## Review Article

# "The Lower Threshold" phenomenon in tumor cells toward endogenous digitalis-like compounds: Responsible for tumorigenesis?

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## Abstract

Since their first discovery as potential anti-cancer drugs decades ago, there is increasing evidence that digitalis-like compounds (DLC) have anti-tumor effects. Less is known about endogenous DLC (EDLC) metabolism and regulation. As stress hormones synthesized in and secreted from the adrenal gland, they likely take part in the hypothalamo–pituitary–adrenal (HPA) axis. In a previous study, we revealed reduced EDLC concentrations in plasma and organs from immune-compromised animals and proposed that a similar situation of a deregulated HPA axis with "adrenal EDLF exhaustion" may contribute to tumorigenesis in chronic stress situations. Here, we put forward the hypothesis that a lowered EDLC response threshold of tumor cells as compared with normal cells increases the risk of tumorigenesis, especially in those individuals with reduced EDLC plasma concentrations after chronic stress exposure. We will evaluate this hypothesis by (a) summarizing the effects of different DLC concentrations on tumor as compared with normal cells and (b) reviewing some essential differences in the Na/K-ATPase of tumor as compared with normal cells (isoform pattern, pump activity, mutations of other signalosome receptors). We will conclude that (1) tumor cells, indeed, seem to have their individual "physiologic" EDLC response range that already starts at pmolar levels and (2) that individuals with markedly reduced (pmolar) EDLC plasma levels are predisposed to cancer because these EDLC concentrations will predominantly stimulate the proliferation of tumor cells. Finally, we will summarize preliminary results from our department supporting this hypothesis.

**Keywords:** Adrenal exhaustion, endogenous digitalis-like compounds, isoforms, Na/K-ATPase, stress, threshold, tumorigenesis

## INTRODUCTION

The endogenous digitalis-like compounds (EDLC) belong

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to a family of steroid hormones, which originally stem from plants and recently have been demonstrated to be synthesized and released mainly in the adrenal gland of different species.[1-10] As "stress hormones" similar to cortisol, they are integrated in the feedback loops of the hypothalamic–pituitary–adrenal (HPA) axis and stimulated by ACTH and Angiotensin II.[11-15] The EDLC are the natural ligands of the Na/K-ATPase (NKA), the classical sodium pump.[16-19] It is nowadays well established that the NKA represents also a signal transducer that is partly independent from its pump

activity.[20-30] Since their first discovery as potential anti-cancer drugs decades ago,[31-34] there is increasing evidence over the last years, *in-vitro* and *in-vivo*, that digitalis-like compounds (DLC) have anti-tumor properties.[35-46] These include, for example induction of apoptosis via $Ca^{2+}$-dependant caspase-3 activation,[47] promotion of cell cycle arrest and cell differentiation via upregulation of the cell cycle inhibitor p21[cip1],[48] cell growth inhibition via downregulation of the NKA-α1 isoform,[49] NF-κB,[50] HIF-1α[51] as well as inhibition of topoisomerase I and II[52] and autophagic cell death via lysosomal membrane permeabilization[53] with interruption of the actin cytoskeleton. Remarkably, inhibition of the NKA by DLC also has been shown to sensitize cancer cells toward anoikosis.[54] Recently, different DLC partly derived from plants (Oleandrin from *Nerium oleander L.*)[55-58] and partly semi-synthetic (UNBS1450 derived from *Calotropis procera*)[50,59,60] entered Phase I trials in solid tumors with, so far, no toxicity. Less is known about the endogenous DLC metabolism and their regulation. As mentioned above, they are part of the HPA axis and thus also depend on the integrity of the thymus. A bidirectional relationship between the thymus and the HPA axis is well established, with mutual dependency in maturation and function.[61-64] As demonstrated in a previous study, nude mice, traditionally used for tumor transplantation, did not only reveal reduced basal EDLC concentrations in the adrenal gland but also did not respond to an acute stress stimulus, even showing decreased plasma EDLC concentrations after additional ACTH application.[15] We proposed that this "adrenal EDLC exhaustion" with gradually decreasing EDLC plasma concentrations in immune-compromised individuals, e.g. after chronic stress exposure, may contribute to tumorigenesis.[65] In the recent years, a lot of evidence has accumulated that exogenous and endogenous DLC are able to induce MAPK signaling pathways via the NKA/Src/epidermal growth factor receptor (EGFR) "signalosome"[66-68] and, hence, lead either to stimulated cell growth (hypertrophy), cell cycle arrest and cell differentiation, or apoptosis.

The kind of interaction between DLC and MAPK signaling pathways is dose- and time dependant, and, moreover, depends critically on the nature of the involved cell membrane receptors, especially the molecular structure (isozymes), activity and cellular amount of the NKA. It also depends on the mutation status of the tyrosine kinase receptors, e.g. the EGF-R.

We put forward the hypothesis that a lowered endogenous DLC response threshold of tumor as compared with normal cells [Figure 1] increases the risk of tumorigenesis, especially in individuals with reduced EDLC plasma concentrations after long stress exposure.

In the first part of evaluation, we will summarize data about either cell growth-stimulating or -inhibiting effects of DLC at different dosages on normal as well as tumor cells.

In the second part, we will recapitulate knowledge about the characteristics of the NKA in tumor as compared with normal cells. Finally, we will develop our hypothesis referring to the data mentioned before.

## SUMMARY OF THE EFFECTS OF DIGITALIS-LIKE COMPOUNDS

### Sub-physiologic EDLC plasma concentrations (1 pM–100 pM)

The *in vitro* studies analyzing the effect of DLC on diverse cell lines scarcely used these low DLC (pmolar) concentrations. Most of the studies start cell treatments at 1 nM–10 nM or 1 nM–100 nM. For instance, *Qiu et al.*[69] did not see any significant impact on cell growth of human umbilical vein endothelial cells (HUVEC) at ouabain concentrations <0.1 nm, i.e. neither cell proliferation nor apoptosis. To our knowledge, no data exist about the effects of pmolar DLC concentrations on malignant cells.

### Physiologic EDLC plasma concentrations (0.1 nM–10 nM; max., 100 nM)

*Effect on normal cells*

*Qiu et al.*[69] exposed HUVEC to different concentrations (0.1 nM–100 nM) of ouabain at 12–48-h intervals. Ouabain stimulated HUVEC cell proliferation at low concentrations (1.0 nM) and induced cell death at markedly higher (>100 nM) concentrations. *Aydemir-Koksov et al.*[70] demonstrated that ouabain at concentrations below those that inhibit the pump, i.e. 0.1 nM and 1.0 nM, induced trans-activation of the EGF-R, resulting in increased proliferation and bromodeoxyuridine (BrdUrd) uptake of canine vascular smooth muscle (VSMC) cells. Interestingly, higher ouabain concentrations (10 nM) had little or no stimulating effect on proliferation. *Winnicka et al.*[71] showed that in human fibroblasts, 30 nM ouabain, digoxin and proscillaridin A induced an anti-apoptotic action by increasing the level of phosphorylated extracellular signal-regulated kinases (p-ERK 1/2). Similarly, *Chueh et al.*[72] demonstrated that ouabain at low nM concentrations promoted cell proliferation in human prostate smooth muscle cells via a Ca(2+)-dependent mechanism and activation of the MEK-p42/44 MAPK pathway. *Li et al.*[73] investigated the effect of low-dose ouabain on the viability of rat renal proximal tubular cells. Ouabain (0.1 nM–10 nM) stimulated the proliferation of kidney cells; interestingly, these effects were abolished when slow calcium oscillations via the IP3R were prevented. *Khundmiri et al.*[74] observed that ouabain induced cell proliferation in

opossium kidney tubular cells involving calcium-dependant phosphorylation of Akt. This effect started at ouabain 1 nM and was maximal at 10 nM–100 nM, whereas at 1 $\mu$M, ouabain decreased Akt-phosphorylation. *Wei et al.*[75] revealed that spiral ganglion neurons exposed to neurobasal medium + 10 nM ouabain had a much lower apoptosis index, increased Bcl-2 levels and, interestingly, longer dendrite growth as compared with neurons exposed to neurobasal medium only. De Rezende Correra *et al.*[76] demonstrated that ouabain significantly increased retinal ganglion cell survival, with a maximum at 3.0 nM, after 48 h in culture. The blockade of protein kinase C activity abolished the ouabain effect.

### Effect on malignant cells

Lopez-Lazaro *et al.*[77] revealed that in three human cancer cell lines – TK-10 (renal), MCF-7 (breast) and UACC-62 (melanoma) – the IC50 values for digitoxin (3 nM–33 nM) were within the concentration range (20 nM–33 nM) seen in the plasma of patients with cardiac disease receiving this glycoside. Specifically, digitoxin at 1 in 30 nM induced levels of DNA-topoisomerase II cleavable complexes similar to etoposide. *Kometiani et al.*[78] explored the mechanism of the growth inhibitory effects of DLC on the estrogen receptor-negative human breast cancer cell line MDA-MB-435. Ouabain concentrations (10 nM and 100 nM) that caused less than 25% inhibition of the NKA pumping function activated Src kinase, stimulated the interaction of Src and Na/K-ATPase with EGF-R, caused a transient and then a sustained activation of ERK1/2 and increased the expression of the cell cycle inhibitor p21[Cip1]. *Winnicka et al.*[79] observed reduced cell viability in another human breast cancer cell line (MDA-MB-231) after applying ouabain, digoxin and proscillaridin A in nmol ranges. They confirmed that cardenolides induce apoptosis in MDA-MB-231 cells by increasing free calcium concentration and by activating caspase-3. Notably, they revealed marked differences in the potency, with proscillaridin A being the most active (IC50 48 ± 2 nM). *Bielawski et al.*[52] evaluated the role of cardenolides in MCF-7 breast cancer cells with special focus on topoisomerases. Both digoxin and ouabain inhibited topoisomerase II catalytic activity at 100 nM. Proscillaridin A was an even more potent poison of topoisomerase I and II activity at 30 nM and 100 nM, respectively. *Mijatovic/Kiss et al.*[53] developed a semi-synthetic cardenolide, UNBS1450, with a markedly higher affinity to the NKA of diverse human cell lines than ouabain or digoxin. UNBS1450 at 10 nM–100 nM induced in A549 NSCLC cells a cell death process associated with dramatic cytoplasmic vacuolization due to increased lysosomal permeabilization. Remarkably, neither signs of apoptotic nor necrotic cell death were seen. *McConkey et al.*[47] demonstrated in two variants (Pro4 and LN4) of the human prostate androgen-independent metastatic adenocarcinoma PC3 pro-apoptotic

effects of cardiac glycosides (oleandrin, ouabain and digoxin). Concentration–response analyses revealed in both cell lines maximal responses of ouabain and digoxin at 100 nM. *Huang et al.*[80] also examined the cytotoxic effects of ouabain on the human prostate cancer cell line PC3. Low concentrations of ouabain (<10 nM) induced the increase of Par-4 expression and sensitized the cells toward cytotoxicity. Higher ouabain concentrations (<100 nM) induced a significant and time-dependent loss of mitochondrial membrane potential (Deltapsim), a sustained production of reactive oxygen species (ROS) and a severe apoptotic reaction. *Smith et al.*[81] examined the relative abilities of oleandrin, ouabain and anvirzel to inhibit FGF-2 export from two human prostate cancer cell lines, DU145 and PC3. Oleandrin (0.1 ng/mL) produced a 45.7% inhibition of FGF-2 release from PC3 cells and a 49.9% inhibition from DU145 cells. Non-cytotoxic concentrations (100 ng/mL) of anvirzel produced a 51.9% and 30.8% inhibition of FGF-2 release, respectively, in the two cell lines. *Li et al.*[82] demonstrated that in a human gastric cancer cell line (MGC803), bufalin at 20 nmol/L induced M-phase cell cycle arrest, whereas at 80 nmol/L, it induced apoptosis via an increased Bax/Bcl-2 ratio and activated caspase-3. Remarkably, these distinct effects were correlated to a transient activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and a complete inhibition, respectively. *Xu et al.*[83] analyzed the combined effects of ouabain (5 nM–1000 nM) and α1-siRNA on human hepatocellular carcinoma (HCC) cells, HepG2. The IC50 of ouabain (at 48 h) in HepG2 cells was 100 nmol/L, and this concentration was shown to induce cell cycle arrest as well as apoptosis. Interestingly, silencing of NKA α1 isoform could enhance the anti-cancer effect of ouabain, in between others, by increasing the expression of p21[Cip1].

## High EDLC plasma concentrations (>100 nM–10 µM)

### Effect on normal cells

*Winnicka et al.*,[71] dealing with the dual role of cardenolides in human fibroblasts, demonstrated that ouabain, digoxin and proscillaridin A only at relatively high concentrations (>300 nM) increased intracellular $Ca^{2+}$ concentration, activated caspase-3 and induced apoptosis in human fibroblasts. Similarly, the group of *Chueh et al.*[72] revealed cytotoxic effects (apoptosis) of ouabain on human prostatic smooth muscle cells only at high concentrations. *Akimova et al.*[84] examined the role of MAPK in the death of ouabain-treated renal epithelial cells. Exposure of C7-MDCK cells to 3 µM ouabain led to phosphorylation of p38 and consequent apoptosis, without a significant impact on phosphorylation of ERK and JNK. In ouabain-resistant smooth muscle cells from rat aorta and endothelial cells from human umbilical vein, no effect of ouabain on p38 phosphorylation was observed.

*Effect on malignant cells*

*Yeh et al.*[85] demonstrated that digoxin, digitoxin and ouabain significantly inhibited the proliferation of human prostate cancer LNCaP, DU145 and PC3 cells at 1 μM or 10 *μM*. In contrast, normal control human glomerular epithelial cells showed no response to digitalis treatment at all tested doses. The discrepancy to the results of other authors (see above) remains to be clarified. *Felth et al.*[38] screened several cardiac glycosides in three human colon rectal cancer cell lines (HT29, HCT116 and CC20). Convallatoxin, oleandrin and proscillaridin A were identified as the most potent test compounds, with IC50 values ranging from 0.007 μM to 0.55 *μM*. Interestingly, the combination of digitoxin and oxaliplatin exhibited synergism in the otherwise highly drug-resistant HT29 cell line.

## COMPARISON OF NA/K-ATPASE CHARACTERISTICS IN TUMOR VS. NORMAL CELLS

After summarizing these effects of DLC on different kinds of cell types in different experimental conditions, it becomes evident that the interaction between NKA and DLC follows a certain scheme according to the (E)DLC plasma concentrations [Figure 1]:

- At low EDLC plasma concentrations with no significant NKA inhibition, cell proliferation via Src/EGF-R/PI3K/Akt and Raf/Ras/MAPK pathways is predominant, resulting in transient ERK1/2 activation, activation of NF-kB and, in addition, recycling of signalosome members (α1, EGF-R).
- At low physiologic EDLC plasma concentrations with only partly (25%) NKA inhibition, cell cycle arrest and cell differentiation via the Src-EGFR-PI3K-Akt pathway are seen, resulting in sustained ERK1/2 activation with upregulation of p21$^{Cip}$ and, in addition, lysosomal degradation of signalosome members.
- At high physiologic EDLC plasma concentrations, mainly "classic" NKA inhibition occurs, while pro-proliferative effects are diminished or blocked.
- At high EDLC plasma concentrations (with >60% NKA inhibition), cell apoptosis is revealed involving high intracellular $Ca^{2+}$, production of ROS and activation of caspase-3.

This "stepwise" reaction scheme of NKA–EDLC interaction seems to be valid for both normal as well as tumor cells. What are the underlying mechanisms driving different cells either into cell growth, cell differentiation, cell cycle arrest or apoptosis when they are exposed to the same (E)DLC plasma concentrations? While dealing with this question, we will show that the "physiologic" EDLC range of normal cells

indeed correlates with a specific response pattern that shifted to a lower "tumor-specific physiologic" range in tumor cells. According to Schoner,[45] "the mechanism by which a normal cell or a tumor cell enters the pathway of differentiation and proliferation or apoptosis seems to be essentially the same for normal and malignant cells." Therefore, the crucial difference is likely to be found at the cell membrane surface. With respect to the DLC (ligands) and their specific receptor (NKA), it is well established that a distinct combination of α- and β-subunits is crucial for the kind of activated response.[86-89] Not only have tissue-specific patterns of NKA isoforms[90-92] been identified but also a switch of isoforms during development from neonatal to adult tissues has been observed.[93-95] *Crambert et al.*[96] mention in a detailed analysis of the human NKA that the isozymes combined with the α1 isoform have the lowest Kd (i.e., the highest affinity for EDLC), the highest sensitivity toward K+ and Na+ and, hence, the greatest turnover, compared with other isozymes. These features reflect the "housekeeping role" of the ubiquitous α1 subunit, as "Na/K-ATPase with such characteristics should work at optimum rates under physiologic conditions but cannot respond to increased physiological demands."[96] This implies that in case of transforming cells with, for example the "leaking-phenomenon" (see below), a "normal-active" NKA cannot respond to the needs of malignant cell transformation, like high intracellular K+- and glucose concentrations. Only after structural and/or conformational changes of the NKA (e.g., switch of isoforms) in the process of malignant transformation, can the pump provide this task, in between others, by developing hyperactivity.

Recent research has shed light on a new, still controversial role of α1 in cancer development and therapy. What seems to crystallize is the observation that in many different cancers (bladder, prostate, gastric), a reduced expression of the α1- and increased expression of the α3-isoform is present.[97-101] The meaning of these switches in α-isoforms in cancer progression is not yet fully understood. On the one hand, the α1 subunit is essential for maintaining the actin cytoskeleton[102] and cell-growth capacity of a cell, most likely as part of the "signalosome" formed in special caveolae.[66-68] *Kiss et al.* called the α1 subunit a new target in cancer therapy, especially in NCSLC and Glioblastoma.[59,60,103] UNBS1450, a semi-synthetic cardenolide, decreased in NSCLC A549 cells both the NF-κB transcriptional activity and the DNA-binding capacity of the p65 subunit.[50] In Gioblastoma cells, the same compound caused ATP depletion, disorganization of the cytoskeleton and, finally, autophagic cell death.[103] *Newman et al.*[42] pointed out that, in addition to its growth-stimulatory effects, the α1 NKA isoform is associated with high intracellular gluthathione levels that prevent or delay

ROS-induced apoptosis. This protective feature is "logic" for normal cells considering their genetic program, which drives them to proliferate unless they are aged, or dysfunctional (by mutation or other DNA damages). *Xie et al*.[49] demonstrated elegantly in a recent study that the knockout for NKA α1 is sufficient to upregulate the cell cycle inhibitor p21[Cip1], leading to cell cycle arrest. Ouabain at low doses (nM) induced NKA endocytosis in both a benign and two malignant cell lines but, however, whereas in normal cells a repletion of NKA α1 at the cell membrane was seen (involving the Src/PI3K/Akt/mTOR pathway), which correlated with cell growth stimulation, in both malignant cell lines the NKA α1 subunits were directed to lysosomal degradation, resulting in cell growth inhibition.[49] The authors admit that the reason for this opposite behavior still needs to be clarified and suggested differences, e.g. in the components of the signalosome (caveolin-1 and cholesterol). We will propose below that mutations or amplifications of the EGF-R (as often found in malignancies) may be crucial determinants of α1 intracellular pathways and, hence, of the cell's fate. In summary, for the sake of tumor growth control, the down-regulation or another form of inactivation of the NKA α1 isoform is "reasonable" and, thus, an important goal in targeted therapies.

On the other hand, as described above, many cancers (i.e. pancreatic) have already down-regulated NKA α1- and upregulated α3 isoforms. Contrary to what we might expect from the recently mentioned data, these cancers are not less aggressive than those with α1 expression. The reasons for this down-regulation of NKA α1 in some cancer types are not fully evaluated; most likely, cytokines are involved. From all available data, we may assume that endogenous DLC are "already-at-work." In other words, one and the same cancer cell could have a different NKA isoform pattern during its life span with high expression of α1 isoforms in early stages and low α1 expression in later stages – in favor of high α3 – when endogenous defense mechanisms, including stress hormones like EDLC, have entered the "battlefield." In any case, a high α3/α1 ratio seems to render cancer cells more sensitive toward cell growth inhibition and/or apoptosis.[42,104] We may say in a literal sense that in cancer therapy, we continue or follow the path that nature itself has already chosen.

Concerning normal tissue, the NKA α3 subunit is predominant (α3/β1) in brain and neuronal tissue. As mentioned above, in cancer, the α3 isoform is often overexpressed. Shibuya *et al*.[100] analyzed the NKA isozyme expression in HCC. Interestingly, the expression levels of the NKA α3 isoform in HCC tissues were not only significantly higher than those in the accompanying non-tumor tissues but also correlated significantly with the NKA activity. The authors suggested that overexpression of α3 increases the Na/K-ATPase activity of HCC cells.[100] One possible interpretation is that in the program of malignant transforming cells, a signal for the need of more nutrition is translated in signaling cascades leading to a special NKA isoform pattern (α3/β2?) fulfilling these needs. But, indeed, the observed "correlation" between a3 expression and NKA activity could also represent a pure epiphenomenon. Remarkably, DLC not only have a high sensitivity to the human α3 subunit but also are able to downregulate the mRNA of the α3-subunit.[105] It is not yet clear whether the NKA α3 subunit *per se* in cancer cells has a specific tumor-promoting effect, but there is evidence that the upregulation of α3 is promoted by the pro-inflammatory surrounding that is typical for tumor formations. Besides, it has been demonstrated that, on the other hand, α3 downregulation is driving human leukemic cells into cell differentiation.[106] Remembering what we said above about the downregulation of α1 "in favor" of α3, you get the impression that the "devil is driven out by the Beelzebub." Moreover, it has been demonstrated that the α3 isoform can substitute α1 in the signalosome and induce downstream signaling pathways. Pierre *et al*.[89] used the baculovirus expression system to determine which subunits of the transporter are required for mediating signal transduction events, e.g. the activation of ERK1/2 by phosphorylation. Interestingly, Sf9 insect cells expressing the NKA α1/β1 isozyme showed under ouabain application a dose-dependent linear increase in p-ERK, with the highest response obtained at 100 µM and 1000 $\mu$M. In contrast, Sf9 cells expressing the α3/β1 isozyme showed a dual response pattern, with increase in p-ERK1/2 only up to 0.1 $\mu$M and, afterwards, a decrease, reflecting an inhibitory effect of ouabain on cell growth-related pathways at higher concentrations.[89] This is in accordance with the known dual activity of DLC, and also supports our lower threshold theory in cancer (see below): the same ERK1/2-activating effect was obtained in α3/β1 (overexpressed in cancer) cells with $10^3$–$10^4$-fold lower ouabain concentrations than in α1/β1 cells. The studies and results from baculovirus expression systems are limited in so far that they work with rodent NKA isoforms, with α1 having a much lower ouabain sensitivity than α2, α3 and α4. In human cells, in contrast, all isoforms are similarly sensitive to ouabain.[96] Assuming a comparable mechanism for the induction of signaling pathways, the data from rodents may be applied to human beings.

With respect to the NKA β subunits, it is accepted that β1-isoform expression in malignant cells is often downregulated, as shown for human clear cell renal,[107] gastric[108] and bladder cancer.[97] This β1-downregulation has been suggested to be associated with the loss of tight junctions and epithelial polarity in cancer cells.[109] It was also

demonstrated that decreased expression of the β1-subunit in poorly differentiated carcinoma cell lines correlated with increased expression of the transcription factor Snail, known to downregulate E-cadherin, with consequent transition from epithelial to mesenchymal phenotypes.[110] Finally, Rajasekaran *et al*.[111] showed that the levels of phosphorylated ERK 1/2 are inversely correlated with the β1 isoform levels in the tumors (MSV-MDCK), indicating a direct tumor-suppressor function of β1 in epithelial cells. Blanco *et al*.[112] analyzed the function of different β isoforms, α3/β1 and α3/β2. Using Sf9 cells, they mentioned that the accompanying β subunit isoform does not drastically affect the properties of the α3 subunit. Both NKA isozymes have similar turnover numbers, affinities for $K^+$ and ATP and comparable high sensitivity to ouabain. Other authors claim that a switch from β1 to β2 may have an impact on tumorigenesis.[90] Here, further studies are needed, assuming that the downregulation of one isoform (e.g., β1) involves the upregulation of another one (e.g., β2). To summarize, in cancer, we often deal with overexpression of the α3 subunit and downregulation of the β1 subunit.

In malignant cells, not only the structure of the Na/K-ATPase is changed but also its dynamics. Decades ago, it was discovered that kinetic changes in the Na/K-ATPase activity are already present at very early stages of tumorigenesis, even long before their morphologic manifestation.[32,113-115] Gonta-Grabriec *et al*.[116] revealed that in both spontaneous and radiation-induced thymomas, 86Rb uptake, ATP hydrolysis and 3H-ouabain binding per cell were higher than in normal thymuses. These changes correlated highly with cAMP content and 3H-thymidine incorporation, taken as indicators of the proliferative activity typical for a pre-leukemic period. Moreover, NKA activity may vary during the lifespan of malignant cells. For example, a depolarization of the plasma cell membrane in chicken embryo fibroblasts, transformed by Rous sarcoma virus, was described, reflecting a reduced NKA activity, maybe shortly before apoptosis.[117] Similarly, Davies *et al*.[118] measured the kinetics of the NKA in distal colonic mucosa of CF1 mice 1 week after injections of the carcinogen 1,2-dimethyhydrazine (DMH) over 4 weeks. The $V_{max}$ of the pump in pre-malignant mucosa was lower (55%–65% of control) for both sodium and potassium substrate activation, correlating with a 50% decreased NKA activity.

The reasons for these discrepancies are not yet clear. In malignant cell transformation, a "leakage phenomenon" was described causing a hyperactivity of the pump to compensate for the loss of potassium (Kaplan, 1978) and providing the tumor cell with nutrition necessary for the aberrant increased metabolism.[114] Whether and when exactly this hyperactive state is preceded (or followed) by a period of reduced pump activity is not known in detail. One possible scenario is that in a very early stage, the pump activity is reduced and only after, e.g. upregulation of the α3 isoform, the pump may switch to a hyperactive form (see: Shibuya). In general, basic kinetic properties of the NKA are modulated by a family of transmembrane spanning FXYD proteins that are colocalized with the NKA αβ subunits in the cell membrane.[119,120] In tumorgenesis, they seem to be upregulated; for instance, in NSCLC, recently, a high overexpression of FXYD5 (related to ion channel) was revealed correlating with increased activity ($V_{max}$) of the pump, loss of TJ, increased cell permeability, impaired attachment and restricted cell movement.[120] Thus, the activity of the NKA might reflect the metabolic needs of a transforming cell, but the NKA that is embedded in the signalosome, is known to transmit signals independently of the pump's activity.

Finally, analyzing the parameters at the cell membrane surface, we have to examine the neighborhood of the NKA. Considering that the NKA is closely interacting with other receptors at the cell surface ("signalosome"), it is reasonable to assume that a structural/functional change in one or more of these receptors will dramatically influence the kind of response to EDLC. This also helps to explain that malignant cells not only respond differently to DLC as compared with normal cells but also the differences in reaction between various cancer cell types.

The best analyzed receptor, EGF-R, is a tyrosinase kinase receptor, and its interaction with NKA in the signalosome is well established.[21,24,26] EGF-R kinase domain mutants found in non-small cell lung cancer (NSCLC) are constitutively active, a trait critical for malignant cell transformation. Chung *et al*.[121] stressed that aberrant trafficking of mutant EGF-R in NSCLC allows a preferential interaction with Src, a critical partner for EGF-R-mediated oncogenesis. Remarkably, mutant EGF-R, but not the wild-type EGF-R, show a perinuclear accumulation and colocalization with recycling endosomal markers such as Rab11 and EHD1 upon treatment of cells with endocytic recycling inhibitor monensin, suggesting that mutant EGF-Rs preferentially traffic through the endocytic recycling compartments. Medts *et al*.[122] aimed to test whether acute Src activation impacts on signalling and trafficking of non-liganded wild-type EGF-R. They found that thermoactivation caused rapid Src recruitment to the plasma membrane, concomitant association with EGF-R and its phosphorylation at Y845 and Tyr1173. Like low EGF concentrations, activated Src triggered EGF-R endocytosis via clathrin-coated vesicles and led to its sequestration in perinuclear/recycling endosomes with avoidance of multivesicular bodies and lysosomal degradation. The activation of Src and consecutive transactivation of (non-

liganded) EGF-R by binding of ouabain to NKA is well documented.[21,68,123,124] We want to add that endogenous EDLC may be the most important natural activators of Src and transactivators of EGF-R, competing with its primary ligand EGF. So far, some of these cited data would speak rather in favor of a tumor-promoting effect of EDLC by (a) contributing to enhanced recycling of the membrane-bound EGF-R and (b) by stimulating/activating the Src-EGFR-PI3K/Akt/mTor and Raf/Ras/MAPK pathways. How can we explain these discrepancies to the anti-proliferative effects described in numerous recent reports? We assumed previously that the mutation status of EGF-R might be a relevant cofactor in determining the direction of DLC-induced pathways. The above-mentioned aberrant trafficking of mutant EGF-R was observed in a setting where mutant EGF-R was stimulated by EGF, the natural ligand.[121] EGF binding induces auto-phosphorylation at Tyr1173 and Y845; ouabain, in contrast, has been shown to transactivate EGF-R by tyrosine phosphorylation at other sites. This could, at least partly, explain why the effects of DLC-transactivated EGF-R are different from EGF-stimulated EGF-R [Figure 2].

## HYPOTHESIS

The facts presented above contribute to the hypothesis that the main reason for the observed phenomenon that tumor cells react differently to DLC as compared with normal cells

is a lowered endogenous response threshold [Figure 1]. Above this threshold, tumor and normal cells reveal a similar response patterns toward (E)DLC, assuming no additional changes of intracellular molecules involved in the signaling cascades. In other words, tumor cells have their individual (tumor-specific) "physiologic EDLC ranges" starting at much lower (pmolar) EDLC concentrations as compared with normal cells:

Sub-threshold EDLC range of normal cells = "low-physiologic" EDLC range of tumor cells.

The reasons for a lowered threshold in malignant cells could be (as discussed above), i.e.

- Tumor-specific NKA pattern (α3/β2) at the cell membrane surface leading to changes in NKA sensitivity and activity
- Aberrations of receptors of the signalosome, e.g. mutant EGF-R
- Other changes in the signalosome, e.g. decrease of caveolin-1 and cholesterol

We assume that under physiologic conditions (normal serum $K^+$ and $Na^+$ concentrations), when the EDLC are binding preferentially to α3 isoforms (see: "$K^+$-antagonism" Crambert), tumor cells with overexpression of α3 become, naturally, the selective target of EDLC while normal cells with predominant α1 isoforms are "protected." This effect may
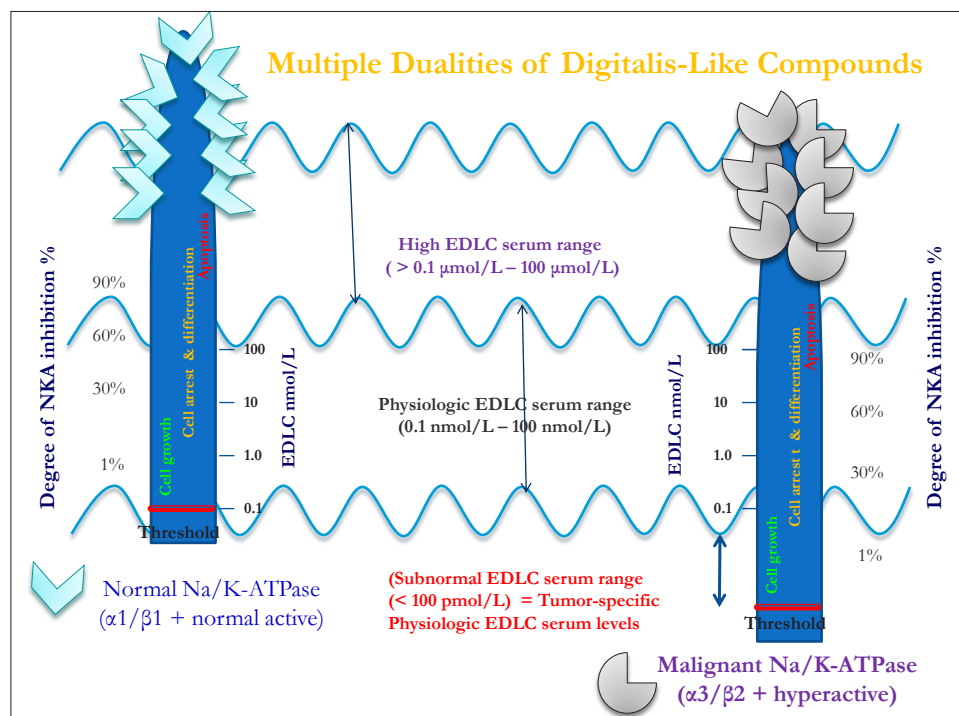


**Figure 1: The lower-threshold theory in cancer.** The dual effects of endogenous digitalis-like compounds (EDLC) are demonstrated, which are, in principle, similar for normal and tumor cells, with the only crucial difference being that the response threshold of the tumor cells toward EDLC shifted to a lower (pmolar) EDLC range
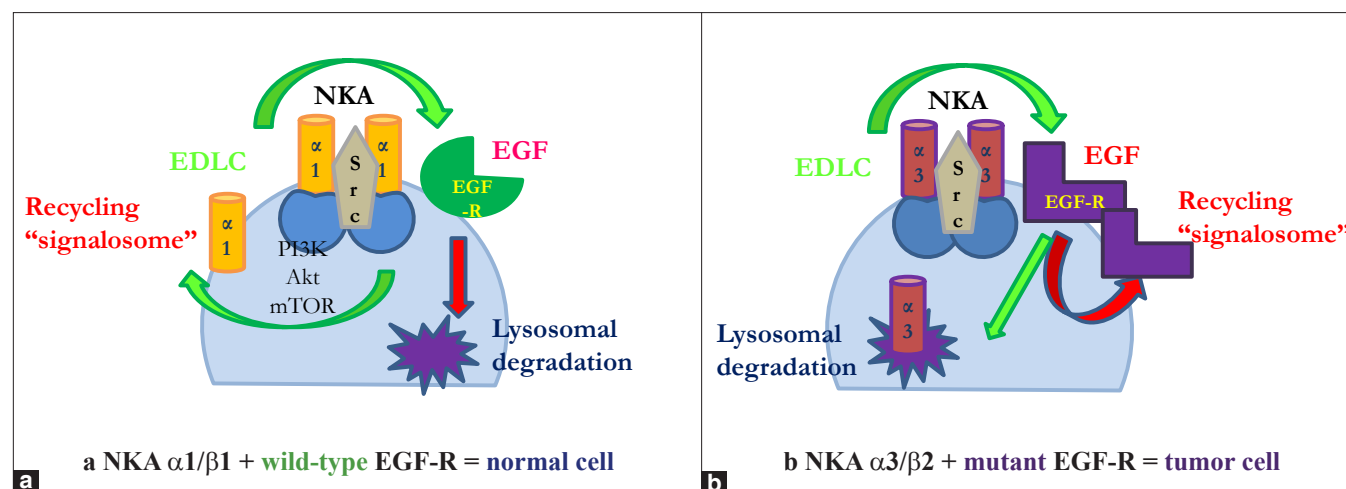
**Figure 2: (a)** Endogenous digitalis-like compounds (EDLC) and epidermal growth factor (EGF) induce different endocytotic trafficking pathways according to EGF-R mutation status and NKA isoforms. Transactivation of wt-epidermal growth factor receptor (EGF-R) by endogenous digitalis-like compounds induces endocytosis of the signalosome and causes recycling of the NKA αI subunit and other signalosome members at the cell membrane via activation of PI3K/Akt/mTor. Activation of wt-EGF-R by EGF, in contrast, results in lysosomal degradation of the signalosome, **(b)** Transactivation of mut-epidermal growth factor receptor (EGF-R) by endogenous digitalis-like compounds induces endocytosis of the signalosome and causes its lysosomal degradation. Activation of mut-EGF-R by EGF, in contrast, results in aberrant endocytic trafficking and recycling of EGF-R, NKA αI/3 subunits and other signalosome members

be even more pronounced in situations of sub-physiologic low EDLC plasma concentrations, e.g. in chronic stress conditions with relative exhaustion of the adrenal gland and inability to maintain normal EDLC plasma concentrations. Very low (pmolar) EDLF plasma concentrations (pmolar) are critical in the following aspects:

- First, they will affect exclusively malignant cells because the pmolar range lies below the response threshold for normal cells.
- Secondly, they will stimulate exclusively their proliferation because this is the main function of DLC close to or above the response threshold [Figure 1].
- Finally, they might trigger or support the above outlined dynamic switch in NKA isoforms with loss of β1 (substituted by β2) and loss of α1 (substituted by α3), resulting in increased invasiveness and hyperactivity of the Na/K-ATPase.

To the best of our knowledge, there are no published data about the effect of pmolar DLC concentrations on malignant human cells. But, regarding the pro-proliferative effects of low DLC concentrations on non-malignant cells, one may expect a similar effect in the lowest "physiologic cancer" EDLC ranges. Here, we also want to mention the dual function of ERK1/2: transient activation leads to cell proliferation while sustained activation promotes cell cycle arrest and cell differentiation.[48] Low-dose ouabain has been shown to induce transient ERK1/2 activation,[28,125,126] whereas higher physiologic doses (≤100 nM) caused a sustained ERK1/2 activation with consequent upregulation of the cell cycle inhibitor p21[cip].[78] Applying this concept on

our theory, we may draw the conclusion that both normal as well as tumor cells react with transient or sustained ERK1/2 activation according to their individual physiologic EDLC ranges [Figure 3].

## SUMMARY OF EVENTS IN EARLY TUMORIGENESIS WITH RESPECT TO THE ROLE OF NKA AND EDLC

- Early changes of cell membrane composition and fluidity, induced either by biophysical factors of the environment (hypoxia, ROS), overexpression of oncogenes (Snail) or parts of the ion channels (FXYD), result in increased cell membrane permeability, downregulation of NKA β1, loss of tight junctions, aggravation of membrane permeability and loss of K+.
- Downregulation of NKA α1 and upregulation of NKA α3 by cytokines from an increasing pro-inflammatory tumor cell environment with compensatory increase in NKA activity result in restored intracellular K+ and nutritional components (glucose, proteins). Other factors, e.g. overexpressed *Bcl-2*, may contribute to a hyperactivity of the NKA.
- High intracellular K+ concentrations promote cell proliferation and also transformation by stimulating the expression of oncogenes (c-myc, c-fos).
- High intracellular glucose concentrations enhance excessive cell growth, especially when the tumor cell switches to aerobic glycolysis (Warburg effect).
- At this point – under normal conditions – upregulation of endogenous defense mechanisms, including the HPA
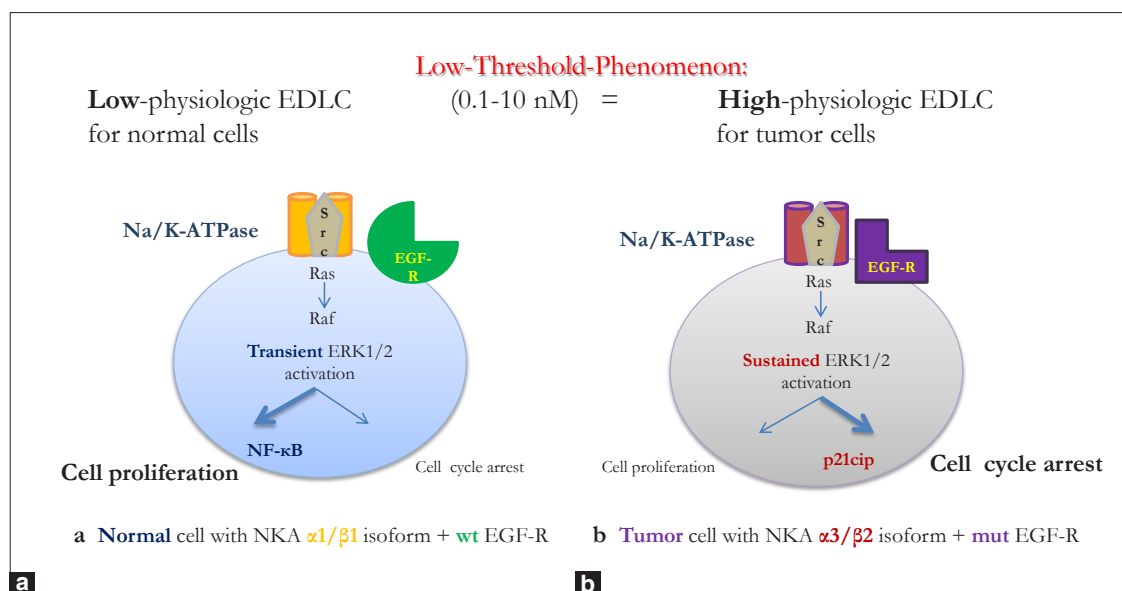
**Figure 3: Induction of different signaling pathways in tumor vs. normal cells: impact of NKA isoforms and epidermal growth factor receptor mutation status. (a)** In normal cells, low-physiologic endogenous digitalis-like compounds plasma concentrations (0.1 nM–10 nM) mainly stimulate cell proliferation, e.g. via transient activation of ERK 1/2 resulting in NF-kB induction, **(b)** For tumor cells, the same endogenous digitalis-like compounds concentrations represent a high-physiologic range and thus stimulate mainly cell cycle arrest via sustained activation of ERK 1/2 resulting in upregulation of Cip21

system with the EDLC, help to re-balance the NKA isozymes and NKA amount at the cell surface membrane and trigger signal cascades leading to cell cycle arrest and apoptosis.

• In case of a deregulated HPA function (de-sensitization) with pathologic low EDLC supply from the adrenal gland, a breakdown of normal cell stability and integrity occurs, with further acquisition of malignant features (loss of adherence, increased invasiveness) and predominance of pro-proliferative effects via EDLC-NKA-Src-EGF-R interaction on tumor cells.

## OUTLOOK: PRELIMINARY RESULTS– FUTURE AIMS

Future clinical studies are necessary to verify this low threshold phenomenon and its tumor-promoting effect in situations of reduced EDLC plasma concentrations. A first step would be to analyze the effects of pmolar DLC concentrations on different cancer cell lines. A second step could include analyzing EDLC plasma concentrations of cancer patients at first diagnosis as compared with healthy persons, the NKA isozyme profile and NKA activity in the tumor tissue of these cancer patients correlated to known markers of tumor aggressiveness and the mutation status of the EGF-R. Furthermore, in order to verify the concept of a deregulated HPA axis ("De-sensitization") as a cornerstone in tumorigenesis, it is warranted to evaluate, in between others, in the same patient and in control groups, the responses to

acute stress (TSST) of both DLC and cortisol and to analyze their correlation.

We put forward the hypothesis that the ratio of these two adrenal stress hormones (DLC/cortisol) is prognostically relevant, rather than their absolute plasma/serum concentrations.

Here, we have to consider two controversial issues: first, the available data about physiologic EDLC plasma concentrations are limited and quite variable due to different methods and protocols[127-130] [Table 1]. Second, the intracellular signaling cascades described in recent studies were often initiated by ouabain concentrations several orders of magnitude higher than the measured human plasma concentrations of putative endogenous ouabain. Hansen draws the conclusion that there is "No evidence for a role in signal-transduction of Na+/K+-ATPase interaction with putative endogenous ouabain."[131] But, remembering that many of the studies used tissues from rodents with a known ouabain-insensitive α1-isoform of the NKA, we may assume that in human tissue, the signaling pathways altogether are triggered at lower DLC concentrations.

In a pilot study, we aimed to analyze in healthy volunteers ($n = 15$) the plasma EDLC concentrations in correlation to cortisol (derived from saliva) by performing the mental stress test (TSST). For the first time, four specific responses ("cluster") of EDLC to stress exposure were revealed [Figures 4a–d]. After establishing the EDLC cluster in healthy individuals, we

**Table 1: DLC and OLC plasma concentrations in human beings**

| Protocol  -  compound | Plasma concentration (nmol/L) | Ref. |
|---|---|---|
| Healthy persons, 15' exercise, OLC | Rest : 2.5 ± 0.5 nmol/L ; exercise : 86 ± 27.2 nmol/L | 127 |
| Critical ill patients,  OLC | 3.59 ± 1.43 nmol/L vs. control 0.38 ± 0.31 nmol/L | 128 |
| Healthy persons, OLC | 0.09 ± 0.009 nmol/L | 129 |
| Patients cardiac insufficient, DLIS | 0.55 ± 0.44 nmol/L (range 0.26 − 1.52 nmol/L | 130 |
| Normotensive patients, EDLF | Basal med. 0.89 nmol/L; after ACTH med. 1.83 nmol/L | 12 |

OLC = Ouabain-like-compounds; DLIS = Digoxin-like-immunoreactive substances; EDLF = Endogenous digitalis-like-factors
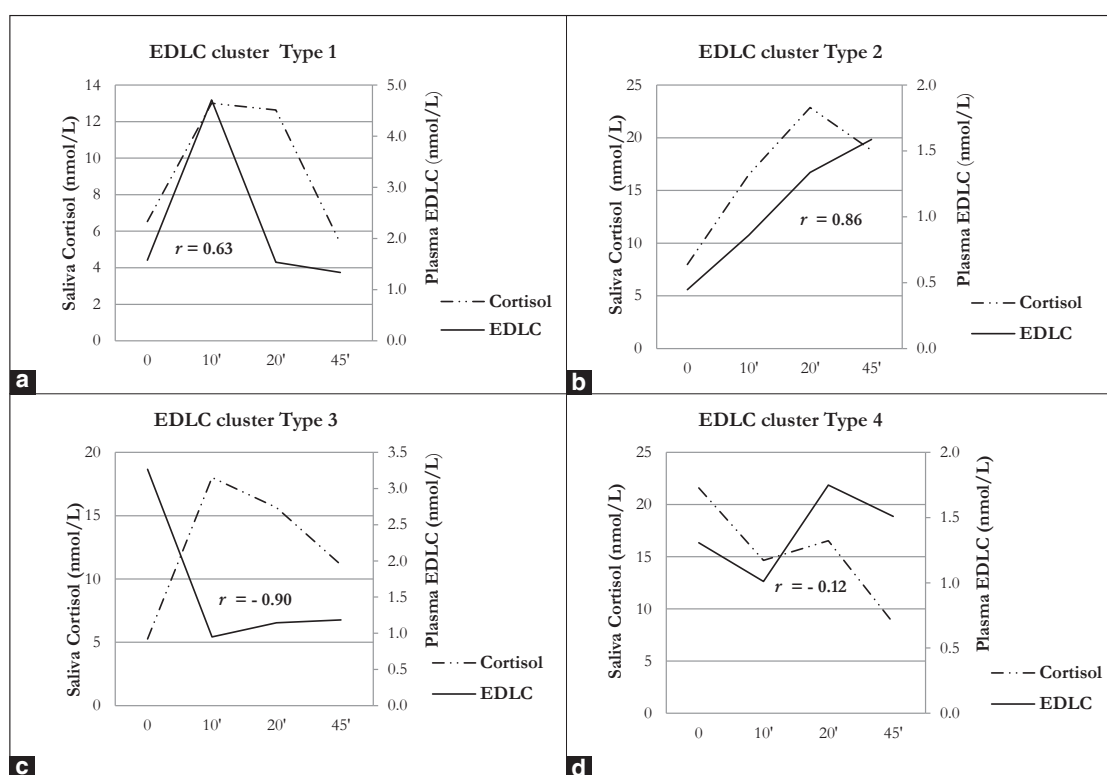


**Figure 4 (a–d): Individual endogenous digitalis-like compounds "cluster" in response to mental stress (TSST) and their correlation to cortisol. (a) Endogenous digitalis-like compounds (EDLC) cluster Type 1 is characterized by normal baseline levels, a rapid and marked increase shortly (10') after the stress test and a similar rapid and marked decrease afterwards. Cortisol in EDLC cluster Type 1 is characterized by normal baseline levels, a moderate increase shortly (10') after the stress test, a plateau phase and a return to baseline at 45'. The correlation to EDLC is moderate ($r = 0.63$), but not significant, (b) Endogenous digitalis-like compounds (EDLC) cluster Type 2 is characterized by rather low baseline levels and a slow but continuous rise after the test (up to 45'). Cortisol in EDLC cluster Type 2 is characterized by normal baseline levels and a continuous increase starting shortly (10') after the stress test, with a high peak at 20'. The correlation to EDLC is strong ($r = 0.86$), but not yet significant, (c) Endogenous digitalis-like compounds (EDLC) cluster Type 3 is characterized by very high baseline levels, a rapid and marked decrease shortly after the test (10') and remaining low to normal levels. Cortisol in EDLC cluster Type 3 is characterized by normal baseline levels, a rapid and quite marked increase shortly (10') after the test and a slow decrease afterwards. Interestingly, there is a strong inverse ($r = - 0.90$) and significant ($P = 0.042$) correlation to EDLC, (d) Endogenous digitalis-like compounds (EDLC) cluster Type 4 is characterized by low to normal baseline levels that do not rise after the test but remain all the time in the same range. Cortisol in EDLC cluster type 4 is characterized by extreme high baseline levels and a stepwise decrease starting shortly (10') after the stress test. The correlation to EDLC is only weak ($r = - 0.12$) and not significant**

analyzed the saliva cortisol concentrations corresponding to each of these EDLC clusters. We also discovered four distinct cortisol response patterns, but, interestingly, not always in positive correlation to EDLC [Figures 4a–d]. These results support our hypothesis that a dysbalance in EDLC/cortisol synthesis and secretion under prolonged stress exposure with inner "competition" may result in individually different risk

patterns for cancer development (see: "EDLC cluster type 3").

In another preliminary trial (Registration ID NCT00310882), we analyzed EDLC plasma and cortisol serum concentrations in breast cancer patients ($n = 22$) at the time of first diagnosis compared with patients with a benign breast disease ($n = 10$) as the control group. A significant positive
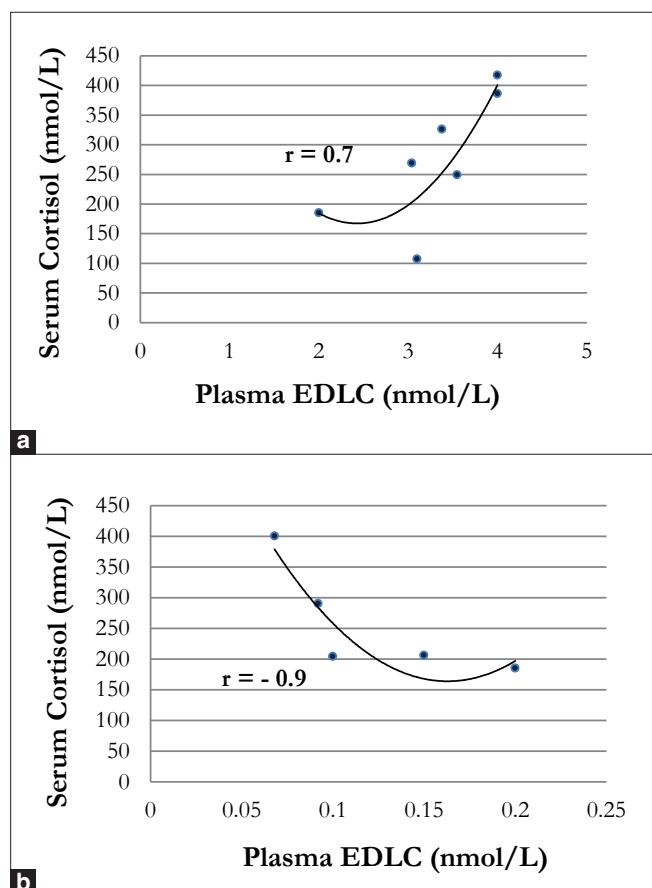
**Figure 5: (a) Positive correlation between endogenous digitalis-like compounds (EDLC) and cortisol in patients with benign/ malignant breast disease. This correlation was only observed in patients with physiologic plasma EDLC (>0.1 nmol/L) and serum cortisol concentrations (r = 0.7, P = 0.05), (b) Inverse correlation between endogenous digitalis-like compounds (EDLC) and cortisol in patients with breast cancer. This negative correlation was observed only in cancer patients with subnormal (<0.1 nmol/L) plasma EDLC concentrations (r = -0.9, P = 0.03)**

correlation between EDLC and cortisol was seen in the control as well as in patients ($r_s = 0.7, P = 0.05$), but only in cases of normal plasma/serum concentrations of both stress hormones [Figure 5a]. Interestingly, in breast cancer patients with very low EDLC plasma concentrations (<0.1 nmol/L), a significant inverse correlation ($r_s = - 0.9, P = 0.03$) was observed [Figure 5b]. This is in accordance with our previous findings and supports our hypothesis that high "tumor-promoting" cortisol concentrations are maintained under chronic stress at the expense of "tumor-protecting" EDLC.

## CONCLUSION

Assuming a lower threshold of malignant cells toward EDLC, it becomes evident that very low EDLC plasma concentrations due to an exhausted HPA system put an individual extremely at risk to develop cancer. It remains a challenging task to analyze in individuals their stress hormone response patterns

and to investigate and develop tools to rebalance disturbed EDLC/cortisol concentrations – e.g., by physical (exercise) and mental (hypnosis, meditation) methods. This task should be started as early as possible in childhood to avoid the development of hormonal dysregulation and to strengthen the individual's self-defense mechanisms against cancer.

## ACKNOWLEDGMENT

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