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Phänotypische Auswirkungen der ClC-Kb^{T481S} Mutation

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard-Karls-Universität
zu Tübingen

vorgelegt von
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aus
Tübingen

2010

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Meinen lieben Eltern

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1 General Introduction

The first part of this doctoral thesis gives a general introduction into chloride channels of the ClC-family and their physiological and pathophysiological relevance.

The second part consists of a study which has been conducted to show the influence of the ClC-Kb^{T481S} mutation on hearing thresholds. This part of the research project has been published in a slightly modified version in the scientific journal of hearing research:

Frey A, Lampert A, Waldegger S, Jeck N*, et al.* Influence of gain of function epithelial chloride channel ClC-Kb mutation on hearing thresholds. *Hear Res* 2006; 214: 68-75.

The third part presents additional findings based on the investigated population. At the end a general summary in English and German versions can be found, bringing together the most important findings from both parts of this doctoral thesis. Since many quotations of the different parts are redundant, one common reference list is given at the end of the document.

1.1 Chloride channels

Biological membranes, which surround all cells of an organism, are a very efficient barrier to ion diffusion. The hydrophobic inner area of the membrane plays a key role for the barrier. It enables ion gradients to form between the cytoplasm and the extra cellular space. Therefore, specific membrane proteins, which are positioned according to their structure within the membrane, mediate the exchange of substances between intra and extra cellular space. (18, 128).

Anionic channels are membrane proteins which represent pores in biological membranes that enable the diffusion of negatively charged ions along with their electrochemical gradient. Although these channels are more permeable for Ions like I^- or NO_3^- than for chloride (Cl^-), they are often called chloride channels because Cl^- is the most common anion in the organism. Chloride channels also exist in intracellularly located membranes of vesicle cell organelles.

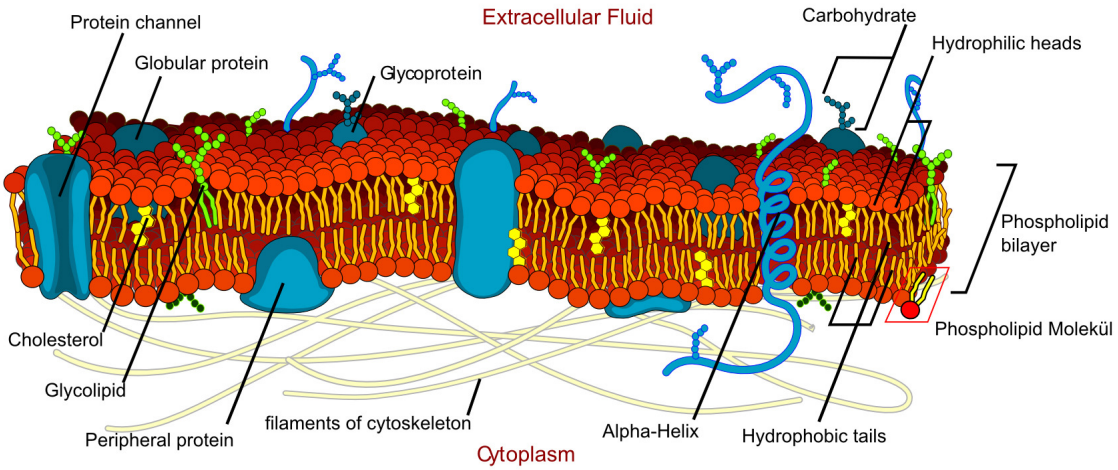

Fig. 1-A

Schematic view of the plasma membrane (110).

There is a variety of different chloride channel types. They differ in activation mechanism, biophysical properties, expression patterns and physiological function. It can be assumed that at the moment entire gene families of anionic channels remain to be discovered (60).

The known chloride channels could be categorized as follows:

- CLC chloride channels
- ligand gated $GABA_A$ and glycine receptors
- cystic fibrosis transmembrane conductance regulator (CFTR)
- bestrophines (140)

According to their various properties, chloride channels are involved in many physiological processes, such as maintaining membrane potential, synaptic inhibition, cell volume regulation, transepithelial transport, extra cellular and vesicular pH-regulation as well as endocytosis.

Within epithelial cells a chloride concentration between 30 and 40 mmol/l is often found, mainly due to the Na-K-2Cl cotransporter. For that reason membrane potentials of around 50 mV, which is typical for epithelia, result in an efflux of Cl^- from the cell. Therefore the localisation (apical or basolateral) of the chloride channels determines whether the epithelium is secreting or resorbing Cl^-.

1.2 Chloride channels of the CLC family

1.2.1 Overview

The primary structure of the ClC chloride channel was first identified by Jentsch, Steinmeyer and Schwartz in 1990 by means of gene expression in *Xenopus laevis* oozytes (61). The so called ClC-0 channel was first isolated from the DNA of *Torpedo marmorata*, the marine electric skate. Beside this species, genes of the CLC family are also found in other animals, plants, yeasts, archebacteria and eubacteria – in nearly all organisms. In mammals CLC Proteins show tissue-specific expression patterns, making distinct cellular purposes possible. This functional variety is represented by the specialized members of the CLC gene family, which consists of 9 known members (57).

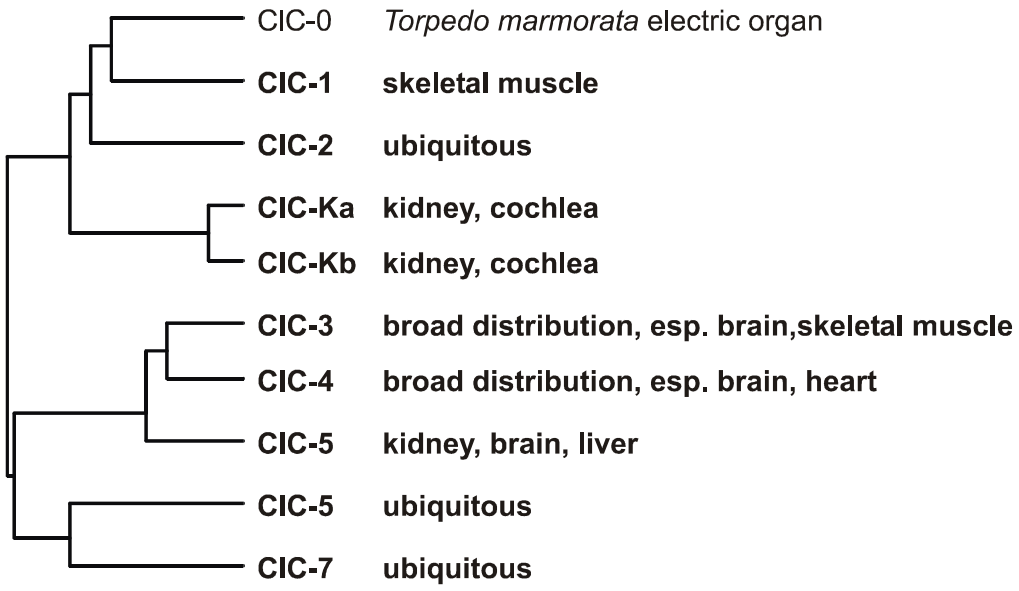

Fig. 1-B

The CLC family of chloride channels: The dendrogram indicates the degree of similarity between different human CLC gene products (indicated in bold). The human CLC channels are grouped into three branches with less than 30% identity between them. The ClC-0 channel is also shown because of its close homology to ClC-1 (56).

1.2.2 Molecular architecture of ClC channels

1.2.2.1 Structure and topology

Eukaryotic ClC protein subunits consist of 18 α-helices (containing 17 transmembraneous helices) and show a complex topology. The c-terminal half

(helices J-Q) represents a structural repetition of the n-terminal half (helices B-I). Within the membrane both parts are oriented symmetrically in opposite directions according to each other. The α-helices are strongly tilted and have variable lengths; many of them span the membrane only partly. Because of this structure the two subunits arrange around their common centre, forming the selectivity filter for Cl^- within the membrane (27).

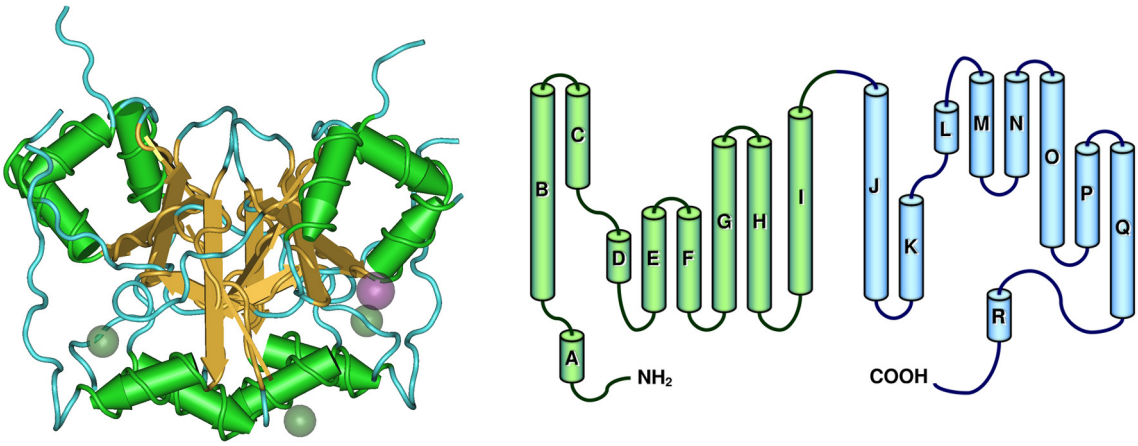

Fig. 1-C

Left: 3-dimensional model of the crystal structure of a ClC-Ka subunit (92). Right: Current model of the ClC subunit membrane topology, α-helices A-R are shown as cylinders (27).

Furthermore, both subunits consist of long intracellular n- and c-termini. Mutations in the c-terminal tail show pronounced effects on the gating properties of ClC channels (45). Thus, the c-terminal tail and the α-helix R could be important for regulatory functions. In particular this section contains two cystathionin-β-synthase units (CBS), which play a role in determining the protein's function (116), its cellular localization (120) and the protein sorting mechanism (21). The CBS units have ATP-binding functions and are thus supposed to provide regulating functions in connection with the cellular energy status (122).

1.2.2.2 Dimeric occurrence

One common feature of ClC channels is their dimeric occurrence (96), whereby each of the pores maintains its individual properties – gating of each pore is independent from the other pore. This independent gating of both protopores is also called "protopore-gating". There is additionally a common gate which could close both pores at the same time (87, 113, 156). Therefore

the effect of heterozygous dominant loss-of-function mutations is small if they affect the protopore gating mechanism, because the function of the individual pores remains intact. Only mutations affecting the formation of the dimers or the common gate can result in dominant negative effects. This is why the pathologies of dominant negative variants of diseases like myotonia congenita (132) or osteopetrosis (70) compared to their recessive counterparts (59) appear rather mild.

It could be assumed that the homodimeric existence of ClC channels represents the most common in vivo variant. However, it has been shown that heterodimers of CLC-0, CLC-1 and CLC-2 as well as CLC-4 and CLC-5 could emerge (86, 97). The flux passing the ClC channels can be modulated by means of various mechanisms: voltage, extra- and intracellular anions (109), pH (30), extracellular Ca^{2+} (143), cell swelling (37), and phosphorylisation (35). The voltage-dependent protopore gating mechanism is based on the movement of the permeating anion as a voltage sensor within the pore. This simple model simultaneously explains the concentration-dependent gating (107). A glutamyl residue plays a key structural role, being located at the central binding site of the chloride ion within the pore (28).

The different members of the ClC channel family can be divided into two groups: plasma membrane chloride channels (ClC-0, ClC-1, ClC-2, ClC-Ka, ClC-Kb), as well as vesicular ClC proteins, which perform tasks within the endosomal / lysosomal system (ClC-3 to ClC-7) (95, 114).

1.2.3 Occurrence and properties of ClC-Ka/ClC-Kb

Two highly homologous CLC channel proteins (ClC-Ka and ClC-Kb) are mainly expressed in the kidney. The orthologous proteins of rodents are called ClC-K1 and ClC-K2. The gene symbols for the human forms ClC-Ka and ClC-Kb are CLCNKA and CLCNKB. Both paralogous genes are 90% identical (4, 65, 142); moreover, they are located just a few kilo bases away from each other on chromosome 1p36 (127). There is an additional β-subunit, Barttin, which is necessary for the genes' functional expression. Barttin always co-localizes with the ClC proteins (30). In the kidney, ClC-Ka is expressed in the ascending thin

limb (ATL) of the Loop of Henle (143). The question whether both the apical and the basolateral localization emerge simultaneously (143) or just the basolateral one alone (145) remains unsettled. Indeed, the crucial β-subunit Barttin has until now only been found on the basolateral cell pole (30). ClC-Kb is expressed at the basolateral pole of cells of the thick ascending limb (TAL) of the Loop of Henle, the distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting duct (CD) (68). In the inner ear, both channels are present in the basolateral plasma membrane of the secretoric epithelia of the organ of Corti and the vestibular organ (30, 89). Both ClC-Ka and ClC-Kb as well as Barttin are too expressed within respiratory epithelia as confirmed for the Calu-3 human airway epithelial cell line (98).

Ion selectivity decreases in the following order for ClC-Ka/Barttin: $Cl^- \geq Br^- > NO_3^- > I^-$ and for ClC-Kb/Barttin $Cl^- > Br^- = NO_3^- \geq I^-$. There is only little voltage-dependent gating because the polar glutamyl residue, which is present in the other members of the ClC family (27, 28), is replaced with valin in ClC-K proteins (152). The flux in both channels can be modulated by means of extracellular calcium concentrations as well as pH. Thus ClC-Kb reacts stronger to pH shifts than ClC-Ka. (30).

1.2.3.1 Barttin

Barttin was discovered by Hildebrandt et al. (30, 48). It is a small membrane protein with a molecular weight of 40 kDa with two transmembrane domains (see Fig. 1-D). Barttin does not belong to a larger gene family; interestingly, it interacts only with ClC-K proteins, i.e., the human ClC-Kb and ClC-Ka. Most importantly, its presence is crucial for the development of transmembrane ionic currents through ClC-K channels. By binding onto the outer lateral surfaces of ClC-K (136), Barttin leads to an increased surface expression of ClC-K Proteins, and thus to an increased ionic flux. A loss of Barttin on the other hand results in an accumulation of ClC-K proteins in the endoplasmatic reticulum and therefore leads to reduced Cl^- conductance (30, 44). As an additional mechanism Barttin also changes the properties of ClC-K currents (118).

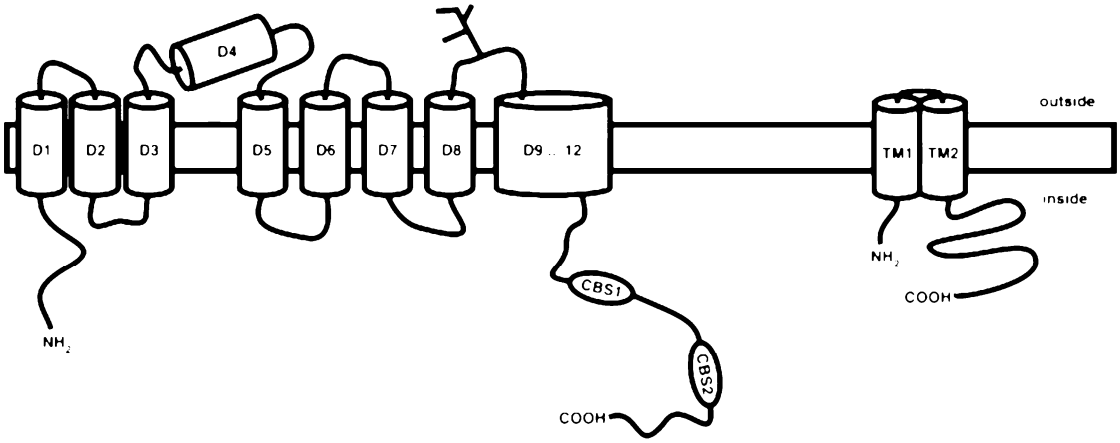

Fig. 1-D

Model of ClC channels (left) and Barttin (right) suggested by Jentsch based on biochemical analyses. In the mean time replaced by X-ray structure analyses (117).

The role of Barttin in endocytosis has also been investigated. Renal epithelial cells use the ubiquitin-proteasome pathway to regulate their protein homeostasis (24). Protein ubiquitin ligases belonging to the *„Neuronal precursor cell expressed, developmentally down-regulated gene 4 isoform family 2"* (Nedd4-2) are involved in these ubiquitinylation processes. The intracellular C-terminus of Barttin contains a PY-motif which enables it to bind to ubiquitin ligases; it thus mediates endocytosis signals (13, 29, 30). This corresponds to the similar endocytosis mechanism as proposed for ClC-5 or ENaC (119, 131). Furthermore, it has been shown that serum and glucocorticoid-induced kinases 1 and 3 (SGK1 and SGK3) influence the described effect of Nedd4-2 on endocytosis. The influence of SGK1 on the abundance of the ClC-Ka/Barttin protein is also visible using the ^{Y98A}Barttin variant where the C-terminal PY-motif is lacking. This suggests a regulation mechanism based on SGK1 without interference from Nedd4-2 (73). These observations support the suggestion that hormones (e.g. glucocorticoids, mineralocorticoids, insuline, IGF-1) that primarily influence SGK1 transcription also indirectly regulate the transepithelial Cl^- transport.

1.2.4 ClC-proteins as antiporters

Newer results indicate that ClC-4 and ClC-5 can function as electrogenic antiporters (103, 114), which allows the concept of ClC-proteins as mere Cl^- channels to be expanded (59). The exchanger activity is compatible with the

role of ClC-4 and ClC-5 in vesicular acidification: Cl^- is driven by the H^+-ATPase dependent H^+-gradient into the vesicles. Within this process the Cl^- transport is directly coupled to the vesicular pH (2, 103, 114). It still remains unknown whether ClC-6 and ClC-7 also act as Cl^- / H^+ exchangers, but it is likely because they share structural patterns with other ClC exchangers (3); likewise, ClC-3 belongs to this category because it shares the same degree of homology and in addition its ion currents are comparable with those of ClC-4 and ClC-5 (77, 78).

1.3 Physiologic and pathophysiologic importance of ClC-proteins

As for all ClC-channels, the physiologic significance of ClC-K/Barttin is shown through genetically determined disorders in humans and mice.

1.3.1 ClC-1 / myotonia congenita

ClC-1 is principally expressed within skeletal muscle; there the resulting conductivity contributes to up to 80% of the resting potential of the cellular membrane (5, 133). This is the reason for the relatively low resting membrane potential of skeleton muscle cells (around -90 mV). Mutations within ClC-1 cause myotonia congenita; its symptoms are muscle stiffness, hypertrophy and delayed relaxation. Mutated ClC-1 variants are responsible for both the dominant variant (Thomsen) and the recessive one (Becker) (47). There are more than 50 different known causal mutations that lead to muscular hyperexcitability (159).

1.3.2 ClC-2

ClC-2 is ubiquitously expressed; it shows expression patterns that overlap with CFTR (99). Furthermore, it shares several biophysical qualities with CFTR such as: similar anion selectivity, non influencability through 4,4´-Diisothiocyanostilbene-2,2´-disulfonacid (DIDA) and regulation possibilities through cAMP (138). It was long speculated that ClC-2 could function as a replacement candidate to enable transepithelial Cl^- transport in case of CFTR deficiency (121); conversely, it has been shown with a mouse model using a combined knock-out (KO) of CFTR and ClC-2 that combined KO leads to a

longer lifespan and no pathologic lung or pancreas alterations (162). Furthermore, isolated ClC-2-KO in mice leads to retinal and testicular degeneration due to pH missregulation between the interstitial space and cytoplasm (17). Interestingly, ClC-2 can modulate the Cl^- concentration in neurons, thus influencing the effect of GABA receptors (130). Though no epilepsy was observed in ClC-2-KO mice (17), three human families with epilepsy and cosegregating ClC-2 mutation have been described (43).

1.3.3 ClC-K

1.3.3.1 ClC-K1

Expression of ClC-K1 in mice (and its ortholog ClC-Ka in humans) is crucial to the Cl^- permeability of the ATL. It enables the establishment of high osmolarity in the renal medulla, thus facilitating the reabsorption of water thus leading to high urine concentration (6). Knock out (KO) of ClC-K1 in mice (93) leads to severe renal salt and water loss. Though a corresponding human disorder is unknown, a family with simultaneous ClC-Ka and ClC-Kb mutations showed symptoms of Bartter syndrome type IV (115).

1.3.3.2 ClC-Ka/Barttin, ClC-Kb/Barttin

K^+ secretion by strial marginal cells and vestibular dark cells into the endolymph requires that Cl^- recycle in the basolateral membrane via a major Cl^- conductance (74). ClC-Ka and ClC-Kb together with Barttin help to recycle Cl^- across the basolateral membrane to maintain the uptake of K^+ via SLC12A2 (NKCC1). Therefore ClC-Ka/Barttin and ClC-Kb/Barttin enable Cl^- conductance and the *Stria vascularis* to secrete K^+ into the *Scala media* (9, 30, 137). High potassium concentrations (~150 mM) in the endolymph of the *Scala media* and the resulting potential (+90 mV) are necessary to force K^+ ions through the apical mechanosensitive cation channels of the sensory hair cells (55).

As both ClC-Ka/Barttin and ClC-Kb/Barttin are expressed within the *Stria vascularis*, Bartter's syndrome type III with isolated loss-of-function mutation of ClC-Kb does not cause deafness like in Bartter's syndrome type IV where Barttin is mutated (13, 30). In turn, simultaneous mutations of CLCNKA and

CLCNKB also lead to Bartter's syndrome type IV (115). This observation is consistent with the finding that CLCNKA and CLCNKB are coexpressed in cells of the inner ear, supplementing each other in terms of Cl^- conductance in the inner ear (108).

On the contrary, in the kidney both channels serve the very different function of basolateral net transport of Cl^- along the ascending thin and thick limb respectively (74). A disruption of ClC-Kb therefore leads to Bartter syndrome type III (127). It causes severe renal salt loss due to sodium chloride (NaCl) reabsorption deficiency in the TAL. NaCl is reabsorbed in this nephron segment by means of secondary active transport by the apical Na-K-2Cl cotransporter (NKCC2). Potassium ions recirculate into the lumen through the ROMK (Kir1.1) potassium channel; but Cl^- leaves the cell basolateral through the ClC-Kb/Barttin channel.

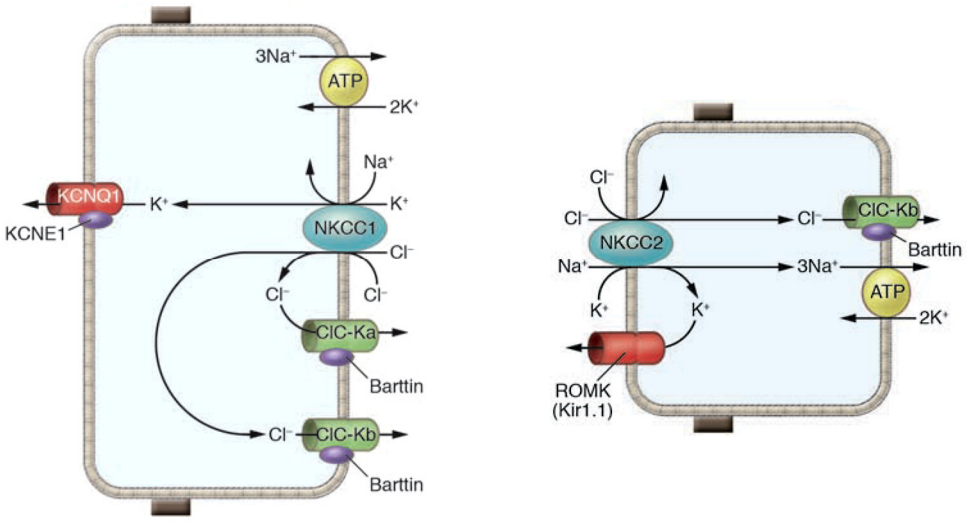

Fig 1-E

Left: Strial epithelial cell. The lumen of the *Scala media* is located on the left side with contact to KCNQ1. Right: Cell of the TAL epithelium. The lumen of the tubule is located on its left side. With friendly permission of T. Jentsch (58).

Mutations of ClC-Kb were also found in a minority of patients with the phenotype of Gitelman syndrome when no mutation of the thiazide-sensitive NaCl cotransporter (NCC, SLC12A3) was present. Gitelman syndrome is characterized by hypokalemic metabolic alkalosis combined with significant hypomagnesaemia and low urinary calcium excretion. It is assumed that the clinical phenotype in patients with ClC-Kb mutations can be highly variable,

from an antenatal onset of Bartter syndrome on one side of the spectrum to a phenotype closely resembling Gitelman syndrome on the other (67).

1.3.3.3 Gain-of-function polymorphism ClC-Kb^{T481S}

Jeck et al., using the heterologous expression of ClC-Kb/Barttin in *Xenopus laevis* oocytes, showed that ClC-Kb^{T481S}/Barttin had a Cl^- conductivity 20 time higher than the wild type variant ClC-Kb^{wt}/Barttin (51). This is the first known gain-of-function mutation within the ClC chloride channel family. This mutation is of particular interest because there is about a 20% frequency of heterozygous carriers in mid-European populations. The frequency varies in other populations: 3% within a Japanese population (69) and 37% within a Ghanaian population (52). The ion selectivity of ClC-Kb^{T481S} equals that of ClC-Kb^{wt}. There are also no differences in surface expression between the two variants (51). The threonine residue at position 481 of ClC-Kb is located at the n-terminal end of the P helix (see Fig. 1-C). This configuration is conserved through all members of the human ClC-family at homologous positions (27). Increased Cl^- currents were also measured with the orthologous ClC-K1^{T481S} in mice (51).

1.3.3.4 Renal blood pressure regulation

Renal blood pressure regulation is principally achieved by controlling tubular transport of Na^+ and Cl^-. Most monogenetic disorders affecting blood pressure regulation affect genes involved in renal salt reabsorption. The kidney filters over 170 litres of blood plasma with an overall amount of around 23 Mol salt. It thus has to reabsorb 99,5% of the filtered salt under normal nutritive conditions, which it accomplishes by a system of various ion channels, exchangers and transporters. 60% of the filtrated sodium is reabsorbed in the proximal tubule of the nephron, 30% in the TAL through the Na-K-2Cl cotransporter, 8% in the DCT through sodium chloride cotransporter and the remaining 2% through the epithelial sodium channel (EnaC) in the cortical collecting duct. Though these last two percentages make up the smallest part, the corresponding nephron segment plays a key role in the renin–angiotensin–aldosterone system (RAAS) (39). Lower salt concentrations in the TAL lead to renin secretion. Renin converts angiotensinogen into angiotensin I, which again is converted into

angiotensin II by the angiotensin-converting enzyme. Angiotensin II engages various mechanisms, which eventually lead to increased blood pressure, including: stimulation of aldosterone secretion, vasoconstriction by stimulating the G_q protein in vascular smooth muscle cells, hypertrophy of cardiomyocytes, increased appetite for salt, and thirst (144). Aldosterone and SGK1, on the other hand, increase the activity of ENaC, thus leading to higher salt reabsorption (83, 150). The Liddle syndrome is an example where the surface expression of ENaC is dysregulated. An alteration in ENaC PY-motive leads to insufficient degradation through the ubiquitin proteasome system (125), leading to hypertension, alkalosis, hypopotassemia and reduced renin and aldosterone levels (82).

1.3.3.5 ClC-Kb^{T481S} and hypertension

As mentioned above, ClC-Kb plays a substantial role in various ion transport processes. It is particularly involved in reabsorbing salt within the distal nephron, where 40% of the NaCl reabsorption takes place, which means that ClC-Kb is one of the potential candidate genes that may cause hypertension. Assuming an increased reabsorption of Cl^-, and hence salt, within TAL and DCT, an increased intravascular volume and possibly hypertension may result. On the other hand, it is unlikely that the basolateral Cl^- conductivity is the only limiting factor for the transepithelial transport of Cl^-, so there probably are other factors influencing Cl^- reabsorption (51). However, the association between ClC-Ka^{T481S} and hypertension was first shown in 2004 within a collective of 220 individuals (52). In this study even heterozygous individuals (ClC-Ka^{T481S}/ClC-Ka^{wt}) showed significant increases in blood pressure levels. Due to the small size of the study sample, further investigations with a larger population sample were necessary to confirm these initial findings (36). No evidence for altered blood pressure levels has been shown in a Japanese Study (69). But the heterozygote ClC-Kb^{T481S} prevalence was anyway only 3% in this population sample (69). In a case control study with 196 samples of essential hypertension and 321 normotensive controls no effect of the mutation could be detected (129). Most importantly however, a study of a large Swedish population with an heterozygote prevalence of 23% and 6055 participants did not show an

association either (32). This study had enough statistical power to detect alterations in blood pressure as small as 1.7 mmHg for systolic and 0.8 mmHg for diastolic values. It is hence likely that the gene variant for hypertension has little effect.

On the other hand other confounding variables that have not been included in the previous studies could be masking the effect of ClC-Kb^{T481S} on blood pressure. There are for example regulative effects that modulate the effect of the ClC-Kb^{T481S} mutation as well; in particular, alterations of SGK1 are associated with increased blood pressure (19). SGK1 stimulates Na/K-ATPase (115), EnaC (150), ClC-Ka (29), ClC-Kb (73) and others. Large studies also showed the association of a common SGK1 variant with hypertension (147). These observations are completely in line with measurements from SGK1-KO mice (49).

Also a (very different) polymorphism in CLCNKA was associated with hypertension (11). Moreover, a Barttin variant (V431I) was identified in African Americans and other populations, but not in Caucasians (126). Upon coexpression with ClC-Kb, this variant showed only about 30% current when compared to WT ClC-Kb/Barttin. However, it was not associated with a protection against high blood pressure (126).

Therefore further research is needed to reveal the influence of ClC-Kb^{T481S} on blood pressure (53).

1.3.3.6 Collecting duct ClC-K channels

The meaning of ClC-Ka and ClC-Kb in the α and β cells of the collecting duct is still only partly understood (30). It has been speculated that ClC-Ka/b in α cells has a similar recirculation function as needed for the KCC4 potassium chloride cotransporter. Though knock out of KCC4 leads to renal tubular acidosis (14), it is hard to detect a disturbance of the acid-base homeostasis in patients with Bartter syndrome type III because of the salt reabsorption that is severely dysregulated (58).

1.3.4 ClC-3 / neurodegeneration

At the moment there is no human disorder caused by mutations of ClC-3; yet a disrupted channel leads to severe neurodegeneration as well as atrophy of the hippocampus and the retina in mice (134). ClC-3 is located in synaptic vesicles and endosomes. Its disruption impairs the acidification of these compartments (40, 161).

1.3.5 ClC-4

ClC-4 is mainly expressed in the brain, kidney and liver; after all, it is the member of the ClC family about which we know the least. Like ClC-3, it plays a role in the acidification of (97); furthermore, it has been assumed that ClC-4 plays a role in copper haemostasis (153).

1.3.6 ClC-5 / Dents syndrome

Dent's disease is an x-chromosomal recessive inherited disorder. It affects some renal tublule functions, leading to low molecular proteinuria, albuminuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and renal failure (88, 158). The disorder results from a mutation in the renal chloride channel gene CLCN5 (160). ClC-5 plays a key role in the acidification of endosomal and lysosomal vesicles in the kidney (38), resulting in disturbed protein endocytosis in the proximal tubule (84).

1.3.7 ClC-6 / neurodegeneration

Defects in ClC-6 cause neurodegeneration similar to malfunctioning ClC-3. It is almost exclusively expressed in neurons of the central and peripheral nervous system; with spinal ganglia showing particularly high levels of expression. ClC-6 is located in late endosomes; thus, neuronal lipofuscines accumulate if the channel is disrupted. Knock out of ClC-6 in mice leads to hypaesthesia and behavioural abnormalities. ClC-6 also contributes to the development of neuronal ceroid lipofuscinoses (NCL) (104, 135).

1.3.8 ClC-7 / osteopetrosis

ClC-7 is widely expressed within various tissues. It is particularly expressed in late endosomes and lysosomes. If it is mutated in humans the phenotype of Osteopetrosis can be observed (70). Today there are around 30 known human ClC-7 mutations that coincide with Osteopetrosis (22). The disease is characterized by reduced bone resorption and reduced bone marrow space. ClC-7 is mainly located within the acid-secreting cellular membrane of osteoclasts. It serves there as an anion shunt, enabling Cl^- to follow the actively transported H^+; it is therefore crucial for the acidification of the resorption lacunas around osteoclasts (70). Furthermore, it leads defective ClC-7 to lysosomal storage disease in mice. The rodent tissue is altered similarly to the human NCL (63).

As an interesting feature, it has been shown that the channel function of ClC-7 is largely influenced by a β-subunit namely Ostm1. The subunit distinctly increases the stability of ClC-7. If Ostm1 is missing, the result is a phenotype comparable to the ClC-7 knock-out variant (76).

1.4 Pharmacology of ClC-channels

Because of the above-shown importance of ClC-K channels in human physiology and disease, a search for pharmacological remedies suggests itself. Specific inhibitors could act as a new class of diuretics (33). Although highly homologous (65), the two CLC-K isoforms show different pharmacological profiles (102). The first known ligand of ClC-K channels is a derivative of 2-p-chlorophenoxypropionic acid (CPP), namely 3-phenyl-CPP. It blocks ClC-Ka, ClC-K1 but not ClC-Kb (81, 102). It is also possible to irreversibly block ClC-Ka with the non-specific Cl^- channel blocking agent 4,40-diisothiocyanatostilben-2,20-disulphonic acid (DIDS) (81, 102, 152).

Niflumic acid, which belongs to the group of nonsteroidal antirheumatic drugs (NSARs), increases ClC-Ka and ClC-Kb channel activity, yet the effect on ClC-Ka is more pronounced (80). Flufenamic acid derivatives produce instead an inhibitory effect on CLC-Ka, but an activating effect on CLC-Kb (80). But it also has been shown that Niflumic acid has an inhibitory effect on ClC-1, which

is responsible for the Cl^- conductance of skeletal muscle cells (79). Further side-effects of these agents cannot be ruled out at the moment.

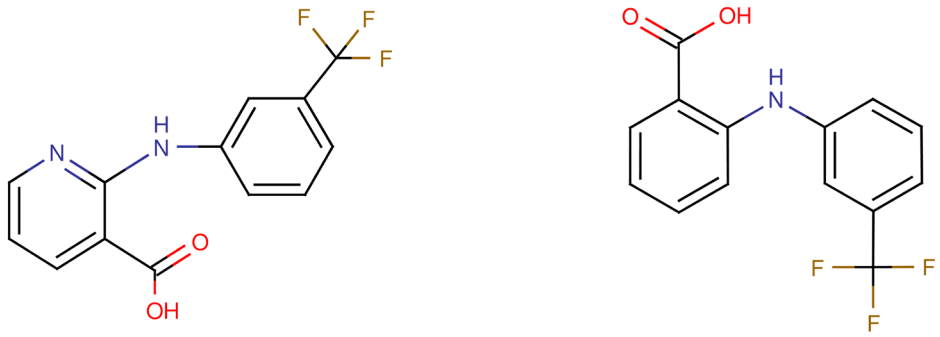

Fig 1-F

Structure formulas. Left: Niflumic acid. Right: Flufenamic acid (124).

Recently a new class of small molecules have been discovered, the polythioureas, which are derived from DIDS hydrolysis. They inhibit ClC-0, ClC-Ka and ClC-ec1 more effectively than DIDS itself. These polythioureas are the highest affinity inhibitors known for the CLC proteins at the moment. They are expected to provide valuable insight into the mechanisms of inhibition and ion transport in CLC proteins (94).

Finally, the discovery of the exact binding site of Barttin to ClC-K has lead to a newly proposed mechanism for diuretics. It is the idea of inhibiting the binding of Barttin to ClC-K, thus reducing the surface expression and therefore the activity of ClC-K (136).

2 Influence of gain of function epithelial chloride channel ClC-Kb mutation on hearing thresholds

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2.1 Abstract

Hearing depends on functional ClC-K-type chloride channels composed of Barttin with ClC-Ka or ClC-Kb. Loss-of-function mutations of the Barttin gene BSND or of both the ClC-Ka gene CLNKA and the ClC-Kb gene CLNKB lead to congenital deafness and renal salt wasting. Recently, we identified the gain-of-function mutation ClC-Kb^{T481S} which is associated with increased blood pressure. To explore the impact of ClC-Kb^{T481S} on hearing, healthy volunteers (n = 329) and individuals suffering from tinnitus (n = 246) volunteered for hearing tests (n = 348) and genetic analysis (n = 575). 19.1% of the individuals were heterozygote (ClC-Kb^{T481S}/ClC-Kb) and 1.7% homozygote carriers. Pure tone average hearing threshold (PTAt) for air conduction was significantly ($p < 0.033$) lower in ClC-Kb^{T481S} carriers (13.2 ± 1.2 dB) than in wild-type individuals (17.1 ± 0.9 dB). The prevalence of ClC-Kb^{T481S} carriers was significantly increased (29.7%) in individuals with PTAt < 15 dB ($p < 0.05$) and significantly decreased (13.2%) in individuals with PTAt > 30 dB ($p < 0.017$). The difference was largely due to the female population. Bone conduction was less affected, pointing to an effect of the mutation on middle ear function. Tinnitus tended to be more

frequent in ClC-Kb^{T481S} carriers, a difference that was, however, not statistically significant. In conclusion, hearing thresholds are slightly lower in carriers of ClC-Kb^{T481S}, i.e., the gain-of-function polymorphism ClC-Kb^{T481S} exerts a subtle but significant protective effect against hearing loss.

2.2 Introduction

The Cl^- channels composed of the pore forming units ClC-Kb (68, 89, 152) or ClC-Ka (9), and the β-subunit Barttin (30, 151) are expressed in the kidney and the inner ear. Defective Barttin (13), ClC-Kb (127), or both, ClC-Ka and ClC-Kb (115) lead to renal salt wasting with severe hypovolemia, defective Barttin (13) and simultaneous defects of ClC-Ka and ClC-Kb (115) result in deafness. Most recently, voltage-clamp experiments disclosed that a naturally occurring variation of the CLCNKB gene (A to T exchange at nucleotide position 1441 starting from the ATG start codon of the GenBank entry NM_000085.1), which leads to the replacement of threonine by serine at the amino acid position 481 of the ClC-Kb protein (ClC-Kb^{T481S}), dramatically increases ClC-Kb chloride channel activity (51). The mutation was expected to stimulate renal tubular NaCl reabsorption, thus leading to extracellular volume expansion and increased blood pressure (51). As a matter of fact, a small but significant increase of blood pressure has been observed in individuals carrying the ClC-Kb^{T481S} mutation (51). The present study aimed to explore whether the mutation may similarly affect the hearing thresholds of affected individuals.

2.3 Materials and methods

2.3.1 Functional analysis of mutated ClC-Kb

To verify the functional significance of the ClC-Kb^{T481S} mutation, *Xenopus laevis* oocytes were injected (149) with cRNA encoding wild-type Barttin (6 ng/oocyte) together with 6 ng/oocyte of either wild-type ClC-Kb (151) or ClC-Kb^{T481S} (52) or with 3 ng/oocyte of both ClC-Kb and ClC-Kb^{T481S}. After three days the currents were determined in two-electrode voltage-clamp experiments with a pulse protocol of 800 ms pulses ranging between -140 mV to +40 mV

increased in 20 mV increments. Data were recorded with a Turbo Tec 10 CX-amplifier (NPI, Tamm, Germany), acquired through the Clampex feature of the pCLAMP 8.0 software (AXON INSTRUMENTS, CA, USA) and analyzed with Clampfit and Origin 6.0. To minimize voltage-clamp errors due to the large amplitude of currents, we used the integrator of the TurboTec 10 CX amplifier. Voltage-clamp control was also checked during the recordings on the PI-controller of the amplifier. Pipettes were filled with 3 M KCl and had resistances of 0.5–1 MX. The bath solution (ND96) contained (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, for a pH of 7.6.

2.3.2 Volunteers

Healthy volunteers (n = 329) and individuals suffering from tinnitus (n = 246) were recruited by advertisement. The individuals volunteered for hearing tests (n = 348, 233 individuals suffering from tinnitus and 115 healthy volunteers) and genetic analysis (n = 575). The study participants were of Caucasian origin. The study was approved by the ethics committee of the University of Tübingen. All volunteers gave their written informed consent.

2.3.3 Determination of hearing thresholds

Pure tone average hearing thresholds (PTAt) for air and bone conduction were determined utilizing a neurootometer from Hortmann (GN Otometrics, Münster, Germany). Air conduction PTAts were determined in both ears for frequencies 0.25 kHz, 0.5 kHz, 0.75 kHz, 1 kHz, 1.5 kHz, and 4 kHz and 8 kHz, bone conduction PTAts for both ears for the frequencies 0.25 kHz, 0.5 kHz, 0.75 kHz, 1 kHz, 1.5 kHz, and 4 kHz, by calculating the arithmetic mean.

2.3.4 Mutational analysis

Genotyping was performed by a 5_0nuclease assay using TaqMan technology as described previously in detail (52).

2.3.5 Statistical evaluation

All data from the hearing tests are provided as means ± SEM. Data were analyzed by parametric or nonparametric methods, depending on whether data

distribution was normal or not normal. Since the prevalence of homozygous carriers for the ClC-Kb$_{481S}$ variant is low (52), wild-type individuals were compared to heterozygous or homozygous carriers of the ClC-Kb^{T481S} variant. For statistical analysis, the Student's t-test, the χ^2-test and the Mann–Whitney U-test were used as appropriate. All statistical tests were two-tailed and a p-value of 60.05 was defined as statistically significant. For all calculations the JMP IN software package version 5.1.2 was used (SAS institute Inc., Cary, NC, USA). All laboratory procedures were carried out blind to case control status.

2.4 Results

In the (94) presence of Barttin, the current induced by ClC-Kb^{T481S} was significantly larger than the current induced by wild-type ClC-Kb. Interestingly, the injection of 3 ng/oocyte ClC-Kb^{T481S} together with 3 ng/oocyte wild-type ClC-Kb mimicking heterozygosity led to similar currents as the injection of 6 ng/oocyte of ClC-Kb^{T481S} mimicking homozygosity of ClC-Kb^{T481S} carriers. Those observations point to saturation of the current leading to similar currents in heterozygosity and homozygosity. In the studied population (n = 575), 19.1% of the individuals were identified as heterozygote carriers (ClC-Kb ^{T481S}/ClC-Kb) and 1.7% as homozygote carriers (ClC-Kb^{T481S}/ClC-Kb^{T481S}). All data were found to be in Hardy–Weinberg equilibrium. The number of homozygote carriers was too small for reliable statistical analysis and thus, heterozygous and homozygous carriers of ClC-Kb^{T481S} were not separated. No significant differences were observed between ClC-Kb^{T481S} carriers and wild-type individuals in age, size, or body weight. Table 2-A shows the results for the population that underwent a hearing test.

Table 2-A

Age, sex, body size, and weight of all volunteers participating in hearing tests.

	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
number	258 (109 ♀, 149 ♂)	90 (48 ♀, 42 ♂)	ns
age (years)	43.1 ± 1.0	40.3 ± 1.8	ns
body size (cm)	173.3 ± 0.6	173.0 ± 1.0	ns
weight (kg)	72.8 ± 0.9	70.3 ± 1.4	ns

The prevalence of ClC-Kb^{T481S} carriers tended to be higher in patients suffering from tinnitus (23.6%) than in healthy individuals (18.8%), a difference that was not, however, statistically significant. Thus, the impact of the mutation on the prevalence of tinnitus is at best mild. The pure tone average hearing threshold (PTAt) for air conduction, however, was significantly ($p < 0.033$) lower in ClC-Kb^{T481S} carriers (13.2 ± 1.2 dB) than in wild-type (17.1 ± 0.9 dB) individuals.

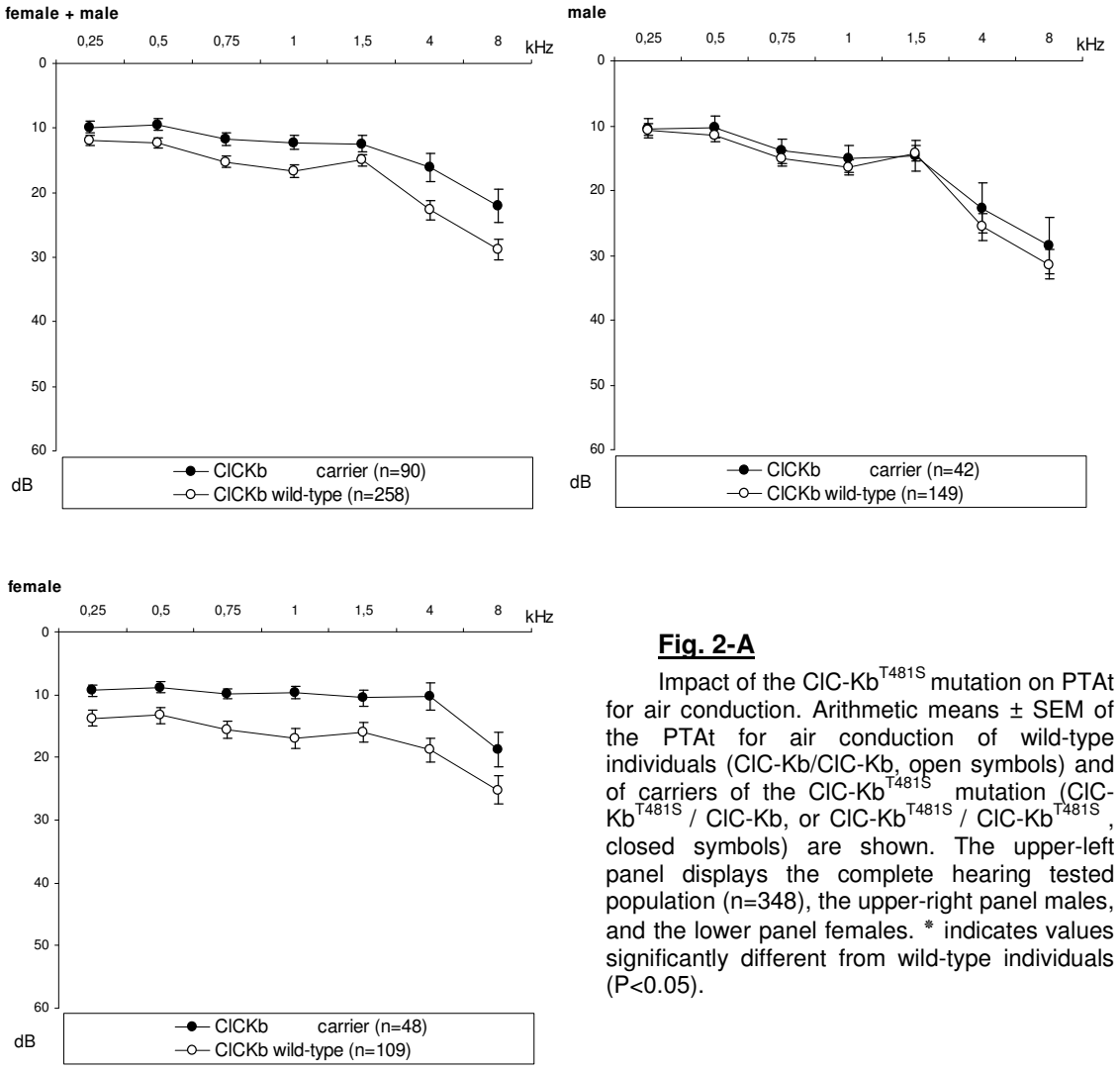


Fig. 2-A

Impact of the ClC-Kb^{T481S} mutation on PTAt for air conduction. Arithmetic means ± SEM of the PTAt for air conduction of wild-type individuals (ClC-Kb/ClC-Kb, open symbols) and of carriers of the ClC-Kb^{T481S} mutation (ClC-Kb^{T481S} / ClC-Kb, or ClC-Kb^{T481S} / ClC-Kb^{T481S}, closed symbols) are shown. The upper-left panel displays the complete hearing tested population (n=348), the upper-right panel males, and the lower panel females. * indicates values significantly different from wild-type individuals ($P<0.05$).

As illustrated in Fig. 2-A, the difference was more prominent at higher frequencies. The slight differences in PTAt between ClC-Kb^{T481S} carriers and wild-type individuals of the complete population (Fig. 2-A, upper panel) was largely due to differences in the female subpopulation (Fig. 2-A, middle panel),

while the difference was not significant in the male subpopulation (Fig. 2-A, lower panel).

Analysis of different female age groups (Fig. 2-B) disclosed that the lower PTAt of ClC-Kb^{T481S} carriers compared to wild-type individuals was particularly apparent at age ≥50 years (Fig. 2-B, lower panel).

The PTAt in bone conduction tended to be lower in ClC-Kb^{T481S} carriers (4.9 ± 0.9 dB) than in wild-type individuals (5.8 ± 0.6 dB), a difference that was not, however, statistically significant. Table 2-B summarizes the bone conduction at different frequencies.

Table 2-B

Hearing thresholds (in dB) of bone conduction in ClC-Kb^{T481S} carriers and wild-type individuals of the complete hearing-tested population (see Table 2-A)

Frequency (Hz)	ClC-Kb/ClC-Kb	ClC-Kb^{T481S}carrier	P values
250	-0.60 ± 0.57	-0.34 + 0.88	ns
500	3.39 + 0.68	3.96 ± 1.02	ns
750	5.44 ± 0.80	4.99 ± 1.13	ns
1000	6.01 ± 0.74	4.4 ± 0.98	ns
1500	9.97 ± 0.85	7.91 ± 1.24	ns
4000	13.76 ± 1.22	9.71 ± 2.01	0.036

Significantly lower hearing thresholds were observed only at 4000 Hz in the complete hearing-tested population (Table 2-B) and the female subgroup (10.49 ± 1.55 dB ClC-Kb wild-type vs. 4.63 ± 1.84 in ClC-Kb^{T481S} carriers, p=0.019). The difference of the mean PTAt over all frequencies between bone conduction and air conduction was again significantly ($p < 0.016$) lower in ClC-Kb^{T481S} carriers (8.2 ± 0.8 dB) compared to wild-type individuals (10.3 ± 0.5 dB, Table 2-C).

Again, the difference was larger in the female subgroup, where the mean difference between air and bone conduction PTAt for all frequencies was significantly ($p < 0.003$) smaller in ClC-Kb^{T481S} carriers (7.0 ± 0.9 dB) than in wild-type individuals (10.7 ± 0.8 dB). In the male population the mean difference between air and bone conduction PTAt for all frequencies was similar ($p < 0.82$) in ClC-Kb^{T481S} carriers (10.0 ± 0.7 dB) and wild-type individuals (9.5 ± 1.4 dB).

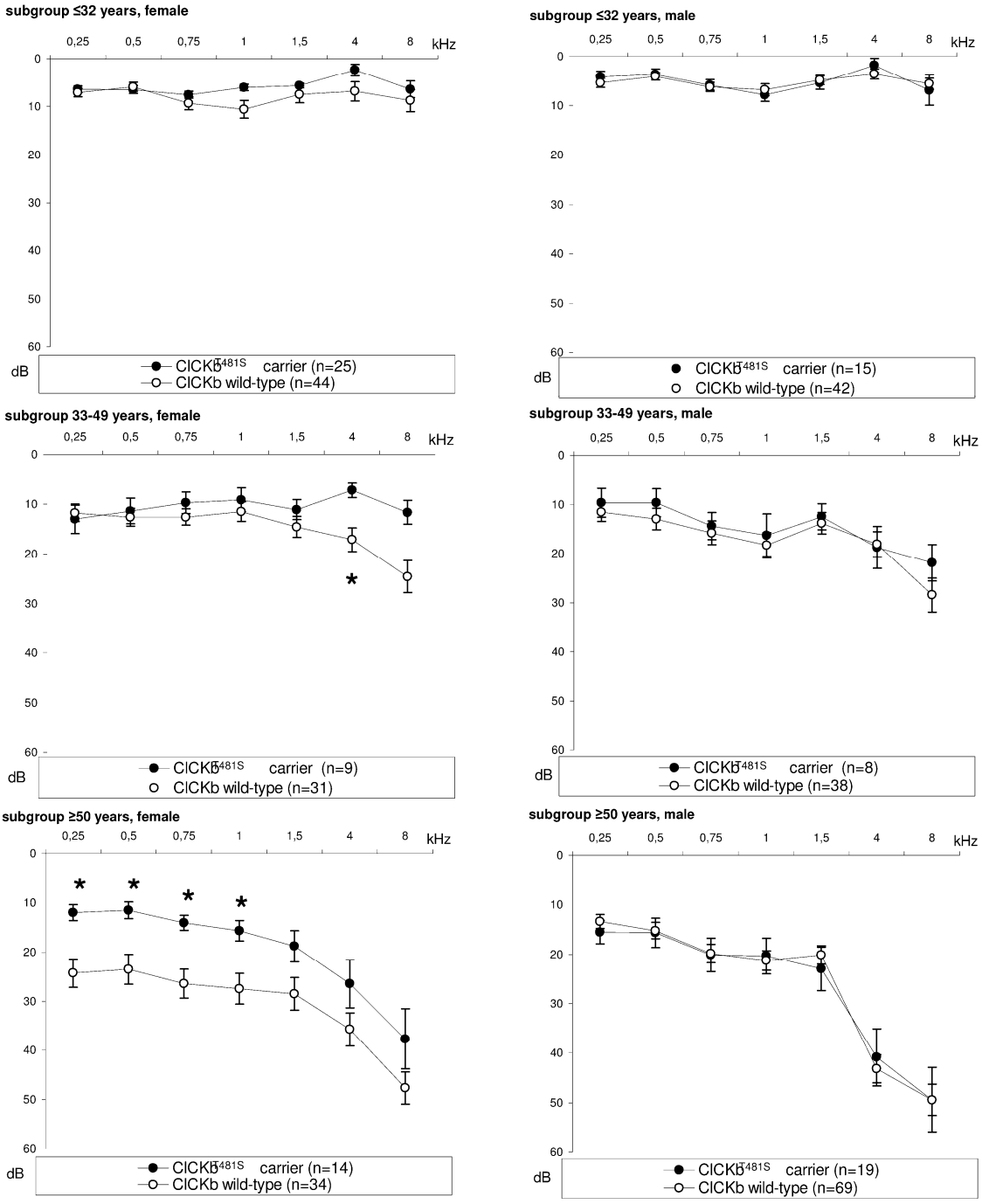


Fig. 2-B

Impact of the ClC-Kb^{T481S} mutation on PTAt for air conduction in females and males of different age groups. Arithmetic means ± SEM of the thresholds for air conduction of wild-type female (left) and male (right) individuals (open symbols) and of carriers of the ClC-Kb^{T481S} mutation (closed symbols) in different age groups are shown. The upper panel displays individuals ≤32 years (n = 25 carrier / 44 wild-type females, 15 carrier / 42 wild-type males), the middle panel 33-49 years (n = 9 carrier / 31 wild-type females, 8 carrier / 38 wild-type males), and the lower panel ≥50 years (n = 14 carrier / 34 wild-type females, 19 carriers / 69 wild-type males). * indicates values significantly different from wild-type individuals ($P<0.05$).

Table 2-C

Difference in hearing levels (in dB) calculated by means of air conduction minus bone conduction in ClC-Kb^{T481S} carriers compared to wild-type individuals of the complete hearing-tested population (see Table 2-A)

Frequency (Hz)	ClC-Kb/ClC-Kb	ClC-Kb^{T481S}-carrier	P values
250	12.07 ± 0.64	10.14 ± 0.94	ns
500	8.19 ± 0.54	5.43 ± 0.94	0.0358
750	9.28 ± 0.59	6.63 ± 0.87	0.0182
1000	9.99 ± 0.78	7.83 ± 0.97	ns
1500	4.32 ± 0.59	4.32 ± 0.81	ns
4000	7.27 ± 0.57	5.74 ± 0.82	ns
All frequencies	10.3 ± 0.5	8.2 ± 0.8	p<0.016

If ClC-Kb^{T481S} carriers are protected against hearing loss, then the prevalence of carriers should be higher in the subpopulation of individuals with a low hearing threshold than in the subpopulation of individuals with a high hearing threshold. This was indeed the case. As compared to the prevalence of ClC-Kb^{T481S} carriers (25.9%) in the complete population analyzed by a hearing test (n = 348), the prevalence of ClC-Kb^{T481S} carriers was significantly higher (29.7%) in individuals with a threshold <15 dB (29.7%, n = 199, p < 0.045) and significantly (p < 0.017) lower (13.2%) in individuals with a threshold >30 dB (n = 53). Again, this difference was largely due to the female population. As compared to the prevalence of ClC-Kb^{T481S} carriers (30.6%) in the complete female population analyzed by a hearing test (n = 157), the prevalence of ClC-Kb^{T481S} carriers was significantly (p < 0.010) higher (37.6%) in female individuals with a threshold <15 dB (n = 101) and significantly (p < 0.003) lower (5.0%) in female individuals with a threshold >30 dB (n = 20).

2.5 Discussion

In this study the relation between the chloride channel mutation ClC-Kb^{T481S} and tinnitus or PTAt was investigated. The present observations confirm the gain-of-function of ClC-Kb^{T481S} shown previously (52). They further demonstrate that chloride currents measured following an injection of 6 ng mRNA encoding ClC-Kb^{T481S} (mimicking homozygous carriers) are similar to the currents

obtained following 3 ng mRNA encoding ClC-Kb^{T481S} together with 3 ng encoding wild-type ClC-Kb (mimicking heterozygous carriers). Thus, at least under the in vitro experimental conditions chosen, the current saturates and does not depend on gene dose. As shown in a previous study (52), there was no evidence that blood pressure was higher in homozygous carriers than in heterozygous carriers of the polymorphism. The number of homozygous individuals was, however, too small to allow for reliable conclusions. Most importantly, the present experiments disclose some impact of enhanced activity of ClC-Kb channels on hearing performance. Individuals carrying the ClC-Kb^{T481S} mutation display significantly lower hearing thresholds. The relevance of ClC-Kb/Barttin for hearing is demonstrated by rare genetic loss-of-function defects of the β-subunit Barttin (13). Interestingly, genetic loss-of-function defects of ClC-Kb alone does not lead to severe hearing loss (127), presumably, since both ClC-Kb and ClC-Ka are expressed in the inner ear and the loss of one of the pore-forming units does not disrupt channel activity. In contrast, both ClC-Ka and ClC-Kb require the β-subunit Barttin to become functional (30, 151) and thus lack of Barttin precludes the function of both ClC-Ka/Barttin and ClC-Kb/ Barttin (13). Similarly, a simultaneous genetic loss of function defect of ClC-Ka and ClC-Kb leads to severe hearing loss (115). The function of ClC-Ka/ClC-Kb channels in the inner ear is still elusive. In theory, the channels may be involved in the generation of the K^+ rich endolymph (155), which is assumed to be accomplished by basolateral Na^+, K^+, $2Cl^-$ cotransport (91), K^+ exit into the luminal fluid across the apical membrane through KCNE1/KCNQ1 (141), and Cl^- recycling through basolateral Cl^- channels (111, 155). The lumen positive transepithelial potential difference is thought to drive Cl^- across the paracellular shunt into the lumen. Water movement is presumably accomplished by water channels (12). Accordingly, hearing is impaired by pharmacological inhibition of the Na^+, K^+, $2Cl^-$ cotransport during excessive doses of loop diuretics (50), following inhibition of KCNE1 (41), after genetic knockout of the Na^+, K^+, $2Cl^-$ cotransporter NKCC1 (25) and with loss-of-function mutations in KCNE1/KCNQ1 (101, 146). The present observations disclose that hearing may be affected by a gain-of-function mutation. It appears

that the polymorphism affects the air conduction without significantly altering bone conduction. As evident from Table 2-C, the gain-of-function significantly increases the difference between air and bone conduction, which should reflect the middle ear function. Thus, the possibility must be kept in mind that the ClC-Kb^{T481S} mutation affects hearing not by altering the inner ear function but by some other mechanism, possibly secondary to altered renal function. In any case, the impact of ClC-Kb^{T481S} is subtle and by no means comparable to the severe hearing loss observed in individuals lacking functional Barttin (13) or both functional ClC-Ka and ClC-Kb (115). In view of the dramatic influence of the amino acid exchange on channel activity (51), it is surprising that the impact of the polymorphism on blood pressure (52) and on hearing threshold remains moderate. Presumably, increased Cl^- channel activity clamps the cell membrane potential to the electrochemical equilibrium for Cl^-. Cytosolic Cl^- activity and thus cell membrane potential and potential sensitive cellular function may then depend on other carriers or channels. Possibly for the same reason, homozygosity has little additional impact on the phenotype. Moreover, the difference is only significant in female individuals. At present, no causal explanation can be provided for the preferential effect of the mutation on females. In theory, wild-type males would not benefit from the polymorphism, if testosterone stimulated ClC-Kb and thus, Cl^- channel activity was no more limiting in males. Alternatively, estrogen-dependent down regulation of ClC-Kb or ClC-Ka in females could account for increased sensitivity to ClC-Kb function. Indeed, estrogen-dependent expression of a close member of the ClC-family has been reported before (100). However, it remains elusive how the mutation affects hearing, and further studies are needed to explore the correlation of hearing with ClC-Kb^{T481S}. It seems reasonable for us to speculate that the gain-of-function of ClC-Kb^{T481S} might cause individuals to be prone to developing tinnitus. In the population studied, no clear evidence supporting this view was found, even though there was a tendency to higher expression of ClC-Kb^{T481S} in the patients suffering from tinnitus (23.6% compared to 18.8% in healthy individuals). In conclusion, the ClC-Kb^{T481S} gain-of-function mutation of the pore-forming ClC-Kb leads to a subtle decrease of the PTAt. Nevertheless, due to

the high prevalence of individuals carrying ClC-Kb^{T481S}, approaching 20% of Caucasians and 40% of Africans (51), the mutation could, in combination with other genetic or environmental factors, significantly modify the development of clinically relevant hearing loss.

2.5.1 Authors' contributions

To this publication A. Frey and A. Lampert contributed equally in carrying out the study and the patient examinations. A. Frey performed the statistical analysis with the assistance of F. Lang. The genetic analysis was performed by M. Schwab and E. Schaeffeler. F. Lang conceived and designed the study and wrote the final manuscript. All authors read and approved the final manuscript version.

2.5.2 Acknowledgements

The authors were supported by the Deutsche Forschungsgemeinschaft and the Robert-Bosch Foundation, Stuttgart.

3 Further influences on phenotype due to the ClC-Kb^{T481S} mutation.

3.1 Introduction

As a part of the research project several more parameters were recorded and analyzed. The investigation was widened to cover the influences of the ClC-Kb^{T481S} variant on blood pressure, excretion parameters, thyroid function parameters as well as personality traits. Therefore the following investigations were performed.

3.2 Material and methods

Basically the materials and methods of this part are the same as those listed in chapter 2.3. Especially the mutational analysis and statistical evaluation as have been described there. Therefore a detailed description of all methods should be unnecessary. Only the additional methods will be described:

3.2.1 Volunteers

The study protocol previously described was continued, which resulted in a total of 586 unrelated volunteers (256 female, 330 male) recruited for this study. A written informed consent was obtained from all participants after a complete description of the study was provided to them. A broad questionnaire about their own and family medical history was completed by 372 subjects. Additionally, the German version of the NEO-FFI (23) was completed by 401 subjects. It consists of 60 items and allows reliable and valid assessment of personality along the dimensions of neuroticism, extraversion, openness to experiences, agreeableness and conscientiousness (16). The study was approved by the ethics committee of the University of Tübingen.

3.2.2 Biometrical data

Blood pressure was determined with automatic cuffs once during the day in sitting position after at least 15 minutes of rest (IntelliSense; Omron Matsusaka, Japan) (n=484), and repeatedly every 30 minutes for 24 hours (TM-2430;

Bosch und Sohn, Jungingen, Germany) for the individuals who volunteered for 24-hour blood pressure measurements (n=348). Urine was collected over 24 hours (n=336). The glomerular filtration rate (GFR) was calculated from the creatinine clearance, whereby creatinine concentrations were determined using a BAYER Advia Centaur analyzer with reagents supplied by the manufacturer (Bayer Leverkusen, Germany). Sodium, chloride and potassium concentrations were determined by the appropriate electrodes (Advia 1650; Bayer Leverkusen, Germany); calcium concentrations were determined by photometric determination of cresolphthalein and ammonium-phosphomolybdate complexes (Advia 1650; Bayer Leverkusen, Germany).

The serum concentrations of thyroid-stimulating hormone (TSH), free T3 (FT3) and free T4 (FT4) were determined utilizing a BAYER ADVIA Centaur immunoassay with direct chemiluminescence technology (BAYER Diagnostics, Fernwald, Germany).

3.3 Results

The number of homozygous carriers of ClC-Kb^{T481S} (n=10, 1.7%) was too small for a separate statistical analysis. Therefore homozygous (ClC-Kb^{T481S}/ClC-Kb^{T481S}) as well as heterozygous (ClC-Kb^{T481S}/ClC-Kb) individuals are summarized under the term '*ClC-Kb^{T481S} carrier*' in the following. The proportion of ClC-Kb^{T481S} carriers was 21.0% in this sample. Table 3-A shows the descriptive data of the population.

Table 3-A

Age, sex, body size of the investigated population. Data are given as means ± SEM.

	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
number	463 (195 ♀, 268 ♂)	123 (61 ♀, 62 ♂)	ns
age (years)	36.0 ± 0.8	37.4 ± 1.7	ns
body size (cm)	173.4 ± 0.4	173.2 ± 0.8	ns
weight (kg)	71.4 ± 0.6	70.4 ± 1.2	ns

3.3.1 Blood pressure

To assess the blood pressure, 484 individuals volunteered for the single measurement and 348 individuals for the long term measurement. The median

duration of the long term measurement was 23.5 hours, the 25% quartile was 23.0 hours, and the 75% quartile 24.0 hours. 346 individuals wore the measurement apparatus during daytime (6-22h), and 339 also wore it during night-time (22-6h). The results of the blood pressure measurements are summarized in Table 3-B.

Table 3-B

Blood pressure (BP) of single and long term measurements given as mean ± SEM (mmHg), whole population.

	ClC-Kb/ClC-Kb	ClC-Kb^{T481S}-carrier	P values
Single systolic BP	130.6 ± 0.9	134.2 ± 1.7	0.0702
Single diastolic BP	81.2 ± 0.6	84.0 ± 1.1	**0.0050**
Systolic average BP 6-22h	128.2 ± 0.8	129.3 ± 1.5	0.4955
Diastolic average BP 6-22h	77.5 ± 0.5	78.9 ± 0.8	0.1856
Systolic average BP 22-6h	112.6 ± 1.0	113.0 ± 1.8	0.8523
Diastolic average BP 22-6h	66.5 ± 0.5	66.8 ± 1.0	0.7869

In the analysis of the subgroup of the 20 to 50 year-old individuals (n=372), the impact of the ClC-Kb^{T481S} genotype on blood pressure (Table 3-C) was more pronounced and the difference was, at least for the single measurement, statistically significant.

The incidence of hypertensive diastolic blood pressure values (≥ 90 mmHg) due to the single measurement was significantly higher in ClC-Kb^{T481S} carriers compared to ClC-Kb/ClC-Kb wild-type individuals with an odds ratio of 1.75 (95% CI 1.08–2.84; p=0.0334). On the other hand, the incidence of systolic blood pressure values in the single measurement was higher in ClC-Kb^{T481S} carriers, the odds ratio was 1.30 (95% CI 0,81–2,08) but it did not reach statistical significantce (p=0.272).

Table 3-C

Blood pressure (BP) of single and long term measurements given mean ± SEM (mmHg), of the subgroup of 20-50 year-old individuals.

	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
Single systolic BP	127.2 ± 1.0	132.8 ± 1.9	**0.0094**
Single diastolic BP	79.0 ± 0.6	84.0 ± 1.3	**0.0002**
Systolic average BP 6-22h	125.8 ± 0.9	127.7 ± 1.7	0.3242
Diastolic average BP 6-22h	76.4 ± 0.6	77.8 ± 1.0	0.1934
Systolic average BP 22-6h	109.9 ± 1.2	112.2 ± 2.1	0.3535
Diastolic average BP 22-6h	65.0 ± 0.6	65.3 ± 1.1	0.8476

3.3.2 Excretion parameters

There were 334 volunteers for the 24h urine collection. They started in the morning by emptying their bladder and then collected their urine during that day into a container provided for them. The exact times of beginning and end of the collection period were recorded. The median collection period was 24.0 hours, the 25% quartile was 23.8 hours and the 25% quartile 24.0 hours. The median collected urine amount was 2000.0 ml, the 25% quartile 1412.5 ml and the 75% quartile 2568.8 ml. The results of the laboratory analysis are presented in Table 3-D.

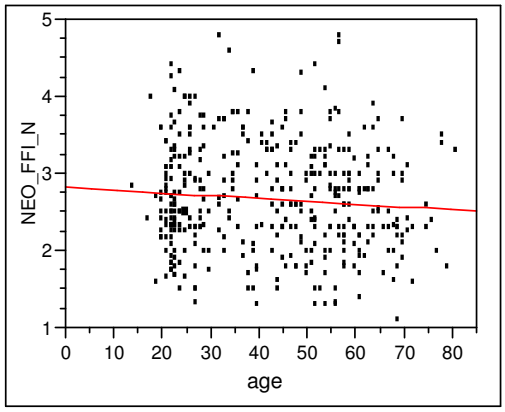
Table 3-D

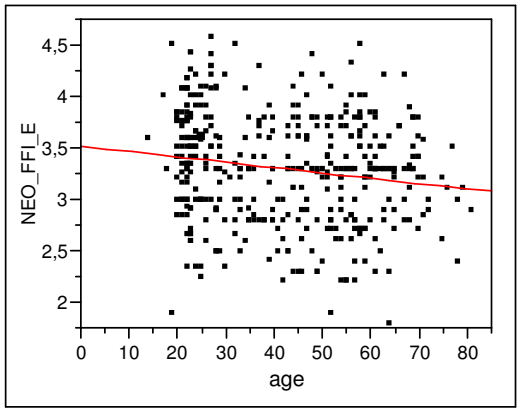
Summary of glomerular filtration rate (GFR), fractional excretion (FE), plasma concentrations ($[X]_P$) and urinary excretion (UV_X). Data are given as means ± SEM.

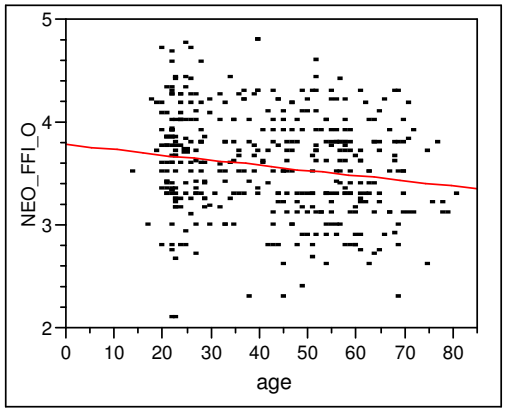
	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
GFR, mL/min	93.3 ± 1.8	86.0 ± 3.0	**0.0382**
FE_{Na}, %	0.93 ± 0.02	0.96 ± 0.03	0.4194
FE_K, %	14.11 ± 0.39	15.53 ± 0.66	0.0647
FE_{Ca}, %	1.60 ± 0.05	1.67 ± 0.08	0.4801
FE_{Cl}, %	1.35 ± 0.05	1.37 ± 0.09	0.3535
$FE_{Cortisol}$, %	0.55 ± 0.04	0.51 ± 0.08	**0.0002**
$[Na]_P$, mmol/l	141.7 ± 0.1	142.0 ± 0.2	0.3327
$[K]_P$, mmol/l	4.3 ± 0.1	4.2 ± 0.1	0.7113
$[Ca]_P$, mmol/l	2.3 ± 0.1	2.4 ± 0.1	**0.0189**
$[Cl]_P$, mmol/l	104.0 ± 0.3	104.7 ± 0.7	0.3509
$[Cortisol]_P$, nmol/l	392.7 ± 9.0	430.2 ± 15.3	**0.0348**
UV_{Na}, mmol/24h	174.4 ± 5.1	165.9 ± 8.6	0.3938
UV_K, mmol/24h	76.7 ± 2.6	73.9 ± 4.4	0.5813
UV_{Ca}, mmol/24h	5.0 ± 0.2	4.8 ± 0.3	0.5722
UV_{Cl}, mmol/24h	186.3 ± 9.3	182.2 ± 19.4	0.5810
$UV_{Cortisol}$, nmol/24h	244.0 ± 15.5	216.0 ± 27.9	0.1324

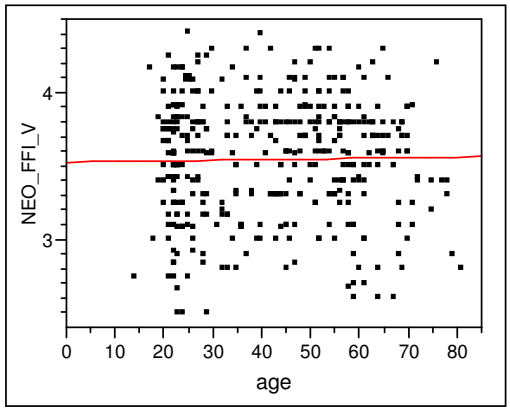
3.3.3 NEO-FFI

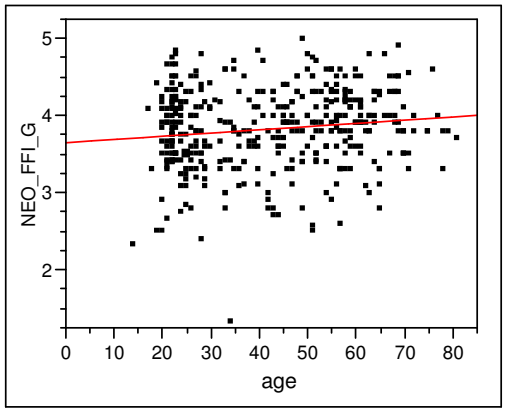
To assess personality traits the NEO-FFI five factor inventory was completed by 401 individuals out of the study population. Eight individuals were excluded from the analysis of the NEO-FFI scores because of known psychiatric disorders. Linear regression showed age-dependent changes within the personality traits (see Fig. 3-A and Table 3-E). Because of the age dependency in the impact of the ClC-Kb^{T481S} mutation, the analysis was split into two separate age groups. The cut-off was set at 50 years. The first group (<50 years) contained 238 individuals, median age 28 years, 25% quartile 23.0 years, 75% quartile 40.0 years. The second group (≥50 years) contained 155 individuals, median age 59 years, 25% quartile 55.0 years, 75% quartile 65.0 years.











	Intercept	P value	X(age)	P value
N	2.83 ± 0.09	**<0.0001**	-0.0036 ± 0.002	0.0841
E	3.52 ± 0.07	**<0.0001**	-0.0051 ± 0.001	**0.0012**
O	3.79 ± 0.07	**<0.0001**	-0.0050 ± 0.001	**0.0008**
V	3.53 ± 0.05	**<0.0001**	0.0004 ± 0.001	0.7128
G	3.66 ± 0.07	**<0.0001**	0.0043 ± 0.001	**0.0052**

Table 3-E

Parameters of the regression formula R(N|E|O|V|G)=Intercept + age * X(age), with appropriate p values. Data are given as means ± SEM.

Fig. 3-A

Linear regression plots of neuroticism (N), extraversion (E), openness to experiences (O), agreeableness (V) and conscientiousness (G) in relation to age, the red line shows the plot of the regression formula (see Table 3-E).

Table 3-F

Measured values for neuroticism (N), extraversion (E), openness to experiences (O), agreeableness (V) and conscientiousness (G) within the two age groups. P values for testing between ClC-Kb wild-type and ClC-Kb^{T481S} carrier individuals. Data are given as means ± SEM.

Age < 50 years	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
N	2.73 ± 0.05	2.74 ± 0.10	0.9680
E	3.36 ± 0.04	3.30 ± 0.07	0.5078
O	3.61 ± 0.04	3.70 ± 0.07	0.2690
V	3.56 ± 0.03	3.46 ± 0.06	0.1095
G	3.81 ± 0.04	3.64 ± 0.08	**0.0266**

Age ≥ 50 years	ClC-Kb/ClC-Kb	ClC-Kb^{T481S} carrier	P values
N	2.65 ± 0.06	2.36 ± 0.11	**0.0200**
E	3.18 ± 0.05	3.40 ± 0.08	**0.0121**
O	3.49 ± 0.04	3.48 ± 0.08	0.9129
V	3.55 ± 0.04	3.57 ± 0.06	0.8892
G	3.95 ± 0.05	4.03 ± 0.08	0.3714

These findings indicate slightly lower levels of conscientiousness for ClC-Kb^{T481S} carriers within the age group of individuals younger than 50 years. In the age group of 50 year-old and older individuals neuroticism was slightly lower and extraversion slightly higher if the ClC-Kb^{T481S} feature was present.

It seemed worthwhile to check whether there was evidence for correlations between tinnitus and personality traits within the sample. The extraversion was clearly lower (3.21 ± 0.03) in persons suffering from tinnitus (n=235) compared to individuals not suffering from tinnitus (3.43 ± 0.04, n=158), the difference was highly significant (p=0.00006). Also, openness to experiences was clearly lower in persons suffering from tinnitus (3.52 ± 0.03) compared to persons not suffering from tinnitus (3.65 ± 0.04), and the difference was again statistically significant (p=0.0103). The other three personality traits covered by the NEO-FFI showed only marginal differences between the two groups, which werew not statistically significant.

There were 375 individuals who provided information about their educational level. The median age of this group was 43 years, 25% quartile

25.0 years, 75% quartile 57.0 years. The comparison of personality traits between individuals with a people with a high school diploma (General Certificate of Secondary Education, GCSE) (n=272) and individuals with less education showed interesting results. Individuals with a GCSE clearly showed lower levels of neuroticism, higher levels of extraversion, higher levels of openness for experiences, but interestingly lower levels of conscientiousness (see Table 3-G).

Table 3-G

Measured values for neuroticism (N), extraversion (E), openness to experiences (O), agreeableness (V) and conscientiousness (G) within the two groups. P values for testing between individuals with a GCSE and individuals without a GCSE. Data are given as means ± SEM.

	GCSE	No GCSE	P values
N	2.63 ± 0.04	2.81 ± 0.07	**0.0211**
E	3.32 ± 0.03	3.19 ± 0.05	**0.0204**
O	3.67 ± 0.03	3.35 ± 0.05	**<0.0001**
V	3.56 ± 0.03	3.55 ± 0.04	0.8505
G	3.79 ± 0.03	3.96 ± 0.05	**0.0036**

3.3.4 Thyroid function parameters

To assess the function of the thyroid gland, hormone levels of free 3,5,3',5'-tetraiodothyronine (FT4), free 3,3',5-triiodothyronine (FT3) and Thyroid-stimulating hormone (TSH) were measured in the plasma of 68 individuals. The average serum values of TSH, FT3 and FT4 were 1.38 ± 0.1 mU/l, 5.18 ± 0.1 pmol/l and 16.9 ± 0.3 pmol/l. TSH was significantly (p=0.0367) lower (1.27 ± 0.14 mU/l) in ClC-Kb^{T481S} carriers compared to ClC-Kb wild-type individuals (1.69 ± 0.14 mU/l). Also FT4 was lower in ClC-Kb^{T481S} carriers (16.7 ± 0.42 pmol/l) compared to (17.10 ± 0.43 pmol/l) in ClC-Kb wild-type individuals. The FT3 levels were lower too (5.01 ± 0.15 pmol/l) in individuals with the ClC-Kb^{T481S} variant compared to wild-type ClC-Kb individuals (5.34 ± 0.14 pmol/l); however, these two latter differences were not significant.

3.4 Discussion

In this study the relation between the chloride channel mutation ClC-Kb^{T481S} and blood pressure, excretion parameters, personality traits as well as thyroid

function parameters was investigated. The number of homozygous carriers was too small for a separate statistical evaluation. Additionally, as has been already shown,there is no evidence that the gene dose influences channel activity (51). Therefore, the population was divided into homozygous (ClC-Kb^{T481S}/ClC-Kb^{T481S}) and heterozygous (ClC-Kb^{T481S}/ClC-Kb) individuals on the one hand, and wild-type (ClC-Kb/ClC-Kb) individuals on the other. The present experiments show that enhanced activity of ClC-Kb channels impacts blood pressure. The influence of the activating mutation ClC-Kb^{T481S} on blood pressure has already been shown (52). There is clear genetic evidence that the presence of ClC-Kb in the ascending thick limb of Henle's loop is important for proper salt reabsorption. Disruption of ClC-Kb leads to Bartter syndrome type III (127) which results in severe renal salt loss. Also disruption of Barttin or both ClC-Ka and ClC-Kb (simultaneously) leads to renal failure (30, 115). Salt reabsorption is also one of the key factors in blood pressure regulation, which is strongly influenced by the renin angiotensin aldosterone system (39). But there are also other polymorphisms, especially a common SGK1 variant which has been associated with increased blood pressure (19, 53). Interestingly, SGK1 is also assumed to regulate ClC-Kb and therefore could influence the effect of ClC-Kb^{T481S} on blood pressure (73). Most recently, data indicated a strong upregulation of both NCC and ClC-K along the DCT in salt-sensitive hypertension, underlining the relevance of ClC-K channels in hypertension (20).

However, the observed influence of ClC-Kb^{T481S} in this study was subtle and only significant for the diastolic values of the single blood pressure measurement. The reason why the long term measurement did not yield significant results could be that the measurements took place during normal daily activities, which were not standardized within the population (54, 62, 123, 139)., Admittance as in-patient or multiple accelerometry combined with interactive monitoring during the measurement period could provide more reliable, long-term results (105). Other investigations with larger populations contradicted the present findings with respect to blood pressure (32, 69, 129). However, results that indicate higher allele frequencies for ClC-Kb^{T481S} in hypertensive subjects were also observed (154).

As evident from Table 3-C the impact on blood pressure was most likely due to the subpopulation of the 20 to 50 year-old individuals. Reduced renal function in patients older than 50 years also decreases the impact of renal tubular blood pressure regulation (1). Hence the difference in blood pressure might be easier to detect in the younger age group. What is more, high blood pressure leads to renal damage (8) as another premise for interpretation of the data. This could explain a lower glomerular filtration rate in ClC-Kb^{T481S} carriers compared to ClC-Kb wild-type individuals. As evident from Table 3-D, the higher values for glomerular filtration rate in ClC-Kb^{T481S} carriers match with previous findings (52). The reduced GFR despite higher blood pressure could of course be the result of enhanced ClC-Kb channel activity, because ClC-Kb is expressed in the macula densa which plays a key role in tubuloglomerular feedback (42).

As to electrolytes, only calcium showed slightly higher plasma levels in ClC-Kb^{T481S} carriers compared to wild-type individuals. However, when ClC-Kb is activated, we should expect to see the opposite of the Bartter syndrome phenotype, which is accompanied by hypercalciuria and nephrolithiasis (46). Interestingly, significantly higher cortisol levels were observed in the group of ClC-Kb^{T481S} carriers. This finding fits very well with the higher plasma calcium levels (90). The fractional excretion of calcium was also higher in ClC-Kb^{T481S} carriers, though the difference was not statistically significant. On the other hand the fractional excretion of cortisol was significantly lower in the group of ClC-Kb^{T481S} carriers. This, in turn, could explain the higher levels of plasma cortisol. However, the reason for lower fractional cortisol excretion in ClC-Kb^{T481S} carriers remains elusive. To clarify this, it could be helpful to also analyze the excretion of cortisol metabolites (64).

The NEO-FFI recordings confirm that neuroticism, extraversion, openness to experiences, agreeableness and conscientiousness, which was described previously (7, 157), are age dependent (Fig. 3-A). Several influences of single nucleotide polymorphisms on NEO-FFI personality traits have already been described (15, 26, 71, 72, 75, 112, 148). Most of them concern variations of neurophysiologcically relevant genes which encode brain-derived neurotrophic factors, serotonine transporters or receptors, neuregulin1, cytocrome CYP2A6

or monoamine oxidase A. But the effect of ClC-Kb on neuronal activity has not yet been sufficiently investigated, though Kieferle did demonstrate in 1994 the presence of ClC-K proteins within the brain tissue of rats (65). ClC-K proteins have also been shown not to be as kidney-specific as initially assumed: meanwhile ClC-Ka and ClC-Kb were detected by Northern Blot analysis within the cochlea (108) human airway epithelial cells (98) and bladder epithelia (142). Most importantly, a study with transgenic mice harbouring the enhanced green fluorescence protein (EGFP) gene driven by a human ClC-Kb gene promoter showed significant presence of ClC-Kb within tissue homogenates from heart and brain (68). Fluorescence microscopy studies verified that ClC-Kb was present within the medullary reticular formation (68). It Therefore seems reasonable to speculate that there might be a physiological explanation for the present findings concerning personality traits. Increased Cl^- conductance in ClC-Kb^{T481S} carriers could promote neuronal hyperpolarisation within the medullary reticular formation, thus leading to decreased activation. Low activity of the medullary reticular formation is assumed to correlate with extraversion (66) according to Eysenck's cortico-reticular activation theory (31). But clearly further studies are necessary to elucidate these coherences, and also to provide explanations for the observed differences in neuroticism and conscientiousness where no explanation can be given at the moment.

Lower TSH levels in ClC-Kb^{T481S} could be related to changes in blood pressure. Yet a large population based study has shown a linear positive correlation of TSH with systolic and diastolic blood pressure (10). The fact that this association is not reflected by our data suggests that the observed difference is due to the ClC-Kb^{T481S} genotype. However, the expression of ClC-Kb within cells of the endocrine regulation of thyroid hormones has not been previously described. The physiological role of ClC-Kb in the observed difference in TSH levels remains therefore elusive.

In summary, the data suggests that enhanced activity of ClC-Kb^{T481S} leads to higher blood pressure values and altered excretion parameters which have already been observed in previous studies. The new observations concerning altered personality traits and thyroid function parameters point to the

significance of ClC-Kb in these areas, too. Further research is clearly necessary to confirm the present findings and to identify possible confounding variables as well as the underlying molecular mechanisms. The conclusions derived from this data should therefore be considered as preliminary.

3.4.1 Error discussion

When analysing a single nucleotide polymorphism in a small population there is a risk of selecting individuals with a genetic background that differs from that of the population as a whole (34). Other confounding factors unique to the selected population could thus influence results. There is also a high rate of false-positive results in studies of single-nucleotide polymorphism associations (85). However, the investigated population was recruited by advertisement, the individuals were free to volunteer for parts of the investigation and the individuals were unrelated. Although, this does not rule out a population-based bias, the biometrical data suggest that the population was not significantly stratified. To proove this, a genomic control method study could be applied to the data in order to quantitatively assess the degree of stratification (106).

The genetic analysis in the study was strictly blinded and took place in other laboratories than those used for measuring other biometrical data. All measurements were performed by the same personnel and the same methods were used to avoid interobserver variability. It is therefore unlikely that the results were biased by the study's design.

4 General summary

This population-based study was performed in order to explore the influences of the gain-of-function ClC-Kb^{T481S} mutation on phenotype in humans. Heterozygous carriers constitute approximately 20% of Caucasian populations; the variant could therefore be considered as common. Since ClC-Kb has been shown to be expressed within kidney, cochlea and other tissues, genetic analysis, pure tone audiometry, 24 hour ambulatory blood pressure measurements, 24 hour urine collection and analysis as well as analysis of serum electrolytes and thyroid function parameters were performed. Additionally, the volunteers completed a NEO-FFI questionnaire as well as questionnaires about their present and past medical history.

The observations disclose that hearing may be affected by the ClC-Kb^{T481S} variant. The pure tone average hearing threshold (PTAt) for air conduction was significantly lower in ClC-Kb^{T481S} carriers than in wild-type individuals. The difference was largely due to the female population ≥50 years. The PTAt in bone conduction tended to be lower in ClC-Kb^{T481S} carriers but was not statistically significant. As compared to the prevalence of ClC-Kb^{T481S} carriers (25.9%) in the complete population analyzed by a hearing test (n = 348), the prevalence of ClC-Kb^{T481S} carriers was significantly higher (29.7%) in individuals with a PTA threshold <15 dB (29.7%) and significantly lower (13.2%) in individuals with a threshold >30 dB. The prevalence of ClC-Kb^{T481S} carriers tended to be higher in patients suffering from tinnitus than in healthy individuals, though this difference was not statistically significant.

This study also showed the influence of ClC-Kb^{T481S} on renal-related parameters. Systolic and diastolic blood pressure values tended to be higher in ClC-Kb^{T481S} carriers. The difference was highly significant for the 20-50 year old subpopulation. The prevalence of hypertensive diastolic blood pressure values was significantly higher in ClC-Kb^{T481S} carriers compared to ClC-Kb/ClC-Kb wild-type individuals. The glomerular filtration rate and fractional cortisol excretion were lower, and plasma cortisol and plasma calcium were higher in ClC-Kb^{T481S} carriers compared to ClC-Kb wild-type individuals.

Interestingly, influences on personality traits were also observed in relation to the NEO-FFI questionnaire. The scores yielded lower levels of conscientiousness for ClC-Kb^{T481S} carriers younger than 50 years. In the age group ≥50 years lower levels of neuroticism and higher levels of extraversion were observed. These observations provide evidence for the first time that ClC-Kb^{T481S} influences personality. On the other side, Individuals suffering from tinnitus showed lower levels of extraversion that were highly significant. Additionally, recordings of TSH showed significant lower plasma levels in ClC-Kb^{T481S} carriers.

Thus, it was shown that the influence of ClC-Kb^{T481S} as a common genetic variant in human beings results in a phenotype which is slightly but distinctly altered from that of the rest of the population. The most important conclusion is that the ClC-Kb^{T481S} gain-of-function mutation of the pore forming ClC-Kb leads to a subtle decrease in pure tone audiometry thresholds. Furthermore, ClC-Kb^{T481S} seems to predispose to hypertension. Individuals with a known predisposition could counteract this with dietary measures or more frequent screening tests. Of course, the relatively small number of individuals studied limits the strength of this hypothesis. Thus studies need to be carried out in other populations to confirm the association between the mutation and hearing, tinnitus, blood pressure, GFR, excretion parameters, personality traits and thyroid function. It would be interesting to carry out a more detailed study in a population of tinnitus patients that thoroughly characterized the tinnitus and included all other possible causes. Since blood pressure measurements are subject to a high variability, more accurate recordings (e.g. including multiple accelerometry) could increase the power of further studies. Finally, the new associations of ClC-Kb with cerebral functions could open new directions for research on ClC-Kb .

5 Abschließende Zusammenfassung

Diese populationsbasierte Studie wurde durchgeführt um die Einflüsse der gain-of-function Mutation ClC-Kb^{T481S} auf den menschlichen Phänotyp zu erforschen. Die Prävalenz der heterozygoten Genträger beträgt circa 20% in mitteleuropäischen Populationen und kann daher als häufig bezeichnet werden. Da nachgewiesen werden konnte, dass ClC-Kb in der Niere, der Cochlea und anderen Geweben nachweisbar ist, wurde eine genetische Analyse, Schwellenwertaudiometrie, 24 Stunden Langzeit-Blutdruckmessungen, 24 Stunden Sammelurin durchgeführt sowie Elektrolyte, Ausscheidungsparameter und Schilddrüsenparameter im Serum gemessen. Zusätzlich wurden ein NEO-FFI Fragebogen sowie Fragebögen zur Anamnese von den Studienteilnehmern ausgefüllt.

Die Beobachtungen enthüllten, dass die Hörfähigkeit wahrscheinlich durch ClC-Kb^{T481S} beeinflusst wird. Die Schwellenwertaudiometrie für Luftleitung lieferte signifikant niedrigere Ergebnisse für ClC-Kb^{T481S} Genträger verglichen mit Wild-Typ Individuen. Der Unterschied ist großteils auf die weibliche Population ≥50 Jahren zurückzuführen. Die Schwellenwertaudiometrie für Knochenleitung neigte ebenfalls zu niedrigeren Werten für ClC-Kb^{T481S} Genträger, der Unterschied war jedoch nicht signifikant. Verglichen mit der Prävalenz von ClC-Kb^{T481S} Genträgern in der gesamten Population (25,9%) welche an der Schwellenwertaudiometrie teilgenommen hat (n=348), ist die Prävalenz von ClC-Kb^{T481S} Genträgern in Individuen mit einem durchschnittlichen Schwellenwert <15 dB signifikant höher (29,7%) und in Individuen mit einem durchschnittlichen Schwellenwert >30 dB signifikant niedriger (13,2%). Die Prävalenz von ClC-Kb^{T481S} Genträgern tendierte unter Patienten die an Tinnitus leiden höher zu sein als unter gesunden Individuen. Dieser Unterschied war jedoch nicht statistisch signifikant.

Ebenfalls wird der Einfluss der ClC-Kb^{T481S} Variante auf renal abhängige Parameter durch diese Studie untersucht. Systolisch und diastolische Blutdruckwerte tendierten zu höheren Werten in ClC-Kb^{T481S} Genträgern. Die Differenz war höchst signifikant innerhalb der 20 bis 50 Jahre alten

Subpopulation. Die Prävalenz von hypertensiven diastolischen Blutdruckwerten war in ClC-Kb^{T481S} Genträgern signifikant höher, verglichen mit ClC-Kb/ClC-Kb Wild-Typ Individuen. In ClC-Kb^{T481S} Genträgern waren die glomeruläre Filtrationsrate und die fraktionelle Cortisolausscheidung signifikant niedriger, sowie der Plasma-Cortisolspiegel und der Plasma-Kalziumspiegel signifikant höher, im Vergleich zu ClC-Kb Wild-Typ Individuen.

Interessanterweise wurden ebenfalls Einflüsse auf Persönlichkeits-eigenschaften durch den NEO-FFI Fragebogen festgestellt. Die Einzelfaktoren ergaben niedrigere Werte für Gewissenhaftigkeit bei ClC-Kb^{T481S} Genträgern die jünger als 50 Jahre sind. In der Altersgruppe ≥50 Jahre wurden niedrigere Werte für Neurotizismus sowie höhere Werte für Extraversion im Vergleich zur normalen Genvariante festgestellt. Diese Beobachtungen beschreiben erstmalig einen Einfluss von ClC-Kb^{T481S} auf die Persönlichkeit. Individuen die an Tinnitus leiden wiesen andererseits höchst signifikant niedrigere Werte für Extraversion auf. Zusätzlich zeigte TSH signifikant niedrigere Werte im Plasma von ClC-Kb^{T481S} im Vergleich mit ClC-Kb Wildtyp Individuen.

Daher konnte die Bedeutung des verbreiteten Polymorphismus ClC-Kb^{T481S} für verschiedene Aspekte des menschlichen Phänotyps gezeigt werden, welche leicht aber deutlich vom Rest der Population verschieden sind. Der wichtigste Schluss ist, dass der ClC-Kb^{T481S} gain-of-function Polymorphismus zu einer subtilen Reduktion von Schwellenwerten in der Reintonaudiometrie führt. Durch die Mutation kann eine Prädisposition für Bluthochdruck angezeigt werden. Individuen mit einer bekannten Prädisposition könnten durch diätetische Maßnamen oder häufigere Kontrolluntersuchungen einem Ansteigen den Blutdruckes gegensteuern.

Selbstverständlich ist eine wichtige Einschränkung der Hypothese die relativ kleine Anzahl von untersuchten Individuen. Daher sind weitere Studien an anderen Populationen notwendig um die Assoziationen zwischen ClC-Kb^{T481S} und Gehör, Tinnitus, Bluthochdruck, glomerulärer Filtrationsrate, Ausscheidungsparametern, Persönlichkeit sowie Schilddrüsen-funktionsparametern zu bestätigen. Insbesondere wäre es interessant eine Studie an Tinnituspatienten durchzuführen die mit einer gründlichen

Charakterisierung des Tinnitus sowie anderer individueller Erklärungsmöglichkeiten für die Erkrankung verbunden ist. Da Blutdruckmessungen einer hohen Variabilität unterliegen, wären auch Studien mit einer erhöhten Genauigkeit der Messwerte, z.B. in Verbindung mit multipler Beschleunigungsmessung ein Weg um die statistische Teststärke zu erhöhen. Schließlich könnten die neuartigen Assoziationen von ClC-Kb mit cerebralen Funktionen die Forschung bezüglich ClC-Kb in eine neue Richtung führen.

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7 Abbreviations

ATL	ascending thin limb
BP	blood pressure
BSND	Barttin gene symbol
cAMP	cyclic
CBS	cyclic adenosine monophosphate
CD	collecting duct
CFTR	cystic fibrosis transmembrane conductance regulator
CNT	connecting tubule
CPP	2-p-chlorophenoxypropionic acid
cRNA	complementary RNA
dB	decibel
DCT	distal convoluted tubule
DIDA	4,4´-Diisothiocyanostilbene-2,2´-disulfonacid
DIDS	4,40-diisothiocyanatostilben-2,20-disulphonic acid
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
FE	fractional excretion
FT3	free 3,3',5-tri-iodothyronine
FT4	free 3,5,3',5'-tetraiodothyronine (Thyroxine)
GABA	gamma-aminobutyric acid
GCSE	General Certificate of Secondary Education
GFR	glomerular filtration rate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hz	Hertz
KO	knock-out
mRNA	messenger RNA
NCC	thiazide-sensitive NaCl cotransporter
NCL	neuronal ceroid lipofuscinoses
NEO-FFI	neuroticism extraversion openness for experiences - five factor inventory
ns	not significant
PTAt	pure tone average hearing threshold
RAAS	renin - angiotensin - aldosterone system
SEM	standard error of the mean
T3	3,3',5-tri-iodothyronine
T4	3,5,3',5'-tetraiodothyronine (Thyroxine)
TAL	thick ascending limb
TSH	thyroid-stimulating hormone (Thyrotropin)
UV	urinary excretion
WT	wild-type

8 Acknowledgements

First of all I would like to thank my supervisor, Prof. Florian Lang, for giving me the opportunity to write this doctoral thesis. I thank him especially for his help with all the scientific aspects as well as with the data analysis. I am very grateful for all the support he gave me during this project. It was also a pleasure to work in his institute as all doors were opened when necessary, and the working atmosphere was overall both open and productive. Special thanks also to Undine Lang who gave me the opportunity to participate in one of her projects. I also thank all the co-authors of the papers we published for their support.

My most devoted thanks goes to Elfriede Faber for her continuous support, for explaining every detail in the lab to me, and for countless pieces of advice. Without her this study would not have been possible.

My honest thanks is due to Lejla Subasic and Tanja Loch who always did everything to help even though they were themselves always busy. Special thanks is due to Peter and Charly who helped by providing their fabulous technical constructions from the workshop. Many thanks are also due to Dr. Apfel who organized the acquisition of many pieces of labroratory equipment. From the institute I also want to thank Adrian Lupescu, Ahmad Akel, Andrea Weible, Britta, Christoph Böhmer, Diana, Elisa Metzger, Faruk Subasic, Ferruh Arthunc, Helga Reimer, Ivonne Gruner, Marion Striegel, Monika Palmada, Monika Weber, Nunzi, Oana Ureche, Perikles Simon, Prof. Gülch, Prof. Heinle, Rexhep Rexhepaj, Stephan Huber, Teresa Ackermann, Ulrike Henrion, Uschi Pascht, Uwe Schüler, and all the others who are not listed here for their warm welcome in the institute, for their help and advice and also their encouraging and cheering conversation.

Most importantly I want to thank my parents for their support and how they believed in me, especially while preparing the manuscript.

And finally, I also want to thank my successors, Andrea Ubbenhorst, Melanie Glocke and Sarah Striebich, for their patience and their commitment. I wish them much succes with their own projects!