Chronic kidney disease

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General discussion

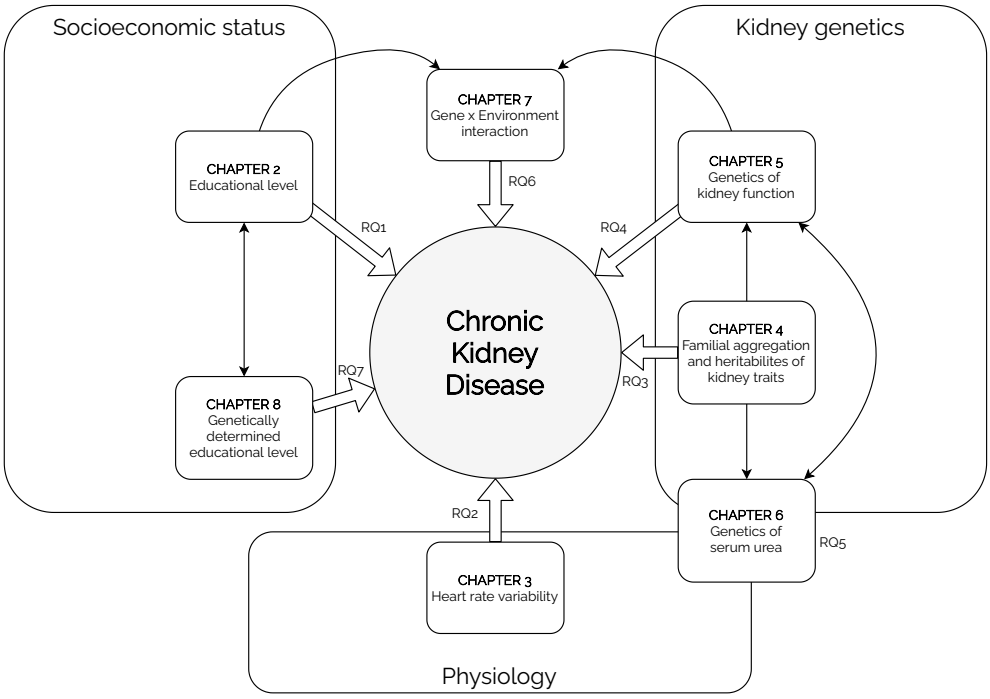
CHAPTER 9

GENERAL DISCUSSION

In this thesis, I examined mechanisms that influence risk of chronic kidney disease (CKD). Of particular interest were socioeconomic disparities in CKD, and how knowledge of the genetics underlying CKD can help in understanding these disparities. In a range of studies, I applied a variety of traditional epidemiological methods as well as genetic epidemiological designs and concepts, such as genetic risk scores (GRS), a family design, a genome wide association study (GWAS), a gene x environment interaction study, and Mendelian randomization (MR). In **Figure 1**, I provide a graphical overview of the different chapters, the research questions (RQs 1-7), and their interrelationship within this thesis.

This chapter contains the general discussion of my findings. First, I reflect on these findings in a broader context, and comment on the methods applied in this thesis. Then, I discuss future perspectives and the implications of my findings for public health, as well as clinical and research practice.

Figure 1. Graphic representation of the research questions (RQs) and their interrelationship in the context of this thesis. White arrows represent hypothesized direction of effect. Black arrows reflect overlap between chapters in use of data, methods, and/or results.



PART I: EVALUATING THE EFFECT OF SOCIOECONOMIC STATUS AND AUTONOMIC DYSFUNCTION ON RISK OF CKD

Chapter 2: Educational attainment is associated with risk of chronic kidney disease in the general population1

The predominance of studies on the relation between socioeconomic status) and CKD are based on cross-sectional, US data[2,3]. In **Chapter 2**, I present longitudinal data from the PREVEND cohort study, a Dutch community-based observational study with serial follow-up, where I used educational attainment (EA) as an indicator of socioeconomic status. In this sample, participants with low EA were at a 25% higher risk of developing CKD, and on average had a 0.15 mL/min/1.73m^2 faster eGFR decline per year compared to those with high EA, taking into account age and sex. Further analysis suggested a mediating role for modifiable factors such as hypertension, diabetes, and anthropometric indices, corroborating several cross-sectional findings from a previous mediation analysis[4].

An important finding from this study is the suggested mediating role of poor diet, i.e. low EA is associated with CKD through poor diet, in particular low potassium intake (i.e. few fruits or vegetables[5]). A role of poor diet in exacerbating CKD risk has long been proposed[6]. However, there is a paucity of data as only one previous study formally tested mediation by poor diet[4], but that study was limited because only questionnaire data on fruit intake was available. In contrast, I assessed diet by examining 24h urine excretions of nutrients as objective measures of nutrient intake (i.e. sodium, potassium, magnesium, and protein).

This study adds to the literature by providing insights into the mechanisms underlying the EA-CKD relation. In my models, the addition of potential mediators did not completely explain the association between EA and eGFR decline. This suggests the existence of non-traditional intermediate factors in the EA-CKD association.

Box 1. Abbreviations
CKD = chronic kidney disease
eGFR = estimated glomerular filtration rate
EA = educational attainment
GRS = genetic risk score
GWAS = genome wide association study
MR = Mendelian randomization
SNP = single nucleotide polymorphism

Future study may focus on identifying these non-traditional factors. In addition, further study of the intermediate pathways is necessary. Importantly, establishing the interrelationship between the different mediating factors (e.g. the effect of poor diet on body-mass index and hypertension, and body-mass index on diabetes, etc.) may help in prioritizing targets for public health interventions to mitigate socioeconomic disparities in CKD.

Chapter 3: Low heart rate variability does not precede chronic kidney disease in the general population[7]

Given that non-traditional risk factors may play a role in the development of CKD, I examined the effect of low heart rate variability, as an index of autonomic dysfunction, on CKD incidence (**Chapter 3**). Low heart rate variability occurs in the presence of an imbalance in autonomic function, when parasympathetic function is reduced relative to sympathetic function. Potentially, autonomic dysfunction leads to renal damage through changes in renal hemodynamics, with some evidence for such a detrimental effect in animal models. This effect of low heart rate variability on kidney health may exist in humans as well. Previously, a community based study in the US reported associations of low heart rate variability with CKD related hospitalization and end-stage renal disease[8]. Thus, the expectation was that low heart rate variability precedes new-onset CKD. In longitudinal analyses of data of 4605 subjects participating in the PREVEND cohort study, I observed a 50-100% higher risk of incident CKD for participants in the lowest quartile of heart rate variability measures, relative to those in the upper three quartiles. However, this association appeared to be completely driven by higher age in those with low heart rate variability and CKD. Thus, I could not corroborate a relation between low heart rate variability and CKD incidence. Rather, in post-hoc analyses, I found evidence suggesting an effect in the opposite direction (i.e. reverse causation), that is, CKD resulting in low heart rate variability, given that low heart rate variability was associated with kidney function only in those with CKD.

PART II: GENETICS OF KIDNEY FUNCTION AND THE TRANSLATION TO CLINICAL AND RESEARCH PRACTICE

Chapter 4: The heritability of kidney traits is considerable, and family history is an important determinant of CKD in the general population

Using the unique multi-generational family design of Lifelines, I estimated the heritability, i.e. the contribution of genetic factors to inter-individual variation in

a number of kidney traits. I observed considerable heritability of eGFRcrea (44%), urinary albumin excretion (20%), and serum urea (31%), among others. Furthermore, I computed the relative risk of developing CKD conditional on affected relatives. Here, I found that compared to the general population, the risk of having CKD for an individual is three times higher in case of a first-degree relative with CKD.
This study is the largest study of familial aggregation of kidney traits to date. An important observation in this study is that a positive family history strongly increases risk of CKD, suggesting a genetic component to kidney health. Furthermore, the heritability estimates provide an upper bound to the proportion of variance in kidney traits that can be explained by genetic factors. Future studies may focus on identifying these genetic factors.

Chapter 5: A genetic risk score based on 53 SNPs associated with eGFRcrea is a useful genetic proxy of kidney function, but possibly not of CKD susceptibility[9]
In addition to traditional clinical risk factors and lifestyle factors, genetic factors play a role in CKD. Recent genome-wide association studies (GWAS) have identified genetic variants associated with the CKD defining traits, eGFR[10] and albuminuria[11]. To date, GWAS for eGFR have been the most successful in terms of number of discovered variants. In the most comprehensive GWAS at the time, 53 SNPs were reported to have associations with eGFR estimated from serum creatinine (eGFRcrea)[12]. Each of these SNP effects were small and therefore unlikely to have meaningful clinical impact. However, it is possible to aggregate all SNP effects into one composite genetic risk score (GRS). Such a GRS may have utility in clinical practice as a risk stratification tool, and in research as a proxy for genetic predisposition. In **Chapter 5**, I evaluated a GRS based on these 53 eGFRcrea SNPs. Using data from 3649 subjects from the PREVEND cohort study, I found modest but robust associations of the GRS with eGFRcrea outcomes. These results were validated using eGFR estimated from cystatin C (eGFRcysc) rather than creatinine; similar associations of the GRS with eGFRcysc were found. This is important, given that eGFRcrea may in part reflect muscle mass rather than kidney function per se. Another important finding is that the GRS was not associated with albuminuria, and had an effect on eGFR independent of the renal risk factors, body-mass index, smoking, hypertension, diabetes, high cholesterol, and history of cardiovascular disease. This indicates that the GRS is a true genetic proxy of kidney function, not of kidney damage or kidney risk factors. However, the GRS only explained 1% in eGFR variance in PREVEND. Furthermore, longitudinal data were inconclusive: the

GRS was not significantly associated with eGFR decline, nor with incidence of CKD conditional on baseline eGFR. I therefore conclude that the GRS is unlikely to have a meaningful role in risk prediction of CKD. However, as a true genetic proxy of kidney function, the GRS may have utility in population level research, and in designs such as Mendelian randomization.

Chapter 6: Genome-wide association study of serum urea in Europeans identifies two novel genetic loci[13]

In **Chapter 5**, I used two different biomarkers for eGFR, namely serum creatinine and serum cystatin C. In **Chapter 6**, I investigated the genetics of serum urea (also known as blood urea nitrogen, BUN, when only the nitrogen component of urea is assayed). Serum urea is another commonly used, diagnostic marker for kidney function that was shown to be heritable in **Chapter 4**. Genetic data on this trait has been derived predominantly from East-Asian populations, where SNPs at 13 loci were known at the time[14-17]. Only few studies investigated this trait in European populations. These studies were either unsuccessful in finding associations[18], or lacked a replication phase for the five associations that were found. I therefore performed the first meta-analysis of GWASs on serum urea in European populations, with a gene discovery phase in 13,312 participants from the Lifelines Cohort, and built-in replication of the findings in 7379 participants from three community based cohorts (PREVEND, NESDA, and EGCUT). I identified replicable associations of six SNPs at the genome-wide level ($p < 5 \times 10^{-8}$), of which two were novel findings (rs2003313 on chromosome 11 near *POU2AF1*, and rs998394 on chromosome 3 in *ADAMTS9-AS2*).Furthermore, all SNPs previously identified in either East-Asians or Europeans were replicated, except for SNPs at three loci that are potentially specific to East-Asians.

I then aimed to identify potential causal genes involved in the pathways underlying urea metabolism and explore potential relevance to kidney function. Of the six identified SNPs, two were novel. Bioinformatics analysis of these two novel loci did not yield a clear relation to urea metabolism or kidney function, and thus, additional functional work is needed. An interesting candidate locus with regards to kidney function and disease is the *MUC1* locus. In kidney biopsy specimens, I found one of the identified SNPs, rs914615, to be an expression quantitative trait locus (eQTL) for *MUC1*, i.e. SNP rs914615 is linked to *MUC1* gene expression. Other SNPs tagging the *MUC1* locus have been consistently associated with serum urea in

previous studies[16]. It is a locus with potential clinical relevance for several reasons: it is involved in ion channels and electrolyte balance; aberrant activation of *MUC1* has been related to CKD development and; a frameshift mutation in *MUC1* causes medullar cystic kidney disease type 1[19,20]. Furthermore, a recent GWAS reported associations of albuminuria with SNPs that tag the *MUC1* locus[11]. Finally, in a recent study, differential expression of *MUC1* in the kidney was suggested to affect eGFR[21], adding evidence for a role of this locus in the development of kidney disease.

Next, I investigated the overlap of my findings with genetic data on kidney function. Overlap can be expected between serum urea and creatinine-based indices of kidney function, as serum levels of both urea and creatinine are influenced by kidney function. In a previous family analysis, a genetic correlation (r_g=0.56) was found between urea and creatinine[18], suggesting pleiotropy between these two traits. The positive direction of the genetic correlation indicates that shared genetic factors between urea and creatinine affect serum levels of both in the same direction (i.e. higher urea is genetically correlated with higher creatinine). Adding to this evidence is my finding that the 53 SNPs associated with eGFRcrea were enriched for associations with serum urea; 14 out of 53 eGFRcrea SNPs were also associated with serum urea, much more than could be expected based on random chance. Furthermore, a GRS based on these 53 eGFRcrea SNPs (the same GRS as in **Chapter 5**) was modestly but significantly associated with serum urea. The effect of this GRS was attenuated after adjustment for eGFRcrea, suggesting that the GRS indeed affects serum urea levels through kidney function.

Notwithstanding these statistically significant results, the clinical utility of these GWAS data on serum urea is rather limited. Together, the identified genetic variants explained no more than 0.56% of serum urea variation. My findings do, however, generate hypotheses for two novel loci (*POU2AF1* and *ADAMTS9-AS2*) with regards to urea and kidney function biology that may be investigated in functional research. Furthermore, the GWAS results on serum urea may be utilized in validating proposed kidney function loci: if a genetic variant is truly a marker of kidney function, the variant is expected to be related to both higher eGFRcrea and lower serum urea (or vice versa). This is exemplified in the most recent GWAS on eGFRcrea[10], in which the authors used GWAS results on BUN (the nitrogen component of urea) as a positive control to validate their findings.

PART III: UTILIZING GENETICS TO EXPLAIN SOCIOECONOMIC

DISPARITIES IN CHRONIC KIDNEY DISEASE

Chapter 7: Low educational attainment amplifies genetic risk of CKD in the general population

In **Chapters 7** and **8**, I applied the knowledge gained in previous chapters to integrate genetic methods with traditional social epidemiological methods. In **Chapter 7**, using data from the PREVEND cohort study, I present evidence for an amplifying effect of low EA on genetic risk of low eGFR. This finding was most pronounced in longitudinal analysis, where I observed an interaction between low EA and a high GRS. This interaction resulted in a more rapid rate of eGFR decline for those with both a high GRS and a low EA with a departure from additivity, meaning that the joint effects of a GRS and EA are larger than the sum of their main effects. Furthermore, these results suggest that high EA the genetic risk of eGFR decline, given that no apparent effect of a GRS was found in this group. This interaction could not entirely be explained by traditional risk factors (body-mass index, smoking, cholesterol, blood pressure, and glucose), suggesting the existence of unmeasured mediating factors whose influence is not captured by traditional factors.

These results add to the literature, as these are the first to provide evidence of a gene-environment interaction effect on kidney outcomes resulting from a modifying effect of EA. Importantly, I found that genetic risk of CKD is equally distributed across strata of EA, suggesting that there is no selection on kidney risk variants in those with low EA. Hence, the higher risk of CKD in those with low EA is attributable to an amplified effect of a GRS due to low EA itself or due to downstream effects of low EA. The results plead against genetic determinism in CKD, i.e. the risk of developing disease is not predetermined based on one's genes. Given that the interaction effect was rather modest and only accounted for ~0.1% of explained variance in rate of eGFR decline, its utility in risk stratification of individuals is negligible. However, if the effect is proven to be replicable in other samples, some benefit is to be expected from population level intervention on EA and its modifiable downstream effects in mitigating genetic risk of eGFR decline. Furthermore, although this study was sufficient powered to identify interaction effects on continuous outcomes, larger numbers are needed to assess whether the interaction effect results in increased risk of CKD, based on clinical cut-off values. Finally, the results warrant further characterization of the

mediating pathways between EA and CKD, and the specific genes involved in these pathways.

Chapter 8: The association between educational attainment and CKD may be confounded

The results in **Chapter 2** suggest a reno-protective effect of higher EA, as higher EA was associated with slower eGFR decline and lower CKD incidence, However, it is uncertain whether this association represents a true causal relation due to the observational nature of the data. In **Chapter 8**, I applied a Mendelian randomization method that uses genetic proxies for EA to minimize bias, thereby strengthening causal inference. For the two-sample MR analysis, I obtained data on 1271 SNPs with known effects on years of schooling[23], and interrogated the effect of these SNPs in genetic summary data from the CKDGen Consortium on eGFRcysc, eGFRcrea, and albuminuria (urinary albumin-to-creatinine ratio). I found that each one sd (4.2 years) higher EA was associated with a 3.2% higher eGFRcysc, consistent with my prior hypothesis of a protective effect. However, I found a null effect on eGFRcrea. A higher EA was even associated with higher urinary albumin-to-creatinine ratio, suggesting that higher EA results in kidney damage. To further investigate this counterintuitive finding, I performed secondary analyses in individual-level data of the Lifelines cohort, in which more detailed albuminuria data are available. I computed a genetic score based on the 1271 SNPs for years of schooling, and used this score as a genetic proxy for years of schooling. The counterintuitive detrimental effect of EA on urinary albumin-to-creatinine ratio found in the two-sample MR analysis was also observed using data of the Lifelines cohort. Here, I corroborated that this was due to higher urinary albumin excretion and not due to lower urinary creatinine excretion, thus not an artifact of lower muscle mass. This suggests that higher EA indeed leads to increased albuminuria.

Given the existing evidence on the protective effects of EA on cardiovascular health[24,25], protective effects on renal health were expected. However, I found inconsistent effects of EA on eGFRcrea and eGFRcysc, and an unexpected detrimental effect on urinary albumin-to-creatinine ratio and urinary albumin excretion. Future study may investigate what mechanisms explain this apparent detrimental effect on albuminuria. Based on these results, I conclude that there is insufficient genetic evidence for a protective causal effect of EA on kidney health. Thus, future studies on disparities in CKD may investigate other potentially

causative socioeconomic factors such as income, occupation and occupational exposures, social deprivation, or area-level indicators of socioeconomic status.

METHODOLOGICAL CONSIDERATIONS

General comments

Important strengths of this thesis include its use of multiple datasets, and the multidisciplinary approach to analyzing these data. I combined the expertise from the fields of nephrology, social epidemiology, and genetic epidemiology. This combination resulted in a wide range of analytic approaches: traditional epidemiological methods in **Chapters 2** and **3**, a family study in **Chapter 4**, genetic risk score application in **Chapters 5** through **8**, a GWAS in **Chapter 5**, a gene-environment interaction study in **Chapter 7**, and a Mendelian randomization study in **Chapter 8**. In this section, I describe the most important data sources and comment on the methods applied in this thesis.

Data sources

The research questions in this thesis were addressed using data from a number of existing sources. Here, I discuss the data sources that contributed most to this thesis, namely the Prevention of REnal and Vascular ENdstage Disease (**PREVEND**) cohort study, the Lifelines Cohort study and Biobank (**Lifelines**), and the Chronic Kidney Disease Genetics (**CKDGen**) consortium.

Data from the **PREVEND** cohort study[26] was used for **Chapters 2**, **3**, **5**, **6**, and **7**, while it contributed in part to **Chapter 8**. This prospective, observational cohort was sampled from the general population of the city of Groningen, the Netherlands. It was originally initiated to study the natural course of albuminuria and its association with renal and cardiovascular outcomes. PREVEND is ideally suited for investigating kidney outcomes due to its substantial follow-up duration (five consecutive examination rounds between 1997 and 2010). Importantly, PREVEND allows for precise measurement of kidney function and damage, with serum creatinine, serum cystatin C, and urinary albumin excretion being available. Furthermore, two 24h urine collections per examination round were available, allowing for optimal evaluation of albuminuria. The baseline sample consisted of ~8600 participants, of which a random sample of ~3500 was genotyped with a genome-wide array.

For **Chapters 4, 6** and **8**, data from **Lifelines**[27] was used. Lifelines is a large,

population-based prospective cohort study sampled from the Netherlands' three northernmost provinces (Groningen, Friesland, and Drenthe). From 2006 to 2013, ~165,000 participants were included and extensively phenotyped. Currently, genotype data is available for ~13,500 participants, which were included for analysis in **Chapters 6** and **8**. For the baseline measurement, 24h urine collections were available, which allows for exact evaluation of urinary creatinine and urinary albumin excretion. However, only two surveys were currently available; follow-up data is therefore limited. Another limitation of Lifelines with regards to kidney research are that measurements of serum creatinine are available but not of serum cystatin C, and that there are no follow-up data on urinary albumin excretion, a determination of urinary albumin was discontinued after the first 60,000 participants were measured at the baseline assessment. For **Chapter 4**, I exploited the multi-generational design in Lifelines to perform the largest family study on kidney outcomes to date, with >29,000 families and up to 4 generations per family.

Another important data source was the Chronic Kidney Disease Genetics consortium (**CKDGen**). CKDGen is an international collaborative effort to investigate the genetics of kidney outcomes. For **Chapter 5**, **6**, and **7**, I constructed a genetic risk score based on the then-known 53 or 63 genome-wide significant SNPs reported by CKDGen in 2016 and 2017, respectively[12,28]. In **Chapter 8**, I used summary statistics derived from a more recent and comprehensive GWAS meta-analysis on $eGFR_{crea}$[10] as well as the latest GWAS meta-analysis on urinary albumin-to-creatinine ratio[11]. It is noteworthy that both **PREVEND** and **Lifelines** have contributed data to CKDGen GWAS meta-analyses, either as discovery or replication cohort.

Measurement of kidney outcomes

In clinical and research practice, kidney function is assessed as glomerular filtration rate (GFR), which is the rate of pre-urine production that is obtained by filtering blood in the glomeruli. The most accurate measurements of GFR are derived from the injection of exogenous markers such as inulin, or radioisotopes such as ^{125}I-iothalamate. These markers are ideal for assessing kidney function, as their rate of excretion is dependent on their filtration through the glomerulus, and not on secretion or reabsorption in the renal tubule. However, the use of these markers for GFR measurement is costly and time-consuming, and therefore currently unavailable for large epidemiological studies. In such studies GFR is

therefore usually not measured but estimated from endogenous filtration markers that can be measured in serum, of which creatinine is the most widely used. However, given that creatinine is a product of muscle metabolism, creatinine-estimated GFR may in part reflect muscle mass rather than kidney function per se, thereby introducing bias in estimates of GFR. An alternative marker is cystatin C. This marker is not sensitive to variations in muscle mass, although other extrarenal, non-GFR factors partly explain cystatin C serum concentration. It has been shown that equations that incorporate both creatinine and cystatin C provide the most reliable estimates of GFR[29,30]. In this thesis, I therefore estimated GFR based on both creatinine and cystatin C whenever possible (**Chapter 2, 3, 5** and **7**). Furthermore, I used cystatin C estimated GFR as a positive control to creatinine-estimated GFR (**Chapter 5** and **8**). However, despite many improvements over the past decade, the accuracy of estimating equations is a debated topic[31-33]. Novel filtration markers such as beta-2-microglobulin, beta-trace-protein, and metabolite profiles, as well as the combination of these markers in novel estimating equations, may eventually result in a more accurate approximation of GFR[34,35]. This will not only lead to improved risk stratification, but also in increased power for (genetic) epidemiological studies with kidney function as trait of interest.

Albuminuria is a measure of kidney damage and a predictor of cardiovascular morbidity and mortality. Measurement of urinary albumin excretion in 24h urine collections is considered the gold standard. However, 24h collections are cumbersome, and therefore not always available in large epidemiological cohorts. A more convenient method to detect albuminuria is to measure albumin and creatinine concentrations in spot urine specimens, and then calculating the urinary albumin-to-creatinine ratio; adjusting for creatinine is a method to take into account variation in albumin concentrations due to concentration/dilution dependent on hydration status. Urinary albumin excretion and urinary albumin-to-creatinine ratio correlate well, although misclassification can occur e.g. due to differences in muscle mass[36]. Due to the poor availability of 24h urine collections for large samples, GWAS on albuminuria have thus far used outcomes based on urinary albumin-to-creatinine ratio[11,37]. A major strength of this thesis is the availability of 24h urine collections, which facilitates gold standard outcome definitions (**Chapter 2, 3, 4, 5**, and **8**), and where necessary, verification of results from urinary albumin-to-creatinine ratio based outcomes (**Chapter 4** and **8**).

Educational attainment as an indicator of socioeconomic status

Socioeconomic status is defined as the social standing or class of an individual or group. In this thesis, I used EA as the main indicator of socioeconomic status. EA is sometimes preferred because of its comparatively easy measurement and high response rate. Education is usually completed in young adulthood and predicts occupation and income, and therefore is expected to show overlap with these indicators of socioeconomic status in their association with CKD. Theoretically however, indicators of socioeconomic status are not interchangeable and have different implications. EA reflects cognitive functioning, material and intellectual resources from the family of origin, and health literacy[38-40] As EA is usually completed in young adulthood, it is unlikely that there is reverse causation by chronic diseases, such as CKD, that usually occur at later age. However some selection may be present as health at young age affects EA. A more direct measure of socioeconomic status is income. Income is a proxy for the material resources an individual can convert to health-enhancing commodities and services, and arguably the best indicator of actual, material living standards[39]. However, income may be sensitive to reporting bias, and income may not necessarily reflect disposable income, which is dependent on household composition, taxations, and hypothecated income (e.g. food stamps) that are difficult to measure. Furthermore, health may directly affect income, and therefore reverse causation may bias the results.

Recent meta-analyses that synthesize the literature on the relation between socioeconomic status and CKD[2,3] show clear associations of socioeconomic indicators, such as low EA, and low household income, with higher prevalence of CKD, lower kidney function measures, and higher levels of kidney damage markers. These meta-analyses also showed large heterogeneity between study populations, which may possibly be explained by between-country differences in lifestyle, ethnicity, educational and healthcare systems, and/or differences in risk factor prevalence. Additionally, the strength of each indicator may vary between countries. For example, it has been demonstrated that in the US, a nation with high income inequality, low income is more strongly associated with CKD than low education. In contrast to the US, health care access is less income-dependent in the Netherlands, which may explain that low income does not seem to result in excess CKD risk in the Netherlands[41]. Given that most of the data I used for this thesis were sampled from the Dutch population, I chose to use EA rather than income as the main indicator of socioeconomic status. To allow comparison with

other countries, I mapped Dutch educational levels to the International Standard Classification of EDucation (ISCED)[42].

EA was not associated with eGFR in cross-sectional analyses conditional on age (**Chapter 7**). Strong confounding with age may explain this lack of cross-sectional association: in the Netherlands, schooling until age 16 has been compulsory by law since 1969 (Dutch: "leerplichtwet"). Since 2007, due to an amendment to the 1969 law, those aged between 16-18 years can drop out only if they have a qualification (Dutch: "kwalificatieplicht") equivalent to, or higher than, secondary vocational schooling (Dutch: MBO ≥ level 2) or higher secondary schooling (Dutch: HAVO/VWO)[43] (ISCED level 3). Before 1969, schooling was only compulsory for children aged 6-14. This policy may explain that in the Netherlands, low EA (ISCED < level 3) is less prevalent among more recent cohorts (e.g. those born after the 1950s), and that those with low EA have higher age on average. In longitudinal analyses however, I observed a convincing educational gradient in eGFR decline (**Chapter 2** and **Chapter 7**) and CKD incidence (**Chapter 2**) independent of age. Future work may include a more in-depth examination of cohort effects. In particular, the cohort effects that relate to past educational policy changes may provide additional insights into the effects of EA on CKD. Potentially, if several methodological challenges can be overcome (e.g. identifying a control group, or an exogenous source of variation in exposure), these policy changes can be analyzed as natural experiments[44-46].

Heritability, GWAS, and genetic scores

Heritability is the fraction of interindividual variation of a trait that can be attributed to genetic factors, in a given population[47]. Studies that yield insights into the heritability of a disease or a trait provide clues regarding their causes, and are a first step towards disentangling genetic and environmental effects. Furthermore, heritability estimates indicate an upper bound of the proportion of phenotypic variance in traits that can be explained by genetic factors. Traditional methods for estimating heritability include the twin study, in which phenotypic similarity of identical (monozygotic) twin pairs is compared with non-identical (dizygotic) twin pairs[48]. However, twin studies potentially overestimate heritability due to unaccounted gene x gene interaction, gene x environment interaction, gene-environment correlation and violations of assumptions[49-52]. Furthermore, obtaining a representative sample of twins is difficult, and may lead to reduced

statistical power and generalizability. An alternative is to recruit families rather than twins. Through leveraging the multigenerational family design of Lifelines, I obtained heritability estimates of a number of kidney traits, including eGFRcrea, albuminuria, and serum urea (**Chapter 4**), and found that the heritability of these traits is considerable. A popular method of identifying potential genetic factors for any heritable trait is the GWAS, a data-driven, hypothesis-free method of skimming the genome for associated genetic variants. Below, I discuss some basic concepts of GWAS and discuss the methods applied in the GWAS I performed in **Chapter 6**.

The human genome consists of >6 billion nucleotide bases (guanine, cytosine, thymine, and adenine; G, C, T, A), arranged in base pairs (G-C or T-A). Most of these are fixed: any random pairing of two individuals will show >99.5% overlap in genomic sequence. The genetic factors underlying differences in traits are believed to reside within the remaining <0.5% of the genome. The most common type of variation is the single-nucleotide polymorphism (SNP), a naturally occurring variation in a single nucleotide base at specific positions in the genome, with an average frequency of ~1 in 1000 nucleotides[53]. As an example, most individuals may have a G nucleotide at a certain position (the reference allele), but in some, the position is instead occupied by an A nucleotide (the alternative allele). SNPs in coding regions potentially affect the protein product of a gene, whereas SNPs in non-coding regions may tag functional SNPs that are in linkage disequilibrium (LD, the non-random association of alleles) in coding regions, or may affect gene expression. Much of the heritability of traits may potentially be traced back to SNPs. Each individual has a paternally and a maternally inherited allele, therefore an individual can have 0 1, or 2 reference alleles of each SNP. In GWAS, these SNP alleles are tested for their association with a trait, assuming allele effects are additive. In a typical GWAS in European samples, ~10^6 independent SNP tests are performed, increasing the risk of false positive findings. To minimize this risk the consensus for genome-wide significance has been set to a strict, Bonferroni adjusted threshold of $p = 0.05/10^6 = 5 \times 10^{-8}$. A source of bias in GWAS estimates of genetic effects is population stratification: genetic drift or ancestry may lead to systematic differences in allele frequencies between subgroups in a sample. These systematic differences may lead to confounded effect estimates. Genetic principal components (PCs) may capture variation due to possible subgroup effects, and I therefore adjusted for these PCs in those studies in which I assessed SNP effects (**Chapter 5** through **8**).

In **Chapter 6**, I performed a GWAS on serum urea, a heritable indicator of kidney function. With GWAS on EA and eGFRcrea reaching sample sizes of over a million participants[10,23], the GWAS in **Chapter 6** is a relatively small study (N = 20,500). This may explain that the SNPs identified in this study only explained ~0.6% of variance in serum urea. In **Chapter 4**, I estimated the heritability of serum urea to be 30%, meaning that many of the genetic factors underlying this trait remain to be discovered. Nevertheless, the results were highly replicable and consistent with previous studies in non-European ancestry samples. The results inform studies that explore the biological functions of the identified genetic loci, and their relevance to urea metabolism and kidney function.

In addition to providing biological insights, GWAS results may be used for trait prediction. Generally, SNPs that are identified in GWAS have small effects. A genetic risk score (GRS, in this thesis also referred to as weighted genetic score, WGS) aggregates these effects, thereby greatly increasing statistical power compared to using single SNP effects. Therefore, the GRS is a practical summary score of genetic predisposition for the traits addressed in this thesis: eGFR in **Chapter 5**, **6**, **7**, and EA in **Chapter 8**. There are however limitations to the GRS. Importantly, the different genetic scores used in this thesis only explain a modest fraction of between-individual variation in traits: a 63-SNP GRS for eGFR explained only 1% in eGFR variance in PREVEND, while a 1271-SNP GRS for years of schooling explained 4% of EA in Lifelines. With ever-increasing sample sizes for GWAS, it is expected that more SNPs will eventually be detected, with effects that are estimated more precisely. Furthermore, up until now GWAS have mostly been limited to study the effects of common SNPs (i.e. SNPs with allele frequencies of ≥1%), as these could be economically genotyped with the usual GWAS arrays. As sequencing techniques become more affordable, whole genome sequencing for large samples will become feasible in the near future. With such whole genome sequence data becoming available, rarer variants (with allele frequencies well below 1%) can be detected that are predicted to have greater effects[54,55]. It is expected that these rare variants will explain a substantial part of the heritability that has thus far been hidden[52,56]. An updated GRS incorporating these rare SNPs may be a more comprehensive summary measure of genetic risk.

The GRSs used in this thesis were comprised of genome-wide significant SNPs. However, non-significant SNPs may contain additional information and thus can contribute to trait and disease prediction. Methods have been developed to

include these non-significant SNPs into genome-wide polygenic scores (PGS). For coronary artery disease, such a PGS has been reported to identify individuals with elevated risk with a predictive power comparable to that of rare monogenic mutations that typically convey a several-fold increase in disease risk[57]. Future work could include the evaluation of such PGSs based on the recent eGFR[10] and urinary albumin-to-creatinine ratio[11] GWASs for kidney outcomes.

With regards to **Chapter 7**, several specific limitations of the GRS need to be addressed. First, by using a GRS in interaction analysis, it is implicitly assumed that all genetic variants included in the GRS have directionally consistent interaction effects with EA. Another implicit assumption is that the same set of genetic variants affect eGFR in each category of EA. To check these assumptions, single SNP interaction effects need to be assessed, but this requires large sample sizes and is therefore beyond the scope of this thesis. Future research may include genome-wide interaction studies (GWIS) to identify the genetic variants whose effects are modified by EA. Similar GWIS have been performed to investigate a range of health behaviors (e.g. smoking, alcohol consumption, physical activity) and their modifying role in genetic effects on blood pressure, lipid levels, and obesity[58-61].

In each study in this thesis, I investigated European ancestry populations, and therefore I cannot generalize my findings to other ethnicities. Of note, disparities in GWAS exist, as currently, most GWAS are performed in European ancestry populations[62-64]. This is also true for the GWAS I performed in **Chapter 6**, and the GWASs that were used to create the GRSs in this thesis. Given that there may be subtle ethnic differences in the genetic architecture of disease and social traits, such as eGFR and EA, a GRS based on data from white populations may not perform similarly in populations with other ethnicities[65]. This is problematic, given that socioeconomic status is closely related to ethnicity[66-68], and that it is likely that ethnic background influences the effect of socioeconomic status on CKD[69,70]. Furthermore, if the ethnicity-gap in genetic knowledge is not bridged, this may in itself contribute to socioeconomic disparities[71]. Future work should therefore include GWAS in a multiethnic context, and expansion of GWAS into non-white populations. This allows for the creation of more inclusive and/or ethnicity-specific GRSs, and thereby allow for a more comprehensive examination of the effects of socioeconomic status on CKD.

Causal inference

In this thesis, I examined several factors for their association with kidney outcomes. Ideally, to establish causality, one would design a controlled experiment. In such a setting confounding bias would be minimized, and differences in outcomes could be attributed to intervention/exposure effects. However, experimentally establishing a causal effect of education on kidney outcomes would be unfeasible due to ethical and practical reasons: participants would have to be randomly assigned to different educational levels at a young age, and undergo follow-up for several decades until CKD occurs in mid-to-late life. Instead, the research in this thesis was based on observational data and thus, conclusions regarding causality should be interpreted with caution. Below, I describe a number of strategies employed in this thesis to strengthen causal claims in observational studies.

In **Chapters 2**, **3**, **5**, and **6**, I applied a longitudinal study design. To a certain extent, a longitudinal design helps in causal inference as it provides evidence of temporality, that is, whether the hypothesized explanatory variable precedes the outcome variable, or whether there is in fact reverse causation. In **Chapter 3**, I performed a replication study of a previous observational study to assess the consistency of the heart rate variability-CKD association, that is, whether I would reach similar conclusions regarding this association in a different, independent sample to the original discovery sample. In **Chapter 6**, replication analyses were built into the GWAS study design to assess consistency of SNP effects, thereby strengthening the conclusions in this chapter.

To minimize confounding bias, I performed multivariable analyses and adjusted my estimates for a number of known risk factors presumably influencing both exposure and outcome. However, estimates will only truly be unbiased if all confounding factors are accounted for and measured precisely, both of which are unverifiable conditions. Because of this, confounding is a threat to observational studies in general and, therefore, also a limitation of the observational studies reported in this thesis.

To examine mechanisms through which EA could affect CKD, I performed mediation analysis. Mediation refers to the mechanism in which the exposure affects the outcome (fully or partly) through a mediator variable, in which

exposure and mediator are on the same causal pathway. In **Chapter 2**, I examined several risk factors presumed to be mediators of the EA-CKD association. To estimate their mediation effects, I applied causal mediation analysis, a method within the counterfactual framework[72]. A counterfactual outcome is the potential outcome that would have occurred if the exposure were different, i.e. counter to fact; with everything else held constant, differences in the outcome can be attributed to differences in the exposure. In the mediation analysis applied in **Chapter 2**, counterfactuals of exposure and mediator variables were simulated from the original data using a bootstrap procedure. Then, from the bootstrap simulations of exposures, mediators, and outcomes, I estimated average direct effects and mediation effects. I examined mediation effects of clinical risk factors (hypertension, diabetes, high cholesterol, overweight) and health behaviors (smoking, alcohol, diet) separately, but not in conjunction with each other. This exploratory approach was chosen given that the theoretical framework regarding the interplay of these different variables is incomplete. Furthermore, time-varying effects of mediators were not considered, as the methodology to incorporate these effects has only recently been developed[73]. Future work may expand the models to include effects of multiple potential mediators, and to include time-varying effects using methods such as structural equation modelling[74].

As previously mentioned, confounding and reverse causation limit causal inference in observational studies. To strengthen causal inference in observational research, methods such as Mendelian randomization (MR) may be considered. In **Chapter 8**, I performed an MR study to assess causal effects of EA on kidney outcomes. MR is a form of instrumental variable analysis, a method applied to minimize confounding in observational studies[75]. Instrumental variables are proxies of a given exposure that must meet the exclusion restriction criterion: the instrument is related to the outcome only through the exposure. It has been proposed that individual genotype can be used as an instrumental variable[76]. Genetic variants are randomly assigned during meiosis, and therefore unrelated to any confounders. Furthermore, given that genetic variants are fixed throughout life, there cannot be reverse causation. MR studies therefore resemble an intention-to-treat analysis of a randomized controlled trial, in which participants are assigned to an intervention group based on random assignment. This randomization procedure ensures equal distribution of confounding factors in each intervention group, thus a difference in outcome between intervention groups can assumed to be due

to exposure to the intervention. A number of methods are available within the MR framework. In **Chapter 8**, I applied a two-sample MR design using summary genetic data[77], as well as a one-sample MR design using a GRS in individual-level data[78]. The potential of MR, as well as its limitations and possible threats, have been extensively described in literature[76,79-83]. Below, I discuss arguably the most important threat to MR, namely violation of the exclusion restriction criterion due to pleiotropy of genetic instruments.

MR provides unbiased causal estimates on a given exposure-outcome relation if assumptions regarding instrument validity are met. An important criterion for validity is that the genetic variant only affects the outcome through the exposure. If the biological function of a genetic variant is well-defined, this strengthens the conclusions drawn from MR. As an example, Holmes et al. used the rs1229984 variant in the alcohol dehydrogenase 1B gene (*ADH1B*) as a genetic instrument to study the effect of alcohol consumption on risk of coronary heart disease[84]. The *ADH1B* gene is known to play a specific role in alcohol metabolism, and certain variants in this gene are known to influence tolerance to alcohol and therefore consumption of alcohol. Hence, individuals are randomly assigned to alcohol consumption based on their genotype for *ADH1B*, and the effect of *ADH1B* gene variants on coronary heart disease can be attributed to alcohol exposure. For a complex trait such as EA, the biological functions of the 1271 genetic variants that have been identified in the most recent GWAS on EA[23] are poorly known. Furthermore, variants identified in GWAS may not to be causal themselves but may be linked to causal variants through linkage disequilibrium. Importantly, it is possible many of these variants have pleiotropic effects that influence risk of CKD not only through EA, but also through other pathways. Such horizontal pleiotropy may result in invalid estimates, in particular when the pleiotropy is unbalanced: unbalanced pleiotropy results in a net positive or negative bias in causal estimates. Methods have been developed that are robust to varying degrees of violations of MR assumptions due to pleiotropy, including MR Egger[85], outlier adjustment, and median- and mode based methods[86,87], and the methodology is quickly advancing. In **Chapter 8**, in case of suspected pleiotropy, I applied a range of complementary MR methods to test the robustness of my findings. In general, these complementary methods yielded results comparable to that of standard inverse variance weighted MR, thus strengthening my conclusions.

As is the case in all observational studies, including MR, selection, or conditioning on a selection variable, may lead to collider bias. A collider is a variable that is causally downstream of both exposure and outcome; conditioning on such a variable may result in biased effect estimates and spurious associations[88,89]. For the MR results in **Chapter 8**, collider bias is a possible explanation of the counterintuitive detrimental effect of EA on urinary albumin-to-creatinine ratio found in two-sample MR. However, the results of two-sample MR were corroborated in the Lifelines cohort. No selection criteria for either EA or kidney traits were applied for recruitment into Lifelines or its genotyped subset. Lifelines was assessed to be generally representative of its source population of the Northern part of the Netherlands, with only slight undersampling of those with low EA[27,90,91]. Therefore, selection bias is likely only minor and hence unlikely to have seriously affected the MR results[92]. Nevertheless, some selection is inherent given that this MR study is based on genetic data sampled from high income countries with relatively few barriers to health care. Inclusion of genetic data sampled from low to middle income countries in future studies may yield more generalizable results.

MR is a powerful method that may resolve a number of problems with causal inference from observational data, especially the problems that arise due to confounding and reverse causation. However, many potential threats (e.g. instrument pleiotropy) affect MR, and thus it is not a panacea. Some have argued that due to its many threats, null MR results are more likely to be true than non-null results[82]. Rather than above described traditional methods, MR may have a place next to these methods. Ultimately, synthesizing evidence from different designs, each with complementary sets of strengths and limitations - an approach coined 'triangulation'[93] - may be the best strategy for drawing conclusions concerning causality.

FUTURE PERSPECTIVES

Towards a better understanding of socioeconomic disparities in CKD

One of the major goals in this thesis was to elucidate the mechanisms underlying socioeconomic gradients in CKD risk, with a focus on the role of EA. Observational data have suggested that low EA is associated with CKD risk through a complex of pathways that include mediation by lifestyle factors and amplification of genetic risk. However, observational and genetic evidence from a MR study did not converge on a convincing protective effect of EA. Thus, much of the observed evidence linking EA to CKD may instead reflect an influence of other socioeconomic factors closely related to EA rather than EA per se, e.g. income, occupational factors, social deprivation, or area-level factors. Future research on socioeconomic gradients in CKD may focus on these factors rather than EA.

The data described in this thesis indicate that EA do not influence CKD risk. However, this data was sampled from high-income populations. Therefore, EA cannot be dismissed as a risk factor in lower income countries where health care access may be more dependent on EA. Inclusion of data from lower income countries, and countries where differences in EA and income are more pronounced, may yield more definitive insights into socioeconomic disparities in CKD.

Public health: opportunities for primary and secondary prevention of CKD

Given the inconsistent evidence for a protective effect of high EA against CKD in this thesis, intervention policies on EA itself, e.g. increasing school-leaving age, may not result in reduced rates of CKD. Nevertheless, low EA groups may still be a target population for preventive policies or screening. In low EA groups, the higher prevalence of modifiable renal risk factors (e.g. poor diet, smoking, high body-mass index, hypertension, and diabetes) could be a target for primary CKD prevention. Furthermore, I demonstrated that family history of CKD and a GRS based on SNPs for eGFR are associated with a higher prevalence of CKD, independent of clinical risk factors. Thus, prediction models for CKD may benefit from the inclusion of family history and/or a GRS. These models could then be used for screening purposes and early detection of CKD. Future studies may evaluate whether specifically targeting low socioeconomic status groups (defined by EA or otherwise) for primary and secondary prevention may be effective in reducing socioeconomic disparities in CKD.

Future genetic studies: bigger, more advanced, more inclusive

Contemporary genetic epidemiology is characterized by great increases in sample sizes and rapid advances in methodology, facilitated by affordable genotyping technology. These trends show no signs of slowing down, and it can be expected that due to decreasing costs, whole genome sequencing will gradually replace the usual GWAS arrays[94-96]. This means more power and more precision to identify common as well as rare genetic variants, leading to improved genetic prediction and possibly genetics-driven personalized medicine. In addition, population-based research is expected to increasingly adopt multigenerational, within-family designs, which allows for examination of, and control for, transgenerational effects[97-100]. These developments hold promise to greatly increase our understanding of the genetic underpinnings of health and behavior. Furthermore, transethnic GWAS are becoming commonplace, and an increasing number of scientists are pushing for genetic studies to be less Euro-centric and more inclusive with regards to ethnicity[62,64,71,101-103]. Thus, there is hope that the ethnicity gap in genetic knowledge will eventually be bridged, allowing a greater diversity of people to benefit from genetic data.

Collaboration

On a more general note, future studies will benefit from the continued collaboration between researchers. The disappointing results from early candidate gene studies and poor replication due to Winner's curse[104-106] has driven genetic epidemiologists to collaborate and share data on a large scale. This facilitated the inclusion of greater samples, harmonization of data, exchange of expertise, advancement of methodology, and systematic replication of results, thereby ensuring high quality, reliable science. In addition, much of the produced genetic data is made publicly available, through platforms such as the GWAS Catalog[107], LD Hub[108], and MR Base[109], which is a major stimulus for follow up study. The genetic studies performed in this thesis (**Chapter 5** through **8**) utilized data that was made possible due to such collaboration. A growing number of researchers, including those from non-genetic fields such as the social and behavioral sciences, continue to follow this example of collaboration and, by doing so, contribute to more efficient allocation of research resources and to solving the replication crisis in science[110,111].

Concluding remarks

The results in this thesis provide valuable insights into the causes of kidney disease. First, I corroborate the existence of socioeconomic disparities in kidney disease, as those with lower education tend to have higher rates of CKD and faster rates of kidney function decline. Second, those with a positive family history have a threefold higher risk of having CKD, and there is strong evidence for a genetic component to kidney traits such as eGFR, albuminuria, and serum urea. Third, genetic risk of CKD may be offset by higher socioeconomic status. Finally, educational level may not be the main driver of socioeconomic disparities in chronic kidney disease, as the genetic evidence for a causal effect of educational level is weak.

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