UNIVERSITY OF LJUBLJANA – FACULTY OF PHARMACY

Investigation of Angptl-4 gene expression in lung cancer tissue and adjacent non-cancer tissue

BACHELOR THESIS

by

Carina Pojer

Supervisor: Assoc. Prof. Dr. Darko Černe

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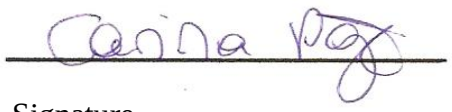
Statutory declaration

I hereby declare that the present bachelor thesis was written by myself under the mentorship of Assoc. Prof. Dr. Darko Černe. All parts of this thesis, which contain direct or indirect thoughts of other sources, are labeled. This work has not been tendered to any other examination board and has not been published so far.



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ABSTRACT

There are many unknown mechanisms concerning lung cancer, which is known to be one of the leading causes of death in the world. Several studies indicate a correlation between tumor progression and LPL (lipoprotein lipase) levels. For instance, in patients with resectable non-small cell lung cancer, LPL activity is higher in cancer tissue than in the surrounding non-cancer lung tissue. There are several possible mechanisms hypothesized trying explain the background being responsible this interrelationship.

The aim of our study was to investigate whether Angiopoietin-like protein 4 is responsible for the change in LPL activity levels. We hypothesized that Angptl-4 gene expression is higher in apparently healthy lung tissue surrounding the tumor than in the lung cancer tissue and that Angptl-4 gene expression negatively correlates with LPL activity (in lung cancer tissue as well as in the apparently healthy lung tissue surrounding the tumor). The study included forty two patients suffering from resectable non-small cell lung cancer. Samples of lung cancer tissue and surrounding, apparently healthy tissue were cut from the excised lung. To test the hypothesis we performed transcription of isolated mRNA to cDNA, real-time PCR (polymerase chain reaction) quantification of Angptl-4 gene expression and statistical analysis of the obtained values.

In contradiction to our expectations, Angptl-4 expression in tumor tissue was 1.48 times higher than in the non - cancer tissue ($p=0.0141$). We found no statistical correlation between Angptl-4 gene expression and LPL activity, neither in tumor tissue, nor in control tissue. In turn, Angptl-4 gene expression in tumor and control tissue positively correlated with fatty acid synthase expression ($p=0.0036$; $p=0.0152$) as well as with LDLR (low density lipoprotein receptor) expression ($p=0.0024$; $p=0.0011$). Furthermore, in control tissue Angptl-4 negatively correlates with serum triglycerides ($p=0.0389$) and serum albumin ($p=0.0068$), and positively with weight loss ($p=0.0070$). These findings suggest that in lung cancer patients Angptl-4 may have an important role in regulation of metabolism. Though we couldn't approve our hypothesis, the found correlations are in accordance with some studies, which also agreed upon the fact that Angptl-4 plays an important role in lipid and energy metabolism. Therefore, Angptl-4 has to be considered as a potential target for the development of new, well-directed therapies. Further studies are needed to find the real regulators of LPL activity in cancer tissue and in tissue surrounding the tumor.

KURZREFERAT

Viele Mechanismen rund um die Entstehung von Lungenkrebs, der zu den häufigsten Todesursachen weltweit zählt, sind noch unerforscht. Studien deuten darauf hin, dass ein Zusammenhang zwischen dem Tumorwachstum und der Höhe des LPL(Lipoprotein Lipase)-Levels besteht. Bei Patienten mit resektablem NSCLC (nicht-kleinzelligem Lungenkarzinom) wurde nachgewiesen, dass die LPL Aktivität im Tumorgewebe höher als im umgebenden, gesunden Gewebe ist. Dazu gibt es mehrere Hypothesen, die versuchen, die Hintergründe dieser LPL-Aktivitätsminderung darzulegen.

Das Ziel unserer Studie war, herauszufinden, ob Angiopoietin-like protein 4 (Angptl-4) verantwortlich für diese Aktivitätsminderung ist. Wir nahmen daher an, dass die Angptl-4 Genexpression im gesunden Gewebe höher ist als im Tumorgewebe, und dass die Angptl-4 Genexpression negativ mit der LPL-Aktivität korreliert (sowohl im Tumor- als auch im umgebenden, gesunden Gewebe).

Die Studie umfasste 42 Patienten, die an resektablem NSCLC litten. Es wurden vom entnommenen Lungengewebe Proben genommen: direkt aus dem Tumorgewebe und auch aus dem gesunden Gewebe. Um die Hypothese zu überprüfen, wurde isolierte mRNA zu cDNA transkribiert, Real-Time PCR (Polymerase-Kettenreaktion) zur Quantifizierung der Angptl-4 Genexpression und die statistische Analyse der erhaltenen Werte durchgeführt.

Im Gegensatz zu unseren Erwartungen war die Angptl-4 Genexpression im Tumorgewebe 1.48fach höher als im gesunden Gewebe ($p=0.0141$). Wir konnten keine statistische Korrelation zwischen der Angptl-4 Genexpression und der LPL-Aktivität nachweisen, weder im Tumor- noch im gesunden Gewebe. Dagegen zeigte sich eine positive statistische Korrelation (in Tumor- und in gesundem Gewebe) zwischen Angptl-4 Genexpression und Fettsäure-Synthase ($p=0.0036$; $p=0.0152$), als auch mit LDL(Lipoprotein niederer Dichte-) Rezeptor ($p=0.0024$; $p=0.0011$). Zusätzlich korrelierte Angptl-4 im gesunden Gewebe negativ mit Serumtriglyceriden ($p=0.0389$) und mit Serumalbumin ($p=0.0068$), als auch ein positiv mit dem Gewichtsverlust der Patienten ($p=0.0070$). Obwohl wir unsere Hypothese nicht bestätigen konnten, stimmen wir mit anderen Studien überein, dass Angptl-4 ein wichtiger Faktor im Metabolismus des Körpers ist. Daher ist Angptl-4 als potentieller Kandidat für die Entwicklung von Therapiemöglichkeiten zu betrachten und weitere Studien sind nötig, um die regulierenden Faktoren der LPL-Aktivität zu finden.

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Abbreviations

WHO	World Health Organization
NSCLC	non-small cell lung carcinoma
SCLC	small cell lung carcinoma
LPL	lipoprotein lipase
VLDL	very-low density lipoprotein
LDL	low density lipoprotein
FFA	free fatty acids
PPAR	peroxisome proliferator-activated receptor
LXR	liver X receptor
RXR	rexinoid receptor
PKC	protein kinase C
HDL	high density lipoprotein
TNF	tumor necrosis factor
IUGR	intrauterine growth restriction
CLL	chronic lymphocytic leukemia
LIF	leukemia-inhibiting factor
Angptl-4	Angiopoietin-like protein 4
PCR	polymerase chain reaction
IUCC	International Union Against Cancer Conference
LDLR	low density lipoprotein receptor
FAS	fatty acid synthases
MAT	Methionine adenosyltransferase
MT	methyltransferase
MS	methionine synthase
BHMT	betaine methyltransferase

SAM	S-adenosyl methionin
SAH	S-adenosylhomocysteine;
SAHH	S-Adenosyl-L-homocyteine hydrolase;
MAT1A	methionine adenosyltransferase I;

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1 INTRODUCTION

1.1 Lung cancer

According to recent studies of the World Health Organization (WHO), cancer is one of the most common causes of death throughout the world. Lung cancer causes approximately 1.3 million deaths each year. Among men, it is the most common form of cancer and among women, it is presently on second place after breast cancer. Although there is progress in the development of therapy, the prognosis for affected patients is still poor. Therefore the WHO predicts an increase of deaths caused by cancer [1]. There are numerous unanswered questions concerning lung cancer which need further elucidation and it is of primary importance for cancer research to identify and describe proteins and enzymes which could be responsible for or participating in the regulation of these malignant tumors.

In general, lung cancer is defined as unregulated growth of cells in lung tissue. The predominant type of lung cancer is carcinoma, a malignant form of tumor which has its origin in epithelial tissue.

There are two central types of lung carcinoma called non-small cell lung carcinoma and small cell lung carcinoma. They are divided in those two groups because they have several characteristics in common.

1.1.1 Non-small cell lung carcinoma (NSCLC)

The three sub-types of non-small cell lung cancer are squamous cell lung carcinoma, adenocarcinoma and large cell lung carcinoma. Their commonness is primarily based on their treatment: If the tumor is in an early stage and not yet metastasizing into other organs beside the lung, it is the best way to remove the tumor to enhance the chance for recovery of the patient [2].

Squamous cell carcinoma, which is the lung cancer sub-type in 25-35%, is mainly placed in the center of the lung and his origin is typically located in the bronchial epithelium.

Accounting for approximately 35-40% of lung carcinomas and therefore the most common ones, adenocarcinomas have their origin mostly in peripheral tissue, evolving of mucus producing glandular tissue. Non-smokers are typically affected by this form of lung carcinoma.

In 5-10% of the cases, it is concerning large cell lung carcinomas. Their only commonality is the size of the tumor cells and in most of the cases they show an aggressive, rapid growth progression.

1.1.2 Small cell lung carcinoma (SCLC)

Small cell lung carcinomas represent approximately 15-20% of lung carcinomas, and nearly all of the concerned patients are smokers or former smokers. This type of carcinoma is known to metastatasize in early stages and surgical removal of the tumor is mostly not possible or of no avail in avoiding tumor progression. Moreover, the average survival rate is considerably low in comparison to NSCLC (non treated SCLC has a survival rate of approximately 6-18 weeks) [3].

Manifestations in lung cancer are multifaceted and they are dependent on various factors, such as location and growth rate of the tumor. Only in 5-15% of the patients, this malignant disease is discovered by routinely performed chest radiography before they are suffering from any symptoms like a persistent cough, which is the most common symptom.

Mostly affected from lung cancer are patients, who are 50 - 70 years old, persons younger than 35 are usually not concerned [4].

Different publications encourage the hypothesis that lipoprotein lipase (LPL) takes a part in the regulation of tumor growth in a not fully established manner. Some studies investigated as case in point the plasma postheparin LPL activity of patients with different forms of cancer, and they found a considerable decrease of activity, especially in lung cancer patients, where they obtained the lowest values with 54% of normal activity [5].

1.2 Lipoprotein Lipase

Lipoprotein lipase (LPL) is the most important enzyme for the hydrolysis of triglycerides, which are circulating in the body. Therefore it is considered to play a primary role in lipoprotein metabolism. For this hydrolysis which is associated with providing free fatty acids, LPL is connected with the lumen of blood vessels. Due to this connection, these free fatty acids can be embodied by adipose and muscle tissue [6].

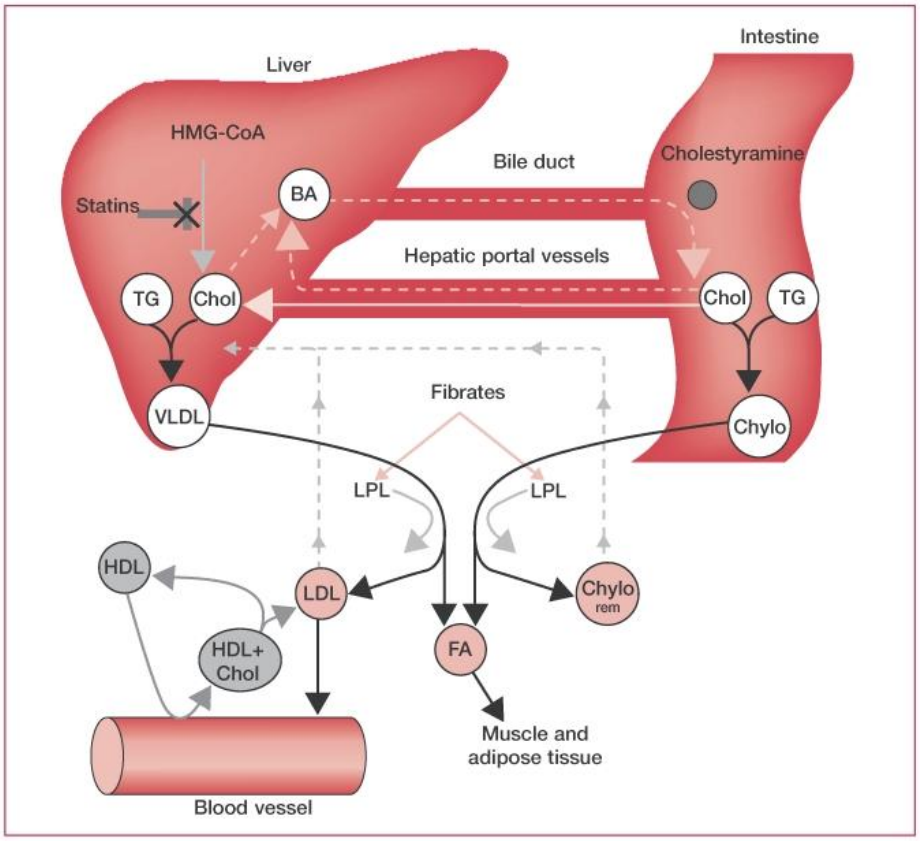


Figure 1: Illustration of the role of LPL in lipoprotein metabolism [7]

As demonstrated in Figure 1, LPL hydrolyzes triglycerides located in circulating triglyceride-rich lipoproteins (VLDL and Chylomicrons), which are then converted into LDL or chylomicron remnants. Then LDL can be attached to a receptor for assimilation. FFA-uptake can now be proceeded by the surrounding muscle and adipose tissue.

As already mentioned above, LPL is mainly detectable in the cardiac and skeletal muscle and in the adipose tissue. In addition, LPL activity is also found in the following tissues:

adrenal, ovary, thoracic aorta, mammary glands, spleen, small intestine, testes, lung, kidney, brain and liver (during neonatal period) and also in differentiated macrophages [8].

Its active form is a non-covalent homodimer. According to recently published findings, which have been obtained by the detection of fluorescence resonance energy transfer between the subunits of LPL, rapid exchange of the subunits happens in this LPL dimer. In addition, it has been shown that the monomers are more disposed to aggregate because their structure offers more binding sites and thereby it is more flexible [9].

LPL acts as a triglyceride hydrolase for very low-density lipoproteins (VLDL) and chylomicrons, but it also has a so-called bridging function, because of which LPL is able to attach lipoproteins to the blood vessel wall which enables an easier particle uptake and hydrolysis. Moreover the specific uptake of vitamin A and E or other lipoprotein associated lipids is provided by this mechanism, without the incorporation of the whole lipoprotein. In addition, the enzyme serves as a ligand for most of the LDL receptors.

The gene, which encodes for LPL is a 28-kb gene which is situated on the long arm of chromosome 8. The mRNA of LPL is 3.5 kb long. The regulation of lipoprotein lipase takes place on different levels: on transcriptional level, on translational and also on posttranslational level [10].

1.2.1 Regulation on transcriptional level:

Several reporter genes are responsible for the activity of the LPL promoter, so transient transfection assays were used to identify different potential cis-regulatory elements. Relating to these findings it is shown that the 5' regulatory area extends up to 4 kb from the transcription start. Within 101 bp upstream of the transcription start site the elementary promoter elements are situated. This basic transcriptional equipment comprises an octamer motif interacting with the proteins Oct-1 and Oct-2, a TATA-Box and a CCAAT box which is able to bind nuclear factor-Y [11]. This above mentioned octamer motif is considered to play a basic role in positioning the preinitiation complex formation [12].

Apart from this elementary transcriptional equipment, there are several other cis-acting elements described: A CT element, which is known as an enhancer and local control

region of genes in general, is found upstream of human LPL gene and it is considered to have an effect on basal promoter activity by attaching to the transcription factors Sp1/Sp3 [13]. A sterol regulatory element, which is located in the same region as the CT element mentioned above, is known to competitively interact with SREBP-1 and also with Sp1. Therefore, this collaboration of the CT element and the sterol regulatory element is hypothesized to offer a way of communication for cholesterol and triglyceride metabolic pathways [14]. These Sp1/Sp3 factors are able to interact with an interferon-γ-responsive element as well. This conclusion was detected in test arrangements where interferon-γ was added to macrophages resulting in decrease of Sp3 and thereby in diminishment of LPL transcription rates [15]. Relating to recent data declaring that tissue specific induction of LPL is interrelated to the concomitance of the corresponding peroxisome proliferator-activated receptors (PPARs), a tissue specific modulation of LPL activity and LPL mRNA levels by respective PPAR activators exists [16]. In addition to those transcription factors, there is also the oxysterol liver X receptor responsive element described. It is located in intron 1 of the LPL gene and it is shown, that this element interacts with the liver X receptor (LXR) and the rexinoid receptor (LXRα/RXR). For this reason it has an impact on tissue specific LPL messenger RNA expression too [17]. Furthermore, there is also the hypothesis that a nuclear factor-1 like motif is involved in the suppression of LPL expression, indicating that this factor may hire another factor by forming a protein complex [18].

The plurality of the factors described above is considered to activate or upregulate LPL transcription or respectively activity, but there are also elements or transcription factors which have a silencing effect on LPL activity. They have been described from -225 to -81 within the LPL promoter [19].

1.2.2 Regulation on translational and posttranslational level:

Even if it is shown that there are many possibilities for the regulation of LPL activity and expression on translational and posttranslational level, the participating processes are barely investigated. For the translational regulation of LPL the involvement of protein kinase C (PKC) is indicated for instance. In several studies decreased LPL expression levels were detected when PKC was inhibited in adipocytes [20].

Moreover there are multiple points of application during the maturation of the functional LPL enzyme. There are many steps involved where posttranslational regulation is possible concerning especially the dimerization and glycosylation in the Golgi complex and in the endoplasmic reticulum. It is not elucidated whether LPL attains its active form already in the endoplasmatic reticulum or whether it has to be transported to the Golgi complex to attain its definite active form [21,22].

There are several transcription factors which can interact on posttranslational level too, such as PPARα and PPARγ agonists. They are known to influence cellular LPL trafficking, which refers to the glucose conditioned translocation of LPL from intracellular to extracellular sites of pancreatic β-cells [23]. This type of translocation was also noticed in cardiomyocytes hypothesized to be dependent on insulin and glucocorticoid secretion. Studies with cytochalasin D disproved the influence of insulin and glucocorticoid, so Ewart et al. published the hypothesis of the participation of the actin cytoskeleton in the regulation of cellular trafficking [24].

Another possible factor in posttranslational regulation of LPL is calcium. Recently published data show that calcium has an influence on refolding of LPL after entire denaturation of the molecule. The first step of refolding is quickly performed, starts from the C-terminal domain and calcium is not needed for this folding of an inactive LPL-monomer. The second step, on the contrary, is triggered by Ca2+. This slow step involves the compact folding of the monomers, so they are able to form dimers. In these experiments, Ca2+ triggers the regaining of activity of before inactive monomers [25].

These numerous factors mentioned above, which are all able to somehow influence the regulation of LPL show that there are multiple, possible ways to change the expression and also the activity of LPL. These data indicate again that further elucidation in this area of research is absolutely needed.

1.2.3 Tissue specific regulation and expression of LPL

LPL is present and also regulated in many different tissues. Therefore it is important to consider the tissue specific expression and activity of LPL, not only the postheparin plasma LPL activity for measuring the LPL activity in the entire body. In addition, tissue specific regulation of LPL offers the possibility for locally regulated assimilation of FFAs, lipids and lipoproteins as a whole. This in turn facilitates the allocation of nutrients in different target tissues [10].

Concerning for example plasma triglyceride levels, variations in LPL levels are strongly associated with hypertriglyceridemia. As it is shown in patients suffering from heterozygous lipoprotein lipase deficiency, they develop severe hypertriglyceridemia and show decreased HDL levels [26]. These heterozygotes are also known to have an increased risk of premature atherosclerotic diseases [27]. In approximately 20% of the cases patients suffering from hypertriglyceridemia are carriers of mutations, mostly concerning common mutations in the LPL gene (like Asp9Asn, Asn291Ser) [28]. Apart from these mutations, there are several other metabolic causes for decreased or increased LPL activity:

Insulin resistance in type 2 diabetes for example is known to decrease LPL activity in adipose tissue and additionally released interleukin-6 and TNFα leads to further decrement [29]. In macrophages, there are also several factors which are known to downregulate or inhibit LPL activity or its gene expression: lipopolysaccharides [30], different cytokines (gamma-interferon, interleukin-1 and interleukin-2) [31] and prostaglandin E2 [32]. Factors that increase LPL levels in macrophages are for instance glucose, which is supposed to involve transcriptional mechanisms [33], or homocystein, which is shown to increase both LPL activity as well as LPL expression [34].

LPL is supposed to play an important role in adipose tissue and also in muscle tissue. Publications indicate that if the ratio adipose tissue to muscle tissue LPL activity is raised, free fatty acids (FFA) can be delivered to the adipose tissue and stored there. This, in turn, is followed by obesity and this whole mechanism is known as the ‘gatekeeping function of LPL’ [35]. Certainly it is also hypothesized that muscle tissue LPL is regulated inversely to whom in adipose tissue. So it is concluded, that inhibiting LPL by factors like TNFα may prevent or work against obesity because the ratio changes towards muscle LPL and

therefore FFAs can be delivered to muscle tissue instead of being stored in adipose tissue [36].

With the aim of detecting mechanisms, which have an influence on the regulation of LPL, a study with lean and obese cats has been carried out. The researchers measured LPL, TNFα and hormone-sensitive lipase in adipose and muscle tissue and they approved, amongst others, with the hypothesis of the 'gatekeeping function' mentioned above. Moreover, they also demonstrated that TNFα influences this mechanism by suppressing adipocyte LPL [37]. This hypothesis, indicating that TNFα has an inhibiting influence on LPL activity and that this mechanism of LPL regulation plays an important role in energy metabolism, was also examined in human beings. There, they also found increased TNFα mRNA levels corresponding to the degree of adiposity. TNFα levels are decreased when LPL levels are elevated (like it is the case in obese patients) and they are increased when LPL is decreased (in case of loss in weight for instance) [38].

LPL plays an important role not only in energy metabolism, but also in carbohydrate metabolism. A study with transgenic mice indicates that insulin resistance combined with increased triglyceride levels and reduced glucose uptake in skeletal muscle results from overexpression of LPL in muscle tissue. This finding is regarded as a further indication that muscle insulin resistance is strongly connected with the contained muscle lipid. By contrast, the role of LPL in insulin resistance concerning the liver is not yet fully elucidated. It is also known that increased expression of liver specific LPL is followed by the decrease of glucose tolerance and this overexpression is also known to interfere with the insulin signaling pathway [39].

Another important aspect is the role of placental LPL in severe intrauterine growth restriction (IUGR). IUGR is a complication during the pregnancy where the fetus is exposed to the risk of starving because of a reduction of nutrients supplied to the fetus. In this study the researchers supported the hypothesis that in cases of severe IUGR placental mRNA gene expression is significantly higher in comparison to levels in pregnancies which go according to plan without severe complications. This increased expression of placental LPL results in augmentation of nutrient transport to the fetus [40].

In addition to the roles of LPL described above and its regulation therein, LPL is indicated to be involved in tumor growth.

1.3 The role of LPL in cancer:

Sakayama and colleagues already provided evidence in 1994 that LPL is present in a number of examined samples of human sarcomas and carcinomas. They also demonstrated, that human osteosarcoma cells produce LPL to make free fatty acids available, which can further be used as components of cell membranes and certainly also for nutrition and development of the tumor. Additionally, they indicated that LPL is not uniformly distributed in tumor tissue and that this inhomogeneity in distribution is associated with the level of proliferation of the examined tumor tissue. Thus, they found increased LPL activity levels in areas of the tumor tissue, which show progressive growth at the time of the measurements [41].

Already in 1967, Arthur Spector made obvious that a tumor needs free fatty acids for its active proliferation. Tumor cells are shown to synthesize fatty acids by transforming for instance glucose, but it is hypothesized that tumor cells are not able to produce enough fatty acids for active proliferation. This hypothesis indicates that most of the available LPL delivered to the tumor is already formed by the host [42]. In 1974, studies in Ehrlich cells show that these cells can make use of triglycerides which are located in Ehrlich ascites plasma VLDL under in vitro conditions. These results lead to the conclusion that one important function of the VLDL, which is present in the ascites plasma, might be to transport FFAs in form of triglycerides from the other body compartments of the host to the tumor tissue. Even if there is a part of the present FFAs delivered from the ascites plasma FFAs, the amount of FFAs which is transported by VLDL in form of triglycerides is considerably large [43].

An additional factor, which clarifies that there exists an influence of LPL on cancer development and accordingly progression, is a study concerning Ser447 stop polymorphism. This stop polymorphism affects synthesis of LPL, because it results in a very rapid processing in the Golgi apparatus and endoplasmic reticulum. The quicker synthesis results in higher activity and this is hypothesized further to increase the risk for developing of prostate cancer [44].

Moreover, it is shown in tumor-bearing rats that there exists a tumor dependent suppression of LPL activity in surrounding non-cancer tissues. With ongoing tumor

progression in one day fasted rats, a considerable decrease of LPL activity in cardiac muscle and epididymal fat tissue was noticeable. After the excision of the tumor the LPL activity switched back to the normal activity [45].

Tumor growth is also shown to be influenced by several drugs. Cortisol, which is already endowed to patients for pre-and postoperative neoadjuvant chemotherapy, is shown to reduce proliferation of human osteosarcoma cells in vitro and this study also indicated an inhibiting effect of cortisol on LPL synthesis which results in a downregulated activity of LPL [46].

Peroxisome proliferator-activated receptor (PPAR) alpha agonists like bezafibrate or PPARgamma agonists like pioglitazone were used in studies with Apc-deficient mice suffering from age-dependent hyperlipidemia which is known to be connected to the development of colon cancer and polyp formation in intestines. Therefore, these mice had decreased levels of LPL mRNA in intestines and liver. It was demonstrated that both drugs mentioned above suppress both hyperlipidemia and, as a further consequence, also colon polyp formation by stimulating increase of mRNA levels [47]. These effects were also shown in Min (multiple intestinal neoplasms) mice, which are known for a high rate of spontaneous tumour development in intestines. The researchers used different doses of another PPARgamma agonist, pioglitazone, depending on the triglyceride and VLDL levels in the Min mice. In this study the increasing effect of the PPARgamma agonist drug on liver-specific mRNA levels and further suppression of colon polyp formation was again demonstrated. Therefore, it was hypothesized that both PPARalpha agonists, as well as PPARgamma agonists might be prospective chemopreventive drugs for colon cancer [48].

Bezafibrate is also known to extensively increase the growth development of the MAC16 tumor in cachectic animals if it is available for tumor tissue. MAC16 is a subcutaneous adenocarcinoma with a slow growth rate in general and it is very debilitative for the host by causing severe secondary actions body, such as cancer cachexia. This increase of the progression rate was not indicated in MAC13 tumor, which generally shows a similar histological structure as MAC16, but is not affecting body compartments and also not inducing cachexia. Therefore, it is again hypothesized that the enabled increasing uptake of FFAs is responsible for the elevating tumor level [49].

Concerning the survival prognosis of cancer patients there are two relevant publications at the moment. The first one is a study which investigated the association of LPL mRNA expression levels and the survival rate of patients with B-cell chronic lymphocytic leukemia (CLL). The research team found that there is a definite correlation of increased LPL expression and reduced survival time without treatment. Due to these data they indicated that LPL is to be regarded as a reliable, prognostic marker. Although in this case should be considered that CLL is not a solid tumor and that the measurements of LPL mRNA expression levels were taken in blood and so the results are not related to specific body compartments [50]. The second publication is a study concerning patients suffering from resectable non-small cell lung cancer (NSCLC). The researchers tested the hypothesis that elevated levels of LPL activity in cancer tissue have a predictive value for shorter patient survival. This hypothesis has been confirmed, but they found no association of LPL expression and survival time and therefore no possible prediction of patient survival. These findings also approve that the tumor needs lipids for its progression. In this trial, they also discovered that the increase of the LPL gene expression in non-cancer lung tissue, which surrounds the tumor and contains obviously healthy cells, is in an unexplainable manner associated with considerably decreased LPL activity in the same tissue. This difference in the levels of expression and activity seems to be somehow influenced or even regulated by the tumor [51].

There are several possible mechanisms described trying to find reasons or responsible influencing variables which could be responsible for the differences in LPL activity levels. One potential mechanism could be the intervention of several inhibitory factors. These factors can either be produced by the host or by the tumor tissue itself, such as cytokines like TNFα or leukemia-inhibiting factor (LIF) or a not yet fully-elucitated 76kDa –protein, which is known to be heat-labile. They all show a noticeable inhibitory effect on LPL activity when conditioned with samples of different human lung cancer cell lines [52].

Another possible explanation could be the intervention of angiopoietin-like protein 4. It is also the aim of the present work to evaluate whether this protein is a possible modulator for lipoprotein lipase activity and whether it is the responsible factor for the decrease of LPL activity in the non-cancer tissue surrounding the tumor.

1.4 Angiopoietin-like protein 4

Up to now, there are already seven members of the angiopoietin-like (Angptl) protein family established. They were named like this, because they have several structures with the angiopoietins in common. For instance, they both have an N-terminal coiled-coil domain as well as a fibrinogen-like domain at C-terminal. Thus, the most important difference separating them from angiopoietins, is, that they do not attach to tie receptors, neither to Tie2 nor to the similar structured Tie1 receptor, which are both known as typical angiopoietin receptors. Three of the Angptls are known to have influence on lipid metabolism, whereof Angptl-3 and Angptl-4 are able to suppress LPL activity which leads to increase of triglycerides in plasma [53]. They differ for instance in their sites of action. Whereas Angptl-3 is predominantly found in liver, Angptl-4 has been detected in various tissues, but mostly in adipose tissue. Furthermore, Angptl-3 is reported to bind to liver X-receptor, in contradiction to Angptl-4 which is known to interact with PPARs [54].

Angptl-4 is reported to show an oligomeric structure with intermolecular disulfide bonds. It is known to form these structures (dimers and tetramers) before the protein is secreted. Cleavage leads in vivo to release of the two domains, whereas the N-domain maintains the oligomeric structure in contrast to the C-domain, which splits to monomers. Thus it has been shown that the suppression of cleavage does not influence the ability of Angptl-4 to inhibit LPL activity. On the other hand, if oligomerization is inhibited, the protein loses this function. Therefore it is indicated, that building the structure of oligomers, and not the cleavage, is the essential step for regulation of the LPL-inhibiting and possible other abilities of Angptl-4 [55,56]

Studies in mice demonstrated, that both Angptl-3 as well as Angptl-4 have the ability to inhibit LPL activity leading to increasing plasma lipid levels, when administered to the animals. The comparison displayed in addition that Angptl-4 increases these levels higher than Angptl-3 [57]. It is also shown that mice with deficiency in Angptl-4 and Angptl-3 have increased levels of LPL and therefore a decrease in triglyceride levels. As well in the inverse case, mice having highly elevated levels of Angptl4 show increase in triglyceride levels and decrease in activity of LPL. These effects are known to become more severe in the fasting state [58].

As it is already mentioned in the studies above, Angptl-4 is being promoted as important regulator of LPL. Therefore, searchers engaged themselves in finding the underlying mechanisms how Angptl-4 acts upon LPL. They found that Angptl-4 acts neither on transcriptional level, nor does it affect the processing or the synthesis of LPL. Surprisingly, they detected that this protein uses a quite uncommon method. It causes a change in the conformation of the LPL molecule, in such a way that LPL switches from his active, dimeric form to a monomeric form, which is known to be the inactive form. This mechanism starts with the binding of the amino-terminal coiled-coil domain of Angptl-4 to the LPL molecule, whereas Angptl-4 is known to have a high bonding capacity for LPL, binding is induced also at low levels of added Angptl-4 [59]. It is indicated that the rapid exchange of monomers respectively subunits in LPL molecules has a supportive effect on this mechanisms [9,59]. Whereas it is not yet known, if Angptl-4 attaches to the dimer itself or if it binds to monomers during the subunit exchange of LPL [59]

Studies with rats showed again that Angptl-4 takes part in the regulation of LPL activity also as a reaction to alterations in nutrition. Food deprivation in rats leads to elevated mRNA levels of Angptl-4 and further suppression of LPL activity. When the rats were allowed to feed again, Angptl-4 mRNA showed a decrease of approximately 75% within 6 hours and LPL activity showed an elevation exceeding 200% [59]

This form of inactivation of the LPL molecule is known to lead to missing catalytic activity and also decrease in affinity for heparin, but it is hypothesized, that the LPL molecule has still elementary functions, because the monomers remain folded and because of that they are supposed to be relatively stable. It is further determined that Angptl-4 does not stay attached to the enzyme after inactivation, but it is able to bind to other LPL molecules and lead to further inactivation. From this basic, it is indicated that the role of Angptl-4 in this context is a catalytic one [9].

The aim of the present work is to investigate whether Angptl-4 takes part in regulation of LPL activity in lung tissue, relating to the study where the differences in the LPL activity levels in tumor tissue and in the surrounding non-cancer tissue of lung cancer patients were determined [51]. Therefore, the gene expression levels of Angptl-4 in lung cancer tissue and also in the surrounding non-cancer tissue were measured.

2 OBJECTIVE

There are many unknown or not well understood facts and mechanisms concerning lung cancer, which is known to be one of the leading causes of death in the world among men, as well as among women [1]. It is supposed, that LPL, which is the most important enzyme for the hydrolysis of triglycerides circulating in the body, plays a considerably important role in the development of certain forms of lung cancer [51]. It is provided evidence in appropriate studies that there is a correlation between tumor progression and LPL levels [41, 44]. It is moreover shown that in patients with resectable non-small cell lung cancer LPL activity is higher in cancer tissue than in the surrounding non-cancer lung tissue [51, 62]. As it is mentioned in the introduction of this text, there are several possible mechanisms described trying to find reasons or responsible influencing variables which could be responsible for the differences in LPL activity levels [47-51].

The aim of this study is to investigate whether Angiopoietin-like protein 4 is connected with the change in LPL activity levels or if there is no correlation between the LPL activity values and this inhibitory factor. We hypothesized:

1.) Angptl-4 gene expression is higher in apparently healthy lung tissue surrounding the tumor than in the lung cancer tissue; and that
2.) Angptl-4 gene expression negatively correlates with LPL activity (in lung cancer tissue as well as in the apparently healthy lung tissue surrounding the tumor).

This would mean that Angptl-4 may regulate LPL activity and thus may modify the LPL – driven influx of lipids to the tumor.

To test the hypothesis we will perform transcription of isolated mRNA to cDNA as well as Real-time PCR quantification of Angptl-4 gene expression and statistical analysis of the obtained values.

To confirm the hypothesis would mean that Angptl-4 is most likely the factor, which regulates LPL activity in this case and potentially offers itself as a target for intervention to develop new methods for inhibiting tumor growth or even development of the tumor.

3 PATIENTS AND METHODS

3.1 Patient Selection and Tissue Sampling

The study includes forty two patients suffering from resectable non-small cell lung cancer in stages I, II and III. These patients have undergone surgical excision of the tumour and at the time of the removal, samples of lung cancer tissue and surrounding, apparently healthy and visually unaffected tissue were cut from the excised lung within 15 minutes of the operation. The tumor tissue samples were taken from the periphery where the tumor was supposed to have the most viability. The non-cancer tissue samples were taken from lung periphery as far away from tumor as possible. These samples, which are supposed to represent normal and healthy lung tissue, were taken from each patient as control tissue.

The obtained tissue samples were stored in liquid nitrogen until analyzing.

Staging was performed according to TNM Classification of Malignant Tumours, 6th edition, International Union Against Cancer Conference (UICC). The histological analysis of the obtained samples was performed according to World Health Organization (WHO) histological classification. Every patient enrolled in the study received 5000 IU dalteparin (Fragmin; Pfizer, New York, USA) subcutaneously 15 to 20 hours before the operation. Furthermore, samples of venous blood were taken before the surgery, after at least 12 hours of fasting. The obtained samples were centrifuged for 10 minutes at 1600 x *g* after coagulation of the blood and the serum was stored at -80° C for analysis. The study was approved by the national ethics committee. Informed consent was obtained from all patients enrolled in the study. [51, 62]

The following of the patients has lasted for four years. The examinations have taken place every three months in the first two years, then, for the following two years, the patients have been examined every six months. For every examination, clinical status and also the x-ray results have been registered. Bronchoscopy, CT scans and scintigraphy have been used in suspicious cases. The patients affected by stage III, have been treated accessorily with chemotherapy after the removal of the tumor.

3.2 RNA Isolation and Transcription to cDNA

RNA of all samples was isolated from tissue with RNeasy Mini Kit (Quiagen, Hilden, Germany) following the manufacturer's protocol. Approximately 30 mg were used for the isolation and homogenized under liquid nitrogen in a highly denaturating guanidine-isothiocyanate-containing buffer, which is responsible for the inactivation of RNAses. The suspension is then passed through a gDNA Eliminator soin column, which enables the removal of genomic DNA. Then ethanol is added to the flow-through, which offers optimal binding conditions for mRNA. The mixture is applied to a RNeasy Spin column and its membrane is able to bind total RNA. Other components are washed away, so genomic DNA contamination is avoided. The obtained RNA isolates were aliquoted into 0.5ml tubes. The RNA quality and amount was estimated with RNA 6000 Nano LabChips (Agilent, Palo Alto, CA), which were run on Agilent 2100 Bioanalyser platform.

For the Real-Time PCR, internal standards for controlling batch to batch variability were mixed out of three different samples of lung tissue and they were treated the same way as the samples. Aliquots of the samples as well as the aliquots of the internal standards were immediately frozen at -86°C to avoid changes in the gene expression profile.

Reverse transcription of RNA to cDNA of all the samples as well as the internal standards was performed with a High-Capacity cDNA Archive Kit (Applied Biosystems Foster City, CA). The reaction mixture contains supplied Reverse Transcription Buffer, 50 U of Multiscribe Reverse Transcriptase, dNTP mixture, random primers, Rnase-free water and 5 µl of RNA inhibitor. The final volume amounts to 100 µl, whereof 50 µl is sample and 50 µl of the above mentioned Mastermix are added. These reaction mixtures were incubated at 25°C for 10 minutes, 37°C for 120 minutes and 95°C for 5 minutes in a polymerase chain reaction thermocycler (MWG AG Biotech Primus 96plus). The obtained cDNA samples were aliquoted and stored at -86°C.

3.3 Real-time PCR Quantification of Angptl-4 gene expression

The measurements for the quantification of Angptl-4 gene expression in the samples mentioned above were taken with TaqMan real-time PCR. One microliter of cDNA was used for each measurement and the Mastermix consisted of 10 µl TaqMan Mix (TaqMan Universal PCR Master Mix, Applied Biosystems), 1µl of primer and probe mix (18

µmol/L and 5 µmol/L, respectively) (TaqMan Gene Expression Assay, Applied Biosystems) and 8µl purified water. From this follows the total volume of 20 µl. The samples were FAM (6-carboxyfluorescin) labeled. To avoid random errors, which could occur accidentally, all analyses were made in triplicate and paired samples, so that tumor tissue and control tissue of the same patient were analyzed at the same plate. The reaction mixtures were analyzed with ABI Prism 7000 Sequence Detection System PCR thermal cycler (Applied Biosystems) in 96-well optical reaction plates. The incubation lasted for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C and 60 seconds annealing/extension at 60°C. For every plate, an internal standard was carried along and according to the measured CT values of the internal standards the elimination of batch to batch variation was enabled. The mean CT value of all internal standard samples was adjusted by manually lowering or raising the threshold value of each batch.

Concerning the increased sensitivity of the quantitative PCR, a suitable internal control gene is required for normalization. Selection of internal control gene was performed with TaqMan Human Endogenous Control Plate (Applied Biosystems), where 11 possible genes were available. These genes are supposed to be transcribed relatively constant and further to not being affected by the experimental conditions. Four pairs of samples (cancer and control-tissue), which are known to be as different as possible, were chosen for the selection of the target gene. Reaction mixtures were prepared according to manufacturer's protocol and run on ABI Prism 7000 Sequence Detection System PCR thermal cycler. In each cycle, the gene which is most inappropriate was excluded. This excluded gene was chosen by comparing the similarity of the Ct - values in all 8 samples. Patterns, which are not similar, indicate that this gene is affected by disease or by different influences during measurement and it is not convenient as an internal control gene. So the selection of this gene was based on the smallest geometric average Ct – values of individual candidates and results from geNorm [60]. Selected as internal control gene was GUS (β-glucoronidase) and it was VIC (6-carboxyrhodamine) labeled.

3.4 Statistical Analysis

Results are presented as medians with 25^{th} and 75^{th} percentiles and range values. Comparisons of parameters between cancer and control tissue samples were evaluated with Wilcoxon matched pairs test and spearman rank order correlations test. Associations between variables were made with Spearman rank order regression analysis. Comparisons between groups of the same parameter were evaluated with Mann-Whitney or Kruskal-Wallis tests. Anova- median test was used to evaluate the correlation between variables and stage of disease. For assessment of the effects of variables on survival Cox regression method has been used.

Statistical analysis was carried out with program package SPSS version 12.0 (SPSS, Chicago, IL, USA). Values of $p < 0.05$ were assumed to indicate statistical significance.

4 RESULTS

During the four years of the study one patient died due to a non-cancer reason and therefore has to be excluded from statistical analysis.

In table 1, the most important characteristics of the remaining forty one participating patients are summarized. It is apparent that the distribution of gender is uneven with males accounting for 73.17 % of cases. At the time of examination, the median age of the patients was 63 years and most of them, 87.80 %, were current or former smokers. The predominant histological type of tumor was squamous cell carcinoma, accounting for 46.34 % of all the cases, followed by adenocarcinoma representing 31.71 % of cases, whereof the stage of disease according to TNM classification of malignant tumours are mostly stage I and stage II, accounting for 41.46% and 34.15%, respectively.

Table 1: Basic characteristics of examined subjects (examined in part in earlier studies) [62, 51]

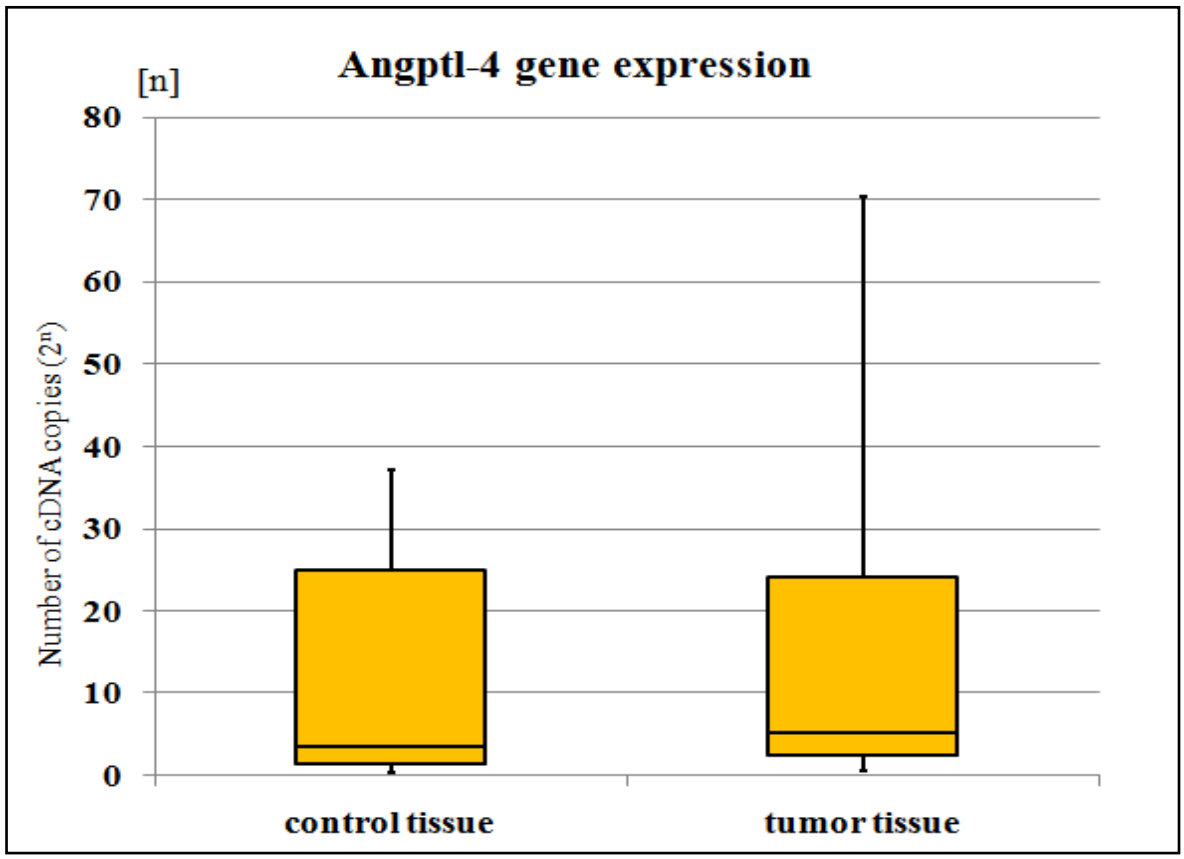
Parameter	**Patient**
Gender (male/female)	30/11
Age (years)	63.0 {53.0/67.0} (44-77)
Body mass index (kg/m²)	24.8 {22.2/28.3} (16.4-46.5)
Stage of disease according to TNM classification of malignant tumors (I/II/III)	17/14/10
Histological classification of tumor (squamous cell/adenocarcinoma/large cell/other)	19/13/5/4
Smoking status (never/current or former)	5/36

Values are median {25th percentile/75th percentile} (range) or number of subjects.

The obtained samples of lung cancer tissue and non-cancer (control-) tissue were stored at -86°C.

Basic laboratory findings are summarized in Figure 2.

Figure 2: Basic experimental results of Angptl-4 gene expression in tumor tissue and in adjacent control tissue.

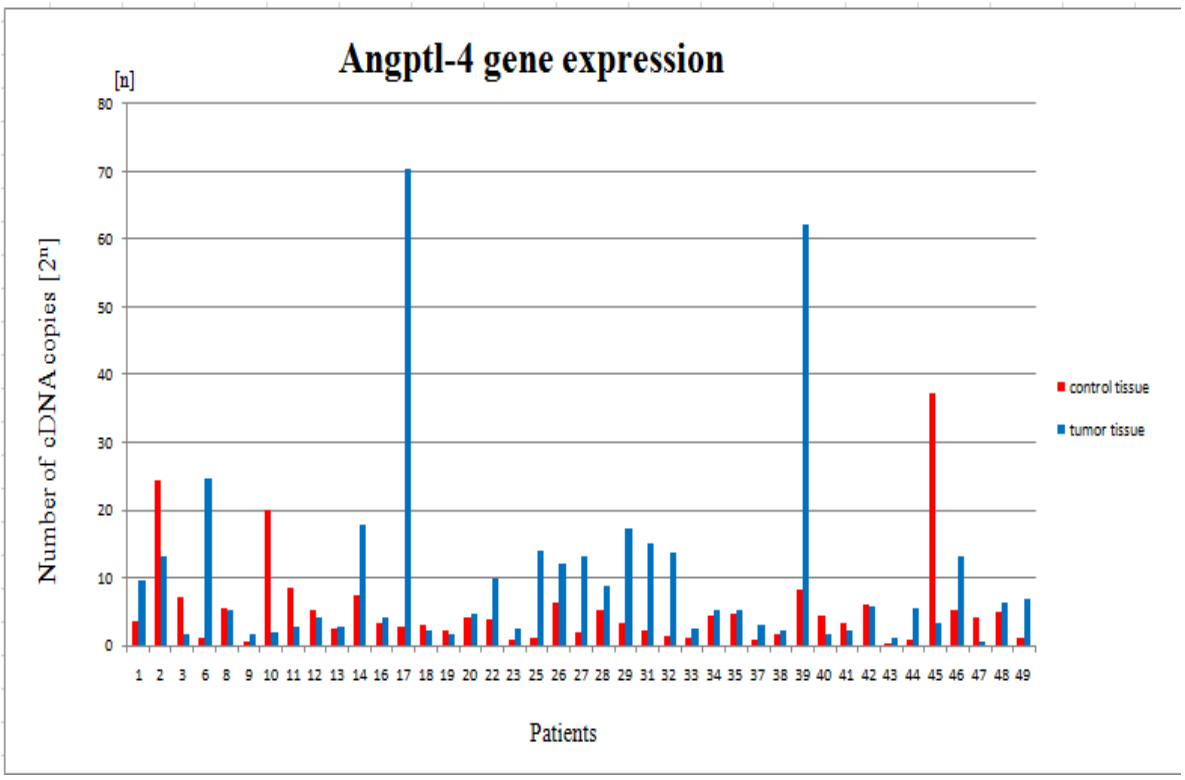


Histograms indicate median {25th percentile / 75th percentile} and bars indicate range.

As it is displayed in Figure 2 and in contradiction to our expectations and the formed hypotheses, Angptl-4 expression in tumor tissue is 1.48 times higher than Angptl-4 expression in the adjacent, non - cancer tissue (named “control tissue” below). The median value of the Angptl-4 gene expression in tumor tissue is 5.33 in comparison to the median value of the control tissue which accounts for 3.60. The p-value accounts for 0.0141, which is on the border to significance, but it is still significant. Also the maximum value in the tumor tissue is considerably higher (70.52 in tumor tissue vs. 37.27 in control tissue). In

turn, the minimum value in control tissue is not that much lower than in tumor tissue (0.63 in tumor tissue versus 0.36 in control tissue).

Figure 3: Results of Angptl-4 gene expression for each patient (in tumor and in control tissue).



As shown in Figure 3, Angptl-4 gene expression is higher in the tumor tissue in 28 cases out of 41, therefore accounting for 68.29 %. In the other 13 cases, gene expression was higher in the control tissue (in 31.71% of cases).

Furthermore, there is no statistical correlation found between Angptl-4 gene expression in tumor tissue and Angptl-4 gene expression in the control tissue.

Cancer stage was distributed almost uniformly from stage I to stage III, as it is shown above in Table 1. Based on this distribution, ANOVA median test was performed to evaluate whether there is a correlation between Angptl-4 expression in tumor tissue and the stage of disease. Similar to the tests performed above, there is no statistical correlation between the stage of disease and the gene expression of Angptl-4 in tumor tissue ($p = 0.0696$). The corresponding p-values and results, respectively, for each stage are shown in Table 2.

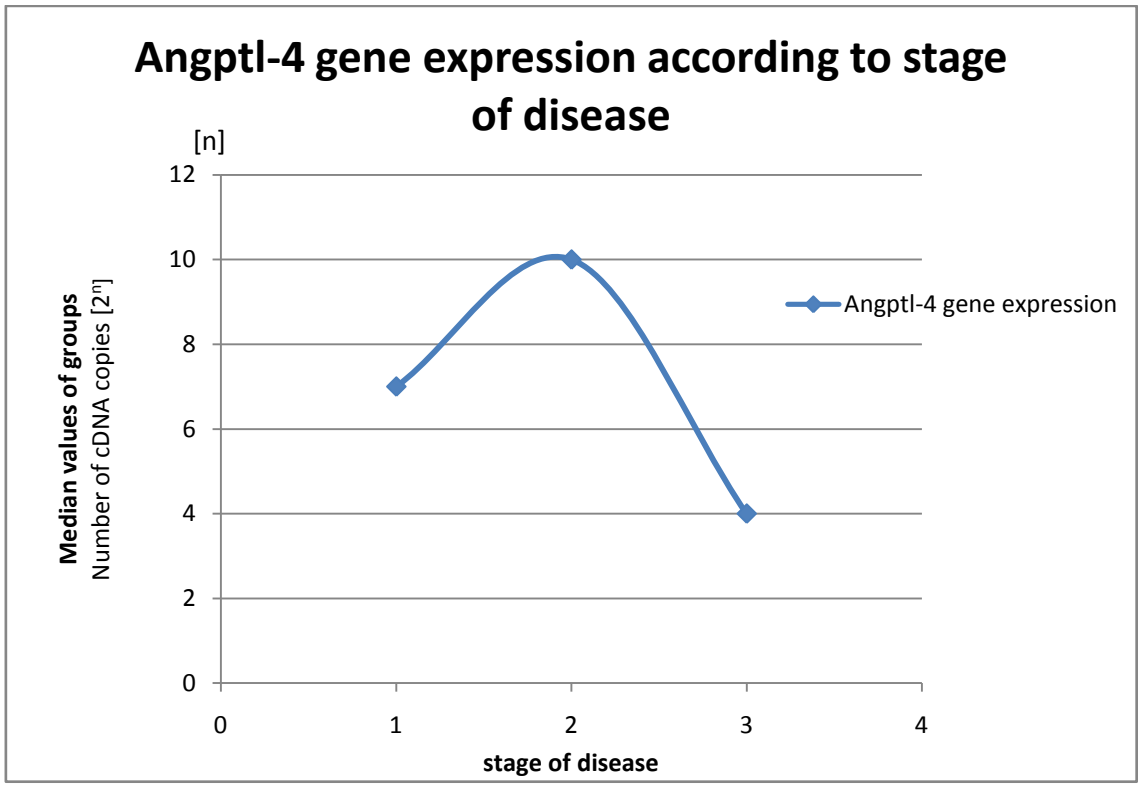
Table 2: Angptl-4 gene expression compared to stage of disease (according to TNM classification of malignant tumors) and corresponding p-values.

Stage of disease	Comparison of mean values	Corresponding p-values
I	stage I > stage II	p = 0.0722
II	stage I > stage III	p = 0.9076
III	stage II < stage III	p = 0.0818.

As it is shown in Table 2, there is no statistical correlation found between the stage of disease and Angptl-4 gene expression in tumor tissue, even if two of the three p-values are near the border to significance. The results illustrate the course of Angptl-4 gene expression according to the different stages.

In Figure 4, there is a possible way which course the Angptl-4 gene expression in tumor tissue takes. It is a schematic design of the course we hypothesized in referral to the results that were gained through the statistical analysis.

Figure 4: Course of Angptl-4 gene expression according to stage of disease.



There are some variabilities in Angptl-4 gene expression between different stages of disease, but they are statistically insignificant. Furthermore, higher stage of disease is definitely not associated with higher Angptl-4 gene expression. As it is shown in Figure 4, we assume that the highest values of Angptl-4 gene expression are found in stage I. The lowest values are supposed to be in stage II and in stage III, a re-increase of Angptl-4 gene expression is assumed.

Due to the relatively high number of patients suffering from the histological types of squamous cell carcinoma and adenocellular carcinoma, an individual statistical analysis of these two histological types of lung cancer was possible. In turn, there was no statistically significant difference of Angptl-4 gene expression in tumor tissue between these two types of tumor ($p= 0.6045$). However, it was not possible to draw firm conclusions, because the clinical stratification of those two subgroups was poor. Namely, there was higher abundance of smokers having a long smoking history in the squamous cell carcinoma subgroup, but on the other hand higher abundance of females in the adenocellular carcinoma subgroup.

During the 4-year follow-up, 21 out of the 41 remaining patients died. Their death was a result of tumor progression. One patient, who died due to a non-cancer reason, was excluded. The Angptl-4 gene expression is shown to not correlate with the 4-year patient survival ($p = 0.987$). As mentioned above, Cox regression method was used to assess the effect of Angptl-4 on survival. Again, four years after operation, there is no statistical difference detectable in Angptl-4 expression between patients who survived and patients who deceased. (in tumor tissue $p = 0.2849$; in control tissue $p = 0.3545$).

Furthermore, the correlation of the measured Angptl-4 gene expression with other clinical data and some other previously measured parameters was evaluated. In the following there are the most important results:

1.) In contrast to our expectations, it is shown that Angptl-4 expression did neither correlate with LPL activity nor with LPL gene expression measured in the same tissues (no correlation in tumor tissue as well as in control tissue).
2.) In turn, Angptl-4 gene expression in tumor tissue positively correlated with FAS expression ($r = 0.4446$; $p = 0.0036$) as well as with LDLR expression ($r = 0.4614$; $p = 0.0024$). These p-values show a relatively strong correlation.
3.) Positive statistical correlation between Angptl-4 gene expression and FAS ($r = 0.3768$; $p = 0.0152$) as well as with LDL receptor ($r = 0.4932$; $p = 0.0011$) was found also in the control tissue.
4.) Angptl-4 gene expression in control tissue correlated positively with weight loss in kg ($r = 0.4145$; $p = 0.0070$) and it is also evident that Angptl-4 gene expression is higher in patients with weight loss than in patients without weight loss. ($p = 0.0099$).
5.) Angptl-4 gene expression in control tissue further statistically correlated with triglycerides in serum ($r = -0.3238$; $p = 0.0389$)
6.) Additionally, there was also a correlation found with albumin in serum ($r = -0.4160$; $p = 0.0068$).

5 DISCUSSION

Our study showed that in resectable non-small cell lung cancer tissue the gene expression of Angptl-4 as a whole is 1.48 times higher in tumor tissue than in the adjacent, apparently healthy lung tissue (control tissue). The finding of the higher gene expression of Angptl-4 in lung cancer tissue corresponds to another study (and their published data) in another form of cancer.

In a French study concerning conventional renal cell carcinoma researchers found higher gene expression as well as protein levels of Angptl-4 in tumor cells especially in the hypoxic areas which surround the necrotic regions of the tumor. The French researchers assumed, according to their findings, for Angptl-4 to be a marker of conventional renal cell carcinoma and they further drew the conclusion that in this case Angptl-4 is induced by hypoxia. Therefore, they suggested a possible use of Angptl-4 levels as a diagnostic factor and a potential target for new therapies, which is able to modulate angiogenesis in tumors as well as in other ischemic tissues, like in critical leg ischemia. Although they found higher levels of Angptl-4 mRNA in tumor cells of conventional renal cell carcinoma, they could not find higher levels of Angptl-4 mRNA in tumor tissue of other benign or malignant renal tumors [61].

Table 3 summarizes the Angptl-4 gene expression in various cancer tissues. As it was mentioned above, high Angptl-4 gene expression is only found in one type of renal cancer: in conventional renal carcinoma. In the other forms of renal carcinomas, expression of Angptl-4 was detectable only in tumoral cells. Furthermore, the researchers of this study reported about Angptl-4 gene expression in hepatocellular carcinoma, in liposarcoma and in brain tumors. In opposition to our results, the researchers of this study reported that Angptl-4 gene expression was detectable only in tumoral cells of adenocarcinomas and not in other histological types of lung cancer [61]. This contradicts our results that there is no difference in Angptl-4 gene expression between adenocarcinoma and squamous cell histological type of lung cancer.

Table 3: Angptl-4 gene expression in different tumor tissues [**Fehler! Textmarke nicht definiert.**]

Organ	Tumor type	Angptl-4 gene expression in normal tissue	in tumoral cells	in peri-necrotic cells
Kidney	Conventional carcinoma	−	+++	+++
	Tubulo-papillary carcinoma	−	+	−
	Chromophobe cell carcinoma	−	+	−
	Composite tumor	−	+	−
Breast	Canalar invasive carcinoma	−	−	−
Lung	Adenocarcinoma	−	+	−
	Squamous cell carcinoma	−	−	
	Small cell carcinoma	−	−	
Brain	Glioblastoma	−	+	−
	Oligodendro-glioma	−	+	−
Soft tissues	Angiosarcoma	−	*	−
	Liposarcoma	++	++	++

Angptl-4 gene expression levels are indicated by + signs; - signs indicate no detected gene expression; * indicates the presence only of very small regions of necrosis, in which Angptl-4 mRNA was barely detected.

Our experimental data showed that only in 13 out of 41 cases, the Angptl-4 gene expression was higher in the control tissue than in the tumor tissue, where we previously had found very low LPL activity levels. In the rest of the cases, accounting for 68.29%, Angptl-4 gene expression was higher in the tumor tissue, where we had previously measured high LPL activity levels. These results do not prove the hypothesis that Angptl-4 is involved or somehow responsible for the changes in LPL activity levels by shifting dynamic equilibrium from dimeric of LPL towards inactive monomeric form. We hypothesized a downregulation of LPL activity by Angptl-4 in the control tissue and

therefore expected increased Angptl-4 gene expression in the control tissue when compared to the tumor tissue.

Additionally, there was no statistical correlation found between Angptl-4 gene expression in tumor tissue and the stage of disease. As it is shown in Figure 4, the Angptl-4 gene expression does not increase or decrease with the increasing or decreasing, respectively, stage of disease. We tried to assume a curve which shows the course of Angptl-4 gene expression according to the different stages of disease. As it is shown, we can conclude that there is definitely no trend of Angptl-4 gene expression with the increased severity of the disease.

Furthermore, Angptl-4 gene expression did not correlate with the histological type of tumor. As mentioned above, there was no difference in Angptl-4 expression in tumor tissue between squamous cell and adenocarcinoma histological type of the tumor. These findings were in accordance with an earlier study concerning the same samples. In this study, they also found that neither LPL activity nor LPL gene expression in tumor tissue correlated with the histological type of the tumor [62].

In addition, the gene expression of Angptl-4 did neither correlate with the 4-year patient survival, nor there was a statistical difference between survived and deceased patients four years after operation. Therefore, according to our results, Angptl-4 has no predictive value in this way.

These results seem to be in opposition to another study concerning tongue cancer. Chinese researchers examined Angptl-4 gene expression in a subtype of tongue cancer by analyzing paraffin-embedded samples of the tumor. They found that high gene expression of Angptl-4 had a predictive value concerning the poor prognosis of the patients suffering from oral tongue squamous cell carcinoma, especially, if there was a concomitant high expression of tenascin-C protein. Hence, they report that Angptl-4 is to consider as an independent prognostic value of poor patient’s survival in cases of tongue cancer which is, in comparison to other prognostic values, not dependent on lymph node metastasis status [63].

In our study, we also found correlations of Angptl-4 gene expression with several other observed parameters. In tumor tissue as well as in control tissue Angptl-4 gene expression correlates positively with FAS expression and LDL receptor expression. The correlations were especially strong in tumor tissue. Fatty acid synthase (FAS) is known as homodimeric, multifunctional structure responsible for the intracellular de novo synthesis of fatty acids [64]. The LDL (Low-Density-Lipoprotein) – receptor family is a family of chimeric proteins, which consists of several distinct domains. The receptor complex is bound to the cell surface of most of the nucleated cells. Those receptors mediate the uptake of LDL through endocytosis and its synthesis is assumed to be controlled in general by the amount of free intracellular cholesterol. It is supposed that if there is an intracellular overdose of cholesterol, the gene expression of the receptor gene is inhibited [65]. Both, LDL receptor as well as FAS, are supposed to be expressed not in macrophages, but rather in tumor cells. Further, they are supposed to act in a combined way and therefore to be an indirect marker for what is happening in tumor tissue. Thus, they are in part responsible for the supply of lipids and energy, respectively, for the cell - whereof the LDL receptor provides lipids from the blood and the FAS are responsible for the de novo intracellular synthesis of fatty acids.

Furthermore, there was also a positive correlation between the Angptl-4 gene expression in control tissue and the weight loss of patients. 17 out of 41 participating patients suffered from weight loss in the last three months (accounting therefore for 41.46 %), whereof the remaining 24 patients had no remarkable weight loss. The statistical analysis showed that Angptl-4 gene expression in control tissue is higher in patients with weight loss than in patients without weight loss.

In addition, there is a statistical correlation of Angptl-4 gene expression with the concentration of triglycerides in serum ($r = -0.3238$; $p = 0.0389$) as well as with the concentration of albumin in serum ($r = -0.4160$; $p = 0.0068$). These results indicate that albumin in serum and triglycerides in serum are decreased while Angptl-4 gene expression is increased.

In summary it can be said that these results of the statistical analysis are not what we had expected, but seen from another perspective, they seem to be quite reasonable. First, the results indicate that our hypothesis cannot be approved. Angptl-4 is therefore assumed to

be not the factor sought-after, which was supposed to down regulate or decrease LPL activity in control tissue. Even though our hypothesis is not approved by the obtained results of the statistical analysis, there seems to be another important role of Angptl-4: its role in lipid metabolism.

According to recently published data, Angptl-4 is supposed to be a novel endocrine factor that highly increases plasma triglyceride levels by inhibiting the clearance of triglycerides. A Dutch study further showed that Angptl-4 levels in plasma show strong variations from human to human, but these levels for the individuals stay relatively stable during the day. In turn, the levels increase considerably in response to fasting, caloric restriction and endurance sports. The researchers of this study further demonstrated that intralipid injection as well as the usage of beta-adrenergic agonist, which both causes an increase of plasma FFA levels, elevates Angptl-4 gene expression too. Therefore, the researchers drew the conclusion that plasma Angptl-4 levels are increased by the factors mentioned above and they also suppose that this is probably mediated by increased plasma FFAs [66]. These findings are somehow in accordance with our results and it might offer a reasonable explanation for the higher Angptl-4 gene expression in tumor tissue: the tumor would need influx of lipids and therefore it is useful to increase the plasma FFAs.

Moreover, another study proposed Angptl-4 again as a strong hyperlipidemia-inducing factor. The researchers examined their hypothesis by intravenous administration of Angptl-4 in KK/San mice. After 30 minutes they analyzed the plasma lipid levels which showed a distinct increment and they further performed an LPL assay for approving the Angptl-4 impact and its inhibiting effect on LPL. This study demonstrated that Angptl-4 should be considered as a strong hyperlipidemia - inducing factor and as a new possible therapeutic agent for hyperlipidemia. So, the results of this study encourage again the importance of the role of Angptl-4 in lipid metabolism [67]. This is confirmed by another study concerning Angptl-4 and its ability of regulation of glucose homeostasis and insulin sensitivity. They showed that adenovirus-mediated expression of Angptl-4 causes hyperlipidemia and potently increases plasma triglyceride levels [68]. This ability of Angptl-4 to regulate triglyceride metabolism is confirmed in another study concerning the effects of transgenic Angptl-4 overexpression and its targeted disruption. They generated mice with a deficiency of Angptl-3 and Angptl-4 and also mice with overexpression of human Angptl-4 in liver. The overexpression of human Angptl-4 in mice caused a

considerable increase of plasma triglycerides and a decrease of postheparin plasma LPL activity. In turn, the Angptl-4 deficient mice suffered from hypotriglyceridemia and increased LPL activity [69]. This hyperlipidemic effect of Angptl-4 was further confirmed in another study which investigated the effect of adeno-virus mediated overexpression of Angptl-4. The researchers of this study also found that this expression led to an increase of plasma triglyceride levels [70]. Hence, these studies demonstrated and approved the effects of Angptl-4 mentioned above in animal models with adeno-virus mediated overexpression as well as with intravenous administration of Angptl-4.

All these studies confirm in general that Angptl-4 is a potent hyperlipidemia-inducing factor and further confirm its potentially important role in lipid metabolism. These findings are somehow in accordance with our results and suggest that cancer tissue may increase Angptl-4 gene expression in order to induce hyperlipidemia and thus improve the supply of lipids for the tumor.

As mentioned above, 17 patients suffered from weight loss. This weight loss leads to a deficit of energy and therefore a shortage of nutrients. The body further tries to compensate this shortage of energy with everything which is available at that moment. This lack of energy leads to the situation that tissues maximize the mechanisms to cover the needs. This might be a reasonable cause for the increase of FAS – and LDL – receptor expression in our study. As we mentioned, these two expressions correlated with the elevated Angptl-4 gene expression in the same tissue.

Furthermore, the low albumin levels could be caused by the lack of energy and the according use of proteins for energy production. For this, albumin is a candidate because it forms a large proportion of all plasma protein and has a high turn-over rate, which means that a big amount of protein can be synthesized in short time – this, in turn, could be used as an energy source by the body. Although we found in our study relatively high statistical correlations between the factors mentioned above, it has to be considered, that we studied tissues as a whole, as a heterogenous cell population. If Angptl-4 is expressed in non-tumor cells of cancer tissue, then it should be released into the bloodstream, to be finally able to modify FAS- and LDL-receptor expression in tumor cells. If we act on the assumption that Angptl-4 is released into the blood stream after expression, it leads to the question, why it doesn’t show an effect on LPL, which is located extracellularly. It is not examined yet, in

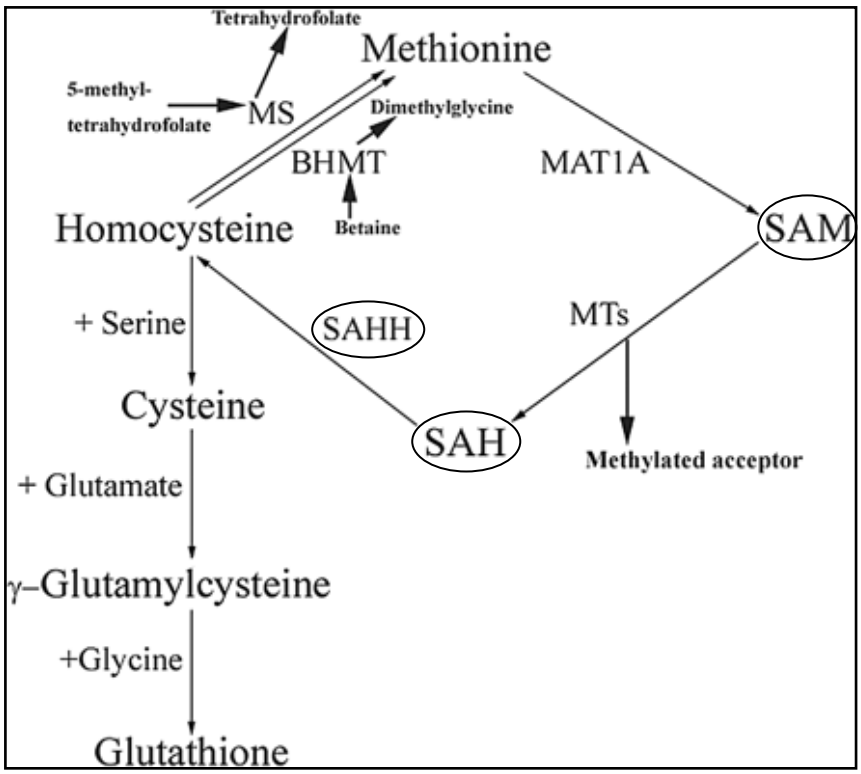
which cell lineAngptl-4 is mainly expressed, even if it is already published that Angptl-4 may be expressed in endothelial cells, adipocytes and smooth muscle cells [61].

In addition to the role of Angptl-4 in lipid metabolism mentioned above, it has to be considered that several studies demonstrated that this protein also plays an important role also in energy metabolism and insulin sensitivity, whereof the underlying molecular actions are not fully elucidated. This second important role of Angptl-4 seems reasonable when taking into consideration that Angptl-4 is known to be the target gene of peroxisome proliferator-activated receptors (PPARs), of both, of the lipid-lowering PPARα agonists, fenofibrates [71], as well as of the insulin-sensitizing PPARγ agonists [72].

A study, where researchers examined protein profiles in the livers of db/db mice treated with Angptl-4 confirmed that Angptl-4 has to be considered as an important protein in energy metabolism. They compared protein liver profile with the protein profile of lean mice and found that 118 proteins were down- or up- regulated in db/db mice. Moreover, they found 14 spots corresponding to the key enzymes of methionine and homocysteine metabolic pathways. Interestingly, they approved that continuous administration of Angptl-4 reversed most of these up- or down- regulated proteins nearly to a level of lean mice [73].

Figure 5 demonstrates the enzymes which are supposed to be involved in the methionine pathway.

Figure 5: Metabolic Pathways of Methionine – Illustration of the deregulated enzymes in db/db mice involved in those pathways [73].



MAT = Methionine adenosyltransferase; MTs = methyltransferase; MS = methionine synthase; BHMT = betaine methyltransferase, SAM = S-adenosyl methionine; SAH = S-adenosylhomocysteine; SAHH = S-Adenosyl-L-homocyteine hydrolase; MAT1A = methionine adenosyltransferase I;

As it is shown in Figure 5, this study proved that several of these up-regulated enzymes are involved in the methionine/homocysteine metabolic cycles and they showed further that treatment with Angptl-4 leads to significantly decreased expression levels of these enzymes. For instance, MAT1A, which is the only enzyme to catalyze SAM synthesis and also SAHH, which is necessary for the cleavage of SAH and the further generation of homocysteine, are shown to be up-regulated in db/db – mice. Interestingly, this increased expression was reversed by Angptl-4 administration. SAM is known as an essential methyl donor for biological methylation reactions of molecules like DNA or RNA for example. According to these results, the researchers assumed that Angptl-4 regulates or is involved in the regulation of methionine/homocysteine cycles in the livers of db/db – diabetic mice [73].

Moreover, according to the fact that most of these dysregulated enzymes in db/db – mice are located in mitochondria and that long-time administration of Angptl-4 also leads to an increase of activity in mitochondria respiratory chain complex II-III and IV in db/db-mice, and thus increases energy production, the researchers of this study suggested that Angptl-4 obtains its metabolic effects through modulation of mitochondria functions. Angptl-4 driver increased energy production in mitochondria is also somehow in correspondence with increased Angptl-4 expression in tumor tissues found in our study. In disagreement with earlier studies, they could not approve that the hypoglycemic effects of Angptl-4 are related to its effects on fatty acids combustion and that there is no distinct relation to peroxisomal fatty acid β-oxidation in particular [73]. This is also in agreement with our results, because Angptl-4 gene expression correlated, as already mentioned, with FAS receptor expression.

In conclusion it has to be said that though we couldn't approve our hypothesis that Angptl-4 is the protein, which is responsible for the decrease of LPL activity in control tissue, we found several other correlations as mentioned above. Therefore, there need for further investigation to find the regulators of LPL activity. The obtained results in this study, even if they are not confirming our hypothesis, are also relevant and may lead to further investigations in this field.

At the moment, there is a variety of hypotheses concerning Angptl-4 and its effectiveness, but its role, especially the mode of action and the underlying molecular mechanisms, are poorly understood. Despite these diversities, all the conclusions drawn from the different studies agreed upon the fact that Angptl-4 plays an important role in lipid metabolism as well as in energy metabolism.

Finally, Angptl-4 has to be considered as a potential target for the development of new, well-directed therapies and further studies are going to inform us about all the possibilities of its action.

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