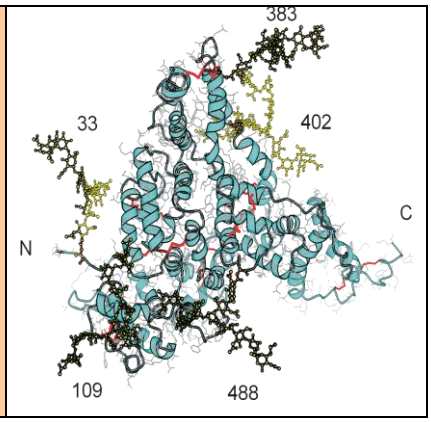
Projects Dieplinger Lab

Work in our research group has concentrated in the past 25 years on the metabolism and genetics of various components of the human lipid/lipoprotein system and their impact on modern widespread diseases such as atherosclerosis, neurodegeneration, and cancer. Recently, we have been focusing on the vitamin E-binding plasma protein afamin and its various functions. In addition, we continue to investigate (patho)-physiological functions of other important components of the lipoprotein family including lipoprotein(a) [Lp(a)] and apolipoprotein A-IV (apoA-IV).

Afamin – a multipurpose protein for all stages of life

Our group previously identified afamin, a novel vitamin E-binding glycoprotein and member of the albumin gene family on chromosome 4q11-q13 (structure model taken from (1)). Afamin is primarily expressed in the liver and secreted into plasma, but also occurs in substantial amounts in extravascular fluids (1, 2). We have been employing cell culture, animal model, biochemical-functional and clinical-epidemiological approaches to focus our research on afamin's possible function in fertility, neuroprotection, cancer and cardiovascular diseases.



Afamin and fertility/infertility: The role of afamin in male and female fertility was discovered with the afamin knock-out mouse model. These mice were completely infertile even at the chimeric stage of afamin gene deletion. Male mice showed, in addition, disrupted testis histology, impaired spermatogenesis and decreased testis organ size (Fig. 1). The central importance of afamin in fertility could be further supported by completely restoring fertility and testes histology after exogenous application of recombinantly expressed mouse afamin in previously infertile chimeric animals.

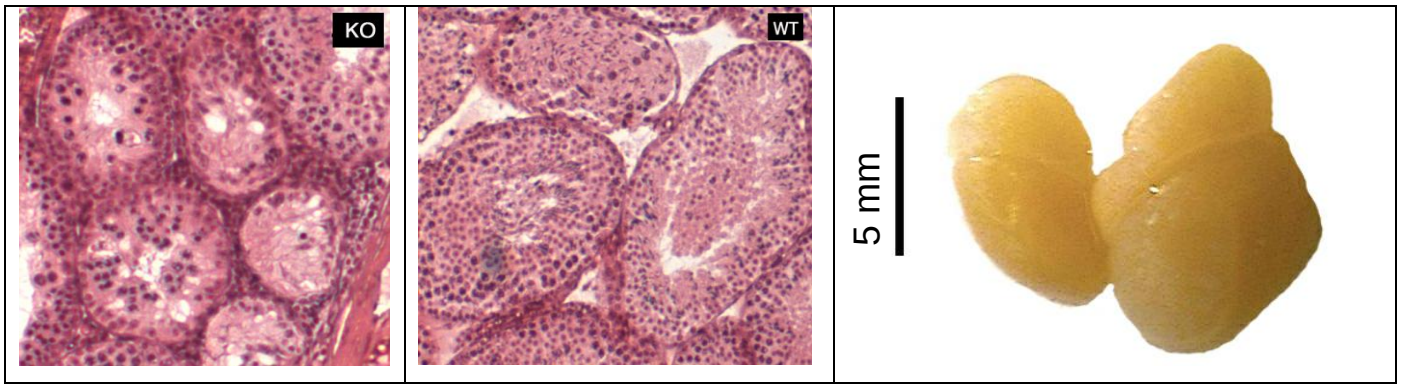


Figure 1: **Substantially impaired spermatogenesis in chimeric afamin knock-out mice (left panel) as compared to wildtype littermates (middle panel). In addition, chimeric animals exhibit grossly diminished testis organ size (left organ) as compared to wildtype littermates (right organ, right panel).**

These results from a gene-deleted mouse model indicate a central role of afamin in fertility possibly due to its vitamin E-binding properties. So far, no association has been found between afamin and infertility in humans.

The neuroprotective role of afamin: In a chicken neuron primary cell culture model, exogenously added vitamin E-loaded afamin was demonstrated to be neuroprotective (3). In vitro transport assays using cell culture models simulating the blood-brain barrier revealed highly facilitated vitamin E transport by afamin and surprisingly also discovered afamin expression in cerebrovascular endothelial cells forming the blood-brain barrier as well as in various other brain-specific cell types (Fig. 2) (4).

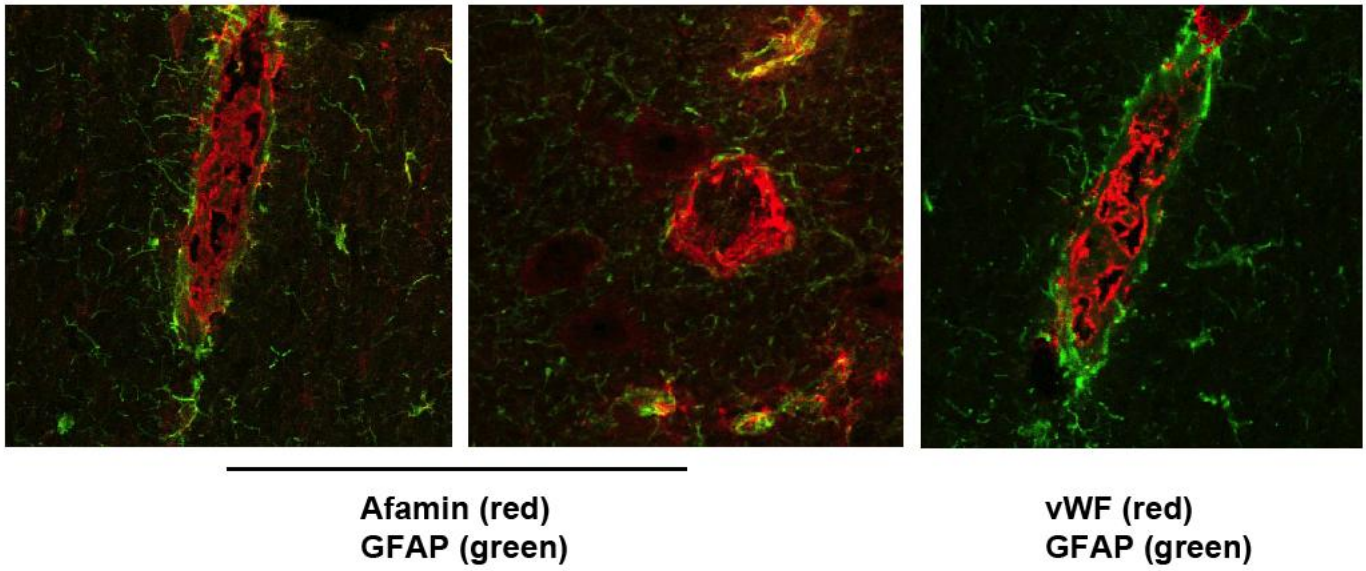


Figure 2: **Double immunofluorescence of porcine brain sections shows co-staining of afamin with GFAP (astrocyte marker) and vWF (endothelial marker) (4).**

Afamin as novel tumor marker for cancers of reproductive organs: Proteomic profiling investigations led to the discovery of afamin as potential biomarker for ovarian cancer. These findings were confirmed by measuring decreased serum concentrations of afamin with specific sandwich-type ELISA in pre-operative patients diagnosed with ovarian cancer in comparison to those with benign gynecological conditions, other cancers and healthy controls (5, 6). Afamin concentrations rose again to levels of healthy individuals after tumor removal and chemotherapy. Reduced afamin serum levels were also found in patients with cervical and testicular cancers but not with benign diseases such as endometriosis (7). Taken together, afamin, possibly in combination with other, established markers, may serve as a novel specific tumor marker for cancers of reproductive organs (see also chapter below on ApoA-IV as novel tumor marker).

Afamin and cardiovascular diseases: In the search for further physiological or pathophysiologic functions of afamin, its plasma concentrations were measured in two large population-based association studies and found to be associated with a variety of biomarkers indicative of cardiovascular disease. These include dyslipidemia, hypertension, glucose intolerance, insulin resistance and obesity. Individuals diagnosed with three or more of these parameters are considered to suffer from the metabolic syndrome. Prevalence of the metabolic syndrome is steadily increasing and,

in the USA alone, has reached 25% of the overall population. In our studies, afamin plasma concentrations were associated with the number of these parameters present in a patient and therefore directly correlated with the metabolic syndrome. Furthermore, basal afamin levels also positively correlated with an increase in the number of parameters over a prospective observation time of five years, suggesting high predictivity of afamin concentrations for developing the metabolic syndrome (Fig. 3).

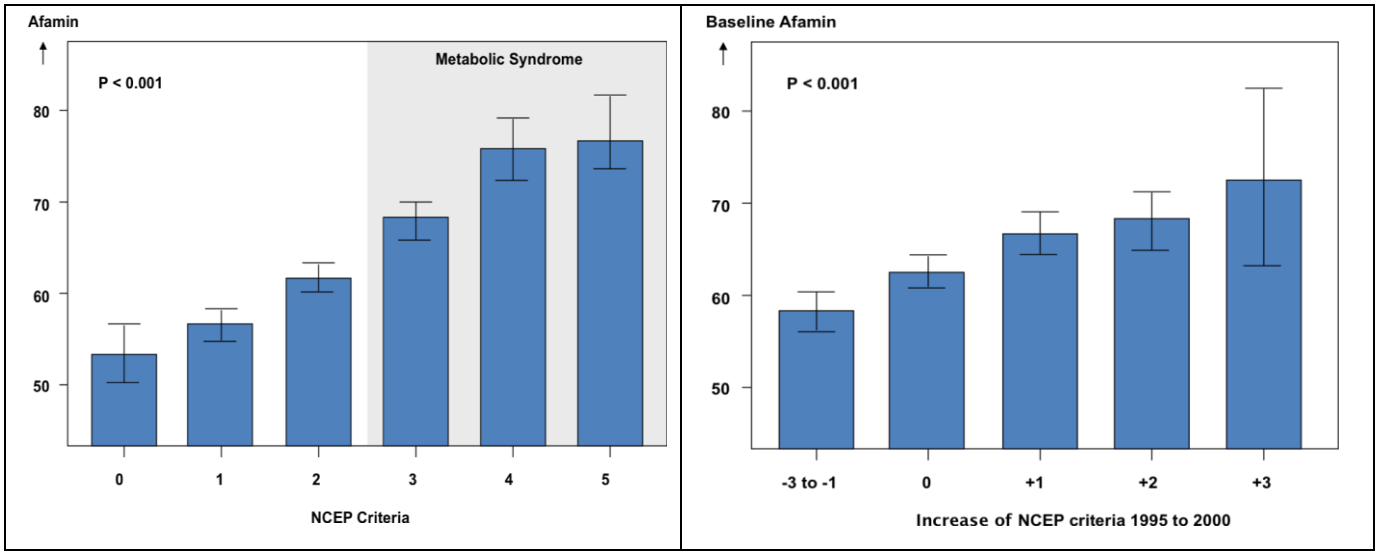


Figure 3: **Baseline afamin plasma concentrations are associated with the number of diagnostic paramters defining the metabolic syndrome (NCEP criteria) as well as with the increase in these criteria over a prospective observation period of five years (unpublished).**

Ongoing or planned work

- We are continuing to investigate several other genetically modified animal models to study various organ-specific functions of afamin. These will include transgenic and conditional knock-out mice.
- We are searching for suitable cell lines to study the regulation of afamin expression. So far, no established cell line has been found to express sufficient amounts of afamin.
- Structure/functional analysis of afamin by crystallography after recombinant expression of afamin followed by crystallisation of purified afamin protein.
- Identifying possible further ligands (by metabolomic approaches) of afamin in search of its functions.
- Investigating the development of afamin in various developmental stages of human and animal life including pregnancy, postnatal stages, childhood.
- Cross-sectional and prospective cohort studies as well as case-control studies of various diseases.
- We aim to identify genes that determine afamin plasma concentrations using genome-wide association studies.

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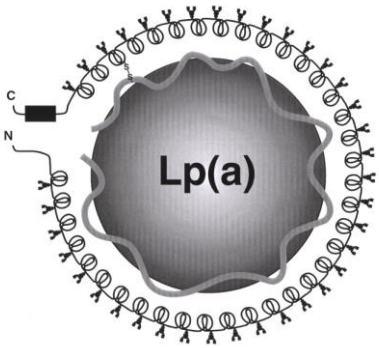
Lp(a) – a mysterious lipoprotein with unusal genetics, biosynthesis and catabolism

Lipoprotein(a) [Lp(a)] is a liver-derived lipoprotein complex in plasma consisting of an LDL particle covalently linked to the glycoprotein apo(a) (structure model taken from (8)). Lp(a) concentrations are largely genetically determined by synthesis and not degradation and are an established risk factor for cardiovascular including peripheral artery disease (9). The physiological function of Lp(a) is unknown (8). Our group has been focusing its research on Lp(a) on the complex assembly mechanism of Lp(a) (10) and the unusual role of the human kidney in its catabolism (11) by employing various cell culture model systems and studies in humans including in vivo kinetic turnover studies.



Lp(a) biosynthesis and assembly. Lp(a) plasma concentrations are inversely correlated with the copy number of transcribed and translated kringle-4 repeats in the apo(a) gene and hence with the molecular weight of the apo(a) isoform (phenotype). This seminal finding has led to the concept of Lp(a) as a genetically determined risk factor for atherosclerotic diseases such as stroke as well as coronary and peripheral artery disease (9) (reviewed in (8)).

The biogenesis of mature circulating Lp(a) particles is a highly complicated, multi-step process that is still controversially discussed. The majority of work addressing this important issue has been performed using primary animal hepatocytes or human HepG2 cells transfected with different recombinant apo(a) constructs (12), but also in-vivo kinetic studies in humans. We have been addressing two major questions regarding Lp(a) biosynthesis: 1) How does variation at the apo(a) locus determine

Lp(a) synthesis/secretion? 2) Where, within or outside the hepatocyte, is the mature Lp(a) particle assembled?

Post-translational mechanisms that significantly influence Lp(a) production rates have mainly been elucidated from studies in primary baboon hepatocytes (reviewed in (13)). We investigated the intracellular metabolism of human apo(a) in stably transfected HepG2 cells and found prolonged retention times of apo(a) precursors in the ER positively correlated with the apo(a) isoform size. Under temperature-blocking conditions, no apo(a)/apoB-100 complexes could be detected within cells (14). We therefore concluded that, in HepG2 cells, the apo(a) precursor, dependent on the apo(a) isoform, is retained in the ER for a prolonged period of time. The assembly of Lp(a) takes place exclusively extracellularly folowing the separate secretion of apo(a) and apoB.

The latter findings and conclusion stand in sharp contrast to several in-vivo kinetic studies (including own unpublished work) using the stable isotope methodology (reviewed in (10)). In these studies, synthesis rates for apo(a) and apoB in Lp(a) were very similar and different from those of LDL-apoB, which argues for different pools for Lp(a) and LDL secretion in line with the concept of an intracellular Lp(a) assembly process. These conflicting results most likely reflect differences among the used model systems.

The role of the human kidney in Lp(a) and LDL metabolism. Numerous clinical investigations using sandwich-type ELISA (15) have revealed significantly elevated plasma concentrations of Lp(a) in patients suffering from various kidney diseases (reviewed in (11), see also „Studies in Patients with Kidney Disease" on this website). In hemodialysed patients with end-stage renal disease, two further interesting observations have been made: first, the apo(a) isoform distribution did not differ from that of healthy controls and second, Lp(a) was significantly elevated only in patients with high-molecular weight (HMW) apo(a), but not in those with low-molecular weight (LMW) phenotype (Fig. 1) (16, 17).

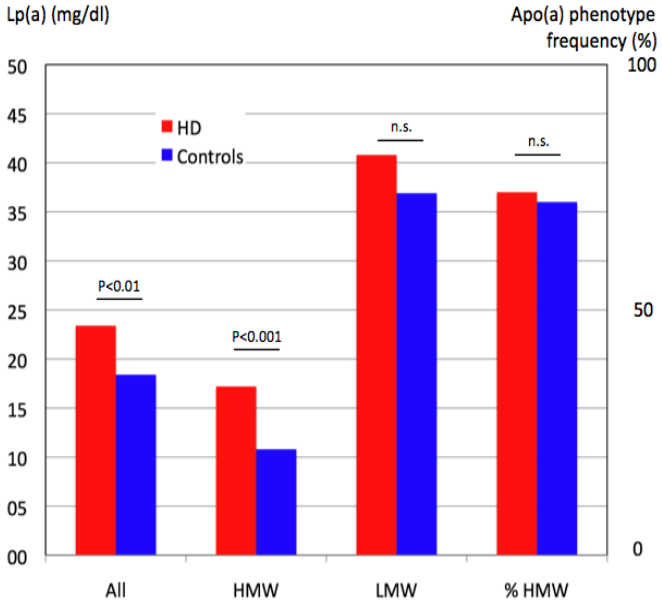


Figure 1: **Significantly elevated mean plasma Lp(a) concentrations in 534 hemodialysis (HD) patients as compared to 256 controls. Phenotype distribution frequency did not differ between patients and controls; significant differences in Lp(a) levels were restricted to patients with HMW apo(a) phenotype (data from (17)).**

While the mechanisms leading to isoform-specific Lp(a) elevations in patients with chronic renal disease are still unknown, the unchangend phenotype distribution in patients indicated a non-genetic origin of the elevated Lp(a) levels, secondary to kidney disease and suggested a possible catabolic role of the human kidney in healthy individuals. This assumption was further supported by a decrease to normal, apo(a) phenotype-adequate Lp(a) plasma concentrations after successful kidney transplantation in patients with chronic kidney disease (18), in that significant arterio-venous plasma concentrations of Lp(a) were observed in subjects with normal kidney function (19) and apo(a) degradation products were identified in human and rat urine (20, 21).

More recently, we investigated the metabolism of Lp(a) in in vivo kinetic studies in seven HD patients and nine healthy controls and compared their fractional catabolic (FCR) and production rates (PR). FCR but not PR of both apo(a) and apoB of Lp(a) were significantly lower in HD patients than in controls, indicating a decreased clearance of Lp(a) in HD patients (22) (Fig 2). The elevated Lp(a) levels in HD patients are therefore caused not by overproduction but by diminished catabolism, again suggesting an acitive role of the human kidney in Lp(a) removal from circulation.

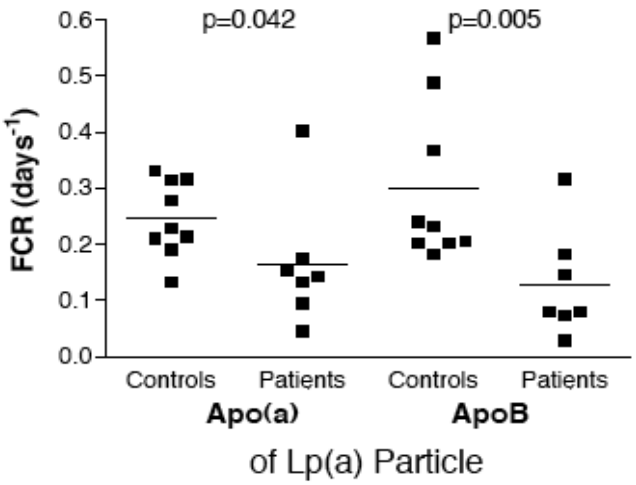


Figure 2: **Significantly decreased FCR of Lp(a)-apo(a) and Lp(a)-apoB in seven HD patients compared with nine healthy controls suggest a catabolic role of the human kidney for Lp(a) (data from (22)).**

Finally, we also investigated the in vivo metabolism of all other apoB-containing lipoproteins in HD patients by addressing one major question: is the in vivo turnover of apoB-containing lipoproteins impaired despite normal LDL levels and could this explain the increased risk for cardiovascular disease in these patients? Comparison of in vivo kinetics of apoB-containing lipoproteins between HD patients and healthy controls revealed a severely impaired catabolism of LDL and IDL masked by normal plasma cholesterol levels (23). The resulting markedly prolonged residence times of both IDL and LDL particles might thus significantly contribute to the well-documented high risk for premature cardiovascular disease in HD patients.

Ongoing or planned work

- Influence of statin therapy on the in vivo kinetics of apoB-containing lipoproteins in HD patients to see whether statins are able to normalise the impaired apoB metabolism.
- Immunochemical localisation of Lp(a) in human kidney sections to further investigate the role of the human kidney in the catabolism of Lp(a).
- Plasma distribution of oxidised phospholipids in relation to Lp(a).
- Therapy of hyperlipidemias with novel antisense agents.

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ApoA-IV – a novel biomarker for atherosclerosis, kidney disease and cancer

Human apolipoprotein A-IV (apoA-IV) is a 46 kD glycoprotein synthesised by the small intestine. It enters the plasma compartment as a structural component of chylomicrons, from where it is transferred to other lipoprotein fractions (see also "Apolipoprotein A-IV in Health and Disease" on this website). ApoA-IV participates in several steps of the "reversed cholesterol transport" pathway considered to be of central importance in protecting against atherosclerotic disease. Accordingly, an inverse correlation between plasma apoA-IV levels and coronary artery disease (CAD) was found in human populations (24). In contrast, elevated apoA-IV levels were described in patients with chronic kidney disease (17, 25) and later confirmed and extended also for patients in early phases of kidney disease (26, 27). These findings not only identified apoA-IV as an early marker of kidney impairment but also, similar as discussed above regarding Lp(a), suggest an important role of the kidney in the metabolism of apoA-IV. Finally, we previously observed in a small pilot study decreased apoA-IV plasma concentrations in kidney cancer patients (28). We have therefore been focusing our research on this multifunctional protein on i) plasma distribution of apoA-IV in CAD patients and healthy controls to see whether differences in apoA-IV subfractions might be functionally related to disease state, ii) localization of apoA-IV by immunohistochemistry in human kidney tissue to investigate the role of the kidney in apoA-IV metabolism, and iii) analysis of plasma concentrations of apoA-IV in patients with various forms of cancer to see whether apoA-IV can serve as a general or organ-specific cancer marker.

Plasma distribution of apoA-IV in CAD patients and controls. Previous studies have shown lower apoA-IV plasma concentrations in patients with CAD (24). The relative contribution of individual apoA-IV-containing plasma fractions to this result was not known since the plasma distribution of apoA-IV was not known for CAD patients. Reports on its distribution in human plasma in healthy subjects are contradictory, ranging from almost entirely bound to HDL to mostly unassociated with lipoproteins at all, depending most likely on the procedure of separating apoA-IV containing plasma fractions. We therefore developed a gentle technique to separate three apoA-IV-containing plasma fractions using a combination of lipoprotein precipitation and immunoprecipitation of apoA-I-containing particles (29): lipid-free apoA-IV (4% of total apoA-IV), apoA-IV bound to apoA-I (LpA-I:A-IV, 12%) and apoA-I-unbound but lipoprotein-containing apoA-IV (LpA-IV, 84%). This distribution pattern did not differ between 52 age- and sex-matched CAD patients and healthy controls suggesting no differences in the anti-atherogenic properties of various apoA-IV-containing plasma fractions.

Role of the human kidney in the metabolism of apoA-IV. Based on the increased levels of apoA-IV in chronic kidney disease we wanted to investigate whether and which part of the healthy human kidney is involved in apoA-IV metabolism and causes the abnormal values seen in cases of kidney impairment. For this purpose, apoA-IV was localised by immunohistochemistry in healthy kidney tissue obtained from patients undergoing nephrectomy (28). ApoA-IV immunostaining was detected in proximal and (much weaker) in distal tubular cells, capillaries and vessels, but not inside glomeruli, suggesting a direct role of the human kidney in apoA-IV metabolism (Fig. 1). The strong signal in the tubular system indicates a rescue function of the kidney for otherwise escaping apoA-IV.

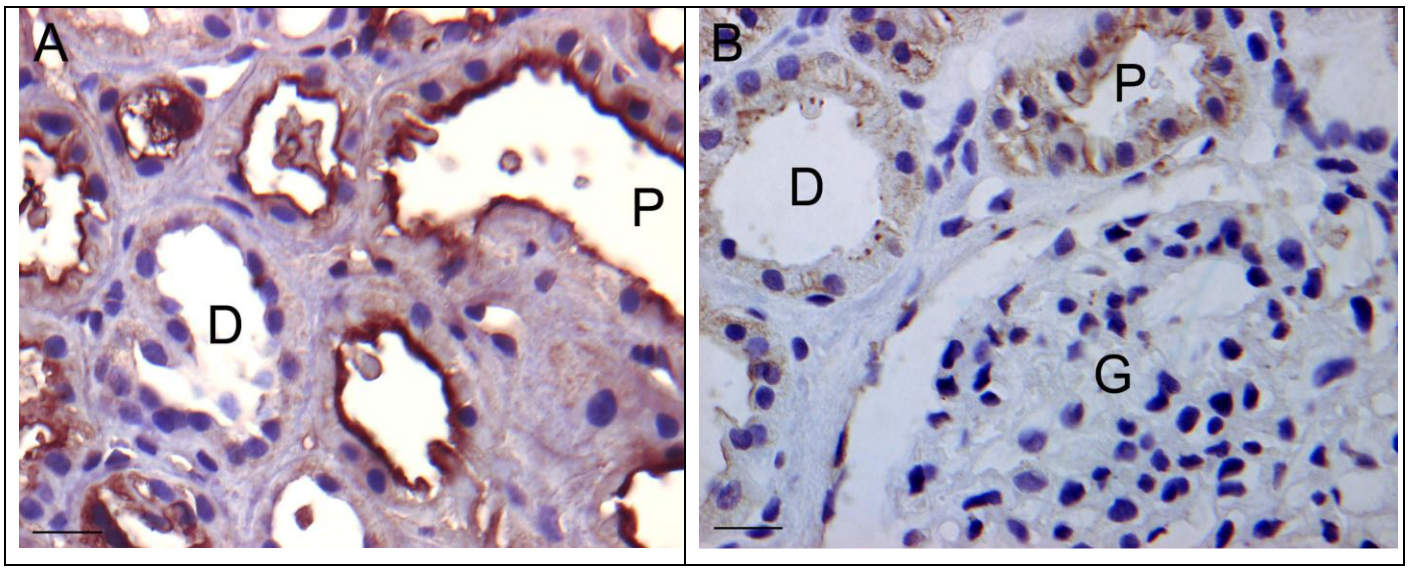

Figure 1: **Immunoreactivity of apoA-IV in normal human kidney tissue. Panels A and B show strong immunostaining in proximal (P) and (much weaker) in distal (D) tubules. In proximal tubules, signals were detected in intracellular granules, on basolateral membranes and faintly in the cytoplasm. No staining was observed in the glomeruli (G, panel B) (28).**

ApoA-IV as novel tumor marker. Based on preliminary findings of decreased ApoA-IV levels in patients suffering from kidney cancer (28), we conducted a large study (n>3000) including various cancer patients with ovarian (OC), cervical, colon, stomach, pancreatic, prostate, kidney and lung cancer as well as benign conditions and healthy controls. ApoA-IV (together with afamin, see respective chapter above)

was measured with sandwich-type ELISA in 181 OC patients in various clinical stages, 399 patients with benign gynecologic disease and 177 controls and compared with the conventional OC marker CA125 (5). ApoA-IV concentrations were 13.0 mg/dl in controls, 11.7 mg/dl in benign patients and 9.4 mg/dl in OC ($p < 0.001$). ROC analysis for differentiating OC patients from healthy controls revealed for a specificity of 90% sensitivity values of 92.4%, 42.4% and 40.8% for CA125, afamin and apoA-IV, respectively. These relatively low sensitivities thus indicate that afamin and apoA-IV alone are not sufficiently suitable as diagnostic markers for OC, but may serve as parts of marker panels together with established tumor markers.

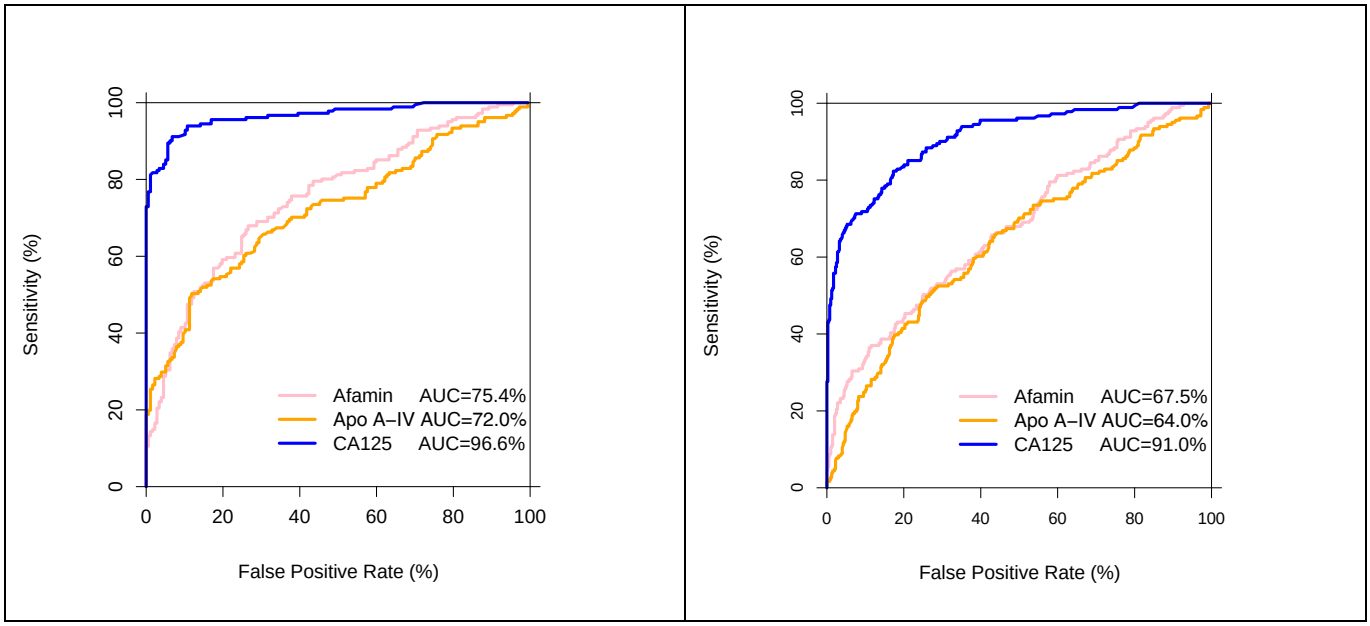

Figure 2: **ROC curves for afamin, apoA-IV and CA125 for differentiating ovarian cancer patients from healthy subjects (left panel) and subjects with benign disease (right panel) (data from (5)).**

Ongoing or planned work

- We are continuing to investigate afamin and apoA-IV as possible tumor marker for several other common human cancers.
- We are studying the association of apoA-IV with cardiovascular disease in follow-up studies.
-

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