The role of EGFR signaling in autosomal dominant polycystic kidney disease (ADPKD)

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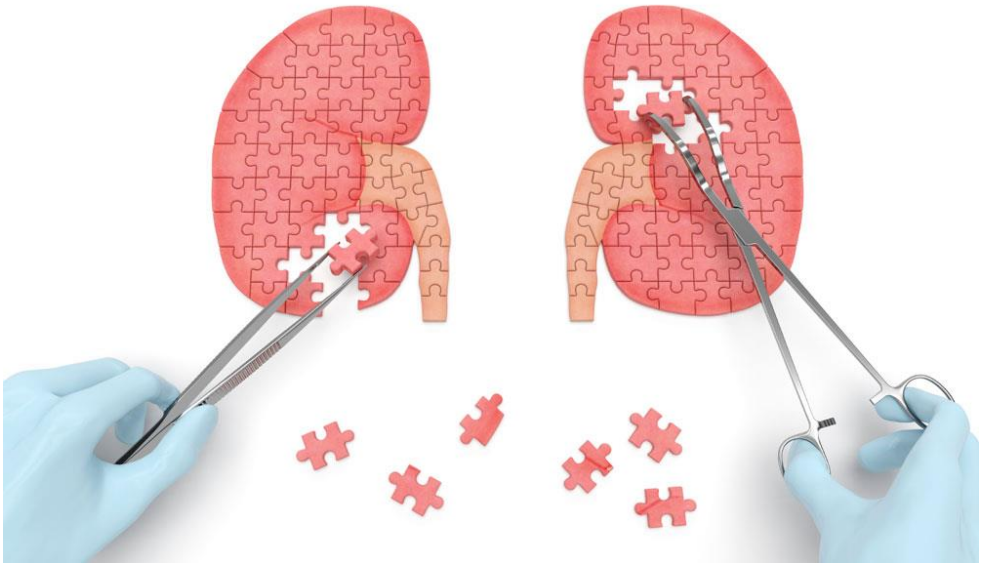

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Preface

This report was written in context of my second research project for the master biomedical sciences at the UMCG department of pathology and nephrology. This report describes the potential role of *HB-EGF* in the pathophysiology of ADPKD via EGFR signaling.

During my internship I have learned different techniques including immunohistochemistry, PCR and qRT-PCR. I liked the combination of tissue staining and genotyping of mice pups. Furthermore, I gained lots of knowledge about ADPKD. Besides my own project, I also attended and presented data at the kidney center meetings and meetings at the pathology department wherein I learned lots of new things about various research topics.

Since I am really glad that I had these experiences, I would like to thank prof. dr. Ron Gansevoort for giving me the opportunity to work on this project. I want to thank prof. dr. Harry van Goor for the supervision. I could always come to your office to for example show my stained slides and you learned me to recognize different parts in renal tissue, thanks for all your help and support.

In special I would like to thank Laura Harskamp. Although, you were not in the UMCG most of the times and you were very busy with your medical internships you always could make time for me, even in the evening at your home. Thanks for your help and support and good luck with continuing this project. You may always contact me if you need some help with the genotyping of your mice or something else.

I also would like to thank some other people involved in this project. First, Marian Bulthuis for teaching me the technique of IHC and cutting frozen tissues by a cryostate. Second, Sippie Huitema for teaching me the PCR/qRT-PCR technique. Third, Niek Casteleijn for building up the ADPKD tissue biobank and helping me with patient related information and finally, nephropathologist Dr. Marius van den Heuvel for judging the stained tissues.

Groningen, June 2016

Summary

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common heritable kidney disease affecting 3-4 in 10000 individuals. It is a proliferative renal disease characterized by the formation and growth of numerous cysts in both kidneys. ADPKD patients have a high likelihood of progression to renal failure for which dialysis is needed. As yet, there is no proven therapy available for clinical use. The proliferative abnormalities in ADPKD, that lead to cyst formation and growth, are potentially mediated via epidermal growth factor receptor (EGFR) signaling, which leads to increased cell proliferation. Since human studies in ADPKD regarding EGFR signaling are sparse, we examined the role of EGFR signaling in tissue of 19 ADPKD patients and compared the results to healthy controls with normal renal function We showed that the active, phosphorylated form of the EGFR (pEGFR) is expressed in distal tubules, collecting ducts, smooth muscle cells and in cyst lining epithelial cells in ADPKD patients. The pEGFR was expressed more strongly in ADPKD patients compared to controls with normal renal function, and apical mispolarization of the pEGFR was detected in ADPKD. This study implies that increased activity of the EGFR in ADPKD is mediated by its ligand *HB-EGF*, since we found co-localization of *HB-EGF* with pEGFR positive cysts. Furthermore, *HB-EGF* was strongly expressed in distal tubules and weakly expressed in proximal tubules. *HB-EGF* was expressed more strongly in ADPKD compared to controls with normal renal function. Moreover, in a previous study we found a positive correlation of urinary *HB-EGF* with ADPKD severity. Taken together, these studies imply that *HB-EGF* may be involved with disease progression in ADPKD, and as such is a potential candidate for therapy. Of note, research showed that *HB-EGF* was also upregulated in other chronic kidney diseases. Therefore, we hypothesize that *HB-EGF* may play a common role in kidney disease progression associated with increased inflammation, fibrosis and tissue repair. Targeting *HB-EGF* may therefore have a broad application in the field of nephrology.

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1 Introduction:

1.1 Polycystic kidney disease pathogenesis

Polycystic kidney disease (PKD) is a proliferative disorder of kidney tubular epithelial cells which is characterized by development and growth of epithelium-lined cysts in both kidneys [1]. The major types of PKD are autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) . The most common form is ADPKD which is caused by a loss of function mutation of the polycystin-1 (*PKD1*) gene located on chromosome 16 or polycystin-2 (*PKD2*) gene located on chromosome 4 [2]. *PKD1* is responsible for 85% of the cases of ADPKD, while the remaining 15% of the patients have a *PKD2* mutation [3]. ADPKD is the most common inheritable renal disease, occurring in 3-4 of 10.000 individuals worldwide [4]. It is characterized by the formation of multiple fluid-filled cysts in both kidneys which originate from proliferating tubular cells, leading to enlarged kidneys, pain and hematuria. The cysts will eventually compromise the normal renal tissue, causing progressive irreversible loss of renal function. As a result, 70% of the patients with ADPKD develop end stage renal disease between the 4th and 7th decade of life and are dependent on renal replacement therapy [5]. Besides renal cysts, most patients also develop cysts in the liver and they have a higher risk for cardiovascular complications compared to the general population [5].

Until recently there was no treatment available for ADPKD. However, researchers identified that increased signaling by the cyclic adenosine monophosphate (cAMP)-dependent pathway is associated with ADPKD disease progression via stimulation of cell proliferation and cyst growth (Figure 1) [6] [7]. These findings resulted in testing of new drugs to inhibit cAMP, including somatostatin analogues and vasopressin 2 receptor antagonists (V2RAs). Although two clinical trials showed promising results, they also suggest that the beneficial effect of somatostatin analogues may be attenuated after two years [8a]. Therefore, the DIPAK-1 study is currently testing the somatostatin analogue lanreotide in a clinical trial trial [8b]. Whereas, the V2RA tolvaptan has been shown to slow the rate of growth in total kidney volume (TKV) and the rate of renal function loss in patients with relatively early ADPKD [9] [10]**.** Recently, tolvaptan has been approved for use in ADPKD by regulatory authorities in Japan, Canada and Western Europe.

Despite these positive results, disease progression still occurs during tolvaptan treatment. Moreover, experimental research suggests that tolvaptan may be less efficacious in later-stage ADPKD. Currently, the efficacy of tolvaptan is being further explored in the REPRISE study, a clinical trial with patients in later-stage ADPKD. Another concern regarding the use of this drug is the severe side effects of tolvaptan with major impact on daily life, including polyuria, nocturia, polydipsia, hypernatremia and hepatotoxicity [11]. This implies that tolvaptan is not tolerated by some patients. Furthermore, tolvaptan is not universally clinically available yet, since the US Food and Drug Administration has not yet approved tolvaptan for treatment of ADPKD. Because of these limitations, there is a need for additional treatments.

Several other signaling pathways are suggested to play a role in the pathogenesis of ADPKD, including the mammalian target of rapamycin (mTOR) pathway and epidermal growth factor (EGFR) pathway (figure 1) [6]. While in *Pkd*1 mice, increased levels of mTOR in the cyst epithelial cells were

found [12], and disease progression was effectively attenuated by inhibition of mTOR , [13] [14] mTOR inhibitors were not beneficial in human ADPKD [15]. However, promising data have been published regarding the role of the EGFR pathway in ADPKD. The EGFR pathway is a logical candidate for research in PKD and may be involved in the pathophysiology of this disease, since EGFR activation is one of the major triggers for tubular cell proliferation. EGFR signaling regulates cell growth, differentiation and proliferation which are all processes that are dysregulated in ADPKD and associated with cystogenesis. The evidence for the importance of the EGFR pathway in ADPKD is, however, scarce. The possibility that this pathway is involved in the pathophysiology of ADPKD needs to be further substantiated in patients as well as in models orthologous for human ADPKD. This pathway is of special interest since agents have been developed that can block activation of EGFR. Therefore, in this research project the role of the EGFR pathway and its ligands in the pathogenesis of human ADPKD will be further investigated [16].

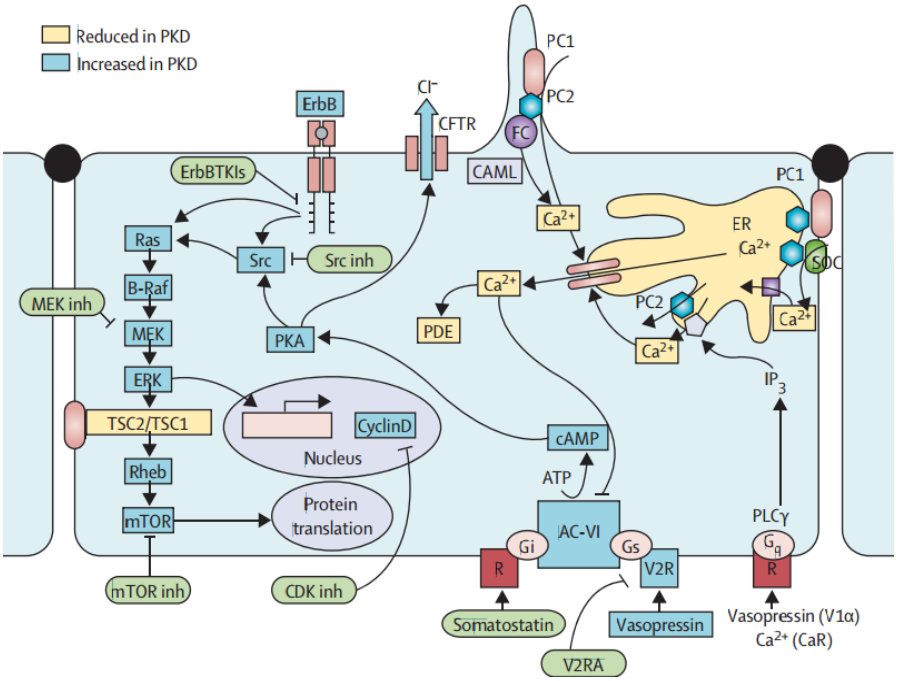


Figure 1: Signaling pathways involved in ADPKD pathogenesis. In ADPKD, cyst formation and cyst growth is induced via a complex dysregulation of multiple signaling pathways, including the cAMP, mTOR and EGFR signaling pathways. Inhibition of these pathways for treatment of ADPKD is explored in clinical trials via the use of somatostatin analogues/vasopressin-2 receptor antagonists, mTOR inhibitors and tyrosine kinase inhibitors [6].

1.2 EGFR pathway

The epidermal growth factor receptor (EGFR) family consists of four different transmembrane receptors including the EGFR or ErbB1 (HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (Figure 2) [17]. All EGFR members belong to the family of tyrosine kinase receptors. We will refer to the EGFR family members as ErbB receptors (ErbBs). The receptors can be activated by binding of various ErbB receptor ligands on the extracellular ligand-binding domain of the receptor. Until now 11 ErbB receptor ligands have been identified, including EGF, TGF-α, *Amphiregulin*, *Epigen*, *HB-EGF*, *Epiregulin*, *Betacellulin* and four different neuregulins (NRGs). All ErbB receptor ligands have their own affinity for binding with a specific ErbB receptor as shown in Figure 2. Most ligands are able to activate multiple ErbB receptors, while others bind specifically to one ErbB receptor to activate this receptor. No ligand exists for ErbB2, while ErbB3 lacks tyrosine kinase activity. This means that ErbB2 and ErbB3 are only functional receptors in combination with another ErbB receptor used for dimerization [17].

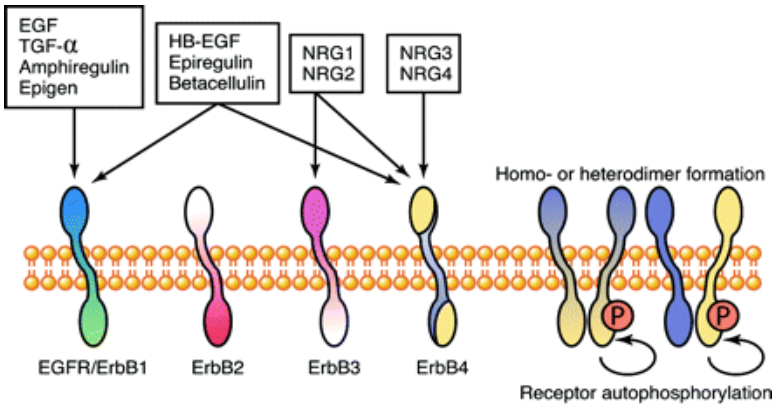


Figure 2: Overview of ErbB receptors and their ligands. The ligands can be divided into four different groups depending on their binding affinity with the receptor. Binding of a ligand to its receptor induces a conformational change resulting in receptor dimerization and autophosphorylation [17].

ErbB receptors can be activated in three different ways (figure 3).

a. ErbB receptors can be activated by direct binding of a soluble ligand to the extracellular binding site of the receptor which is the main way of ErbB receptor activation. All ErbB ligands exist in an inactive precursor form (proligand) in the cell membrane and need to be cleaved of their ectodomain by ADAM (A disintegrin and metalloproteinase) for release as active soluble ligand Binding of a soluble ligand to an ErbB receptor results in endocrine (activation of distant cells), paracrine (activation of adjacent cells) or autocrine (activation of the cell itself) activation of the receptor. See Figure 3, part a.

b. ErbB receptors can also be activated by ligands which are attached to the cell membrane. This results in juxtacrine signaling which means that a membrane anchored ligand on one cell activates ErbB receptors present on neighbouring cells. See Figure 3, part b.

c. EGFRs can also be activated in an indirect manner via activated G-protein coupled receptors (GPCR), which is called EGFR transactivation. Activation of the GPCR by its agonists (like

angiotensin II) , results in upregulation of ADAM molecules. The presence of more ADAM molecules leads to the release of more soluble ligands. [18]. See Figure 3, part c.

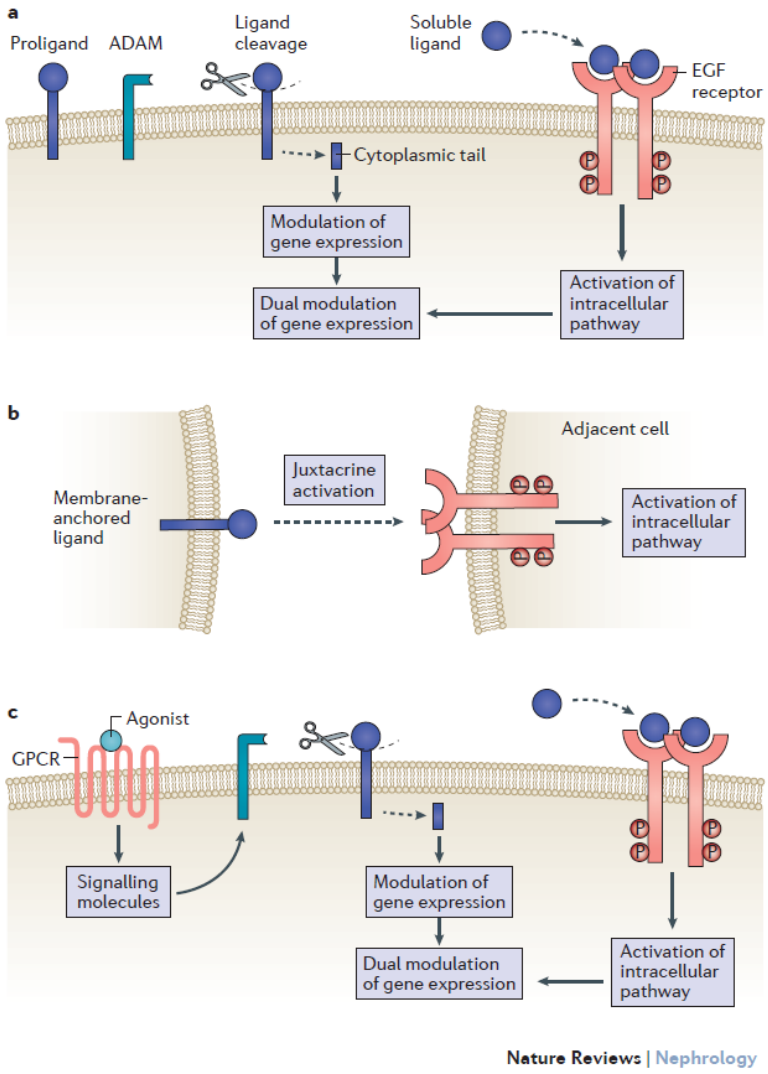


Figure 3: ErbB receptor activation can be in three different manners: by biding with their soluble ErbB ligands (a), membrane anchored ligands (b) or GPCR transactivation (c) [18].

Ligand binding induces homo- or heterodimerization of the receptor. Upon dimerization the cytoplasmic part of the receptor is activated via autophosphorylation of specific tyrosine amino acids [19]. This will lead to activation of intracellular signaling cascade via phosphorylation of other signaling proteins. Important intracellular signaling pathways which can be activated by EGFRs are the mitogen activated protein kinase (MAPK), JAK/STAT and phosphatidylinositol-3 kinase (P13K) pathways. These cytoplasmic pathways translate signals to the nucleus, changing the activity status of transcription factors, and thereby modify gene transcription and thus cellular behavior like proliferation and migration. The outcome of ErbB receptor activation is variable and dependent on the receptor type, receptor homo/hetero dimerization and the type of ligand which binds to the receptor.

1.3 Expression of ErbBs and their ligands in nephrogenesis

Nephrogenesis includes the phases of pronephros, mesonephros and metanephros respectively [20]. There is compelling evidence that EGFR signaling is crucially involved in nephrogenesis. First, its importance during embryogenesis is confirmed by the finding that knockout mice that lack the EGFR die during or three weeks after gestation owing to impaired epithelial development in several organs, including the kidney that show hypoplastic renal papillae, widespread apoptosis of tubular cells and widened renal collecting ducts [21]. *In vitro* experiments with embryonic kidney cells lacking EGFR activity also showed impaired cellular processes such as inhibition of cell proliferation and inhibition of ureteric bud morphogenesis. In line with these findings, addition of EGFR ligands to a coculture system of murine collecting duct cells with embryonic kidney cells stimulates cell proliferation and ureteric bud morphogenesis, branching and tubulogenesis [22].

Second, since ErbBs are widely expressed in different tissues, including renal tissue, EGFR activation can lead to various effects during normal development and during diseases. Third, important signaling pathways which can be activated by the EGFR are the MAPK, JAK/STAT and P13K pathways which regulate cell proliferation, migration, differentiation, cellular survival, apoptosis and tissue repair. These are all vital processes for embryogenesis in which the EGFR family is highly involved [18].

Finally, expression of different ErbB receptors and their ligands during nephrogenesis in different kidney segments is determined. In human embryos the EGFR is predominantly expressed in the collecting duct, while ErbB2 is expressed in the proximal tubules as well as in the collecting ducts of embryos. Expression of ErbB3 and ErbB4 during human embryogenesis is unknown. Although, a study in rat embryos found expression of ErbB4 in the ureteric bud and tubules [17]. Of the ErbB receptor ligands, *EGF* and *TGF-α* are expressed in proximal- and distal tubules and glomeruli, while *Amphiregulin* is only expressed in glomeruli during nephrogenesis [17]. The expression pattern of other ErbB receptor ligands during nephrogenesis is unknown. Figure 4 provides an overview of the expression of the ErbB receptors and their ligands during human nephrogenesis [17]. These findings suggest the important role of EGFR signaling during nephrogenesis.

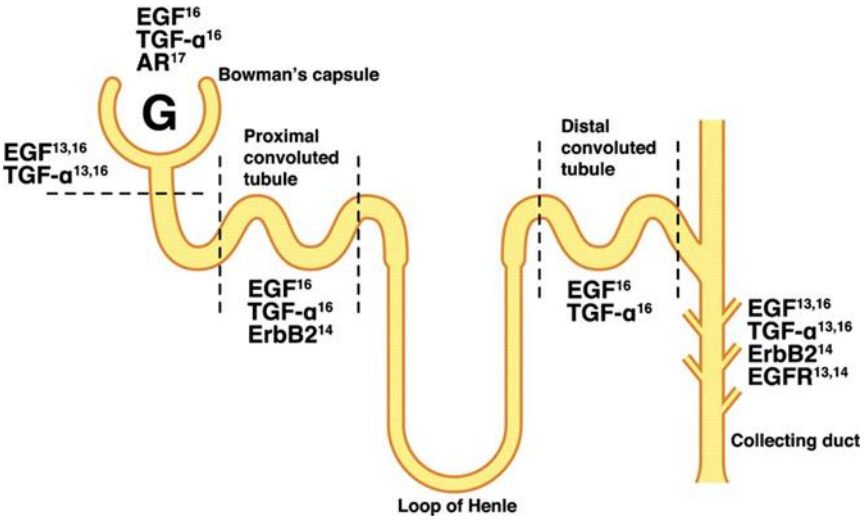


Figure 4: The expression of the ErbB receptors and their ligands during human nephrogenesis [17].

1.4 Expression of ErbBs and their ligands in the healthy adult kidney

The expression level and pattern of ErbB receptors and their ligands undergo changes after embryogenesis. During embryogenesis ErbBs are located at the apical side of epithelial cells, while in adult kidneys the ErbB receptors are all translocated to the basal side of epithelial cells (figure 5AB) [20]. Figure 6 shows the expression of various members of the EGFR family and their ligands in a healthy human adult kidney [18]. From all receptor types the EGFR (ErbB1) is most abundantly expressed within the kidney. Although, expression levels are lower compared to embryogenesis. The EGFR (ErbB1) is expressed in the glomeruli, distal and proximal tubuli, loop of Henle, and collecting ducts [23] [24] [25]. Expression of EGFR is determined in multiple cell types of the kidney including epithelial cells, podocytes, endothelial cells of glomeruli, mesangial cells and medullary interstitial cells. One research group determined that ErbB2 is expressed in collecting ducts [26]. However, others found no expression of ErbB2 in the adult kidney, while ErbB2 was present in fetal kidneys [27] (figure 5DE). ErbB3 is detected in distal tubules [28]. ErbB4 is expressed within distal and proximal tubuli, loop of Henle and collecting ducts. ErbB4 expression levels are lower in the adult kidney compared to the fetal kidney [29].

The main EGFR ligands: EGF, *HB-EGF* and *TGF-α* are expressed at the basal and apical site of renal epithelial cells [20]. Although most Immunohistochemistry studies of *HB-EGF* expression are performed in rodent kidneys, some studies used human kidneys. They identified *HB-EGF* expression in tubules and vascular smooth muscle cells in healthy human kidneys [30]. Others showed expression of *HB-EGF* in the cytoplasm of distal and proximal tubules in human kidney donor biopsies [31]. In human kidneys, *HB-EGF* expression was shown in the tubules as well as in mesangial cells of the glomeruli [32]. The EGFR ligand *EGF* is expressed within the glomerulus and in proximal tubules and *TGF-α* is expressed in the distal tubules [18]. The expression pattern of other ErbB receptor ligands in human kidneys is still unknown.

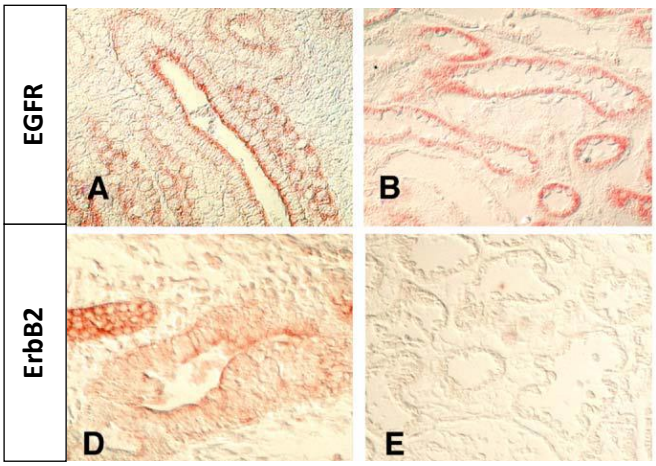


Figure 5: The differences in expression patterns between EGFR and ErbB2 in fetal kidneys (A,D) and adult kidneys (B,E) [27].

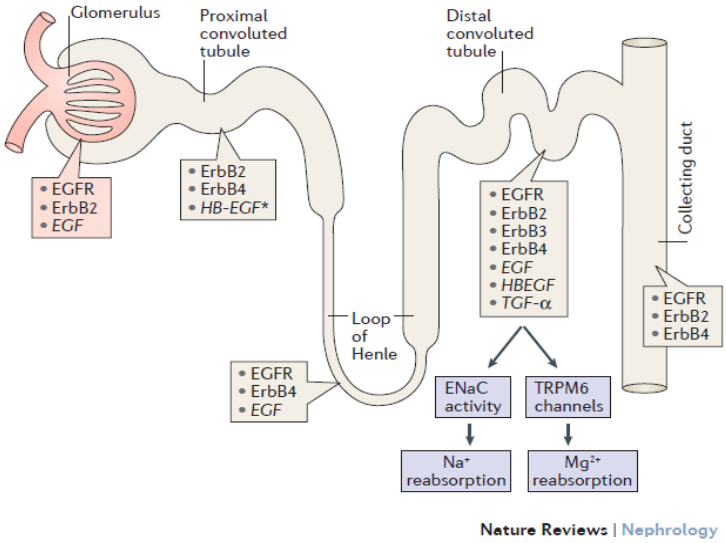


Figure 6: Expression of EGFR and their ligands in the healthy human kidney [18]

1.5 EGFR signaling in the healthy adult kidney

In addition to the importance of EGFR signaling in nephrogenesis, EGFR signaling also plays an important role in renal physiology including, the repair of kidney damage and in renal electrolyte handling.

Various studies state that EGFR signaling is involved in the repair of acute kidney damage during normal physiology. For instance *in vitro*-studies showed that the EGFR ligand *HB-EGF* might be produced in the plasma by monocytes or direct production within renal tissue by tissue resident macrophages, since *HB-EGF* is a growth factor molecule produced by monocytes or macrophages. However, the exact location of *HB-EGF* production is still unclear. In addition, in a rat model *HB-EGF* production was increased after ischemia/reperfusion injury [33] [34] [31]. These findings indicate that *HB-EGF* plays a role in tubular repair by proliferation of tubular cells in response to renal injury. Moreover, *HB-EGF* works protective against apoptosis as shown in obstructed rat kidneys [35]. This suggests that the activation of the EGFR by its ligands might be renoprotective in response to acute injury.

An important function of the kidney is to regulate electrolyte levels. The EGFR pathway also contributes to electrolyte handling, since EGFR signaling in the kidney is essential for the regulation of calcium, sodium and magnesium concentrations. Concentrations of these electrolytes can be regulated via controlling the activity of the cation channels, including TRPC5 and PC-2 [18]. Also specific ion channels are involved in homeostasis of electrolytes for example calcium channels and furthermore, sodium levels are regulated via the epithelial sodium (ENaC) channels [36]. The critical role of the EGFR pathway in electrolyte homeostasis has been illustrated in various studies, including research showing that *EGF* increased TRPM6 expression and enhanced magnesium reabsorption in renal epithelial cells [37]. Other researchers showed that magnesium reabsorption from pro-urine back to the blood is mediated by EGF-EGFR signaling. Furthermore, it was found that binding of *EGF* to the EGFR activates TRPM6 channels within distal tubules which induces magnesium reabsorption [17]. Finally, additional evidence regarding the role of EGFR signaling in electrolyte handing showed that inhibition of the EGFR in mice resulted in hypomagnesemia [38], and patients with colorectal cancer who were treated with an anti-EGFR antibody showed severe hypomagnesaemia [39]. To our knowledge, data regarding the effects of other ErbB receptors and ligands in electrolyte handling is lacking.

1.6 EGFR signaling in experimental ADPKD

Whereas the initial task of EGFR signaling is meant to be helpful in the repair of acute kidney damage, it has been suggested that dysregulation of EGFR signaling might contribute to chronic renal diseases. Excessive EGFR signaling is seen in renal inflammation and fibrosis which are general processes associated with chronic kidney disease. EGFR signaling is associated with the pathology of various other kidney diseases than PKD including, rapidly progressive glomerulonephritis, diabetic nephropathy and in chronic allograft nephropathy [40] [30] [41] [42].

The EGFR pathway is a logical subject of research in PKD which is characterized by hyperproliferation of renal tubular cells as activation of EGFR is one of the major triggers for tubular cell proliferation. Approximately 20 years ago researchers showed that cyst formation in mice was induced via activation of the EGFR pathway by *TGF-α* [43]. *In vitro* studies in human renal epithelial cells also

showed increased cyst formation during exposure to EGFR ligands *EGF* and *TGF-α* [44]. In addition, EGFR activation by EGFR ligands *HB-EGF* and *Amphiregulin* resulted in cyst formation *in vitro* and *in vivo* [45] [46]. Furthermore, it has been shown that the mRNA levels of EGFR are increased in ADPKD, as well as the protein levels of EGFR and EGFR tyrosine kinase activity [47] [48] [49] [50]. These studies imply that the EGFR pathway plays an important role in the pathogenesis of ADPKD.

EGFR is of specific interest in PKD research because of its disturbed cellular localization during this disease. In normal renal physiology, tubules and cysts consists of an epithelial cell layer and the epithelial cells of tubules have apical-basolateral cell polarity [51]. Controversially, in ADPKD apical and basolateral cell polarity is disturbed resulting in changes in protein and lipid expression patterns including the EGFR [27]. Mislocalization of the EGFR was detected in kidneys of human ADPKD patients via 125-I labeled *EGF* binding assays. Binding of *EGF* to the basolateral side occurred with a high affinity in both ADPKD kidneys and control kidneys, while binding of *EGF* to the apical side only occurred with a high affinity in ADPKD kidneys [52]. *Wilson et al.* also showed this type of localization in human kidneys via immunohistochemistry [27]. This finding implies that the EGFR ligands present in the lumen of tubules or secreted in cysts are also able to activate EGFR signaling in ADPKD, because of the apical localization of the receptor (Figure 4), in contrast to healthy kidneys, where the epithelial cell layers are intact and the EGFRs are located at the basal side.

Besides mislocalization of the EGFR in ADPKD, the expression of the ErbB receptors is different from the healthy human kidney (figure 7). In normal fetal kidneys, the EGFR and ErbB2 receptors are both highly expressed at the apical membrane and will change towards basal expression of EGFR solely after development of the kidneys. In ADPKD, the EGFR and ErbB2 receptors both remain highly expressed at the apical membrane, which is similar in fetal kidneys. Therefore, EGFR signaling is thought to be upregulated in ADPKD.

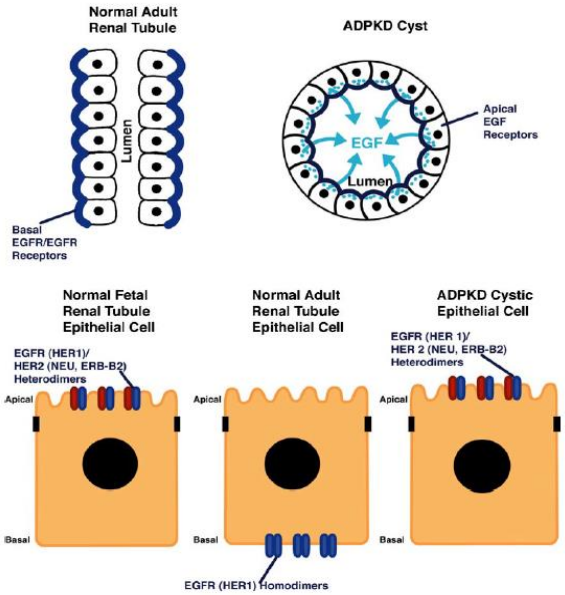


Figure 7: Disturbed cell polarity in ADPKD results in apical localization of the EGFR as well as the ErbB2 receptor [27].

In addition to the EGFR, the ErbB2 receptor might also be involved in PKD, since transgenic mice which overexpressed ErbB2 developed cysts within the kidney [20]. Research showed that treatment of *Pkd* null -/+ mice with an ErbB2 inhibitor reduced the cystic phenotype in these mice [27]. It is still unknown whether ErbB3 plays a role in the pathophysiology of PKD. However, different results are reported for the role of the ErbB4 receptor in PKD. Experiments with pax8-Cre-mediated conditional ErbB4 overexpression and ErbB4 knockout mice suggest that ErbB4 plays a role in proliferation, epithelial cell polarity and the development of collecting ducts. Cortical tubular cysts were detected in the mice which overexpressed ErbB4, while lacking ErbB4 resulted in kidney abnormalities including defects in polarization and larger ducts [53]. Moreover, ErbB4 deletion in a *cpk* mice model of ARPKD resulted in accelerated disease progression [54].

As EGFR knockout mice, full knockout mice that are lacking the ErbB2 or ErbB4 receptor also die at (mid)gestational age [20]. In contrast to ErbB receptor knockout mice, mice lacking EGFR ligands only showed minor phenotypes and were fertile. *TGF-α* knockout mice only showed some abnormalities in the eyes and skin, while transgenic mice that overexpress *TGF-α* showed renal enlargement, glomerular mesangial expansion and renal cyst formation [43]. Overexpression of TGF-a in *pcy* mice, a slowly progressive model of PKD, resulted in increased progression of cystic kidney disease [55]. Other researchers crossbred TGF-a knockout mice with *bpk* mice, a model for ARPKD and showed that TGF-a was not required for cystogenesis [56]. Knockout mice of the EGFR ligand *EGF* and *Amphiregulin* showed no phenotype. Moreover, different combinations of double and a triple knockout mice of EGF, TGF-a and *Amphiregulin* showed no abnormalities in kidney histology. These triple mutants were healthy and survived for more than one year [57]. *HB-EGF* knockout mice also showed minor phenotypes [31]. The minor phenotypes of EGFR ligand knockout mice compared to the EGFR knockout mice indicates the high redundancy in ErbB receptor signaling. It might be hypothesized that the lack of some ligands and the EGFR itself can be compensated via ErbB signaling with other ErbB receptors and ligands.

Several *in vitro* and *in vivo* studies showed that cyst formation and growth can be attenuated via the performance of interventions to inhibit the EGFR signaling pathway. *In vitro*, inhibition of cyst growth in a murine organ culture system was reduced by the addition of *EGF* and EKI-785 compared to *EGF* stimulated kidney organ cultures [58] [59]. In Bpk mice, a model of ARPKD, tyrosine kinase inhibitor EKI-785 which inhibits EGFR and ErbB2 showed a decrease in cyst formation. Moreover, the treated mice had less tissue fibrosis, improved renal function and an increased lifespan compared to the untreated control groups [60]. Similar results were obtained in the Han-SPRD rat model of ADPKD treated with EKI-785 [61]. These studies suggest EGFR and its ligands as a potential therapeutic target in ADPKD.

1.7 EGFR signaling in human ADPKD

Despite the positive experimental data of the EGFR pathway in ADPKD, intervention studies in human ADPKD are limited. Previous results of our research group examined the levels of EGFR ligands in plasma and urine of 27 ADPKD patients. An increase of urinary *HB-EGF* excretion was found in ADPKD patients at baseline compared to controls (Figure 8). Moreover, urinary *HB-EGF* excretion was positively correlated with disease severity, given as measured glomerular filtration

rate and total kidney volume. The EGFR ligand, *TGF-α* showed similar concentrations in ADPKD patients as in controls [65].

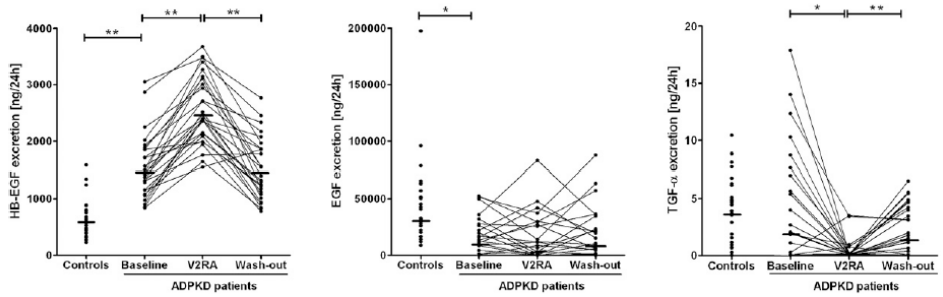


Figure 8: EGFR ligand excretion in urine of ADPKD patients compared to controls. *HB-EGF* excretion is increased in ADPKD patients, while EGF is decreased and TGF-a is similar in patients compared to controls. [65].

Urinary *EGF* excretion was significantly decreased in ADPKD patients compared to controls, which has been seen in other chronic kidney diseases. A large study examining 261 patients showed that the concentration of *EGF* in urine is reduced in patients with various kidney diseases and correlated with decreased intrarenal *EGF* mRNA expression. In addition, low urinary *EGF* excretion was found to predict accelerated loss of renal function in three independent cohorts of patients with chronic kidney disease [66].

Research showed that EGFR ligand *Amphiregulin* mRNA is upregulated in a human *Pkd1*kidney cell line, and that inhibition of *Amphiregulin* reduced cyst formation in these cells. Moreover, *Amphiregulin* gene expression was increased in tissues derived from human ADPKD patients with end stage renal disease, and furthermore, stimulated EGFR signaling which contributed to cyst formation and growth [46]. These studies show that *HB-EGF* and *Amphiregulin* might be possible therapy targets for treatment of ADPKD.

1.8 Potential targets for medical intervention in the EGFR pathway

Inhibition of EGFR signaling in ADPKD could be achieved in multiple ways, and can be distinguished in compounds that directly target the receptor or compounds that indirectly modify receptor activity by targeting the activity of EGFR ligands. The potential targets for medical intervention in the EGFR pathway are listed below [18].

1. Targeting the ErbB receptor
    - Tyrosine kinase inhibitors, which target one or several ErbB receptors inhibit phosphorylation of the receptor(s) necessary for activation of downstream pathways
    - Monoclonal antibodies against an ErbB receptor can either block the interaction between the receptor and its ligand or prevent receptor dimerization.

2. Targetting EGFR ligands
    - Ligand neutralizing antibodies which decrease ligand availability
    - ADAM inhibitors which decrease ligand availability

At the moment these drugs are not used in the clinics for treatment of kidney diseases. However, tyrosine kinase inhibitors, are currently used in two clinical trials in patients with ADPKD. The first is an ongoing phase Ib/IIa trial investigating the effects of nonspecific TKI tesevatinib given to ADPKD with an impaired kidney function. The primary outcome of the phase I trial is to determine the safety, plasma pharmacokinetics, and maximum tolerated dose of this drug. Prelimanary results show that low dosages of this drug were tolerated well, whereas higher dosages caused severe side effects including skin rash, heart problems and diarrhea. Treatment with low dose of tesevatinib will be continued in the ADPKD patients for 24 months to determine the effects on renal function (phase IIa) [18]. The second study compared 24 months of treatment with the nonspecific TKI bosutinib versus placebo in patients with ADPKD with a normal renal function and high total kidney volume. Participants were divided into three groups: placebo, 200 mg bosutinib and 400 mg bosutinib. Participants in the 400 mg were down-titrated to 200 mg, possibly due to the high withdrawal rate in the 400 mg group. Results of this trial showed that the change in kidney volume was significantly in favour of bosutinib 200 mg and 400/200 mg versus placebo. However, after completion of the trial, there was no difference in change in kidney function between the 200 mg group and placebo, however the effect was worse for the 400/200 mg group when compared to placebo [18].

The first study showed that nonspecific targeting of the EGFR leads to serious adverse events. Although the second study does not provide us with information regarding adverse events, these can be expected to be considerable, given the high treatment withdrawal rate. Also, clinical trial in the field of oncology regarding nonspecific interventions in the EGFR pathway demonstrated a number of systemic adverse effects. Taken together, these studies suggest that TKIs might be a potential future treatment option for patients with ADPKD.

Interventions in the EGFR in the field of nephrology are limited, while the EGFR pathway is extensively studied within the field of oncology. Dysregulation of the EGFR pathway can result in excessive cell proliferation and tumor growth which is seen in various types of epithelial cancer, for example in breast, lung and colorectal cancer [62]. Compounds that target the EGFR pathway are already in clinical use for treatment of cancer or have been evaluated in clinical trials for treatment of cancer, like the EGFR and ErbB2 receptor. For example, Herceptin is used for the treatment of ErbB2 positive breast cancer [63]. Multiple EGFR targeting compounds are FDA approved for the use in cancer treatment, like monoclonal antibodies gefitinib, erlotinib, cetuximab, and panitumumab [64].

Since, targeting the ErbB receptors is effective in treating cancer, it is suggested that they can also be effective in another proliferative disease like ADPKD. Tyrosine kinase inhibitors (TKI) are distinguished in specific and aspecific drugs. Specific TKI only inhibit one ErbB receptor, while aspecific drugs can inhibit multiple ErbB receptors. In most cases, inhibition of ErbB receptors is nonspecific due to the wide expression of ErbB receptors in different tissues. Therefore, the main disadvantages of the use of ErbB TKIs are their severe side effects, like edema, nausea, vomiting, diarrhea, generalized rash and new onset proteinuria. As mentioned before, treatment with nonspecific TKI directed against the EGFR used in treatment for colorectal cancer resulted in severe hypomagnesemia. Because severe side effects of TKI, long term treatment with these drugs might not be tolerated in patients [18]. Therefore, a more targeted approach is required to inhibit EGFR signaling in kidney disease like ADPKD, like targeting EGFR ligands by ADAM inhibitors or neutralizing antibodies against ErbB ligands.

1.9 Aim of this project

As discussed above, the EGF receptor pathway may be involved in the pathophysiology of ADPKD. Since, EGFR activation promotes cell proliferation. Furthermore, EGFR ligands *EGF* and TGF-α, induced cyst formation *in vitro*. Research regarding the importance of the EGFR pathway in the pathophysiology of human ADPKD is very limited. Therefore the relevance of the EGFR pathway in ADPKD needs to be further substantiated. This pathway is of special interest since agents have been developed that can block activation of EGFR. For these reasons, we determined the expression of the EGFR and its ligands in renal tissue of ADPKD patients. Because our previous study showed a correlation of urinary *HB-EGF* with ADPKD disease severity, we are particularly interested in the EGFR ligand *HB-EGF*.

Overall hypothesis:
The EGFR pathway as determinant of rate of renal tubular cell proliferation is involved in the pathophysiology of ADPKD.

Specific study questions regarding the EGFR:

- Is the expression of phosphorylated EGFR higher in renal tissue of patients with ADPKD compared to controls with a normal renal function and impaired renal function?
- Are there differences in staining between ADPKD tissue and controls with normal and impaired renal function?
    - What is the localization of the phosphorylated EGFR staining?
    - Is there colocalization with *HB-EGF*?
    - Is there colocalization with specific tubular markers: THF, LRP2 and AQP2?
- Are EGFR relative mRNA levels higher in ADPKD tissues compared to controls with a normal renal function?

Specific study questions regarding *HB-EGF*:

- Is the expression of *HB-EGF* higher in renal tissue of patients with ADPKD compared to controls with a normal renal function and impaired renal function?
- Are there differences in staining between ADPKD tissue and controls with normal and impaired renal function?
    - What is the localization of the *HB-EGF*?
    - Is there colocalization with phosphorylated EGFR?
    - Is there colocalization with specific tubular markers: THF, LRP2 and AQP2?
- Are *HB-EGF* relative mRNA levels higher in ADPKD tissues compared to controls with a normal renal function?

2 Materials & methods:

2.1 Study design

The objective of this study was to determine the role of EGFR signaling in the pathophysiology of human ADPKD by investigating the expression of the EGFR and its ligand *HB-EGF* in renal tissue of patients with ADPKD compared to controls. Therefore, human paraffin-embedded kidney tissues were obtained from patients with ADPKD(N=19) from the biobank of the UMC Groningen. These tissues were previously collected from ADPKD patients who have underwent a nephrectomy between 2013-2015 in the Netherlands. The majority of patients (80%) underwent nephrectomy prior to kidney transplantation to make space for graft implantation, while the remaining patients underwent nephrectomy because of cyst bleeding, mechanical issues or a disturbed nutritional state. All of the patients with ADPKD suffered from severe impaired kidney function and most patients had reached end stage renal disease (ESRD) at the moment of nephrectomy. The mean age of the patients at the moment of nephrectomy was 50 ± 10 years.

Paraffin-embedded control tissues were obtained from the biobank of the department of pathology at the UMCG. Two types of human renal control tissues were included: controls with a normal renal function (N=12) and controls with impaired renal function (N=5). Normal human kidney tissue was derived from the healthy parts of kidneys from patients with renal cell carcinoma with normal renal function. Controls with impaired kidney function were taken into account to investigate whether the (possible) differences in EGFR signaling might be ADPKD specific or whether these differences can be explained as dependent on kidney function. These controls were selected from patients with hydronephrosis and reflux nephropathy, since dysregulated EGFR signaling is not reported in these renal diseases in contrast to many chronic kidney diseases besides ADPKD. In this study the following approach was applied to obtain qualitative data and quantitative data. Qualitative data was obtained with immunohistochemistry for the phosphorylated (activated) EGFR and its ligand *HB-EGF*. Moreover, quantitative mRNA expression data of different ErbB receptors and their ligands were obtained with qRT-PCR.

2.2 Tissue preparation & histology

Multiple pieces of tissues were collected from each kidney from the cortex, medulla to pyelum in three regions of the kidney as indicated in figure 9. Material was collected as frozen tissues and as formalin fixed tissues embedded in paraffin. A selection procedure was performed to identify suitable tissues for immunohistochemistry and RNA extraction. ADPKD tissues were included into the study based on the following requirements: presence of multiple cysts, tubules and glomeruli.

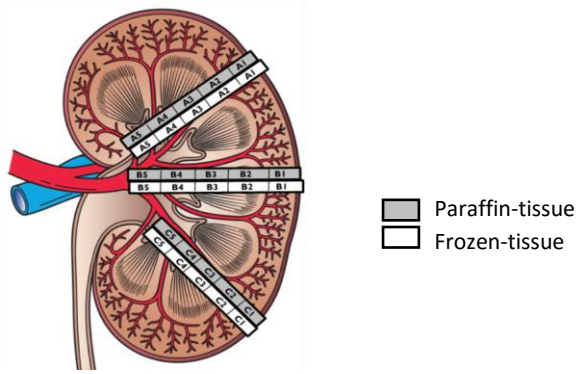


Figure 9: The pieces of kidney tissues were derived from different parts of the kidney: region A, B and C. From each region tissue pieces are obtained from cortex, medulla to pyelum (1-5) for frozen and paraffin tissue.

Exclusion criteria for tissues were, absence of cysts, the tissue piece consisted for a major part of fat or muscle tissue, pyelum tissue or a lot of blood was present in the tissue.

To determine if the selected tissue pieces satisfied the inclusion criteria a hematoxylin eosin (HE) staining was performed. HE staining,is a standardized staining to visualize cytoplasm with eosin (orange) and cell nuclei with hematoxylin (purple). After this selection procedure, we determined whether paraffin embedded tissues or frozen tissues would give the best staining results. Since paraffin embedded tissue showed optimal results for staining in combination with a clearer overview of the morphology of the tissue, we have chosen to use paraffin embedded tissue for our final staining. Before staining of tissues is possible, paraffin should be removed via xylene and different alcohol percentages as shown in figure 10 for paraffin embedded tissues. After 10 minutes exposure to hematoxylin, slides were washed with running tap water for 10 minutes. Then eosin was added followed by a dehydration process. Tissue slides were provided with a cover glass using mounting medium.

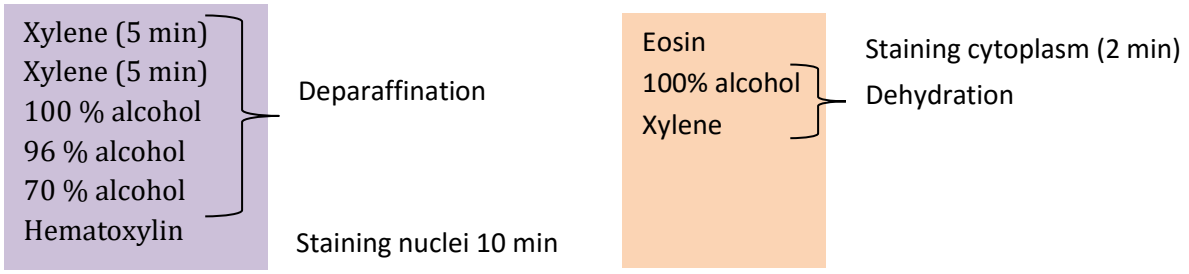


Figure10: Hematoxylin eosin staining

2.3 Immunohistochemistry

Immunohistochemistry was performed to identify the localization of the activated phosphorylated EGFR (pEGFR) and its ligand *HB-EGF* in human ADPKD tissue compared to controls with normal renal function and impaired renal function. First, immunohistochemistry protocols were optimized by testing several antibodies against pEGFR and *HB-EGF*, as well as different methods to handle the tissue, including several antigen retrieval methods. In table 1 we have given an overview of the used antibodies for the final results using immunohistochemistry. Of note, we used three different antibodies for the staining of *HB-EGF* since one of the antibodies against *HB-EGF* showed a specific kind of staining pattern for *HB-EGF* in contrast to the other antibodies *HB-EGF*. For comparison of the localization of *HB-EGF* localization, we used three different kinds of antibodies against *HB-EGF*. To determine the exact origin of the positive staining in these tissues, several general antibodies were used as markers for different parts of the kidneys (table 1). Tamm-Horsfall protein (THP) also called uromoduline was used as a marker for distal tubuli and thick ascending limb of henle, whereas LRP2 or megalin was used as a marker for proximal tubules. Moreover, aquaporin-2 was used which is a marker for the collecting duct. Consecutive sections were stained for the phosphorylated (p)EGFR, the three markers for different parts of the nephron, followed by staining with the three independent antibodies of *HB-EGF*.

For each staining 3 uM thick tissue sections were used. Before staining, paraffin was removed via deparaffination as discussed before. Heat induced antigen retrieval was performed to minimalize

crosslinking within the tissue to obtain optimal staining results. Therefore, several antigen retrieval buffers were tested including 10 mM citrate (pH = 6), 1 mM EDTA (pH = 8), 0,1 mMtris/HCL (pH = 9), 10 mMTris/ 1 mM EDTA (pH = 9). Heat induced antigen retrieval was performed at a power of 500 watt within the microwave for 15 minutes.Tris/HCL was also incubated overnight at 80 degrees. Moreover, 1% protease was also tested as antigen retrieval method incubated for 30 minutes at room temperature. Since most tissues express endogenous peroxidases which might interfere with staining results, endogenous peroxidases were blocked via 500 ul 30% H_2O_2 in 50 ml phosphate buffered saline (PBS) for 30 minutes. Thereafter, the primary antibody was diluted in PBS + 1% bovine serum albumin and applied for 1 hour at room temperature. Optimal antibody dilution and optimal antigen retrieval method was determined for each antibody. Table 1 provides information regarding the primary antibodies and their optimal dilution and antigen retrieval method that was used for the final staining.

Table 1: Antibodies used for immunohistochemistry

Primary antibody	Company	Host/Clonality	Dilution	Antigen retrieval	Secondary antibodies
Phospho-EGF receptor (Tyr1068) (1H12)	Cell signaling technology	Mouse Monoclonal antibody	1:200	Citrate	RAMpo GARpo
HB-EGF AF-259-NA	R&D systems	Goat IgG Polyclonal antibody	1:50	Tris/EDTA	RAGpo GARpo
HB-EGF (E10) sc-74526	Santa cruz biotechnology	Mouse Monoclonal antibody	1:20	Tris/EDTA	RAMpo GARpo
HB-EGF (HPA053243)	Atlas antibodies	Rabbit IgG Polyclonal antibody	1:40	Tris/EDTA	GARpo RAGpo GARpo
LRP2 (HPA064792)	Atlas antibodies	Rabbit Polyclonal antibody	1:750	Tris/EDTA	GARpo RAGpo
Tam horsfall protein (clone 10.32A)	Cedarlane	Mouse IgG2b Monoclonal antibody	1:1000	Without	RAMpo GARpo
Aquaporin 2	Merck Millipore (calbiochem)	Rabbit	1:2000	EDTA	GARpo RAGpo

Depending on the host of the antibody the appropriate peroxidase labeled secondary antibodies were used. All secondary antibodies were diluted 1:100 in PBS + 1% bovine serum albumin + 1% human serum and incubated for 30 minutes at room temperature. Between every step, slides were washed with PBS for 15 minutes. Staining was visualized by using 3,3-diaminobenzidine tetrachloride (DAB) as a substrate for the peroxidase enzymes which develops into a brown pigment. Cell nuclei were counterstained with hematoxylin, and thereafter tissues were dehydrated with a serie of alcohol solutions and covered with a cover glass by using mounting medium. Digital images of the stained tissues were obtained with Aperio or Hammamatsu scanner and were viewed with ImageScope version 12.01.

2.4 RNA isolation & qRT-PCR

Quantitative real time pcr analysis (qRT-PCR) were performed to identify the quantitative amount of the phosphorylated EGFR and one of its ligand *HB-EGF* in human ADPKD tissue compared to controls. Therefore, RNA was isolated from approximately 10 frozen tissue sections of 10 uM thickness by using trizol (Ambion/life technologies) from ADPKD kidney tissues (n = 18) and normal

functioning kidneys as control (n = 4). Tissues were collected in RNAse/DNAse free tubes and stored at -80 °C until further use.

The samples were thawed on ice and incubated at room temperature for five minutes. Then, 200 µl chloroform was added to each sample and they were mixed for 15 seconds. For phase separation the samples were untouched for 2-3 minutes. Subsequently, the tubes were centrifuged at 12000 g for 15 minutes at 4 °C. After these proceedings three separate phases can be distinguished: aqueous phase which contains RNA, interphase which contains DNA and an organic phase which contains proteins and lipids (figure 11). The aqueous phase was carefully collected and transferred into a new 1,5 ml tube without touching the interphase or organic phase.

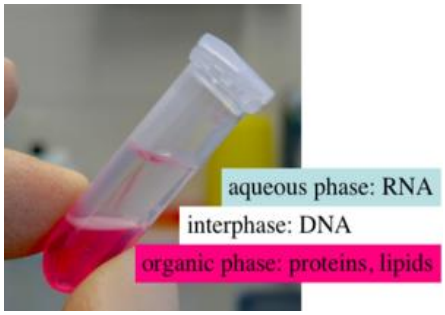


Figure11: Phase separation

The RNA was mixed with 500 µl isopropanol and left at room temperature for 10 minutes to precipitate the RNA. Afterwards the tubes were centrifuged again at 12000 g for 10 minutes at 4 °C. The remaining pellet was washed with 75% ethanol. After centrifugation, the pellet was dried to the air and resuspended in 30 µl RNase free water and stored at -20 °C. RNA concentrations were measured with a nanodrop spectrophotometer. 0,5 µg from the total isolated RNA was transcribed into complementary DNA (cDNA).

cDNA was generated because it is much more stable than RNA. First the RNA was mixed with 1 µl random hexameres and 1 µl dNTPs. This was filled up with water until the total volume was 10 µl. After mixing well, the samples were incubated at 65 °C for 5 minutes which allows annealing of the random hexamere oligonucleotide primers at multiple random places within the mRNA. These primers provide starting points for cDNA synthesis. At the meantime, the RT-master mix was prepared which contains the following: 4,0 µl 5X first strand buffer, 2 ul 0,1 u/Mol DTT, 1 ul RNase out recombinant ribonuclease inhibitor (40 units/ul) and 1,0 µl superscript II. Subsequently 8 µl of this mix was added to the samples which were then properly mixed and incubated into a thermocycler with heated lid for 10 minutes at 25 °C and 50 minutes at 42 °C and 15 minutes at 70 °C. RNase out was used to inhibit RNases and the buffer was used to catalyze the reaction wherein the enzyme reverse transcriptase converts RNA to cDNA by using dNTPs. cDNA samples were diluted to a concentration of 2 ng/ul which was directly used in the qRT-PCR.

Quantitative real time pcr analysis was performed in 384 wells plates. All samples were analyzed as triplicates. 2,5 ulof the cDNA sample was used for each reaction. Water was used as a negative control to check for DNA contamination. Gene expression was determined with Taqman gene assays (applied biosystems) (table 2). These assays consist of a primer set and a sequence specific probe to detect the target gene with high specificity and sensitivity. The genes examined were: ErbB receptor

ligands *HB-EGF*, TGF-α, EGF, *Amphiregulin*, NRG1-4, as well as ErbB receptors EGFR, ErbB2, ErbB3 an ErbB4. For each reaction 8 ul mastermix was used containing 0,5 ul of the taqman assay, 5 ul complete mastermix with Rox and 2 ul water per reaction. Pipetting was performed with a pipetting robot. After pipetting, the plate was covered with seal, centrifuged and analyzed with the taqmanqRT-PCR machine at ERIBA. The qPCR program is shown in figure 12. Data were analyzed with SDS 2.2 (applied biosystems) according to the ΔΔCt method. This method determines the relative mRNA expression of the gene compared to the housekeeping gene TBP as endogenous control.

Table 2: Taqman gene expression assays (applied biosystems)

Target genes	Taqman assay
HB-EGF	Hs00181813_m1
TGF-α	Hs00608187_m1
EGF	Hs01099999_m1
Amphiregulin	Hs00155832_m1
EGFR	Hs01076090_m1
ErbB2	Hs01001580_m1
ErbB3	Hs00951455_m1
ErbB4	Hs00171783_m1

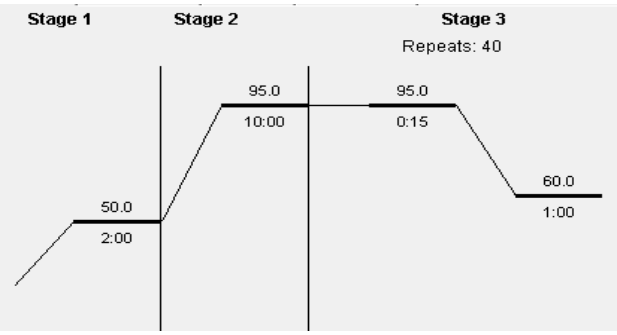


Figure 12: qRT-PCR program

2.5 Statistical analysis

Characteristics are shown for patients with ADPKD and controls. Parametric variables are displayed as mean ± SD. Differences in characteristics between patients and both control groups were calculated with a Mann–Whitney *U* test in case of nonparametric data. Differences in RT-PCR between patients and controls were calculated with a Mann-Whitney *U* test, because of the skewedness of the data. All statistical analyses were performed using SPSS software, version 22.0 (IBM, Inc., Armonk, NY). A *P* value <0.05 was considered to represent statistical significance, and all statistical tests were two tailed.

3 Results

3.1 Histological analysis identified suitable renal tissues of ADPKD patients and controls

Hematoxylin eosin (H&E) staining was performed to select tissues for immunohistochemistry. The selection procedure resulted in a suitable piece of tissue for most patients (figure 13). Most tissues which fulfilled the inclusion criteria were derived from the renal cortex (region A1, B1, C1). The tissue of one patient did not meet the inclusion criteria, because blood was present. Therefore, tissue of this patient was excluded from the study.

Tissue from patients with ADPKD consisted for large parts of fibrotic and inflammatory tissue with cysts in different sizes from dilated tubules (±100 um), small cysts (1 mm) to large cysts (±10 mm). Cysts are aligned with a layer of epithelial cells, which varies from cubic, columnar to in most cases flattened epithelial cells. Only minor amounts of normal renal tissue, consisting of glomeruli and tubuli, was present in ADPKD tissue samples. Most glomeruli present in ADPKD showed atrophy of glomerular content. In contrast, control kidneys derived from patients with normal renal function contained many glomeruli and tubuli without the presence of inflammation and fibrosis (Figure 13). However, tissue derived from the control group consisting of patients with impaired renal function showed inflammation and fibrosis due to obstruction comparable to ADPKD.

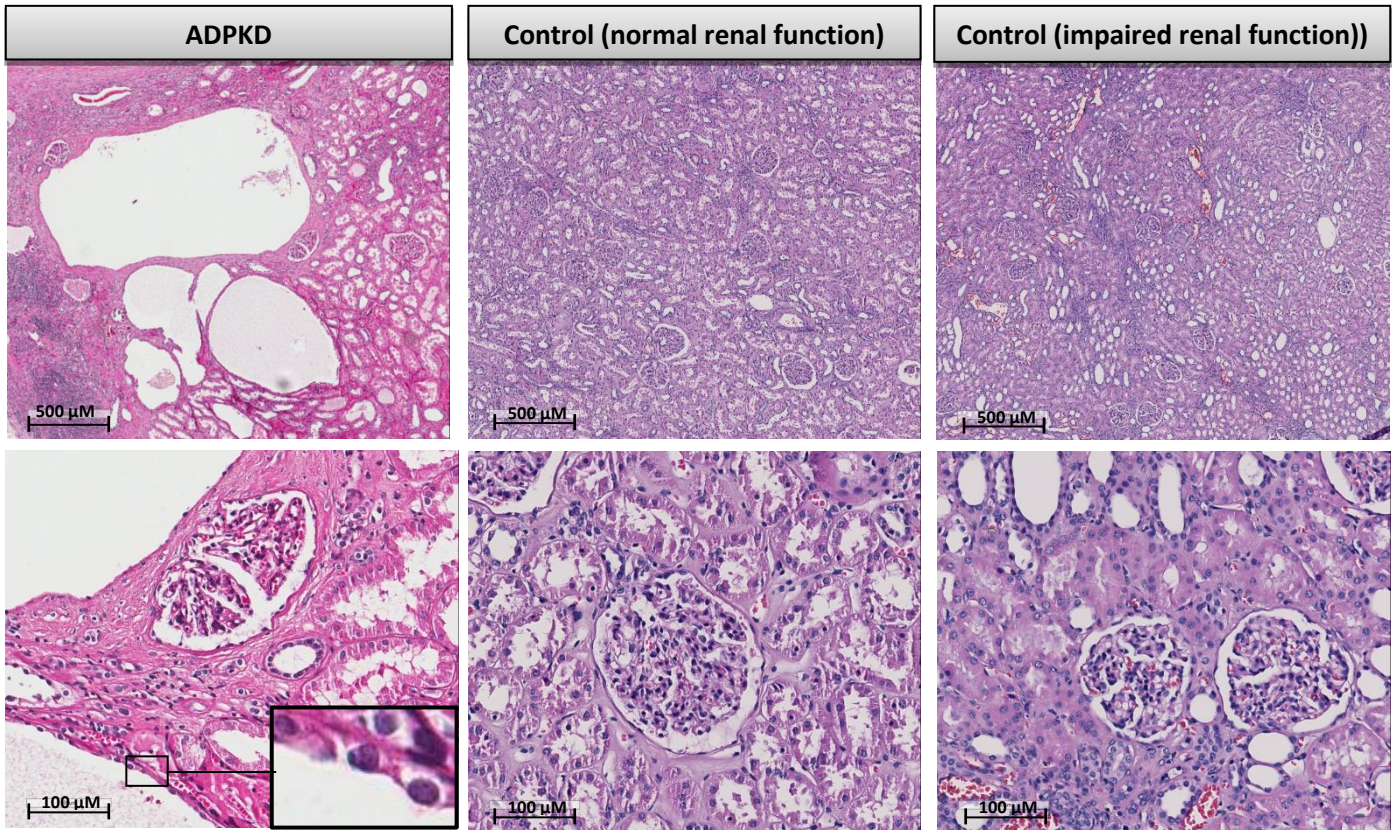


Figure 13: Hematoxylin eosin staining of human renal tissue from ADPKD patients compared to controls. ADPKD tissues containing multiple cysts, tubuli and glomeruli were included into the study. Controls with impaired kidney function contained besides glomeruli and tubuli, inflammation and fibrosis which is also present in ADPKD but absent in control tissues from patients with normal functioning kidneys.

3.2 Characteristics of ADPKD patients and controls

Characteristics of ADPKD patients and controls prior to nephrectomy are shown in Table 4. We included 19 patients with ADPKD and as controls 12 participants with a normal renal function and 5 participants with an impaired renal function. There were significant differences between patients with ADPKD and controls with a normal renal function regarding renal function given by a higher serum creatinine level and higher kidney weight in patients, reflecting their disease status. Moreover, ADPKD patients significantly differ from controls with impaired renal function, since patients have a higher kidney weight and a higher serum creatinine. There were no differences between controls with normal renal function and impaired function, except for kidney weight. Kidney weight in controls with normal renal function is higher than the average weight of a normal kidney due to parts which are infiltrated with tumor tissue.

Table 3: Characteristics of ADPKD patients and controls

Characteristics	ADPKD patients	Control group 1 (normal function)	Control group 2 (impaired function)
Participants (n)	19	12	5
Men (%)	72	33	80
Age (years)	50 ± 10	60 ± 11	40 ± 29
Serum creatinine (µmol/l)	504 ± 199	90 ± 14	141 ± 62
eGFR (ml/min per 1.73 m²)	13 ± 10	-	-
Kidney weight (g)	3627 ± 2862	475 ± 332	347 ± 290
**Values are given as means ± SD, eGFR: estimated glomerular filtration rate.*			

3.3 pEGFR is expressed in cysts of ADPKD patients

Immunohistochemistry was performed on consecutive tissue sections of kidneys from ADPKD patients and controls to examine the localization of the pEGFR and *HB-EGF*. Furthermore, kidney segmental markers were used to determine cyst origin. Representative images of the stained tissues are shown in figure 14-15. The results as presented in this report are an overview of the observations and discussion during a microscope session with dr. M. van den Heuvel, nephropathologist at the UMCG. Extensive studying of the immunohistochemistry data are beyond the scope of this report and will be executed in the future.

No staining was observed in all negative controls, meaning that the secondary antibodies cause no background staining itself. As shown in Figure 14A, the expression of the pEGFR, which is the active form of the EGFR, is localized at cyst lining epithelial cells of most cysts. However, not all cysts were positive for the pEGFR. Expression of the pEGFR was not detected in glomeruli in ADPKD, whereas some distal tubuli or dilated tubuli and collecting ducts or dilated collecting ducts also expressed the pEGFR. In tissue from controls with normal renal function, activity of the EGFR was absent in most cases (Figure 15). In tissues from patients with impaired renal function pEGFR expression was detected in urothelium cells and in the collecting ducts.

pEGFR is also expressed in stromal cells of both ADPKD tissues and controls. Figure 14B presents expression of the pEGFR in smooth muscle cells within a blood vessel wall. This serves as an internal positive control.

3.4 pEGFR is localized at the apical plasma membranes of ADPKD cysts & collecting ducts

Since previous research showed that the EGFR is mislocalized in ADPKD, we identified the localization of the pEGFR. The expression pattern for the pEGFR in ADPKD was classified as diffuse cytoplamatic. Although, expression was mainly localized towards the apical cell membrane in ADPKD cysts, observed at places where the cyst lining-epithelial cells were not stretched out and where the cell layer was completely intact. This was also observed in collecting ducts (figure 14D) for the pEGFR and AQP2.. Apical localization of the pEGFR receptor was not observed in control tissues from patients with normal renal function.

3.5 pEGFR expression colocalizes with *HB-EGF* expression

Activation of the EGFR can be achieved by binding with one of its ligands, including *HB-EGF*. Staining of *HB-EGF* was performed with three different antibodies: HPA053243, AF259-NA and E10 sc-74526 respectively. *HB-EGF* was weakly expressed in proximal tubuli and stronger expressed in distal tubules of control kidneys with normal renal function. In tissue from controls with impaired renal function *HB-EGF* was also weakly expressed in proximal tubules, while expression in distal tubules at the first sight seems to be stronger compared to healthy control tissue. Besides *HB-EGF* positive cyst-lining epithelial cells, *HB-EGF* is predominantly expressed in distal tubules and only weakly expressed in proximal tubules in ADPKD compared to controls with impaired kidney function. Glomeruli are negative for *HB-EGF* in all tissues. These results were found with all antibodies tested for *HB-EGF*. The *HB-EGF* antibodies HPA053243 and AF—259-NA gave comparable results. However, AF-259-NA resulted in the strongest positive staining of cyst lining epithelial cells. The staining pattern of *HB-EGF* detected with those antibodies can be classified as diffuse cytoplasmic. As shown in Figure 14, *HB-EGF* antibody E10 sc-74526 showed a remarkable coarse-grained staining pattern, interpreted as a lysosomal pattern, with less positively stained cells compared to the other two antibodies . Cysts which are positively stained for the pEGFR co-localize with *HB-EGF* expression (Figure 14C and 15).

3.6 Cyst origin in ADPKD can be heterogeneous

Immunohistochemistry showed that the pEGFR was not present in all cysts which could be due to a distinct origin of the cysts which are positive for the pEGFR. To determine if there is a common pattern in cyst origin in combination with pEGFR positive cysts, tissues were stained for kidney segment specific markers. THF-protein, LRP2 and AQP2 are markers which are known to be expressed in healthy adult kidney segments: distal tubules/limb of Henle, proximal tubules and collecting ducts respectively. All antibodies used as kidney specific markers gave clear and specific staining results as shown in renal control tissues (Figure 15). Results of consecutive stains in ADPKD tissue showed that the origin of cysts can be very heterogeneous and even variates within one patient. However, at first sight the majority of the cysts which are positive for the pEGFR seem to be derived from collecting ducts as there was colocalization of the pEGFR with collecting duct marker AQP2. Examples of cysts derived from collecting ducts are shown in Figure 14C and 15. It was observed that a few other cysts which were positive for the pEGFR colocalized with distal tubule marker THF-protein and therefore, these particular cysts seem to be derived from distal tubules or limb of Henle. In contrast, tissue from ADPKD patients stained for proximal tubule marker LRP2 did not show positive staining within cyst lining epithelial cells which indicates that cysts are not derived from proximal tubuli. Taken together, these data suggest that cysts originate from distal

tubules/limb of Henle or collecting ducts. However, from most cysts the origin is still unclear, because these cysts do not express any of the kidney specific segmental markers.

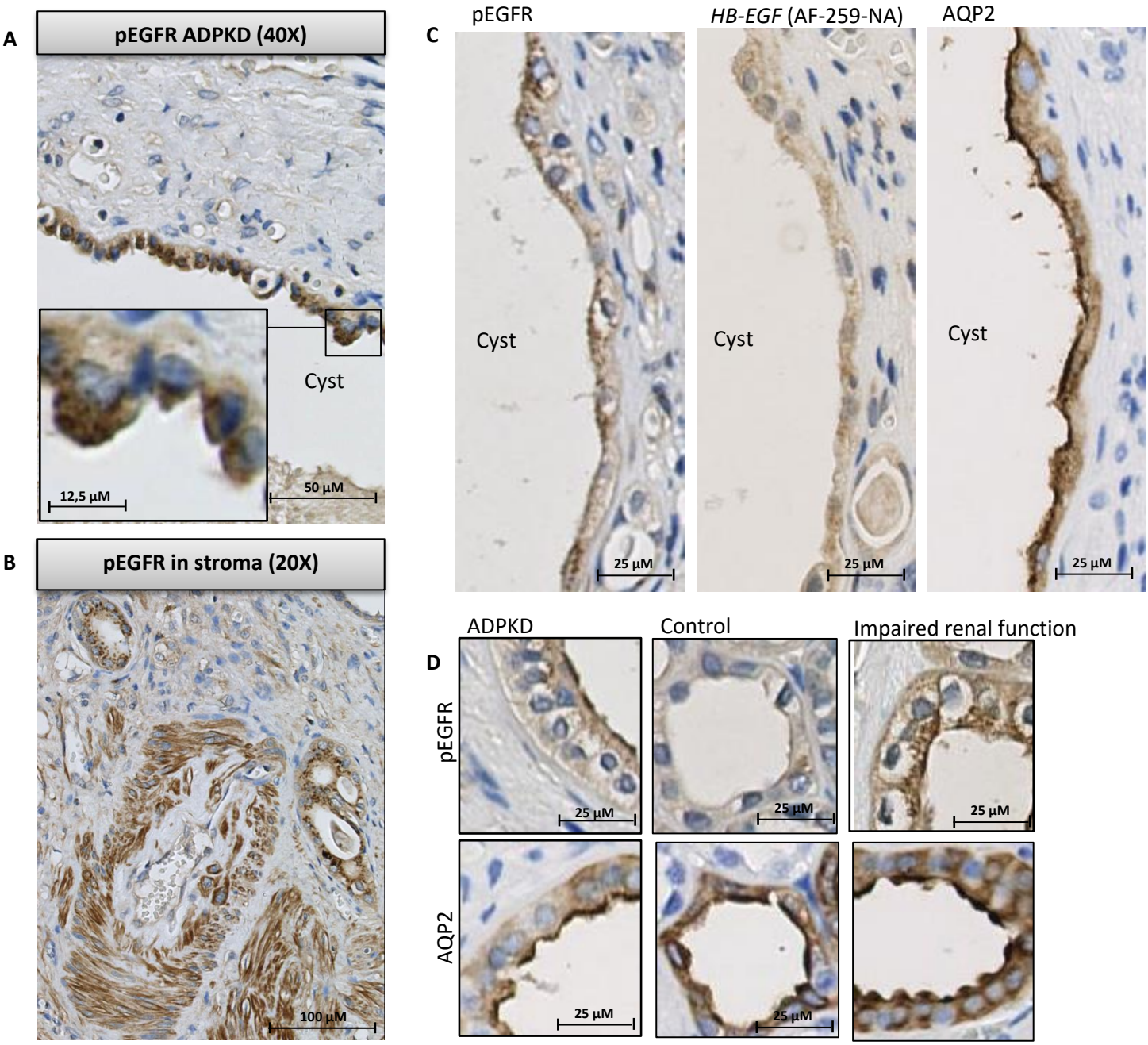


Figure 14: Activity of EGFR signalling in ADPKD. A: pEGFR is expressed in cyst lining epithelial cells. B: pEGFR is expressed in smooth muscle cells of blood vessels. C. Expression of pEGFR in cyst lining epithelial cells co-localize with expression of *HB-EGF*. Moreover, this cyst originates from the collecting duct because the cyst is positive for AQP2. D. pEGFR and AQP2 is expressed on the apical side of collecting ducts in ADPKD.

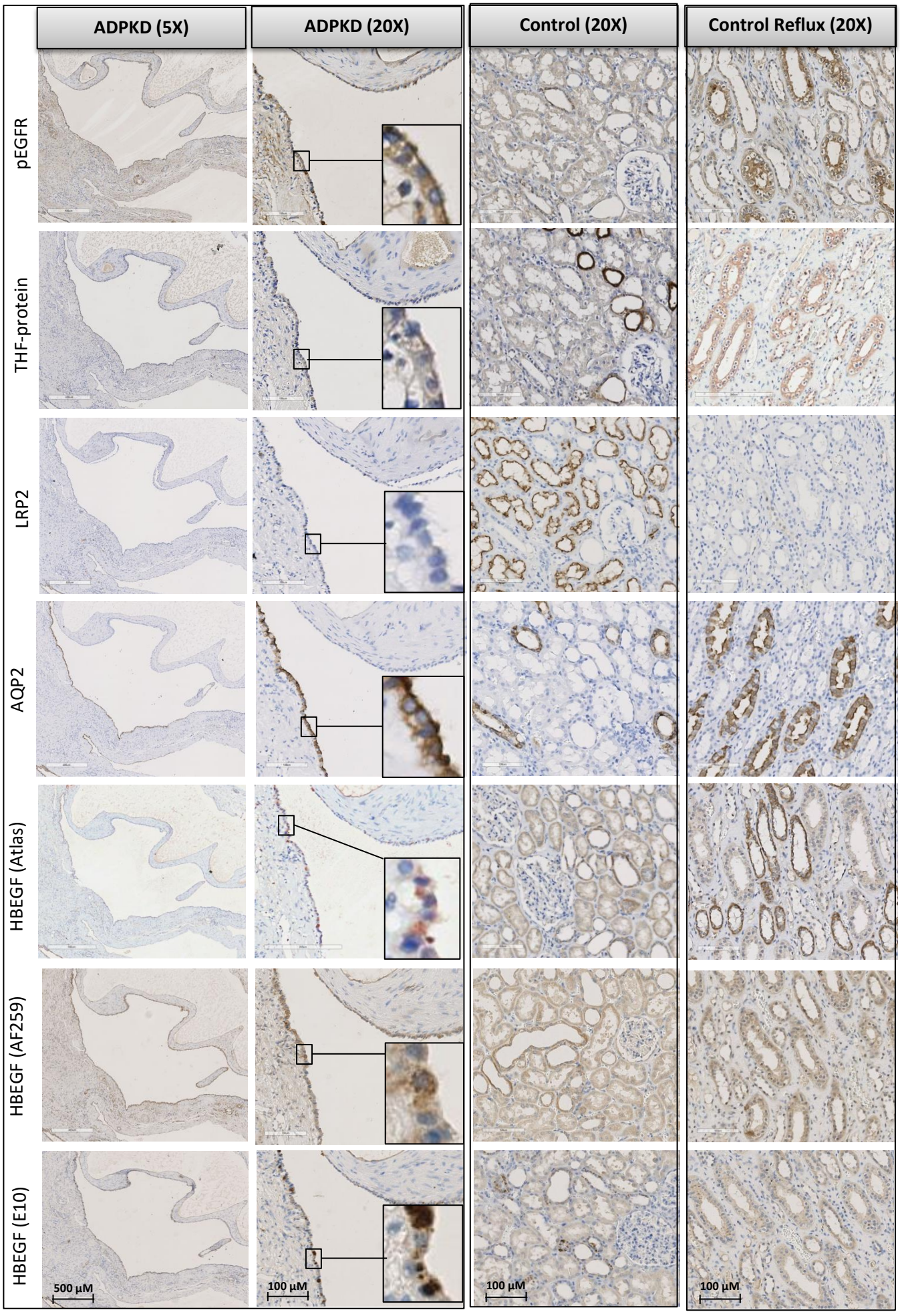


Figure 15: Overview of consecutive sections of human ADPKD tissues and controls stained with antibodies against pEGFR, THF-protein, LRP2, AQP2, *HB-EGF* atlas, *HB-EGF* AF-259 and *HB-EGF* E10 respectively. Images of the cysts in ADPKD were taken at similar regions within the tissue. The cyst lining epithelial cells stained positive for the pEGFR, AQP2 and *HB-EGF*.

3.7 No increase in ErbB receptor mRNA levels in ADPKD

To examine the quantity of the four ErbB receptors, renal tissues of ADPKD patients (n = 18) and controls with normal renal function (n = 4) were analyzed using quantitative RT-PCR. Relative mRNA levels are given as means ± SD compared to the housekeeping gene TBP. In ADPKD patients, mRNA levels of the different ErbB receptors were not significantly increased compared to controls. Moreover, mRNA levels of the ErbB2 receptor even were significantly decreased in ADPKD patients compared to controls (Figure 16).

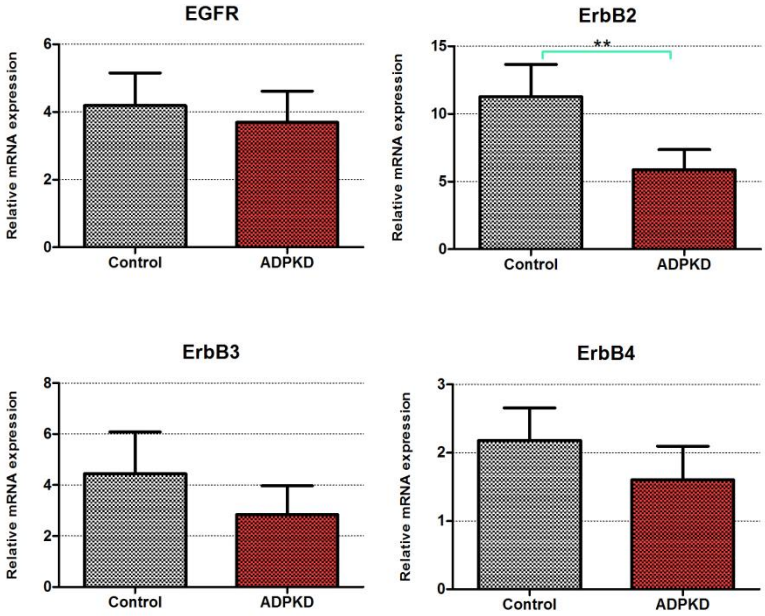


Figure 16: ErbB receptor mRNA levels in ADPKD patients compared to controls in human renal tissue. EGFR mRNA levels are similar in ADPKD patients and controls, whereas mRNA levels of ErbB2 were significantly decreased in ADPKD.

3.8 Changed pattern of EGFR ligand mRNA levels in ADPKD

In addition, we measured the mRNA levels of seven ErbB ligands with specificity to the EGFR and/or ErbB4 receptor. For the comparison of the mRNA expression of the ligands between ADPKD patients and controls, we refer to table 5. We found that the mRNA level of *HB-EGF* was higher in ADPKD compared to controls, although the difference did not reach formal statistical significance ($p>0.05$) (Figure 17A). In contrast, mRNA levels of *TGF-α* mRNA levels showed a minor decrease in ADPKD tissue compared to controls (figure 17B), while mRNA levels of *EGF* were almost absent in renal tissue of ADPKD patients compared to high *EGF* mRNA levels in control tissue (figure 17C), $p>0.05$ and $p<0.001$, respectively.

Interestingly, additional findings shown in appendix I revealed significantly increased mRNA levels of ErbB ligands *Amphiregulin*, *Epiregulin*, and *Epigen* in ADPKD patients compared to controls, $p<0.05$, $p<0.01$, $p<0.05$, respectively. Finally, ErbB ligand *Betacellulin* was significantly decreased in ADPKD patients versus controls ($p<0.01$).

Table 4: mRNA expression pattern of EGFR ligands in ADPKD compared to controls

EGFR specific ligands	Result	EGFR & ErbB4 ligands	Result
EGF	↓↓↓	*HB-EGF*	↑↑
TGF-α	↓	*Epiregulin*	↑
Amphiregulin	↑	*Betacellulin*	↓
Epigen	↑		
** ↓= downregulation in ADPKD, ↑ = upregulation in ADPKD*			

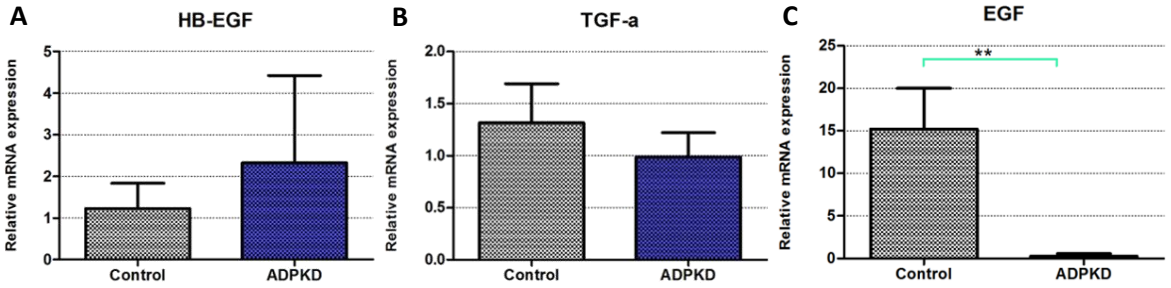


Figure 17: Relative mRNA levels of the EGFR ligands *HB-EGF*, *EGF* and *TGF-α* in ADPKD compared to control renal tissues. *HB-EGF* mRNA levels were increased in ADPKD, while a *EGF* was absent in ADPKD compared to controls. *TGF-α* levels showed a minor decrease in ADPKD ** p<0.01.

4 Discussion/conclusion

The Epidermal Growth Factor Receptor (EGFR) pathway is important for regulation of renal tubular cell proliferation in normal physiology. Autosomal Dominant Polycystic Kidney Disease (ADPKD) is characterized by unrestricted tubular cell proliferation, leading to cyst formation and growth. Activation of the EGFR pathway by binding with its ligands, results in activation of down-stream signaling pathways that cause proliferation. Given these considerations, we hypothesized that both the EGFR and its ligand, *HB-EGF*, could play a role in the pathophysiology of ADPKD. Therefore, in this project the role of EGFR signaling was examined in renal tissue of patients with ADPKD compared to controls.

Our study shows that the activated (phosphorylated) EGFR and *HB-EGF* were both highly expressed and co-localized in in cyst lining epithelial cells. As hypothesized, expression of the pEGFR and its ligand *HB-EGF* was higher in ADPKD compared to controls with normal kidney function. In ADPKD, expression of the EGFR was localized at the apical membrane of most cysts and collecting ducts, while *HB-EGF* co-localized with pEGFR positive cysts. In addition, mRNA levels of the EGFR were not increased in ADPKD, whereas mRNA levels of *HB-EGF* were found to be upregulated in ADPKD patients.

4.1 ErbB receptor signaling in PKD

We observed no expression of the pEGFR in control tissues derived from patients with normal kidney function. Staining which was present was regarded as aspecific, and therefore these controls can be classified as negative. These results are in contrast to those of other studies which showed that the EGFR was highly present in human renal control tissues localized to basolateral cytoplasmic membranes of epithelial cells. They found that the EGFR is expressed in the glomeruli [23] [25], distal tubules [23] [25], distal tubules [24] [23] [25] [67], proximal tubules [67], thick ascending limb of Henle [25], capillaries/arteriolar walls [25] and collecting ducts [24] [23]. There are three possible explanations for these differences. First, all studies described above detected the inactive form of the EGFR, while we detected the active pEGFR. Second, these studies are outdated, and methodology that was used was not optimal compared to present techniques for tissue handling and staining. For example improvements are made in protein conjugation, tissue fixation methods, detection labels and microscopy. Finally, these studies show other limitations. For instance *Yoshioka et al*. conducted a small study using only 2 control tissues. Furthermore, the study of *Nakopoulou et al.* showed positive staining in only 3 out of 10 control tissues, that were moreover derived from renal cancer or trauma patients .

In our study, compared to the controls with normal renal function, increased expression of the pEGFR was observed in ADPKD in the distal tubules, collecting ducts, smooth muscle cells and in cyst lining epithelial cells with apical membrane localization. This is in line with the studies mentioned above, except for the absence of pEGFR in glomeruli. Since the EGFR is detected in glomeruli by two outdated small scaled studies, the evidence regarding the existence of the EGFR in glomeruli is very doubtful. Furthermore, apical membrane localization of the pEGFR was detected in ADPKD, in line with other research. While basolateral membrane expression is reported to occur in controls. These observations suggest that localization and activities of the EGFR at specific polarized membranes of the tubular epithelial cell surface area are essential for cystogenesis. Mislocalization of the pEGFR in

ADPKD gives access to luminal EGFR ligands leading to increased EGFR signaling which might contribute to disease progression. The last decades various experimental studies have been published investigating the role of EGFR and its ligands in PKD models. In line with our findings, these studies describe increased activity of the EGFR in kidneys of an ARPKD mice model [50] and in human polycystic kidney cells [68]. Besides increased EGFR activity, the localization pattern in ARPKD mice [50] and in human ADPKD tissues was similar as in our study [52] [27].

As mentioned, we found that the pEGFR is expressed in cyst-lining epithelial cells. However, not all cysts were positive for the pEGFR. A possible explanation could be a distinct origin of pEGFR positive cysts. In general it is known that cysts are formed from kidney epithelial cells. Although, it is still unclear from which part of the nephron cysts develop. Cystogenesis starts with the proliferation of a single epithelial cell, leading to a cyst which is separated from the original kidney segment. Because most cysts are thought to be clones from one single cell, each cyst should be derived from a specific nephron segment. Our studied showed that the pEGFR positive cysts were derived predominantly from collecting ducts or from distal tubules/limb of Henle, since there was co-localization with kidney markers AQP2 and THF. Of some cysts the origin remained unidentified, since there was no co-localization with specific kidney markers. Other studies also state that cyst origin can be heterogenous. For example *Devuyst et al*, which showed that two third of the cysts expressed either AQP1 (marker for proximal tubules) or AQP2 (marker for collecting duct), whereas one third of the cysts remained of unknown origin [69]. Others, also found cysts derived from the collecting ducts, although many cysts remained unstained indicating a mixed origin of cysts as in our study [70] The fact that the origin of some cysts remained unidentified requires additional research with different nephron segmental markers. Most likely, cysts do not express similar nephron markers as in adult kidneys which could explain the unidentified origin throughout literature.

In addition, a current study of *Wilson et al.* suggested that specifically in ADPKD the EGFR will form a dimer with the Erbb2 receptor. These authors indeed showed that both the ErbB2 receptor as well as the EGFR were upregulated in ADPKD, while ErbB2 was absent in normal adult kidneys. Therefore, the forming of this dimer might be involved in disease progression instead of the EGFR solely [27]. In contrast, we found a significant downregulation of the ErbB2 relative mRNA levels in ADPKD. Different findings might be due to different methodology. We only identified ErbB2 by qRT-PCR, while *Wilson et al.* performed immunohistochemistry to identify both the EGFR and ErbB2 receptor in human renal tissue. Because only a limited number of studies are available regarding ErbB2 in ADPKD, additional research is needed to establish the exact role of ErbB2 in human ADPKD.

In contrast with increased expression of the pEGFR, we identified that mRNA levels of the EGFR were not increased in ADPKD patients which is in discrepancies with existing literature [68]. These differences might again be caused by differences in methodology. *Klingel et al.* used northern analysis which is an essentially different detection method compared to the method we used: qRT-PCR. The latter is described as being a more sensitive method. On the other hand, the technique of qRT-PCR is only able to identify total mRNA quantity of the receptors, which is unrelated to EGFR phosphorylation. Therefore mRNA levels will provide no information about the actual activity of the receptors. Similar EGFR mRNA expression could still result in higher EGFR activity due to the presence of EGFR ligands. Therefore, our finding that EGFR mRNA levels were not affected in ADPKD might suggest that increased activity of the EGFR is not due to an increase in the amount of individual EGFRs but this is rather caused by a change in EGFR ligands.

4.2 EGFR ligands in PKD

EGFR ligands can be produced within the renal tissue itself or they can be plasma-derived. Measuring EGFR ligands in plasma and urine of patients therefore reflects the activity of EGFR signaling. Our previous study in ADPKD patients found that urinary *HB-EGF* concentrations positively correlated with ADPKD disease severity, while this was not observed in plasma [65]. Furthermore, compelling evidence showed that the EGFR ligand *EGF* is a local intrarenal production. Since, these results suggest that EGFR ligands might be locally produced in the kidney and reflect the activity of the EGFR pathway, we identified the expression of EGFR ligand *HB-EGF* in human renal tissue.

We found a diffuse cytoplasmic staining pattern of *HB-EGF* in tissue of controls with normal kidney function, localized to the distal and proximal tubuli. This distribution pattern of *HB-EGF* throughout the kidney was also reported by other human studies, in which *HB-EGF* expression was also detected in distal and proximal tubules [30] [31] [32]. Increased *HB-EGF* staining was observed in ADPKD patients compared to controls with normal kidney function. Expression of *HB-EGF* in ADPKD patients localized predominantly in the cytoplasm of distal tubules and cyst lining epithelial cells and *HB-EGF* was also expressed in proximal tubules. Experimental data in a mice model for PKD also showed accelerated disease progression by *HB-EGF* induced EGFR signaling [45]. Our findings of *HB-EGF* expression in cyst lining epithelial cells are in line with our previous human study which determined increased urinary *HB-EGF* excretion in ADPKD which correlated with disease severity [65]. To our knowledge, other publications regarding *HB-EGF* in ADPKD patients are lacking.

In renal tissue, *HB-EGF* is described as being a more potent mitogen than *EGF* [71] [72]. Furthermore, *HB-EGF* was reported as being the major ligand for the EGFR and not *EGF* in renal cysts from a *Pkd1* mice model of ADPKD [73]. This is in line with our finding that relative *EGF* mRNA levels were almost completely absent in ADPKD tissues. Recently, it was reported that mRNA *EGF* levels were decreased in other chronic kidney diseases and can be used as a biomarker to predict renal function decline. In this study low *EGF* was associated with impaired renal function, in line with the findings in the ADPKD patients in our study [66]. Together, these studies indicate that *HB-EGF* and not *EGF* might play an important role in activation of the EGFR contributing to ADPKD progression.

Although our focus lays mainly on *HB-EGF* as player in cystogenesis, the change in mRNA expression of other EGFR ligands is interesting. We found that mRNA levels of some of the other EGFR ligands (*Amphiregulin*, *Epigen* and *Epiregulin*) were also upregulated in ADPKD, whereas *Betacellulin* and *EGF* were downregulated. Upregulation of *Amphiregulin* is also reported as potential ligand which enhances PKD progression [47] [48] [49] [50]. Therefore, *Amphiregulin* might also play a role in EGFR activation and disease progression. To our knowledge, no studies are performed in ADPKD patients regarding *Epigen* and *Epiregulin*. Therefore, the role of these ligands in ADPKD is still unknown. Moreover, the mRNA levels of *Epigen*, *Amphiregulin* and *Epiregulin* in ADPKD patients lay around 0,5, while the levels of *HB-EGF* lay around 4. This might imply that *HB-EGF* is more important compared to the other ligands. Although, the exact contribution of other EGFR ligands in human ADPKD is yet unknown and still needs to be investigated.

4.3 *HB-EGF* in ADPKD and other chronic kidney diseases

In line with other research, our results showed apical localization of the pEGFR in ADPKD patients. However, we also found apical pEGFR localization in controls with impaired renal function, in contrast to literature. Furthermore, these controls also had upregulated expression of the pEGFR

and *HB-EGF*. These findings suggest that increased EGFR signaling may be a more common pathway associated with tissue repair, inflammation and fibrosis in chronic kidney diseases.

Various types of kidney diseases already have been associated with increased EGFR signalling [40] [30] [41] [42]. Obviously, chronic kidney disease causes tissue damage which sequentially will activate repair pathways including EGFR signalling. Within the kidney, *EGF* and *HB-EGF* both play a role in tissue regeneration and repair by activation of the EGFR [74]. Renal repair is meant to be beneficial. However, chronic kidney damage induces prolonged phosphorylation of the EGFR which is known to accelerate disease. Moreover, more macrophages are attracted to the site of tissue damage which might produce increased amounts of *HB-EGF*. *HB-EGF* in turn activates more EGFRs which in turn promote fibrosis as a vicious circle.

Figure 18 presents a possible mechanism for the role of *HB-EGF* in ADPKD with tissue repair as initial player. Tissue repair has been shown to accelerate cystogenesis, because repair requires tubular cell proliferation [75]. First, tissue injury might accelerate cyst initiation by EGFR signaling. Second, newly formed cysts cause additional local injury which may activate the EGFR and in turn trigger new cyst formation. Third, recruitment of macrophages to the site of injury is reported to accelerate disease progression in human PKD [76] [77], while macrophage depletion in mouse models of PKD attenuates cystic disease. One reason why macrophages accelerate PKD could be because of increased production of *HB-EGF*, since it is known that macrophages or monocytes produce *HB-EGF*. Together, these studies provide evidence that exaggerated tissue repair by *HB-EGF*-EGFR signaling is detrimental in ADPKD.

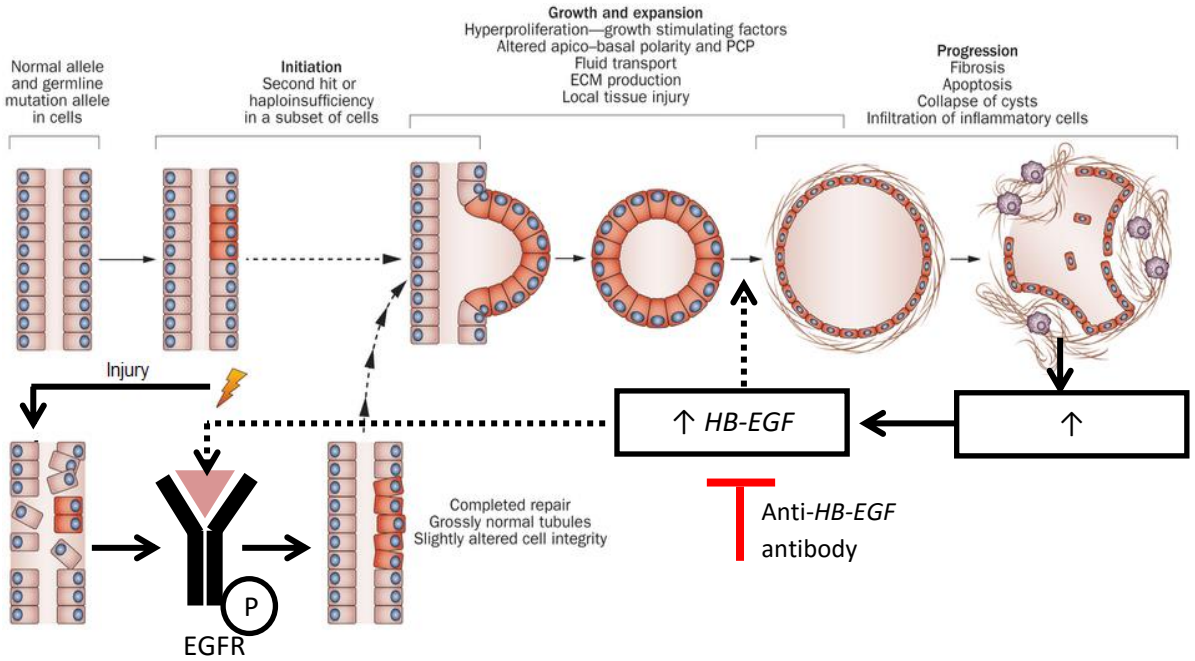


Figure 18: Possible role for *HB-EGF* in the pathophysiology of ADPKD. Presence of a PKD1 or PKD2 mutation makes cells prone to form cyst. Actual initiation of cyst formation might be triggered by renal injury. Injury leads to activation of the EGFR which result in repair. In ADPKD repair might result in newly formed cells with altered cell integrity. Growth of one cyst will cause additional local injury which activates additional repair and might trigger new cyst formation. Macrophages are recruited at the sites of injury. These cells probably produce more *HB-EGF* which is in turn able to activate EGFR signaling and thereby stimulating cyst formation and cyst growth. Therefore, neutralizing *HB-EGF* antibodies might be a promising future therapy in ADPKD. Figure adapted from [75].

4.4 Limitations and strengths of this study

Some limitations of this study need to be addressed. First, renal tissues were derived from ADPKD patients with end stage renal disease. Therefore, all patients have impaired renal function. It is known that cysts will grow constantly with different growth rates ranging from 2–70% per year. Since it is suggested that EGFR signaling stimulates cyst growth, the pEGFR should be detectable especially in cysts with high growth rates. However, in the ADPKD tissue we used cysts may be in different growth stages, and most might actually be in low a growth rate, which might explain why not all cysts showed positivity for the pEGFR. Unfortunately, a distinction between cysts with high or low growth rates cannot be made based on the method we used. Future studies might possibly investigate this issue. Second, qRT-PCR determined mRNA levels in total kidney tissue including fibrotic and inflammatory areas varied with regions of normal-like renal tissue in ADPKD, while they were compared to normal tissue of controls. Therefore, total mRNA also includes mRNA expressed by fibroblasts and cannot detect the exact mRNA levels present solely in the cysts. Moreover, the size and quantity of cysts present in a tissue piece derived from ADPKD patients varies, despite tissue selection. This might be a possible explanation for the high variation found in *HB-EGF* mRNA levels between ADPKD patients. In the future, using laser guided tissue microdissection might solve this problem. Third, *HB-EGF* specifically binds to both EGFR as well as the ErbB4 receptor. Therefore, ADPKD disease progression might also be due to increased *HB-EGF*-ErbB4 signaling instead of *HB-EGF*-EGFR signaling or a combination of both. The first possibility might be less likely, because ErbB4 knockout mice actually showed an accelerated cyst progression and renal function deterioration. However, knowledge regarding the role of ErbB4 in human ADPKD is lacking. In the future, determination of the expression of the ErbB4 receptor in human ADPKD tissue might shed light on this issue.

This study has also strengths that include amongst others the use of an additional control group with renal tissues from patients with impaired renal function. Using this control group allowed us to distinguish whether differences are ADPKD specific or caused by renal failure. Moreover, tissue derived from 19 ADPKD patients and 17 controls were included in the study. The power of this study is therefore better than that of previous studies, as reasoned above. Finally, three independent antibodies gave comparable staining results regarding *HB-EGF* localization. One of the antibodies gave an interesting course-grained staining pattern of *HB-EGF* in line with another study [33], while the other two antibodies showed a cytoplasmic staining pattern, which is in line with the expected distribution of a growth factor within the renal tissue and also observed in other studies [32] [31].

4.5 Conclusions

Both the phosphorylated EGFR and *HB-EGF* were highly expressed in renal tissues derived from ADPKD patients compared to controls. Whereas in ADPKD tissues the pEGFR was expressed in cyst-lining epithelial cells and collecting ducts, in controls with normal renal function the pEGFR was not expressed. Furthermore, pEGFR expression localized at the cytoplasm and apical membranes of cysts. Moreover, pEGFR expression co-localized with cytoplasmic expression of *HB-EGF*. Since pEGFR and *HB-EGF* were also upregulated in control tissues with impaired renal function, increased EGFR signaling via *HB-EGF* might be mediated by tissue repair and this may contribute to disease progression in ADPKD.

5 Future perspective

This project gives rise to many unanswered questions. For instance, what is the exact percentage of cysts positive for the pEGFR and *HB-EGF* and the ratio of cyst origin? What is the role of *Epigen*, *Epiregulin* and *Amphiregulin* which were also found to be upregulated in ADPKD? Is the ErbB4 receptor also involved in ADPKD disease progression? What will be the effect of *HB-EGF* inhibition in ADPKD? Additional research is required to answer these questions. Ideas for additional experiments are listed below.

1. Our current stains should be analyzed in detail to determine the exact percentage of pEGFR/*HB-EGF* positive cysts and the expression level should be scored. For example, by giving different numbers to the staining observed in each cyst wherein 0 = negative, 1=weakly positive and 2= strong positive. Moreover, cyst origin should be determined wherein the fraction of cysts derived from distal tubules, proximal tubules, collecting ducts and unknown origin should be given.

2. Determination of pErbB4 stains in human ADPKD tissues compared to controls with normal and impaired renal function should be performed to determine receptor localization. Moreover co-localization of pErbB4 with *HB-EGF* should be determined to see whether *HB-EGF* is predominantly signaling by the EGFR, or ErbB4 receptor or by regulating both receptors in ADPKD.

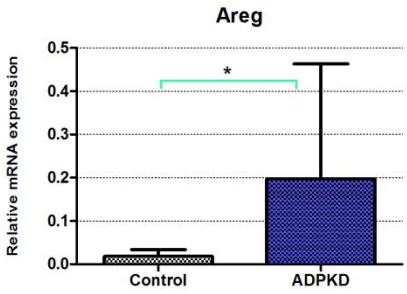
3. More evidence of increased phosphorylation of the EGFR and ErbB4 can be collected by measuring protein levels of the pEGFR and pErbB4 by Western blot analysis which is a better method to measure quantity of a phosphorylated receptor compared to immunohistochemistry.

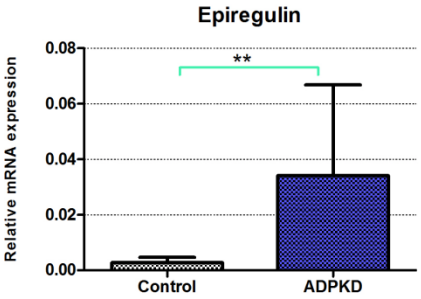
4. Urinary excretion of *Amphireglulin*, *Epiregulin*, *Betacellulin* and *Epigen* could be determined in patients with ADPKD, in healthy controls and in patients with other chronic kidney diseases by ELISA. Furthermore, expression of these ligands in human ADPKD tissues can be determined via immunohistochemistry.

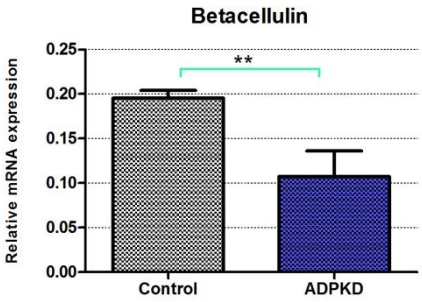
5. A culture system of human cystic cells can be used for future research to study the role of all EGFR ligands in ADPKD. EGFR ligands can be added to the culture medium individually or in combination to see the effect on cyst growth. In contrast, EGFR inhibitors or inhibitory antibodies directed to one or multiple EGFR ligands can be added to examine the effect on cyst growth. This system could also be used to look at the other ErbB receptors.

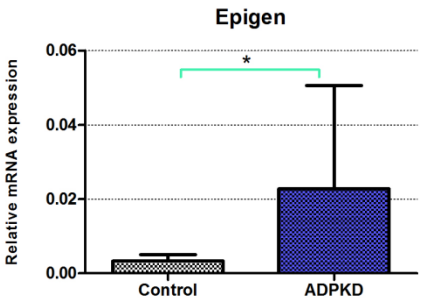
6. Finally, ADPKD transgenic mice models which lack or overexpress *HB-EGF* can be used to study the effect of *HB-EGF* on ADPKD pathogenesis.

Appendix I: qRT-PCR









6 References

[1] Rizk D, Chapman AB: Cystic and inherited kidney diseases, Am J Kidney Dis. 2003. 42(6):1305-1317.

[2] Chebib FT, Torres VE.Autosomal Dominant Polycystic Kidney Disease: Core Curriculum 2016, Am J Kidney Dis. 2016 May;67(5):792-810.

[3] Ong AC, Devuyst O, Knebelmann B, Walz G; Diseases, ERA-EDTA Working Group for Inherited Kidney. Autosomal dominant polycystic kidney disease: the changing face of clinical management, Lancet. 2015 May 16;385(9981):1993-2002.

[4] Neumann HP,et al. Epidemiology of autosomal-dominant polycystic kidney disease: an in-depth clinical study for southWestern Germany., Nephrol Dial Transplant 2013 Jun;28(6):1472-1487..

[5] Spithoven EM, Kramer A, Meijer E, Gansevoort RT et al.Renal replacement therapy for autosomal dominant polycystic kidney disease (ADPKD) in Europe: prevalence and survival--an analysis of data from the ERA-EDTA Registry, Nephrol Dial Transplant. 2014 Sep;29 Suppl 4:iv15-25.

[6] Torres VE, Harris PC, Pirson Y.Autosomal dominant polycystic kidney disease, Lancet. 2007 Apr 14;369(9569):1287-30.

[7] Alberts B, Johnson A, Lewis J, Raff M et al. Molecular biology of the cell. Chapter 15: Mechanisms of cell communication. 2008. Garland Science 5th edition.

[8] a. Chapman A.B, Rahbari-Oskoui F, Bennett W. Course and treatment of autosomal dominant polycystic kidney disease. UpToDate.

b. Meijer E, Drenth JP, d'Agnolo H, Casteleijn NF et al. Rationale and design of the DIPAK 1 study: a randomized controlled clinical trial assessing the efficacy of lanreotide to Halt disease progression in autosomal dominant polycystic kidney disease, Am J Kidney Dis. 2014 Mar;63(3):446-55.

[9] Torres VE, Chapman AB, Devuyst O, Gansevoort RT, Grantham JJ, Higashihara E, Perrone RD, Krasa HB, Ouyang J, Czerwiec FS; TEMPO 3:4 Trial Investigators. Tolvaptan in patients with autosomal dominant polycystic kidney disease, N Engl J Med. 2012 Dec 20;367(25):2407-18.

[10] Torres VE, Higashihara E, Devuyst O, Chapman AB, Gansevoort RT, Grantham JJ, Perrone RD, Ouyang J, Blais JD, Czerwiec FS.Effect of Tolvaptan in Autosomal Dominant Polycystic Kidney Disease by CKD Stage: Results from the TEMPO 3:4 Trial, Clin J Am Soc Nephrol. 2016 Feb 23.

[11] Baur BP, Meaney CJ.Review of tolvaptan for autosomal dominant polycystic kidney disease, Pharmacotherapy. 2014 Jun;34(6):605-16.

[12] Shillingford JM, Murcia NS, Larson CH, et al. The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease, Proc Natl Acad Sci U S A 2006;103:5466–71.

[13] Zafar I, Belibi FA, He Z, et al. Long-term rapamycin therapy in the Han:SPRD rat model of polycystic kidney disease (PKD), Nephrol Dial Transplant 2009;24:2349–53. 92.

[14] Wahl PR, Serra AL, Le Hir M, et al. Inhibition of mTOR with sirolimus slows disease progression in Han:SPRD rats with autosomal dominant polycystic kidney disease (ADPKD), Nephrol Dial Transplant 2006;21:598–604.

[15] Canaud G, Knebelmann B, Harris PC, et al. Therapeutic mTOR inhibition in autosomal dominant polycystic kidney disease: what is the appropriate serum level?, Am J Transplant 2010;10: 1701–6.

[16] Mohieldin AM, Upadhyay VS, Ong ACM and Nauli SM. Autosomal Dominant PolycysticKidney Disease: Pathophysiology and Treatment, 2013 Nova Science Publishers.ISBN: 978-1-62808-760-4.

[17] Melenhorst WB, Mulder GM, Xi Q, Hoenderop JG, Kimura K, Eguchi S, van Goor H. Epidermal growth factor receptor signaling in the kidney: key roles in physiology and disease, Hypertension. 2008 Dec;52(6):987-93.

[18] Harskamp L.R, Gansevoort R.T,Meijer E. The Epidermal Growth Factor Receptor Pathway in chronic kidney diseases, Nature nephrology 2016 (article in press).

[19] Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling, Mol Syst Biol. 2005;1:2005.0010.

[20] Zeng F, Singh AB, Harris RC. The role of the EGF family of ligands and receptors in renal development, physiology and pathophysiology, Exp Cell Res. 2009 Feb 15;315(4):602-10.

[21] Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, et al.Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype, Science. 1995 Jul 14;269(5221):230-4.

[22] Sakurai H, Tsukamoto T, Kjelsberg CA, Cantley LG, Nigam SK.EGF receptor ligands are a large fraction of in vitro branching morphogens secreted by embryonic kidney, Am J Physiol. 1997 Sep;273(3 Pt 2):F463-72.

[23] Yoshioka K, Takemura T, Murakami K, Akano N, Matsubara K, Aya N, Maki S. Identification and localization of epidermal growth factor and its receptor in the human glomerulus, Lab Invest. 1990 Aug;63(2):189-96.

[24] Nakopoulou L, Stefanaki K, Boletis J, Papadakis J, Kostakis A, Vosnides G, Zeis PM. Immunohistochemical study of epidermal growth factor receptor (EGFR) in various types of renal injury, Nephrol Dial Transplant. 1994;9(7):764-9.

[25] Gesualdo L, Di Paolo S, Calabró A, Milani S, Maiorano E, Ranieri E, Pannarale G, Schena FP. Expression of epidermal growth factor and its receptor in normal and diseased human kidney: an immunohistochemical and in situ hybridization study, Kidney Int. 1996 Mar;49(3):656-65.

[26] Press MF, Cordon-Cardo C, Slamon DJ: Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues, Oncogene 5(7):953-962, 1990.

[27] Wilson SJ, Amsler K, Hyink DP, Li X, Lu W, Zhou J, Burrow CR, Wilson PD. Inhibition of HER-2(neu/ErbB2) restores normal function and structure to polycystic kidney disease (PKD) epithelia, Biochim Biophys Acta. 2006 Jul;1762(7):647-55.

[28] Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ: Expression of the c-erbB-3 protein in normal human adult and fetal tissues, Oncogene 7(7):1273-1278, 1992.

[29] Srinivasan R, Poulsom R, Hurst HC, Gullick WJ: Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumour types, J Pathol 185(3):236-245, 1998.

[30] Bollée G, Flamant M, Schordan S, Callard P, Endlich N, Tharaux P.L et al. The Epidermal Growth Factor Receptor Promotes Glomerular Injury and Renal Failure in Rapidly Progressive Crescentic Glomerulonephritis, Nat Med. 2011 Oct; 17(10): 1242–1250.

[31] Mulder GM, Nijboer WN, Seelen MA, Sandovici M, Bos EM, Melenhorst WB, Trzpis M, Kloosterhuis NJ, Visser L, Henning RH, Leuvenink HG, Ploeg RJ, Sunnarborg SW, van Goor H. Heparin binding epidermal growth factor in renal ischaemia/reperfusion injury, J Pathol. 2010 Jun;221(2):183-92.

[32] Nakamura Y, Handa K, Iwamoto R, Tsukamoto T, Takahasi M, Mekada E. Immunohistochemical distribution of CD9, heparin binding epidermal growth factor-like growth factor, and integrin alpha3beta1 in normal human tissues, J Histochem Cytochem. 2001 Apr;49(4):439-44.

[33] Homma T, Sakai M, Cheng HF, Yasuda T, Coffey RJ Jr, Harris RC. Induction of heparin-binding epidermal growth factor-like growth factor mRNA in rat kidney after acute injury, J Clin Invest. 1995 Aug;96(2):1018-25.

[34] Kirkland G, Paizis K, Wu LL, Katerelos M, Power DA.Heparin-binding EGF-like growth factor mRNA is upregulated in the peri-infarct region of the remnant kidney model: in vitro evidence suggests a regulatory role in myofibroblast transformation., J Am Soc Nephrol. 1998 Aug;9(8):1464-73.

[35] Nguyen HT, Bride SH, Badawy AB, Adam RM, Lin J, Orsola A, Guthrie PD, Freeman MR, Peters CA. Heparin-binding EGF-like growth factor is up-regulated in the obstructed kidney in a cell- and region-specific manner and acts to inhibit apoptosis, Am J Pathol. 2000 Mar;156(3):889-98.

[36] Pavlov TS, Levchenko V, O'Connor PM, Ilatovskaya DV, Palygin O, Mori T, Mattson DL, Sorokin A, Lombard JH, Cowley AW Jr, Staruschenko A. Deficiency of renal cortical EGF increases ENaC activity and contributes to salt-sensitive hypertension, J Am Soc Nephrol. 2013 Jun;24(7):1053-62.

[37] Ikari A, Sanada A, Okude C, Sawada H, Yamazaki Y, Sugatani J, Miwa M.Up-regulation of TRPM6 transcriptional activity by AP-1 in renal epithelial cells, J Cell Physiol. 2010 Mar;222(3):481-7.

[38] Dimke H, van der Wijst J, Alexander TR, Meijer IM, Mulder GM, van Goor H, Tejpar S, Hoenderop JG, Bindels RJ. Effects of the EGFR Inhibitor Erlotinib on Magnesium Handling, J Am Soc Nephrol. 2010 Aug;21(8):1309-16.

[39] Tejpar S, Piessevaux H, Claes K, Piront P, Hoenderop JGJ, Verslype C, Van Cutsem E. Magnesium wasting associated with epidermal-growth-factor receptor-targeting antibodies in colorectal cancer: a prospective study, Lancet Oncol. 2007;8:387–394.

[40] Minner S, Rump D, Tennstedt P et al. Epidermal growth factor receptor protein expression and genomic alterations in renal cell carcinoma, Cancer. 2012 Mar 1;118(5):1268-75.

[41] Wassef L, Kelly DJ, Gilbert RE.Epidermal growth factor receptor inhibition attenuates early kidney enlargement in experimental diabetes, Kidney Int. 2004 Nov;66(5):1805-14.

[42] Sis B, Sarioglu S, Celik A, Zeybel M, Soylu A, Bora S. Epidermal growth factor receptor expression in human renal allograft biopsies: an immunohistochemical study, Transpl Immunol. 2004 Nov;13(3):229-32.

[43] Lowden DA, Lindemann GW, Merlino G, Barash BD, Calvet JP, Gattone VH 2nd. Renal cysts in transgenic mice expressing transforming growth factor-alpha, J Lab Clin Med. 1994 Sep;124(3):386-94.

[44] Neufeld TK, Douglass D, Grant M, Ye M, Silva F, Nadasdy T, Grantham JJ. In vitro formation and expansion of cysts derived from human renal cortex epithelial cells, Kidney Int. 1992 May;41(5):1222-36.

[45] MacRae Dell K, Nemo R, Sweeney WE Jr, Avner ED. EGF-related growth factors in the pathogenesis of murine ARPKD, Kidney Int. 2004 Jun;65(6):2018-29.

[46] Aguiari G, Bizzarri F, Bonon A,et al. Polycystin-1 regulates *Amphiregulin* expression through CREB and AP1 signaling: implications in ADPKD cell proliferation, J Mol Med (Berl). 2012 Nov;90(11):1267-82.

[47] Richards W.G, Sweeney W.E, Yoder B.K, Wilkinson J.E, Woychik R.P and Avner E.D. Epidermal growth factor receptor activity mediates renal cyst formation in polycystic kidney disease, J Clin Invest. 1998 Mar 1; 101(5): 935–939.

[48] Avner, E.D., and W.E. Sweeney. Apical epidermal growth factor receptor expression defines a distinct cystic tubular epithelial phenotype in autosomal, Pediatric Res. 1995. 37:359A .

[49] Wilson, P.D., J. Du, and J.T. Norman. Autocrine, endocrine and paracrine regulation of growth abnormalities in autosomal dominant polycystic kidney disease, Eur. J. Cell Biol. 1993. 61:131–138.

[50] Orellana, S.A., W.E. Sweeney, C.D. Neff, and E.D. Avner. Epidermal growth factor receptor expression is abnormal in murine polycystic kidney, Kidney Int. 1995. 47:490–499.

[51] Singh B, Coffey RJ.Trafficking of epidermal growth factor receptor ligands in polarized epithelial cells, Annu Rev Physiol. 2014;76:275-300.

[52] Du J, Wilson PD.Abnormal polarization of EGF receptors and autocrine stimulation of cyst epithelial growth in human ADPKD, Am J Physiol. 1995 Aug;269(2 Pt 1):C487-95.

[53] Veikkolainen V, Naillat F, Railo A, Chi L, Manninen A, Hohenstein P, Hastie N, Vainio S, Elenius K. ErbB4 modulates tubular cell polarity and lumen diameter during kidney development, J Am Soc Nephrol. 2012 Jan;23(1):112-22.

[54] Zeng F, Miyazawa T, Kloepfer LA, Harris RC. Deletion of ErbB4 accelerates polycystic kidney disease progression in cpk mice, Kidney Int. 2014 Sep;86(3):538-47.

[55] Gattone VH 2nd, Kuenstler KA, Lindemann GW, Lu X, Cowley BD Jr, Rankin CA, Calvet JP. Renal expression of a transforming growth factor-alpha transgene accelerates the progression of inherited, slowly progressive polycystic kidney disease in the mouse, J Lab Clin Med. 1996 Feb;127(2):214-22.

[56] Nemo R, Murcia N, Dell KM. Transforming growth factor alpha (TGF-alpha) and other targets of tumor necrosis factor-alpha converting enzyme (TACE) in murine polycystic kidney disease, Pediatr Res. 2005 May;57(5 Pt 1):732-7. Epub 2005 Mar 17.

[57] Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC.Targeted inactivation of the EGF and *Amphiregulin* genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development, Development. 1999 Jun;126(12):2739-50.

[58] Sweeney WE, Futey L, Frost P, Avner ED. In vitro modulation of cyst formation by a novel tyrosine kinase inhibitor, Kidney Int. 1999 Aug;56(2):406-13.

[59] Pugh JL, Sweeney WE Jr, Avner ED.Tyrosine kinase activity of the EGF receptor in murine metanephric organ culture, Kidney Int. 1995 Mar;47(3):774-81.

[60] Sweeney WE, Chen Y, Nakanishi K, Frost P, Avner ED. Treatment of polycystic kidney disease with a novel tyrosine kinase inhibitor, Kidney Int. 2000 Jan;57(1):33-40.

[61] Torres VE, Sweeney WE Jr, Wang X, Qian Q, Harris PC, Frost P, Avner ED. EGF receptor tyrosine kinase inhibition attenuates the development of PKD in Han:SPRD rats, Kidney Int. 2003 Nov;64(5):1573-9.

[62] Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy, Pharmacol Ther. 2004 Apr;102(1):37-46.

[63] Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J.Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast

cancer cells, Cancer Res. 2001 Jun 15;61(12):4744-9.

[64] Dutta PR, Maity A.Cellular responses to EGFR inhibitors and their relevance to cancer therapy, Cancer Lett. 2007 Sep 8;254(2):165-77.

[65] Harskamp LR, Gansevoort RT, Boertien WE, van Oeveren W, Engels GE, van Goor H, Meijer E. Urinary EGF Receptor Ligand Excretion in Patients with Autosomal Dominant Polycystic Kidney Disease and Response to Tolvaptan, Clin J Am Soc Nephrol. 2015 Oct 7;10(10):1749-56.

[66] Ju W, Nair V, Smith S, Zhu L, Shedden K, Song PX, Mariani LH, Eichinger FH, Berthier CC, Randolph A, Lai JY, Zhou Y, Hawkins JJ, Bitzer M et al. Tissue transcriptome-driven identification of epidermal growth factor as a chronic kidney disease biomarker, Sci Transl Med. 2015 Dec 2;7(316):316ra193.

[67] Thomasson M, Hedman H, Guo D, Ljungberg B, Henriksson R.LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT--PCR and immunohistochemical analysis, Br J Cancer. 2003 Oct 6;89(7):1285-9.

[68] Klingel R, Dippold W, Störkel S, Meyer zum Büschenfelde KH, Köhler H. Expression of differentiation antigens and growth-related genes in normal kidney, autosomal dominant polycystic kidney disease, and renal cell carcinoma, Am J Kidney Dis. 1992 Jan;19(1):22-30.

[69] Devuyst O, Burrow CR, Smith BL, Agre P, Knepper MA, Wilson PD.Expression of aquaporins-1 and -2 during nephrogenesis and in autosomal dominant polycystic kidney disease, Am J Physiol. 1996 Jul;271(1 Pt 2):F169-83.

[70] Verani RR, Silva FG.Histogenesis of the renal cysts in adult (autosomal dominant) polycystic kidney disease: a histochemical study, Mod Pathol. 1988 Nov;1(6):457-63.

[71] Horikoshi S, Kubota S, Martin GR, Yamada Y, Klotman PE.Epidermal growth factor (EGF) expression in the congenital polycystic mouse kidney., Kidney Int. 1991 Jan;39(1):57-62.

[72] Aplin AE, Howe A, Alahari SK, Juliano RL.Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins, Pharmacol Rev. 1998 Jun;50(2):197-263.

[73] Jiang ST, Chiou YY, Wang E, Lin HK, Lin YT, Chi YC, Wang CK, Tang MJ, Li H. Defining a link with autosomal-dominant polycystic kidney disease in mice with congenitally low expression of Pkd1., Am J Pathol. 2006 Jan;168(1):205-20.

[74] Tang J, Liu N, Zhuang S. Role of epidermal growth factor receptor in acute and chronic kidney injury., Kidney Int. 2013 May;83(5):804-10.

[75] Happé H, Peters DJ.Translational research in ADPKD: lessons from animal models, Nat Rev Nephrol. 2014 Oct;10(10):587-601.

[76] Swenson-Fields KI, Vivian CJ, Salah SM, Peda JD, Davis BM, van Rooijen N, Wallace DP, Fields TA.Macrophages promote polycystic kidney disease progression, Kidney Int. 2013 May;83(5):855-64.

[77] Karihaloo A, Koraishy F, Huen SC, Lee Y, Merrick D, Caplan MJ, Somlo S, Cantley LG.Macrophages promote cyst growth in polycystic kidney disease, J Am Soc Nephrol. 2011 Oct;22(10):1809-14.

[78] Laplante M, Sabatini DM. mTOR signaling in growth control and disease, Cell. 2012 Apr 13;149(2):274-93.

[79] LaRiviere WB, Irazabal MV, Torres VE. Novel therapeutic approaches to autosomal dominant polycystic kidney disease, Transl Res. 2015 Apr;165(4):488-98.

[80] Hwang JH, Park HC, Jeong JC, Ha Baek S, Han MY, Bang K, Cho JY, Yu SH, Yang J, Oh KH, Hwang YH, Ahn C. Chronic asymptomatic pyuria precedes overt urinary tract infection and deterioration of renal function in autosomal dominant polycystic kidney disease, BMC Nephrol. 2013 Jan 7;14:1.