responsible for renal disease are closely linked. In contrast, similar transfer of *Rf-5* has no effect on autoregulation or any other indices of renal disease. The data suggest that the *Rf-1* QTL harbors one or more genes influencing renal autoregulation. Further studies in double or multiple congenic strains are needed to provide direct evidence for a role of *Rf-5* in the development of renal damage. Finally, renal autoregulation may need to be examined in patients showing linkage to the human homologous genomic region.

Acknowledgements

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Chapter 4

Interaction between *Rf-1* and *Rf-4* QTLs increases susceptibility to renal damage in double congenic rats

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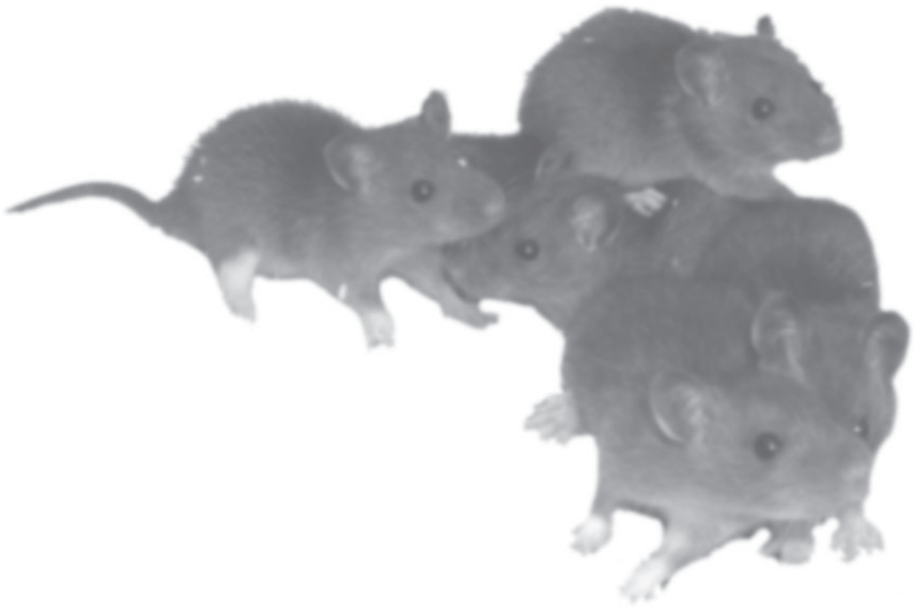


Abstract

Background. Five quantitative trait loci (QTLs), *Rf-1* to *Rf-5* were found in FHH (Fawn Hooded Hypertensive) rats influencing susceptibility to renal damage. Previously we found that single transfer of the *Rf-1* QTL from FHH rats onto the renal resistant ACI (August x Copenhagen Irish) strain caused a small increase in renal susceptibility. To investigate the separate role of the *Rf-4* QTL and its interaction with *Rf-1*, we generated a single congenic strain carrying *Rf-4* and a double congenic carrying both *Rf-1* and *Rf-4*.

Methods. Differences in renal susceptibility between ACI, *Rf-1A*, and *Rf-4* single congenics and *Rf-1A+4* double congenics were assessed using four different treatments: control (2K), 2K with L-NAME induced hypertension, unilateral nephrectomy (UNX), and UNX+L-NAME. In separate experiments, renal blood flow (RBF) autoregulation was compared between two-kidney ACI and congenic rats.

Results. Compared to ACI, *Rf-1A* rats developed more renal damage, while *Rf-4* rats did not. The most severe renal damage was found in the *Rf-1A+4* double congenic rats. Analysis of variance demonstrated a significant interaction between the *Rf-1A* and *Rf-4* QTLs. The magnitude of the interaction varied with the type and duration of the treatment. The RBF autoregulation was impaired in *Rf-1A* single and *Rf-1A+4* double congenics, while in *Rf-4* single congenics it was similar to that of ACI controls.

Conclusion. These findings indicate that the *Rf-1* QTL directly influences renal susceptibility and autoregulation. In contrast, the *Rf-4* QTL shows no direct effects, but significantly increases susceptibility to renal damage via an interaction with *Rf-1*.

Introduction

Chronic kidney disease is assumed to be a complex polygenic disease.[33,129,201,219,220] Chromosomal loci, as well as specific genes have been identified in various inherited forms of renal disease.[194,295] However, finding the genes involved in the more complex forms of human ESRF has been more arduous. Linkage analysis has identified some chromosomal regions possibly involved in diabetic and non-diabetic forms of nephropathy, while candidate gene analyses have tested several genes with limited success.[38,81,201] Studies in inbred rat strains might help to decrease the number of candidate gene, which could facilitate finding genes in human studies.

Inbred rat strains also vary widely in their susceptibility to develop renal damage. Our studies involve the Fawn Hooded Hypertensive (FHH) rat, prone to develop mild systolic hypertension and marked proteinuria (UPV), albuminuria (UAV), and focal and segmental glomerulosclerosis (FGS) at relatively young age. Male FHH rats die of ESRF within a year and a half if not treated.[280,281,282] The FHH strain is well characterized by numerous physiological and histological studies.[132,133,198,238,239,272,273,279] Crosses between FHH and the proteinuria resistant ACI rat revealed the presence of five QTLs linked to UPV and other parameters of renal damage. These QTLs were named *Renal failure-1* (*Rf-1*) to *Rf-5*.[25,234] It is surmised that each of these QTLs contains gene(s) that play a role in the development of progressive renal damage in the FHH rat.

Even though the nature of the genes have not yet been identified, the separate role of each QTL in the initiation and progression of renal damage can be studied in congenic rat strains that have a *Rf*-region of the FHH rat introgressed into the genomic background of the proteinuria resistant ACI rat. Next, interactions between the QTLs can be studied in double and multiple congenic strains. Previously, we reported about the susceptibility to renal damage in ACI.FHH-*Rf-1B* (*Rf-1B* for short) and ACI.FHH-*Rf-5* (*Rf-5* for short) single congenic rats.[199,267] Since the ACI rat is resistant to renal damage even when made hypertensive we need to stress the kidney to initiate the renal failure phenotype and be able to study the effect of a single QTL in the ACI background. The *Rf-1B* congenic rats developed significantly more UPV and UAV than the ACI progenitor strain. This difference was most pronounced following unilateral nephrectomy (UNX) combined with L-NAME induced hypertension.

In *Rf-1B* congenic rats, renal autoregulation was impaired to the same extent as the parental FHH rat.[272] In the FHH the impaired renal autoregulation, resulting in an elevated intra-glomerular pressure (P_{GC}) is thought to be an important mechanism in the early development of renal damage.[133,238,273] In contrast to the *Rf-1B* congenic rats, the *Rf-5* single congenic strain showed no increase in renal susceptibility and a normal RBF autoregulation.[267] In the *Rf-1B* congenic, the levels of UPV and UAV following UNX and L-NAME treatment were much less than those found in FHH.[239] This indicates that the *Rf-1* QTL only accounts for part of the renal damage in FHH rats. Our previous linkage analysis suggested complex interactions between the QTLs. With the exception of *Rf-1*, the other *Rf*-QTLs by themselves showed little effect on UPV. However, a marked increase in UPV level was noted when these QTLs were combined with *Rf-1*.[234] Direct evidence for such an interaction can be obtained by studying double congenic rats. Interactions between blood pressure QTLs in rats have been described previously.[167,206]

In the present experiments we wanted to test the presence of an interaction between the *Rf-1* and *Rf-4* QTLs, as predicted from the linkage analysis.[234] Therefore, we compared the renal susceptibility between the ACI progenitor strain and three congenic strains, i.e. ACI.FHH-*Rf1A,* ACI.FHH-*Rf-4* single congenics, and ACI.FHH-*Rf1+4* double congenics. We tested the hypothesis that a gene-gene interaction occurs between the *Rf-1* and *Rf-4* QTLs increasing the susceptibility to renal damage.

Methods

Congenic and control rat strains

For the experiments, single congenics ACI.FHH-(*D1Rat298-D1Rat90*) (*Rf-1A* for short), ACI.FHH-(*D14Mit11-D14Rat82*) (*Rf-4* for short), double congenic ACI.FHH-(*D1Mit18-D1Rat90*)/(*D14Mit11-D14Rat33/D14Rat65-D14Rat90*) (*Rf-1A+4* for short) rats and ACI control rats were used. All breeding was performed at the Animal Research Centre at Erasmus MC, Rotterdam, Netherlands. Animals were housed in individually-ventilated cages under SPF-conditions as previously described.[180,267] The protocol received approval from the animal ethical committee of Erasmus MC.

Congenic rat strains were generated using a speed congenic strategy as previously described for the *Rf-1B* strain by Provoost et al.[199] A schematic view of the introgressed *Rf*-regions of the various congenic strains is presented in Figure 1. The congenics generated here contain the earlier reported 95% C.I. for *Rf-1* and *Rf-4*. In the *Rf-1A* single congenic strain, the congenic region is on chromosome 1 between markers *D1Rat298* (227.2 Mb) and *D1Rat90* (267.3 Mb), spanning 41.5 Mb, about 15% of the chromosome. The *Rf-1A* region is about 17 Mb larger than the region in the *Rf-1B* strain.[199]

In the *Rf-4* single congenic strain, the region is on chromosome 14 between markers *D14Mit11* (5.0 Mb) and *D14Rat82* (30.3 Mb), spanning 25.3 Mb, about 23% of the chromosome. In the *Rf-1A+4* double congenic rat the *Rf-1* congenic region is between *D1Mit18* (224.6 Mb) and *D1Rat90* (267.3 Mb), spanning 42.7 Mb. In the *Rf-1A+4* double congenic rat the *Rf-4* region is between the markers *D14Mit11* (5.0 Mb) and *D14Rat33* (29.3 Mb), spanning 24.3 Mb, i.e. 1.0 Mb smaller than in the *Rf-4* single congenic. In addition, a second region of chromosome 14 from FHH has been introgressed between markers *D14Rat65* (36.4 Mb) and *D14Rat90* (74.0 Mb), spanning 37.6 Mb. This region, however, is way outside the 95% C.I. for the *Rf-4* QTL (between 5-20 Mb) on chromosome 14. A whole genome scan with 140-150 genetic markers on these three congenic strains showed that there was no detected FHH genomic contamination on other chromosomes.

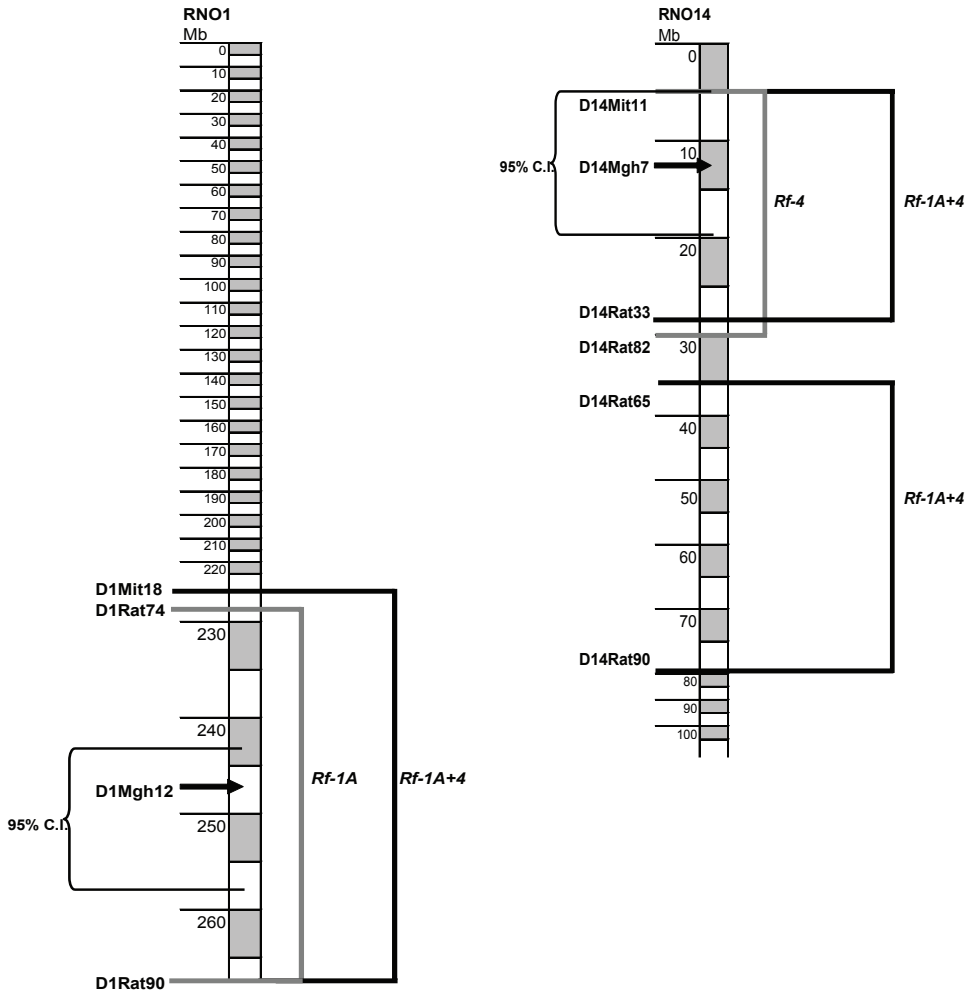


Fig 1. Genetic maps of rat chromosomes 1 and 14 depicting the homozygous FHH regions introgressed on the ACI background in the *Rf-1A*, *Rf-4*, and *Rf-1A+4* congenic strains. The areas homozygous FHH in the congenic strains are indicated by the solid and striped lines. The arrows indicate the locations of the *Rf-1* and *Rf-4* QTL peaks found in previous studies, i.e. *D1Mgh12* for *Rf-1*, and *D14Mgh7* for *Rf-4*.[25,234] Distances are given in mega-base pairs (Mb). 95% C.I. represents the 95% confidence interval of the QTLs.

Renal damage susceptibility

Experiments for assessment of renal damage susceptibility were performed on 164 animals starting from the age of 6-7 weeks. Per strain, the animals were randomly divided over four treatment groups (Table 1). The first received no treatment, remaining with two kidneys (2K), i.e. control situation. The second remained with 2K and was chronically treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) (2K+L-NAME) to induce systemic hypertension. The third treatment consisted of renal mass reduction by unilateral nephrectomy (UNX), the fourth of UNX+L-NAME-induced hypertension.

Table 1: Number of rats studied in the renal susceptibility experiments.

	2K	2K+L-NAME	UNX	UNX+L-NAME
ACI	6	6	10	9
Rf-1A	11	12	12	12
Rf-4	9	9	10	10
Rf-1A+4	12	10	12	14, 14, 10*

* Number of rats at first, second and third follow-up, respectively. 2K, 2-kidneys; 2K+L-NAME, 2K+L-NAME-induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, UNX+L-NAME-induced hypertension.

Surgery for UNX and L-NAME treatment were performed as previously described.[274] There was some variation in the amount of L-NAME intake. However, Figure 3 shows that blood pressure levels were equivalent among the different strains of rats within the different treatment groups. Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period. Following the urine collection, SBP was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained, but trained rats, as described previously.[199]

Shortly after the last series of urine collections and SBP measurements the animals were sacrificed as previously described.[267] Kidneys were collected and weighed. The left kidney was used for histological examination. The extent of glomerular damage was determined in 1-µm sections stained with periodic acid-Schiff (PAS) reagent. In each animal, 50 glomeruli of the left kidney were examined for the presence of sclerotic lesions, *i.e.*, segmental glomerular scarring, obliteration of glomerular capillaries, mesangial matrix expansion, and adhesion formation between tuft and Bowman's capsule. The extent of glomerular damage was expressed as the percentage of the glomeruli (%FGS) showed one or more of these features.[267]

Renal blood flow autoregulation

Experiments for assessment of renal blood flow autoregulation were performed on 54 animals (15 ACI, 12 *Rf-1A*, 15 *Rf-4*, and 12 *Rf-1A+4* rats) with an age of 13-15 weeks. To get an indication of the presence of renal damage, UPV and UAV were assessed using a 24-hr sample obtained before the autoregulation experiments, while at the end of the evaluation both kidneys were collected and weighed and the left kidney was used to determine the %FGS, as previously described.[267]

Animals were anaesthetized with a mixture of 3% Isoflurane®, 30% N_2O, and 60% O_2 and surgically prepared for autoregulation studies.[267,272] After surgery and a 10-min equilibration period, the relationship between the left kidney RBF and the renal perfusion pressure (RPP) was determined. The RBF was recorded as the RPP was lowered from 150 to 80 mm Hg in 10 mm Hg steps by tightening a clamp around the aorta, followed by a 3-min equilibration period. To normalize the outcome of the individual rats, the RBF at a RPP of 100 mm Hg (RBF_{100}) was considered to be 100%. Renal autoregulatory indexes (RAIs) over the range of pressures from 80 to 150 mm Hg were calculated by the method of Semple and de Wardener.[228] A RAI of 0 indicates perfect autoregulation of RBF, and a RAI of 1 indicates that there is no autoregulation present due to a fixed renal vascular resistance.

Analytical procedures

Plasma and urinary samples were analysed with an ELAN system (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Total urinary protein was determined colorimetrically with pyrogallol red-molybdate complex.[286] Plasma and urinary albumin levels were determined with bromocresol green.[62] Plasma and urinary creatinine levels were determined with the Jaffé method without deproteinisation.[240]

Statistics

Data are presented as mean ± SEM, unless stated otherwise. Statistical differences in mean values between groups were compared using one-way analysis of variance (ANOVA), followed by the Bonferroni-test to determine which pairs were significantly different. These tests were performed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996).

For different parameters (X) the magnitude of the effects of the presence of the *Rf-1* or *Rf-4* QTL on the ACI background and their interaction was calculated as follows:
Effect of *Rf-1* equals ($X_{Rf\text{-}1A} - X_{ACI}$), effect of *Rf-4* QTL equals ($X_{Rf\text{-}4} - X_{ACI}$), and the interaction between the *Rf-1* and *Rf-4* QTLs equals ($X_{ACI} + X_{Rf1+4} - X_{Rf\text{-}1} - X_{Rf\text{-}4}$). In these formulas X_{strain} is the mean value of the parameter under investigation at the different time-points for the various strains and treatments. The standard deviation (SD) was calculated as the weighed SD of the variables in the formulae.

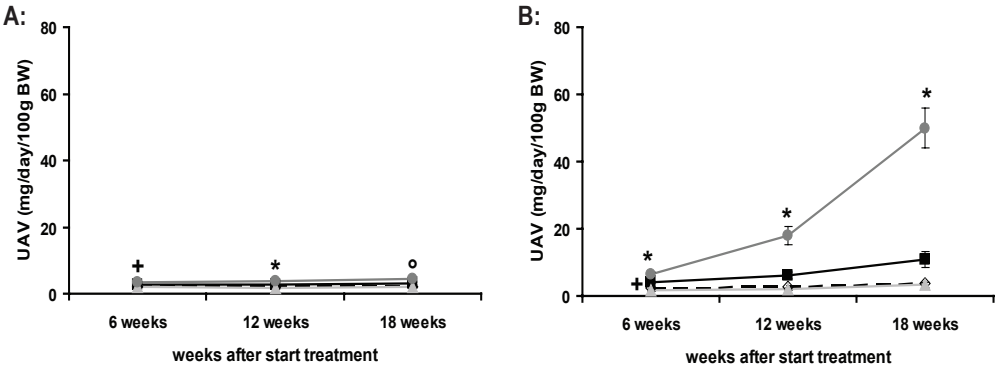
Statistical significance of the main effect of the *Rf-1* and *Rf-4* QTLs and their interaction was calculated using a 2x2 factorial ANOVA procedure provided by VassarStats (http://faculty.vassar.edu/lowry/anova2x2.html). In all tests, a p-value <0.05 was considered to be statistically significant.

Results

Animal survival and albuminuria

All 2K, 2K+L-NAME, and UNX rats survived the 18-week follow-up period. Following UNX+L-NAME treatment, four of the fourteen *Rf-1A+4* double congenic rats did not survive up to the third measurement. Data obtained from these four rats are included in the results for the first and second measurements.

Both UPV and UAV values were assessed, and no remarkable differences were found between the two values. Mean values for UAV during follow-up from the various treatments are presented in Figure 2A-2D. In the 2K-situation, UAV was significantly higher in *Rf-1A+4* double congenic rats compared to *Rf-4* single congenics at all time points, to ACI at the 2nd and 3rd time point, and to *Rf-1A* single congenic rats at the 2nd time point (Figure 2A). Following 2K+L-NAME treatment, UAV in *Rf-1A+4* double congenic rats was significantly increased at all time points compared to ACI, *Rf-4* and *Rf-1A* single congenic rats (Figure 2B). Following UNX, UAV was at all time points significantly higher in *Rf-1A+4* double congenics compared to ACI, or *Rf-4* and *Rf-1A* single congenic rats. The level of UAV in *Rf-1A* single congenic rats was significantly higher compared to *Rf-4* single congenics at all time points and to ACI rats at the third time point (Figure 2C). When treated with UNX+L-NAME, UAV in *Rf-1A+4* double congenic rats at all time points was significantly increased compared to ACI, *Rf-4* and *Rf-1A* single congenic rats. In *Rf-1A* single congenics rats this treatment led, at the second and third evaluation, to a significantly increased UAV compared to ACI and *Rf-4* single congenic rats (Figure 2D). Regardless of treatment or time-point, no statistically significant differences in UAV were found in the *Rf-4* single congenic rats when compared to ACI (Figure 2A-2D).



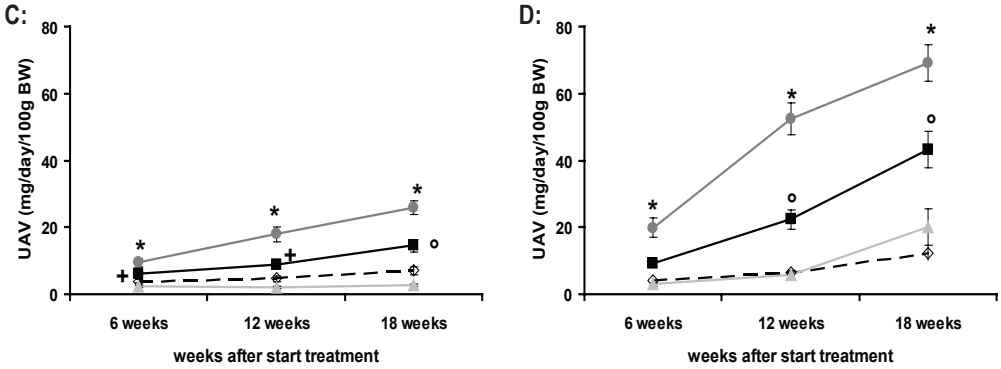


Fig 2. Albuminuria (UAV) after 6, 12, and 18 weeks of follow-up during four treatments ACI (◇), *Rf-1A* (■), *Rf-4* (▲), and *Rf-1A+4* (●) rats. **A**: 2K (2-kidneys); **B**: 2K+L-NAME (2K+L-NAME-induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME (UNX+L-NAME-induced hypertension). Values (mg/day per 100 g BW) are given as mean ± SEM, number of rats is given in Table 1. *: $p < 0.05$ vs. ACI, *Rf-1A* and *Rf-4*; °: $p<0.05$ vs. ACI and *Rf-4*; $^{+}$: $p < 0.05$ vs. *Rf-4*.

Systolic blood pressure

Values for SBP are presented in Figure 3A-3D. In the 2K-situation, *Rf-1A* single congenic rats compared to ACI showed a small, but significant increase in SBP at the first and second evaluations (Figure 3A). After UNX, a slightly higher SBP was present in the *Rf-4* single congenics compared to ACI at the second time point (Figure 3C). Chronic L-NAME treatment increased SBP in all strains. At the second and third evaluations, SBP in *Rf-1A+4* double congenic rats with 2K+L-NAME treatment was significantly increased compared to ACI and *Rf-1A* single congenic rats. At the first time point, following 2K+L-NAME treatment, *Rf-4* single congenic rats had a significantly lower SBP compared to ACI and *Rf-1A*. This difference was not seen at the second and third evaluations (Figure 3B). The *Rf-1A+4* double congenics with UNX+L-NAME treatment, showed a higher SBP at the first and second evaluations compared to ACI and to *Rf-1A* single congenics at the second evaluation. However, at the final evaluation there was no difference present (Figure 3D).

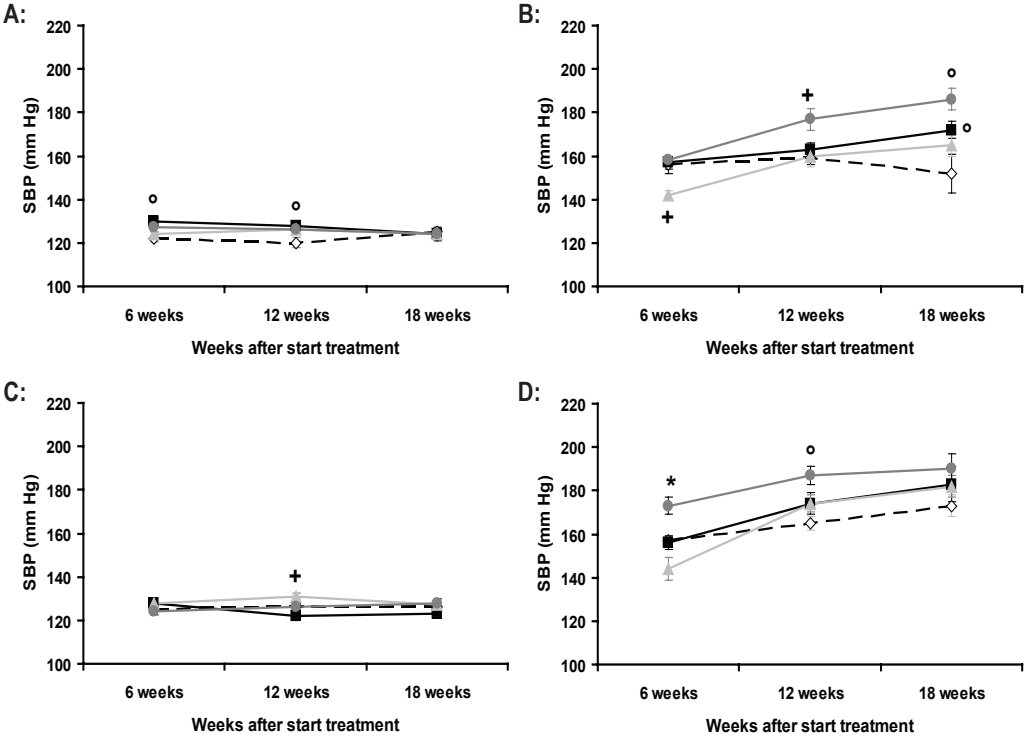


Fig 3. Tail-cuff blood pressure at 6, 12, and 18 weeks in ACI (◇), *Rf-1A* (■), *Rf-4* (▲), and *Rf-1A+4* (●) rats. **A**: 2K (2-kidneys); **B**: 2K+L-NAME (2K+L-NAME-induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME (UNX+L-NAME-induced hypertension). Values (mm Hg) are given as mean ± SEM; number of rats is given in Table 1. *: $p < 0.05$ vs. ACI, *Rf-1A* and *Rf-4*; ⁺: $p < 0.05$ vs. ACI and *Rf-1A*; °: $p < 0.05$ vs. ACI.

Gene-gene and gene-treatment interactions

The results of the 2x2 factorial ANOVA analyses showed the presence of significant gene-gene interactions. While *Rf-4* alone showed no effect on UAV when compared to ACI, combining *Rf-4* with *Rf-1A* always resulted in a further increase in UAV when compared with *Rf-1A* single congenics. Furthermore, the magnitude of these interactions was significant in all four treatments. However the greater the haemodynamic stress upon the kidney, the greater the synergistic effect.

An example is presented in Figure 4, for the UAV at the second time point, after 12 weeks of treatment. It is shown that changing the *Rf-1* genotype from homozygous ACI (AA) to homozygous FHH (FF), while the *Rf-4* genotype remains AA, induced an increase in UAV per 100g BW in all treatment groups. In contrast, changing the *Rf-4* genotype from AA to FF, while the *Rf-1* genotype remains AA, showed almost no change in UAV. Assuming an additive effect on UAV when both *Rf-1* and *Rf-4* change from AA to FF, an expected change in UAV can be calculated. In all four situations, the observed changes in UAV significantly exceeded the expected change. The difference, being the interactive effect between *Rf-1* and *Rf-4* depended on the experimental situation, but was significant in all treatment groups. The magnitude

was small with 2K (+1.5 mg/day/100g BW), intermediate with 2K+L-NAME (+12.5 mg/day/100g BW) and UNX (+11.7 mg/day/100g BW) and large in the UNX+L-NAME situation (30.9 mg/day/100g BW).

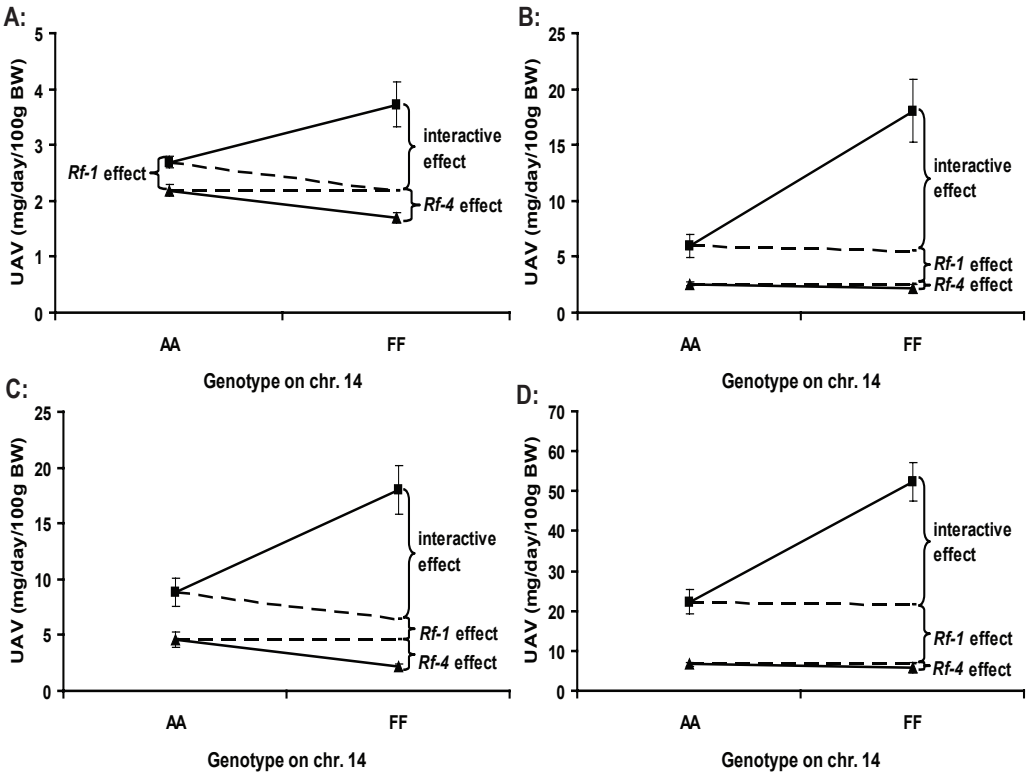


Fig 4. The effects of the *Rf-1* and *Rf-4* (chr. 14) genotype and their interaction of *Rf-1* and *Rf-4* on UAV after 12 weeks of treatment (M2). **A**: 2K (2-kidneys); **B**: 2K+L-NAME (2K+L-NAME-induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME (UNX+L-NAME-induced hypertension); AA, homozygous ACI; FF, homozygous FHH. Values for UAV (mg/day per 100 g BW) are mean ± SEM. Statistics are given in Table 2.

Additional information about the effects of the *Rf-1* and *Rf-4* QTLs and their interactions on UAV at all three time-points is provided in Table 2. The major finding being that the magnitude of the interaction between *Rf-1* and *Rf-4* not only depended on the type of treatment but also on the duration of the treatments, i.e. the longer the treatment the larger the interaction.

Table 2: Effect of the *Rf-1* and *Rf-4* QTLs and their interaction on albuminuria (UAV).

	6 weeks of treatment	2x2 factorial ANOVA P	12 weeks of treatment	2x2 factorial ANOVA P	18 weeks of treatment	2x2 factorial ANOVA P
2K						
Rf-4 effect	-0.3±0.9	P=0.476	-0.5±0.4	P=0.234	-0.3±0.6	P=0.178
Rf-1 effect	+0.6±0.5	P<0.001	+0.5±0.4	P<0.001	+0.9±0.7	P<0.001
Interaction of *Rf-1* and *Rf-4*	+0.8±0.8	P=0.072	+1.5±0.8	P=0.009	+1.6±1.3	P=0.045
2K+L-NAME						
Rf-4 effect	-0.8±1.0	P=0.116	-0.5±0.9	P=0.002	-0.3±2.1	P<0.001
Rf-1 effect	+1.6±1.3	P<0.001	+3.4±2.9	P<0.001	+7.1±6.9	P<0.001
Interaction of *Rf-1* and *Rf-4*	+3.3±1.4	P<0.001	+12.5±5.1	P<0.001	+39.5±10.7	P<0.001
UNX						
Rf-4 effect	-1.5±1.4	P=0.155	-2.5±1.7	P=0.010	-4.5±2.8	P=0.011
Rf-1 effect	+2.2±2.0	P<0.001	+4.2±3.6	P<0.001	+7.4±5.2	P<0.001
Interaction of *Rf-1* and *Rf-4*	+4.8±2.6	P=0.004	+11.7±4.7	P<0.001	+15.9±5.2	P<0.001
UNX+L-NAME						
Rf-4 effect	-1.0±1.4	P=0.003	-0.9±3.3	P<0.001	+7.7±13.0	P<0.001
Rf-1 effect	+5.1±2.7	P<0.001	+15.7±6.9	P<0.001	+30.9±14.7	P=0.012
Interaction of *Rf-1* and *Rf-4*	+11.8±6.4	P=0.005	+30.9±11.3	P<0.001	+18.2±16.1	P=0.009

For each treatment and time-point the *Rf-4* effect ($UAV_{Rf-4} - UAV_{ACI}$), the *Rf-1* effect ($UAV_{Rf-1A} - UAV_{ACI}$), and the interaction ($UAV_{Rf-1A+4} - UAV_{Rf-1A} - UAV_{Rf-4} + UAV_{ACI}$) were calculated. Data represent change in UAV (mg/day per 100 g BW) ± SD. Statistical significance of the overall (main) effect of *Rf-1* and *Rf-4* and their interaction were calculated using 2x2 factorial ANOVA. 2K, 2-kidneys; 2K+L-NAME, 2K+L-NAME-induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, UNX+L-NAME-induced hypertension.

Findings at end of experiment

The incidence of focal glomerulosclerosis (%FGS), creatinine clearance per 100 g BW (Cc/100) and plasma albumin (Palb) level are summarized in Table 3. The %FGS was significantly higher in *Rf-1A+4* double congenic rats compared to ACI, *Rf-4* and *Rf-1A* single congenic rats, regardless of treatment. Following both 2K+L-NAME and UNX+L-NAME, the %FGS was significantly higher in *Rf-1A* single congenics compared to ACI rats. Irrespective of the treatment no statistically significant differences in %FGS were found between *Rf-4* single congenics and ACI rats.

As with UAV, a significant interactive effect upon the %FGS was present between the *Rf-1* and *Rf-4* QTLs. The magnitude of the interaction differed per treatment. It was low (+9±6%, p=0.021) for 2K, intermediate (+21±10%, p=0.002) for 2K+L-NAME and (+24±10%, p<0.001) for UNX, and most pronounced (+49±12%, p<0.001) for UNX+L-NAME treated rats.

In the 2K- and UNX-situation, Cc/100 level was significantly lower in *Rf-1A* single congenic and *Rf-1A+4* double congenic rats compared to *Rf-4* single congenics. Following 2K+L-NAME, Cc/100 was

lower in *Rf-1A+4* double congenics compared to all other strains, while in *Rf-1A* single congenic rats it was lower compared to *Rf*-4 single congenic rats. After UNX+L-NAME treatment, Cc/100 of *Rf-1A+4* double congenics and *Rf-1A* single congenics was lower than that of *Rf-4* single congenics and ACI rats. At the end of the follow-up, Palb level was significantly decreased in *Rf-1A+4* double congenic rats compared to all other strains after 2K+L-NAME or UNX treatment, and compared to ACI and *Rf-4* single congenics after UNX+L-NAME treatment. Following UNX+L-NAME treatment, *Rf-1A* single congenics also showed a lower Palb level in comparison to ACI and *Rf-4* single congenic rats.

Table 3: Measurements at end of follow-up

2K	n	BW (gram)	FGS (% glom.)	Cc/100 (ml/min/100gBW)	Palb (g/l)
ACI	6	303 ± 4	10 ± 2	0.55 ± 0.05	28.8 ± 0.9
Rf-4	9	317 ± 7	8 ± 1	0.66 ± 0.05	28.9 ± 0.6
Rf-1A	11	326 ± 5	15 ± 2 °	0.45 ± 0.03 $^{+}$	28.9 ± 0.5
Rf-1A+4	12	327 ± 5 $^{\#}$	22 ± 2 *	0.44 ± 0.03 $^{+}$	28.6± 0.5
ANOVA		P = 0.037	P < 0.001	P<0.001	P = 0.977
2K+L-NAME					
ACI	6	312 ± 8	14 ± 4	0.54 ± 0.02	29.1 ± 0.2
Rf-4	9	309 ± 4	6 ± 1	0.62 ± 0.03	29.3 ± 0.3
Rf-1A	12	326 ± 4	26 ± 2 °	0.47 ± 0.02 $^{+}$	27.7 ± 0.5
Rf-1A+4	10	278 ± 6 *	39 ± 5 *	0.34 ± 0.03 *	25.8 ± 0.8*
ANOVA		P < 0.001	P < 0.001	P<0.001	P < 0.001
UNX					
ACI	10	309 ± 6	18 ± 2	0.50 ± 0.03	27.8 ± 0.4
Rf-4	10	298 ± 4	6 ± 2	0.56 ± 0.03	28.2 ± 0.3
Rf-1A	12	324 ± 6 °	26 ± 3 °	0.46 ± 0.02 $^{+}$	26.3 ± 0.5
Rf-1A+4	12	331 ± 5 °	38 ± 5 *	0.45 ± 0.02 $^{+}$	25.0 ± 0.4*
ANOVA		P < 0.001	P < 0.001	P=0.014	P < 0.001
UNX+L-NAME					
ACI	9	301 ± 10	20 ± 3	0.52 ± 0.03	27.2 ± 0.6
Rf-4	10	283 ± 6	16 ± 6	0.51 ± 0.02	27.2 ± 0.8
Rf-1A	12	320 ± 5 °	42 ± 3 °	0.37 ± 0.02 °	23.2 ± 1.0 °
Rf-1A+4	14	258 ± 10 °	69 ± 3 *	0.34 ± 0.04 °	23.5 ± 0.7 °
ANOVA		P < 0.001	P < 0.001	P<0.001	P < 0.001

Abbreviations: 2K, 2-kidneys; 2K+L-NAME, 2K+L-NAME-induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, UNX+L-NAME-induced hypertension; BW, body weight (gram); FGS, incidence of glomerulosclerosis (% glomeruli); Cc/100, creatinine clearance (ml/min per 100gBW); Palb, plasma albumin level (g/l). Values are given as mean ± SEM; n is number of rats. $^{\#}$: p<0.05 vs. ACI; $^{+}$ p < 0.05 vs. *Rf-4*; ° p < 0.05 vs. ACI and *Rf-4*; * p < 0.05 vs. ACI, *Rf-4* and *Rf-1A*.

Assessment of renal blood flow autoregulation

No differences were found in the absolute values of the RBF at 100 mm Hg between ACI, *Rf-1A*, *Rf-4*, and *Rf-1A+4* rats. Despite the relatively large variations within each strain, a significantly higher RBF at a RPP of 150 mm Hg was present in the *Rf-1A* and *Rf-1A+4* rats in comparison with ACI rats (data not shown). After normalizing the RBF, more significant differences were revealed (Figure 5). From 130-150 mm Hg, mean %RBF values were significantly higher in the *Rf-1A* single congenics compared to ACI rats. At 150 mm Hg, mean %RBF value of *Rf-1A+4* double congenics was significantly higher compared to ACI rats. This points to an impairment of the RBF autoregulation in *Rf-1A* and *Rf-1A+4* rats. Over the pressure range 100-150 mm Hg, the RAIs were in the order of 0.2 –0.3 in ACI as well as in *Rf-4* single congenics, indicating a normal renal autoregulation in these strains. In contrast, RAI values were significantly increased to levels of about 0.4-0.7 in *Rf-1A* single and *Rf-1A+4* double congenic rats, indicating an impaired renal autoregulation.

No marked renal damage was present in the rats used for the autoregulation experiment. In all four strains, the average UAV level was about 2-4 mg/day/100g BW, while the percentage of injured glomeruli was in the order of 2-6%.

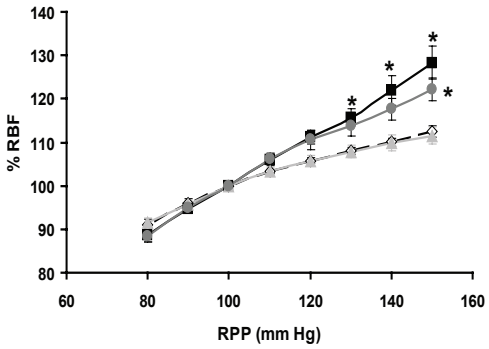


Fig 5: Renal blood flow autoregulation curves in two-kidney ACI (◇, n=15), *Rf-1A* (■, n=12), *Rf-4* (▲, n=15), and *Rf-1A+4* (●, n=12) rats. Values (as %RBF at RPP of 100 mm Hg) are given as means ± SEM (error bars); %RBF, relative renal blood flow; RPP, renal perfusion; * $p < 0.05$ vs. ACI and *Rf-4*.

Discussion

The major finding of our present study is the presence of a powerful synergistic interaction between the *Rf-1* and *Rf-4* QTLs that is markedly enhancing the susceptibility to renal damage as measured by the levels of UAV during and the %FGS at the end of the follow-up. The magnitude of the interactive effect differs between the various treatments, and appears to depend on the intensity and the duration of the exposure to the various stressors. The largest effect is observed when a reduction in renal mass by UNX is combined with L-NAME induced hypertension. A moderate effect is noted following UNX or L-NAME induced hypertension. Even in the 2K-situation there appears to be a small synergistic interaction.

The interpretation of the present finding of an interaction between the *Rf-1* and *Rf-4* QTL is complicated by the presence of a second part of the FHH chromosome 14 introgressed in the *Rf-1A+4* double congenic rats. However, this extra part located between 36.4-74.0 Mb is way off the 95% C.I. of the *Rf-4* QTL (i.e. between 5 and 20 Mb). In theory, a gene or genes in this additional part could account for the increased susceptibility of the double congenic strain. Preliminary data, obtained by H.J. Jacob and M.M.

Lutz (MM Lutz et al, manuscript in preparation), exclude a role of the distal part of chromosome 14. In order to narrow down the interval of the *Rf-4* QTL, a panel of subcongenic strains was generated from the *Rf-1A+4* and *Rf-1A* strains tested in the present experiments. One of the subcongenic strains (*Rf1A+4_by*) was identical to the *Rf-1A+4* with regard to the distal part of chromosome 14, but the FHH genotype between the markers *D14Mit11* (4.9 Mb) and *D14Mit2* (19.1 Mb) was replaced by ACI. In these strains susceptibility to renal damage was compared with *Rf-1A* single congenics. Following 8 weeks of UNX and L-NAME treatment UAV corrected for BW in the *Rf-1A+4_by* subcongenic (34±6 mg/day, n=6, mean±SEM) was significantly less that that of *Rf-1A+4* double congenics (98±10 mg/day, n=20), but not different from that of *Rf-1A* single congenics (31±4 mg/day, n=6). These findings indicate that the gene(s) responsible for the interaction between the *Rf-1* and *Rf-4* QTLs is located on chromosome 14 in the 4.9 and 19.1 Mb interval. Future studies aim to further reduce the size of the *Rf-4* interval and positionally clone the *Rf-4* gene(s).

Nature of the interaction

In recent years, there is an increasing awareness that genetic and environmental factors play an important role in common complex diseases in humans, such as diabetes, hypertension, and mental disorders.[169,246,257] Double congenic rat strains have been studied to establish interactions between blood pressure QTLs.[167,206] To our knowledge, the present findings are the first to directly show a QTL interaction increasing susceptibility to renal damage. Such an interaction between the *Rf-1* and *Rf-4* QTLs was predicted in an F2 population from a cross of FHH and ACI rats subjected to UNX.[234] The synergistic interaction is extremely powerful in this protective genomic background, particularly in the presence of kidney stressors. This observation could be important in understanding the variability found in the human situation.

Gene-gene interactions may have different phenotypic implications.[292] For example, the interaction could be additive, where the combined effect of *Rf-1* and *Rf-4* in the double congenic strain would roughly be equal to the sum of the effects of *Rf-1* and *Rf-4* on the phenotype. Irrespective of treatment the effect of *Rf-4* on UPV and UAV was only marginal, and therefore additivity can be excluded for UPV and UAV, but not for FGS. The UPV and UAV results clearly show that the gene-gene interaction has a powerful synergistic effect, consistent with epistasis for complex traits.[30] Our findings fit this concept. The effects of changing the genotype of *Rf-1* and *Rf-4* from homozygous ACI to homozygous FHH, has a powerful and significant effect on the pathophysiological parameters of renal damage. In addition, the complexity of the gene-gene interaction is further distended by the fact that the effects on renal damage of changing the *Rf-1* and *Rf-4* genotypes also depends on the severity of the (haemodynamic) strain put upon the kidney and the length of the exposure to the harmful stimuli. The renal damage parameters (UAV and FGS) are distant traits, i.e. not likely to be directly related to a gene defect. With the generation of the *Rf-1A+4* double congenics we have constructed a relatively simple two-locus model that results in an increased susceptibility to renal damage. One effect of *Rf-1* appears to be an impairment of the renal autoregulation, which might result in an increased P_{GC} when faced with systemic hypertension and/or reduced renal mass. The effect of *Rf-4* appears to be on a different level and only becomes detectable presence two copies of the *Rf-1* QTL from FHH. We can only speculate about possible mechanism(s) of action of *Rf-4*. It is conceivable that *Rf-4* as well as other *Rf*-QTLs play a role in protecting the integrity of the glomerular filtration barrier when exposed to an increased P_{GC}. Although this limits the number of possible candidates, it still leaves a wide range of

molecular pathways important in the biology of the podocyte and the glomerular barrier.[9,164,174,191]

Next to five QTLs in FHH, numerous QTLs influencing parameters of renal damage have been detected in other strains.[84,175,195,224,225,235] The Rat Genome Database (http://rgd.mcw.edu) has collected over 90 QTLs associated with renal damage or renal function components, some of them overlapping with the five *Rf*-QTLs. As far as renal damage is concerned, it looks as if 15-20 different loci might be involved in the various rat models.[129] Should a similar number of QTLs also play a role in humans, the number of possible interacting gene combinations that can be derived from these loci becomes tremendous. Inbred rat models remain relevant for gene identification and gene interaction, as the number of gene combinations per strain is relatively small. With the detection of an interaction between *Rf-1* and *Rf-4*, we continue to investigate the interaction between *Rf-1* and the three other *Rf*-QTLs. The ultimate goal, of course, is to identify the genes in the various *Rf*-QTLs, as recently accomplished for *Rf-2*[204], and establish how they affect susceptibility to renal damage and how they interact.[116] Subsequently, these genes and gene-gene interactions can be tested in humans. With the recent completion of the sequencing of the rat genome[87] it has become feasible to make a rat, mouse, and human genome comparison.[261] Analysis of the genes present in the various *Rf*-regions with the Ensembl Genome Browser (http:/www.ensembl.org) indicated that the 25 Mb *Rf-1* interval on rat chromosome 1 contains about 200 known and predicted genes with homologies in both mouse and human. The 25 Mb *Rf-4* interval on rat chromosome 14 contains about 130 known and predicted genes. Currently, 15-25% of the genes are predicted, novel, genes without a description of the gene product, while the description of others is vague. Eventually, shortening the congenic region will further decrease the number of candidate genes.

Conclusion

The present studies show that there is an interaction between the *Rf-1* and *Rf-4* QTL, which is markedly enhancing the susceptibility to renal damage in ACI genomic background that is ordinarily protective for proteinuria. It appears that the *Rf-1* QTL of the FHH rat contains one or more genes directly influencing renal susceptibility, possibly by impairing the efficacy of renal autoregulation. The *Rf-4* QTL also contains one or more genes that influence renal susceptibility. However, the effect of the *Rf-4* QTL can only be observed in the homozygous presence of the *Rf-1* QTL from the FHH rat.

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Chapter 5

Synergistic QTL interactions between *Rf-1* and *Rf-3* increase renal damage susceptibility in double congenic rats

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Abstract

The FHH (Fawn-Hooded Hypertensive) rat is a well established model of hypertension-associated chronic kidney damage (CKD). Multiple interacting genes determine the high renal susceptibility in this inbred rat strain. Previously we reported a synergistic interaction between *Rf-1* and *Rf-4*. In the present study we tested the presence of an interaction between the *Rf-1* and *Rf-3* QTLs.

The experiments were carried out in ACI.FHH-(*D1Rat298-D1Rat90*) (*Rf-1A* for short), ACI.FHH-(*D3Wox2-D3Rat59*) (*Rf-3* for short), and ACI.FHH-(*D1Rat475-D1Rat90*)/(*D3Rat84-D3Rat59*) (*Rf-1A+3* for short) congenic rats and ACI control rats. Rats were randomly divided over four treatment groups, i.e. two-kidney control (2K), 2K plus L-NAME induced hypertension (2K+L-NAME), unilateral nephrectomy (UNX), and UNX+L-NAME. Proteinuria (UPV), systolic blood pressure (SBP) were assessed 6, 12 and 18 weeks after starting treatment. The incidence of focal glomerulosclerosis (%FGS) was assessed at the end of the experiment. In the *Rf-1* and *Rf-3* single congenics, small increases in renal susceptibility were found following UNX+L-NAME treatment. However, when *Rf-1* and *Rf-3* were combined a major synergistic increase in renal susceptibility was found with all four treatments.

Autoregulation was assessed in 13-15 week old ACI, *Rf-1A*, *Rf-3* and *Rf-1A+3* congenic rats. Both *Rf-1A* and *Rf-1A+3* congenic rats had an impaired renal autoregulation. In contrast, the *Rf-3* had a normal autoregulation, similar to that of the ACI rat.

In conclusion, this study provides evidence that both *Rf-1* and *Rf-3* slightly increase the susceptibility to the development of renal damage. However, a synergistic interaction between these two QTLs is markedly enhancing renal susceptibility. Also, the *Rf-3* region does not carry any genes influencing renal autoregulation.

Introduction

Chronic kidney disease (CKD) is an important risk factor for the progression to end-stage renal failure (ESRF), cardiovascular disease, and overall mortality.[44,161,177] A large majority of CKD and ESRF is not associated with primary renal disease, but with systemic conditions like diabetes and hypertension.[263] However, only a minority of patients with diabetes and/or hypertension develops CKD, indicating that other aspects, such as genetic factors, also determine susceptibility to progressive renal disease.[220] Finding genes involved in these complex forms of nephropathy in humans has been more arduous. Linkage analysis has identified several chromosomal regions possibly involved in diabetic and non-diabetic forms of nephropathy, while candidate gene analyses have tested several genes with limited success.[38,81,219]

Inbred rat strains also vary widely in their susceptibility to develop renal damage and may be helpful to elucidate the genetics of progressive nephropathy. Our studies involve the FHH (Fawn-Hooded Hypertensive) rat, a well characterized model of hypertension-associated proteinuria and ESRF.[133,198,238,239,272,273,279] Crosses between FHH and the renal resistant ACI (August x Copenhagen Irish) rat revealed the presence of five QTLs, named *renal-failure*-1 (*Rf-1*) to *Rf-5*, linked to proteinuria (UPV) and other parameters of renal damage.[25,234] It is surmised that each of these QTLs contains gene(s) influencing the susceptibility to progressive renal damage in the FHH rat.

Although the nature of the genes is still unknown, the role of each QTL can be studied in congenic rat strains that have an *Rf*-region of the FHH rat introgressed in the genomic background of the renal-resistant ACI rat. Next, interactions between QTLs can be studied in double and multiple congenic strains. Since ACI is resistant to UPV even when made hypertensive, we stress the kidney to initiate the renal failure phenotype and be able to study the effect of a single QTL in the ACI genomic background. Previously, we reported about the susceptibility to renal damage in strains of ACI.FHH-*Rf1*, ACI.FHH-*Rf4*, and ACI.FHH-*Rf5* single congenics.[199,267,270] Following unilateral nephrectomy (UNX) combined with L-NAME induced hypertension, ACI.FHH-*Rf1* congenic rats developed significantly more UPV and albuminuria (UAV) than the ACI progenitor strain. In contrast, the ACI.FHH-*Rf4* and –*Rf5* single congenic rats showed no significant increase in renal susceptibility. In *Rf-1* congenic rats, renal autoregulation was impaired to the same extent as the parental FHH rat, while a normal renal blood flow (RBF) autoregulation was present in *Rf-4* and *Rf-5* single congenics.[267,270,272]

Despite the lack of a direct effect on renal susceptibility in the *Rf-4* single congenic rats, the combined presence of both the *Rf-1* and *Rf-4* QTLs increased renal damage susceptibility.[270] The scale of interaction depended on the experimental treatment. It was small in the normotensive two-kidney (2K) situation, intermediate following L-NAME treatment in 2K rats and in normotensive rats following UNX. The largest interaction was noted in the L-NAME treated UNX rats.[270] These experiments provided direct evidence for an interaction between the *Rf-1* and *Rf-4* QTLs, already suggested in the linkage analysis.[234]

In the present experiments we tested the presence of an interaction between the *Rf-1* and *Rf-3* QTLs. Therefore, we compared the renal susceptibility between the ACI progenitor strain and three congenic strains, i.e. ACI.FHH-*Rf1A*, ACI.FHH-*Rf-3* single congenics, and ACI.FHH-*Rf-1A+3* double congenic, in four experimental situations. Similar to the combination of *Rf-1* and *Rf-4*, we found evidence for a significant synergistic gene-gene interaction between *Rf-1* and *Rf-3* increasing the susceptibility to renal damage. Again, the magnitude of the interaction depended on the experimental treatment. We also examined the

renal autoregulation in two-kidney ACI, ACI.FHH-*Rf1A*, ACI.FHH-*Rf3* single congenics, and ACI.FHH-*Rf1A+3* double congenic rats. Results showed that renal autoregulation was impaired in ACI.FHH-*Rf1A* single congenics and ACI.FHH-*Rf1A+3* double congenic rats. Renal autoregulation was normal in ACI.FHH-*Rf3* single congenics, similar to the ACI rats.

Methods

Congenic and control rat strains

For the experiments, ACI.FHH-(*D1Rat298-D1Rat90*) (*Rf-1A* for short), ACI.FHH-(*D3Wox2-D3Rat59*) (*Rf-3* for short), and ACI.FHH-(*D1Rat475-D1Rat90*)/(*D3Rat84-D3Rat59*) (*Rf-1A+3* for short) congenic rats and ACI control rats were used. All breeding was performed at the Animal Research Center at Erasmus MC, Rotterdam, the Netherlands. Animals were housed in individually-ventilated cages under SPF-conditions[180] as previously described.[267] The protocol received approval from the animal ethical committee of the Erasmus University.

Congenic rat strains were generated using a speed congenic strategy as described for the *Rf-1B* strain by Provoost et al.[199] Since the single and double congenic strains were generated in parallel, the size of the introgressed congenic regions (homozygous for FHH) slightly differs between the strains. A schematic view of the introgressed *Rf*-regions of the various congenic strains is presented in Figure 1. The introgressed *Rf-1* region in the double congenic rats is about 13 Mb shorter than in the single congenic. The introgressed *Rf-3* region in the double congenic rats is about 7 Mb shorter than in the single congenic. A whole genome scan with 150 genetic markers on these three congenic strains showed that there was no FHH genomic contamination on other chromosomes.

Renal damage susceptibility

Experiments for assessment of renal damage susceptibility were performed on 184 animals starting from the age of 6-7 weeks. Per strain, the animals were randomly divided over four treatments (Table 1). The first received no treatment, remaining with two kidneys (2K), and was considered to be the control situation. The second remained with 2K and was chronically treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) (2K+L-NAME) to induce systemic hypertension. The third treatment consisted of UNX to reduce renal mass (UNX), while the fourth treatment consisted of UNX and receiving L-NAME (UNX+L-NAME).

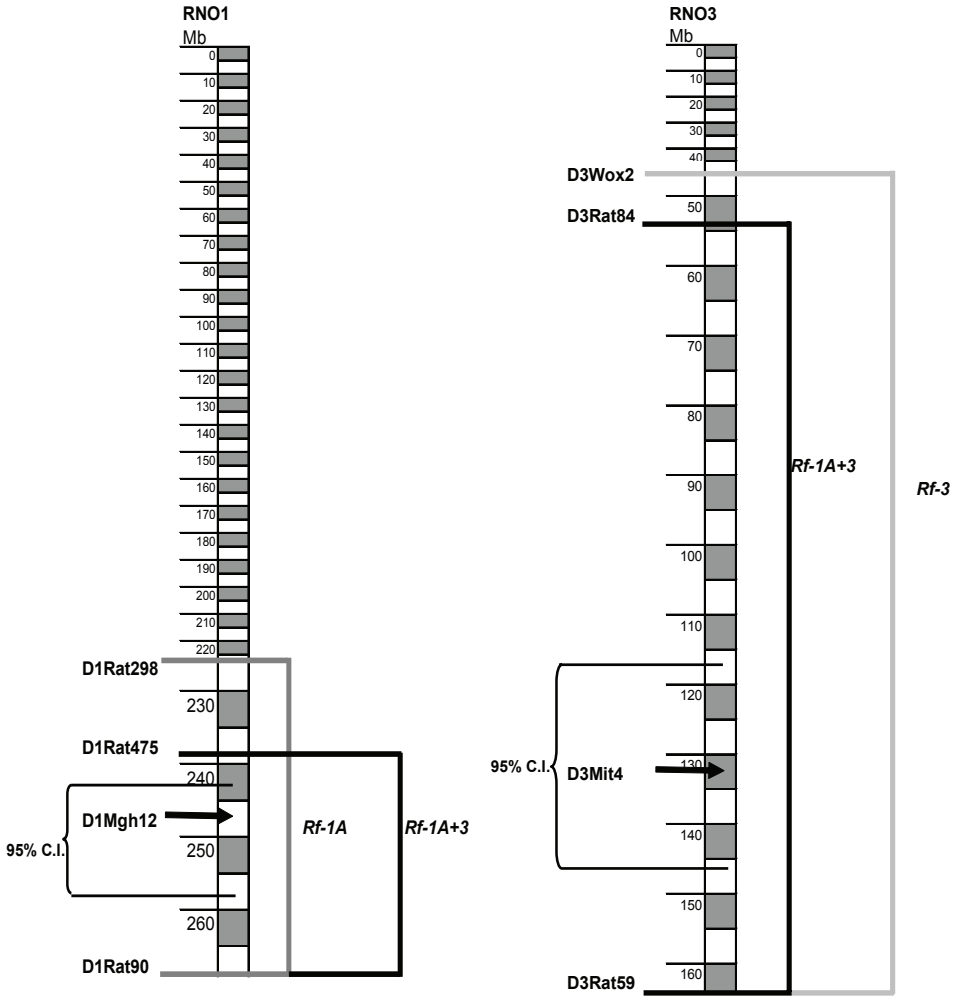

Figure 1: Genetic maps of rat chromosomes 1 and 3 depicting the homozygous FHH regions introgressed on the ACI background in the *Rf-1A*, *Rf-3* single, and *Rf-1A+3* double congenic strains. The areas homozygous FHH in the congenic strains are indicated by the solid and striped lines. The arrows indicate the locations of the *Rf-1* and *Rf-3* QTL peaks found in previous studies, i.e. *D1Mgh12* for *Rf-1*, and *D3Mit4* for *Rf-3*.[25,234] Distances are given in mega-base pairs (Mb). The 95% C.I. represents the 95% confidence interval of the QTLs.

Table 1: Number of rats studied in the renal susceptibility experiments.

	2K	2K+L-NAME	UNX	UNX+L-NAME
ACI	12	12	12	12
Rf-3	10	10	9	11
Rf-1A	12	12	12	12
Rf-1A+3	12	12	12	12,10,5*

* Number of rats at first, second and third follow-up, respectively.

Surgery for UNX and L-NAME treatment were performed as previously described.[274] Actual L-NAME intake was calculated in mg/kg from the fluid intake after 6, 12 and 18 weeks of treatment. The L-NAME intake was in the order of 8-12 mg/day/kg BW in the 2K+L-NAME situation, and 9-17 mg/day/kg BW in the UNX+L-NAME situation.

Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period. Besides the amount of urine excretion, food and fluid intake was also measured. Following the urine collection, systolic blood pressure (SBP) was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained rats, as described previously.[199]

Shortly after the last series of urine collections and SBP measurements, the rats were sacrificed as previously described.[267] Both kidneys were weighed, and left kidneys were used for histological examination. In a PAS-stained slide a total of 50 glomeruli were examined to determine the incidence of focal glomerular sclerosis (%FGS) as previously described.[267]

Renal blood flow autoregulation

Experiments for assessment of renal blood flow autoregulation were performed on 64 animals (21 ACI, 12 *Rf-1A*, 15 *Rf-3*, and 16 *Rf-1A+3* rats) with an age of 13-15 weeks. To get an indication of the presence of renal damage, UPV and UAV were assessed using a 24-hr sample obtained before the autoregulation experiments, while at the end of the evaluation both kidneys were collected and weighed and the left kidney was used to determine the %FGS, as previously described.[267]

Animals were anaesthetized with a mixture of 3% Isoflurane®, 30% N_2O, and 60% O_2 and surgically prepared for autoregulation studies.[272,273] After surgery and a 10-min equilibration period, the relationship between the left kidney RBF and the renal perfusion pressure (RPP) was determined. The RBF was recorded as the RPP was lowered from 150 to 80 mm Hg in 10 mm Hg steps by tightening a clamp around the aorta, followed by a 3-min equilibration period. To normalize the outcome of the individual rats, the RBF at a RPP of 100 mm Hg (RBF_{100}) was considered to be 100%. Renal autoregulatory indexes (RAIs) over the range of pressures from 80 to 150 mm Hg were calculated by the method of Semple and de Wardener.[228] A RAI of 0 indicates perfect autoregulation of RBF, and a RAI of 1 indicates that there is no autoregulation present due to a fixed renal vascular resistance.

Analytical procedures

Plasma and urinary samples were analysed with an ELAN system (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Total urinary protein was determined colorimetrically with pyrogallol red-molybdate complex.[286] Plasma albumin was determined with bromocresol green.[62] Plasma and urinary creatinine levels were determined with the Jaffé method without deproteinisation.[240]

Statistics

Data are presented as mean ± SEM. Statistical differences in mean values between groups were compared using one-way ANOVA. In both studies the ANOVA was followed by the Bonferroni test to determine which pairs were significantly different. The experimental design was also analysed as a 2x2 factorial analysis of variance (ANOVA) providing an evaluation of main effects and the interaction between the genotypes of *Rf-1* and *Rf-3*, being either homozygous ACI or homozygous FHH. In all tests, a p-value <0.05 was considered statistically significant. One-way ANOVA, followed by Bonferroni test were performed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996). The 2x2 factorial ANOVA was performed by using the statistics program at http://faculty.vassar.edu/lowry/anova2x2.html.

Results

Animal survival

All 2K, 2K+L-NAME, and UNX rats survived the 18-week follow-up period. Following UNX+L-NAME treatment, two of the twelve *Rf-1A+3* double congenic rats did not survive up to the second measurement, and another three of the remaining ten *Rf-1A+3* double congenics did not survive to complete the third measurement. Another two did not eat and suffered from severe weight loss, and were excluded from the analysis.

Proteinuria

Mean values for UPV during follow-up from the various treatments are presented in Figure 2A-2D. At all time points, regardless of treatment, UPV was significantly higher in *Rf-1A+3* double congenic rats compared to ACI, *Rf-1A* and *Rf-3* single congenic rats. In the 2K situation, *Rf-3* single congenics show an increased UPV compared to ACI at the second measurement. At the final measurement following L-NAME treatment in 2K-rats, a slight albeit not significant increase in UPV was found in *Rf-1A* and *Rf-3* single congenics compared to ACI. In the UNX model, *Rf-1A* single congenics showed an increase in UPV at the third time point compared to ACI and *Rf-3* congenic rats. After UNX+L-NAME treatment, both *Rf-1A* and *Rf-3* single congenics have a significantly higher UPV compared to ACI at the third time point.

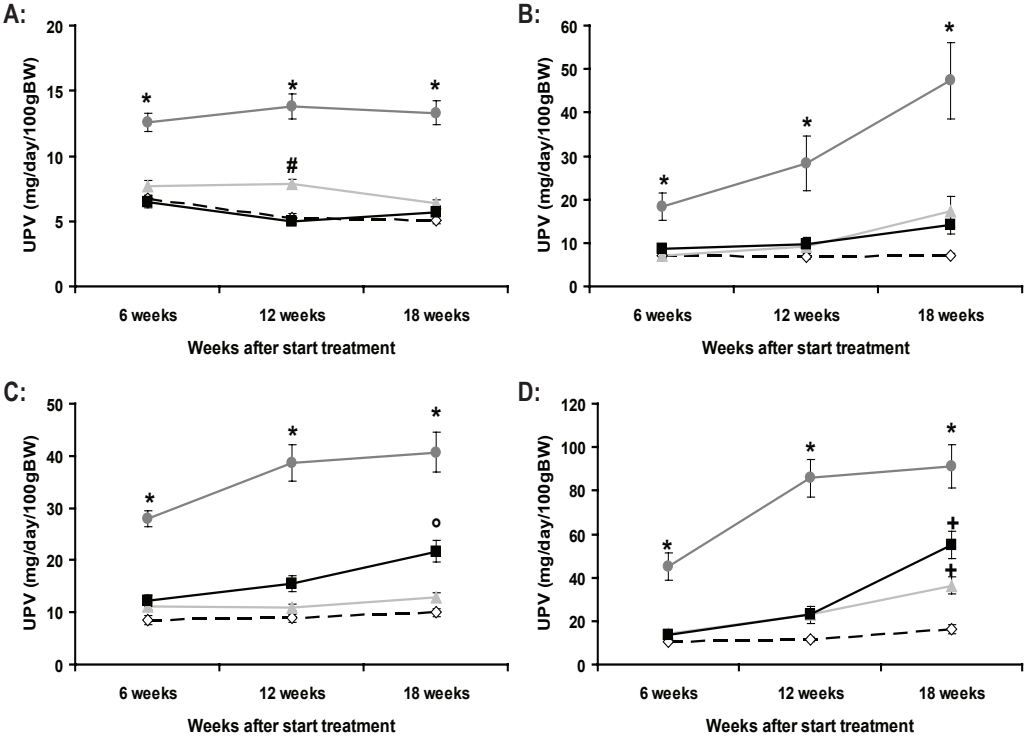

Figure 2. Proteinuria in ACI (◇), *Rf-1A* (■), *Rf-3* (▲), and *Rf-1A+3* (●) rats after 6, 12, and 18 weeks of follow-up during four treatments, values are given as mean ± SEM; **A**: 2K (2 kidneys); **B**: 2K+L-NAME (2K + L-NAME induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME; +: P<0.05 compared to ACI; *: P<0.05 compared to ACI, *Rf-1A*, and *Rf-3*; °: P<0.05 compared to ACI and *Rf-3*; #: P<0.05 compared to ACI, *Rf-1A*, and *Rf-1A+3*.

SBP

Values for SBP are presented in Figure 3A-3D. In the 2K-situation all four rat strains remained normotensive. At the first measurement, the congenic strains showed a small but significantly higher SBP compared to ACI. However, this difference was no longer present in the second and third measurements. After UNX all strains remained normotensive (Figure 3C), but a slightly higher SBP was present in the *Rf-1A+3* double congenics compared to the *Rf-1A* and *Rf-3* single congenic rats at the third time point. In the 2K-situation, chronic L-NAME treatment increased SBP in all strains. At the first time point, SBP in *Rf-1A+3* double congenic rats, and *Rf-1A* and *Rf-3* single congenic rats with 2K+L-NAME treatment were significantly increased compared to ACI rats. At the second and third time point, following 2K+L-NAME treatment, *Rf-1A+3* double congenic rats had a significantly higher SBP compared to ACI, *Rf-1A*, and *Rf-3* single congenic rats. Following UNX+L-NAME treatment, the *Rf-1A+3* double congenics had a higher SBP compared to the other strains at the first and second time points.

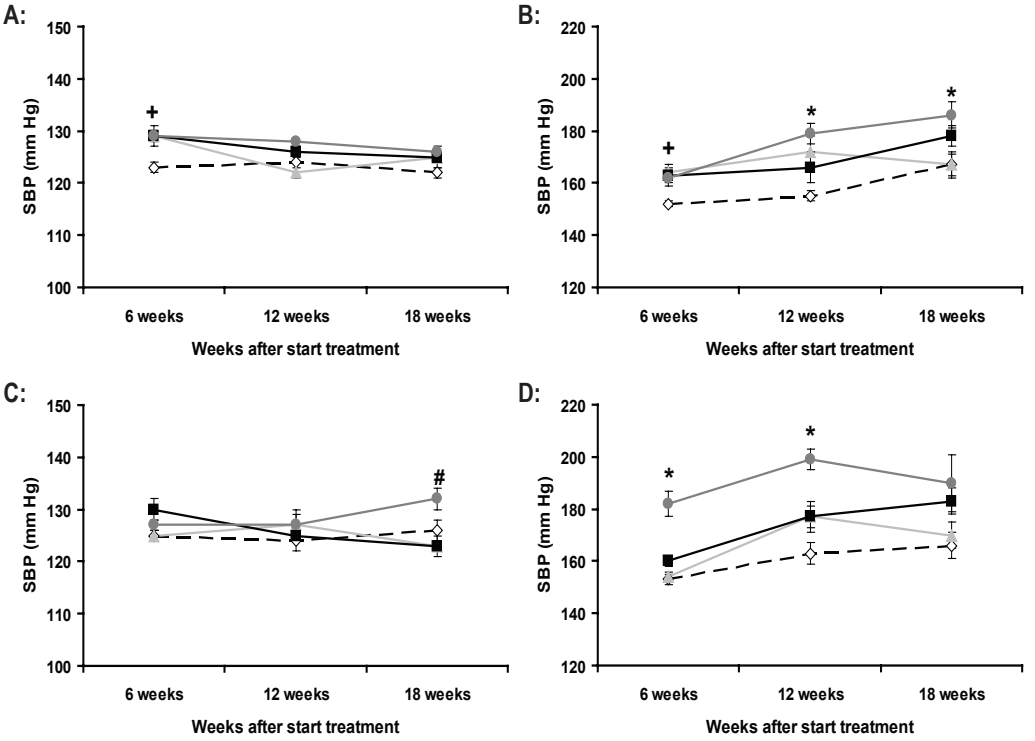

Figure 3. Systolic blood pressure in ACI (◇), *Rf-1A* (■), *Rf-3* (▲), and *Rf-1A+3* (●) rats after 6, 12 and 18 weeks of follow-up during four treatments; values are given as mean ± SEM; number of rats are the same as given in Figure 2 **A**: 2K (2 kidneys); **B**: 2K+L-NAME (2K + L-NAME induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME; +: P<0.05 compared to ACI; *: P<0.05 compared to ACI, *Rf-1A*, and *Rf-3*; °: P<0.05 compared to ACI and *Rf-3*; #: P<0.05 compared to *Rf-1A*, and *Rf-3*.

Gene-gene and gene-treatment interactions

The results of the 2x2 factorial ANOVA analyses showed the presence of significant gene-gene interactions. The *Rf-1* and *Rf-3* QTLs each showed to have an effect on UPV compared to ACI. Combining *Rf-3* with *Rf-1A* always resulted in a further increase in UPV when compared to *Rf-1A* and *Rf-3* single congenics. Furthermore, the magnitude of these interactions was significant in all four treatment groups, however the greater the haemodynamic stress upon the kidney, the greater the synergistic effect. (Table 2)

Table 2: Effects of the *Rf-1* and *Rf-3* QTLs and their interaction on proteinuria (UPV).

	6 weeks of treatment	Oneway ANOVA P	2x2 factorial ANOVA P	12 weeks of treatment	Oneway ANOVA P	2x2 factorial ANOVA P	18 weeks of treatment	Oneway ANOVA P	2x2 factorial ANOVA P
2K									
Rf-3 effect	+1.0±1.3	Ns	<0.001	+2.7±1.2	<0.05	<0.001	+1.3±0.9	<0.05	<0.001
Rf-1 effect	-0.2±1.5	Ns	<0.001	-0.2±1.2	Ns	<0.001	+0.6±1.2	Ns	<0.001
Interaction of *Rf-1* and *Rf-3*	+5.3±1.8	N/a	<0.001	+6.1±2.1	N/a	<0.001	+6.3±1.9	N/a	<0.001
2K+L-NAME									
Rf-3 effect	-0.2±2.3	Ns	0.005	+2.3±2.4	Ns	0.002	+10.0±10.3	<0.05	<0.001
Rf-1 effect	+1.7±2.6	Ns	0.002	+2.7±4.3	Ns	0.004	+7.0±7.5	Ns	<0.001
Interaction of *Rf-1* and *Rf-3*	+9.2±6.2	N/a	0.010	+15.4±12.2	N/a	0.035	+23.2±15.1	N/a	0.030
UNX									
Rf-3 effect	+2.6±2.4	Ns	<0.001	+2.0±2.7	Ns	<0.001	+2.9±3.0	Ns	<0.001
Rf-1 effect	+3.8±3.5	<0.05	<0.001	+6.6±4.7	<0.05	<0.001	+11.7±5.2	<0.05	<0.001
Interaction of *Rf-1* and *Rf-3*	+13.0±3.9	N/a	<0.001	+21.1±7.4	N/a	0.021	16.1±8.0	N/a	<0.001
UNX+L-NAME									
Rf-3 effect	+3.6±3.8	Ns	<0.001	+11.4±10.1	<0.05	<0.001	+20.2±10.5	<0.05	0.002
Rf-1 effect	+3.3±3.3	Ns	<0.001	+11.9±6.1	<0.05	<0.001	+38.8±16.8	<0.05	<0.001
Interaction of *Rf-1* and *Rf-3*	+27.6±11.9	N/a	<0.001	+50.0±16.1	N/a	<0.001	+16.2±17.3	N/a	0.038

For each treatment and time-point the *Rf-3* effect ($UPV_{Rf\text{-}3} - UPV_{ACI}$), the *Rf-1* effect ($UPV_{Rf\text{-}1A} - UPV_{ACI}$), and the interaction ($UPV_{Rf\text{-}1A+3} - UPV_{Rf\text{-}1A} - UPV_{Rf\text{-}3} + UPV_{ACI}$) were calculated. Data represent change in UPV (mg/day per 100 g BW) ± SD. Statistical significance of *Rf-1* and *Rf-3* effects alone were calculated comparing the *Rf-1* and *Rf-3* strains against the ACI controls by one-way ANOVA followed by the Bonferroni test. Statistical significance of the overall (main) effect of *Rf-1* and *Rf-3* and their interaction were calculated using 2x2 factorial ANOVA. 2K, 2-kidneys; 2K+L-NAME, 2K + L-NAME-induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, UNX + L-NAME-induced hypertension; N/a: not applicable; Ns: not significant.

An example is presented in Figure 4, for UPV at the second time point, after 12 weeks of treatment at the age of about 18 weeks. It is shown that changing the *Rf-3* genotype from homozygous ACI (AA) to homozygous FHH (FF), while the *Rf-1A* genotype remains AA, induced a slight increase in UPV per 100g BW in all four treatment groups. Changing the *Rf-1A* genotype from AA to FF, while the *Rf-3* genotype remains AA, also an increase in UPV is seen. Assuming an additive effect on UPV when both *Rf-1A* and *Rf-3* change from AA to FF, an expected change in UPV can be calculated. In all four situations, the observed changes in UPV induced by changing both *Rf-1A* and *Rf-3* from AA to FF, significantly exceeded the expected calculated change in UPV. The difference, being the interactive effect of *Rf-1A* and *Rf-3* depended on the experimental situation, but was significant in all four situations. The magnitude of the interaction was small with 2K (+6.1 mg/day/100g BW), intermediate with 2K+L-NAME (+15.4 mg/day/100g BW) and UNX (+21.1 mg/day/100g BW) and large in the UNX+L-NAME situation (50.0 mg/day/100g BW).

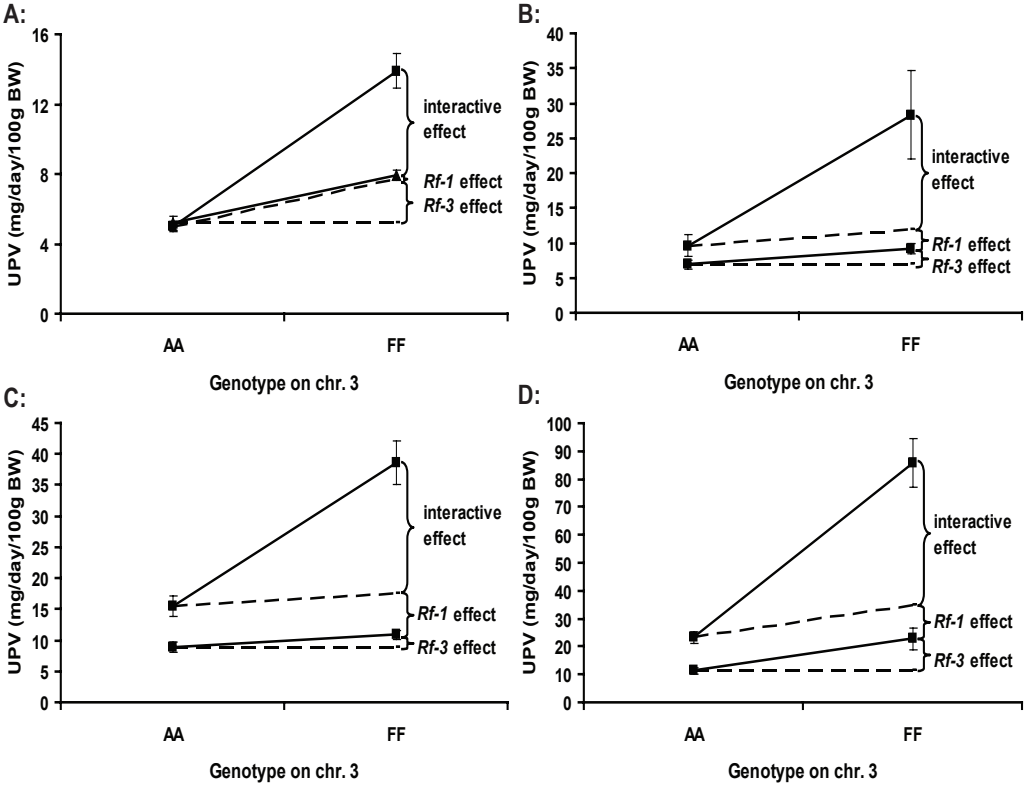

Figure 4: The effects of the *Rf-1* and *Rf-3* (chr. 3) genotype and their interaction of *Rf-1* and *Rf-3* on UPV after 12 weeks of treatment (M2). **A**: 2K (2 kidneys); **B**: 2K+L-NAME (2K + L-NAME induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME; AA, homozygous ACI; FF, homozygous FHH. Values for UPV (mg/day per 100 g BW) are mean ± SEM. Statistics are given in Table 2.

Findings at end of experiment

Body weight (BW), the incidence of focal glomerulosclerosis (%FGS), the creatinine clearance (Cc/100), and plasma albumin level (Palb) are summarized in Table 3. The %FGS was significantly higher in *Rf-1A+3* double congenic rats compared to ACI rats, following 2K+L-NAME, UNX, or UNX+L-NAME treatment. Following UNX+L-NAME treatment %FGS in *Rf-1A+3* double congenics was also significantly increased compared to *Rf-1A* and *Rf-3* single congenics. Following UNX+L-NAME treatment, the incidence of FGS was significantly higher in *Rf-1A* and *Rf-3* single congenic rats compared to ACI rats.

In *Rf-3* single congenic rats, Cc/100 was significantly increased compared to ACI and *Rf-1A* single congenics in all treatment groups. Following UNX, *Rf-1A+3* double congenics also showed an increased Cc/100 compared to ACI and *Rf-1A* single congenics. Plasma albumin (Palb) levels were slightly higher in *Rf-3* single congenic rats in the 2K, 2K+L-NAME and UNX treatment groups. Following 2K+L-NAME, UNX, or UNX+L-NAME treatment, Palb levels were decreased in *Rf-1A+3* double congenic rats. In the UNX situation, Cc/100 in *Rf-1A+3* double congenics is slightly increased compared to ACI rats. Following UNX+L-NAME treatment, *Rf-1A* single congenics have a decreased Palb compared to ACI and *Rf-3* single congenics.

Renal blood flow autoregulation

After normalizing the RBF, significant differences were revealed (Figure 5). From 110-150 mm Hg, mean %RBF values were significantly higher in the *Rf-1A* single congenics compared to ACI rats. From 130-150 mm Hg, mean %RBF value of *Rf-1A+3* double congenics were significantly higher compared to ACI rats. This points to an impairment of the RBF autoregulation in *Rf-1A* and *Rf-1A+3* rats. Over the pressure range 100-150 mm Hg, the RAIs were 0.25±0.02 in ACI and 0.28±0.03 in *Rf-3* single congenics, indicating a normal renal autoregulation in these strains. In contrast, RAI values were significantly increased in *Rf-1A* single (0.57±0.08) and *Rf-1A+3* double (0.49±0.05) congenic rats, indicating an impaired renal autoregulation.

No marked renal damage was present in the rats used for the autoregulation experiment. In all four strains, the average UAV level was about 2-7 mg/day/100g BW, while the percentage of injured glomeruli was in the order of 2-6%.

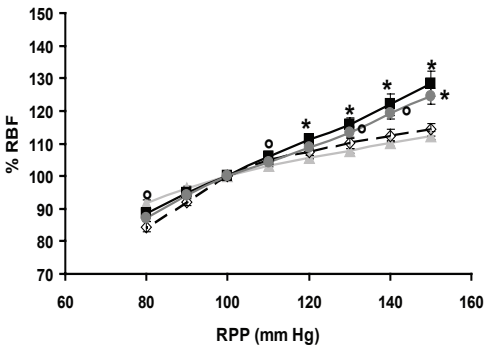

Figure 5: Renal blood flow autoregulation curves in two-kidney ACI (◇, n=21), *Rf-1A* (■, n=12), *Rf-3* (▲, n=15), and *Rf-1A+3* (●, n=16) rats. Values (as %RBF at RPP of 100 mm Hg) are given as means ± SEM (error bars); %RBF, relative renal blood flow; RPP, renal perfusion; * $p < 0.05$ vs. ACI and *Rf-3*, °: $p < 0.05$ vs. ACI.

Table 3: Measurements at end of follow-up.

	n	BW (gram)	FGS (% glom.)	Cc/100 (ml/min/100gBW)	Palb (g/l)
2K					
ACI	12	309±4	9±2	0.46±0.03	28.6±0.4
Rf-3	10	316±3	13±2	0.72±0.04 °	31.7±0.3 °
Rf-1A	12	333±7 #	14±2	0.51±0.03	29.0±0.6
Rf-1A+3	12	311±3	14±2	0.56±0.02	27.9±0.4
ANOVA		P=0.002	P=0.238	P<0.001	P<0.001
2K+L-NAME					
ACI	12	319±5	11±1	0.50±0.02	28.0±0.3
Rf-3	10	321±4	18±2	0.81±0.03 °	29.7±0.4 °
Rf-1A	12	328±3	23±2	0.49±0.01	28.0±0.4
Rf-1A+3	12	296±6 *	31±6 +	0.52±0.04	26.4±0.5 *
ANOVA		P<0.001	P=0.001	P<0.001	P<0.001
UNX					
ACI	12	315±3	15±2	0.41±0.01	27.8±0.2
Rf-3	9	304±3	20±2	0.58±0.02 ●	28.6±0.4 □
Rf-1A	12	330±6 ■	23±3	0.46±0.02	26.3±0.4
Rf-1A+3	11	304±5	27±3 +	0.58±0.02 ●	23.6±0.6 *
ANOVA		P<0.001	P=0.015	P<0.001	P<0.001
UNX+L-NAME					
ACI	12	314±6	15±1	0.46±0.05	27.3±0.3
Rf-3	11	301±6	39±5 +	0.63±0.04 °	27.2±0.4
Rf-1A	12	319±6	37±4 +	0.40±0.03	25.6±0.3 ▲
Rf-1A+3	5	263±12 *	64±11 *	0.31±0.06	23.0±0.4 *
ANOVA		P<0.001	P<0.001	P<0.001	P<0.001

Abbreviations: 2K, 2-kidneys; 2K+L-NAME, 2K + L-NAME-induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, UNX + L-NAME-induced hypertension; BW, body weight (gram); FGS, incidence of glomerulosclerosis (% glomeruli); Cc/100, creatinine clearance (ml/min per 100gBW); Palb, plasma albumin level (g/l). Values are given as mean ± SEM; n is number of rats; +: P<0.05 compared to ACI; ●: P<0.005 compared to ACI and *Rf-1A*; #: P<0.05 compared to ACI and *Rf-1A+3*; □: P<0.05 compared to *Rf-1A* and *Rf-1A+3*; ■: P<0.05 compared to *Rf-3* and *Rf-1A+3* °: P<0.05 compared to ACI, *Rf-1A*, and *Rf-1A+3*; *: P<0.05 compared to ACI, *Rf-1A*, and *Rf-3*; ▲: P<0.05 compared to ACI and *Rf-3*.

Discussion

The major finding of the present study is that introgression of the *Rf-1* and *Rf-3* QTLs on the ACI genomic background have a synergistic effect markedly increasing susceptibility to renal damage. This synergistic interaction between the *Rf-1* and *Rf-3* QTLs found in the *Rf-1A+3* double congenic rat is quantitatively comparable with the synergistic effect of *Rf-1* and *Rf-4* QTLs found earlier in *Rf-1A+4* double congenics.[270] Synergistic interactions between *Rf-1* and other *Rf*-regions suggested by Shiozawa et al.[234] were based on linkage analyses in an ACIxFHH F2-cross following UNX.[234] Our studies have now provided direct evidence for gene-gene interaction between *Rf-1* and *Rf-3*, as well as *Rf-1* and *Rf-4*. Interestingly, the magnitude of the interaction depended on the experimental situation, i.e. the presence or absence of hypertension and/or reduced renal mass.

In contrast to the parental FHH strain, the single and double congenics in the 2K-control group are normotensive, providing evidence that the *Rf-1* and *Rf-3* loci do not carry genes responsible for hypertension. To detect changes in renal susceptibility resulting from the introgression of the *Rf*-QTLs on the ACI background, we had to raise systemic blood pressure. Based on our earlier experience we used chronic L-NAME treatment to raise systemic blood pressure.[274,276] As indicated in our previous studies, using chronic L-NAME treatment has some disadvantages. It is difficult to match SBP between the various strains at a level normally present in the FHH rat. Chronic L-NAME treatment may directly affect the vascular structure in the kidney, independent of its blood pressure effects.[306] Furthermore, reducing endothelial NO-synthase activity by L-NAME may have a negative effect on the protective action of NO in organs that are targets of hypertensive injury.[101] Thus, differences in renal damage between *Rf-1A*, *Rf-3* single congenics, and *Rf-1A+3* double congenics and ACI rats may be partly due to an increased susceptibility to L-NAME.

Loss of functional renal mass is another way to induce progressive renal damage.[21] Therefore, we also tested if the renal disease was simply the result of a decrease in renal mass, mimicked by UNX. It should be noted that the ACI was almost completely protected from renal damage, despite the reduction in renal mass and/or presence of hypertension. Therefore, any change in renal damage found in the congenic rats must be the result of FHH genomic regions introgressed into the ACI genome.

A surprising finding was the presence of an increased Cc/100 in the *Rf-3* single congenic rats. This would indicate the presence of one or more genes in this region influencing the glomerular filtration rate. However, an increased Cc/100 in the *Rf-1A+3* double congenics was only seen following UNX and not in the 2K-situation and L-NAME treated rats. No good explanation is yet available, and further studies using better GFR methodologies need to be performed.

In previous studies we found that rats carrying the *Rf-1* QTL had an impaired renal autoregulation.[267,270] The *Rf-1A+3* double congenics also showed to have an impaired renal autoregulation, similar to that of the *Rf-1A* single congenic rat. In contrast, the *Rf-3* single congenic has a normal autoregulation, similar to that of the ACI rat. These findings indicate that the *Rf-3* region of the FHH rat does not influence renal autoregulation.

With the completion of the sequencing of the human, mouse and rat genome, genomic comparisons between these three species are greatly facilitated.[87,135,278,287] The 95% confidence interval of the *Rf-3* region (3: 117-146 Mb) is homologous to a part of human chromosome 20 (~1-33 Mb) and mouse chromosome 2 (126-155 Mb)(http:/www.ncbi.nlm.nih.gov/projects/Homology). Surprisingly, the *Rf-3* homologous region in human and mouse appear both to be involved in renal disease. Linkage studies in Pima Indians suggest the presence of a gene on chromosome 20 influencing diabetic nephropathy.[111] The homologous region in the mouse has recently been linked to albuminuria in KK/Ta mice.[233] The presence of QTLs linked to nephropathy in rat, mouse and human make the *Rf-3* region very interesting for further investigation.

In conclusion, this study provides evidence that *Rf-1* and *Rf-3* alone slightly increase the susceptibility to the development of renal damage. However, a synergistic interaction between these two QTLs does markedly enhance renal susceptibility.

Acknowledgements

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Chapter 6

Absence of interactions between the *Rf-1* and *Rf-5* QTLs influencing susceptibility to renal damage in rats

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Abstract

Previous studies in *Rf*-double congenic rats showed an increase in renal damage susceptibility when compared to *Rf-1* single congenics. Combining the *Rf-1* and *Rf-3* or *Rf-1* and *Rf-4* regions of the FHH on the ACI genomic background induced synergistic interactions markedly enhancing renal susceptibility. In the present study we wanted to determine whether such an interaction was also present between the *Rf-1* and *Rf-5* QTLs.

Renal damage susceptibility was assessed in *Rf-1B+5* and *Rf-1B+4* double congenics and compared to *Rf-1B* single congenics in four situations, i.e. two-kidney control (2K), unilateral nephrectomy (UNX), L-NAME induced hypertension (2K+L-NAME) and UNX+L-NAME. Albuminuria (UAV) and systolic blood pressure (SBP) were regularly measured. In a separate experiment renal autoregulation was assessed in 2K *Rf-1B*, *Rf-1B+5*, and *Rf-1B+4* rats.

The *Rf-1B+4* rats developed more UAV than the *Rf-1B* and *Rf-1B+5* rats following UNX, 2K+L-NAME and UNX+L-NAME. No differences were found in UAV between *Rf-1B* and *Rf-1B+5* rats regardless of treatment or time. Following UNX and 2K+L-NAME treatment no differences in SBP were noted between the three strains. With UNX+L-NAME treatment, the SBP in both double congenic strains was increased compared to *Rf-1B* single congenics. Autoregulation was impaired to a similar extent in the three strains.

We conclude that the *Rf-5* region does not influence renal damage susceptibility, neither alone nor in the presence of *Rf-1B*. We therefore assume that the *Rf-5* region does not contain genes influencing renal susceptibility.

Introduction

Chronic kidney disease (CKD) is assumed to be a complex polygenic disease[19], limiting the rate of success in finding genes involved in the development of CKD in human studies.[292,136,90] Multiple interacting genes may enhance the susceptibility to renal disease, as well as interactions between genes and the environment. Therefore, single gene mutations could be present in humans without showing an increased susceptibility to renal disease.[292]

Inbred rat strains also vary in their development of renal damage. The FHH (Fawn-hooded hypertensive) rat develops severe renal damage and hypertension at a relatively young age. Linkage analysis of crosses of FHH and the renal-resistant ACI (August x Copenhagen Irish) rat revealed five QTLs, named *Renal failure-1* (*Rf-1*) through *Rf-5*.[25,234]

Several congenic rats were generated carrying a single *Rf*-QTL region of the FHH onto an ACI genomic background. Previously we reported that transfer of the *Rf-1* region of the FHH onto the ACI genomic background increases susceptibility to renal damage, while transfer of *Rf-4* or *Rf-5* alone did not.[267,270] Furthermore, we reported synergistic interactions between the *Rf-1* and *Rf-4* as well as the *Rf-1* and *Rf-3* region of the FHH rat. In the *Rf-1A+4* and the *Rf-1A+3* double congenic rat susceptibility to renal failure was markedly, compared to the various single congenic strains.[269,270]

Influences of the *Rf-5* region on renal susceptibility still remain to be discovered. This can be done by studying the presence or absence of an interaction between the *Rf-5* and *Rf-1* regions in a double congenic rat. For this we generated double congenic rats carrying the *Rf-1* region and *Rf-5* regions. In the present study we investigated the susceptibility to the development of renal damage in *Rf-1B+5* and *Rf-1B+4* double congenics compared to *Rf-1B* single congenics. Renal susceptibility was tested as previously described.[267,269,270] In a separate experiment the efficacy of the renal blood flow (RBF) autoregulation was assessed in the three strains.

Methods

Congenic rat and control strains

For the experiments, ACI.FHH-(*D1Rat384-D1Rat156*) (*Rf-1B* for short), ACI.FHH-(*D1Mit18-D1Mit8*)/(*D14Mit11-D14Hmgc14b/D14Rat65-D14Rat90*) (*Rf-1B+4* for short), and ACI.FHH-(*D1Rat324-D1Rat452)*/(*D17Rat61-D1Arb5)(D17Rat51*) (*Rf-1B+5* for short) congenic rats were used. All breeding was performed at the Animal Research Centre at Erasmus MC, Rotterdam, the Netherlands. Animals were housed in individually-ventilated cages under SPF-conditions[180] as previously described.[267] The protocol received approval from the animal ethical committee of the Erasmus University.

Congenic rat strains were generated using a speed congenic strategy as described for the *Rf-1B* strain.[199] Since the single and double congenic strains were generated in parallel, the size of the introgressed congenic regions (homozygous for FHH) differs between the strains. A schematic view of the introgressed *Rf*-regions of the various congenic strains is presented in Figure 1. A whole genome scan with 150 genetic markers on these three congenic strains showed that there was no FHH genomic contamination on other chromosomes.

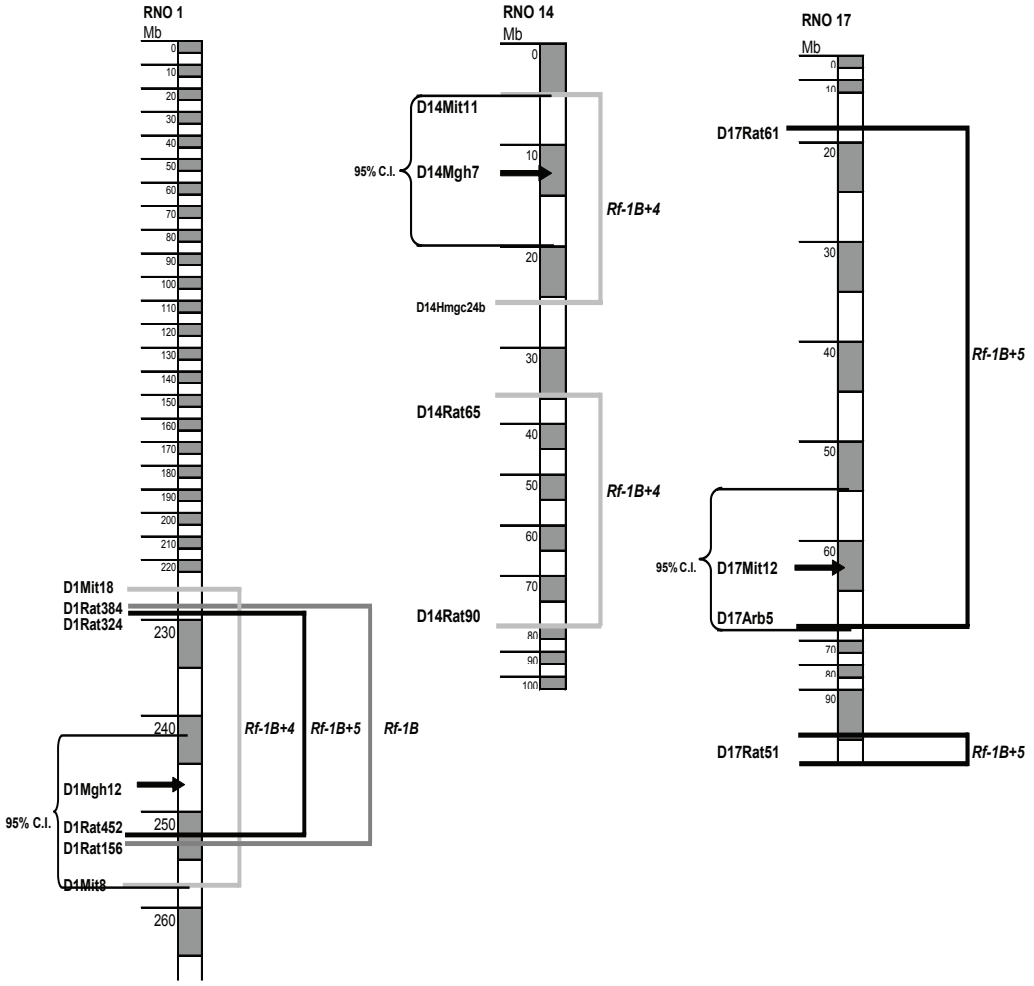

Figure 1: Congenic regions (homozygous FHH) in the *Rf-1B*, *Rf-1B+4*, and *Rf-1B+5* strains. The whole genomic background of these congenic rats is ACI, except for the areas shown at the right hand side above with the different markers. These areas are homozygous for FHH, and contain the QTL peak. The arrows at the left hand side indicate the locations of the QTL peaks plus 95% C.I. found in previous studies, i.e. *D1Mgh12* for the *Rf-1* QTL, *D14Mgh7* for the *Rf-4* QTL, and *D17Mit12* for the *Rf-5* QTL.[25,234]

Renal damage susceptibility

Experiments for assessment of renal damage susceptibility were performed on 132 animals starting from the age of 6-7 weeks. Per strain, the animals were randomly divided over four treatments (Table 1). The first received no treatment, remaining with two kidneys (2K). This was considered to be the control situation. The second remained with 2K and was chronically treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) (2K+L-NAME) to induce systemic hypertension. The third treatment consisted of UNX to reduce renal mass (UNX), while the fourth treatment consisted of UNX and receiving L-NAME (UNX+L-NAME).

Table 1: Number of rats studied in the renal susceptibility experiments.

	2K	2K+L-NAME	UNX	UNX+L-NAME
Rf-1B	12	12	12	12,12,11*
Rf-1B+4	12	12	12	12, 10, 2*
Rf-1B+5	8	10,10,9*	9,8,7*	9,9,7*

* Number of rats at first, second and third follow-up, respectively.

Surgery for UNX and chronic L-NAME treatment were performed as previously described.[274] Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period. Besides the amount of urine excretion, fluid intake was also determined. Actual L-NAME intake was calculated in mg/kg from the fluid intake after 6, 12 and 18 weeks of treatment. The L-NAME intake was in the order of 9-16 mg/day/kg BW in the 2K+L-NAME situation, and 10-21 mg/day/kg BW in the UNX+L-NAME situation.

Following the urine collection, SBP was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained rats, as described previously.[199]

The rats were sacrificed shortly after the last series of urine collections and SBP measurements as previously described.[267] Kidneys were weighed, and left kidneys were used for histological examination. In a PAS-stained slide a total of 50 glomeruli were examined to determine the incidence of focal glomerulosclerosis (%FGS) as previously described.[274]

Renal blood flow autoregulation

Experiments for assessment of renal blood flow autoregulation were performed on 37 animals (13 *Rf-1B*, 12 *Rf-1B+4*, and 12 *Rf-1B+5* rats) at the age of 13-15 weeks. To get an indication of the presence of renal damage, UAV was assessed using a 24-hr sample obtained before the autoregulation experiments.

The determination of renal blood flow autoregulation was performed as previously described.[269,270,272] Renal autoregulatory indexes (RAI) over the range of pressures from 130 to 150 mm Hg were calculated by the method of Semple and de Wardener.[228]

At the end of the autoregulation evaluation, both kidneys were weighed, and left kidneys were used for histological examination, as previously described.[274]

Analytical procedures

Plasma and urinary samples were analysed with an ELAN system (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Plasma and urinary albumin was determined with bromocresol green.[62] Plasma and urinary creatinine was determined with the Jaffé method without deproteinisation.[240]

Statistical analysis

Data are presented as mean ± SEM. Statistical differences in mean values between groups were compared using one-way analysis of variance (ANOVA), followed by the Bonferroni test to determine which pairs were significantly different. These tests were performed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996). In all tests, a p-value <0.05 was considered to be statistically significant.

Results

Animal survival

All 2K and UNX rats survived the 18-week follow-up period. Following 2K+L-NAME treatment, one *Rf-1B+4* double congenic rat did not survive the total follow-up period. Following UNX+L-NAME treatment, two of the twelve *Rf-1B+4* double congenic rats did not survive up to the second measurement, and another eight of the remaining ten *Rf-1B+4* double congenics did not survive up to the third measurement. One of the *Rf-1B* single congenics did not survive the follow-up period after UNX+L-NAME treatment. Data obtained from these twelve rats are included in the results for the first and, if measured, for the second measurements.

Albuminuria

Mean values for UAV during follow-up from the various treatments are presented in Figure 2A-2D. In the 2K-situation, *Rf-1B+4* showed an increase in UAV compared to *Rf-1B* at all time points, however it was only statistically significant at the first measurement. After both 2K+L-NAME and UNX, UAV was significantly increased in *Rf-1B+4* double congenic rats compared to *Rf-1B* rats at all time points. Following UNX+L-NAME treatment, *Rf-1B+4* double congenics developed significantly more UAV compared to *Rf-1B* single congenics rats at the first and second time point. At the third time-point, only two *Rf-1B+4* double congenics survived, not allowing a statistical analysis. No differences were found in UAV in the *Rf-1B+5* double congenic rat compared to *Rf-1B* single congenic rats, regardless of treatment or time.

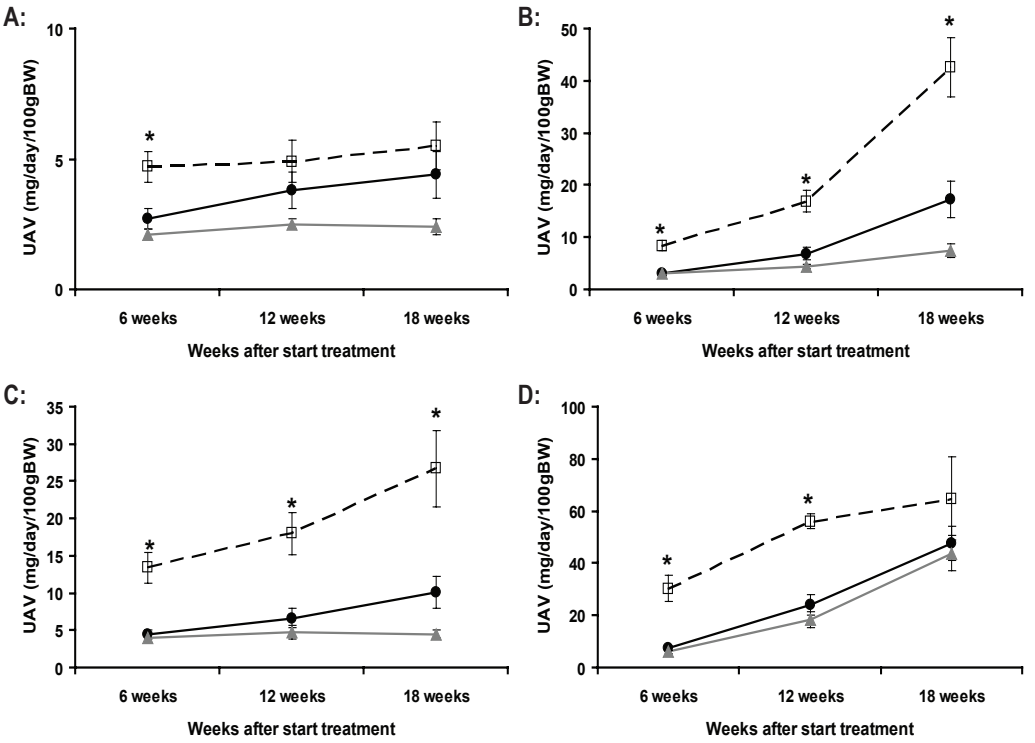

Figure 2. Albuminuria in *Rf-1B* (●), *Rf-1B+4* (□) and *Rf-1B+5* (▲) rats after 6, 12, and 18 weeks of follow-up during four treatments; values are given as mean ± SEM; **A**: 2K (2 kidneys); **B**: 2K+L-NAME (2K + L-NAME induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME, statistical analysis at third time point only between *Rf-1B* and *Rf-1B+5*; *: $p < 0.05$ versus *Rf-1B*.

Systolic blood pressure

Values for SBP are presented in Figure 3A-3D. In the 2K-situation, the *Rf-1B+4* double congenic rats showed a small but significantly lower SBP compared to *Rf-1B* at the first measurement. This difference was not present in the second and third measurements. After UNX (Figure 3C), no significant differences were found in SBP between the three strains. Chronic L-NAME treatment increased SBP. No differences were found following 2K+L-NAME treatment. However, following UNX+L-NAME treatment SBP was significantly increased in *Rf-1B+4* (first and second time point) and *Rf-1B+5* (at all time points) double congenic rats compared to *Rf-1B* single congenic rats.

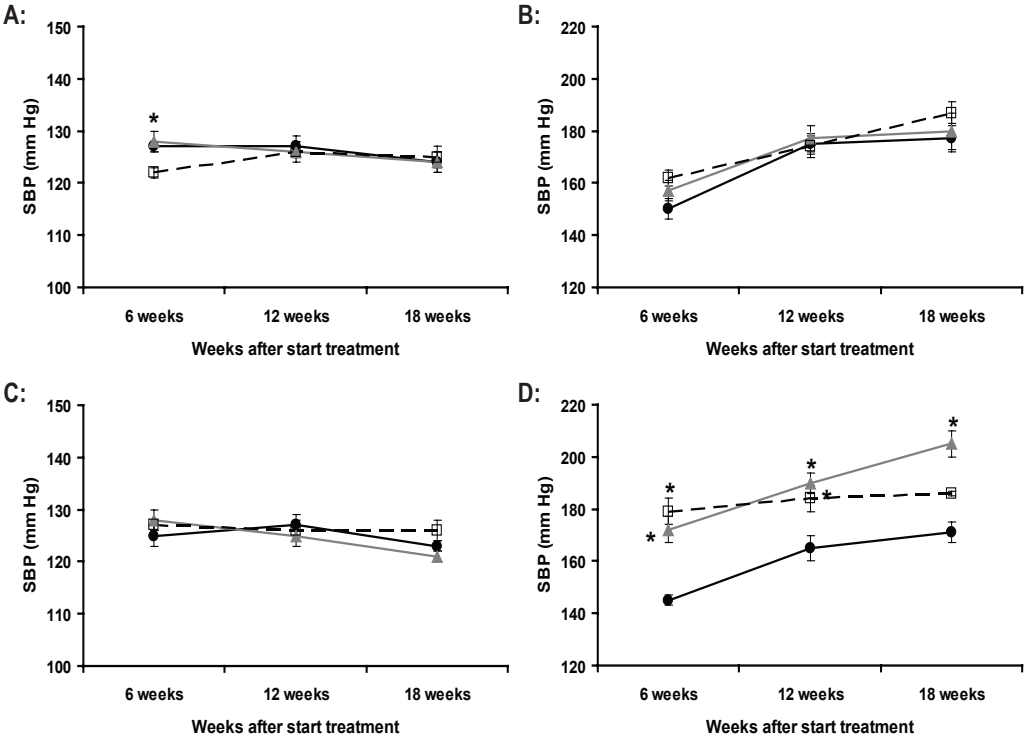

Figure 3. Systolic blood pressure (SBP) in *Rf-1B* (●), *Rf-1B+4* (□) and *Rf-1B+5* (▲) rats after 6, 12, and 18 weeks of follow-up during four treatments; values are given as mean ± SEM; **A**: 2K (2 kidneys); **B**: 2K+L-NAME (2K + L-NAME induced hypertension); **C**: UNX, (unilateral nephrectomy); **D**: UNX+L-NAME, statistical analysis at third time point only between *Rf-1B* and *Rf-1B+5*; *: $p < 0.05$ versus *Rf-1B*.

Measurements at end of follow-up

No differences were found in body weight (BW), %FGS, and plasma albumin (Palb) in the 2K-situation. Creatinine clearance (Cc/100) was slightly decreased in the *Rf-1B+4* and *Rf-1B+5* double congenics compared to *Rf-1B* single congenics. Following 2K+L-NAME treatment an increase in %FGS was found in *Rf-1B+4* double congenics compared to *Rf-1B* single congenics. After UNX, %FGS was increased in *Rf-1B+4* double congenics, and Palb was increased in *Rf-1B+5* double congenics compared to *Rf-1B* single congenics. Following UNX+L-NAME treatment, Palb was decreased in *Rf-1B+4* double congenics compared to *Rf-1B* single congenics (Table 2).

Table 2: Measurements at end of follow-up.

	n	BW (gram)	FGS (% glom.)	Cc/100 (ml/min/100gBW)	Palb (g/l)
2K					
Rf-1B	12	311±6	16±2	0.67±0.03	28.5±0.4
Rf-1B+4	12	305±2	21±3	0.52±0.04*	25.5±1.8
Rf-1B+5	8	312±5	8±2	0.54±0.03*	28.7±0.6
ANOVA		P=0.516	P=0.006	P<0.001	P=0.151
2K+L-NAME					
Rf-1B	11	294±5	20±3	0.55±0.05	27.7±0.9
Rf-1B+4	11	287±8	33±3*	0.46±0.04	26.2±0.6
Rf-1B+5	9	286±7	15±3	0.53±0.03	28.8±0.5
ANOVA		P=0.672	P<0.001	P=0.407	P=0.051
UNX					
Rf-1B	12	305±8	13±2	0.54±0.04	26.4±0.6
Rf-1B+4	12	304±4	30±3*	0.50±0.03	25.3±0.5
Rf-1B+5	7	303±2	12±2	0.50±0.07	28.8±0.3*
ANOVA		P=0.997	P<0.001	P=0.738	P<0.001
UNX+L-NAME					
Rf-1B	11	272±11	39±6	0.47±0.04	25.5±0.4
Rf-1B+4	2	286±0.4	59±11	0.37±0.04	23.6±0.4
Rf-1B+5	7	276±7	36±5	0.40±0.04	25.0±1.0
ANOVA		P=0.742	P=0.666	P=0.160	P=0.511

Values are given as means ± SEM. 2K, 2 kidneys; 2K+L-NAME, 2K + L-NAME induced hypertension; UNX, unilateral nephrectomy; UNX+L-NAME, unilateral nephrectomy with L-NAME induced hypertension; BW, body weight; FGS, incidence of glomerulosclerosis; Pcreat, plasma creatinine level; Palb, plasma albumin level; *: $p < 0.05$ compared to *Rf-1B*.

Renal blood flow autoregulation

The RBF autoregulation curves are presented in Figure 4. No statistical differences in RBF were present between the three rat strains. In addition, no statistical differences were found in RAI indexes for the 130-150 mm Hg RPP range. The RAI values amounted to 0.59±0.05 in *Rf-1B*, 0.45±0.07 in *Rf-1B+4*, and 0.45±0.03 in *Rf-1B+5* rats. The magnitude of the RAI values indicated that autoregulation is impaired to a similar extent in *Rf-1B* single and *Rf-1B+4* and *Rf-1B+5* double congenic rats.

No marked renal damage was present in the rats used for the autoregulation experiment. In all three strains, the average UAV were ~1-3 mg/day/100g BW, while the percentage of injured glomeruli was in the order of 4-10%.

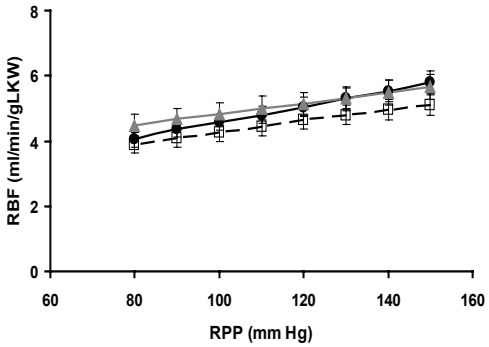

Figure 4: Relationship between renal blood flow (RBF) and renal perfusion pressure (RPP) in *Rf-1B* (●, n=13), *Rf-1B+4* (□, n=12) and *Rf-1B+5* (▲, n=12) rats; Values are given as means ± SEM (error bars). No significant differences were present.

Discussion

The present study fails to demonstrate an effect of the *Rf-5* QTL on the susceptibility to develop renal damage. Renal susceptibility in the *Rf-1B+5* double congenic rats did not differ from that observed in *Rf-1* single congenics. It is therefore likely that the *Rf-5* QTL does not contain genes influencing renal damage susceptibility. In contrast, the *Rf-1B+4* double congenic rats show an increased susceptibility to renal damage, comparable with the previously reported results in the *Rf-1A+4* double congenic strain.[270]

Following UNX+L-NAME treatment, *Rf-1B+4* double congenics did develop significantly more renal damage than the *Rf-1B* single congenics. Because of this increased susceptibility to renal damage, the majority of the *Rf-1B+4* double congenics did not survive the complete 18-week follow-up period. Based on the similarities of the present findings in the *Rf-1B+4* and our previous findings in the *Rf-1A+4* double congenic rats, we confirm the presence of a synergistic interaction between the *Rf-1* and *Rf-4* QTLs.[270]

No differences in the severity of renal damage induced by the various procedures could be detected when comparing the *Rf-1B+5* double congenics to the *Rf-1B* single congenics. While studies demonstrated that the presence of the *Rf-3* or *Rf-4* QTL besides the *Rf-1* increases renal susceptibility, the *Rf-5* QTL fails to do so. Following 2K, UNX, and 2K+L-NAME, no marked differences in SBP were found between the three strains. Thus the absence of an effect of *Rf-5* cannot be explained by difference in SBP. To the contrary, following UNX+L-NAME the SBP was significantly higher in the *Rf-1B+5* double congenics compared to *Rf-1B* single congenics, but no differences in renal damage susceptibility were present. The differences in SBP were due to an increased L-NAME intake in *Rf-1B+5* double congenics. The results also indicate that differences in L-NAME intake by itself have little effect on the development of renal damage.

We reported earlier that congenic rats carrying the *Rf-1* QTL region from the FHH on the ACI background do have an impaired renal autoregulation similar to FHH parental rats.[267,270] This study shows that renal autoregulation is also impaired in the *Rf-1B+4* and *Rf-1B+5* double congenic rats. Despite similar degrees of impaired renal autoregulation renal damage varies markedly between the different strains, underscoring that an impaired renal autoregulation alone is not sufficient to induce severe renal damage. Even in the presence of hypertension or reduced renal mass renal damage is limited in the absence of other *Rf*-QTLs, i.e. additional susceptibility genes. We surmise that the genes play a role in maintaining the integrity of the glomerulus or the glomerular filtration barrier when facing an increased hemodynamic stress. On the other hand, they could be involved in the renal handling of filtered proteins, like albumin. Further

studies will be needed to substantiate these assumptions, and eventually explain the mechanisms of the high susceptibility to develop renal damage seen in the FHH rat.

The evidence for a role of the *Rf-5* QTL in renal damage susceptibility remains meagre. The present study does not present any evidence, while a previous study showed that the *Rf-5* region alone does not influence renal susceptibility. Furthermore, the LOD-score of the *Rf-5* QTL was only suggestive for linkage (2.99).[234] Currently, a panel of consomic rat strains is being generated at the Medical College of Wisconsin (http://pga.mcw.edu). In these consomic strains each FHH chromosome is, one by one replaced by the corresponding chromosome of the BN strain. In the FHH-17^{BN} strain, FHH chromosome 17 containing the *Rf-5* QTL is substituted. The FHH-17^{BN} rats showed no difference in either UAV or SBP when compared to FHH rats. The results of the consomic studies strengthen our conclusion that the *Rf-5* QTL of the FHH does not contain genes influencing renal damage susceptibility. However, the situation might be different in other rat strains. Yagil et al. reported that chromosome 17 of the SBH/y carried genes influencing the development of proteinuria in the salt-susceptible Sabra rat strain.[299]

In conclusion, the present study confirms that the *Rf-4* region interacts with the *Rf-1* region to enhance renal susceptibility. The *Rf-5* region does not influence renal damage susceptibility when combined with *Rf-1*. We therefore assume that the *Rf-5* QTL from FHH does not contain genes influencing the susceptibility to develop renal damage.

Acknowledgements

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Chapter 7

Renal damage susceptibility in *Rf-1A+2* double congenic rats compared to FHH and FHL rats

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Abstract

Linkage analysis of a cross of FHH and the renal-resistant ACI (August x Copenhagen Irish) rats revealed five QTLs, named *Rf-1* through *Rf-5*. A double congenic rat was generated carrying *Rf-1* and *Rf-2*, the ACI.FHH-(*D1Mit34-D1Rat90*)(*D1Rat101-D1Rat120*) or (*Rf-1A+2*) for short.

Renal damage susceptibility was assessed in *Rf-1A+2* double congenic, FHL and FHH rats in two situations, i.e. two-kidney control (2K), and unilateral nephrectomy (UNX). After 6, 12, and 18 weeks after treatment, albuminuria (UAV) and systolic blood pressure (SBP) was measured. Renal autoregulation was assessed in two-kidney *Rf-1A+2*, FHL and FHH rats at an age of 13-15 weeks. A global genome scan on FHH and FHL was performed.

The FHH develops significantly more UAV (2K: 51.4±5.3, UNX: 166.8±9.2) compared to *Rf-1A+2* and FHH, regardless of treatment or time. Following UNX an increase in UAV is found in *Rf-1A+2* and FHL (33.8±4.6 and 52.8±5.2, respectively). The FHH are hypertensive (SBP ~160 mmHg), FHL are normotensive (SBP ~130 mm Hg), and SBP of the *Rf-1A+2* is intermediate (~145 mm Hg). The *Rf-1A+2*, FHL and FHH all have an impaired renal autoregulation. In the *Rf*-regions, FHH and FHL rats are identical for 86%, over all chromosomes it is 75%.

The *Rf-1* and *Rf-2* regions carry genes that influence renal susceptibility, and the *Rf-2* region carries a gene that increases blood pressure. Differences in renal damage found between FHH and FHL can be partly explained by the differences in SBP, and partly by differences in genotype of the *Rf*-regions.

Introduction

The FHH (fawn-hooded hypertensive) rat is an inbred rat strain that is well characterized as a model for hypertension-associated renal failure. The FHH rat is a result of inbreeding of a random-bred FH strain. The FHH rats develop hypertension, progressive proteinuria (UPV), focal glomerulosclerosis (FGS), and finally end-stage renal failure (ESRF) at a relatively young age.[198,200,238] During inbreeding of the FHH rat, another strain was selected for its low blood pressure and relatively low proteinuria, i.e. the FHL (fawn-hooded low blood pressure) rat. This FHL rat showed to be less susceptible to the development of ESRF, which could be partly explained by the absence of hypertension.[198,275]

Linkage analysis of crosses of FHH and the renal-resistant ACI (August x Copenhagen Irish) rat revealed the presence of five QTLs, named *Renal failure-1* (*Rf-1*) through *Rf-5*.[25,234] It is surmised that each of these QTLs contain genes that influence the susceptibility to the development of renal damage. The *Rf-1* QTL had by far the highest LOD-score (16.9) for albuminuria (UAV), and was therefore the most promising QTL. The *Rf-2* QTL peak had a LOD-score of 5.4 for UAV. In the *Rf-2* region, a QTL was embedded suggestively linked to blood pressure, which was named *Blood pressure fawn-hooded* (*Bpfh-1*).[25,234] A recent study by Matsson et al describes a consomic rat strain with chromosome 1 of the FHH, carrying *Rf-1*, *Rf-2* and *Bpfh-1*, being replaced by that of the BN (Brown Norway) strain. This FHH.BN1 strain had a significantly lower blood pressure and less renal damage than he FHH parental strain after L-NAME treatment.[158] These results support our previous linkage results, indicating that FHH chromosome 1 carries genes influencing blood pressure and the development of renal damage.

Previously we reported that the transfer of just *Rf-1* from FHH onto the genetic background of the ACI rat, increased renal damage susceptibility after UNX+L-NAME treatment.[267] The effect of *Rf*-1 alone was small, but when *Rf-1* was combined with either *Rf-3* or *Rf-4*, a significant interaction was found to markedly increase renal damage susceptibility.[269,270] Thus it appears that next to *Rf-1* the presence of another QTL is needed to enhance renal susceptibility. Therefore, we studied the effect of the combined presence of *Rf-1* and *Rf-2* on blood pressure and the development of renal damage. We therefore generated a congenic rat strain carrying both QTLs from FHH on the ACI background. This double congenic strain, ACI.FHH-(*D1Mit34-D1Rat90*)(*D1Rat101-D1Rat120*) or (*Rf-1A+2*) for short, was studied in the two-kidney (2K) situation and following unilateral nephrectomy (UNX). The effects in the *Rf-1A+2* strain were compared to those of FHH and FHL rat. We also performed a global genome scan comparing the inbred FHH and FHL strains to find genetic differences that might help to explain the difference in blood pressure and renal damage susceptibility between these two strains.

Methods

Congenic rat and control strains

For the experiments, *Rf-1A+2* double congenic rats, FHH, and FHL rats were used. All breeding was performed at the Animal Research Centre at Erasmus MC, Rotterdam, the Netherlands. Animals were housed in individually-ventilated cages under SPF-conditions[180] as previously described.[267] The protocol received approval from the animal ethical committee of the Erasmus University. It was anticipated that in some strains the experimental procedures could induce severe distress demanding to end the experiment. Criteria to remove animals were severe weight loss (>15% within a week), paralysis or the presence of glaucoma.

The congenic rat strain was generated using a speed congenic strategy as described for the *Rf-1B* strain by Provoost et al.[199] A schematic view of the introgressed *Rf*-regions of the *Rf-1A+2B* congenic strain is presented in Figure 1. The congenic generated here encompass the earlier reported 95% C.I. for *Rf-1* and *Rf-2* and a major part of the *Bpfh-1* QTL.[25,234] A whole genome scan with 150 genetic markers on the congenic strain showed that there was no detected FHH genomic contamination on other chromosomes.

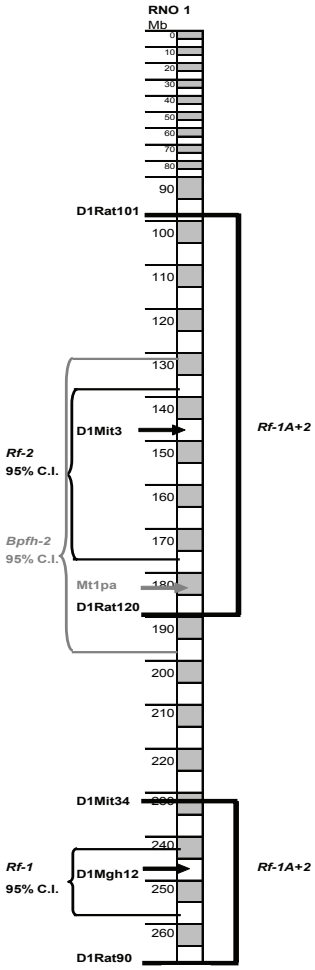

Figure 1: Congenic regions (homozygous FHH) in the *Rf-1A+2* strain. The whole genomic background of these congenic rats is ACI, except for the areas shown at the right hand side above with the different markers. These areas are homozygous for FHH, and contain the QTL peak. The arrows at the left hand side indicate the locations of the QTL peaks plus 95% C.I. found in previous studies, i.e. *D1Mgh12* for the *Rf-1* QTL, and *D1Mit3* for the *Rf-2* QTL, and in gray, *Mt1pa* for *Bpfh-1*.[25,234]

Genetic heterogeneity between FHH and FHL rats

Genomic DNA was extracted from spleens of FHH and FHL rats. Genotyping with simple-sequence length polymorphisms (SSLPs) was performed.[115] Randomly divided over all rat chromosomes, 124 SSLPs were tested on DNA obtained from FHH an FHL. Of these 124 SSLPs, 21 were located in the five *Rf*-regions of the FHH rat.

Susceptibility to L-NAME in *Rf-1A+2* double congenics

Previously we observed that both FHH and FHL rats suffered a high mortality following chronic L-NAME treatment, especially following UNX.[275,276] Based on these findings, susceptibility to L-NAME of the *Rf-1A+2* double congenic rats was also tested in the 2K-situation (n=5) and in combination with UNX (n=6).

Renal damage susceptibility in two-kidney rats and following UNX

Experiments for assessment of renal damage susceptibility in 2K and UNX were performed on 61 animals starting from the age of 6-7 weeks. Per strain, the animals were randomly divided over two treatments (Table 1). The first received no treatment, remaining with two kidneys (2K), which was considered to be the control situation. The second treatment consisted of UNX to reduce renal mass (UNX). UNX was performed as previously described.[274]

Table 1: Number of rats studied in the renal susceptibility experiments.

	2K	UNX
Rf-1A+2	11	12
FHL	10	9
FHH	10	9,9,6*

* Number of rats at first, second and third follow-up, respectively.

Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period. Besides the amount of urine excretion, fluid intake was also determined.

Following the urine collection, SBP was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained rats, as described previously.[199]

The animals were sacrificed shortly after the last series of urine collections and SBP measurements as described previously.[267] Kidneys were weighed and left kidneys were used for histological examination. In a PAS-stained slide a total of 50 glomeruli were examined to determine the incidence of focal glomerulosclerosis (%FGS) as previously described.[267]

Renal autoregulation experiment

Experiments for assessment of renal blood flow autoregulation were performed on 24 animals (8 *Rf-1A+2*, 8 FHH, and 8 FHL rats) with an age of 13-15 weeks. To get an indication of the presence of renal damage, UAV was assessed using a 24-hr sample obtained before the autoregulation experiments.

The determination of renal blood flow autoregulation was performed as previously described.[267,270,272] Renal autoregulatory indexes (RAI) over the range of pressures from 80 to 150 mm Hg were calculated by the method of Semple and de Wardener.[228]

At the end of the autoregulation evaluation, both kidneys were weighed, and left kidneys were used for histological examination, as previously described.[274]

Analytical procedures

Plasma and urinary samples were analysed with an ELAN system (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Plasma and urinary albumin was determined with bromocresol green.[62] Plasma and urinary creatinine was determined with the Jaffé method without deproteinisation.[240]

Statistical analysis

Data are presented as mean ± SEM. Statistical differences in mean values between groups were compared using one-way analysis of variance (ANOVA), followed by the Bonferroni test to determine which pairs were significantly different. These tests were performed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996).

Results

Genetic heterogeneity between FHH and FHL rats

Genetic heterogeneity in FHH and FHL rats was tested in order to reveal differences between these two strains. In total, 124 SSLPs were tested, of which 31 (25%) had different lengths in FHL compared to FHH. Of the 21 SSLPs located in the *Rf*-regions of the FHH rat, only three had a different length in FHL (14%). In the *Rf-1* and *Rf-3* regions no polymorphisms were found between FHH and FHL. In the *Rf-2*, *Rf-4* and *Rf-5* regions only one SSLP in each region was different in FHL compared to FHH. Overall, 75% of the SSLPs are identical in FHL and FHH, while in the *Rf*-region identity increases to 86%.

Susceptibility to L-NAME and survival

Mean survival time of the *Rf-1A+2* double congenic rats on L-NAME in the 2K-situation was 18.7±1.7 weeks, i.e. after ~12 weeks of treatment. The mean survival time on L-NAME when combined with UNX was reduced to 14.0±4.0 weeks of age, i.e. after about 7 weeks of treatment.

Survival of animals in the 2K and UNX situation

All rats survived the follow-up period of the 2K-control situation. After UNX, all FHL and *Rf-1A+2* double congenic rats survived the entire follow-up period. Three out of nine FHH rats did not survive the entire follow-up period after UNX. Data obtained from these three rats are included in the results of the first and second measurements.

Albuminuria and systolic blood pressure

In the 2K-situation the *Rf-1A+2* double congenic rats develop slightly more UAV than the FH. However this is not statistically significant. The FHH rats develop considerably more UAV compared to both FHL and *Rf-1A+2* double congenics. Following UNX, the *Rf-1A+2* double congenic rats developed UAV than FHL rats. However this difference was again not statistically significant. The FHH rats develop a progressive increase in UAV values, being significantly different from both FHL and *Rf-1A+2* double congenics (Figure 2).

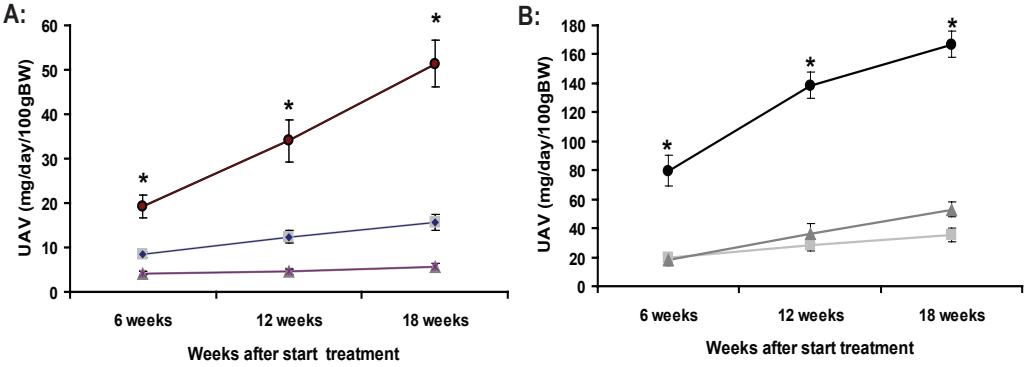

Figure 2. Albuminuria in FHH (●), FHL (▲) and *Rf-1A+2* (■) rats after 6, 12, and 18 weeks of follow-up during two treatments, values are given as mean ± SEM; **A**: 2K (2 kidneys); **B**: UNX, (unilateral nephrectomy); *: P<0.05 compared to *Rf-1A+2* and FHL.

The FHL rats showed to have a normal blood pressure of around 120-125 mm Hg, regardless of treatment or time. In the 2K-control situation and following UNX, SBP in FHH rats averaged at about 150-160 mm Hg. The SBP of *Rf-1A+2* double congenic rats was above that of FHL and below that of FHH, averaging at 140-145 mm Hg. With one exception, differences in SBP between FHL, FHH, and *Rf-1A+2* were statistically significant (Figure 3).

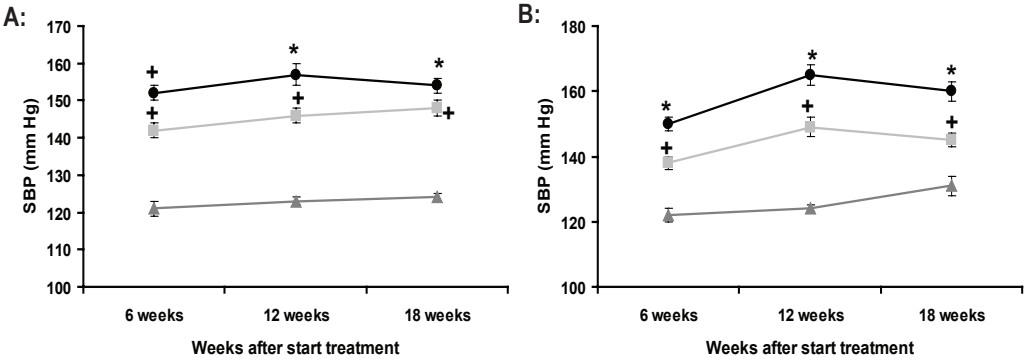

Figure 3. Systolic blood pressure (SBP) in FHH (●), FHL (▲) and *Rf-1A+2* (■) rats after 6, 12 and 18 weeks of follow-up during two treatments; values are given as mean ± SEM; **A**: 2K (2 kidneys); **B**: UNX, (unilateral nephrectomy); *: P<0.05 compared to *Rf-1A+2* and FHL; $^{+}$: P<0.05 compared to FHL.

To enable a comparison between the outcome of the present experiment with the UAV and SBP values of the ACI parental strain and *Rf-1A* single congenic strains data at 18 weeks of follow-up are presented in Table 2. The UAV and SBP values for ACI and UAV are derived from a previous study. The comparison shows that in the 2K situation and following UNX the UAV and SBP values of *Rf-1A+2* double congenics are significantly higher than those of ACI and *Rf-1A* single congenics rats.

Table 2: Comparison of UAV and SBP values at 18 weeks of follow-up.

	n	BW (gram)	UAV (mg/day/100 g BW)	SBP (mm Hg)
2K				
ACI	12	305±4	1.9±0.1	122±1
Rf-1A	12	329±7*	2.7±0.2	125±2
Rf-1A+2	11	301±4	15.7±2.5#	148±2#
ANOVA		0.005	<0.001	<0.001
UNX				
ACI	12	310±3	5.4±0.6	126±2
Rf-1A	11	325±6	16.5±2.0+	123±2
Rf-1A+2	12	299±7	35.5±4.8#	145±2#
ANOVA		0.041	<0.001	<0.001

Values are given as means ± SEM. 2K, 2 kidneys; UNX, unilateral nephrectomy; BW, body weight; UAV, urinary albumin excretion; SBP, systolic blood pressure; * P<0.05 compared to ACI, *Rf-1A+2*; # P<0.05 compared to ACI and *Rf-1A*; + P<0.05 compared to ACI.

Measurements at end of follow-up

Data obtained at the end of the follow-up are depicted in Table 3. The BW of the *Rf-1A+2* double congenics is significantly lower compared to FHH and FHL. After UNX, BW of FHH rats is also have a lower than that of FHL. The %FGS is significantly lower in FHL rats in the 2K-control situation compared to FHH and *Rf-1A+2* double congenic rats. Following UNX the %FGS in FHH significantly higher compared to FHL and *Rf-1A+2* double congenics rats. The %FGS in *Rf-1A+2* is significantly higher compared to FHL. Plasma albumin is significantly lower in FHH rats in both the 2K as the UNX situation. In the 2K-situation, creatinine clearance (Cc/100) in *Rf-1A+2* double congenics is significantly lower compared to FHH and FHL rats. In the UNX-situation, Cc/100 in *Rf-1A+2* double congenics is significantly lower compared to FHL rats

Table 3: Measurements at end of follow-up.

	n	BW (gram)	FGS (% glom.)	Cc/100 (ml/min/100gBW)	Palb (g/l)
2K					
Rf-1A+2	11	303±3	35±3	0.52±0.02	26.0±0.4
FHL	10	388±6*	11±1*	0.89±0.06*	29.6±0.8*
FHH	10	385±8*	26±2#	0.91±0.03*	24.5±0.4+
ANOVA		P<0.001	P<0.001	P<0.001	P<0.001
UNX					
Rf-1A+2	12	300±6	48±5	0.44±0.02	24.0±0.5
FHL	9	384±5*	30±3*	0.70±0.03*	26.6±0.6*
FHH	6	323±14+	95±3#	0.51±0.18	19.8±1.4#
ANOVA		P<0.001	P<0.001	P=0.034	P<0.001

Values are given as means ± SEM. 2K, 2 kidneys; UNX, unilateral nephrectomy; BW, body weight; FGS, incidence of glomerulosclerosis; Cc/100 creatinine clearance; Palb, plasma albumin level; *: P<0.05 compared to *Rf-1A+2*; +: P<0.05 compared to FHL; # P<0.05 compared to *Rf-1A+2* and FHL

Assessment of renal blood flow autoregulation

Renal autoregulation curves depicting the relative RBF against the RPP are presented in Figure 4. The RBF at an RPP of 150 mm Hg was increased by 25-27% compared to the RBF measured at 100 mm Hg. No significant differences were found between the *Rf-1A+2*, FHL, and FHH rats. The RAI values over the 100-150 mm Hg pressure range were 0.55±0.03 for FHH (n=8), 0.51±0.06 for FHL (n=8), and 0.49 ±0.06 for *Rf-1A+2* (n=8) congenic rats. There were no statistically significant differences in RAI values between the strains. The magnitude of the RAI values indicated that all three strains do have an impaired renal autoregulation.

In the *Rf-1A+2* double congenic and FHL rats used for the autoregulation experiment no marked renal damage was present. The average UAV was ~5-7 mg/day/100g BW, while the %FGS was in the order of 3-11%. The FHH rat used for the renal autoregulation did show some degree of renal damage, UAV ~29 mg/day/100g BW and ~14%FGS.

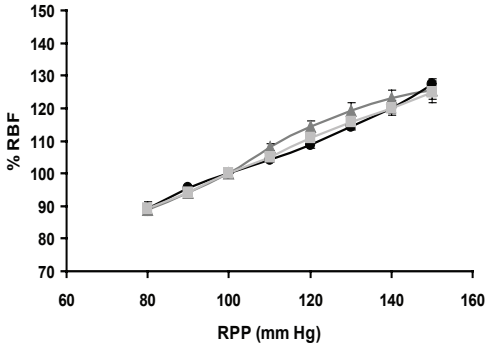

Figure 4: Relationship between percentage of renal blood flow and renal perfusion pressure in 13 to 15 week old FHH (●, n=8), FHL (▲, n=8) and *Rf-1A+2* (■, n=8); Values (as %RBF at RPP of 100 mm Hg) are given as means ± SEM (error bars); %RBF, relative renal blood flow; RPP, renal perfusion; no significant differences were present.

Discussion

The present studies shows that *Rf-1A+2* double congenics rats show an increased susceptibility to renal damage and a higher level of SBP in comparison with ACI and *Rf-1A* single congenic rats. This indicates that the additional presence of the *Rf-2* and *Bpfh1* QTL from FHH on top of the *Rf-1* QTL affects both renal susceptibility and SBP. However, compared to the FHH parental strain renal susceptibility and SBP are less *Rf-1+2* double congenics. Compared to FHL rats, that share about 75% of the FHH genome, SBP is higher in the double congenics. Remarkably, UAV levels in *Rf-1A+2* double congenic rats are higher than FHL in the 2K-situation, but lower following UNX. Similar to FHH, RBF autoregulation was impaired in *Rf-1A+2* double congenic and FHL rats.

In contrast to our previous studies in *Rf-1A+3* and *Rf-1A+4* double congenic rats, renal susceptibility could not be tested in *Rf-1A+2* double congenics following L-NAME-induced hypertension. All *Rf-1A+2* rats died prematurely during L-NAME treatment. This was not a complete surprise, as we previously reported high mortality rates in L-NAME treated FHH and FHL rats.[237,275,276] Such a high early mortality during L-NAME treatment was not present in *Rf-1*, *Rf-3*, *Rf-4*, and *Rf-5* single congenics or *Rf-1+3* and *Rf-1+4* double congenic rats.[199,267,269,270] It is highly likely that the high mortality during L-NAME is due to the presence of the *Rf-2* QTL, probably related to bleeding disorder gene located in that region.[50] The bleeding disorder found in FH rats resembles the platelet-storage pool deficiency in humans.[50,204] The gene responsible for the bleeding disorder is also responsible for the coat color dilution in FH rats.[197] This gene was recently reported to be the *Rab38* gene.[187] *Rab38* is a member of the Rab family of small GTPases that regulate intracellular vesicle formation and trafficking.[192] In addition, *Rab38* is a strong candidate for the *Rf-2* gene affecting proteinuria and albuminuria in the FHH rat.[204]

Interestingly, SBP in intact *Rf-1A+2* double congenics is about 15-20 mm Hg higher compared to FHL but still about 10-15 mm Hg below that of FHH. The effect on SBP is most likely due to the simultaneous introgression of the *Bpfh1* QTL that is embedded in the *Rf-2* QTL. The SBP level in the *Rf-1A+2* double congenics equals that of rats homozygous for *Bpfh1* or *Rf-2* in our original linkage analysis of an FHHx(ACIxFHH)F1 backcross.[25] The lower SBP in FHL might indicate that *Bpfh1*, present in FHH and *Rf-1A+2* double congenics, is lacking in FHL. Comparing the genotypes of FHH and FHL indicated that all 5 SSLP markers in the *Rf-1* and 4 out of 5 markers in the *Rf-2* region were identical in the two

strains. However, one marker in the *Rf-2* and *Bpfh-1* region was different between FHL and FHH, i.e. *D1Mit2*. Extensive genotyping of the region surrounding this marker should give more detailed information about the genetic differences between FHH and FHL and be helpful to find the gene(s) responsible for the differences in SBP between these strains. Our present findings support the supposition that *Rf-2* influencing renal damage and *Bpfh-1* influencing SBP are separate genes, a distinction that could not be definitively made in our previous linkage analyses.[25,234] Studies in different rat strains support our findings. Crosses of WKY with SHRSP, BN/SsNHsd with SS/JrHsdMcwi, and SBH/Ygl with SBN/Ygl all had a blood pressure QTL with a peak at marker *D1Mit2*.[73,156,176,216,249] This indicates that most likely the region around *D1Mit2* on rat chromosome 1 carries a gene that influences blood pressure. Further research should be conducted to reveal the actual gene.

Autoregulation of the RBF is influenced by the *Rf-1* QTL. We previously reported that RBF autoregulation in *Rf-1* carrying single congenic rats is impaired to the same extent as in FHH parental rats.[267,270] Our present findings show no differences in RBF autoregulation between FHH, FHL, and *Rf-1A+2* double congenics, indicating that the functional aspects of *Rf-1* are also present in FHL. These findings together with the equal genotype of 5 SSLP markers in FHL and FHH made us conclude that *Rf-1* is also present in FHL.

Comparing the differences in renal susceptibility between *Rf-1A+2* double congenics, FHH and FHL shows that the FHH rats are the most susceptible strain. The difference between the *Rf-1A+2* double congenics and FHH may be partly due to the difference in SBP. However, it is more likely that it due to the absence of the *Rf-3*, *Rf-4*, and *Rf-5* QTLs that are expected to further increase renal susceptibility in the FHH strain. Additional studies in multiple congenic strains are needed to provide evidence for this assumption. A comparison between the *Rf-1A+2* double congenics and FHL rats is complicated. In the 2K-situation the level of UAV and FGS is higher in the double congenic. This may be due to the difference in SBP that in the presence of an impaired renal autoregulation would result in a higher intra-glomerular pressure and eventually more renal damage. On the other hand, based on the genotype comparison between FHL and FHH it is likely that FHL also carries the *Rf-3* QTL from FHH, as no genotype differences in the *Rf-3* region were present between both strains. Since genotype differences were present between FHL and FHH in the *Rf-4* and *Rf-5* regions we cannot be certain about the presence of the *Rf-4* or *Rf-5* QTL in FHL. However, the additional presence of *Rf-3* in the FHL rat is likely to augment renal susceptibility. An augmented UAV response is seen in FHL following UNX. Although SBP is lower than that of the *Rf-1A+2* double congenics the level of UAV is higher. Strangely enough this is not mirrored by a similar difference in FGS. To the contrary, the incidence of FGS in the *Rf-1A+2* double congenics is significantly higher than that of FHL. This points to a discrepancy between the severity of the functional damage (albuminuria) and structural damage (glomerulosclerosis). Further studies will be needed to explain such a discrepancy.

The combination of the *Rf-1* and *Rf-2* QTL is the third combination that results in an augmentation in renal susceptibility when compared with *Rf-1* single congenic rats. Previously we reported that combining *Rf-1* with *Rf-3* or *Rf-4* also increases renal susceptibility. Remarkably, the levels of renal damage seen in the three combinations are of the same order of magnitude.[269,270] In contrast, renal susceptibility the combination of *Rf-1* and *Rf-5* did not differ from that of *Rf-1* single congenics, indicating that the contribution of *Rf-5* in determining the renal susceptibility in FHH is probably small.[266] Future studies, will involve multiple

congenic strains carrying three or more of the *Rf*-QTL, in order to reconstruct the full renal susceptibility of the parental FHH strain.

Acknowledgements

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Chapter 8

Efficacy of the rat remnant kidney model to assess differences in renal susceptibility

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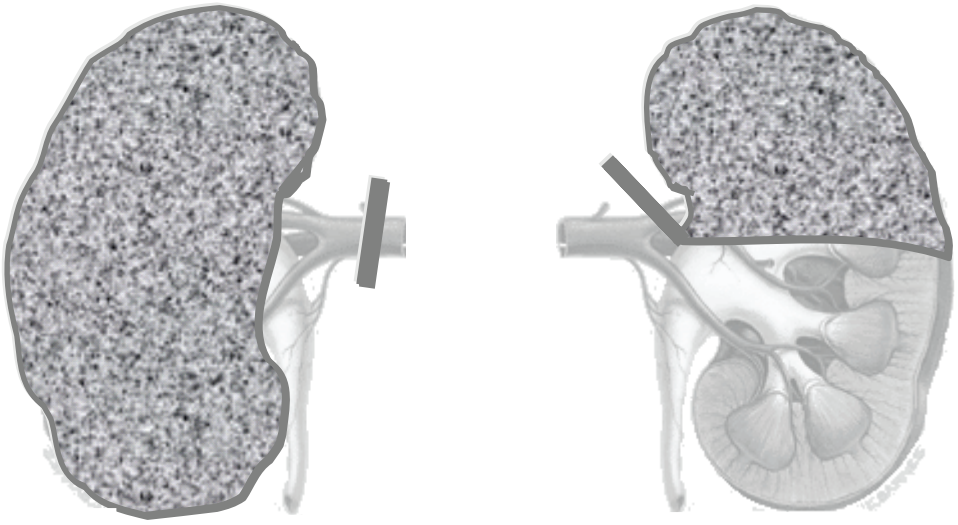

Abstract

Renal damage susceptibility varies among inbred rat strains. The FHH (Fawn-Hooded Hypertensive) is a well-characterized inbred rat strain, which develops hypertension and end-stage renal failure at a relatively young age. Linkage studies in crosses of FHH and renal-resistant ACI (August x Copenhagen Irish) revealed five QTLs linked to proteinuria (UPV), named *Renal failure-1* (*Rf-1*) through *Rf-5*.

In previous studies we determined renal damage susceptibility in FHL, FHH, ACI rats, and ACI. FHH-Rf congenics using unilateral nephrectomy (UNX) and L-NAME induced hypertension. Our goal was to determine if remnant kidney model (RKM) is a suitable model to test renal susceptibility in ACI, FHH, FHL and ACI.FHH-*Rf* congenic rats.

Three experiments were performed. Firstly, FHH and FHL were compared to ACI. In the second set-up, *Rf-1B* and *Rf-5* single congenics were compared to ACI rats. In the third set-up, *Rf-1A* and *Rf-4* single and *Rf-1A+4* double congenics were compared with ACI. At the age of 6 weeks, RKM started. After three and six weeks proteinuria levels (UPV) and systolic blood pressure (SBP) were measured.

In the first set-up, none of the FHH survived for to the first evaluation. FHL developed severe renal damage. In the second set-up, *Rf-1B* developed significantly more UPV than ACI and *Rf-5*, although both ACI and *Rf-5* already developed considerable renal damage. The *Rf-1A+4* developed significantly more UPV compared to only ACI and *Rf-4* rats.

In conclusion, our findings question the suitability of the RKM as a model to rapidly assess differences in susceptibility to develop renal damage. Although it could still be useful in strains that markedly differ in susceptibility, its value to detect differences between congenic rat strains was poor. The UNX model either alone or in combination with L-NAME induced hypertension, despite the longer duration, appears better suitable.

Introduction

The incidence and progression of kidney damage, eventually resulting in end-stage renal failure (ESRF) forms an increasing health care burden.[103,160,244,263,285] To reveal pathophysiological mechanisms behind the development of ESRF, experimental research using various animal models may be helpful.[25,84,175,195,224,225,234,235] Susceptibility to develop progressive renal damage varies among inbred rat strains. This can be used to identify genes involved in renal damage susceptibility via linkage analyses. Through rat-human homology this may lead to the identification of genes involved in human forms of ESRF.[11,78,220,226]

There are several ways to assess susceptibility to renal damage in rat. Differences can be spontaneously present, when comparing strains of inbred rats.[10,20,66,95,99,215,238,245,283] In addition, renal mass reduction, hypertension and diabetes, well-known risk factors to develop renal damage in humans, have been used to induce or accelerate chronic renal damage in rats. Unilateral and subtotal nephrectomy are methods to reduce renal mass.[16,97,130,239,291] Hypertension and diabetes may occur spontaneously or can be induced chemically or pharmacologically.[119,275,276] We previously applied four treatments to detect differences in renal susceptibility between various rat strains.[267,270,275,276] Next to the two-kidney (2K) control situation, they included 2K rats with L-NAME-induced hypertension, renal mass reduction by unilateral nephrectomy (UNX), and the combination of UNX and L-NAME induced hypertension. Recently, these models have been successfully applied to detect differences in renal susceptibility between the relatively resistant (August x Copenhagen Irish hooded) ACI rat and various ACI.FHH-*Rf* congenic rats.[267,270] However, it did take a considerable time to detect significant differences between these strains, markedly slowing the research progress.[199,275,276,274,267,270]

The remnant kidney model (RKM), produced by the removal of over 75% of the functional renal mass, is a frequently used model to induce chronic renal failure in rats.[16,97,130] The RKM has been generated by two means, i.e. ligation of renal artery branches or resection of the upper and lower kidney poles.[32,68,173] In contrast to the resection model, the ligation model also induces a marked increase in systemic blood pressure[31,93,118], thus combining renal mass reduction and hypertension.

The aim of our present studies was to test the efficacy of the RKM to more rapidly detect differences in renal susceptibility between inbred strains. We therefore mimicked previous experiments comparing the effect of the RKM in ACI and other rat strains that were previously shown to differ or not to differ in their susceptibility to develop renal damage.[199,267,270,275,276]

Methods

Animals

For the different experiments we used 11 ACI, 7 FHH (Fawn Hooded Hypertensive), 12 FHL (Fawn Hooded Low blood pressure), 13 ACI.FHH-(*D1Rat384-D1Rat156*) (*Rf-1B*) congenics, 11 ACI.FHH-(*D17Rat112-D17Arb5/D17Rat180-D17Rat51*) (*Rf-5*) congenics, 12 ACI.FHH-(*D1Rat298-D1Rat90*) (*Rf-1A*) congenics, 11 ACI.FHH-(*D14Mit11-D14Rat82*) (*Rf-4*) congenic, and 11 ACI.FHH-(*D1Mit18-D1Rat90*)/(*D14Mit11-D14Rat33/D14Rat65-D14Rat90*)(*Rf-1A+4*) double congenic rats. Congenic rat strains were generated using a speed congenic strategy, as described for the *Rf-1B* strain by Provoost et al.[199] The

congenic regions introgressed from the FHH into the ACI genomic background in the various ACI.FHH-*Rf* congenic rats have been previously described.[267,270]

All breeding was performed at the Animal Research Centre at the Erasmus MC, Rotterdam, the Netherlands. Animals were housed in micro-isolators under SPF-conditions.[180] Lights were on from 7.00 a.m. to 7.00 p.m. Standard commercial rat chow containing 44% carbohydrates, 29% digestible animal protein, 7% fat, 4% fibre and 8% minerals (SRM-A; Hope Farms, Woerden, the Netherlands) and drinking fluid (tap water, acidified to pH 2.4-2.8) were provided ad libitum. The protocol received approval from the animal ethical committee of Erasmus MC. It was anticipated that in some strains the RKM could induce severe distress demanding to end the experimental procedures. Criteria to remove animals from the experiment were severe weight loss (>15% within a week), paralysis or the presence of glaucoma.

Experimental set-up

Three previously published experimental set-ups were mimicked. In the first, we compared the ACI, the FHL, and FHH strains known to markedly differ in renal damage susceptibility.[275,276] In the second, we compared ACI controls and *Rf-1B* and *Rf-5* single congenic rats. We previously showed that renal susceptibility was somewhat increased in *Rf-1B* congenics rats following UNX+L-NAME treatment, while *Rf-5* congenics did not differ from ACI.[199,267] In the third experiment we compared ACI controls with *Rf-1A*, *Rf-4* single congenics and *Rf-1A+4* double congenic rats. We previously showed that renal susceptibility was somewhat increased in *Rf-1A* congenics rats while *Rf-4* congenics did not differ from ACI. In addition renal susceptibility was markedly enhanced in *Rf-1A+4* double congenic rats indicating a synergistic gene-gene-interaction between *Rf-1* and *Rf-4*.[270]

Surgical procedure

For the surgical procedure, rats were anaesthetized with a mixture of 3% isoflurane®, about 30% N_2O, and about 60% O_2, and were placed on a heated surgical table to maintain the body temperature at approximately 36ºC. The RKM was produced by total removal of the right kidney and ligaturing two branches of the left renal artery with a silk suture (8.0, Braun AG, Melsungen, Germany). The wound was closed using a dissolvable suture (4.0, Braun AG, Melsungen, Germany). The rats were allowed to recover from surgery in a warmed cage for half an hour.

Urine collection, proteinuria and systolic blood pressure measurement

Urine of individual rats was collected at 3 and 6 weeks after surgery to determine the level of UPV. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during two consecutive days after a three-day adaptation period. Total urinary protein was determined using an ELAN system (Eppendo*Rf*-Merck, Hamburg, Germany) with the with pyrogallol red-molybdate complex colorimetric method.[286]

Following the urine collection, systolic blood pressure (SBP) was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA) in awake, restrained, but trained rats, as described previously.[275,276]

Statistics

Data are presented as mean ± SEM. Statistical differences in mean values between ACI and FHL were compared by Students-t-test. Statistical differences in mean values between rat strains within the two other experimental set-ups were compared using one-way analysis of variance (ANOVA). The ANOVA test was followed by the Bonferroni test to determine whether strains were significantly different from each other. In all tests, a p-value <0.05 was considered statistically significant. All tests were peRformed using the Primer of Biostatistics for Windows program (Version 4.0, McGraw Hill, 1996).

Results

Experiment 1

Survival

All seven FHH rats died prior to the first evaluation. Mean survival time was only 10±1 days after inducing the RKM. Out of the 12 FHL rats, ten could be measured at the first and nine at the second evaluation. All 11 ACI rats did not survive the complete 6-week follow-up period.

Proteinuria and systolic blood pressure

Values for UPV and SBP during follow-up are presented in Figure 1. Because of differences in BW between strains, UPV values are presented as mg/day/100g BW. Both at 3 and 6 weeks, UPV and SBP values were significantly higher in FHL compared to ACI.

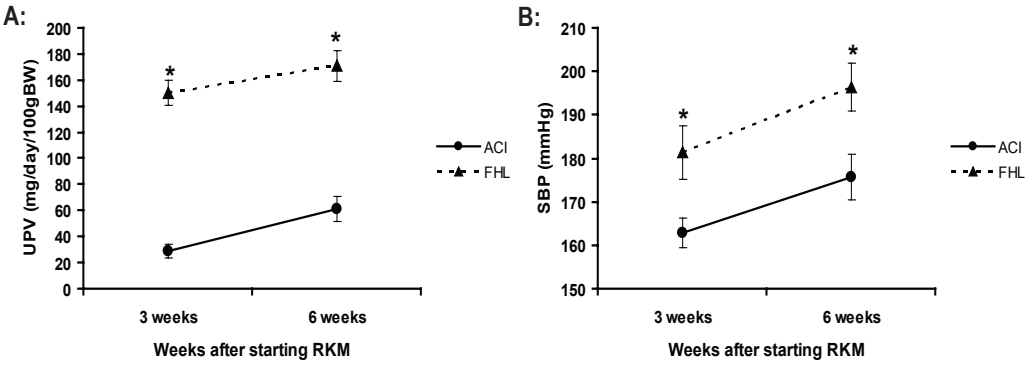

Figure 1: Proteinuria (UPV) and systolic blood pressure (SBP) after 3 and 6 weeks of follow-up in ACI and FHL rats. Values (mg/day per 100 g BW) are given as mean ± SEM, number of rats is given in Table 1. **A**: UPV **B**: SBP; *: P<0.05 compared to ACI.

Experiment 2

Survival, proteinuria and systolic blood pressure

All ACI, *Rf-1B*, and *Rf-5* single congenics survived the entire follow-up period. Values for UPV and SBP during follow-up of all the rats are presented in Figure 2. At 3 weeks mean UPV level in *Rf-1B* congenic rats was significantly higher compared to ACI rats, while at 6 weeks mean UPV level in *Rf-1B* was higher compared to both ACI and *Rf-5* single congenic rats. No significant differences in mean UPV level were noted between ACI and *Rf-5* single congenic rats. No significant differences in mean SBP level were noted between the three strains.

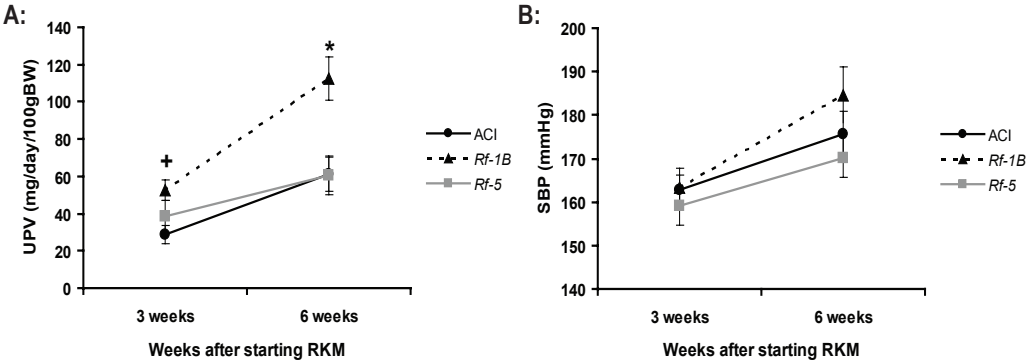

Figure 2: Proteinuria (UPV) and systolic blood pressure (SBP) after 3 and 6 weeks of follow-up in ACI, *Rf-1B* and *Rf-5* rats. Values (mg/day per 100 g BW) are given as mean ± SEM, number of rats is given in Table 1. **A**: UPV **B**: SBP; *: P<0.05 compared to ACI and *Rf-5*; $^{+}$: P<0.05 compared to ACI.

Experiment 3

Survival, proteinuria and systolic blood pressure

All *Rf-1A*, and *Rf-4* single and *Rf-1A+4* double congenics survived the entire follow-up period. Values for UPV and SBP during follow-up are presented in Figure 3. At 3 weeks mean UPV level was significantly higher in *Rf-1A+4* double congenics compared to ACI rats, while at 6 weeks mean UPV level in *Rf-1A+4* double congenics was higher compared to both ACI and *Rf-4* single congenic rats. No significant differences in mean UPV level were noted between *Rf-1A+4* double and *Rf-1A* single congenics and between ACI and *Rf-4* single congenic rats. No significant differences in mean SBP level were noted between the four strains.

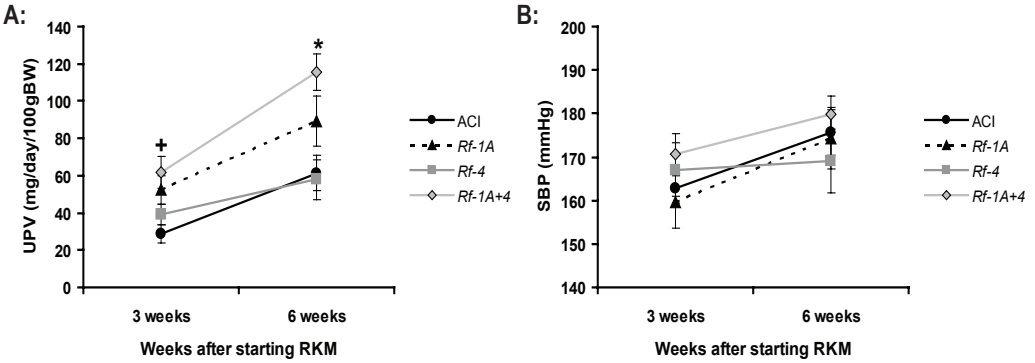

Figure 3: Proteinuria (UPV) and systolic blood pressure (SBP) after 3 and 6 weeks of follow-up in ACI, *Rf-1A*, *Rf-4* and *Rf-1A+4* rats. Values (mg/day per 100 g BW) are given as mean ± SEM, number of rats is given in Table 1. **A**: UPV **B**: SBP; *: P<0.05 compared to ACI and *Rf-4*; +: P<0.05 compared to ACI.

Discussion

This study shows that the RKM is extremely powerful in inducing chronic renal damage. Even the ACI rat, shown to be relatively resistant to other procedures [199,267,270,275,276], rapidly develops severe renal damage. The severity of the renal damage observed in ACI will influence the ability to detect differences in renal susceptibility in other strains. Besides, we observed a large variability in the degree of renal damage induced by the RKM. Comparing the outcome of the present experiments with that of our previous studies shows that some findings, but not all, could be reproduced by the RKM. This makes the RKM not better suitable than the ones we previously applied to detect differences in renal susceptibility between inbred rat strains.

In the first experiment, we confirmed the high renal susceptibility of the FHH and FHL strains. Mean survival time after surgery of the FHH rats was only 10 days. The FHL strain is less susceptible than FHH and the majority of the FHL rats did survive the complete follow-up period. However, severe renal damage developed in this very short period. These findings are in line with previous studies looking at the effects of UNX and UNX+L-NAME treatment in FHH and FHL rats, where severe renal damage rapidly developed. Therefore, RKM is suitable to assess differences in renal susceptibility between the ACI and the two FH rat strains.

In the second, a study was mimicked that had shown that *Rf-1B* single congenics do develop more renal damage than ACI rats, especially when haemodynamic stress is put upon the kidney by the combination of UNX and L-NAME induced hypertension. In contrast, renal damage in *Rf-5* single congenics following this procedure did not differ from that of ACI parental rats.[267] Similar differences were observed in the present study, employing the RKM. Significantly more renal damage was found in *Rf-1B* single congenic rats when compared to ACI rats. In contrast, the renal damage in *Rf-5* single congenics did not differ from that in ACI rats. The level of SBP was not significantly different between the three strains. Thus, differences in renal damage cannot be explained by differences in systemic blood pressure. The absence of any difference in SBP and renal damage between ACI and *Rf-5* single congenic rats also indicates that the introgressed FHH congenic regions in the *Rf-5* congenic strain apparently do not contain genes directly

influencing SBP or renal damage susceptibility. Taken together, the RKM appears suitable to rapidly assess differences in renal susceptibility between single congenic rat strains.

In the third experiment, another study was mimicked that had revealed the presence of a synergistic interaction between *Rf-1A* and *Rf-4* markedly increasing the susceptibility to develop renal damage. The presence of a synergistic interaction could not be confirmed in the RKM. Although a similar trend was present, i.e. the highest level of renal damage in *Rf-1A+4* double congenics, followed by *Rf-1A* single congenics. In contrast, renal damage in *Rf-4* damage did not differ from that of ACI parental rats. The level of SBP was not significantly different between the four strains. Thus, differences in renal damage cannot be explained by differences in systemic blood pressure. Similar to the findings in *Rf-5* single congenics, the absence of any difference in SBP and renal damage between ACI and *Rf-4* single congenic rats indicates that the introgressed FHH congenic regions in the *Rf-4* congenic strain apparently do not contain genes directly influencing SBP or renal damage susceptibility. In contrast to our previous findings, the presence of a synergistic interaction between *Rf-1A* and *Rf-4* was not detected with the RKM. At 6 weeks, renal damage in *Rf-1A+4* double congenics was significantly different from that of ACI and *Rf-4* single congenics, but not from that of *Rf-1A* single congenics. Furthermore, in the present set-up no significance difference in renal damage was found between *Rf-1A* and *Rf-4* single congenics, thus failing to confirm our previous findings in less severe models of chronic renal damage.[270] To our opinion, the failure to detect the earlier found interaction between *Rf-1A* and *Rf-4* can be explained by large variation in UPV value within each rat strain, weakening the power of the statistical analysis. From this experiment we may conclude that the RKM is not suitable to rapidly assess differences in renal susceptibility between single and double congenic rat strains and to reveal the presence of synergistic interactions between congenic regions.

The RKM has been an important, widely used model to study the mechanisms of progressive renal damage[110,148,182], the consequences of chronic renal failure[16,96], as well as the effects of pharmacologic interventions to prevent or treat the development of chronic renal damage.[41,153,162] To detect differences in susceptibility between strains, the RKM has major disadvantages compared to the previously studied UNX or UNX+L-NAME models. The main disadvantage is the poor reproducibility of the model. In contrast to the UNX where 50% of the renal mass is reduced, the amount of renal mass reduction in the RKM is quite variable. This is mainly due to differences in the anatomy of the renal blood supply between individual rats. Tying of two or three branches of the renal artery, will infarct different amounts of renal tissue. While this is anticipated, it is hard to reliably estimate how much renal tissue is actually excluded. One can look at the colour change of the kidney surface, but it could well be that the surface has a good blood supply while the inner part is infarcted. Therefore, a visual estimation of the renal mass reduction could be entirely wrong. A second disadvantage is the poor control of the systemic blood pressure. The RKM rats uniformly develop marked hypertension shortly after surgery. However, large individual differences were present. These differences may, at least partly, be related to differences in the amount of infracted renal tissue. Previous studies have shown that an increase in blood pressure often occurs in RKM with branch ligation, but is less seen with the pole resection method.[92] The consequence of the poor reproducibility of RKM is the large variability of the degree of renal damage induced within each strain, resulting in large standard deviations, markedly reducing the chances of finding statistically differences between rat strains.

In conclusion, our findings question the suitability of the RKM as a model to rapidly assess differences in susceptibility to develop renal damage. Although it could still be useful in strains that markedly differ in susceptibility, its value to detect differences between congenic rat strains was poor. The UNX model either alone or in combination with L-NAME induced hypertension, despite the longer duration, appears better suitable.

Acknowledgements

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Chapter 9

General Discussion

Perspectives

Conclusions

9. General discussion

Human chronic kidney disease (CKD) is an international health care burden.[263] Several studies indicate that CKD in humans is a polygenic disease.[292,129] Experimental studies in rats and mice may be helpful to unravel the complex genetics of CKD.[17,25,69,82,84,88,100,109,130,152,172,175,185,195,224,225,234,235,249,307] Linkage analysis of a cross of ACI and FHH rats revealed five QTLs linked to renal damage, named *Renal failure-1* (*Rf-1*) through *Rf-5*. The *Rf-1* QTL had by far the highest LOD-score and was our main interest.[25,234] We have studied the role of the *Rf-1* to *Rf-5* QTLs in congenic rat strains. Congenic strains are useful for studying the effects of specific genes or genomic regions against a common inbred background. The use of congenic strains has evolved during the past decade as the major working algorithm used by researchers in their attempts to narrow down the span of QTLs.[136,300] Several congenic rats were generated carrying one or two *Rf*-regions of the FHH rats onto an ACI genomic background.[271] The experiments described in this thesis study the susceptibility to the development of renal damage in ACI.FHH-*Rf* single and double congenic rats.

Previous studies have shown that the FHH rat has an impaired renal autoregulation. An impaired renal autoregulation is thought to contribute to the susceptibility to renal damage in FHH rats. The impaired renal autoregulation allows the increased systemic pressure present in FHH rats to enter the glomeruli, which increases hemodynamic stress upon the glomeruli. Therefore, renal autoregulation has also been tested in the ACI.FHH-*Rf* congenic rats.

In the general discussion the major findings presented in this thesis will be reviewed. In addition, an attempt is made to translate the experimental findings to the human situation, and perspectives are given to further unravel the genetic mechanism behind the development of renal damage. This chapter ends with the overall conclusion derived from the different studies.

9.1 Difference in susceptibility to develop renal damage

For the various experiments presented in this thesis renal susceptibility has been tested in 13 different rat strains. To allow for a comparison between these strains, indices of renal damage obtained after 18 weeks of treatment are summarized in Table 1-4. Table 1 and 2 summarize the findings of renal damage indices in ACI, five ACI.FHH-*Rf* single congenics, five ACI.FHH-*Rf* double congenics, as well as FHL and FHH rats in 2K controls and following UNX. Table 3 and 4 summarize the renal damage indices in ACI and ACI.FHH-*Rf* single congenics rats following 2K+L-NAME or UNX+L-NAME treatment.

Table 1: Renal damage indices of two-kidney (2K) ACI, FHH, FHL, and ACI.FHH single and double congenics at 18 weeks of treatment.

2K	n	BW (g)	UPV (mg/day/ 100gBW)	UAV (mg/ day/ 100gBW)	SBP (mm Hg)	TKW (g/ 100gBW)	FGS (%)	Cc/100 (ml/min/ 100gBW)
ACI	30	307±3	5.2±0.2	2.1±0.1	122±1	0.78±0.00	8±1	0.56±0.03
Rf-1A	21	328±5	5.8±0.3	2.9±0.2	125±2	0.73±0.01	14±1	0.51±0.03
Rf-1B	21	310±4	7.1±0.7	4.0±0.6	124±2	0.74±0.00	13±1	0.62±0.03
Rf-3	9	311±3	6.4±0.2	2.3±0.1	125±1	0.74±0.01	13±2	0.72±0.04
Rf-4	9	317±6	4.0±0.4	1.9±0.2	124±3	0.74±0.01	8±1	0.66±0.05
Rf-5	7	273±8	4.6±0.5	0.9±0.2	126±3	0.71±0.01	2±1	0.57±0.05
Rf-1A+2	11	301±4	23.2±2.5	15.7±1.8	148±2	0.78±0.01	35±3	0.52±0.02
Rf-1A+3	12	304±4	13.3±0.9	7.2±0.8	126±2	0.75±0.01	14±2	0.56±0.02
Rf-1A+4	12	322±6	6.5±0.7	4.4±0.6	124±1	0.72±0.01	22±2	0.44±0.03
Rf-1B+4	12	303±3	9.1±1.1	5.5±0.9	125±2	0.71±0.01	21±3	0.52±0.04
Rf-1B+5	8	304±5	6.0±0.6	2.4±0.3	124±2	0.71±0.01	8±2	0.54±0.03
FHH	24	389±4	84.4±5.7	73.6±6.3	158±2	0.85±0.02	35±4	0.73±0.05
FHL	18	396±4	12.4±0.7	6.4±0.6	122±1	0.75±0.01	9±1	0.69±0.06

Values are given as mean±SEM; *Rf*, congenic strain; ACI, August x Copenhagen Irish; FHH, Fawn Hooded Hypertensive; FHL, Fawn Hooded Low blood pressure; n, number of rats used; BW, body weight; UPV, proteinuria; UAV, albuminuria; SBP, systolic blood pressure; TKW, total kidney weight; FGS, focal glomerulosclerosis; Cc/100, creatinine clearance.

Table 2: Renal damage indices of unilateral nephrectomized (UNX) ACI, FHH, FHL, and ACI.FHH single and double congenics at 18 weeks of treatment.

UNX	n	BW (g)	UPV (mg/day/ 100gBW)	UAV (mg/day/ 100gBW)	SBP (mm Hg)	TKW (g/ 100gBW)	FGS (%)	Cc/100 (ml/min/ 100gBW)
ACI	32	306±3	9.2±0.7	5.0±0.6	125±1	0.54±0.01	13±2	0.50±0.02
Rf-1A	23	321±4	21.8±2.5	16.8±2.3	124±1	0.57±0.01	26±3	0.46±0.01
Rf-1B	14	301±7	12.7±2.0	9.3±1.9	122±1	0.53±0.01	12±2	0.54±0.04
Rf-3	9	302±4	12.9±0.9	6.0±0.7	123±1	0.58±0.01	20±2	0.58±0.02
Rf-4	10	294±3	6.1±0.5	2.6±0.3	127±2	0.54±0.02	6±2	0.56±0.03
Rf-5	10	290±4	3.9±0.4	1.4±0.2	121±2	0.51±0.01	2±1	0.50±0.04
Rf-1A+2	12	299±7	45.1±5.3	35.5±4.8	145±2	0.64±0.02	48±5	0.44±0.02
Rf-1A+3	11	295±5	40.7±3.9	31.5±6.0	132±2	0.69±0.02	27±3	0.58±0.02
Rf-1A+4	12	328±5	31.2±2.2	25.9±2.0	128±2	0.58±0.01	38±5	0.45±0.02
Rf-1B+4	12	294±9	37.0±6.3	26.7±5.1	126±2	0.58±0.01	30±3	0.50±0.03
Rf-1B+5	7	295±2	9.3±1.0	4.5±0.5	121±1	0.53±0.01	12±2	0.50±0.07
FHH	6	346±13	201.7±19.4	166.8±9.2	160±3	0.84±0.04	95±3	0.51±0.18
FHL	9	387±6	71.2±6.3	52.8±5.2	131±3	0.59±0.01	30±3	0.70±0.03

Values are given as mean±SEM; *Rf*, congenic strain; ACI, August x Copenhagen Irish; FHH, Fawn Hooded Hypertensive; FHL, Fawn Hooded Low blood pressure; n, number of rats used; BW, body weight; UPV, proteinuria; UAV, albuminuria; SBP, systolic blood pressure; TKW, total kidney weight; FGS, focal glomerulosclerosis; Cc/100, creatinine clearance.

Table 3: Renal damage indices of 2K+L-NAME induced hypertension ACI and ACI.FHH single and double congenics at 18 weeks of treatment.

2K+L	n	BW (g)	UPV (mg/day/ 100gBW)	UAV (mg/day/ 100gBW)	SBP (mm Hg)	TKW (g/ 100gBW)	FGS (%)	Cc/100 (ml/min/ 100gBW)
ACI	32	301±5	7.0±0.3	3.4±0.2	164±3	0.70±0.00	11±1	0.59±0.03
Rf-1A	21	320±3	14.2±2.0	10.2±1.8	175±3	0.73±0.00	24±2	0.50±0.02
Rf-1B	11	292±4	23.2±3.9	17.2±3.5	177±5	0.72±0.01	20±3	0.52±0.04
Rf-3	10	317±3	17.2±3.5	7.5±2.9	167±5	0.75±0.01	18±2	0.81±0.03
Rf-4	9	310±3	7.3±1.1	3.3±0.9	165±5	0.68±0.01	6±1	0.62±0.03
Rf-5	9	288±9	5.2±1.0	2.7±0.8	176±4	0.69±0.01	5±2	0.49±0.04
Rf-1A+3	12	289±6	47.4±8.8	34.9±7.0	186±5	0.86±0.03	31±6	0.52±0.04
Rf-1A+4	10	274±6	57.3±5.7	50.0±5.8	186±5	0.78±0.01	39±5	0.34±0.03
Rf-1B+4	12	283±9	59.0±7.9	42.5±5.7	187±4	0.76±0.01	34±3	0.44±0.04
Rf-1B+5	9	295±6	12.9±1.6	7.5±1.3	180±7	0.71±0.01	15±3	0.53±0.03

Values are given as mean±SEM; *Rf*, congenic strain; ACI, August x Copenhagen Irish; n, number of rats used; BW, body weight; UPV, proteinuria; UAV, albuminuria; SBP, systolic blood pressure; TKW, total kidney weight; FGS, focal glomerulosclerosis; Cc/100, creatinine clearance.

Table 4: Renal damage indices of UNX+L-NAME induced hypertension ACI and ACI.FHH single congenics at 18 weeks of treatment.

UNX+L	n	BW (g)	UPV (mg/day/ 100gBW)	UAV (mg/day/ 100gBW)	SBP (mm Hg)	TKW (g/ 100gBW)	FGS (%)	Cc/100 (ml/min/ 100gBW)
ACI	35	300±4	18.0±1.8	12.4±1.4	169±3	0.55±0.01	17±1	0.50±0.02
Rf-1A	20	316±5	53.9±4.6	45.4±3.9	180±5	0.60±0.01	43±3	0.38±0.02
Rf-1B	12	269±9	56.6±6.8	48.3±6.2	171±3	0.63±0.02	39±5	0.46±0.05
Rf-3	11	298±6	36.3±3.8	25.3±3.5	170±5	0.63±0.01	39±5	0.63±0.04
Rf-4	10	279±6	23.8±4.9	20.0±5.2	182±5	0.57±0.03	16±5	0.51±0.02
Rf-5	9	285±7	12.1±3.1	8.5±3.3	179±7	0.50±0.01	8±2	0.46±0.04

Values are given as mean±SEM; *Rf*, congenic strain; ACI, August x Copenhagen Irish; n, number of rats used; BW, body weight; UPV, proteinuria; UAV, albuminuria; SBP, systolic blood pressure; TKW, total kidney weight; FGS, focal glomerulosclerosis; Cc/100, creatinine clearance.

9.1.1 Differences in renal susceptibility between ACI, FHL, and FHH

The FHH rat is a well-characterized model of hypertension associated renal damage and together with the renal resistant ACI strain they form the foundation of the present studies. The FHL rat, like the FHH generated during the inbreeding process from a random bred FH strain, was selected for its normal blood pressure and lower level of UPV. In the 2K-control situation, the FHH develops mild hypertension of about 160 mmHg and significant amounts of UPV and UAV. Glomerulosclerosis is present, but still mild. Despite a similar level of SBP, UPV and UAV are higher in FHL compared to ACI rats, demonstrating that the FHL strain is more susceptible to renal damage than the ACI strain. Interestingly, compared to ACI both FHL and FHH show glomerular hyperfiltration indicated by the increased creatinine clearance.

When hemodynamic stress upon the kidney is increased by reducing renal mass, a marked further increase in UPV, UAV and FGS is seen in FHH. The FHL strain too shows marked increases in renal damage following UNX. In contrast, only a small increase in UPV and UAV is seen in ACI. In all three strains the level of SBP is similar to the levels found in the 2K-situation.[268] Thus, clear differences in renal susceptibility are present. The FHH strain is clearly the most susceptible followed by the FHL strain, while the ACI strain is quite resistant to develop renal damage.

The origin of the differences in susceptibility to develop progressive renal damage between the FHH and FHL strain are not clear yet. Five *Rf*-QTLs are thought to contribute to the increased susceptibility to renal damage in the FHH rat. A crude genome scan revealed that FHH and FHL were genetically identical for 75%. When looking at the five *Rf*-regions, they were identical for 86%. Markers tested in the *Rf-1* and *Rf-3* regions were identical in FHL and FHH, while marker differences were present in the *Rf-2*, *Rf-4* and *Rf-5* regions. [268] Thus, differences in *Rf-2*, *Rf-4*, and *Rf-5* may account for the difference in renal susceptibility between FHL and FHH.

Differences in systemic blood pressure between FHH and FHL may also play a role. Interestingly, the *Rf-2* region also includes the *Bpfh-1* QTL, previously linked to SBP in the FHH rat.[25,234] A marker difference in the *Bpfh-1* QTL could explain the difference in SBP between the FHH and FHL rats. Unfortunately, the effects of L-NAME-induced hypertension could not be tested. Induction of hypertension by administering L-NAME leads to a high mortality rate in both FHL and FHH.[275,276] Whether this is linked to the high renal damage susceptibility, the bleeding disorder that resembles the platelet-storage pool deficiency in humans[50,204], or another mechanism still remains unclear and should be further studied.

In summary, the difference in susceptibility to renal damage between FHL and FHH rat can be partly explained by difference in SBP, but also partly by genetic differences in some of the *Rf*-regions. A detailed genome scan comparing the FHH and FHL rats in the five *Rf*-regions will delineate the genetic differences between the stains and may be helpful to further narrow down the regions involved in determining the differences in SBP and renal susceptibility.

9.1.2 Differences in renal susceptibility between ACI and single congenics

Congenic rat strains give us an unique opportunity to detect an effect of a QTL-region linked to a disease trait on a genomic background that does not develop the disease trait.[154,300] Where human studies fail because of the huge genetic heterogeneity, studies in congenic animals are more successful because of their genetic homogeneity.[292] The congenic rats carry the QTL region of the FHH but are for over 95% genetically identical to the renal resistant ACI progenitor strain.[271] In this thesis, we described the first congenic rats generated for unraveling the mechanisms behind the development of renal damage. It was surmised that each of the five *Rf*-QTLs had a role in the development of renal damage. This assumption has been tested in single *Rf*-congenic rats.

Single congenic rats were generated carrying the *Rf-1*, *Rf-3*, *Rf-4* or *Rf-5* QTL of the FHH on an ACI genomic background. Each of these single congenic rats have been studied for their susceptibility to renal damage using four models. The control model consisted of two-kidney rats. In another model, renal mass was reduced by unilateral nephrectomy (UNX). Since all tested single congenics were normotensive, an increase in SBP was needed to compare the results with the hypertensive FHH. The 2K+L-NAME model consisted of two-kidney rats with L-NAME induced hypertension, and the last model was a combination of UNX and L-NAME induced hypertension.

Following UNX, 2K+L-NAME, or UNX+L-NAME, both *Rf-1* and *Rf-3* single congenics had significantly higher UPV and UAV values when compared to ACI rats.[267,269] In contrast, rats carrying either the *Rf-4* or the *Rf-5* QTL did not show to have an increased renal susceptibility.[267,270] It is clear that the *Rf-4* and *Rf-5* regions do not have a direct effect on renal susceptibility. In contrast, both the *Rf-1* and *Rf-3* regions appear to directly influence renal damage susceptibility. The role of the *Rf-2* region on the renal susceptibility has not been established yet since the *Rf-2* single congenic rat only recently has been generated.

9.1.3 Differences in renal susceptibility between *Rf-1* single congenics and double congenics

Chronic kidney damage is thought to be a polygenic disease, where multiple genes are interacting to increase renal susceptibility.[129,292] The presence of five QTLs linked to renal damage susceptibility underscores the complexity of CKD. Linkage studies revealed that *Rf-1* had by far the highest LOD-score and studies in *Rf-1* single congenics showed that this region must contain genes influencing renal susceptibility.[25,234] To find out if interactions are present between the different *Rf*-QTLs, double congenics were generated carrying besides the *Rf-1* region, also another *Rf*-region. In this thesis we describe the first double congenics created for unraveling the mechanism behind the development of CKD.

Renal damage susceptibility was assessed in five ACI.FHH-*Rf* double congenic rat strains, i.e. *Rf-1A+2*, *Rf-1A+3*, *Rf-1A+4*, *Rf-1B+4*, and *Rf-1B+5* double congenics. The *Rf-1A+2* double congenic was the only double congenic with an increased blood pressure.[268] The induction of hypertension by L-NAME led to an increased mortality rate in all double congenics. Especially in combination with UNX survival, was low. In this discussion only a comparison is made between the double congenics and the *Rf-1B* single congenics using the 2K and UNX model. Following UNX, increased UPV and UAV values were found in the *Rf-1A+2*, *Rf-1A+3*, *Rf-1A+4* and *Rf-1B+4* double congenics when compared with ACI and *Rf-1* single congenics.[266,268,269,270] Also in the two-kidney model, differences in UPV and UAV values were found between

double congenics and ACI rats. Statistical analysis of the *Rf-1A+3* and *Rf-1A+4* revealed significant interactions between the *Rf-1* and *Rf-3*, and *Rf-1* and *Rf-4* QTLs, explaining the markedly increase in renal damage susceptibility.[269,270] Remarkably, the levels of renal damage seen in the *Rf-1A+2*, *Rf-1A+3*, *Rf-1A+4* and *Rf-1B+4* combinations are of the same order of magnitude.[266,268,269,270] In contrast, renal susceptibility in the *Rf-1B+5* double congenics did not differ from *Rf-1B* single congenics, indicating that the contribution of *Rf-5* in determining the renal susceptibility in FHH is probably small.[266]

9.2 Assessment of renal susceptibility

9.2.1 Comparison of renal damage parameters

Comparing UPV and UAV values is an often used method to compare renal susceptibility.[52,274,276,299] These values will give an assessment of renal function. In a normal situation, glomeruli are relatively impermeable for proteins. However, when renal failure is present, the glomerular membrane becomes more permeable and proteins will leak through to the urine, resulting in elevated levels of UPV and UAV.[258] The incidence of glomerulosclerosis (%FGS) can be assessed at the end of the experiment and will give an indication of structural damage present in the kidney.[52,133] Another renal damage parameter is creatinine clearance (Cc/100). The Cc/100 will give an estimation of the glomerular filtration rate (GFR).[42] These four parameters are often related to each other. The majority of proteins leaking through the membranes are albumin, therefore UAV values are close to the UPV values. Increased UPV and UAV values is often linked to an increased %FGS. In the end, a decrease in functioning glomeruli can lead to a decreased Cc/100.

In most of the tested congenic rats a relation between the renal damage parameters is seen. However, in the *Rf-1A+2* double congenic rat it appears that this relation is absent. Compared to FHL, *Rf-1A+2* double congenic rats develop less UAV following UNX. Strangely enough this is not mirrored by a similar difference in FGS. To the contrary, the incidence of FGS in the *Rf-1A+2* double congenics is significantly higher than that of FHL.[268] This points to a discrepancy between the severity of the functional damage (albuminuria) and structural damage (glomerulosclerosis). Further studies will be needed to explain such a discrepancy.

9.2.2 Role of systemic blood pressure

The FHH rat develops hypertension at a relatively young age. The hypertension present in this rat contributes to the development of renal damage. Three possibilities exist regarding the role of hypertension in CKD: (1) hypertension is necessary and sufficient to produce CKD; (2) hypertension is necessary but not sufficient to produce CKD, other risk factors are also needed; (3) hypertension is neither necessary nor sufficient to produce CKD, but increases the risk in individuals who are otherwise predisposed or increases the rate of progression of CKD. In the first situation, CKD is hypertension-induced, while in the latter two situations CKD will be hypertension-associated.[25]

Since hypertension is a very important risk factor for developing CKD, congenic rats should be tested in both a normotensive as well as a hypertensive situation. Based on our earlier experience we used chronic L-NAME treatment to raise systemic blood pressure.[275,276] As indicated in our previous studies, using chronic L-NAME treatment has some disadvantages. It is difficult, if not impossible, to match SBP between the various strains at a level normally present in the FHH rat. Chronic L-NAME treatment may

directly affect the vascular structure in the kidney, independent of its blood pressure effects.[305] Furthermore, reducing endothelial NO-synthase activity by L-NAME may have a negative effect on the protective action of NO in organs that are targets of hypertensive injury.[101] Thus, differences found in renal damage between *Rf-1B* congenics and ACI may be partly due to an increased susceptibility to L-NAME. However, when comparing the *Rf-1B* single and *Rf-1B+5* double congenics, results suggest that L-NAME is not influencing renal susceptibility. Following UNX+L-NAME, *Rf-1B+5* double congenics had a significantly higher SBP compared to *Rf-1B*, caused by an increase in L-NAME intake. Despite the increase in SBP, renal damage susceptibility in *Rf-1B+5* double congenics was the same as in the *Rf-1B* single congenics, indicating that an increase in SBP alone is not likely to increase renal damage susceptibility.

Renal susceptibility could not be tested in *Rf-1A+2* double congenics following L-NAME-induced hypertension. All *Rf-1A+2* rats died prematurely during L-NAME treatment. This was not a complete surprise, as we previously reported high mortality rates in L-NAME treated FHH and FHL rats.[237,275,276] Such a high early mortality during L-NAME treatment was not present in *Rf-1*, *Rf-3*, *Rf-4*, and *Rf-5* single congenics or *Rf-1A+3*, *Rf-1+4*, and *Rf-1B+5* double congenic rats.[199,267,269,270] It is highly likely that the high early mortality during L-NAME is due to the presence of the *Rf-2* QTL. In conclusion, although L-NAME has disadvantages, it is good way to induce hypertension in congenic rats not carrying the *Rf-2* region.

In this thesis we also described a study where we tried to find out if renal damage susceptibility can be assessed using RKM. We mimicked our previous studies where rats were treated with UNX+L-NAME induced hypertension. With RKM the FHH rats did not survive for more than two weeks because of total renal failure. The FHL rats did survive but developed severe renal damage. Differences could be found between the normally renal resistant ACI rat strain and the FHL rat, although it should be taken into account that the ACI rat also developed a considerable amount of renal damage. In earlier studies we demonstrated that *Rf-1* increases renal susceptibility to renal damage, and *Rf-5* did not have an effect at all. When using RKM, the difference between ACI, *Rf-5* and *Rf-1B* is found, although ACI and *Rf-5* both developed considerable renal damage using RKM. While following UNX+L-NAME, both ACI and *Rf-5* rats showed to be resistant to the development of renal damage. Comparing *Rf-1A+4* double congenics with ACI, and *Rf-1A* and *Rf-4* single congenics in the RKM, differences in susceptibility to renal damage were not as clear as when using the UNX+L-NAME model. The synergistic interaction between *Rf-1A* and *Rf-4* could not be detected in RKM, mainly because of huge variations within each rat strain.

To detect differences in susceptibility between strains, the RKM has major disadvantages compared to the UNX or UNX+L-NAME models. The main disadvantage of RKM is the poor reproducibility of the model. In contrast to the UNX model where 50% of the renal mass is reduced, the amount of renal mass reduction in the RKM is quite variable. This could be due to differences in the anatomy of the renal blood supply between individual rats. Another disadvantage is the poor control of the systemic blood pressure. The RKM rats uniformly develop marked hypertension shortly after surgery. However, large individual differences were present.

Our findings question the suitability of the RKM as a model to rapidly assess differences in susceptibility to develop renal damage. The RKM is extremely powerful in inducing chronic renal damage. Even the ACI rat, shown to be relatively resistant to other procedures [199,267,270,275,276], rapidly develops severe renal damage. Although it could still be useful in strains that markedly differ in susceptibility, its value to

detect differences between congenic rat strains was poor. The UNX model either alone or in combination with L-NAME induced hypertension, despite the longer duration, appears better suitable.

9.3 Renal physiology

9.3.1 Renal blood flow autoregulation

In a normal situation, an adequate autoregulation of the renal blood flow (RBF) is able to protect the glomerular capillary structures from injury due to systemic hypertension. Impaired autoregulation increases susceptibility for renal damage in various rat models including FHH.[15,35,94,272,273] Because of the impaired renal autoregulation, hypertension increases intraglomerular pressure. A high P_{GC} is the predominant cause of UPV and FGS in the FHH rat.[237] When the renal perfusion pressure (RPP) in FHH rats is increasing, the P_{GC} is increasing in the same extent because of an increased efferent arteriolar resistance and a decreased afferent arteriolar resistance.[273] In another study it was found that UPV was directly dependent on the changes in RPP in FHH rats. The mechanism involved in this unusual pressure proteinuric response may be dependent on the GFR in response to elevations in RPP. Thus the high baseline filtered load of protein and the increase seen when RPP is elevated likely increased the delivery load of protein to a level that exceeds the transport maximum for reabsorption for protein in the proximal tubule.[279]

Two mechanisms contribute importantly to the regulation of preglomerular tone: the tubuloglomerular feedback and the myogenic response. Besides a reduced regulation of preglomerular tone, an increased tone of the efferent arterioles (EAs) may also contribute to the increased susceptibility of FHH rats to develop renal disease. A high tone of the EAs will retain an elevated glomerular pressure in the capillary network.[210] The first observation of Verseput et al.[279] was that the TGF system in the FHH rat is intact, despite the fact that the FHH rat has a characteristically low afferent arteriolar resistance as compared to other hypertensive rats. Their second observation was that the FHH rat has a normal or even enhanced TGF system following prolonged administration of an ACE inhibitor (ACE-i). These latter findings indicate that the reduction of P_{GC} achieved by ACE-i is not offset by a concomitant attenuation of TGF function. The results of another study by van Dokkum et al.[273] indicated that the myogenic response of preglomerular renal arteries is impaired in FHH rats and that they exhibit an impaired ability to buffer changes in intraglomerular pressure, especially in response to rapid fluctuations in arterial pressure. This defect in the myogenic response of the preglomerular vasculature in combination with the previously reported increased efferent vascular resistance that elevated baseline P_{GC}[237,238], and the tendency of these animals to develop systolic hypertension promote the transmission of elevated pressures to the glomerulus.

Renal blood flow autoregulation has been tested two-kidney ACI, FHH, FHL, four ACI.FHH-*Rf* single congenics and five ACI.FHH-*Rf* double congenics and results are depicted in Table 5. It shows the average RBF at a RPP of 100 mm Hg, and renal autoregulatory index (RAI) of RPP ranges of 130-150 mm Hg and 100-150 mm Hg. The RAI gives an indication of the renal autoregulation function of the depicted renal perfusion pressure range. A RAI of 0 indicates perfect autoregulation of RBF, and a RAI of 1 indicates that there is no autoregulation present due to a fixed renal vascular resistance. Previous studies were not able to localize impaired autoregulation to a particular chromosomal region. Table 5 shows that all rats carrying the *Rf-1* QTL have an increased RAI, indicating an impaired renal autoregulation. In this thesis we demonstrate that the *Rf-1* region of rat chromosome 1 contains one or more gene(s) responsible for

impairing autoregulation of the RBF before the development of severe renal damage.[267] It still remains to be determined if it is the same gene that is responsible for both a lack of autoregulation and renal disease. In contrast to the findings in congenic rats carrying *Rf-1*, the *Rf-3*, *Rf-4* and *Rf-5* single congenics were shown to have a normal autoregulation, comparable to that of ACI rats (Table 5). Therefore, we can conclude that the *Rf-3*, *Rf-4* and *Rf-5* regions do not contain genes that influence renal autoregulation. The role of *Rf-2* on renal autoregulation has not yet been investigated.

Table 5: Renal blood flow autoregulation of two-kidney (2K) ACI, FHH, FHL, and ACI.FHH single and double congenics at the age of 13-15 weeks.

	n	RBF_{100}	$RAI_{130-150}$	$RAI_{100-150}$
ACI	21	4.63±0.24	0.26±0.04	0.25±0.02
Rf-1A	12	5.22±0.36	0.69±0.10	0.57±0.08
Rf-1B	13	4.58±0.25	0.59±0.05	0.53±0.05
Rf-3	15	4.58±0.36	0.24±0.02	0.28±0.03
Rf-4	15	5.36±0.43	0.21±0.03	0.23±0.04
Rf-5	15	5.90±0.28	0.17±0.02	0.17±0.02
Rf-1A+2	8	4.64±0.31	0.51±0.07	0.49±0.06
Rf-1A+3	16	4.97±0.23	0.63±0.07	0.49±0.05
Rf-1A+4	12	5.45±0.27	0.47±0.03	0.44±0.50
Rf-1B+4	12	4.28±0.30	0.45±0.07	0.42±0.05
Rf-1B+5	12	4.82±0.35	0.45±0.03	0.36±0.03
FHH	8	5.50±0.34	0.73±0.04	0.55±0.03
FHL	8	4.11±0.20	0.36±0.03	0.51±0.06

n: amount of rats; RBF_{100}: renal blood flow (ml/min/gKW) at a renal perfusion pressure of 100 mm Hg; $RAI_{130-150}$: Renal Autoregulatory Index at renal perfusion pressure range of 130 to 150 mm Hg; $RAI_{100-150}$: Renal Autoregulatory Index at renal perfusion pressure range of 100 to 150 mm Hg; *Rf*: congenic strains; ACI: August x Copenhagen Irish; FHH: Fawn Hooded Hypertensive; FHL: Fawn Hooded Low blood pressure.

9.3.2 Creatinine clearance

Creatinine clearance (Cc/100) is used a measurement of GFR.[42] Previous studies demonstrated that FHH and FHL rats are hyperfiltrating, indicated by an increased Cc/100.[51] Renal susceptibility studies in single ACI.FHH-*Rf* congenics revealed the presence of an increased Cc/100 in the *Rf-3* single congenic rats.[269] This would indicate the presence of one or more genes in this region influencing the GFR. However, an increased Cc/100 in the *Rf-1A+3* double congenics was only seen following UNX and not in the 2K-situation and L-NAME treated rats (Table 1-4). No good explanation is yet available, and further studies using better GFR methodologies need to be performed to unravel the genetics behind the increased GFR.

9.4 Translation from rat to human

9.4.1 Complexity

The results from the numerous genomic studies in humans as well as rodent models together indicate that differences in renal susceptibility result from complex interactions between genes and the environment. In humans and rats multiple chromosomal regions have been linked with parameters of renal damage. However, studies with different forms of nephropathy in humans and different rat models yield different QTLs influencing renal susceptibility (Table 4, 5, 6 and Figure 1 of Chapter 1), suggesting an enormous genetic heterogeneity. Studies in congenic rats indicate that the effects of single QTLs may be small or absent. However, when various QTLs are combined in double congenic strains, strong gene-gene (epistatic) interactions do occur, markedly enhancing renal susceptibility. The complexity of the gene-gene interaction is further distended by the fact that the renal damaging effects of changing the genotypes also depends on the severity of the hemodynamic stress put upon the kidney, as well as the duration of the exposure.

Epistasis is an old term to describe a masking effect whereby a variant or allele at one locus prevents the variant at another locus from manifesting its effect. Others defined it as a deviation from adding up the effects of alleles at different loci with respect to their contribution to a quantitative phenotype. More recent approaches dealing with epistasis have focused on the concept that the effect of a gene needs to be estimated in a specific context.[193] The idea is to have a specific model of how genes function and interact and then construct a building-block description of the phenotype based on the genetic composition, as suggested by Cheverud and Routman[36], in what they call physiological epistasis.

Proteinuria, albuminuria, and glomerulosclerosis are all distant traits, i.e. not directly related to a single gene effect. With the generation of the different double congenics we have constructed a relatively simple two-locus model that resulted in an increased susceptibility to renal damage. Next to QTLs in FHH, numerous QTLs influencing parameters of renal damage have been detected in other strains (Table 5 of Chapter 1). It may well be that 10-15 candidate loci are involved in the various rat models of renal damage. Should a similar number of QTLs also play a role in humans, the number of possible gene pairs, trios or quartets that can be derived from these loci becomes tremendous. With n loci, there are [n(n-1)/2] gene pairs, [n(n-1)(n-2)/6] trios, and [n(n-1)(n-2)(n-3)/24] quartets. With 5 loci, the number of pairs, trios and quartets will be 10, 10, and 5 respectively. With 10 loci, these numbers increase to 45, 120, and 210, while with 15 loci there will already be 105, 455, 1365 possible combinations. As the various combinations may also interact, the number of partitions that one has to deal with statistically becomes gigantic.

It may still be feasible to dissect and reconstruct the genetic components of inbred rat models with a limited number of homozygous QTLs involved. However, unraveling the genetic components of susceptibility to renal damage in humans becomes a tremendous task. With a large number of candidate genes and gene combinations involved, it might well be that each of them contributes only marginally to the total ESRF population. For instance, if diabetic nephropathy results from a three-locus model involving 12 candidate genes with equal disease allele frequencies, than 220 possible three-gene combinations account for 30-35% of diabetics that eventually develop diabetic nephropathy. Each three-gene combination, however, only has to account for 0.45% of the cases. Even with a simpler two-locus model, there are 66 possible two-gene combinations and each of them only has to account for 1.5% of the cases.

The involvement of many loci may be one of the reasons that so many human studies fail to replicate animal findings.[292] Consider the testing of one gene polymorphism out of twelve candidate genes in a three-locus model. Supposing equal frequencies of the various possibilities, the gene tested will be present in 55 of the 220 (25%) possible trios. Thus, in 75% of the cases, the disease allele of the tested candidate gene will not be involved. Furthermore, the susceptibility allele of the tested gene may also be found in the control population. If disease alleles or other candidate genes are not present, or if harmful environmental conditions, like hypertension, diabetes, etc., are absent, the disease allele of the tested gene will have a high frequency also in the controls. The involvement of many candidate genes may also account for the inconsistencies in replicating genetic linkage and association studies in humans.[292]

The presence of gene-gene interactions calls for new approaches to analyze the genetics underlying complex diseases in humans.[168,178,292] Recent studies have analyzed the involvement in coronary heart disease of 18 polymorphisms in six candidate susceptibility genes[178], or the role in hypertension of 13 polymorphisms in eight candidate genes that play a role in blood pressure regulation.[293] Both studies indicated that combinations of loci better predicted the trait variation than the marginal single locus effects. However, the computational burden of the analyses was considerable. Multigene approaches also emerge in studying CKD in humans.[70] To achieve enough statistical power, however, future studies should strive for large collaborations to enable sample sizes that are sufficient to simultaneously analyze multiple gene loci.[163]

9.4.2 Comparative genomics

The enormously expanding collection of data generated from genetic and genomic research efforts in human, mouse, and rat emphasizes the fundamental need for integrating bioinformatics systems. Such systems are under permanent development in the USA as well as Europe/UK.[260,28] The Rat Genome Database (RGD, http:/rgd.mcw.edu) is an NIH-funded collaboration between the Bioinformatics Research Center at the Medical College of Wisconsin, the Jackson Laboratory and the National Center for Biotechnology Information. The aims of RGD are to collect, consolidate and integrate data generated from ongoing rat genetic and genomic research efforts and make these data widely available to the scientific community. The rat is uniquely suited to its role as a model of human disease and the primary focus of RGD is to aid researchers in their study of the rat and in applying their results to studies in a wider context. This is achieved by providing a high-quality disease-centric resource, applicable to human, mouse, and rat via comparative tools. Thus, RGD is not only a valuable resource for those working with the rat but also for researchers in other model organisms wishing to harness the existing genetic and physiological data available in the rat to complement their own work. Integrating rat physiology with mouse genetics and clinical results from human by using the respective genomes provides a novel route to capitalize on comparative genomics and the strengths of model organism biology.[116,260,261]

RGD is continuously expanding and improving its activities. Recent developments can be categorized into three groups. (i) Improved data collection and integration to match increased volume and biological scope of research. (ii) Knowledge representation augmented by the implementation of a new ontology and annotation system. (iii) The addition of quantitative trait loci data, from rat, mouse and human to our advanced comparative genomics tools, as well as the creation of new, and enhancement of

existing, tools to enable users to efficiently browse and survey research data. The emphasis is on helping researchers find genes responsible for disease through the use of rat models.[55]

The T1DBase (http://T1Dbase.org) is a public website and database that is currently focused on the molecular genetics and biology of type 1 diabetes (T1D) susceptibility and pathogenesis. It includes annotated genome sequence for human, rat and mouse; information on genetically identified T1D susceptibility regions in human, rat and mouse, and genetic linkage and association studies pertaining to T1D. Although the site is focused on T1D, the system is applicable to any genetic study of complex disease, of either large or small scale.[28,241]

A recent development is the Human Phenome Database (HPD, http://hpd.mcw.edu), which was created to help researchers identify the underlying genetics responsible for complex multifactorial human diseases. Their aims is to consolidate and integrate phenotype data generated from ongoing research efforts and make these data widely available to the scientific community. Data from model organisms, specifically rat and mouse, for comparative analysis as well as a suite of tools that integrate the data and allow interspecies comparisons are provided by HPD to facilitate analysis. The development of HPD will be very helpful for comparative genomics.

The search for genetic factors influencing susceptibility to renal damage in humans and rodent studies has revealed that multiple genes are involved acting in complex gene-gene and gene-environment interactions. Despite the progress, this has not yet resulted in a better understanding of the mechanisms of renal susceptibility. The state of affairs mimics the search for the genetic basis of hypertension[56,301] and diabetes[91,124,190] two important risk factors for CKD.

However, with the completion of the sequencing of the human, mouse and rat genome, genomic comparisons between these three species are greatly facilitated.[87,135,278,287] A linkage study in humans has revealed the presence of QTL linked to renal damage on chromosome 10q, which is homologous to the rat *Rf-1* QTL.[78] The 95% confidence interval of the *Rf-3* region (3: 117-146 Mb) is homologous to a part of human chromosome 20 (~1-33 Mb) and mouse chromosome 2 (126-155 Mb) (http://ncbi.nlm.nih.gov/projects/Homology). Surprisingly, the *Rf-3* homologous region in human and mouse appear both to be involved in renal disease. Linkage studies in Pima Indians suggest the presence of a gene on chromosome 20 influencing diabetic nephropathy.[111] The homologous region in the mouse has recently been linked to albuminuria in KK/Ta mice.[233] The presence of QTLs linked to nephropathy in rat, mouse and human make the *Rf-3* region very interesting for further investigation.

9.5 Perspectives

9.5.1 Additional model systems

Genetic manipulations (i.e. reporter-gene constructs, gene transfer, transgenic models, gene knockouts, consomics and congenics) can generate new strains of animals that are important not only for physiological genomics, but also for targeted functional study at the protein level.

At the moment, we have assessed renal susceptibility in *Rf-1*, *Rf-3*, *Rf-4* and *Rf-5* single congenics, and *Rf-1+2*, *Rf-1+3*, *Rf-1+4* and *Rf-1+5* double congenics. For future studies, the *Rf-2* congenic rat strain should be phenotyped. Results showed that *Rf-5* alone or in combination with *Rf-1* did not have any influence on renal susceptibility. Since *Rf-5* does not have a role in renal damage susceptibility, the focus will be on generating multiple congenic rats of the *Rf-1*, *Rf-2*, *Rf-3* and *Rf-4* regions. At the moment, triple congenic rats are being generated by crossing double congenic rats. The *Rf-1+3+4* triple congenic rat is now ready to be phenotyped. The other two triple congenics, *Rf-1+2+3*, and *Rf-1+2+4* are currently being generated. Our final goal would be generating a quadruple congenic strain carrying *Rf-1*, *Rf-2*, *Rf-3* and *Rf-4*. Ideally, this congenic rat strain should be studied physiologically with four models, i.e. the 2K control situation, UNX, and 2K+L-NAME induced hypertension. The UNX+L-NAME model will not be used because of the high mortality rate in double congenics.

Using the congenic rats described in this thesis, subcongenics can be generated. By making overlapping subcongenics, the QTL region can be narrowed down to a few Mb. By narrowing down the QTL region, the chance of finding genes increases. At the moment, several subcongenics have been generated for the *Rf-1* and *Rf-4* regions and are being tested for their renal damage susceptibility.

Consomic rats are yet another tool to help identifying genes influencing complex traits. The main principle of consomic rats is that the phenotype might be rescued by replacing an entire chromosome from a disease model by one from a healthy control strain.[45] Further, the consomic rats can be used for generating congenic strains. In this way, the generation of congenic rats takes less time compared to the conventional method. Single consomic strains are also useful for generating multiple consomics, which can be used to reveal interactions between genes on different chromosomes.

The Program for Genomic Applications (PGA) generates two panels of consomic rat strains by replacing each individual chromosome of either the FHH or the SS strain by that of the BN strain. Currently, 19 SS.BN and 19 FHH.BN consomics have been generated and tested for several disease traits. Of the available SS.BN consomics, the SS.BN^{5}, SS.BN^{6}, SS.BN^{7}, SS.BN^{8}, SS.BN^{13} and SS.BN^{18} strains are the most promising as they all confer protection from renal damage (Chapter 1, Table 6). Currently, the SS.BN^{13} strain is being studied to unravel the mechanisms behind the protection from salt induced hypertension and renal damage.[47,146]

With regard to our studies, it will be interesting to compare the results obtained with the ACI.FHH-*Rf* congenics with those from the FHH.BN^{1}, FHH.BN^{3}, FHH.BN^{14} and FHH.BN^{17} consomic strains. Replacement of chromosome 1 of FHH by BN led to a partial rescue of the renal damage phenotype. These results underscore the significance of chromosome 1 of the FHH, harboring both *Rf-1* and *Rf-2*. Replacing chromosome 14 of the FHH by BN results in a significant decrease in UPV, confirming a possible role for *Rf-4* in influencing renal susceptibility. Finally, the replacement of chromosome 17 did not have any effect on the renal damage phenotype. This finding is in line with our results indicating that the *Rf-5* region did

not significantly influence renal damage susceptibility. An FHH.BN[3] consomic strain to confirm a role for the *Rf-3* region has not been tested yet. The PGA data support the findings of the ACI.FHH-*Rf* congenic rats presented in this thesis. In the future, double consomics, i.e. FHH.BN[1,3], FHH.BN[1,14], and FHH.BN[3,14] can be generated and compared to the ACI.FHH-*Rf* double congenics. The generation of a multiple consomic where chromosome 1, 3, and 14 of the FHH are replaced by BN could be very interesting. It is expected that the renal damage phenotype in this triple consomic will be totally rescued.

Several genomic studies are carried out in mice. Currently, the total number of knockout mice described in the literature corresponds to about 10% of the ~25-30,000 mouse genes. Large consortia plan to generate comprehensive series of mouse gene knockout strains[5], strains with ENU-mutated genes[6,208], and a Collaborative Cross[40] to speed up biomedical discovery. The aim of the Knockout Mouse Project is "to produce and phenotype knockouts for all mouse genes, and place these resources in the public domain".[5] An academic/industry consortium in the UK has initiated a phenotype-driven mutagenesis program to generate new mouse models of human disease and for gene function assignment.[208] The objective of the European Mouse Mutagenesis Consortium is "to establish and integrate mutagenesis platforms, gene expression resources and phenotyping units, and bioinformatics resources" in order to "accelerate our understanding of gene function and of human health and disease".[6] The Collaborative Cross is an initiative of the Complex Trait Consortium aiming "to promote the development of resources that can be used to understand, treat and ultimately prevent pervasive human diseases." The Collaborative Cross in itself is a panel of eight-way recombinant inbred (RI) mouse strains, derived from a genetically diverse set of founder strains and designed specifically for complex trait analysis.[40] The long-term expectations of all these initiatives are huge. Whereas the knockout and mutagenesis consortia aim at mutating all ~ 28,000 mouse genes, the collaborative cross thinks "of a set of 1,000 fully phenotyped RI strains and more than one million potential isogenic and completely defined F_1 hybrids".[40]

The transgenic and knockout technology that has been flourishing in the mouse has been less successful in the rat. Only a few transgenic rat strains are of interest to study the mechanisms of progressive renal damage. One of them is a strain of rats overexpressing the mouse ren-2 gene (TGR[mREN2]27). This strain was developed to study primary renin-dependent hypertension, but they also develop proteinuria associated with glomerulosclerosis as early as 8 weeks of age.[7,137,214] Deterioration of renal function is accelerated in subtotally nephrectomized (remnant kidney) transgenic rats [TGR(mREN2)27] compared with that in comparably hypertensive stroke-prone SHR.[284] A transgenic rat overexpressing the angiotensin II type 1 (AT1) receptor in podocytes was recently developed. It was found that increased AT1 receptor signaling in podocytes leads to protein leakage and structural podocyte damage progressing to focal and segmental glomerulosclerosis.[105]

The embryonic stem cell technology to produce knockout rats is not yet established. Methods to produce gene-disrupted knockout rats are greatly needed. Recently protocols have been developed for creating ENU-induced germline mutagenesis.[242,304] The ENU mutagenesis technology has been successful in generating knockout rats as well as rats with mutations in genes of interest. No studies related to kidney damage employing this technology have yet been published. However, a program is being developed to generate gene knockouts in the SS, BN, and FHH strains that may be of interest for the renal research community (http://pga.mcw.edu).

9.5.2 More physiology

In order to totally unravel the mechanisms behind the development of renal damage, more renal physiological studies must be conducted. For instance, in Chapter 5 an increased creatinine clearance (Cc/100) is described in single congenic rats carrying the *Rf-3* region. This may point to the presence of an increased GFR. However, the 24-hour creatinine clearance is a rather crude estimate of the real GFR. Other, more GFR specific methods should be used to identify the presence of real differences in GFR between ACI, FHH and *Rf-3* single congenic rats.

The *Rf-4* region is most likely responsible for an increased glomerular permeability.[231] Further studies into glomerular permeability, including electron microscopic pictures of glomeruli should be made to detect differences in glomerular structure, which might explain the differences in glomerular permeability.

Previously, it was reported that the impaired renal autoregulation in FHH was caused by an impaired myogenic response, whereas the TGF was normal.[272,273] Therefore, renal micropuncture studies should be performed in *Rf-1* single congenics to find out if the impaired renal autoregulation in this congenic rat is also due to an impaired myogenic response.

9.5.3 Transcriptome analysis

In the current era, it is not all about genomics. Several other “omics” have been created to help reveal the mechanisms behind a disease trait. Genomics looks at DNA level, while at mRNA level it is called transcriptomics, based on mRNA being the transcript of DNA. Another very important development is proteomics, where levels of proteins are defined. Genomics, transcriptomics and proteomics are “omics” that have been extensively used to several areas of biomedical research. All these techniques combined may contribute to a better understanding of the pathogenesis and pathophysiology of new therapeutic targets, biomarker discovery, prediction of therapeutic response, personalized treatment regimens, better therapeutic outcome and ultimately prevention of a disease.[145,150,255]

Transcriptome analysis, a high-throughput analysis of gene expression at the mRNA level in a (near)-genome scale has become one of the most widely used approached in biomedical research. Gene expression studies are used to detect differences in gene activity between two different samples. The microarray technique is the most often used transcriptome technique. In the last few years, an enormous amount of data has been collected in kidney research using transcriptome techniques. Besides microarray, other techniques like serial analysis of gene expression (SAGE), sequencing of expressed sequence tags (EST) and real-time polymerize chain reaction (RT-PCR) give an estimation of the absolute or relative mRNA levels for all genes. The suppressive subtractive hybridization and differential display techniques are different because they will only identify mRNA that differs quantitatively between the tested samples.[145]

In summary, the sequence of a gene can be assessed by genomics. The expression of a gene, i.e. the mRNA level can be assessed by transcriptomics. Analysis of proteins is called proteomics. It should be noted that one should be cautious when extrapolating mRNA to protein. Especially when mRNA and/or protein abundance is low it could give different quantitative estimates. Transcriptomic analysis alone will not be sufficient for characterizing complex networks of biological regulation. Measurement of proteins will complement mRNA data or provide a validation of the mRNA data.[145]

For future studies, it would be very interesting to find out if there are genes that are differentially

expressed (up or down-regulated) in the kidneys of the FHH rat compared to the ACI rat. Since ACI and FHH are two different inbred rat strains, it is expected that a lot of genes will be different in their activity. Results should be focused on genes that lie within the *Rf*-regions of the FHH rats. A better option would be to compare kidney tissue of ACI with ACI.FHH-*Rf* congenic rats. The ACI.FHH-*Rf* congenic rats are for 95-99% identical to the ACI rat, therefore limiting the total number of genes that will be differentially expressed. The specific changes in gene expression of each *Rf*-QTL can be measured by comparing the various ACI.FHH-*Rf* single congenic strains with the ACI parental strain. Comparing single and double congenics could reveal alterations in gene expression caused by gene-gene interactions. These gene expression studies will result in the discovery of candidate genes that can be linked to the development of renal damage.

9.5.4 Gene discovery

Discovering a gene causing or influencing the susceptibility to develop a disease is not simple. Genetic mapping of QTLs has resulted in several CKD-related QTLs, however a specific set of genes causing CKD has not been established yet. One of the reasons is that QTLs include large areas of a chromosome, incorporating a large set of genes. Congenic animals are most often generated in order to reduce this huge set of genes.[205] The disadvantage of congenic animals is that it is time-consuming and will cost a lot to generate and maintain these animals. Transcriptomic analysis is another strategy to reveal the genetic basis of complex diseases. It will generate massive amount of data, where most of the genes do not have any relevance to the disease. When integrating the genomic strategy with the transcriptomic strategy, it is most likely to find genes that are involved in the pathophsyiology of the disease.[298] This method was used by Yagil et al, who were able to narrow down the number of candidate genes for salt-susceptibility and hypertension in the rat from 1102 to 7 genes.[298]

Integrating transcriptomic, genomic and proteomic analysis in studying the *Rf*-QTLs will most likely yield interesting findings. Such studies should also be performed with the various single and multiple ACI.FHH-*Rf* congenic rats. The ACI.FHH-*Rf* congenic rat strains could be compared to the ACI rat. Differences in gene expression between the congenics and ACI could help explain the mechanism behind the development of CKD. Hopefully, this type of research will hopefully lead to the identification of genes influencing differences in susceptibility to develop CKD.

9.6 Overall conclusions

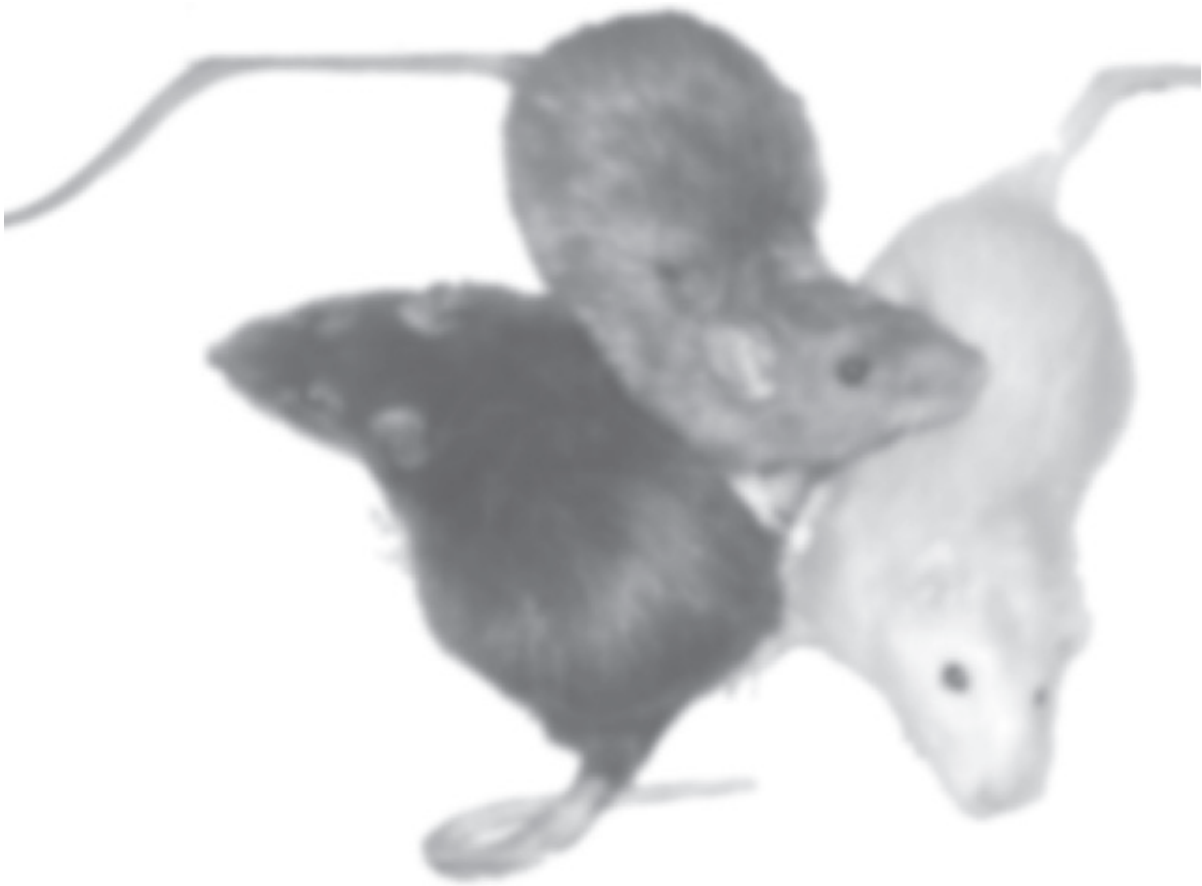
Four aims were formulated in Chapter 1.4. The first aim was to directly assess the role of each of the five *Rf*-QTLs in determining susceptibility to renal damage. The second aim was to directly assess the interaction between *Rf-1* and the four other *Rf*-QTLs. The third aim was to assess whether the effects if the individual *Rf*-QTLs depended on treatment model. The fourth aim was to establish the role of the *Rf*-QTLs in the autoregulation of the renal blood flow. The outcome of the various physiological genomic studies presented in this thesis regarding the rat renal failure QTLs *Rf-1* through *Rf-5* leads to the following conclusions:

1. The *Rf-1* region of the FHH contains one or more genes directly influencing renal susceptibility and impairing renal autoregulation. Whether these genes are the same needs to be further investigated.
2. The direct effect of the *Rf-2* region could not yet be tested. When the *Rf-2* region is combined with the *Rf-1* region, a significant increase in renal damage is seen when compared with *Rf-1* alone. Furthermore, the *Rf-2* region, harboring *Bpfh-1*, increases blood pressure.
3. The *Rf-3* region contains genes directly influencing renal susceptibility. Renal damage susceptibility in *Rf-3* single congenics is similar to that of *Rf-1* single congenics. When *Rf-1* and *Rf-3* are combined a significant synergistic interaction is present. The *Rf-3* congenic rats do have a normal renal autoregulation. The mechanisms of how *Rf-3* influences the development of renal damage still remains to be detected.
4. Genes in the *Rf-4* region do not directly influence renal susceptibility. However, when combined with *Rf-1* a significant synergistic interaction is present. The *Rf-4* congenic rats do have a normal autoregulation. The mechanisms of how *Rf-4* indirectly influences the development of renal damage is still unknown and should be further investigated.
5. The *Rf-5* region does not contain genes that directly influence renal susceptibility. In addition, the *Rf-5* region shows no synergistic interaction with the *Rf-1* region.
6. The Remnant Kidney Model (RKM) is not a very suitable model to analyze renal susceptibility in rats carrying *Rf*-regions of the FHH rats. The four models we used, especially the UNX and 2K+L-NAME model appear more suitable to assess differences in susceptibility to develop renal damage.

Chapter 10

Summary

Samenvatting



Summary

Chapter 1 introduces the reader into this thesis and gives an overview on the present knowledge of the role of genetic factors in the development of chronic kidney disease (CKD). It also describes the rat renal failure QTLs *Rf-1* through *Rf-5* and the possibilities to test their role in the susceptibility to develop CKD. In this chapter, four aims are formulated. The first aim is to directly assess the role of each of the five *Rf*-QTLs in determining susceptibility to renal damage. The second aim is to directly assess the interaction between *Rf-1* and the four other *Rf*-QTLs. The third aim is to assess whether the effects if the individual *Rf*-QTLs depends on treatment model. The fourth aim is to establish the role of the *Rf*-QTLs in the autoregulation of the renal blood flow.

Chapter 2 describes the generation of the various ACI.FHH-*Rf* congenic rat strains that are used for this thesis. These congenic rats carry one or more chromosomal region(s) of the FHH harboring one or more *Rf*-QTLs, on an ACI genomic background. Studies in congenic rats can provide direct evidence for an effect of a single QTL or the interaction between two QTLs on the development of CKD.

Chapter 3 describes the susceptibility to develop of renal damage in ACI, *Rf-1B*, and *Rf-5* congenic rats using four test-models. The first was the two-kidney control model (2K), and in the second model hypertension was induced by L-NAME. The third model consisted of unilateral nephrectomy (UNX), while the fourth was a combination of UNX and L-NAME induced hypertension. The renal damage-resistant ACI rat develops little renal damage, regardless of treatment. The *Rf-1B* single congenic developed significantly more renal damage compared to ACI, which was most pronounced following UNX+L-NAME. In contrast, in the *Rf-5* congenic rats renal damage did not differ from that of ACI. Studies of the renal blood flow (RBF) autoregulation revealed that it was impaired in *Rf-1B* single congenics. In contrast, renal autoregulation in *Rf-5* single congenics was normal, similar to that of the ACI rat. From this study it is concluded that the *Rf-1* region contains one or more genes directly influencing renal susceptibility and renal autoregulation, while the *Rf-5* region has no direct effect.

Chapter 4 describes the renal susceptibility in ACI, *Rf-1A* and *Rf-4* single and *Rf-1A+4* double congenic rat, using the four renal damage models mentioned above. regardless of treatment, renal damage in the *Rf-4* single congenic did not differ from that of ACI rats. However, the *Rf-1A+4* double congenic developed significantly more renal damage compared to *Rf-1A* single congenics. Statistical analysis revealed a significant interaction between the *Rf-1* and *Rf-4* region of the FHH rat. Renal autoregulation studies showed that *Rf-1A+4* and *Rf-1A* congenic rats had a similarly impaired renal autoregulation. In this study it is reconfirmed that the *Rf-1* region carries one or more genes influencing renal susceptibility and renal autoregulation. In addition, the *Rf-4* region carries a gene(s) indirectly influencing renal susceptibility by interacting with the *Rf-1* region.

. **Chapter 5** describes the effect of the *Rf-3* region alone and in combination with *Rf-1* on renal damage susceptibility. Again, four models were used to assess renal damage susceptibility as previously described. Following UNX+L-NAME, both *Rf-1A* and *Rf-3* single congenics developed significantly more renal damage compared to ACI. However, in the *Rf-1A+3* double congenics a significant further increase in renal damage was found compared to the *Rf-1* and *Rf-3* single congenics. Statistical analysis revealed the presence of an interaction between *Rf-1* and *Rf-3* with all treatments, similar to the interaction found between *Rf-1* and *Rf-4*. This study concludes that the *Rf-3* region also carries genes influencing renal susceptibility, and that an interaction is present between the *Rf-1* and *Rf-3* regions of the FHH rat, augmenting renal susceptibility.

Chapter 6 describes renal damage susceptibility and renal autoregulation experiments in *Rf-1B+5* and *Rf-1B+4* double congenic rats compared to *Rf-1B* single congenic rats. Renal damage susceptibility was assessed by using the 2K, UNX, 2K+L-NAME and UNX+L-NAME models. Results showed that *Rf-1B+5* double congenics did not develop more renal damage than the *Rf-1B* single congenics, regardless of treatment. The *Rf-1B+4* double congenic developed significantly more renal damage than the *Rf-1B* single congenic rat, similar to the *Rf-1A+4* double congenics. This study and the data presented in Chapter 3 indicate that the *Rf-5* region does not influence renal damage susceptibility, neither alone nor in combination with *Rf-1*. We therefore assume that the *Rf-5* region does not contain genes influencing renal susceptibility.

Chapter 7 describes the renal damage susceptibility of *Rf-1A+2* double congenic, FHH, and FHL rats. In addition, a global genome scan was performed on FHH and FHL rats to look for genetic differences. Administration of L-NAME led to a high mortality rate in FHH, FHL and *Rf-1A+2* rats and therefore only the 2K and UNX models were used.. The *Rf-1A+2* double congenics developed significantly more renal damage than the *Rf-1A* single congenics. The *Rf-1A+2* double congenic had an elevated blood pressure at a level between the normotensive FHL and hypertensive FHH rats. Some renal damage was present in FHL, but not as severe as in the FHH rat. A global genome scan revealed polymorphisms between FHL and FHH in the *Rf-2*, *Rf-4* and *Rf-5* regions. The *Rf-1* and *Rf-3* regions in FHL are identical to the FHH rats. We conclude that the *Rf-1* and *Rf-2* regions carry genes that influence renal susceptibility, while the *Rf-2* region also carries a gene that increases blood pressure, confirming a direct role of *Bpfh-1*. Differences in renal damage between FHH and FHL can be partly explained by the differences in SBP, and partly by differences in genotype of the *Rf*-regions.

Chapter 8 describes the suitability of the remnant kidney model (RKM) to rapidly assess differences in renal susceptibility between rat strains. The RKM was tested in several *Rf*-congenic rat strains, ACI, FHH and FHL rats. The FHH did not survive this severe model for more than a week. Comparison of ACI and FHL rats showed that differences in renal susceptibility between the renal resistant ACI and renal susceptible FHL rats could easily be detected. A second comparison between ACI, *Rf-1B* and *Rf-5* single congenic rats indicated that significantly more renal damage was present in *Rf-1B* compared to ACI and *Rf-5*, thus confirming the findings of Chapter 3. However, both ACI and *Rf-5* developed a considerable amount of

renal damage. A third comparison was made between ACI, *Rf-1A*, *Rf-4* and *Rf-1A+4* double congenics. The *Rf-1A* and *Rf-1A+4* developed significantly more renal damage. However, the previously found interaction between *Rf-1* and *Rf-4* (Chapter 4) could not be detected when using RKM. We concluded that our findings question the suitability of the RKM as a model to rapidly assess differences in susceptibility to develop renal damage. Although it could still be useful in strains that markedly differ in susceptibility, its value to detect differences between congenic rat strains was poor. The UNX, 2K+L-NAME and even the UNX+L-NAME models appear better suitable, despite the longer duration.

Chapter 9 contains the general discussion, some perspectives for further research and overall conclusions of this thesis. The outcome of the various physiological genomic studies presented in this thesis regarding the rat renal failure QTLs *Rf-1* through *Rf-5* leads to the following conclusions:

1. The *Rf-1* region of the FHH contains one or more genes directly influencing renal susceptibility and impairing renal autoregulation. Whether these genes are the same needs to be further investigated.
2. The direct effect of the *Rf-2* region could not yet be tested. When the *Rf-2* region is combined with the *Rf-1* region, a significant increase in renal damage is seen when compared with *Rf-1* alone. Furthermore, the *Rf-2* region, harboring *Bpfh-1*, increases blood pressure.
3. The *Rf-3* region contains genes directly influencing renal susceptibility. Renal damage susceptibility in *Rf-3* single congenics is similar to that of *Rf-1* single congenics. When *Rf-1* and *Rf-3* are combined a significant synergistic interaction is present. The *Rf-3* congenic rats do have a normal renal autoregulation. The mechanisms of how *Rf-3* influences the development of renal damage still remains to be detected.
4. Genes in the *Rf-4* region do not directly influence renal susceptibility. However, when combined with *Rf-1* a significant synergistic interaction is present. The *Rf-4* congenic rats do have a normal autoregulation. The mechanisms of how *Rf-4* indirectly influences the development of renal damage is still unknown and should be further investigated.
5. The *Rf-5* region does not contain genes that directly influence renal susceptibility. In addition, the *Rf-5* region shows no synergistic interaction with the *Rf-1* region.
6. The Remnant Kidney Model (RKM) is not a very suitable model to analyze renal susceptibility in rats carrying *Rf*-regions of the FHH rats. The four models we used, especially the UNX and 2K+L-NAME model appear more suitable to assess differences in susceptibility to develop renal damage.

Samenvatting

De FHH (Fawn Hooded Hoge bloeddruk) rat is een rattenstam die een matig verhoogde bloeddruk en ernstige nierschade ontwikkelt op een relatief jonge leeftijd. Koppelingsonderzoek in een kruising van de FHH rat en de nierschade-resistente ACI (August x Copenhagen Irish) rat heeft 5 "quantitative trait loci" (QTLs) opgeleverd die gekoppeld zijn aan parameters van nierschade. Deze QTLs hebben we *Renal failure-1* (*Rf-1*) tot en met *Rf-5* genoemd. Met behulp van enkel congene ratten kan de rol van ieder QTL afzonderlijk bekeken worden. Dubbel congene ratten worden gebruikt om eventuele gen-gen interacties aan te tonen. De congene ratten bestudeerd in dit proefschrift hebben de genetische achtergrond van de ACI rat waarvan een klein gedeelte vervangen is door het genoom van de FHH rat waarin een of meerdere QTLs zitten.

Het ontstaan van nierschade wordt beïnvloedt door verschillende risicofactoren. Een verminderde niermassa en een hoge bloeddruk zijn twee van deze risicofactoren. Het onderzoek naar de gevoeligheid van nierschade is uitgevoerd met behulp van vier modellen die rekening houden met de bovengenoemde risicofactoren. Het eerste model is het controle model waarbij geen interventie plaatsvindt. Deze ratten hebben twee nieren en een normale bloeddruk (2K). Het tweede model bestudeert het effect van een hoge bloeddruk, wat geïnduceerd wordt door L-NAME (2K+L-NAME). Bij het derde model wordt de niermassa verminderd door middel van een eenzijdige verwijdering van de nier (unilaterale nefrectomie, UNX). Als laatste wordt de combinatie van UNX en L-NAME geïnduceerde hoge bloeddruk uitgevoerd (UNX+L-NAME).

Eerder onderzoek heeft aangetoond dat de FHH rat een verminderde autoregulatie van de nierdoorbloeding heeft. De nierdoorbloeding wordt door twee systemen geregeld, t.w. de myogene respons en de tubuloglomerulaire feedback (TGF). De TGF bleek normaal in de FHH rat, maar de myogene respons was verminderd, resulterend in een verminderde autoregulatie van de nierdoorbloeding. In de FHH rat leidt een verhoogde bloeddruk in combinatie met een verminderde autoregulatie tot een verhoogde druk in de glomeruli, de filtereenheden van de nier. De glomeruli kunnen deze verhoogde druk niet aan en dit zal uiteindelijk leiden tot ernstige schade van de glomeruli (glomerulosclerose, FGS)

In **Hoofdstuk 1** wordt beschreven wat er op dit moment bekend is over de invloed van genetische factoren op het ontstaan en de ontwikkeling van nierschade. De *Rf-1* tot en met *Rf-5* QTLs worden hier besproken en daarmee de mogelijkheden om de gevoeligheid voor het ontstaan van nierschade te testen. In dit hoofdstuk zijn vier doelstellingen geformuleerd. De eerste doelstelling is het aantonen van een directe rol van ieder QTL in de gevoeligheid voor het ontstaan voor nierschade. De tweede doelstelling is het aantonen van een interactief effect tussen *Rf-1* en een van de andere vier *Rf*-QTLs. Het derde doel is uit te zoeken of de effecten van alle individuele *Rf*-QTLs afhankelijk is van de behandeling. De vierde doelstelling is het vaststellen van de rol van *Rf*-QTLs in de autoregulatie van de nierdoorbloeding.

Congene ratten geven de unieke mogelijkheid om de rol van een QTL in het ontstaan van een ziekte te testen. In de ACI.FHH-*Rf* congene ratten wordt een gedeelte van het FHH genoom met het desbetreffende *Rf*-QTL overgebracht naar de nierschade resistente genetische achtergrond van de ACI rat.

Dit proefschrift beschrijft de gevoeligheid voor het ontstaan voor nierschade in ACI.FHH-*Rf* congene ratten. Hiervoor zijn enkel congene ratten voor het *Rf-1, Rf-3, Rf-4* en *Rf-5* QTL gegenereerd en dubbel congene ratten die naast het *Rf-1* QTL ook het *Rf-2, Rf-3, Rf-4* of *Rf-5* QTL bevatte. In **Hoofdstuk 2** is beschreven hoe de verschillende congene stammen gefokt zijn en welke chromosomale gebieden van de FHH naar de ACI zijn overgebracht.

In **Hoofdstuk 3** wordt een studie beschreven die het ontstaan en de ontwikkeling van nierschade bestudeerd in *Rf-1B* en *Rf-5* enkel congene ratten met behulp van de bovengenoemde vier modellen. De *Rf-1B* congene rat liet een verhoogde gevoeligheid tot het ontstaan van nierschade zien ten opzichte van de ACI rat. De *Rf-5* congene rat daarentegen had geen verhoogde gevoeligheid tot het ontwikkelen van nierschade. Onderzoek naar de autoregulatie functie toonde aan dat de *Rf-1B* enkel congene rat een verminderde autoregulatie heeft, vergelijkbaar met de eerder aangetoonde verminderde autoregulatie van de FHH rat. De *Rf-5* congene rat had een normale autoregulatie functie, vergelijkbaar met die van de ACI rat. Uit deze studie kan geconcludeerd worden dat het *Rf-1B* gebied een of meerdere genen bevat die autoregulatie van de nierdoorbloeding en de gevoeligheid voor het ontstaan van nierschade beïnvloeden.

In **Hoofdstuk 4** zijn de *Rf-1A* en *Rf-4* enkel congenen en *Rf-1A+4* dubbel congeen bestudeerd ten opzichte van de ACI rat. Hierbij werd aangetoond dat de *Rf-1A* enkel congeen gevoelig is voor het ontstaan van nierschade. De *Rf-4* enkel congeen daarentegen ontwikkelde geen nierschade. De gevoeligheid voor nierschade in de *Rf-1A+4* dubbel congene rat bleek verrassend hoger te liggen dan verwacht. Statistische analyse toonde aan dat er een interactie aanwezig was tussen het *Rf-1* en *Rf-4* gebied, wat leidde tot een verhoogde gevoeligheid voor het ontstaan van nierschade. Autoregulatie studies toonde aan dat de autoregulatie functie was verminderd in zowel de *Rf-1A* als de *Rf-1A+4* congenen. De *Rf-4* congeen had net als de ACI rat een normale autoregulatie. De conclusies uit deze studies waren dat *Rf-1* genen bevat die direct de gevoeligheid voor het ontstaan van nierschade kan beïnvloeden. Het *Rf-4* gebied bevat genen die alleen indirect, in de aanwezigheid van *Rf-1*, de nierschade gevoeligheid beïnvloedt.

De nierschade gevoeligheid van de *Rf-3* enkel congenen en de *Rf-1A+3* dubbel congenen wordt beschreven in **Hoofdstuk 5**. Net als in voorgaande hoofdstukken werd de gevoeligheid voor nierschade getest met de vier eerder genoemde modellen. De *Rf-3* enkel congene rat bleek net als de *Rf-1* rat gevoelig te zijn voor het ontstaan van nierschade. De *Rf-1A+3* dubbel congene rat ontwikkelde veel meer nierschade en statistische analyse toonde aan dat er sprake was van een interactie tussen het *Rf-1* en *Rf-3* QTL, vergelijkbaar met de eerder aangetoonde interactie tussen *Rf-1* en *Rf-4*. Opvallend was dat ratten die het *Rf-3* gebied hadden, een lichte stijging in de kreatinine klaring toonde, wat neerkomt op een verhoogde glomerulaire filtratie snelheid. Autoregulatie studies toonde aan dat zowel *Rf-1A* als *Rf-1A+3* congene ratten een verminderde autoregulatie hadden. Daarentegen had de *Rf-3* congene rat een normale autoregulatie, vergelijkbaar met de ACI rat. Uit deze studie kan geconcludeerd worden dat zowel het *Rf-1* als het *Rf-3* QTL een direct effect hebben op de gevoeligheid voor het ontstaan van nierschade, en dat een interactie tussen deze twee QTLs leidt tot een verhoogde nierschade gevoeligheid. Daarnaast bevat het *Rf-3* QTL geen genen die de autoregulatie beïnvloeden.

De *Rf-1B+4* en *Rf-1B+5* dubbel congenen zijn vergeleken met de *Rf-1B* enkel congeen in **Hoofdstuk 6**. Alle vier de modellen zijn gebruikt om de nierschade gevoeligheid te testen. Ook is er gekeken naar de autoregulatie van de nierdoorbloeding. De *Rf-1B+4* ontwikkelde nierschade vergelijkbaar met de *Rf-1A+4* dubbel congene rat. De *Rf-1B+5* dubbel congene rat daarentegen, was even gevoelig voor nierschade als de *Rf-1B* enkel congene rat, onafhankelijk welk model er gebruikt werd. In alle ratten bleek de autoregulatie van de nierdoorbloeding vermindert. Conclusies van deze studies was dat het *Rf-5* gebied geen invloed heeft op de nierschade gevoeligheid, alleen of in combinatie met *Rf-1*. We gaan er daarom van uit dat er geen genen in het *Rf-5* gebied aanwezig zijn die de gevoeligheid voor het ontstaan van nierschade kunnen beïnvloeden.

In **Hoofdstuk 7** is de nierschade gevoeligheid in *Rf-1A+2* dubbel congenen vergeleken met de FHH en FHL rat. Ook is er een algemene genoom scan uitgevoerd op de FHH en FHL ratten. De nierschade gevoeligheid is alleen maar getest met het twee-nierig en UNX model, omdat uit eerdere studies is gebleken dat L-NAME in deze ratten tot een hoge sterfte leidt. De *Rf-1A+2* dubbel congeen is de enige dubbel congeen die een verhoogde bloeddruk heeft. De waarde (~145 mmHg) ligt tussen die van de FHL (lage bloeddruk) en de FHH (hoge bloeddruk) in. De FHH ontwikkelt veel nierschade, zeker na het verwijderen van een nier (UNX). De FHL ontwikkelt ook enige nierschade, echter een stuk minder dan de FHH rat. De *Rf-1A+2* dubbel congene rat heeft ook een verhoogde nierschade gevoeligheid, vergelijkbaar met de FHL rat. Een genoom scan toonde aan dat de FHH en FHL ratten voor 75% identiek aan elkaar zijn. In de *Rf*-gebieden zijn ze zelfs voor 86% identiek. Er zijn verschillen gevonden in de *Rf-2*, *Rf-4* en *Rf-5* gebieden, maar de *Rf-1* en *Rf-3* gebieden zijn identiek aan elkaar. We kunnen concluderen dat het *Rf-2* gebied een gen bevat dat de bloeddruk beinvloedt maar ook de nierschade gevoeligheid verhoogt. Het verschil van nierschade gevoeligheid tussen FHH en FHL wordt naast een verschil in bloeddruk ook nog verklaard door een verschil in *Rf*-gebieden.

In **Hoofdstuk 8** is de toepasbaarheid van het “remnant kidney model” (RKM) getest. In het RKM wordt een nier verwijderd en bij de overblijvende nier wordt 1/2 tot 2/3 van de nierfunctie weggehaald door het samenbinden van een gedeelte van de nierslagaderen. Dit model is in diverse *Rf*-congenen, ACI, FHH en FHL ratten uitgevoerd. Als eerste werd er gekeken of er verschillen aantoonbaar gemaakt konden worden tussen de ACI, FHL en FHH rat. De FHH overleefde dit model niet, maar er konden wel duidelijk verschillen aangetoond worden tussen de ACI en FHL ratten. De FHL ontwikkelde veel meer nierschade dan de ACI rat. Als tweede werd er gekeken of er verschillen waren tussen nierschade gevoeligheid tussen de ACI, *Rf-1B* en *Rf-5* enkel congene ratten. De *Rf-1B* enkel congeen ontwikkelde veel nierschade en was significant verschillend. Echter, zowel de *Rf-5* als de ACI ontwikkelde al een redelijk hoog niveau van nierschade. De laatste vergelijking werd gemaakt tussen de *Rf-1A*, *Rf-4*, en *Rf-1A+4* ten opzichte van de ACI rat. Zowel de *Rf-1A* en *Rf-1A+4* ontwikkelde meer nierschade dan de ACI rat, maar het interactieve effect tussen *Rf-1* en *Rf-4* was niet meer aan te tonen met RKM. De conclusie van deze studie is dat geschiktheid van RKM als een model om snel verschillen in gevoeligheid voor het ontstaan van nierschade aan te tonen in twijfel genomen kan worden. Het UNX met of zonder L-NAME geinduceerde hoge bloeddruk is, ondanks een langere duur van het experiment, meer geschikt.

Hoofdstuk 9 bevat de algemene discussie, enkele potentiële studies voor de toekomst en de uiteindelijke conclusies van dit proefschrift. Het fysiologisch genoom onderzoek van de rat nierschade QTLs *Rf-1* tot en met *Rf-5* leidt tot de volgende conclusies:

1. Het *Rf-1* gebied van de FHH bevat een of meerdere genen die de gevoeligheid voor het ontstaan van nierschade direct beïnvloeden en een verminderde autoregulatie van de nierdoorbloeding veroorzaken. Of deze genen dezelfde zijn moet nog verder onderzocht worden.
2. Een direct effect van *Rf-2* hebben we nog niet kunnen testen. Als het *Rf-2* gebied met het *Rf-1* gebied is gecombineerd zoals in de *Rf-1A+2* dubbel congeen, dan is er een significante stijging in nierschade te zien ten opzichte van de *Rf-1A* enkel congeen. Het *Rf-2* gebied, waar ook *Bpfh-1* in zit, verhoogt de bloeddruk.
3. Het *Rf-3* gebied van de FHH bevat een of meerdere genen die de gevoeligheid voor het ontstaan van nierschade direct beïnvloeden. Als het *Rf-3* gebied met *Rf-1* gecombineerd wordt is een synergistische samenwerking te zien die leidt tot een verhoogde nierschade gevoeligheid. De *Rf-3* enkel congene ratten hebben een normale autoregulatie. Het exacte mechanisme van *Rf-3* in het beïnvloeden van nierschade gevoeligheid moet nog ontdekt worden.
4. Het *Rf-4* gebied van de FHH bevat geen genen die de gevoeligheid voor het ontstaan van nierschade direct beïnvloeden. Daarentegen is er een synergistische interactie aanwezig wanneer *Rf-4* met *Rf-1* gecombineerd wordt. De *Rf-4* enkel congene ratten hebben een normale autoregulatie. Het exacte mechanisme van *Rf-4* in het beïnvloeden van nierschade gevoeligheid moet nog ontdekt worden.
5. Het *Rf-5* gebied bevat geen genen die direct de gevoeligheid voor het ontstaan van nierschade beïnvloeden, niet op zichzelf en niet in combinatie met *Rf-1*.
6. De geschiktheid van RKM als een model om snel verschillen in gevoeligheid voor het ontstaan van nierschade aan te tonen kan in twijfel genomen worden. De vier modellen die hiervoor gebruikt zijn, met name UNX en 2K+L-NAME, zijn ondanks een langere duur van het experiment, meer geschikt voor het bepalen van verschillen in nierschade gevoeligheid.

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Curriculum Vitae

Dankwoord

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Curriculum Vitae

Sabine Jolanda van Dijk werd geboren op 9 juli 1980 te Leidschendam. Zij volgde het Voortgezet Wetenschappelijk Onderwijs aan het Erasmus College in Zoetermeer, en behaalde haar diploma in 1998. Hierna heeft zij de opleiding Hoger Laboratorium Onderzoek (HLO) gedaan met als afstudeerrichting Medische Biologie. Voor haar afstudeerstage verrichtte ze onderzoek op het Gaubius Laboratorium van TNO Preventie & Gezondheid onder de begeleiding van Ir. D.J.M Delsing. De titel van het onderzoek luidde: "The role of apolipoprotein C1 in the development of atherosclerosis". Zij behaalde haar HLO diploma in juni 2001. In september 2001 begon zij aan haar promotieonderzoek getiteld: "Functional genomics of the rat renal failure genes" op de afdeling Kinderheelkunde van het Erasmus MC. Voor dit onderzoek zijn diverse congene ratten gegenereerd en getest op hun gevoeligheid voor het ontstaan van nierschade. Daarbij is er ook gekeken naar de autoregulatie van de nierdoorbloeding in iedere congene rat. Van september 2003 tot en met maart 2004 heeft zij onderzoek verricht op de Human and Molecular Genetics Center, Medical College of Wisconsin in Milwaukee, WI, USA. In deze periode zijn meer dan 200 genetische markers getest op polymorfische eigenschappen tussen de ACI en FHH rat. Met behulp van deze informatie zijn alle tot nu toe gegenereerde *Rf*-congene ratten genetisch in kaart gebracht. Daarnaast heeft zij ook tijd besteed aan het uitvoeren van Subtractive Suppressive Hybridization op nierweefsel van ACI ratten en *Rf-1* enkel congene ratten. De jaren van onderzoek aan zowel de Erasmus MC als de Medical College of Wisconsin leidde tot dit proefschrift. In september 2005 is zij begonnen aan de SUMMA geneeskunde opleiding te Utrecht.

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