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**Molecular Studies of Complications in  
End Stage Renal Disease  
–Focus on Expression and Variations of Candidate  
Susceptibility Genes**

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**Molecular Studies of Complications in End Stage Renal Disease  
–Focus on Expression and Variations of Candidate Susceptibility Genes**

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*“När du verkligen vill någonting  
verkar hela universum  
för att du ska få din önskan uppfylld”*

*(ur Alkemisten, Paulo Coelho)*

**To my wonderful family**

## ABSTRACT

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End stage renal disease (ESRD) is a complex multifactorial disorder, where both environmental and genetic factors are contributing to the phenotype. The most common complications seen in ESRD patients are malnutrition, inflammation and atherosclerosis and the coexistence of these complications is denoted the MIA syndrome, which is associated with inexplicably high mortality. However, due to big inter-individual differences not all patients have the same risk of developing the different complications of the MIA-syndrome.

The objective of this thesis is to contribute to the development of an approach on how to use genetics as an instrument for identifying “high risk” ESRD patients at an early stage and to provide the tools that can help to develop more accurate and individually tailored treatment strategies in the future. With this aim, several susceptibility genes and proteins have been investigated with relation to the MIA syndrome and mortality in ESRD. They are presented in the following papers:

**Paper I;** Myeloperoxidase (MPO) is suggested to be one link between inflammation, oxidative stress and endothelial dysfunction in uremic patients. The effect of a functional single nucleotide polymorphism (SNP) -463 G/A, that induces a 25-fold transcription enhancement, was analyzed in relation to the MIA complications and oxidative stress in ESRD patients. The G/G genotype was associated with higher prevalence of inflammation and CVD, as well as with higher levels of an oxidative stress marker, a finding that replicates other studies.

**Paper II;** The role of the anti-atherosclerotic protein adiponectin was studied in ESRD and the impact of variations of the adiponectin gene (ApM1) on plasma adiponectin levels was investigated. In this study we found that plasma levels of adiponectin were markedly increased in ESRD patients. Additionally, patients with insulin dependent diabetes mellitus (DM) had even higher levels as compared to both non diabetic and non insulin dependent DM patients. Among the four SNPs that were studied (-11391G/A, -11377C/G, 45T/G, 276G/T) only the -1377 C/C genotype was significantly associated with a lower prevalence of CVD as well as lower triglyceride levels. The low impact of the individual SNPs on plasma levels and presence of the MIA-complications shows the importance of analyzing and identifying risk-haplotypes that may have greater impact on the phenotype.

**Paper III;** Since adiponectin levels are markedly elevated in ESRD, we analyzed the expression of ApM1 in fat tissue from patients with a high prevalence of MIA complications, patients who had few complications and healthy matched controls. We found that the ApM1 gene expression was markedly decreased in ESRD patients as compared to controls, but no significant difference was noted between the two groups of patients. The decreased ApM1 expression could be explained by a negative feedback regulation due to the high levels of circulating adiponectin.

**Paper IV;** Plasma levels of interleukine-6 (IL-6) and C-reactive protein (CRP) are highly elevated in ESRD patients and predict mortality in both healthy and uremic individuals. Hence putative SNPs that may regulate plasma levels of these proteins were investigated (IL-6 SNPs: -597G/A, -174G/C, 5014A/G, Phe201Phe C/T and CRP SNPs: -286C/T/A, 1059G/C). We found that a genotype combination (-597G, -174G, 5014A) was associated with inflammation in ESRD patients. However, the lack evident impact on plasma levels, which is seen in other studies, may be explained by the strong effect of a promoter haplotype. Hence, further studies are necessary to evaluate how genotype combinations regulate the plasma levels of IL-6 and CRP.

**Paper V;** Resistin is a newly discovered protein that in rodents may promote insulin resistance. Resistin may have a direct pro-inflammatory effect on vascular endothelial cells, which could enhance the development of atherosclerosis. The role of resistin on insulin resistance was investigated in ESRD patients as well as the impact of a functional -180 C/G SNP on resistin plasma levels. We found that circulating levels of resistin were highly elevated in patients and that plasma levels correlated with glomerular filtrations rate. However, resistin did not seem to be associated with insulin resistance. Plasma resistin was associated with both adhesion molecules and inflammatory markers. Our findings suggest that resistin plasma levels should always be corrected for GFR in all study populations.

The recent development in the field of genetics has made it possible to understand the impact of genotype in disease development and progress. It seems conceivable that in near future, prognostic or predictive multigene DNA assays will provide the nephrological community with a more precise approach for the identification of “high risk” ESRD patients and the development of accurate individual treatment strategies.

**Keywords:** End Stage Renal Disease (ESRD), Single Nucleotide Polymorphism (SNP), gene expression, *in situ* hybridization, myeloperoxidase (MPO), adiponectin, ApM1, C-reactive protein (CRP), interleukine 6 ( IL-6), resistin.

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This thesis is based on the following publications, which will be referred to by their Roman numerals.

- I. Pecoits-Filho R, Stenvinkel P, **Marchlewska A**, Heimbürger O, Barany P, Hoff C, Holmes C, Suliman M, Lindholm B, Schalling M and Nordfors L. A functional variant of the myeloperoxidase gene is associated with cardiovascular disease in end-stage renal disease patients. *Kidney Int Suppl*, 2003, (84), 172-6.
- II. Stenvinkel P, **Marchlewska A**, Pecoits-Filho R, Heimbürger O, Hoff C, Holmes C, Axelsson J, Arvidsson S, Schalling M, Barany P, Lindholm B, Nordfors L. Adiponectin in renal disease: Relationship to phenotype and genetic variation in the gene encoding adiponectin. *Kidney Int.*, 2004, (65), 274-281.
- III. **Marchlewska A**, Stenvinkel P, Lindholm B, Danielsson A, Schalling M, Heimbürger O, Nordfors L. Reduced gene expression of adiponectin in fat tissue from patients with end-stage renal disease. *Kidney Int.*, 2004, (66), 46-50.
- IV. **Bergsten A**, Stenvinkel P, Kockum I, Pecoits-Filho R, Barany P, Hoff C, Holmes C, Jönsson E, Lindholm B, Nordfors L, Heimbürger O. Genetic influence on inflammatory biomarkers in ESRD patients: a study of CRP and IL-6 single nucleotide polymorphisms. (Manuscript)
- V. Axelsson J\*, **Bergsten A\***, Heimbürger O, Barany P, Lönnqvist F, Lindholm B, Nordfors L, Alvestrand A, Stenvinkel P. Elevated resistin levels in chronic kidney disease are associated with decreased glomerular filtration rate and inflammation, but not with insulin resistance. (Manuscript)

\* *First authorship*

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## ABBREVIATIONS

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|                 |                                                        |
|-----------------|--------------------------------------------------------|
| ApM1            | Adipose tissue Most Abundant factor 1                  |
| BMI             | Body Mass Index                                        |
| CAD             | Coronary Artery Disease                                |
| CHD             | Chronic Heart Disease                                  |
| CKD             | Chronic Kidney Disease                                 |
| CRF             | Chronic Renal Failure                                  |
| CRP             | C-reactive protein                                     |
| CVD             | Cardiovascular Disease                                 |
| DM              | Diabetes Mellitus                                      |
| DNA             | Deoxyribonucleic Acid                                  |
| ELISA           | Enzyme-Linked Immunosorbent Assay                      |
| ESRD            | End Stage Renal Disease                                |
| GFR             | Glomerular Filtration Rate                             |
| HD              | Hemodialysis                                           |
| H-W equilibrium | Hardy Weinberg equilibrium                             |
| hsCRP           | high sensitivity CRP                                   |
| IL              | Interleukine                                           |
| kDa             | kilo Dalton                                            |
| LD              | Linkage disequilibrium                                 |
| LDL             | Low Density Lipoprotein                                |
| MIA syndrome    | Malnutrition Inflammation and Atherosclerosis Syndrome |
| MPO             | Myeloperoxidase                                        |
| mRNA            | messenger Ribonucleic Acid                             |
| PCR             | Polymerase Chain Reaction                              |
| PD              | Peritoneal Dialysis                                    |
| RRT             | Renal Replacement Therapy                              |
| SNP             | Single Nucleotide Polymorphism                         |
| TNF- $\alpha$   | Tumor Necrosis Factor $\alpha$                         |
| VNTR            | Variable Number of Tandem Repeats                      |

## INTRODUCTION

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End stage renal disease (ESRD) is a complex multifactorial disorder with high mortality. The most common complications seen in ESRD patients are malnutrition (M), inflammation (I) and atherosclerosis (A) and the coexistence of these complications is denoted the MIA syndrome, which is present in about thirty percent of the patients [1-3]. The MIA syndrome is associated with poor outcome, with mortality rates similar to those of most metastatic malignancies. Interestingly there are big inter-individual differences and not all ESRD patients develop these devastating complications.

Since traditional risk factors such as hypertension, dyslipidemia and smoking can only explain a part of the inter-individual differences, non traditional risk factors i.e. inflammation, oxidative stress, vascular calcification and genetic factors must also be considered. Despite that the last few decades of research has focused on developing and improving renal replacement therapies (RRT), the trend of increased morbidity and mortality, mainly due to cardiovascular disease (CVD), has not been altered [4].

It is important to elucidate the role of the genetic background behind the susceptibility and the development of MIA complications in ESRD. To identify the genetic risk factors in renal disease is as great a challenge as it is indispensable for the patients suffering from kidney failure since the only true remedy today is kidney transplantation. The general aim of this thesis was to contribute to the development of an approach on how to use genetics as an instrument for identifying “high risk” ESRD patients at an early stage and to be able to develop more accurate and individually tailored treatment strategies in the future.

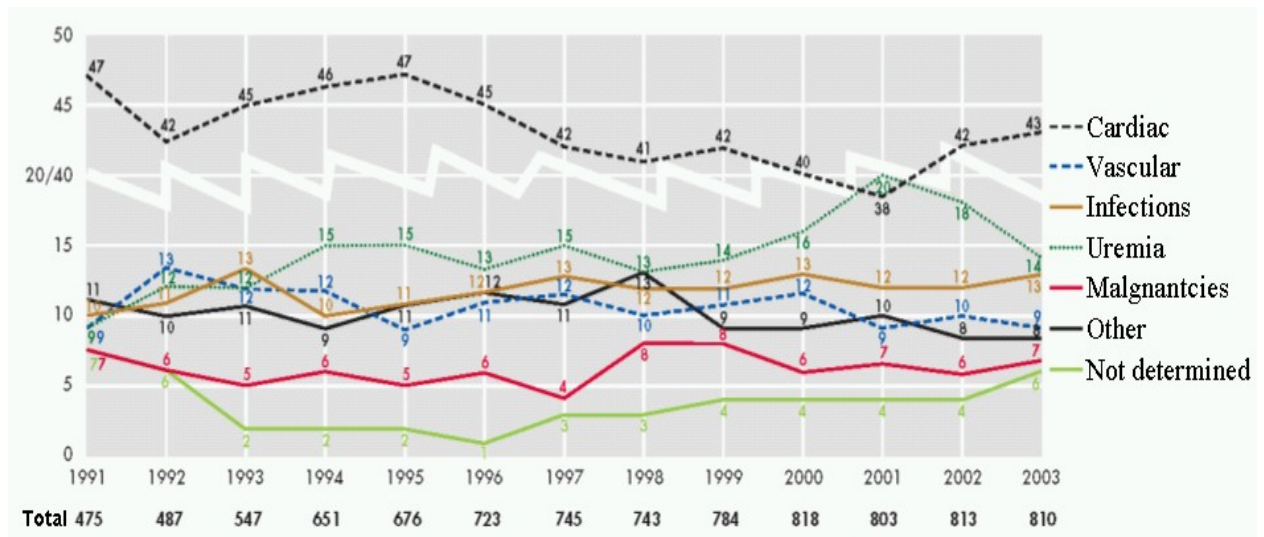
### **End stage renal disease – clinical background**

The role of the kidneys is mainly to process the plasma and remove substances such as metabolic waste products and foreign chemical compounds and excreting them through urine. The kidneys also regulate inorganic ion concentration and volume of the internal environment. Another important function is gluconeogenesis and secretion of hormones [5].

End stage renal disease is the last stage of kidney failure before initiation of dialysis treatment, or renal transplantation. ESRD is also called chronic kidney disease (CKD) stage 5. There are many underlying causes leading to impaired kidney function, the most common being glomerulonephritis and diabetes.

### ***Kidney failure in Sweden***

The number of patients in Sweden that are undergoing RRT has grown continuously and has reached a number of 6950 patients (dialysis (n=3273) and transplantation (n=3677)) in 2003. During 1991-2003 the annual mortality rate was estimated to be 28% (ranging from 23-30%). The main cause of mortality is CVD followed by uremia, infections, other complications and malignancies [4]. See **figure 1**.



**Figure 1.** Mortality rates of dialysis patients during the years 1991-2003. The causes of mortality are grouped individually and presented in percent (n=9075). (The figure was kindly provided by S. Schön, *Aktiv uremivård i Sverige, 1991-2003.*)

### ***Prevalence of the MIA complications in other countries***

The majority of the studies describing the presence of complications in the MIA syndrome have been performed in western industrialized countries and in Asia. Epidemiological studies on both renal and non-renal populations show that there are clear ethnical differences in the prevalence of malnutrition, inflammation and atherosclerosis. For example, the occurrence of malnutrition ranged from 30 to 44% in Swedish [6], Spanish [7] and in Dutch [8] predialysis patients and in Asian ESRD populations malnutrition is reported to range from 42% to 77% [9]. However, the prevalence of inflammation in ESRD patients has been documented to be significantly lower in East Asian countries as compared to western countries [10].

Despite the differences seen in the above mentioned complications between geographical regions, the main cause of mortality among dialysis patients is CVD. Even though the industrialized countries have in the recent 30-40 years shown a decline in CVD mortality, there is a discordant trend seen in the developing countries [11]. It has been speculated that the introduction of a western lifestyle may lead to an altered nutritional intake and lifestyle changes, thereby contributing to the increased prevalence of CVD [11].

Furthermore, chronic infections such as HIV, hepatitis B and C as well as malaria are more common in the developing countries and it has been suggested that presence of an infection may accelerate the progress of atherosclerosis, suggesting that there may be an important link between infectious diseases and both inflammation and atherosclerosis. Indeed, the presence of *helicobacter pylori* DNA has been found in atherosclerotic plaques [12] and in non renal patients *helicobacter pylori* infection causes a chronic inflammation that induces overproduction of cytokines. Interestingly, studies in uremic patients have shown that *helicobacter pylori* is associated with anorexia, inflammation and malnutrition [13]. It is consequently of great interest to broaden the knowledge on how chronic infections may affect atherogenesis in populations with increased risk of CVD [10].

### ***Treatment of renal insufficiency***

Although transplantation is the optimal treatment this is not available for all patients, why most ESRD patients need dialysis, temporarily or lifelong. Most nephrologists believe that peritoneal dialysis (PD) and hemodialysis (HD) are of equal merit to restore effective blood purification.

During PD, dialysis fluid is administered to the peritoneal cavity through a catheter, which is surgically inserted into the abdomen where the waste products are exchanged into the dialysis fluids through osmosis in the fine vessels of the stomach cavity. The dialysis fluid is changed 4-5 times a day and can be performed by the patient. Hemodialysis is the most common treatment and is usually performed at hospitals during 3-4h sessions and repeated 3 times a week. During HD the blood is circulated through a dialyze filter and the waste products are removed whereafter the purified blood is returned.

To investigate the adequacy of PD and HD two large clinical trials (ADEMEX and HEMO) have been performed. Interestingly, inadequate dialysis is apparently not the problem since it was found that currently recommended doses are adequate and that improved dialysis does not alter the mortality rate [14].

## **Complications of the MIA syndrome in ESRD**

### ***Malnutrition***

Malnutrition is commonly seen in patients with ESRD and although being one of the strongest predictors of mortality it is not believed to be the prominent underlying cause of death in this patient group. Malnutrition may exist in two fundamentally different types, where the first type may be related to the uremic syndrome *per se* with low protein and energy intake while the second type of malnutrition may be associated with significant comorbidity and inflammation [15]. The most evident cause of malnutrition in dialysis patients is low protein and energy intake. This may partly be explained by the uremic toxicity caused by inadequate dialysis. Other factors that may contribute to the development of malnutrition are acidosis, infections, comorbidity, psychological factors, physical inactivity but also the presence of inflammation [16]. Patients with chronic kidney disease (CKD), who are malnourished, usually show signs of an ongoing acute-phase response and/or carotid plaques, suggesting that malnutrition is closely associated with atherosclerosis and increased inflammatory response [17]. Chronic inflammation, as determined by increased levels of proinflammatory cytokines such as interleukine-6 (IL-6) and the acute phase protein C-reactive protein (CRP) is a common feature in ESRD patients. Although the proinflammatory cytokines stimulate the acute phase response in the liver [18] and induce the production of CRP [19], it has been suggested that IL-6 may also be involved in satiety signal transduction and hence play a role in malnutrition [20].

### ***Inflammation***

It has been suggested that reduction in kidney function *per se* may lead to a chronic inflammatory response [21]. An acute phase response induced by inflammatory stimuli lasts only a few days, while in some ESRD patients, the acute phase response is chronic. A commonly used inflammatory marker is CRP. The amount of CRP in the blood is proportional to the pro-inflammatory response mediated by cytokines such as IL-1, IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and which mirrors the magnitude of the inflammatory status [22]. C-reactive protein has recently attracted much interest since current studies show that CRP is a powerful predictor of cardiovascular risk and mortality, in both non-renal and uremic patients [19, 23]. C-reactive protein is hence no longer considered only as a marker of inflammation but may actually play an active role in the development of atherosclerosis [23]. In the uremic patient, the CRP levels fluctuate markedly over time [24] and a clinical cut off for plasma CRP levels, indicating an ongoing inflammation, has been estimated to >10-15mg/l [23]. It is questioned whether the presence of chronic inflammation may contribute to the development of vascular

disease or if inflammation is a result of vascular injury [23]. The combination of an impaired immune response together with constant immune stimulation (by for example dialysis treatment) may lead to low-grade systemic inflammation and altered cytokine balance which in turn may partly explain the increased risk of CVD.

### ***Atherosclerosis***

Cardiovascular disease, the single largest cause of mortality in the general population, is also the main cause of death in uremic patients and accounts for the premature death in more than 50% of the patients from Western Europe and North America undergoing regular dialysis [25]. The magnitude of cardiovascular complications across different age-groups of patients with uremia is significantly different from that in the general population. Thus, a marked inconsistency between the vascular and chronological age is seen and the risk to develop CVD in a 30 year old ESRD patient is similar to the calculated risk of a 70-80 year old individual from the general population [23].

It has been shown that the atherosclerotic process accelerates at the initiation of RRT but the reasons for the dramatic increase are not fully understood. One cause might be inflammation, which is associated with stimulated production of soluble adhesion molecules. Levels of adhesion molecule ICAM-1 are significantly elevated in CRF patients with CVD and plasma levels of adhesion molecules have been shown to predict mortality [26]. Other factors that may contribute to the development of atherosclerosis is oxidative stress, vascular calcification as well as genetic background [27].

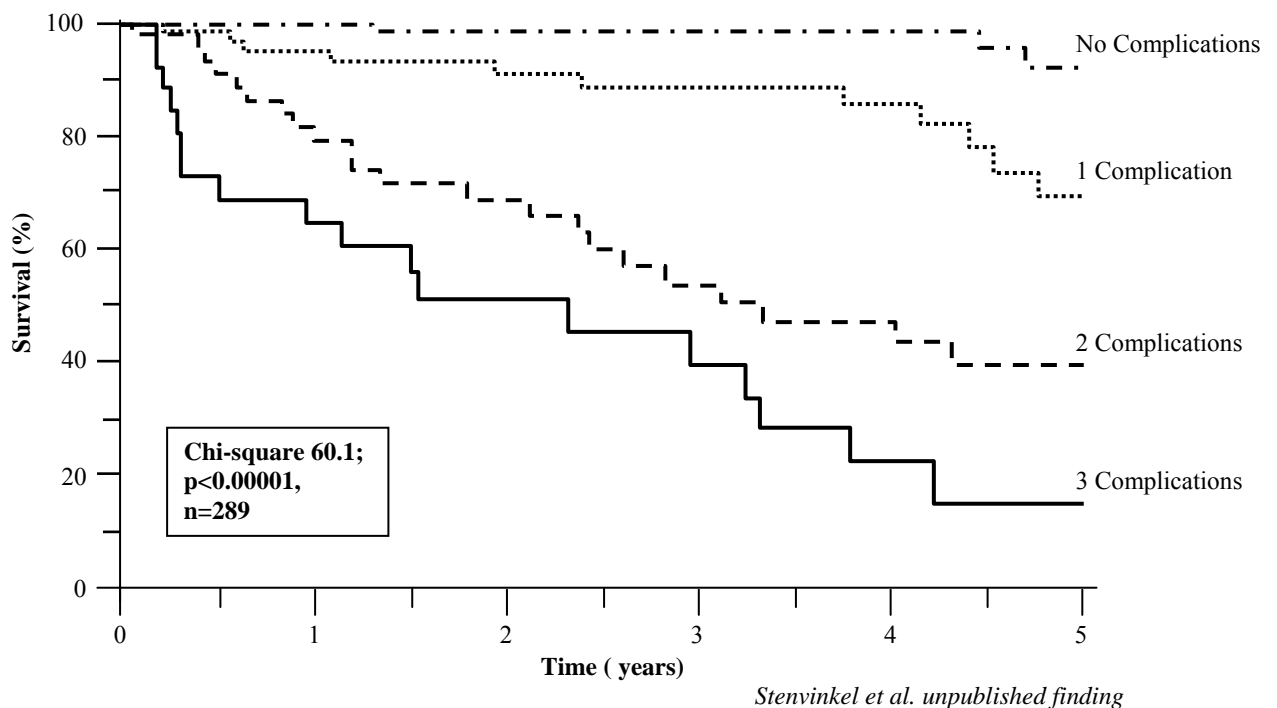
### **Oxidative stress**

Oxidative stress (or oxidant-derived tissue injury) may accelerate the process of atherosclerosis in the uremic patient and takes place when the production of oxidants exceeds the local antioxidant capacity [28]. The imbalance between endothelial damage and the capacity to repair the vascular wall plays a role in many diseases. However, this imbalance is particularly important in ESRD since the uremic milieu *per se* may enhance the generation of pro-oxidants and decrease serum levels of antioxidants which in turn increases the oxidative burden as the kidney failure progresses [28]. Oxidative stress is proceeding in phagocytes where four enzymes are involved in this process i.e. nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, a superoxide producer, superoxide dismutase, a hydrogen peroxide producer, nitric oxide synthase (NOS), a hydrogen peroxide producer and myeloperoxidase (MPO), a hypochlorous acid producer [28]. These enzymes can oxidize low density lipoprotein (LDL) *in vitro* and are expressed in atherosclerotic lesions [27]. There are several clinical markers that can be used for

the evaluation of oxidative stress, which most often are end products of the multiple reactions where MPO is involved, i.e. chlorinated tyrosines, arachidonic acid derivatives and reactive aldehydes to mention a few. Reactive aldehydes can be formed as the end product of different oxidative reactions. One such reaction is the myeloperoxidase-catalyzed oxidative modification of amino acids, where reactive aldehyde compounds, such as glyoxal and pentosidine, are formed. The formation of atherosclerotic plaque is accelerated by the process of oxidative stress. It could be speculated that inflammation, chronic infection, malnutrition and accelerated atherosclerosis are linked via oxidative stress.

### ***The MIA-syndrome***

Based on the strong associations observed between malnutrition, inflammation and atherosclerosis in CKD patients it has been proposed that all these three features constitute a specific syndrome (MIA) [3, 17]. The coexistence of these complications in ESRD patients is associated with high mortality. See **figure 2**.



**Figure 2.** Malnutrition, inflammation and atherosclerosis are associated with a poor prognosis in ESRD. *Reproduced with permission from P. Stenvinkel.*

## **Common molecular genetics**

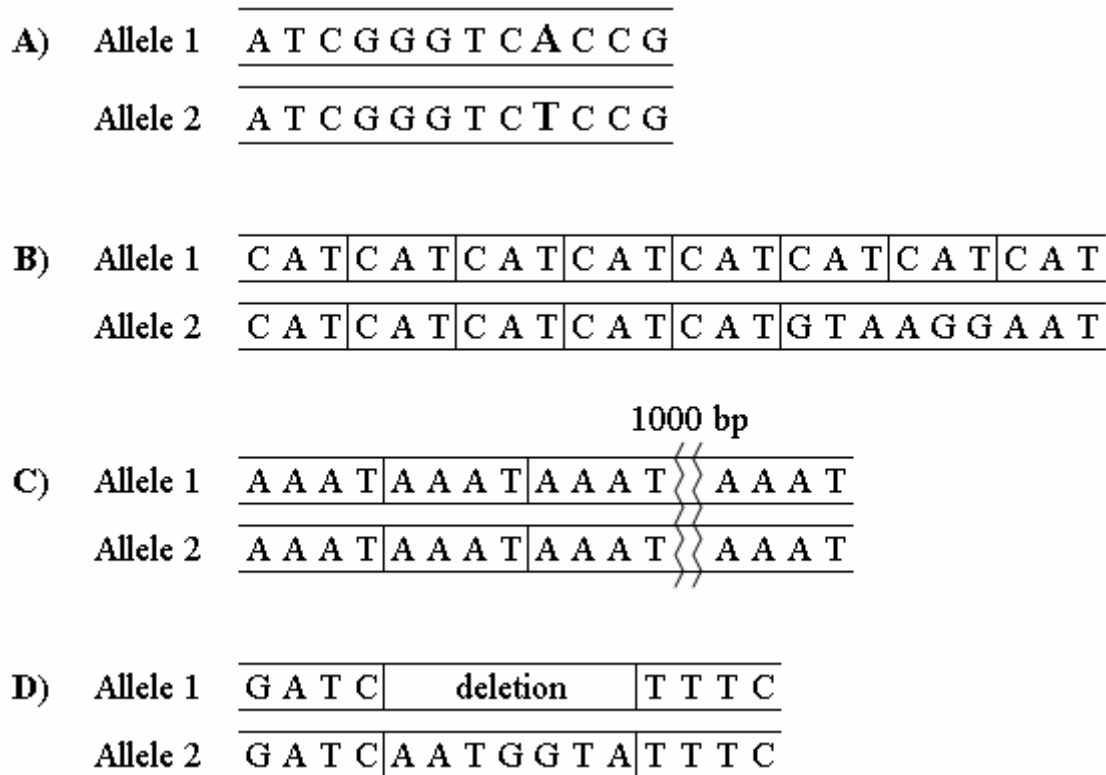
A common disease rarely results from a single mutation in a single important key regulating gene. A specific phenotype may be resulting from the impact of several minor polymorphisms e.g. SNPs, which modulate the risk of developing a disease in each individual. In the absence of this kind of genomic differences between individuals we would all respond in a similar way to environmental exposure and the risk of developing a disease would probably be directly related to the magnitude of environmental exposure. We know, however, that this is not the case. Not all ESRD patients develop the MIA complications and the individual risk of premature mortality differs markedly.

Since ESRD is a multifactorial disorder, with both environmental and genetic factors contributing to the phenotype, a single mutation may have a modest effect in individuals who have low environmental risk, but a major effect when exposed to a particular environment.

### ***Genetic polymorphisms***

Two unrelated individuals share 99.9% of the genome, [29] meaning that we are heterozygous at approximately every 1000 base pairs. A variation is denoted a polymorphism when it is found in more than 1% of the chromosomes in the general population. There are several types of polymorphisms with more than one allele at significant frequencies in the population, e.g. single nucleotide polymorphisms (SNP), mini and microsatellites including variable number of tandem repeats (VNTRs) and insertion/deletion polymorphisms. See **figure 3**.





**Figure 3.** A) Single base substitution B) Microsatellite DNA (less than 0.1kb) moderate arrays of a repeat unit length of 1-4bp, includes VNTRs which constitutes of 5-64bp long repeats, C) Minisatellite DNA (0.1-20kb) an intermediate size array of short tandemly repeated DNA sequences D) insertion/deletion of a DNA sequence.

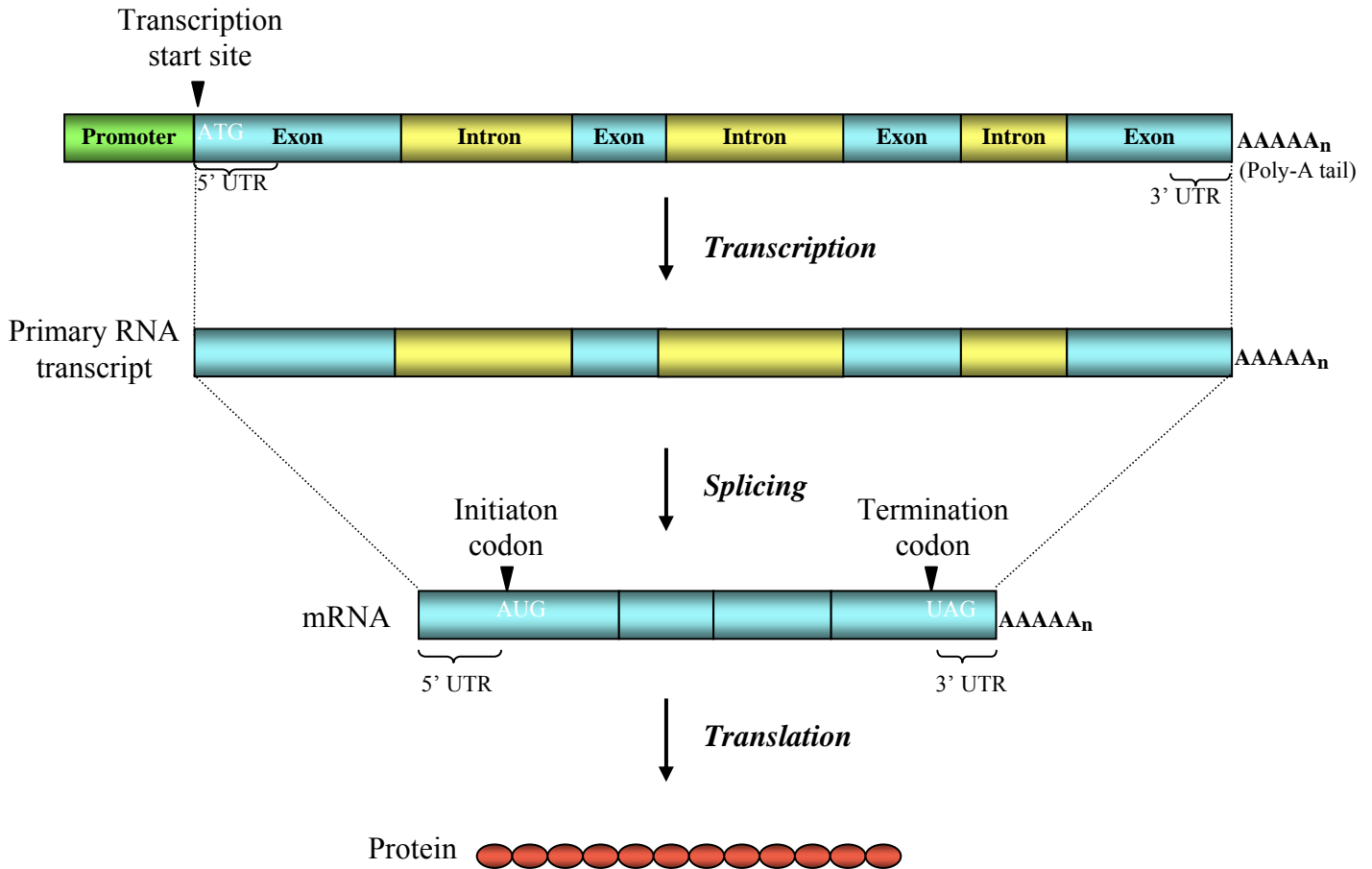
Depending on where in the human genome a SNP is placed, the genetic structure, genetic expression and/or protein conformation can be altered. **Figure 4** is showing a schematic overview of the fundamental steps in the process from a gene sequence to the gene product.

A SNP that is positioned in the promoter region can disturb transcription binding sites and thereby affect transcriptional activity and hence the gene product levels. SNPs are denoted differently depending on their position within the coding sequence. When a SNP changes an amino acid (as for example Ala→Val) it is called a non synonymous or missense mutation. Such mutation can alter the protein structure and/or function. A synonymous mutation is also called a silent mutation, meaning that the SNP does not alter the amino acid for example (Leu→Leu) and thereby does not directly affect the protein. When a SNP alters the genetic code and induces a premature stop codon, it is called a nonsense mutation, resulting in a truncated gene product with no or altered function.

Polymorphisms that are located in non coding regions such as introns or untranslated regions (UTRs) are more likely to affect the stability of the mRNA and the processing of the molecule.

It is important to keep in mind that one gene can encode several gene products due to alternative splicing. In addition one promoter can regulate the transcription of several genes while several promoters can be involved in transcription of only one single gene. This means that the polymorphisms positioned within the regulating regions can influence genes that are not directly downstream of the affected promoter and also that genes that are not in the immediate vicinity of the regulating region can be affected. To determine if a polymorphism is truly contributing to the phenotype, functional studies such as *in vitro* expression and animal studies must be performed.

There are several other elements that can affect the transcription and translation of a protein. A combination of short sequence elements positioned upstream of a gene, so called cis-acting elements, act as recognition signals for transcription factors that in turn bind to deoxyribonucleic acid (DNA) to initiate the transcription of a gene. Such short sequences are the GC box, the TATA box and CAAT box to mention a few. These elements constitute the promoter of a gene. However, to initiate transcription, transcription factors are needed. The transcription factors are called trans-acting elements, meaning that they are synthesized by genes which are remotely located. The associated trans-acting factor for the GC box is for example Sp1, while C/EBP activates the CAAT box. Other factors that can affect the transcription and translation of a gene are enhancers, which increase the transcriptional activity and silencers which inhibit the transcription. Enhancers and silencers are usually positioned at major distances from the transcriptional start site of the gene that they are affecting.



**Figure 4.** The gene structure and the fundamental steps in formation of the protein are shown in this figure. Gene expression is initiated by the binding of transcription factors to specific sites in the promoter. Transcription starts at the transcription start site (ATG on gene level) and exons, including the 5'- and 3' untranslated regions (UTRs) and introns are included in the primary RNA transcript. The primary transcript is spliced into mRNA that only consists of exons. Translation is initiated at the translation start site (an AUG codon at mRNA level) and the coding exons will serve as templates determining the amino acid sequence of the protein until reaching one of the three stop codons (UAA,UAG,UGA). The 5'- and 3' UTRs are excluded during translation. (Reproduced with permission from Nordfors et al 2005.)

### ***Hardy-Weinberg equilibrium***

In the general population the genotype distribution of a single nucleotide polymorphism should always be in Hardy-Weinberg (H-W) equilibrium.

The law of H-W equilibrium was founded in 1908, by Godfrey H Hardy and Wilhelm Weinberg and is basically stating that in a randomly mating population the relative frequencies of heterozygotes and homozygotes are mathematically related and that allele frequencies will remain constant from generation to generation provided that none of the alleles is under positive or negative selection. Several criteria must be fulfilled for a polymorphism to be in H-W equilibrium, namely random mating, absence of selection and gene flow, a large enough population, no change of mutation frequency and finally no chromosomal rearrangements

A SNP should always be tested for H-W equilibrium, both in the patient and control group, since it may reveal diversions from any of the criteria above or a selective genotyping error. The equilibrium can easily be tested by using the H-W equation:  $(p+q)^2 = p^2+2pq+q^2 = 1$ , where  $p$  is the allele frequency of allele 1 and  $q$  is the allele frequency of allele 2 and  $2pq$  is the frequency of heterozygotes. The observed allele frequencies are calculated and used to find the expected genotype frequencies. The observed and expected genotype frequencies are compared using the  $\chi^2$  test. A  $p$  value that is  $<0.05$ , indicates that a polymorphism is not in H-W-equilibrium, which means that the observed and expected genotype distribution values differ significantly.

If a SNP does not follow the H-W law in the patient group it could be interpreted to strengthen the evidence of association between the SNP and the phenotype because of increased frequency of a certain genotype that is associated with the phenotype. On the other hand, H-W equilibrium must always be obtained in the control group when performing association studies.

One other explanation why a SNP does not follow the H-W equilibrium law could be complex SNP related sequence variations, a recently discovered form of genome polymorphisms, called multisite variations (MSVs) that usually do not follow the H-W equilibrium. An MSV can masquerade as a SNP, but may in reality be a duplicon marker that arises from segmental duplications of a sequence. MSVs represent the sum of the signals from many individual duplicon copies that vary in sequence content due to duplication, deletion or gene conversion [30].

Other recently discovered variations in our genome are large scale copy number variations (LCVs). LCVs are gains and losses of genomic DNA ranging from several to hundreds of kilobases and are found in phenotypically normal

individuals. Over 200 LCVs are described in the human genome where Iafrate et al. shows that there are 24 common LCVs that were present in >10% of the subjects and six of these variants were present in >20% of the subjects [31].

Our genome is interspersed with duplicons of sequences, which results in multiple similar sequences that contain SNPs. Hence all the variations in an analyzed sequence will sum up when genotyping a specific SNP in such duplicates, which may lead to inaccurate results about an individual's true genotype [30]. MSVs must also be considered when DNA is pooled for SNP analysis. Since there are inter-individual differences in the number and size of the duplicates the results from a pooled material may be misleading, not representing the correct genotype frequencies. Further, LCVs that contribute to the differences in our genome may complicate the genetic analyses of complex phenotypes greatly, especially if the candidate genes are positioned within an LCV. When conducting future genetic studies it will be necessary to correlate the obtained genetic experimental data with the LCVs database (the Genome Variation Database, <http://projects.tcag.ca/variation/>) [31].

The recent finding of MSVs and LCVs is highly important, since it shows us that there is still much to learn about our genome and that the current dogma can and will be questioned.

### ***Linkage disequilibrium***

Markers, such as SNPs, that are positioned on the same chromosome can segregate together more often than expected and are then said to be in linkage disequilibrium (LD). Linkage disequilibrium can be calculated and is dependent on allele frequencies as well as of recombination. The two most common measures of LD are Lewontin's LD parameter  $D'$ , which is a measure of recombinations, and  $r^2$ , which is a measure of correlation. When  $D'=1$  the two markers are in total LD, meaning that there is no recombination and when  $r^2=1$  the allele frequencies of the markers are equal and there is no recombination meaning that the markers are in total LD. The two LD measures do not have to follow each other, i.e.  $D'=1$ , but  $r^2=0.6$  for the same two markers means that the markers segregate together more often than expected, but the allele frequencies are not equal. Linkage disequilibrium is an association between polymorphisms at the population level, where alleles in close proximity of each other are often inherited together, since they originate from a common chromosome. A polymorphism can be in LD with another polymorphism on the same chromosome, which is associated with a trait and thereby act as a marker for that particular disease.

## Association studies in ESRD

Association studies assess the correlation between genetic variants (alleles) and disease and have a considerably higher resolution in the mapping of disease genes as compared to linkage methods. Current strategies to map common multifactorial disease genes are first to perform linkage analysis to locate the correct loci and then to perform association analysis of SNPs to narrow down the region. For the association analysis, detection of polymorphisms, determination of LD patterns and construction of haplotypes, are the preferred approaches.

When performing association studies in a complex disease, the size of the study population is very important and has to be large enough to reach sufficient power to detect even small effects of single SNPs. The effect of a candidate gene or polymorphism is analyzed in a well established population carrying the disease or trait and is compared with appropriate healthy controls.

The selection of candidate SNPs is based on biological applications which increases the probability of finding an association. In ESRD the number of genes that may be considered as candidates are numerous due to complex gene-gene and gene-environment interactions.

There are methodological cautions and considerations that are crucial when performing association studies, namely phenotype definition, choice of appropriate controls, correction for population stratification and adjustment for multiple comparisons [32].

A SNP provides a marker function of a particular disease locus if it is in linkage disequilibrium with a functional genetic polymorphism. The SNP may be functional itself and an association to a phenotype can be physiologically relevant. The functionality of a polymorphism could be demonstrated both in *in vitro* studies as well as by replication in other populations. An association with multiple linked SNPs in the same region, defined as a haplotype block, provides even stronger evidence. **Table 1** is showing a checklist when performing association studies in complex phenotypes.

**Table 1.** Important questions that should be considered when analyzing association between a polymorphism and a complex phenotype

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- Is the material large enough to be sufficiently powered?
  - Have controls been evaluated?
  - Are the SNPs positions clearly defined?
  - Has the H-W equilibrium been calculated?
  - Has linkage disequilibrium (LD) between different SNPs on the same chromosome been evaluated?
  - If the material has sufficient power - has a haplotype analysis been performed?
  - Has the protein product(s) been analyzed?
  - Is the function(s) of the SNP(s) described?
  - Has a multiple regression analysis been performed accounting for the impact of age, race, gender and co-morbidity?
  - Are the results confirming the results in other patient groups?
- 

There are four critical cornerstones when performing association studies in an ESRD population. The first cornerstone is to have a clinically well characterized patient material, as well as serum and plasma samples for measuring different protein levels, blood for DNA preparation and tissue biopsies for expression studies. It is important that the sample size is large enough to establish small effects of SNPs, a large enough control group, preferably that can be used for comparisons of clinical parameters as well as genetic factors. A second cornerstone is to carefully identify candidate genes and polymorphisms, which requires thorough understanding of the involved pathophysiologic pathways and in this case, careful background work about the genes and proteins that are related to the MIA-complications. The third cornerstone is to link the genetic variation(s) to the phenotype, where functional experiments are needed as well as replications of the results in other study populations. A fourth and final cornerstone is application, how to use the obtained information as a tool for early risk stratification, prediction

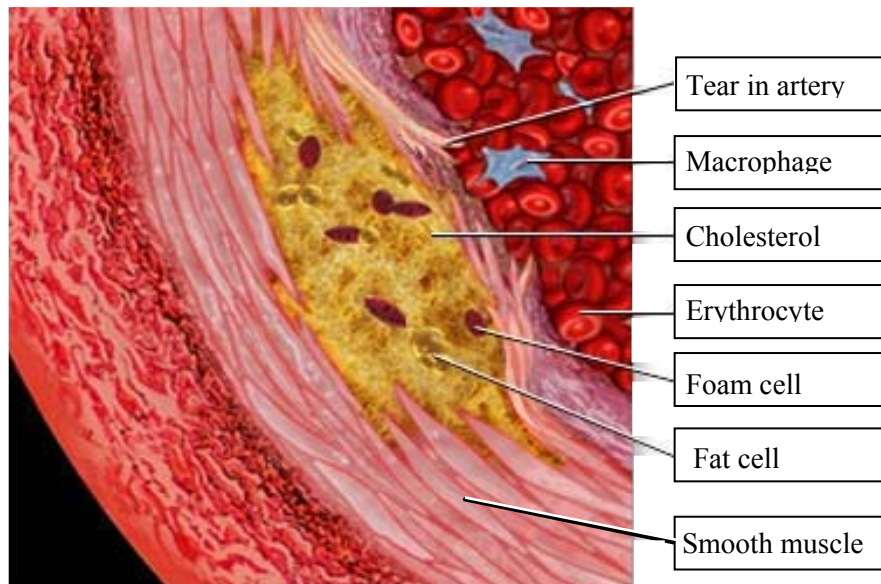
of response to specific treatment, in pharmacogenetics and for the design of clinical trials.

## **Susceptibility genes and proteins in ESRD**

### ***Myeloperoxidase (MPO)***

MPO is an abundant hemoprotein, released by activated neutrophils, monocytes and tissue-associated macrophages. The production of MPO is activated by acute and chronic inflammatory stimuli [33] and its main biological function is in the defense of the organism against foreign substances, such as bacteria. This powerful source of antimicrobial activity can be harmful to the vascular wall both due to the generation of diffusible oxidants that are involved in lipid peroxidation and due to the consumption of nitric oxide. The increased levels of MPO may thereby significantly contribute to endothelial dysfunction and hence to the development of premature atherosclerosis. MPO is involved in the production of free radicals, which also are damaging the vascular wall and hence affect the development of atherosclerotic plaque [33]. In fact, these oxidative agents contribute to the decreased NO bioavailability in the tissue and may thereby affect the vascular reactivity [34] by impairing the endothelium-dependent relaxant response [35]. Another pro-atherogenic activity of MPO includes the acting as a catalytic converter in the production of hypochlorous acid (HOCl a potent pro-oxidant) and in inducing foam cell formation [36] which occurs when macrophages ingest oxidized LDL. See **figure 5**.





**Figure 5.** Damaged endothelium induces the synthesis of adhesion molecules, promoting the permeability for circulating macrophages and LDL to penetrate the artery wall. In the atherosclerotic plaque LDL is oxidized and causes local endothelial dysfunction and injury. Oxidized LDL is engulfed by macrophages that are transformed into foam-cells. Foam-cells accumulate in the plaque and stimulate surrounding cells to produce proinflammatory cytokines leading to attraction of more macrophages.

### MPO in relation to ESRD

MPO may be one of the many factors that link oxidative stress with the development of atherosclerosis in ESRD patients. Increased oxidative stress and chronic inflammation are important contributors to uremic cardiovascular risk. Indeed, it has been shown that elevated levels of leukocyte- and blood MPO are associated with coronary artery disease (CAD) [37] and also that plasma levels of MPO act as strong predictors of cardiovascular events in a non renal population [38]. Further, distinct products of MPO, such as 3-chlorotyrosine, are enriched in human atherosclerotic lesions and in LDL recovered from human atheroma [39]. However, inflammation is commonly seen in the atherosclerotic patient and the distinct inflammatory pathways that are involved in increased oxidative stress in ESRD remain to be evaluated. Hence, it can be speculated that MPO might function as one link between inflammation, oxidative stress and endothelial dysfunction in uremic patients [34].

### Genetic background of MPO

A functional -463 G/A polymorphism has been detected in the promoter of the MPO gene and has been shown to be associated with lung cancer [40, 41], Alzheimer's disease [42] and the age of onset of renal disease [43]. In order to determine the role of this polymorphism in atherosclerosis a study on French-Canadian atherosclerotic patients was performed that showed that the -463 A-allele was less frequent among patients and when the SNP was tested in a recessive and a dominant model it was found that the presence of the A-allele is protective against CAD [44]. This promoter polymorphism was found to disturb a Sp1 transcription factor site and transfection assays showed that the presence of the G-allele resulted in a strong Sp1 binding site which increased the transcription of the MPO gene 25-fold [45]. It might be suggested that due to the strong effect of this promoter polymorphism, this SNP could be used as a stratifying marker for the detection of ESRD patients that are at high risk to develop cardiovascular disease.

### ***Interleukine-6 (IL-6)***

The gene encoding IL-6 is positioned on chromosome 7p21 and is encoding a 26kDa protein. This proinflammatory cytokine is produced in leukocytes, adipocytes, endothelial cells, fibroblasts and monocytes. While many of the other proinflammatory cytokines, such as IL-1 and TNF- $\alpha$ , also are involved in the regulation of the hepatic synthesis of CRP and the hepatic acute phase response, the role of IL-6 in this process is especially significant [46]. In fact, although most other cytokines act via paracrine/autocrine mechanisms, the major effects of IL-6 are due to its concentration in the systemic circulation [46]. Plasma levels of IL-6 has been shown to be independently associated with an increased risk of death in elderly non renal patients [47]. IL-6 has been suggested to have pro-atherogenic effect since elevated IL-6 levels are predicting myocardial infarction in healthy men [48].

### IL-6 in relation to ESRD

IL-6 plasma levels are highly elevated in ESRD patients and since this cytokine is directly stimulating the acute phase response it orchestrates the plasma levels of CRP. IL-6 levels has been shown to be independent predictor of mortality in patient starting dialysis [49]. It has been suggested that IL-6 is involved in the progression of atherosclerosis. Interleukine 6 has been found in the atherosclerotic plaque and is expressed by macrophage foam-cells and smooth muscle cells (SMC) [46], hence it could be suggested that high levels of IL-6 may accelerate atherogenesis in ESRD patients.

### Genetic background of IL-6

There are several polymorphisms reported in the gene encoding IL-6. Due to the strong association of high plasma levels of IL-6 and mortality in different populations, it is of great interest to identify the regulatory genetic factors affecting the true plasma levels of this cytokine. The first studies have been performed on the -174 G/G polymorphism and IL-6 plasma levels. Many studies have been performed analyzing the -174 SNP and though this polymorphism is affecting a transcription binding site, the results have been very conflicting. Fishman et al. showed that healthy subjects carrying the C-allele had lower IL-6 levels and also that expression of IL-6 in HeLa cells containing vectors with the G-allele was higher due to stressful stimuli. However, Brull et al. showed that 6 hours after coronary artery bypass graft surgery (CABG) the highest peak levels of IL-6 were found in -174 C/C carriers [50]. Interestingly, a similar study was performed on a -373 A<sub>n</sub>T<sub>n</sub>, in the promoter region of IL-6. Kelberman et al., who also analyzed IL-6 levels following CABG, showed that individuals carrying the A9T11 genotype had significantly higher post-operative IL-6 levels [51] independently of the other three polymorphisms (-597 G/A, -572 G/C, -174 G/C) that were studied.

The four main polymorphisms in the IL-6 gene (-597 G/A, -572 G/C, -373 A<sub>n</sub>T<sub>n</sub>, -174 G/C) have been analyzed, where the effects of different haplotypes on reporter gene expression were investigated. It was found, that although transcription varied between the cell lines that were used (ECV304 and HeLa, suggesting tissue specific regulation) promoter transcription was highest in a clone containing the GG9/11G haplotype and lower in the clone containing the AG8/12G haplotype which was very rare, the more common haplotype AG8/12C was also significantly associated with lower promoter activity as compared to the GG9/11G haplotype. However, when the effect of the polymorphisms was tested individually, discrepant results were obtained, suggesting a strong haplotype-dependent effect on the transcription level of the IL-6 gene [52]. Since *in vitro* experiments can not fully reflect the conditions *in vivo*, the impact of haplotypes that contain the promoter polymorphisms -597 G/A, -572 G/C and -174 G/C was investigated in human leukocytes. It was shown that the GGG-haplotype was associated with the highest IL-6 levels after stimulation by endotoxin, there was however no association with the -373A<sub>n</sub>T<sub>n</sub>, probably due to the low number of subjects [53]. Although the finding by Terry et al. who found the impact of the -373A<sub>n</sub>T<sub>n</sub> tract on IL-6 levels and which was replicated by Kelberman et al. the transcription levels result from a combination of the A<sub>n</sub>T<sub>n</sub> tract and the promoter polymorphisms [52] which additionally may also vary between different tissues, hence only genotyping for one of the polymorphism is inadequate.

Thereby, the discrepant results obtained from diverse studies where effect of IL-6 promoter polymorphism on plasma levels was studied, may partly be explained by this strong effect of the haplotype that has been proved both *in vivo* and *in vitro*.

### ***C-reactive protein (CRP)***

CRP is an acute phase protein that is produced in the liver and is circulating in a pentamer form consisting of five 23-kDa subunits. Since CRP plasma levels are rapidly increased due to stimulation by for example an infection, CRP is the most commonly used acute phase reactant measured frequently today and it has for a long time been used as a marker for systemic inflammation.

An inflammatory response, triggered by for example an infection, causes the release of proinflammatory cytokines such as IL-1, IL-6 and TNF- $\alpha$  which increase the synthesis and release of proteins such as CRP, fibrinogen and serum amyloid A, thereby decreasing the synthesis of negative acute phase reactants as albumin [54]. It has been suggested that this protein is not only a marker, but also a mediator, of atherogenesis. CRP is one of the strongest predictors of the risk of future cardiovascular events in healthy adults and only a small elevation in baseline levels eminently increase the risk for cardiovascular disease [55]. Indeed, CRP mRNA has been found in smooth muscle like cells and macrophages in the thickening intima of plaques [56] suggesting that expressed CRP could act locally, in an autocrine/paracrine manner, promoting atherogenesis. However, the actual pro atherogenic effect of CRP is not yet full clarified.

### CRP in relation to ESRD

Elevated plasma levels of CRP in uremic and atherosclerotic patients are associated with oxidative stress, vascular calcification and endothelial dysfunction [23]. Several studies have shown that elevated CRP levels predict both all-cause and cardiovascular mortality in HD and PD patients [23]. It has also been recently suggested that there is a gender-difference in serum levels of CRP among ESRD patients, since it was shown, in a large study, that inflamed males had a significantly higher mortality rate than inflamed females [57].

### Genetic background of CRP

Since the CRP baseline plasma levels are apparently up to 40% heritable [58] it is of great interest to investigate how and which genetic polymorphisms in the CRP gene influence the basal levels, as well as levels after stimuli, of this acute phase protein.

The CRP gene is located on chromosome 1q23 and the promoter contains both IL-1 and IL-6 responsive elements [59], meaning that the polymorphisms in the promoters regulating the IL-1 and IL-6 genes may indirectly influence the CRP

synthesis in both healthy individuals [60, 61] and in patients with coronary heart disease (CHD) [62].

A study performed by Kovacs et al was designed to elucidate if there are common promoter polymorphisms in the CRP gene that are associated with CRP plasma levels and indeed, two polymorphisms were identified, a three allelic SNP -286 (C/T/A) and a -717 A→G substitution. However, only the three allelic variant was associated with CRP plasma levels in CHD patients where the A-allele carriers had significantly higher levels of CRP [63]. In another recent study [64] a bi allelic variant -409 (G/T) and the three allelic variant -390 (C/T/A) (referred to as -286 (C/T/A) by Kovacs et al.) were found to be positioned within the transcription factor binding E-box elements. Individuals carrying the -490G/-390T haplotype (high transcription factor binding version in both E-box 1 and 2) had the highest baseline serum CRP levels after correction for age, sex and race. The authors further investigated if the transcription factor binding was affected by these polymorphisms by creating firefly luciferase reporters and found that a promoter carrying the -490G/-390T haplotype had the highest baseline activity as compared to other haplotype combinations. Another variation that has been significantly associated with CRP plasma levels is the 1059 G/C (Leu184Leu) where higher levels were found among G-allele carriers, an association that persisted after correcting for vascular disease [65].

Although these polymorphisms have been associated with plasma levels of CRP, it could not be excluded that they may be in linkage disequilibrium with another mutation directly affecting the transcription levels. Because of the strong impact of baseline levels of CRP and risk of CVD [66], it is of great interest to understand the genetic background of CRP, with the aim of finding the “at risk” haplotype.

### ***Adiponectin (ApM1)***

Adiponectin was initially identified as a gelatin-binding protein of 28 kDa (GBP28) and is secreted from human adipose tissue [67]. The corresponding cDNA was cloned and described as a novel adipose specific collagen-like factor, ApM1 (AdiPose Most abundant Gene transcript 1) [68]. Plasma adiponectin are significantly lower in individuals with higher cardiovascular risk, such as obese subjects [69], CAD patients [70] and type 2 diabetics [71, 72]. In a study by Hotta et al., significantly lower levels of adiponectin as well as leptin were found in patients with diabetes mellitus (DM) and CAD [71]. Adiponectin exact protective role against atherosclerosis may be elucidated by an *in vitro* study on human aortic endothelial cells (HAECs) that showed that adiponectin had an inhibitory effect on TNF- $\alpha$  induced monocyte adhesion and adhesion molecule expression [70, 73].

### Adiponectin in relation to ESRD

Plasma levels of adiponectin are substantially elevated in ESRD patients [74, 75] and there is no clear difference when comparing serum adiponectin levels in patients treated with PD or HD [76]. The kidneys seem to play an important role in adiponectin biodegradation and elimination since adiponectin levels significantly decreased in HD patients that underwent a kidney transplant [77].

It may be hypothesized that high levels of adiponectin are protective against development of atherosclerosis. In a study by Zoccali et al., the role of adiponectin in the development of new cardiovascular events in uremic patients was investigated during a follow-up period (31±13 months). It was shown that adiponectin levels were significantly lower in HD patients that experienced new cardiovascular events compared to event free patients and there was a 3% risk reduction for each 1µg/ml increase in plasma adiponectin levels [74].

However adiponectin levels may have gender-specific anti-atherogenic effects, since adiponectin levels did not predict future risk of CHD in a study performed in women [78]. Further, a recent case-control study showed that high plasma adiponectin concentrations were associated with lower risk of myocardial infarction in men, independent of inflammation and glycemic status [79].

### Genetic background of adiponectin

The adiponectin gene is positioned at the human susceptibility locus for type 2 diabetes on chromosome 3q27 [80]. It has been suggested that plasma levels of adiponectin are highly heritable [81] and several polymorphisms in the adiponectin gene have been detected. The promoter polymorphisms -11391 G/A, -11377 C/G and the +45 T/G SNP in exon 2 as well as the +276 G/T SNP in intron 2 are the most studied SNPs in the adiponectin gene [80]. The polymorphisms have mainly been analyzed in diabetic populations where the G-allele in both SNP +45 and SNP +276 has been shown to be significantly associated with increased risk of type 2 diabetes [82]. However, haplotype analysis reveal that the putative effect of SNP +45 and +276 may primarily result from linkage disequilibrium with SNP -11391 or SNP -11377. A GG risk- haplotype for SNPs -11391 and -11377 was found to be associated with low plasma adiponectin levels and T2DM in French-Caucasians [83]. However a study on Japanese families showed that the -11377 C/C genotype was significantly associated with type 2 diabetes [84] and hence it seems that the G-allele is the risk allele among Caucasians whereas it is the C-allele in the Japanese population. This discrepancy could be explained by that this polymorphism is in LD with another functional mutation that may vary between different populations, but also that there may be multigenic control of this protein. Interestingly, it has been suggested that variations in the adiponectin gene may

only contribute partly to the regulation of adiponectin plasma levels and that a locus on 14q13 appears to be linked with adiponectin plasma levels [85].

### ***Resistin***

The gene encoding resistin is positioned on chromosome 19 and is producing a 12.5 kDa cysteine rich protein. Ever since resistin was identified (2001), extensive animal studies have been performed. Stepan et al. showed that resistin is produced and secreted from adipocytes. In genetically obese mice, circulating resistin levels were increased and administration of resistin to normal mice impaired the glucose intolerance and insulin action [86]. In humans, however, resistin expression is almost undetectable in adipose tissue, while it is most highly expressed in bone marrow and resistin mRNA was widely expressed in human monocyte derived macrophages, however lower levels were found in lung, spleen, liver, kidney and placenta [87]. In plasma, resistin circulates in two distinct assembly states with different levels of bioactivity [88]. Moreover, resistin levels seem to be gender-dependent, with higher levels in women [89].

According to a number of animal studies, resistin is an obvious candidate link between obesity and insulin resistance. Multiple studies have been performed to prove this association also in humans. However, discrepant results [90] have been obtained. A study performed on plasma resistin levels in individuals with diabetes type 1 or 2 as well as healthy controls, could not show upon any relation between plasma levels of resistin and diabetes or BMI [91]. Further it was shown that resistin mRNA expression in primary cultured human adipocytes from healthy women was not associated with insulin resistance or BMI [92].

Although detailed animal experiments and *in vitro* studies on human cells have revealed some of the functions of resistin, further studies are needed to clarify the complex biology of this protein [86].

### **Resistin in relation to ESRD**

The kidneys seem to be an important site for resistin elimination as resistin levels increase with progression of renal failure. Resistin plasma levels are highly elevated in ESRD patients and has been shown to be associated with glomerular filtration rate (GFR), but not with BMI, waist-hip ratio, leptin, glucose or insulin sensitivity [93]. Plasma levels of resistin as well as adiponectin and leptin were analyzed in ESRD patients on HD or PD and were compared to levels in pre-dialysis patients. Resistin levels were significantly higher in ESRD patients (both PD and HD), as compared to pre-dialysis patients, leptin levels were significantly higher in PD patients as compared to HD and pre-dialysis individuals whereas plasma adiponectin levels did not vary between the groups, [94]. The role of resistin in the insulin resistance syndrome seen in ESRD patients is yet to be

clarified. However, in future studies when resistin levels are analyzed it is important to correct for GFR (paper V). This is in particular of interest when performing studies in diabetic populations, since diabetic nephropathy is a common disorder in these individuals.

#### Genetic background of Resistin

There are several polymorphisms that have been identified in the resistin gene and most of the studies have analyzed the impact of polymorphisms on plasma levels and relation to obesity and/or type 2 diabetes. Among the identified SNPs the functional -180 G/C promoter SNP is especially interesting since the G/G-genotype creates gain of recognition binding sites for transcription factors Sp1 and Sp3 [95]. Functional studies, using vectors showed that the G/G genotype resulted in higher promoter activity and additionally, resistin gene expression in fat tissue was significantly higher in obese individuals carrying the G/G genotype [96].

Another polymorphism that may affect plasma levels of resistin is a -420 G/C SNP. The -420 G/G genotype has been associated with higher resistin levels in Japanese type 2 diabetics [95] interestingly in a Korean study, a haplotype containing -420G and -537A was associated with higher plasma resistin levels but not with diabetes or obesity [97]. Functional studies revealed that the -420 G/C polymorphism is positioned within an Sp1/3 transcription factor binding site and that the G-allele is inducing the promoter activity [98]. Osawa et al. showed that mRNA resistin expression in monocytes from healthy controls, was higher in G/G homozygotes and correlated with serum resistin levels and moreover that higher plasma levels were seen in T2DM patients who were G/G homozygous [95, 99].

The discrepant results suggest that the significant effect of this functional polymorphism may influence plasma levels, but the association to diabetes and obesity must be further evaluated. Also, the connection between these two functional SNPs (-180 G/C, -420 G/C) must be further evaluated, to investigate the effects of a putative haplotype on resistin plasma levels.



## **AIMS OF THE STUDY**

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The objective of this thesis was to contribute to the development of an approach on how to use genetics as an instrument for identifying “high risk” ESRD patients at an early stage and to provide the tools that can help to develop more accurate and individually tailored treatment strategies in the future.

The main aim was to evaluate new genetic variants involved in ESRD and its complications. There is a close association between malnutrition, increased inflammatory activity and atherosclerotic CVD, which often coexist in these patients and has been denoted the MIA syndrome.

This study was designed to:

- investigate the effect of a functional polymorphism in the MPO gene on oxidative stress and atherosclerosis in ESRD.
- study the role of elevated adiponectin-, resistin-, C-reactive protein- and IL-6 levels in end stage renal disease and their relation to the MIA syndrome.
- investigate the ApM1 gene expression in ESRD patients and the relation to plasma levels.
- investigate if single nucleotide polymorphisms in the ApM1, resistin and C-reactive protein gene as well as IL-6 genotype combinations affect plasma levels of the respective proteins in ESRD.

## MATERIALS AND METHODS

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### Study populations (papers I-V)

This ongoing prospective study was performed to evaluate risk factors behind the development of complications in ESRD patients. All ESRD patients were recruited shortly before the initiation of renal replacement therapy. The glomerular filtration rate (GFR) was estimated by the mean of urea and creatinine clearances. Information on CVD was obtained from a detailed medical history, including a history of ischemic heart disease, cerebrovascular disease or peripheral artery disease and symptoms of the same. Malnutrition was defined by subjective global assessment (SGA) with a score >1 and inflammation was defined as CRP>10mg/L. SGA is a tool that has been recommended by the National Kidney Foundation (NKF) Kidney Disease/Dialysis Outcomes and Quality Initiative (K/DOQI) for nutritional assessment in adult dialysis patients. SGA used in our studies included six subjective assessments. Three were based on the patient's history of weight loss, incidence of anorexia and incidence of vomiting and three were based on the subjective grading of muscle wasting, the presence of edema and the loss of subcutaneous fat. Based on three assessments, each patient was given a score reflecting the nutritional status as follows; 1= normal nutritional status, 2= mild wasting, 3= moderate wasting, 4= severe wasting.

All studies have been approved by the Ethics Committee of the Karolinska University Hospital. Informed consent was obtained from all subjects.

#### *Patients included in papers I, II, IV and V*

The study exclusion criteria were age >70 years, overt infectious complications and unwillingness to participate in the study. Most of the patients were on antihypertensive medications as well as on other drugs commonly used in ESRD, such as phosphate and potassium binders, diuretics and vitamin B, C and D supplementation.

In **paper I** 155 ESRD patients (age  $52 \pm 2$  years, 62% males) were included. The primary cause of renal disease was diabetic nephropathy (23%, n=35), chronic glomerulonephritis (26%, n=40) patients), polycystic kidney disease (10%, n=16) and other, or unknown, causes in 64 patients (41%). Cardiovascular atherosclerotic disease, as defined by medical history, was present in 46 (30%) of the patients.

In **paper II** 204 ESRD patients (age  $52 \pm 1$  years, 62% males) were included. Fifty nine (29%) of the ESRD patients had diabetes mellitus, including 29 with DM type 1 and 30 with DM type 2. CVD was present in 63 patients who had a clinical

history of cerebrovascular, cardiovascular and/or peripheral vascular disease. In **paper IV**, 229 ESRD patients, age  $52 \pm 12$  years, 62% males were included.

**Paper V** included 239 CKD patients (age  $53 \pm 1$ , 66% males) of which 204 had CKD stage 5 (mean age  $54 \pm 1$  years, 65% males) and 35 were prevalent patients, from the hospital renal outpatient clinic, with CKD stage 3-4 (mean age  $54 \pm 2$  years, 71% males).

The study exclusion criteria were age below 18 years or above 70 years, clinical signs of acute infection, active vasculitis or liver disease at the time of evaluation as well as unwillingness to participate in the study. The causes of CKD (n=74) were chronic glomerulonephritis, diabetic nephropathy (n=38), polycystic kidney disease (n=17) and other (n=110). Fifty-nine (25%) patients were diagnosed as type I diabetics and 35 (15%) had type II diabetes. The majority of patients were on antihypertensive medications and 14 patients were on lipid-lowering medication. The patients were divided into two groups according to the degree of renal failure using the staging according to K/DOQI Guidelines [100].

#### ***Controls included in papers I, II, IV and V***

Genotype controls, used in **papers II, IV and V**, were unrelated Caucasians living in Stockholm County (62% males, mean age  $40 \pm 1$  years). In **paper II**, a control group of 36 (64% males, mean age,  $52 \pm 2$  years) healthy individuals was used for analyses of plasma adiponectin levels. Twenty five controls (64% male, median age 60 years) were used for both biochemical and metabolic comparative analyses in **paper V**.

#### ***Subjects in paper III***

The 18 patients included in this study were divided in two groups, a MIA- and a non-MIA group. The patients were selected based on the presence of malnutrition, inflammation and atherosclerotic cardiovascular disease.

Nine patients (7 males;  $60 \pm 8$  years) with 2 or 3 of the MIA complications were included in the MIA group and 9 patients (7 males;  $55 \pm 9$  years) with none or one complication were included in the non-MIA group.

The controls used in this study were nine healthy subjects undergoing elective laparoscopic cholecystectomy at the Karolinska University Hospital, Huddinge.

The MIA- and non-MIA patients as well as the healthy controls were matched for age, gender and BMI. All patients were free of clinically overt infectious complications. In the MIA group, six had diabetes mellitus, one had polycystic kidney disease, one a post renal etiology of ESRD and one had chronic renal

disease of unknown origin. In the non-MIA group (7 males mean age 55±9 years), one had DM type I, two had polycystic kidney disease and six had chronic glomerulonephritis.

### **Clinical measurements (papers I-V)**

Blood samples were taken in the morning after an over night fast and were stored at -70°C, if not analyzed immediately.

#### ***Paper I***

Plasma IL-6 was measured using ultrasensitive enzyme-linked immunosorbent assay (ELISA) kits (Boehringer Mannheim, Mannheim Germany). Pentosidine was measured using reverse phase high-pressure liquid chromatography (HPLC) and serum albumin levels were measured with the bromcresole purple method. Chlamydia pneumoniae, IgG and IgA antibodies were analyzed using the microimmunofluorescence method with elementary anti-bodies of C. pneumoniae strain IOL 207 which was used as a representative for C. pneumoniae.

#### ***Paper II, III, IV and V***

The analysis of high sensitivity C-reactive protein (hs-CRP) (nephelometry) (**papers II, IV, V**), UAE, fibrinogen, HbA1c and serum albumin in **paper II** and **IV** (bromcresole purple) as well as blood lipids (cholesterol, triglycerides, HDL cholesterol) (**paper II**) was performed at the Department of Clinical Chemistry at Karolinska University Hospital, using routine methods.

Plasma leptin levels (**paper II**) were measured by a radioimmunoassay kit (RIA) (Linco Research, Inc) and plasma adiponectin (**papers II and III**), IL-6 (**paper IV**) as well as plasma resistin levels (**paper V**) were measured by high-sensitivity photometric enzyme-linked immunosorbent assay (ELISA), (Boehringer Mannheim, Mannheim Germany (paper II and II), LINCO Research, St Charles, MS, USA (paper V). In **paper V**, the plates were read using ELISA VERSA max reader™ (Molecular Devices Corporation, Sunnyvale, CA, USA) and the data was analyzed with the SoftmaxPRO® software (Molecular Devices Corporation, Sunnyvale, CA, USA). Lean body mass (LBM) (**papers II and V**), total fat mass (FM) (**paper II**) and truncal FM (**paper V**) was estimated using dual energy x-ray absorptiometry (DEXA) with a DPX-L device (Luna corp., Madison WI, USA).

In **paper V**, nutritional status was estimated by subjective body assessment (SGA) and body mass index (BMI) was calculated as weight (in kg) divided by height (in square meters).

In non-diabetic patients, insulin resistance was estimated by quantitative insulin-sensitivity check index (QUICKI:  $1/[\log(\text{fasting plasma insulin } (\mu\text{U/mL})) + \log(\text{fasting plasma glucose } (\text{mmol/L}))]$ ) [101] and by the homeostasis model assessment for insulin resistance (HOMA-IR:  $\text{fasting serum insulin } [\mu\text{U/ml}] * \text{fasting plasma glucose } [\text{mmol/l}] / 22.5$ ) [102]. High-density (HDL) cholesterol was determined after precipitation of apolipoprotein (Apo) B-containing lipoproteins by phosphotungstic acid and the levels of Apo A and Apo B were determined using an immunonephelometric procedure (Behring AG, Marburg, Germany).

All biochemical analyses, such as serum cholesterol and triacylglycerols as well as plasma insulin and serum levels of ICAM1 and VCAM1 were analyzed by standard procedures at the department of Clinical Chemistry at the Karolinska University Hospital, Huddinge.

### ***In situ* hybridization (paper III)**

#### ***Adipose tissue biopsies***

For analysis of the ApM1 gene expression fat tissue biopsies were taken from both ESRD patients and healthy controls. The biopsies were taken from the abdominal subcutaneous adipose tissue and on ESRD patients the operation was performed following local anesthesia and after an overnight fast. The fat biopsy was taken in connection with the surgical insertion of a PD catheter. In controls, who were undergoing elective laparoscopic cholecystectomy but otherwise healthy, the biopsies were taken from the abdominal subcutaneous adipose tissue after administering intravenous saline. The adipose tissue specimens ranged from 200 mg to 1000 mg and were immediately frozen unfixed on dry ice in the operating room, where after they were stored at  $-70^{\circ}$  until sectioned. The fat tissue biopsies were cut to a thickness of  $14 \mu\text{m}$  in a cryostat (CM 3000; Leica Instruments GmbH, Nussloch, Germany) at  $-35$  to  $-40^{\circ}\text{C}$  and thawed onto electrically charged Polysine™ microscope slides (Menzel-Gläser, Germany). The slides were thereafter processed for *in situ* hybridization.

#### ***Probe preparation for in situ hybridization***

The probes for ApM1 used in **paper III** were designed by hand to be GC rich and 44-46 bases long, ending with a guanine or cytosine for stable binding. The probes, ApM1 sense, ApM1 anti-sense, gamma-actin and CCK, were labeled by mixing  $2 \mu\text{l}$  oligonucleotide ( $40 \text{ng}/\mu\text{l}$ ),  $1,5 \mu\text{l}$   $5 \times \text{TdT}$

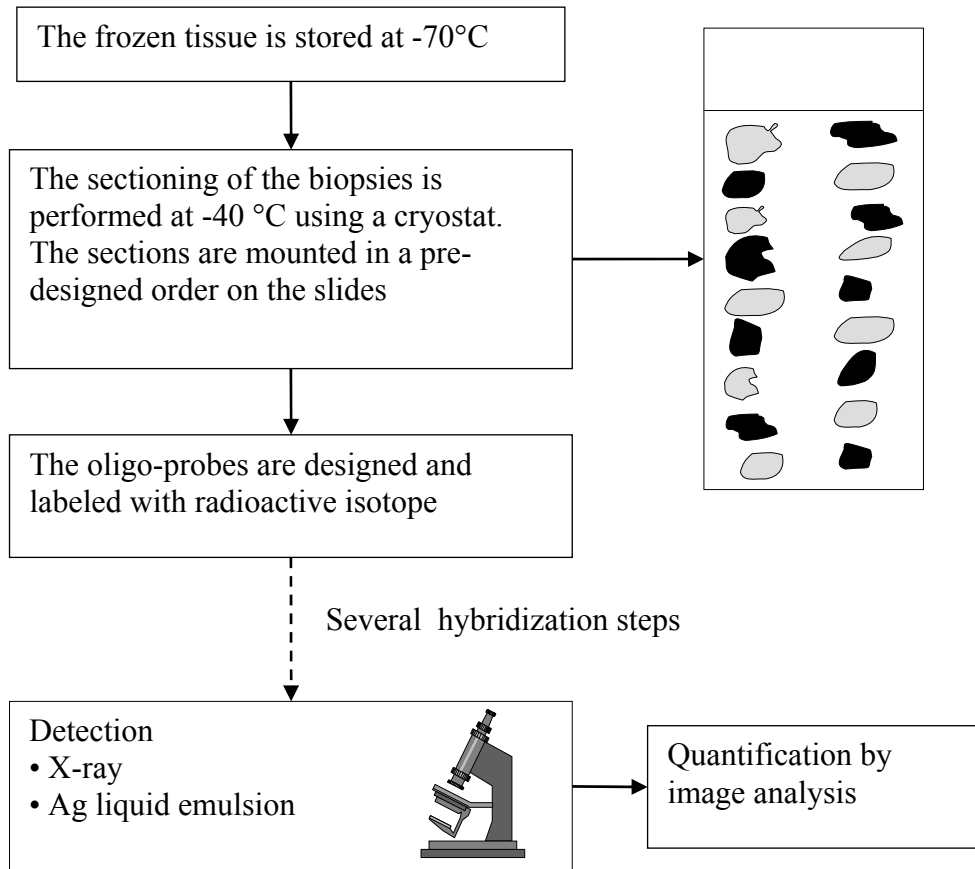
(Amersham Pharmacia Biotech Inc.), 3 $\mu$ l distilled H<sub>2</sub>O, 6 $\mu$ l <sup>33</sup>P (NEG/312H, Perkin Elmer Life Sciences Inc. Boston, USA) and the reaction volume was incubated for 1 hour at 37°C. The labeled probes were cleaned according to the protocol using the QIAquick Nucleotide Removal Kit (250), (QIAGEN, Germany). Probe sequences for ApM1 sense and anti-sense were 5'-GGCCTCTTTTGGCCTCAGTTGACTCTCTCTGTGCCTCTGGTTCCAC-3' and 5'-CCGGAGAAAACCGGAGTCAACTGAGAGAGACAGGGAGACCAAGGTG-3', respectively (MedProbe, Oslo, Norway). The oligonucleotide probes for gamma-actin was as previously described [103]. CCK is strongly expressed in certain structures of the mouse brain and was used as a positive control for optimization of the hybridization steps as well as for the developing procedure. Probes were labeled with <sup>33</sup>P alpa-dATP at the 3' end of the molecule. The quantification of <sup>33</sup>P labeled probes was performed using a scintillation counter, where two aliquots of the probe were measured and a mean value per  $\mu$ l was calculated. Depending on the magnitude of the radiation, the correct amount of each probe was calculated and added to the hybridization cocktail.

### ***In situ hybridization procedure***

Sections from MIA, non-MIA patients and controls were placed onto the slides in a preplanned order and all slides that were used in the study were prepared in triplicate. To obtain an equal exposure all slides were handled simultaneously as a single batch in all *in situ* hybridizations steps. The *in situ* experiment was repeated and the obtained results from the first experiment were reproduced.

In brief, the sections were incubated at 42°C for 15 to 18 hours with 10<sup>6</sup> cpm of <sup>33</sup>P labeled probe solution containing formamide, SSC, Denhardt's solution, sarcosyl, sodium phosphate, dextran sulfate mixed with 500 $\mu$ g/ml sonicated salmon sperm DNA and dithiothreitol. The different ingredients are added because of several special qualities. Formamide lowers the hybridization temperature which leads to better preserved morphology of the tissue. SSC is a salt solution which is used to wash out the excess probe cocktail from the slides. The concentration and temperature of SSC can be optimized to reach the perfect stringency. Dextran sulfate is added because of its ability to push aside water molecules, thereby increase the probe concentration and accelerate the hybridization. Salmon sperm DNA consists of 100-200 base pair long DNA that bind to unspecific DNA binding sites and thereby reduce the background noise.

The slides were rinsed in 1× SSC at an optimized temperature (ranging between 55- 65°C) and dehydrated in EtOH. The sections were dried and exposed to Amersham Hyperfilm β-max x-ray film for 10 days before development with Kodak LX24 X-Ray developer and fixation with Kodak AL4 X-Ray fixer. The hybridization procedure is shown in **figure 6**.



**Figure 6.** A schematic outline of the *in situ* hybridization procedure. Frozen tissue sections are mounted onto slides that are exposed to radioactively labeled probe for hybridization. The sections are washed and the signal is detected by x-ray autoradiography.

### ***Ag-dipping***

Slides were dipped in NTB2 nuclear track emulsion (Kodak, Rochester, NY, USA) and developed after 5-6 weeks and analyzed in a Zeiss dark-field microscope (Axiophot, Carl Zeiss, Oberkochen, Germany).

### ***The choice of control***

*In situ* hybridization is a method for detection, localization and quantification of specific mRNA in various tissues.

A probe can hybridize to unspecific GC-rich regions as well as to homologous sequences in a tissue which may result in unspecific binding of the probe and thereby high background noise, which in turn may lead to overestimated mRNA expression and false positive results. Careful optimization of the technique is crucial and a good control is necessary. There are several types of positive and negative controls that can be used.

As a negative control, a sense probe is commonly used and is designed 5'-3' of a known sequence. The probe sequence is thereafter carefully checked for any homologous sequence in the human genome since it is specifically made not hybridize to the tissue. Sense probes give a smooth and equal background and should be comparable in all samples of the same tissue.

Another negative control is to use sections of a tissue that does not express the particular mRNA being investigated. But, in such cases, it should be considered that there are homologous sequences that may give false positive expression signal. There are a number of positive controls that can be used in *in situ* hybridization. Multiple-probes of a single gene is one example. Multiple probes are a number of sequences that are designed to be none overlapping and bind to the same mRNA sequence, resulting in an equal expression for each probe in the sections of the same tissue. Probes which are designed to bind to housekeeping genes can also be used as positive controls. However, it must be considered that the expression of housekeeping genes may vary between different individuals [104]. Usually, more than one positive and negative control are used simultaneously, to confirm that the hybridization took place correctly and for optimization of the technique, since the sought mRNA expression may be hard to predict and detect.

### ***Quantification of in situ hybridization autoradiographs***

Autoradiographic <sup>14</sup>C microscale strips ARC-146C (Amersham Radio labeled Chemical Inc.LAND) ranging from 0.017 to 3.0 µCi/g were used as standards by co-exposure with the tissue sections. The signals were measured from high-resolution x-ray films, where sections had been co-exposed with radioactive standards. The quantification was performed on a Macintosh Iix equipped with a



Quick Capture frame grabber board, a Northern Light precision illuminator and a Hamamatsu CCD camera equipped with a Nikon 55 mm lens. To process the images, Image software was used. Each frame was digitized to a  $512 \times 512$  matrix with 256 gray levels for each picture element. The gray-levels, corresponding to the 9 standards lying within the exposure range of the film, were determined and used in a third-degree polynomial approximation to construct a gray-level to activity transfer function. Each section was measured individually and the actual measure-value was obtained by subtracting the background from the measured value from each section. Since all sections were in triplicate, the mean value for each individual was calculated.

## **SNP analysis by Pyrosequencing<sup>TM</sup> (papers I, II, IV and V)**

### ***DNA purification and preparation***

Peripheral blood was drawn in a 5mL EDTA sample at recruitment and DNA was extracted using QIAamp<sup>®</sup> DNA kit. All DNA samples were aliquoted, master plates were designed and stored at  $-20^{\circ}\text{C}$ . Separate master plates were made for control DNA.

### ***Primer design and polymerase chain reaction***

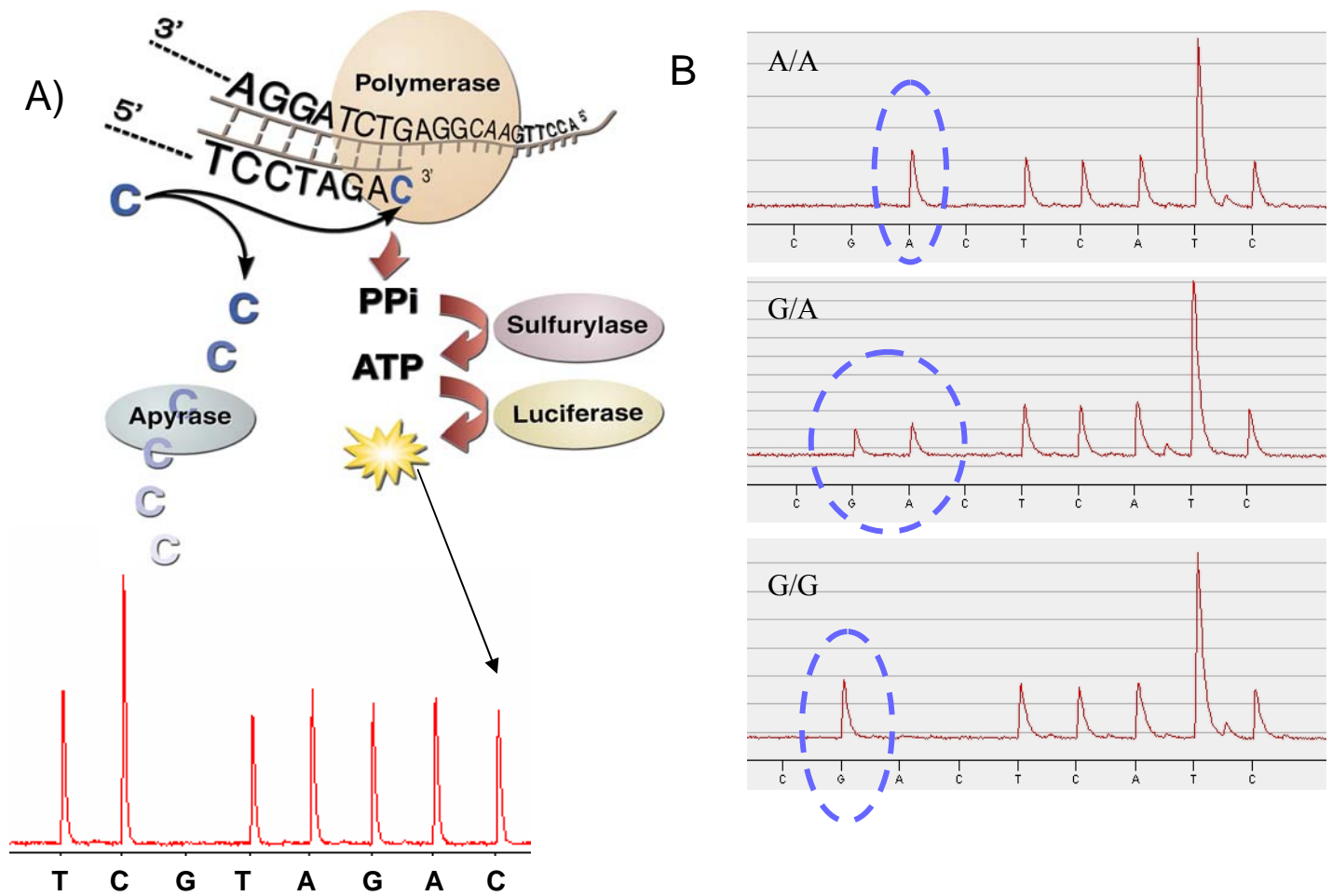
For preparation of pyrosequencing templates, PCR was performed on genomic DNA from patients and controls for all SNPs analyzed in paper I, II, IV and V. PCR primers were designed using the software Primer Designer 4<sup>®</sup> for Windows (version 4.1) and were synthesized by Thermohybrid<sup>®</sup> (Ulm, Germany). One primer in each pair was biotinylated. The PCR reaction volume was 50  $\mu\text{l}$ , containing 20-50 ng of DNA, 10 pmoles of each forward and reverse primer, as well as dNTPs, DyNAzyme<sup>TM</sup> II (DNA Polymerase, Finnzymes, California, USA), Tris-HCl,  $\text{MgCl}_2$ , KCl and 0.1 % Triton X-100. Thermal cycling was performed at  $95^{\circ}\text{C}$  for 5 min, followed by 50 cycles at  $95^{\circ}\text{C}$  for 30s, annealing temperature ranging between  $60-80^{\circ}\text{C}$ , for 60 s, followed by a final extension at  $72^{\circ}\text{C}$  for 5 min. The thermal cycling steps have been optimized when needed to obtain enough of clean PCR product for the pyrosequencing procedure.

### ***The pyrosequencing procedure***

The biotinylated PCR product was immobilized to streptavidin-modified paramagnetic beads (Dynabeads<sup>R</sup> M-280 Streptavidin, Dynal A.S, Oslo, Norway) which were dissolved in 50  $\mu\text{l}$  1 X Binding-Washing buffer (2 X BW-buffer II, pH 7.6, 19mM Tris-HCl, 2M NaCl, 1mM EDTA, 0.1% Tween 20). The PSQ<sup>TM</sup> 96

plate was placed in a shaker at 800rpm for 15 minutes at 65°C. The template was treated with 0.50 M NaOH for separation of the DNA strands. Samples in the PSQ plate were transported using a device equipped with 96 magnetic ejectable microcylinders, covered with a disposable plastic cover. The beads were transferred to a solution containing 4 µl/sample of the sequencing primer (10pmol) and 40µl/sample of 1 X Annealing buffer (10 X Annealing buffer pH 7.6, 200mM Tris-Acetate, 50 mM MgAc<sub>2</sub>) for hybridisation of the single-stranded DNA template, followed by annealing for 1 min at 95°C and cooling to room temperature. The four separate deoxynucleotides, DNA polymerase, luciferase, ATP sulfurylase and the nucleotide-degrading enzyme apyrase as well as substrate luciferin were contained in a cassette which was put into the pyrosequencer.

For each SNP to be analysed a specific dispensation order was pre-programmed and selected using the SNP Entry module of the SNP Analysis Software. The PSQ plate containing the annealed samples was put in the pyrosequencer. The nucleotides were added cycle-wise and each incorporation of a nucleotide resulted in the release of one pyrophosphate (PPi) which was converted to ATP by ATP sulfurylase. Visible light was produced in the following luciferase catalysed reaction and detected with a CCD camera, while unincorporated nucleotides were degraded by apyrase between each cycle. The output was monitored as a pyrogram showing peak heights proportional to the number of nucleotides incorporated per cycle. The pyrosequencing reaction and a pyrogram is shown in **figure 7**. The SNP analysis was performed using the PSQ™96 SNP software and hetero- and homozygous samples were viewed separately. The quality control involved checking that the amplified DNA could not sequence itself by DNA looping, that the sequencing primer could not prevent the sequencing process by primer dimer formation and identification of the predicted sequence.



**Figure 7.** A) The figure is showing the pyrosequencing reaction. Nucleotides are added stepwise to the DNA template. The PPi released during the DNA polymerase reaction is converted to ATP by sulfurylase. Visible light is produced in a luciferase catalyzed reaction and after each cycle the unincorporated nucleotides are degraded by apyrase.

B) The pyrogram is showing an A/A homozygote, a G/A heterozygote and a G/G homozygote. The height of the peaks are proportional the light that is produced in the pyrosequencing reaction and hence the number of incorporated nucleotides.

## RESULTS AND DISCUSSION

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### **A functional variant of the Myeloperoxidase gene is associated with cardiovascular disease in end stage renal disease patients (paper I)**

MPO is an abundant hemoprotein, released by activated neutrophils, monocytes and tissue associated macrophages and the main role of MPO is to take part in the immune defense of the organism [27]. It has been suggested that increased activity of MPO may enhance the development of oxidative stress and endothelial dysfunction in ESRD. An important polymorphism in the promoter region of the MPO gene has been identified and recent studies have shown that the -463 G/A SNP is associated with transcriptional enhancement of the gene [45]. This highly functional SNP alters the transcription binding site for SP1 transcription factor, where presence of a guanine residue results in a 25-fold transcription enhancement of the gene [45]. This polymorphism was investigated in 155 ESRD patients in order to test the effect of this polymorphism on the MIA complications and oxidative stress.

#### ***-463 SNP, inflammation and CVD***

The patients were genotyped using Pyrosequencing™ and we found that the G/G genotype was associated with a higher prevalence of positive serology for *Chlamydia pneumoniae*, a marker of inflammation (due to infection), as compared to carriers of the A-allele ( $p < 0.05$ ). The prevalence of CVD was lower in the A/A (0%) and G/A carriers (18%), compared to the G/G homozygotes (35%) and this association persisted after correction for age, diabetes, smoking, malnutrition and inflammation. When additional patients were included ( $n=257$ ) the significant association with CVD persisted, furthermore 207 controls were genotyped and were shown to be in H-W equilibrium and the genotype frequencies did not differ significantly between patients and controls (unpublished data).

According to a study on atherosclerotic patients, where homozygote A-allele carriers were most protected against CAD [44] our findings suggest that the -463G→A transition may result in lower MPO levels and perhaps also activity, which might explain the lower prevalence of CVD in ESRD patients carrying the A-allele.

It is important to mention that information on CVD obtained from a detailed medical history may underestimate the true prevalence and incidence of atherosclerosis in ESRD patients [105]. Ultrasonographic evaluation of the thickness of the carotid intima media can be used as a complement tool to reach a better accuracy [27].

### ***Relation to oxidative stress***

Since MPO has been suggested to be a link between inflammation, oxidative stress and endothelial dysfunction in uremic patients [34] it is of interest to analyze the relation between MPO and oxidative stress. We found that the concentration of plasma pentosidine (a surrogate marker of oxidative stress) was higher in the G/G homozygotes (28.4 pMol/mg albumin, range 8.5-123 pMol/mg albumin) when compared to the G/A and A/A groups combined (21.4 pMol/mg albumin, range 7.6-384 pMol/mg albumin;  $p < 0.05$ ). Pentosidine circulates in a bound form to albumin, hence the correction for albumin levels. There are several clinical markers that can be used for evaluation of oxidative stress, which most often are end products of the multiple MPO reactions, i.e. chlorinated tyrosines, arachidonic acid derivatives and reactive aldehydes to mention a few. In this study pentosidine was used as a marker of oxidative stress. Pentosidine is a reactive aldehyde that is an end product of the MPO-catalyzed oxidative modification of amino acids, where both glyoxal and pentosidine are formed.

### **Adiponectin in renal disease: Relationship to phenotype and genetic variation in the gene encoding adiponectin (paper II)**

Adiponectin is an adipocytokine that may have significant anti-inflammatory and anti-atherosclerotic effects since low adiponectin levels have previously been found in patients with high risk for CVD [106]. Since the prevalence of CVD and inflammation is high in patients with ESRD the role of adiponectin in the MIA complications as well as the impact of variations of the ApM1 gene on plasma adiponectin levels were investigated.

In a cohort of 204 (62% males) ESRD patients aged  $52 \pm 1$  years the following parameters were studied: presence of CVD, body composition, plasma adiponectin ( $n=107$ ), cholesterol, triglycerides, HDL-cholesterol, serum leptin, high-sensitivity CRP (hs-CRP) and urinary albumin excretion (UAE). Four SNPs (-11391 G/A, -11377 C/G, 45 T/G, 276 G/T) were analyzed using Pyrosequencing™. A small group of gender-matched,  $n=36$  ( $52 \pm 2$  years) individuals served as control subjects for plasma adiponectin analysis whereas the control subjects for ApM1 genotyping were 209 unrelated Caucasians living in Stockholm County (62% males; mean age,  $40 \pm 1$  years).

### ***Plasma levels of adiponectin***

We found that plasma levels of adiponectin were markedly ( $p < 0.0001$ ) increased in ESRD patients (22.2  $\mu\text{g/mL}$ ), as compared to controls (12.2  $\mu\text{g/mL}$ ). An unexpected finding was that IDDM patients had significantly elevated adiponectin

levels (36.8 µg/mL) as compared to both controls and patients with NIDDM. In a stepwise (forward followed by backward) multiple regression model only IDDM ( $p<0.001$ ) and visceral fat mass ( $p<0.05$ ) were independently associated with plasma adiponectin levels. It is not clear why IDDM patients have elevated adiponectin levels as compared to NIDDM patients, but since visceral fat mass tended to be higher in NIDDM, a difference in body composition may be a reason. It might be speculated that high visceral fat correlate with low adiponectin plasma levels whereas low visceral fat mass with high adiponectin levels. However, the significant association between plasma adiponectin and IDDM prevailed following the correction for the impact of visceral fat mass. Another reason why IDDM patients had elevated adiponectin levels as compared to NIDDM patients might be a difference in U-albumin excretion.

***The impact of genotype***

There was strong linkage disequilibrium between the promoter SNPs -11391 and -11377 and between SNPs 45 and 276 ( $D\geq 0.999$ ) in both patients and controls as shown in **table 2**. The calculated LD presented in table 2 agree well with that found in a French-Caucasian population [83].

**Table 2.** Estimated linkage disequilibrium in the adiponectin gene.

Kidney patients  $D'$ (p-value )

|        | -11391          | -11377          | 45             |
|--------|-----------------|-----------------|----------------|
| 276    | 0.59 (p=0.0005) | 0.87 (p<0.0001) | 0.99 (p<0.001) |
| 45     | 0.06 (p=0.5349) | 0.31 (p=0.3478) |                |
| -11377 | 1.00 (p=0.0100) |                 |                |

Controls  $D'$ (p-value)

|        | -11391          | -11377          | 45              |
|--------|-----------------|-----------------|-----------------|
| 276    | 0.56 (p=0.0017) | 0.49 (p=0.0018) | 1.00 (p=0.0001) |
| 45     | 0.17 (p=0.8443) | 0.15 (p=0.6935) |                 |
| -11377 | 0.99 (p=0.0053) |                 |                 |

Although SNPs -11391 and -11377 were in total LD and segregate together in a haplotype block [107], only the -11377 C/C genotype was significantly associated with a lower prevalence of CVD ( $p < 0.05$ ) as well as lower triglyceride levels ( $p < 0.05$ ). Seven patients with the ApM1 276 T/T genotype had significantly ( $P < 0.05$ ) higher median plasma adiponectin levels. SNPs 45 and 276 have also been shown to belong to a separate haplotype block [107] and in our study, these two polymorphisms were in total LD.

In ESRD patients, SNP -11391 deviated from Hardy Weinberg equilibrium ( $p < 0.01$ , chi square=13.4), but in this case it was not a result of an association between a specific genotype and the ESRD phenotype. Lewontin's LD parameter  $D'$  is a measure of recombination, whereas  $r^2$  is a measure of correlation between two markers.  $D'$  between the promoter polymorphisms was estimated to be 1.0 in patients and 0.99 in controls (see table 2). However, the  $r^2$  measure was 0.02 in both patients and controls (not published results). The low  $r^2$  value may explain the inconsistency seen in association to only one of the promoter polymorphisms, since  $r^2$  indicates that these two polymorphisms differ in allele frequency.

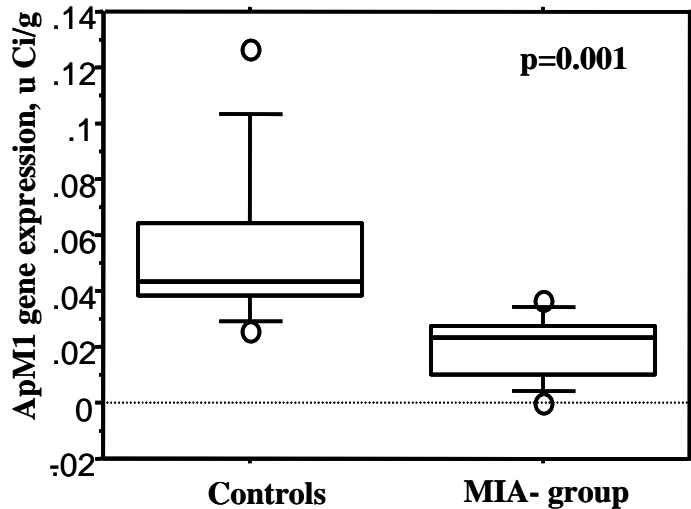
### **Reduced gene expression of adiponectin in fat tissue from patients with end-stage renal disease (paper III)**

A recent study has shown that decreased adiponectin plasma and mRNA ApM1 levels in obese women are associated with higher levels of hs-CRP and IL-6, two inflammatory mediators and markers of increased cardiovascular risk [108]. Hence, it was of interest to investigate the expression of ApM1 in ESRD patients, especially since inflammation plays a key role in the development of both atherosclerosis and malnutrition. Circulating adiponectin levels are elevated in ESRD patients (paper II) and this adipokine is believed to have anti-inflammatory and anti-atherogenic characteristics. We analyzed the ApM1 gene expression in adipose tissue from 18 ESRD patients of whom 9 had a high prevalence of MIA complications and 9 age and gender matched ESRD patients had few MIA complications. The results were compared to 9 healthy controls matched for age, gender and BMI.

#### ***mRNA expression of ApM1***

Gene expression analysis was performed using the *in situ* hybridization technique and the ApM1 gene expression was found to be lower in ESRD patients compared to healthy controls ( $p = 0.001$ ), as shown in **figure 8**. However, when comparing the gene expression between the two groups including ESRD patients with and

without MIA complications, no significant difference in the ApM1 gene expression was detected.



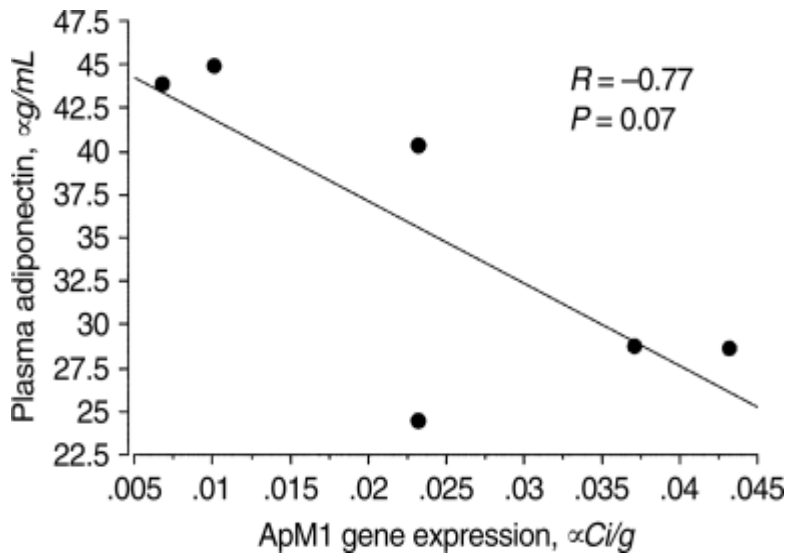
**Figure 8.** ApM1 mRNA expression in fat tissue from 18 ESRD patients and 9 healthy matched controls.

We suggest that the decrease in expression may be the result of a negative feedback regulation, because of elevated levels of circulating adiponectin. It has been shown that adiponectin enhances insulin sensitivity [109] through increased fatty acid oxidation and inhibition of the hepatic glucose production and additionally increased levels of IL-6 and TNF- $\alpha$  seem to have a down-regulative effect on mRNA levels of adiponectin [110]. Indeed in a study investigating adiponectin mRNA levels in obese and lean subjects, adipose tissue was cultured for 72 h with the proinflammatory cytokine IL-1 $\beta$ , which resulted in a significant reduction (80-90%) of mRNA levels in subcutaneous fat tissue from both obese and lean subjects [110]. Hence, the highly elevated plasma levels of both proinflammatory cytokine IL-6 and TNF- $\alpha$  seen in ESRD patients may influence the low mRNA levels of ApM1.

#### ***Correlation between ApM1 and plasma adiponectin levels***

Although the number of patients where both adiponectin mRNA and plasma levels were investigated was only 6, we observed a close to significant correlation, as shown in **figure 9**.





**Figure 9.** Relation between mRNA ApM1 gene expression and plasma adiponectin levels in 6 ESRD patients.

The reason(s) why ESRD patients have increased plasma adiponectin levels are not clear. However, as the present study demonstrated lower adiponectin gene expression in adipose tissue, it seems reasonable to speculate that reduced renal clearance and/or other factors associated with chronic renal disease contribute. However, it could be speculated that elevated plasma levels of adiponectin caused by renal failure induce a negative feedback mechanism resulting in a proportional adjustment of the mRNA expression and hence the observed correlation between the mRNA ApM1 and plasma levels (**figure 9**).

Insulin dependent DM (IDDM) and visceral fat mass was negatively associated with log adiponectin in paper II. This could be an effect of high metabolic activity of visceral fat depots. There is a significant biological difference between subcutaneous and visceral fat mass. Human studies of fat tissue from obese and lean individuals have shown that adiponectin mRNA levels are markedly lower in visceral as compared with subcutaneous fat tissue [111]. It would be of great interest to compare the adiponectin gene expression in visceral and subcutaneous fat tissue from ESRD patients. It might be hypothesized that visceral fat is the main producer of insulin-sensitizing adiponectin and that the gene expression is stable and hard to alter by other factors, on the other hand, subcutaneous adipose tissue might be secreting adiponectin that is involved in short term peripheral adipocyte signaling [112] and hence that the gene expression is more vulnerable and easy to influence.

## **Genetic influence on inflammatory biomarkers in ESRD patients: a study of CRP and IL-6 single nucleotide polymorphisms (paper IV)**

The main role of the proinflammatory cytokines is stimulating the acute phase response in the liver [18] and thereby inducing CRP production [19]. The proinflammatory cytokine IL-6 is markedly elevated and predict mortality in ESRD patients [113]. Plasma levels of CRP are mirroring levels of IL-6 and it has been shown that elevated plasma levels of CRP also have an predictive effect of all-cause and cardiovascular mortality in ESRD patients [23].

The development of the low-grade systemic inflammation and altered cytokine balance is commonly seen in ESRD patients and it has been shown that even a small elevation of circulating IL-6 levels predict CVD in apparently healthy subjects [48]. Hence, it is of great interest to evaluate variations in genes of these two key regulating proteins, with the intend of finding functional mutations that can be used in stratification for the “high risk” patients.

The aim of this study was to elucidate the impact of genetic factors on plasma levels of IL-6 and CRP and the presence of malnutrition, inflammation and atherosclerosis in ESRD patients.

Plasma levels of IL-6, hsCRP and albumin were analyzed and information on presence of malnutrition, inflammation and atherosclerosis was obtained as mentioned previously. We analyzed the IL-6 promoter polymorphisms –597 G/A, –174 G/C, 5014 A/G SNP in intron 4, Phe201Phe C/T SNP in exon 6 as well as a promoter -286 C/T/A SNP and a 1059 G/C polymorphism in exon 2 in the CRP gene. The SNPs were genotyped in 229 ESRD patients and in 207 healthy subjects using the pyrosequencing method.

### ***Correlation between IL-6 and CRP***

As expected log plasma levels of hsCRP correlated well with those of log IL-6 levels ( $p < 0.0001$ ). Moreover, plasma levels of hsCRP and IL-6 were significantly ( $p < 0.0001$ ) associated with the presence of malnutrition and CVD.

### ***Associations to the genotypes***

Although all SNPs in the IL-6 gene as well as the 1059 SNP followed the H-W law, the CRP -286 C/T/A SNP significantly deviated in the patient group ( $p < 0.01$ ). The IL-6 -597, -174 and 5014 SNPs were in almost total linkage disequilibrium in both patients and controls. The most common IL-6 genotype combination was -597G, -174G, 5014 A which was significantly associated with inflammation in patients ( $p = 0.046$ ). However, a study by Kelberman et al. analyzing the –597 and –572 SNPs, an AnTn tract and the -174 SNP [114] in patients undergoing coronary artery bypass graft surgery (CABG), showed that a GG(A10T11)G haplotype was

associated with higher IL-6 levels as compared a GG(A9T11)G haplotype, suggesting that the impact of the promoter polymorphisms is complex [114].

CRP levels have been shown to be highly heritable [58] and elevated CRP levels predict both all-cause and cardiovascular mortality in hemodialysis and peritoneal dialysis patients [23]. Thus, it should be of great interest to evaluate the effect of SNPs on CRP plasma levels. Polymorphisms that are positioned in the promoter of the CRP gene are of particular interest since the promoter contains both IL-1 and IL-6 responsive elements [59], suggesting that polymorphisms in the promoters may regulate the IL-1 and IL-6 genes and indirectly influence the CRP synthesis, an effect that is seen in our material. A significant ( $p=0.027$ ) association was observed between the IL-6 -597 A/A genotype and lower hsCRP levels. Also the -174 G/C genotype tended to be associated with hsCRP levels ( $p=0.08$ ) with lower levels among C/C homozygotes.

The CRP -286 C/T/A SNP deviated significantly from Hardy Weinberg equilibrium in the patient group ( $p<0.01$ ) which might be a result of an association between a specific genotype and the ESRD phenotype. Indeed, we saw a tendency towards higher hsCRP in A- allele carriers as compared to C/C homozygotes ( $p=0.09$ ) an observation that has previously been reported in CHD patients, where the A-allele carriers had higher CRP levels [115].

### **Elevated resistin levels in chronic kidney disease are associated with decreased glomerular filtration rate and inflammation, but not with insulin resistance (paper V)**

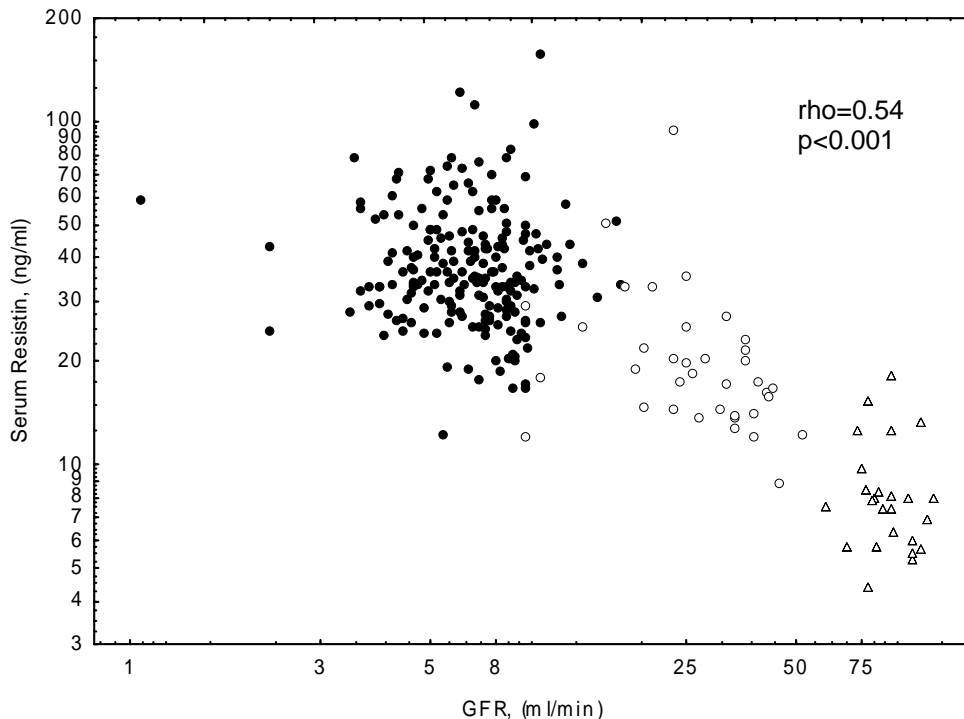
Resistin (named after its ability to induce insulin resistance in mice), is a recently detected protein expressed mainly in human bone marrow [87]. In genetically obese mice, circulating resistin levels are increased and administration of resistin to normal mice impairs the glucose tolerance and insulin action [86]. The -180 G/C promoter SNP has been shown to be functional since the G/G-genotype creates a gain of recognition in binding sites for transcription factors Sp1 and Sp3 and induces the promoter activity considerably [95].

The role of resistin was investigated in ESRD patients and the impact of the -180 C/G SNP on resistin plasma levels in our population was studied. The study included 239 prevalent CKD patients with varying degrees of renal function impairment which were compared with an age and gender-matched selected control group of 25 healthy individuals. The ESRD patients were divided by degree of GFR, where the mean GFR was significantly lower in CKD 3-4 ( $30\pm 2$  ml/min) and CKD 5 patients ( $7\pm 1$  ml/min) than in the age and gender-matched

controls ( $90\pm 3$  ml/min). Plasma analysis was performed of resistin, blood lipids, insulin, glucose, as well as of inflammatory markers (hsCRP, IL-6, TNF- $\alpha$ ) and adhesion molecules (VCAM, ICAM). The analysis of the polymorphism was performed in 168 patients and 205 controls using Pyrosequencing<sup>TM</sup>.

### ***Resistin correlates with GFR***

We found that plasma levels of resistin were elevated in CKD 5 patients ( $39.9\pm 1.3$  ng/mL) as compared to both CKD 3-4 patients ( $23.2\pm 1.0$  ng/mL;  $p<0.05$ ) and controls ( $8.5\pm 0.7$  ng/mL;  $p<0.001$ ) and that plasma levels correlated well with GFR ( $p<0.001$ ), as shown in **figure 10**.



**Figure 10.** Plasma levels of resistin correlate with GFR in 204 CKD 5 patients (●), 35 CKD 3-4 patients (○) and 25 age and gender-matched controls (Δ).

Furthermore, we observed a significant relationship between plasma resistin levels and insulin resistance, which was lost following the correction for GFR. Hence resistin is not a likely mediator of insulin resistance in CKD patients. Our results replicate other studies where, for example, resistin levels were associated with diabetic patients but not with insulin resistance *per se* [116]. This observation was also documented in a study on Pima Indians, who have a high prevalence of

obesity, where resistin was shown to be associated with obesity, but not with obesity-associated insulin resistance [117]

### ***Impact of resistin levels on adhesion molecules and inflammation***

Resistin is suggested to have a direct pro-inflammatory effect on vascular endothelial cells, which could enhance the development of atherosclerosis [118]. Indeed, it has been shown in a study on cultured human vascular endothelial cells that resistin is inducing the expression of adhesion molecules VCAM-1 and ICAM-1, an effect that is inhibited by adiponectin [119]. In this context, it should be of great interest to investigate the relationship between resistin and adhesion molecules in ESRD patients. Indeed, we found a positive association between resistin and both ICAM and VCAM levels. There was also a significant ( $p < 0.001$ ) association between resistin plasma levels and the inflammatory markers hsCRP and IL-6. The association between resistin plasma levels and inflammation is partly in line with the findings of Reilly et al., demonstrating that plasma resistin levels correlate with markers of inflammation in a study on asymptomatic members of the Study of Inherited Risk of Coronary Atherosclerosis [120]. However Reilly et al. also found that resistin levels have a predictive power of inducing coronary atherosclerosis independently of CRP [120].

### ***Impact of the -180 polymorphism***

The -180 G/C polymorphism in the promoter region of the resistin gene can affect transcription factor binding [97] and influence mRNA levels [96, 97]. We speculated that this SNP may also affect the circulating plasma levels of resistin in CKD patients. We found a modest association ( $p < 0.05$ ) between the genotype and plasma resistin levels, where heterozygotes had lower resistin levels as compared to the other genotypes. However, other studies have shown an association with higher plasma resistin levels and the G-allele [95, 121]. The small effect of the SNP on plasma levels may be overshadowed by the elevated resistin levels and the clinical significance of this genetic variation in uremic patients should be further evaluated.

## Limitations of the studies

Some limitations of the studies presented in this thesis should be taken into account.

Our study is limited in patient number. However, the material is well characterized regarding the phenotype and a large number of important clinical parameters are known. The thorough characterization of the patient material makes it possible to find the impact of small variations in a complex phenotype.

The diagnosis of the MIA complications may be insufficient. CVD was based solely on clinical grounds (using symptoms, signs and medical history), an approach which may underestimate the true prevalence of CVD. To complement the medical history, intima media thickness could be measured.

Inflammation was defined as CRP >10mg/L and based on one measurement. Since CRP levels can vary significantly over time, one single measurement of the CRP levels may be inadequate. However, one single measurement of CRP has been shown to correlate well ( $Rho=0.75$ ;  $p<0.0001$ ) with mean value from 12 measurements during 3 months (Stenvinkel. Unpublished observation 2005).

Malnutrition was estimated with SGA. Although SGA seems to have a high precision in determining the presence of malnutrition, but the detection of severity and cause is not fully sufficient. Since there may be two types of malnutrition, the first type related to the uremic syndrome *per se* and the second type associated with significant comorbidity and inflammation [15] it is of importance to estimate the level and cause of malnutrition more accurately.

Overall, the patient material was heterogeneous with variations in comorbidity, age, renal function, duration of renal disease as well as different cause of renal disease.

The large control population used in papers II, IV and V served for comparisons of deviations from the genotype distribution as well as H-W equilibrium tests. However, measurements of clinical parameters in control subjects would be preferable, enabling comparisons of genotype impact on clinical parameters in both patients and healthy controls.

The analysis of polymorphisms was performed using Pyrosequencing<sup>TM</sup>. The accuracy of the pyrosequencer was compared to TaqMan<sup>®</sup> which has been the golden standard for genotyping. The pyrosequencing method was found to be not only 100% accurate but also fast and flexible [122]. The possibility to attain fast

results and the ability to run several SNPs per 96-wells plate made it suitable for our studies.

The *in situ* technique is a sensitive but semi-quantitative way to detect mRNA in tissues, and to localize in which cells in the tissue the gene in question is expressed. A difficulty with this technique is that it often gives a high background that may lead to false positive results, especially when working with adipose tissue biopsies. However, this can be avoided by careful optimization of the hybridization steps, using negative and positive controls as well as replication of the results. A great advantage compared to other methods for mRNA measurements, such as Northern blots and real time methods, is that it leaves the cells and tissues intact, enabling the identification and the localization of the gene expression. Another advantage is that small amounts of tissues is required. The tissue biopsies obtained from patients and controls are very valuable, since the mortality is high, it is hard to get additional samples. The fact that the technique was set up and was running at our laboratory with access to highly experienced people of the method, made the choice of method obvious.

## CONCLUDING REMARKS AND FUTURE STUDIES

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The number of ESRD patients is rapidly increasing, partly due to the obesity-epidemic that is seen in the world, since the main cause of kidney failure today is diabetic nephropathy. The research need to focus on new areas to allow better prevention and treatment of ESRD and its complications.

The complexity of the disease suggests that factors such as infections, oxidative stress and endothelial dysfunction together with genetic variations in key regulating genes, may not only be involved in the progression of ESRD but also in the development of the MIA-complications.

Currently, the diagnosis of renal disease and the choice of treatment have exclusively been based on clinical observations of risk parameters. However, genetic studies of genes and the polymorphisms involved will lead to a better understanding of the different phenotypes observed in ESRD and may hopefully be used as a tool to determine if a patient is genetically predisposed to develop the different complications seen in ESRD.

The enormous progress in medical genetics and the development of high throughput techniques for SNP analysis enables researchers to obtain large amounts of data at a fraction of time needed only few years ago, but also leads to problems in data handling. Consequently, new advanced bioinformatics and statistical procedures are required to be able to perform simultaneous analysis of several polymorphisms at different loci and perform tests under a wider range of assumptions.

There are several important points that need to be considered when evaluating genes that can be used for the identification of “high risk” patients.

- Complex human traits are usually a result of a complex variety of genetic changes.
- Many genes may interact to result in one phenotype.
- One gene may give rise to several phenotypes.
- A single genetic change will most probably be modestly contributing to a phenotype.
- Gene-gene and gene-environment interactions.
- Large, well characterized populations are necessary to reach sufficient power to detect an effect of single genetic polymorphisms.



The impact of genetic variability on the development of complications in progressive kidney disease is becoming clearer and emphasizes the need to elucidate the genetic basis of renal disease and its complications. In the papers included in this thesis, we suggest that genetic variations may indeed affect the phenotype of this patient group. Hence, it is conceivable that genetic factors may explain some of the differences in for example inflammatory response and the development of atherosclerosis and malnutrition.

Development of a prognostic and predictive tool will provide the nephrological community with a more precise approach for identification of “high risk” ESRD patients and provide a possibility to develop more accurate individual treatment strategies.

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*Much has been learned from the genetic studies of ESRD and all observations that have been found provide pieces of a puzzle. However, the task of laying a puzzle without seeing the resulting picture is explicitly hard, and is similar to the story of six blind men palpating an elephant, told in a poem by John Godfrey Saxe. The blind men were asked to tell of their experiences when examining different parts of an elephant, without seeing the entire animal!*

**The Blind Men and the Elephant**  
**John Godfrey Saxe**  
**(1816-1887)**

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