Angiotensin-converting enzyme gene insertion/deletion polymorphism and renal disease
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Chapter 7

Summary and general discussion
SUMMARY

One of the main features in chronic renal failure is progressive renal function loss over the years [1-3]. Much scientific effort has been put in elucidating the mechanisms of progressive renal function loss in patients with renal disease. Reduction of high blood pressure and proteinuria are main intermediate targets to prevent progressive renal failure [2,3]. Unfortunately, not all patients have the same benefit from these renoprotective measures, even if reduction in blood pressure and proteinuria appears effective. Therefore, individual factors, such as genetic factors, may be important determinants in outcome of both the natural course of renal disease and the response to renoprotective therapy.

Recent advances in molecular genetic techniques have provided us tools to investigate the role of genetic factors in the multifactorial process of progressive renal failure. The angiotensin-converting enzyme gene insertion/deletion (I/D) polymorphism is the first genetic polymorphism that received great attention with respect to renal disease [4-6]. Since the discovery of this polymorphism numerous studies have been devoted to study the role of ACE I/D gene polymorphism with respect to the susceptibility for acquiring renal disease, the influence on the natural course of chronic renal failure and the response to renoprotective strategies. Unfortunately, many contradictory studies were published on the role of the ACE I/D polymorphism in renal disease. This has resulted in a cautious attitude towards studies on this subject among many investigators in the field.

The present thesis aims to provide new data concerning the impact of the ACE I/D polymorphism on the progression of renal disease and the effect of renoprotective therapy. We will especially put emphasis on the presently underemphasized role of gene-environment interactions. Directions will be given to help the reader to interpret present and future studies on this subject and to provide suggestions for further studies that are needed in the future.

Chapter 2

In chapter 2 we provide an overview on the vast amount of data that have been published on ACE I/D polymorphism and renal disease. The prevalence of the D-allele in a population appears to vary between subjects of different ethnic origin. In Caucasian populations approximately 36% have the DD genotype, 48% the ID genotype and 16% the II genotype [4,7]. This is in contrast with Asian populations that have a relatively high frequency of II genotype, up to 40% [4,8,9]. High ACE activity in DD genotype compared to ID and II genotype has been demonstrated not only in plasma but also in several tissues, including the heart and kidney [10-12]. The exact role of the specific insertion/deletion basepair fragment in the regulation of ACE activity remains to be clarified. The fragment is located on an intron and thus cannot
affect the expression of mRNA directly. It is hypothesized that the insertion/deletion fragment is in linkage disequilibrium with a still unknown DNA fragment that acts as a silencer fragment. In this way ACE I/D polymorphism is a marker to detect the silencer fragment that possibly inhibits ACE mRNA translation [4,5]. Differences and discrepancies in literature between studies that investigated populations of different ethnic origin could be partly explained by a variable relationship between ACE I/D polymorphism and this silencer fragment.

Almost all studies have shown that ACE I/D polymorphism is not associated with a higher risk to acquire a certain type of renal disease [4,5]. However, in patients with diabetes the discussion whether the DD genotype imposes a risk to develop diabetic nephropathy has not ended yet. Several studies showed a relation between DD genotype and the development of diabetic nephropathy in type I and type II diabetes [13-16], but other studies refuted these findings [17,18]. Most studies evaluated relatively small populations with short follow-up. Pooled data could not solve this problem as one meta-analysis on 4773 patients found an association between the D-allele and diabetic nephropathy [19], but two subsequent meta-analyses only showed a comparable association in Japanese subjects [20,21]. Recently, the first prospective study was published. In 310 patients with type I diabetes, a clearly increased risk was found for patients with DD genotype to develop microalbuminuria and also to progress to more advanced stages of the disease [22]. Obviously, it is very difficult to delineate the impact of ACE I/D polymorphism on a multifactorial disease such as diabetic nephropathy. Most studies have to cope with the flaws of a post-hoc analysis and the pitfalls in performing association studies. This is further discussed in the general discussion section.

Many studies have demonstrated a more progressive renal function decline in patients with DD genotype and chronic renal failure. This has been demonstrated in both diabetic [4,5,22-25] and non-diabetic [4,5,26-33] chronic renal failure. Some studies have disputed this association [34-37]. Unfortunately, the contrasting studies are hard to compare. Heterogeneity in ethnic background, inaccurate definition of renal disease phenotype i.e. differences in proteinuria and pre-existing rate of renal function loss and small sample size all contribute to this problem. Some studies suggest that DD genotype can have impact on rate of renal function loss but that the impact of the genotype can be obscured by other, stronger risk factors [38,39]. Possible interactions with other genetic or environmental renal risk factors could also play a role in discrepancies between studies on the role of ACE genotype rate of renal function decline in different patient populations.

Treatment with ACE-inhibitors has become a main tool in the prevention of progressive renal failure, especially in patients with overt proteinuria [3,40-42]. The interindividual variability in the efficacy of ACE-inhibition has been the major drive to study the role of ACE I/D polymorphism on therapeutic benefit. Disappointingly, it has not become clear whether DD genotype is associated with a better, equal or worse therapeutic benefit as compared to ID and II genotype [43-48]. Most problems
discussed for the studies on the natural course of the disease also apply to studies on therapy response. Environmental factors, such as sodium intake, may have an even larger impact here, as addressed in chapter 5 and 6 of this thesis.

An important shortcoming in the currently available scientific explorations on ACE I/D polymorphism is the paucity of studies on the pathophysiological implications of the polymorphism. The hypothesis that higher ACE activity in DD genotype results in higher angiotensin II production is often used to justify the great effort that has been put in association studies. However, only a few studies tried to test this hypothesis. Several studies showed an increased pressor response to angiotensin I in DD genotype [49-51], some also found corresponding higher angiotensin II levels [49,50]. Not all studies however confirmed these findings [52,53]. Potential explanations to clarify these discrepancies are further discussed in chapter 6.

Chapter 3

In the study described in chapter 3 we investigated the impact of ACE I/D polymorphism on the progression in rate of renal function decline. In a previous study of our department in 81 patients with chronic renal failure, patients with the DD genotype had a significant steeper slope of renal function loss compared to patients with the ID and the II genotype during intervention treatment [33]. Reduction in blood pressure and proteinuria versus baseline were comparable for all genotype groups. This suggested that patients with DD genotype were resistant to renoprotective therapy. To test this hypothesis, in the present study, we assessed the rate of renal function decline in the same study population before they entered this intervention trial. In accord with the findings during intervention, patients with the DD genotype had a more progressive rate of renal function loss over time. However, the difference in renal function loss between the pre-intervention period and the intervention period was far more greater in the DD genotype group as compared to the ID and the II genotype patients. In ID and II genotype, the improvement in slope was only modest. Thus, renoprotective benefit in the DD genotype patients was considerable. This provides evidence that renoprotective therapy in DD patients is even relatively more effective, which prompts for a different interpretation of our previous study. Only the absolute effect appears to be insufficient to result in comparable rates of renal function decline in different genotypes. Another interesting finding in this study is that the pre-intervention rate of renal function decline determines the benefit of renoprotective therapy. Patients with a fast renal function decline showed relatively large improvement in rate of renal function decline, whereas patients with slow progressive renal failure show only modest (but still important) improvement. The predictive value of pre-intervention rate of renal function decline for treatment benefit is of obvious relevance for future studies on renoprotective intervention, as it indicates that it would be important to consider prior
rate of renal function loss as a randomization parameter, as randomization on cross-sectional parameters may not always ensure a good match for risk of renal function loss. Also, prior rate of renal function loss could help to define the renal phenotype more accurately in studies on ACE I/D polymorphism and the effect on natural course of disease and effect of renoprotective strategies. A rapid rate of renal function loss can identify patients that may particularly benefit from intervention treatment.

Chapter 4

In this study we investigated the effect of ACE I/D polymorphism on the short-term response to ACE-inhibition in 61 patients with proteinuria. A good short-term response to ACE-inhibition is an important predictor of a favourable long-term renal outcome [42]. In chapter 3 we demonstrated that patients with DD genotype have a worse long-term renal prognosis despite treatment with ACE-inhibitors. It was hypothesized that short-term response to ACE-inhibition was less effective in DD genotype. However, in this study we demonstrated an equal short-term response to ACE-inhibition for blood pressure, proteinuria and renal hemodynamic parameters for all three genotypes. The results of this study can however not be easily translated to the population studied in chapter 3. Patients from chapter 3 had only modest or no proteinuria. The patients studied in chapter 4 had far more significant proteinuria but on the other hand a stable renal function within the time frame of the protocol, which is in contrast to the data in the population in chapter 3. The outcome of other studies that investigated the impact of ACE I/D polymorphism on short-term antiproteinuric response to ACE-inhibition have been variable [43-48]. Differences in study protocols, patient characteristics and other exogenous factors are probably responsible for these differences. This suggests future studies should focus on the interaction between ACE genotype and other factors that determine therapy response. Also, studies should be sufficiently powered to detect differences in therapy response, as the number of patients analysed in these studies, including ours, were limited.

Chapter 5

In this study we investigated the role of sodium intake on the short-term effect of blood pressure and proteinuria to ACE-inhibition in relation to ACE I/D polymorphism. Activation of the RAAS improves the antiproteinuric response to ACE-inhibition by either sodium restriction or co-treatment with a diuretic [53-54]. In the present analysis we demonstrated an association between sodium intake and the short-term renal response in DD genotype. We studied the short-term response of blood pressure and proteinuria in 88 patients with stable renal function and
proteinuria (> 1gr/day) in relation to sodium status as assessed by urinary sodium excretion. In patients with ID and II genotype this short-term response did not correlate with sodium intake over a wide range of sodium intakes. In contrast, the short-term response to ACE-inhibition in DD genotype showed a significant correlation with urinary sodium excretion. In DD patients with high sodium intake the responses of both blood pressure and proteinuria were relatively poor. This study is one of the first to demonstrate the importance of a genetic factor (ACE genotype) in interaction with an environmental factor (sodium intake). However, as important as this finding may seem, these results do not directly implicate that response to ACE-inhibition is sensitive to sodium intake only in patients with the DD genotype. Due to the post-hoc design of the study, the impact of sodium intake was studied in relation to inter-individual differences rather than by prospectively altering sodium intake with each patient as his own control. From the point of view of clinical applicability, it would be of practical use to estimate the benefit of sodium restriction in individual patients. Therefore, this is the subject of a current study at our department to obtain this prospective confirmation. Moreover, we sought to establish proof of the principle for gene-environment interaction between sodium status and ACE genotype in healthy volunteers, as is described in the next chapter.

Chapter 6

In this chapter we describe our study that aims to prove the hypothesis that subjects with DD genotype have enhanced angiotensin I conversion to angiotensin II. We infused both angiotensin I and, after a washout period, also angiotensin II in healthy subjects to study the response of blood pressure, renal hemodynamics and hormonal parameters. In order to detect gene-environment interaction with sodium status, this experiment was performed twice, on two consecutive days after one week of sodium restriction and liberal sodium intake respectively, in randomized order. In DD subjects we demonstrated increased response of blood pressure, glomerular filtration rate and aldosterone secretion to infusion of angiotensin I as compared to ID and II subjects. Angiotensin II infusion elicited similar responses in the three genotypes, indicating a similar angiotensin II sensitivity in the three genotypes. This strongly suggests that the enhanced angiotensin I responses are the result of a higher ACE activity leading to increased angiotensin II formation. However, we could not demonstrate a difference in angiotensin II levels. A conclusive explanation for these findings cannot be given but it is possible that increased conversion to angiotensin II at tissue level is present. Several other studies also found evidence for an increased response to angiotensin I in DD genotype [49-51]. Some of them found higher plasma angiotensin II levels but these used doses of angiotensin I higher than ours. This could explain the difference with our findings as increased angiotensin II levels in DD subjects were only reported in studies were a relatively high dose of
angiotensin I were used [49,50]. Moreover, these studies already find an increased blood pressure response to angiotensin I during an angiotensin I dose that was lower than the dose required to demonstrate a higher angiotensin II plasma level. Thus, the blood level of angiotensin II does not directly correspond with the pressor effect.

Most remarkably, the increased response to angiotensin I in DD genotype was only demonstrated during liberal sodium intake. Sodium restriction completely abolished the differences between the genotypes, for blood pressure, renal function, as well as aldosterone. Again this implicates interaction between sodium intake and ACE I/D polymorphism, but the mechanism remains obscure. Liberal sodium intake will lead to angiotensin II receptor upregulation, it is possible that only under these circumstances small differences in local angiotensin II concentrations can become apparent. In contrast, downregulated angiotensin II receptor concentration during sodium restriction could abolish the impact of small differences in local angiotensin II concentration. Sodium intake might also affect local uptake or degradation of angiotensin II, or influence ACE activity itself. Presently, no human data are available to support this hypothesis.

GENERAL DISCUSSION

New genetic technologies have recently contributed to the unraveling of the human genome nucleotide sequence and prompted studies of comparative genomic diversity in the human population across the globe. These studies will not only provide information on the origins of genetic variation, but also will provide a basis for understanding the genetic basis for complex diseases and traits. As genotyping techniques are technically simple and become more widely available, studies on gene polymorphisms have therefore become increasingly popular as a tool to associate specific polymorphisms with the occurrence or course of disease. Geneticists are aware of the limitations of investigations based on polymorphisms and call for caution against abuse of association studies. However, many clinical investigators have overlooked or misunderstood the wariness of this approach [56]. It is for this reason that polymorphism association studies are probably used much too extensively without any in-depth knowledge of its underlying theory. ACE I/D polymorphism is the first polymorphism that was intensively studied in cardiovascular and renal disease. As can be anticipated, many problems were encountered in the interpretation of these studies as is discussed below.
ACE I/D gene polymorphism; can we freely associate?

Since the first discovery of ACE I/D polymorphism by Rigat et al [7] and the first major association study that reported an association between the D-allele and myocardial infarction [39], an impressive volume of data on this subject has been published. In the years 1995 and 1996 more than 50,000 individuals were genotyped, an enormous clinical and scientific effort and also an expensive one [56]. However, this fascinating wave of studies became to a tide. The striking phrase of Pinto and van Gilst adequately states the current status of ACE I/D polymorphism; ”after a Good start, conflicting results gave the genotype a Bad reputation, and the stage now looks rather Ugly ” [57]. Thus, it seems that ACE I/D polymorphism is a victim of its own success. As more novel or discrepant associations are published, the less likely it will be that a unifying conclusion on the role of ACE I/D polymorphism will become available. What went wrong in our approach? The explanation can be twofold; first, association studies have not always been conducted according to currently available standards. Second, almost shameful little attention has been paid to the pathogenetic mechanisms that may link ACE I/D polymorphism and disease.

What are the conditions for a good association study?

In order to adequately interpret association studies on ACE I/D polymorphism we must first understand the problems of such studies and define criteria for valid studies. First, a disease locus should be identified. Common inherited disease or risk factors are however difficult to study as a combination of various genes and different environmental factors are often involved. Complex diseases are often thought to be inherited as they tend to run in families, but they do not show mendelian pedigree patterns of inheritance. These non-mendelian diseases may depend on more than one susceptibility locus, with a variable contribution and interaction of environmental factors. The identification of such loci can be pursued by different strategies. Studies in families, either by segregation analysis or by linkage analysis to candidate genes or markers can be used to localize a gene. This could be followed by positional cloning and in the general population by association studies in homogeneous populations. However, segregation analyses are prone to bias. In case of linkage analysis studies, a precise genetic model including disease frequency and the penetrance of each genotype are needed. Thus, population association studies are mostly used for the detection of susceptible loci. In association studies we test whether a genetic marker occurs more frequently in cases than in controls. If so, the polymorphism is either the susceptibility locus itself, or in linkage disequilibrium with this locus. In both cases the polymorphism should identify people at risk to develop disease or to develop complications such as progressive renal failure in pre-existing renal disease.
Several confounding factors have been recognised in this type of study. First, the definition of the phenotype for cases and controls is often variable due to different definitions and also to the heterogeneity of the disease phenotype. Second, genetic background of the cases and controls are often mixed, especially in multicultural areas. This phenomenon elicits the risk of so-called population admixture, which can cause erroneous association if a study includes genetically distinct subpopulations. Some ethnic subpopulations can coincidentally display both a higher frequency of disease and certain allelic variants. Thus, in this case a biologic effect of the genetic variant itself is not present. In view of differences in genetic background it is interesting to note that most studies that found positive association between the D-allele and cardiovascular disease and risks are of European origin. Large sample studies from the US often failed to demonstrate an association. However, the increase in circulating ACE activity in the DD genotype is a consistent finding in European study populations, in contrast to US study populations for which such data are lacking [57]. In support with this assumption it was found that the correlation between ACE activity and the D-allele was not present in an population of afro-american origin, a substantial part of the population in US study samples [58]. It is feasible that the ACE I/D polymorphism has lost its linkage to the functional gene in subjects of afro-american origin. The heterogeneous background of this population may therefore explain the less increased risk of this ancestral gene variation in contrast to a population with low migration rate. Thus, only in homogeneous populations correlations can be found, the so-called founder effect. This loss of the hypothesized linkage with the unknown functional DNA fragment was first hypothesized to be due to the moderate physical distance between the ACE I/D polymorphism locus and this functional DNA fragment. However, also the different frequencies of the genotypes in the different subpopulations [4], genetic mixture due to cultural diversity within one population and also the age (time of origin in a phylogenetic sense) of the allelic variant can determine loss of linkage in certain populations [59]. In conclusion, if positive study results are not replicated in different populations, this could lead to a rejection of a true association recorded in a specific population. Third, methodological difficulties play a role. Studies should have adequate genotyping methods and use appropriate statistical methods with sufficient power and preferably low P values. Fourth, publication bias is a major concern as studies that find a positive association are preferentially published, leading to an overestimate of the significance of a specific polymorphism. In conclusion, considering all these problems, we are in urgent need to define a limited and generally accepted set of methods and criteria that allow appropriate assessment of association studies in the future and help with the comparison of individual study results. We should however not be discouraged by these problems as our standards will evolve as knowledge improves on complex traits and on appropriate strategies for conducting association studies.
ACE gene I/D polymorphism; does it have functional consequences?

As mentioned earlier, we first need to answer basic pathophysiological questions to be able to understand the impact of ACE I/D polymorphism in renal disease. The question is whether there is a plausible credibility that ACE DD genotype is associated with increased risk for progressive renal failure or difference in response to renoprotective therapy. In other words, does it makes biological sense that the D-allele affects the gene product in an meaningful way? Before discussing the current concept and knowledge we have on the pathophysiological consequences of ACE I/D gene polymorphism, we should first realize that the relatively high ACE activity in DD genotype is the central concept in our current running hypothesis on its deleterious effects in cardiovascular and renal disease. Although the I/D locus is in strong disequilibrium with the elevated ACE levels in many cases, recent data showed that ACE I/D polymorphism is not always a clear determinant of ACE activity. The discrepant results in populations of different ethnic origin already pointed forward to recent observations that the level of linkage disequilibrium can vary. Indeed, the I/D locus is strongly linked to ACE activity in Caucasian subjects but recent genetic studies found no association in afro-american and afro-caribbean subjects [58,60]. Differences in ACE activity between populations of variable ethnic origin also appeared to be functionally relevant [61]. Absence of differences in plasma ACE activity between ACE genotypes, as found in blacks, results in a diminished difference in vasodilator response to bradykinin between ACE genotypes. In contrast, in whites with DD genotype, the higher ACE activity was accompanied by a clearly attenuated response to bradykinin, with remarkable differences compared to white ID and II genotype subjects. Perhaps this finding may even have therapeutical implications in understanding the observed ethnic variation in the effect of antihypertensive efficacy of ACE-inhibition. We are therefore in great need to identify polymorphic DNA fragments that are more closely associated with ACE transcription. Two studies aimed to track down such alternative polymorphisms on the ACE gene [60, 62]. In these studies, it was shown that the I/D locus is in linkage disequilibrium with ACE activity in Caucasian subjects but not in Afro-Caribbean subjects. Two single-nucleotide polymorphic sites were identified that were in strong linkage disequilibrium in both populations [60], as another study found a comparable third locus [62]. This major breakthrough is an important step to determine where the quantitative trait locus that determines ACE level itself is exactly positioned. In the near future we may be able to use more different polymorphisms of the ACE gene to identify patients at risk for progressive renal disease or therapy resistance.

At present, we use DD genotype and the accompanying higher ACE activity, as found in Caucasian subjects, as the central component in our pathophysiological concept. The mechanisms by which this variability in ACE level is caused were not precisely defined after the first report of Rigat et al [10]. Subsequent studies showed that ACE activity was also elevated in tissue [11,12].
Summary and conclusions

DNA/mRNA level ACE expression was variable in ACE I/D polymorphism. This hypothesis was confirmed in two studies on ACE mRNA level in human left ventricular tissue which demonstrated increased expression of ACE mRNA in DD genotype patients with heart failure [63,64]. However, these differences are not uniformly present in all tissues as similar ACE mRNA expression was found in human atria [65,66]. Thus, regulation of gene expression appears to be cell or tissue specific. In the current concept of increased angiotensin I conversion in the kidney and the hypothesized repercussions on renal disease, the findings of Mizuiri et al are important [67]. In their recent study it was first demonstrated that increased ACE mRNA level expression in human renal tissue is present in DD genotype with a 3- to 6 fold higher ACE transcripts compared to ID and II genotype respectively.

As a physiological basis for the difference in plasma and tissue ACE (including the kidney) was recently demonstrated, the next point of discussion is whether high ACE activity has functional consequences and leads to increased conversion of angiotensin I. The enzymatic activity (K_M) of ACE was shown not to be affected by ACE genotype [68]. Thus, high ACE activity itself must be responsible for increased angiotensin I conversion. However, it is usually assumed that renin is rate-limiting for the generation of angiotensin II. At regional (tissue) level, membrane-bound ACE is responsible for conversion of angiotensin I [69]. This process follows a first-order kinetics, since angiotensin I levels are approximately six orders of magnitude below the K_M for angiotensin I [70,71]. This will even apply at angiotensin levels that are 10,000-fold higher than normal. In accord with these findings, conversion to angiotensin II was found to be similar over a wide range of arterial angiotensin I levels, in both animal and human studies [69,72,73]. Angiotensin I-induced blood pressure responses in rats of two strains with low and high ACE activity, was shown to be similar despite a two to three fold difference in ACE activity [74]. Transgenic rats that have a 40-fold ACE overexpression also had a normal angiotensin II level [75]. On the other hand, there is contradictory evidence that variable ACE expression and activity can determine angiotensin II formation to some extent. Increased local angiotensin II production in response to increased ACE expression was shown both for infused and locally formed angiotensin I in two experimental models [76,77]. Because of these contradictory data, the hypothesis of increased formation of angiotensin II in humans with DD genotype is highly controversial. Unfortunately, only limited data from human studies are available. In humans with increased ACE expression during an episode of unstable angina, not only de novo angiotensin I production was found to be increased but also the fractional conversion to angiotensin II [78]. In contrast, regional angiotensin I to II conversion in the human forearm and leg did not parallel the previously described DD genotype related differences in ACE activity [79]. This is in accordance with two previous studies that studied the blood pressure response to angiotensin I in healthy volunteers [52,53], although these studies did not investigate regional conversion rates. Ueda et al performed two studies, both providing evidence for increased pressor response to
angiotensin I in DD genotype [49,50]. In both studies angiotensin II levels were significantly higher during angiotensin I infusion, but only during high dose angiotensin I administration. In our own study described in chapter 6, we report comparable findings, but differences in angiotensin II levels were not observed, possibly because the dose of angiotensin I was not as high as in the studies of Ueda et al. [49,50].

Not only at the level of angiotensin II formation but also at the level of aldosterone secretion, recent data on ACE I/D polymorphism became available. Our findings are in line with an experimental study of Ueda et al. who studied the effect of an acute dose of ACE-inhibition in healthy subjects with different ACE genotypes [50]. In subjects with DD genotype, after the drug induced initial fall, the rise in angiotensin II plasma levels occurred significantly earlier as compared to subjects with ID and II genotype. Thus, even during ACE-inhibition, a higher ACE activity in DD genotype results in apparent differences in RAAS parameters. This finding strongly suggests the importance of ACE I/D polymorphism in terms of therapy response. This could be in line with our own data on facilitated aldosterone stimulation discussed in chapter 6. With regard to clinical relevance, this finding seems important as higher aldosterone levels are important in the process of myocardial and vascular fibrosis [80]. Also, inhibition of aldosterone action by spironolactone is associated with reduced risk in morbidity and death in patients with heart failure [81]. A recent study in heart failure patients revealed that DD genotype was associated with aldosterone escape during ACE-inhibition [82]. Thus, increased aldosterone production or and escape in a therapeutical setting could be a mechanism that is involved in increased cardiovascular risk in DD genotype. Unfortunately, there are no data on possible deleterious effects of aldosterone in human renal disease.

Feedback regulation in the RAAS should also be considered. If increased angiotensin I conversion will result in higher angiotensin II levels, a negative feedback loop via a decrease in renin release should compensate for this effect. Such a mechanism could therefore reduce the rate of angiotensin II generation to normal levels in steady state conditions. A large epidemiological study showed that the downregulation of renin, prorenin and aldosterone was not significantly different between the three genotypes [83]. In our own study discussed in chapter 6, a distinct trend of higher PRA suppression in DD genotype during angiotensin I infusion was found, but due to the very low levels of PRA this study was not sufficiently powered to resolve this question. Barlassina et al. studied 145 non-treated hypertensive subjects in a sodium-repleted state [84]. A significantly lower PRA level in DD subjects as compared to II genotype was detected, with intermediate levels (as expected by the current hypothesis) in heterozygotes.

In this respect, it would be very interesting to investigate the presence of feedback within the RAAS system in tissue. In mice, having one, two or three copies of the "Ace" gene (ACE gene in rodents), a linear relationship was found between
ACE activity in serum and the number of gene copies [85]. As a result of apparent negative feedback, kidney renin mRNA was reciprocally more downregulated in mice with more Ace copies. This indicates the presence of negative feedback at tissue level. This probably explains why blood pressure was similar in all groups. However, despite this negative feedback, a higher Ace activity seems to be of functional significance as the pressor response to angiotensin I and the depressor response to bradykinin were clearly attenuated in the group with reduced Ace gene function in contrast to the group with increased Ace gene function [86]. However, this was found in an experimental and pharmacological setting. Thus, the functional consequences at tissue level can not be extrapolated from such data. Kidney and heart weights were found similar between mice with a different number of Ace copies [85]. Remarkably, renal tubulointerstitial volume decreased significantly with increasing Ace copy number. Pinto et al. found no increased angiotensin II in heart tissue in a transgenic model resulting in a 40-fold higher ACE expression in the hearts, but nevertheless found more fibrosis in this group [75]. Thus, in models with high ACE activity, despite negative feedback at tissue renin mRNA level, high ACE appears to have effects at tissue level. This clearly illustrates that we should not just focus on hormonal plasma and tissue levels alone but also on structural changes when formulating a hypothesis that explains the impact of AC I/D polymorphism in renal disease.

In summary, there are data indicating that high ACE activity is associated with increased angiotensin II formation and also with increased aldosterone secretion. The data are however inconsistent. First, we should be aware that in these experimental animal studies, a direct comparison and extrapolation to humans must be made with caution, as marked species differences for the vascular angiotensin II forming pathways can be present between humans and rodents. The scarcely available and contradictory data of clinical studies point out that we still must further explore the clinical relevance of ACE I/D polymorphism. Until now, apart from studies of the association type, most studies have focused on experimental and pharmacological study designs. Angiotensin II and also aldosterone have however many effects on a structural-tissue level. Therefore, secondly, we must also focus on this issue, as it is already known that chronic effects on vascular or cardiac tissue may precipitate even when differences in plasma are minute and barely detectable [87]. Finally, we should not forget that conversion of angiotensin I to angiotensin II is not the only action of ACE. ACE degrades a wide variety of substrates including bradykinin, substance P and the hemopoietic stemcell regulator AcSDKP. In addition, ACE activity may also decrease angiotensin 1-7 levels, which may be a modulator of RAAS activity.
Is there evidence for gene-gene interaction?

We can conclude that after a large number of confusing data from association studies, a number of pathophysiological studies has been performed that may allow to discern the functional role of ACE I/D polymorphism. It is clear that we still do not have all the answers as there are still contradictory results. What can be the cause of that? First, we have to consider the potential interaction with other relevant genetic factors. As was explained earlier, we should keep in mind that whenever an association is found between a genetic polymorphisms and a phenotype, the possibility that another gene polymorphism in close proximity to the one studied is the real causal gene cannot be excluded. The demonstration of synergistic or interactive effects of two or more genes on different chromosomes, preferably both with a plausible role in causing the phenotype, would not only strongly reinforce the likelihood of their pathophysiological involvement, but also help us to understand the complexity of the genetic architecture. Some studies have been addressing the effect of other genetic polymorphisms of the RAAS, such as the angiotensinogen (AGT) M235T gene polymorphism and the angiotensin II type I receptor A1166C gene polymorphism [4]. The angiotensin II type I receptor A1166C gene polymorphism appeared to be associated with an increased response to angiotensin II in isolated human arteries [88]. A subsequent study investigated the increased risk for ischaemic cardiac events in relation to combined RAAS polymorphisms and found interaction between ACE DD genotype and AT(1)R-CC genotype [89]. As to renal diseases, Pei et al. correlated rapid progression of renal function loss in IgA nephropathy in patients with the AGT T allele irrespective of ACE genotype, while AGT MM homozygotes had rapid progression only when DD genotype was present concomitantly [35]. Severity of renal involvement in diabetic subjects with the AGT MM to TT genotype was increased in patients also having DD genotype [90], but interaction was not confirmed in patients with polycystic kidney disease [32]. Not surprisingly, these association studies were also hampered by problems of methodological origin, as discussed previously. This is complicated by the fact that the genetics of the RAAS are much more diverse than just one or two polymorphism for each component. With respect to the ACE gene, more than 78 polymorphisms have been described, with a similar diversity as to the other components [91]. Also, the RAAS not only has multiple components and genes that define its activity but, non-RAAS systems can also enhance or counteract this system. Just as one example of interacting non-RAAS systems, it was shown that vascular contraction was more dependent of nitric oxide in vascular rings of DD genotype subjects [51]. Interestingly, in a subanalysis of our data from chapter 6, we found that during high sodium intake, plasma nitric oxide availability was lower in DD genotype as compared to the ID and II subjects. During low sodium intake, plasma nitric oxide levels were comparable [92]. To give an example of other genetic polymorphisms, Barlassina and coworkers have recently shown that, with an appropriate hypothesis and
experimental setup, it is feasible to recognize interacting genes in a more logical sense [84]. A potential interaction between ACE I/D polymorphism and the α-adducin gene polymorphism was studied in 145 non-treated hypertensive subjects on a liberal sodium diet. This interaction was hypothesized from the assumed functions of the genes. An increased angiotensin II level, as hypothesized in DD genotype, is known to reduce the steepness of slope of pressure-natriuresis relationship, causing salt-sensitivity. Adducin is a cytoskeleton protein with different isoforms, differentially affecting sodium-potassium pump activity on the basolateral tubular membrane, which is the driven force of overall tubular Na+ reabsorption. Hypertensive patients with the 460Trp genotype of the α-adducin gene are salt-sensitive, and have a steeper pressure natriuresis relationship and increased tubular reabsorption [93-95]. The interactive effects of the two polymorphisms were examined by studying the pressor response to a saline load. It was hypothesized that the degree of suppression of tissue angiotensin II under saline (RAAS suppression) conditions in DD genotype would be less effective than in ID/II genotype, and that this functional characteristic might be enhanced by the presence of the α-adducin 460Trp allele. When analysed for the two genes separately, the pressor response to saline was not significantly affected by the DD genotype, as it was in the the α-adducin 460Trp allele group. However, statistical analysis showed that the two genotypes interacted epistatically in the pressor response to saline loading, providing evidence for a causal interaction. This is one of the first elegant examples of the functional consequences of gene-gene interaction in gene polymorphism research demonstrating the potential of hypothesis-driven studies with a solid pathophysiological basis. It is clear that without prior knowledge of the potential role of these interacting genes, it will be extremely difficult to prove that a certain interaction is present for a given phenotype (risk or therapy response). In the near future, we should therefore focus on potential gene-gene interaction, not only in fundamental pathophysiological studies, but also in studies on disease outcome and response to therapy. If we make this effort, this will allow us to determine which polymorphisms act synergistically or on the other hand compensate for the individual impact of other polymorphisms, which is crucial for their clinical significance.

**Is there evidence for gene-environment interaction?**

It is well-known that the setpoint of the RAAS is modified by several exogenous factors. Sodium intake, age, gender and disease state can all modify RAAS activity. In this thesis special emphasis was laid on sodium intake. Sodium restriction activates the RAAS, which has several therapeutic implications. In chapter 5 we described a cross-sectional study that suggested that in DD genotype patients with proteinuria, response to ACE-inhibition was significantly more modified by variable
sodium intake as compared to patients with ID and II genotype. In chapter 6 we found that the response to angiotensin I infusion was enhanced in DD genotype subjects on a liberal sodium intake. This effect was abolished by adherence to sodium restriction. This finding indicates that sodium intake should be taken into account in future studies on ACE I/D polymorphism, both in clinical or also fundamental pathophysiological studies.

The mechanisms that explain the impact of sodium intake are not thoroughly explored. We could speculate that sodium intake influences ACE activity itself and thus has greater impact on DD genotype. It was recently found that high salt intake increased not only blood pressure but also ACE expression in the hypothalamus in Dahl S rats [96]. Dietary sodium loading also stimulated ACE expression in left ventricular tissue in SHRSP rats [97]. In human subjects it was found that fractional angiotensin I conversion in the peripheral vascular bed was higher during liberal sodium intake as compared to low sodium intake [98]. This could be in line with our findings described in chapter 6. Unfortunately, the impact of variable sodium intake on ACE genotype, in specific relation with plasma or tissue ACE activity have not yet been subject of study. The discrepancies in the many studies on ACE I/D polymorphism could be partly explained by differences in sodium intake, but this is hard to prove in most studies, as they are not standardized to sodium intake. It is interesting to review the sodium intake in studies that investigated the angiotensin I to angiotensin II conversion. The previously discussed positive studies on bradykinin response and angiotensin I infusion [49,61], as well as the study on synergistic effect of adducin [84] were all performed in sodium repleted subjects. In two negative studies sodium intake was not protocolized [52,53]. Lachurié et al. blocked the RAAS by renin-inhibition [52] but it is not known how this maneuver affects tissue ACE level. In view of the contrasting effect of sodium intake on tissue and circulating RAAS, Danser et al. found no evidence for increased angiotensin II formation in human DD genotype subjects but in many patients the RAAS was activated by the use of diuretics which could have obscured these results [79].

Another important exogenous factor that has to be considered is blockade of the RAAS by ACE-inhibition. In many clinical studies patients have used ACE-inhibition. For several reasons it is very difficult to determine the differences in effects for different genotypes, especially if ACE-inhibitors are not standardized by dose, type or duration of therapy. It was demonstrated that the percentage of pre-treatment ACE activity that is blocked is similar for all the three genotype groups given a fixed ACE-inhibitor dose [50]. However, the absolute residual degree of ACE activity is variable with higher ACE activity in DD genotype after the acute effect of intravenously administered ACE-inhibitor weans off [50,99]. One hour after the administration of an ACE-inhibitor, the rise of plasma angiotensin II during angiotensin I infusion was prevented in both II and DD genotype. After ten hours however, plasma angiotensin II levels increased during repeated angiotensin I infusion only in DD subjects, indicating that after this time period the inhibition of
ACE had weaned only in DD genotype. Also, long-term blockade of the RAAS usually results in an upregulation of several RAAS components by strong feedback mechanisms. It is currently not known whether RAAS upregulation caused by institution of ACE-inhibition is genotype dependent. If so, we should take this into account in the interpretation of studies where ACE I/D polymorphism is studied in relation with therapeutic efficacy.

Patient characteristics should also be considered. First, as discussed earlier, risk factors with strong impact, such as hypertension or proteinuria, can obscure the effect of a risk factor of lesser strength. Also, the overall clinical condition in subjects can play a role. The relative risk reduction in subjects with an overall worse prognosis or progressive course of disease will likely be of clinical relevance, as we also found in our study described in chapter 5. In adriamycin nephrotic rats, more renal damage is associated with a higher renal ACE activity, with concomitant worse response to ACE-inhibition or angiotensin II blockade [100]. Baseline ACE activity is also found to be higher in diabetic subjects, even in normotensive and normoalbuminuric subjects [101]. Thus, we should also consider differences in baseline severity of disease and other relevant disease conditions, such as diabetes, as well. Finally, we should not forget to pay attention to the gender of subjects included in studies. In sodium-repleted women, glomerular filtration rate decline to angiotensin II infusion is more pronounced, but decrease in renal plasma flow was more blunted as compared to men [102]. Aldosterone baseline levels were lower in women compared to men. This indicates gender specific differences in the RAAS. Only limited clinical data are available. Recently, females with chronic renal failure appeared to be at lower risk to develop end stage renal disease as compared to men [103]. In this study, ACE-inhibition was uniformly renoprotective in women regardless of their ACE genotype. However, male subjects only showed beneficial effects in DD subjects. Unfortunately, data on pre-existing renal failure, described in chapter 3, were not available. Nevertheless, it is clear that gender is an important confounder in studies on RAAS disparities and ACE I/D gene polymorphism.

Conclusions

The clinical genetics of complex diseases is a young discipline. ACE I/D gene polymorphism was the first polymorphism that has been thoroughly studied in cardiovascular and renal disease. The many studies in the beginning were fashionable and got great attention, but we have now come to a point that we have to move further to discern the role of genetic polymorphisms in general and ACE I/D gene polymorphism in particular in clinical medicine. However, we shall make little headway if we do not answer the basic questions on pathophysiological significance of the genetic polymorphisms, with special emphasis on the presence of gene-gene interaction and gene-environment interaction. Only then we will eventually be able to
translate this knowledge into clinical benefit. Despite the major efforts, this path is still poorly lit. With the increasing knowledge on molecular genetics we will be able to create a catalogue of common-coding-sequence variants in human genes and test directly for association with a phenotype. With a genome-wide map of known polymorphisms we can explore the genome for marker-disease association. After a burst of association studies it is very promising that more recently, new data became available on the functional impact of ACE I/D polymorphism. If we continue this effort, determination of ACE genotype in an individual patient might be helpful in the future, to identify patients at risk for progressive renal disease and also to develop specific treatment strategies to modify the course of disease.
REFERENCES


Summary and conclusions


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