DIPLOMA THESIS

Expression of different adipocytokines in breast cancer, adjacent and distant fat tissue

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ABSTRACT

Introduction:

Adipose tissue is an important endocrine organ producing bioactive peptides (proteins) known as adipocytokines (e.g.; leptin, interleukin-6, tumor necrosis factor-α, adiponectin, resistin, serum amyloid A, etc.) with various metabolic roles. Deregulation of adipocytokines may lead to an elevated cancer risk. Overweight and obese postmenopausal women have a higher risk developing breast cancer. The question rises up how different adipocytokines are expressed in breast tumor tissue in comparison to adjacent and distant fat tissue.

Materials & Methods:

For this retrospective study frozen breast fat tissues (adjacent to breast tumor and ≥2 cm away from the tumor) and breast tumor tissues from 5 female patients were used and high-quality RNA was isolated. For quantitative mRNA expression analyses of different adipocytokines SYBR Green-based qRT-PCR was used and data were analysis by relative quantification using ΔΔCt method. The target genes were normalized to the housekeeping gene β2-microglobulin. Data for tumor and adjacent fat tissue samples were further normalized to the distant fat, defined as healthy tissue. Statistics and graphics were edited in Excel.

Results:

We observed that in tumor tissue samples following adipocytokines were down-regulated: leptin, adiponectin, IL-6 and serum-amyloid. Whereas in tumour tissue IL-6 was downregulated, it was up-regulated in all adjacent fat tissue samples. Serpin expression was up-regulated in tumour tissues in four patients.

Discussion & Conclusion:

In most cases we observed a down regulation of adipocytokines in tumor tissue in comparison to the distant fat tissue. Data for adipocytokines expressions in adjacent fat tissue samples were inconsistent, showing the large biological heterogeneity between different patients. Because of the limited number of samples it is difficult to make a clear statement. However, our data indicate that cancer cells might effect adipocytokin expression not only in the tumor but also in the adjacent fat tissue. In context with different patho/physiological roles of adipocytokines this might influence tumor development and growth.

ZUSAMMENFASSUNG

Einleitung:

Das weiße Fettgewebe (WFG) ist ein wichtiges endokrines Organ. Im WFG werden Adipocytokine exprimiert wie: Leptin, Interleukin-6, Tumor-Necrosis-Faktor-α, Aniponectin, Serum amyloid A1, Resistin. Es wird spekuliert, dass eine Dysregulation dieser Adipocytokine zu einem erhöhten Risiko für die Entstehung von Krebs führt. So haben zum Beispiel adipöse postmenopausale Frauen ein erhöhtes Risiko an einem Mammakarzinom zu erkranken. Die Frage ist nun wie diese Adipocytokine im Tumorgewebe, im Vergleich zum Fettgewebe, nahe zum Tumor und entfernt vom Tumor (gesundes Fettgewebe), exprimiert sind.

Material und Methode:

Für diese retrospektive Studie wurde operativ gewonnenes Fettgewebe (Fettgewebe nahe des Mammakarzinoms und Fettgewebe, welches sich ≥ 2cm weg vom Tumor befindet) und Mammakarzinomgewebe (unterschiedliche histologische Grade) von 5 Patientinnen verwendet. RNA wurde aus den gefrorenen Gewebeproben isoliert. Die quantitative mRNA-Expressionsanalyse der unterschiedlichen Adipocytokine in den 3 verschiedenen Geweben wurde mittels quantitativer SYBR Green PCR gemacht. Die Daten wurden nach der relativen Quantifizierung berechnet. Die Diagramme wurden in Excel aufbereitet.

Resultate:

Folgende Adipocytokine waren im Tumor runterreguliert: Leptin, Adiponectin, IL-6 und Serum-amyloid A. Im Gegensatz zum Tumorgewebe war IL-6 bei allen Patientinnen im nahe gelegenen Fettgewebe hinaufreguliert. In vier Patientinnen konnte eine klare Hinaufregulierung von Serpin beobachtet werden.

Diskussion und Zusammenfassung:

Im Vergleich zum entfernten Fettgewebe wurde mehrheitlich eine Runterregulation der Adipocytokine im Karzinomgewebe beobachtet. Die Daten für die Adipocytokinexpression im Fettgewebe nahe dem Mammakarzinom waren sehr inkonsistent. Aufgrund der minimalen Probenzahl ist es schwierig eine klare Aussage über die Expression der Adipocytokine im Krazinomgewebe und im nahen Fettgewebe zu treffen. Unsere Daten zeigen, dass die Krebszellen die Adipocytokinexpression möglicherweise sowohl im Mammakarzinom als auch im nahegelegenen Fettgewebe beeinflussen.

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ABBREVIATIONS

ACE	angiotensin-converting enzyme
ADSF	adipocyte specific secretory factor
BMI	body mass index = kg/m^2
BRCA-1	breast cancer type 1
BRCA-2	breast cancer type 2
β_2M (β2-MG)	β_2-microglobulin
cDNA	complementary DANN
C_t	threshold cycle
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynukleosidtriphosphate
ELISA	enzyme-linked immunosorbent assay
FIH-1	factor inhibiting hypoxia-inducible factor 1
G	earth’s gravitational acceleration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HER2/neu	Human Epidermal growth factor Receptor 2
HGF	hepatocyte growth factor
HIF-1	hypoxia inducible factor-1
IL-6	interleukin-6
kDa	kilo Dalton
ObR	a leptin receptor
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
pH	percent hydrogen
qPCR	quantitative real time polymerase chain reaction

RAR	retinoic acid receptor
RAS	renin-angiotensin system
RAAS	renin-angiotensin-aldosterone system
RBP	retinol binding protein
RNA	ribonucleic acid
Rpm	revolutions per minute
SAA	serum amyloid A
SHBG	sex-hormone binding globulin
TE buffer	Tris- EDTA buffer
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

1 Introduction

1.1 Breast cancer

Breast cancer is still the most common cancer diagnosed in women. About 5000 women in Austria were newly diagnosed with breast cancer [STATISTIK AUSTRIA, 2011]. In contrast to this, male breast cancer is very rarely diagnosed. Breast cancer is defined as a malignant tumor of epithelial ductal or lobular cells. This is a heterogeneous disease with different prognosis, courses of disease and different responses to treatment [Böcker, Denk, Moch, 2008, p.1011].
Breast cancer is a severe illness and one third of patients suffering from breast cancer die. Moreover, it has a huge impact on life-quality considering that such a diagnosis can lead to a mastectomy. [STATISTIK AUSTRIA, 2011].

Epidemiology

Breast cancer is the most frequently diagnosed cancer among women between 35 and 55 years in the western world. One in eight has a lifetime risk of developing breast cancer. The risk rises with ageing. Very young women are rarely affected by breast cancer, but if they are affected, it is usually a quite aggressive form. Five year survival rate accounts 75% and ten year survival rate accounts 60%. 1% of all breast cancers are detected in male [Stauber and Wyerstahl, 2007, p.371].
The risk for women to die from breast cancer before they turn 75 decreased from 2.1% to 1.6% in the last ten years. This decrease was partially ascribed to opportunistic screening. The screening helped to detect breast cancer more often and in an earlier stage [STATISTIK AUSTRIA, 2010].

Figure 1: Development of malignant breast cancers over time. Standardized by age to 100.000 people (WHO world population, 2001). The scheme was adapted from [STATISTIK AUSTRIA, 2010. Available on: http://www.statistik.at/web_de/statistiken/gesundheit/krebserkrankungen/brust/020511.html [30.3.2011].

Anatomy

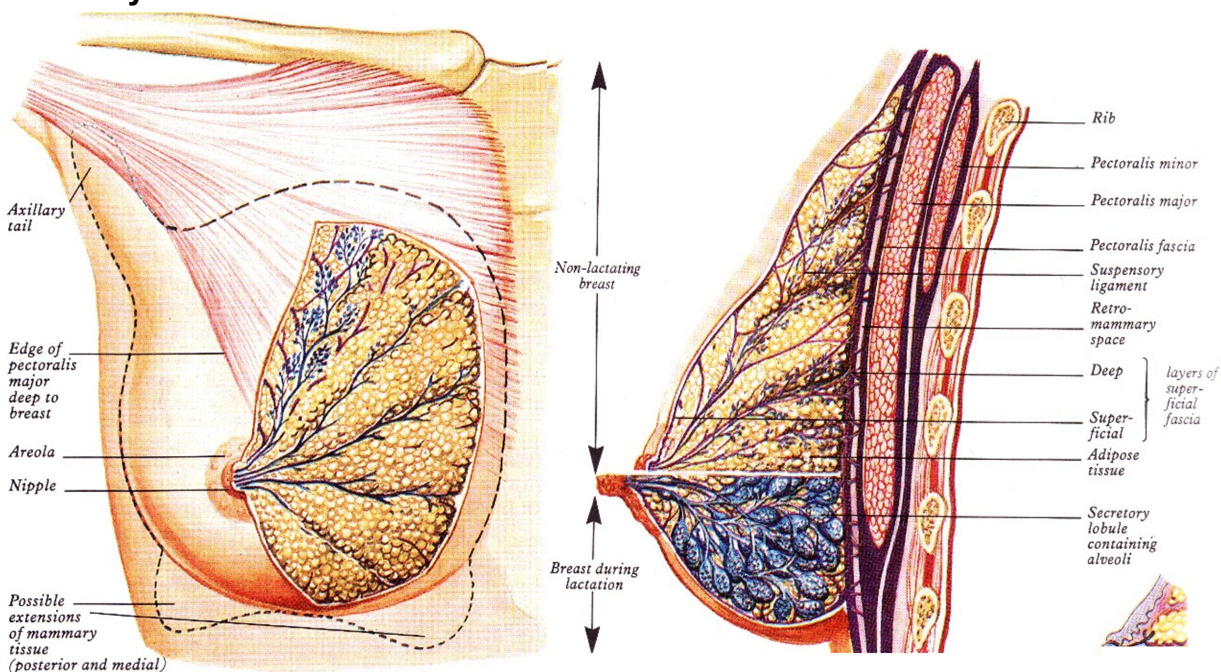

Figure 2: **Breast anatomy.**
The scheme was adapted from [Bannister et al., 1995, p. 419].

Aetiology and risk factors

The aetiology of breast cancer is still not clear. Furthermore it is still unknown why there are global incidences and ethic differences.

- It is known that breast cancer may occur on the basis of an inherited genetic mutation. For example mutations of the tumor suppressor genes BRCA-1 and BRCA-2 can be inherited. They play a prominent role in the pathogenesis of breast cancer. Female carriers of a BRCA 1/2 mutation have a lifetime risk up to 80% for development of breast cancer and a higher risk for development of ovarian carcinoma. BRCA-2 is associated with female breast and ovarian carcinoma as well as male breast cancer. However, the group of women dying of sporadically mutated breast neoplasias is still larger than that group with a hereditary background.
- In general, family history is a risk factor and in this context that means that if one or more of female relatives have or had breast cancer, the risk for female offspring to die from is higher [Stauber and Wyerstahl, 2007, p.371].
- Hormone replacement therapy was also considered as a risk factor. Hormone therapy, which is used to treat menopausal complaints, is containing a combination of oestrogen and progesterone. It turned out that hormone replacement therapy for no longer than 5 years is not a risk factor, whereas hormone replacement therapy longer than five years increases the breast cancer risk from 1.2 % to 1.4 % [Stauber and Wyerstahl, 2007, p.371].
- An early menarche under age twelve and late menopause after age fifty-five are risk factors because consequently there is a longer oestrogen influence on the breast.
- Women who give birth to a child after their thirty-fifth birthday raise their breast cancer risk by two to three times as well as women who have never given birth.
- The risk rises with ageing.
- Breast lesions such as ductal hyperplasia, lobular intraepithelial neoplasias and ductal intraepithelial neoplasia are risk factors. [Böcker, Denk, Moch, 2008, p.1011].
- Drinking more than twenty gram alcohol per day increases breast cancer risk from 1.5 to 2 times.

- Overweight and obese postmenopausal women have a two to three times higher breast cancer risk and progression. Adiposity is defined as BMI over 25 [Stauber and Wyerstahl, 2007, p.372], whereas values between 18.5 and 25 indicate normal weight. One explanation is that after the menopause the ovaries stop their oestrogen production, but in the adipose tissue C19 steroid androstenedione is aromatised to oestrone and oestradiol and consequently there are elevated oestrogen levels in obese postmenopausal women [Vona-Davis and Rose, 2007].

  It has been recognized that obesity is not equal obesity. The risk developing breast cancer for premenopausal women is quite contrary to the risk of postmenopausal women. The more body weight premenopausal women have the lower might be their risk developing breast cancer. Furthermore, abdominal fat is more dangerous than waist fat. In obese women the binding of the plasma oestradion to the sex-hormone binding globulin (SHBG) is also affected. Less SHBG would mean freer and therefore available oestradion [Housa, Housova, Haluzik, Vernerova, 2006].
- Postmenopausal women suffering from diabetes mellitus type 2 have a moderately increased breast cancer risk [Rose, Komninou, Stephenson, 2004].

Pathogenesis

Activation of oncogenes and inactivation of tumor suppressor genes leads to abnormal cell growth. As a result *in situ* carcinoma develops. Carcinoma *in situ* is confined to the ducts or to the lobules and has not broken through the basement membrane. The in situ carcinoma can lead to an invasive carcinoma and if the carcinoma gets contact to lymphatic or blood vessels, cells might break away and lead to metastasis. Moreover, other genes are involved in tumorgenesis too. For example, the already mentioned BRCA-gene, which is a DNA-repairing gene, as well as the E-Cadherin, which regulates the cell-cell and cell-matrix interactions are involved in tumorgenesis. Loss of these genes means losing cell adherence. Usually the cancer is located either in the lobules or the ducts [Böcker et al., 2008, p.1012].

Classification

<u>Lobular carcinoma</u>

- Lobular carcinoma *in situ*
- Invasive lobular breast cancer (ca. 10%-20%)

<u>Ductal carcinoma</u>

- Ductal carcinoma *in situ*
- Invasive
  - Invasive ductal carcinoma (approximately 60%)
  - Medullary carcinoma (<1-7%)
  - Mucinous carcinoma (2%)
  - Tubular carcinoma (2-7%)
  - Papillary carcinoma (<1-2%)
  - Inflammatory carcinoma
  - Paget's disease of the nipple with invasive ductal carcinoma [Böcker et al., 2008, p. 1017]

Invasive breast cancer

Invasive breast cancer develops from an *in situ* carcinoma. It is defined as an invasive growing, malignant tumor of epithelial ducts cells or lobules cells, which can metastasize. The invasive breast cancer is mainly located in the upper outer quadrant (50%). The percentage in the other quadrants are each 10% and for central location behind the mamilla 20%. 30% to 40% of patients die of this cancer [Böcker et al., 2008, p. 1016].

Histology

The macroscopic picture appearance of breast cancer can be very different. It can appear as white bordered radiated tumor or as diffuse infiltrated tumor without building a lump [Böcker et al., 2008, p. 1016].

Invasive lobular carcinoma

About 10% to 15% of breast cancers are invasive lobular carcinomas. Because of the loss of the adherence molecule E-Catherin, they are characterised by their single file pattern (the tumor cells are behind one another) or target pattern (tumor cells are located circularly around the duct) [Böcker et al., 2008, p. 1017].

Invasive ductal carcinoma

60% of the invasive ductal carcinomas are not otherwise specified because histologically the invasive ductal carcinoma has no specifical histological characteristic such as the lobular carcinoma or the special form. The cell structure of this adenocarcinoma can be tubular, trabecular or solid. It is common that there are also parts where an intraductal carcinoma is detected. [Böcker et al., 2008, p. 1017].

1.2 Adipose tissue - an endocrine organ

Prevalence of people suffering from overweight and obesity and their sequel turns out to become one of the biggest health problems worldwide. Considering that exceeded body weight is a risk factor for many diseases it is obvious that obesity leads to changes in the physiological function of adipose tissue. Obesity can lead to insulin resistance and consequently to diabetes mellitus type 2. Moreover obesity is associated with an increased risk of cardiovascular disease and with some types of cancer such as endometrial, colorectal and postmenopausal breast cancer [Van Kruijsdijk et al., 2009].
White adipose tissue is a metabolic organ containing adipocytes, connective tissue matrix (e.g. fibroblasts), nerve tissue, vascular cells and immune cells (e.g. macrophages). Moreover, it is also an important endocrine organ with adipocytes producing adipocytokines and metabolising sex-steroids and glucocorticoids [Kershaw and Flier, 2004]. It is expected that dysfunctions in the adipocyte are responsible for the increased cancer risk [Van Kruijsdijk et al., 2009]. With their autocrine and paracrine functions adipocytes not only affect the metabolism of lipids and carbohydrates but the adipose tissue derived hormones are also

involved in many other processes in the body. Leptin, the most studied one, and adiponectin are the classical adipocytokines secreted from adipocytes of the white adipose tissue. But not only the fat tissue and adipocytes itself produce adipocytokines. These hormones can also be produced by other tissues and cells such as immune competent cells [Housa et al., 2006]. Except leptin and adiponectin, 90% of the adipocytokines are also secreted by non-fat cells. There is a positive correlation between the BMI and the amount of plasma concentration of most of the adypocytokines [Vona-Davis and Rose, 2007].

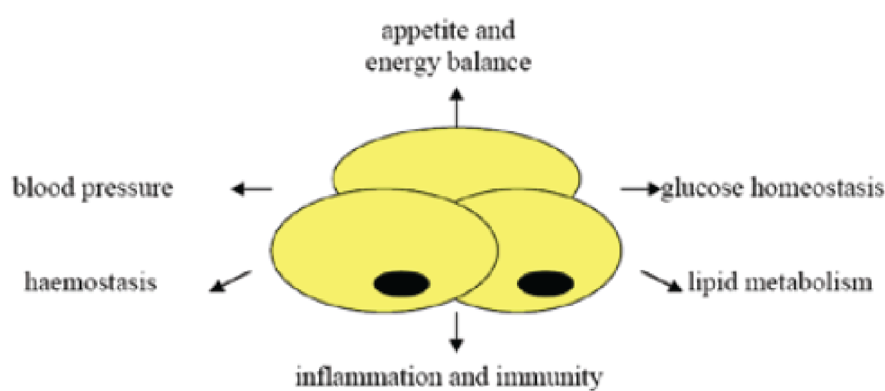

Figure 3: Physiological and metabolic processes in which white adipose tissue is involved through the secretion of adipokines. The scheme was adapted from [Gnacińska et al., 2009].

Leptin

The 16-kilo Dalton (kDa) large adipocytokin leptin is known since 1994 as a product of the obesity gene on chromosome 7q31 [Gnacińska et al., 2009]. Regarding the plasma concentration, there is a gender difference. Women have two to three times higher levels than men matched for age and BMI [Housa et al., 2006]. Leptin is involved in the regulation of many physiological processes. The name leptin comes from the Greek word *leptos* which means slim (thin) and characterises the function as a metabolic signal very good. Leptin regulates food intake by decreasing appetite. Furthermore, this hormone increases energy balance, regulates the immune response, fertility and haematopoiesis. In addition, leptin influences insulin sensibility, whereas a lack of leptin would lead to insulin

resistance [Gnacińska et al., 2009]. There is a direct correlation between the amount and the concentration of leptin and increasing adipose tissue mass [Ray and Cleary, 2010]. At the same time obese people develop leptin resistance. By losing weight, leptin levels go down immediately.

Beside the function on the endocrine system and effects on energy homeostasis, leptin has also effects on the neuroendocrine system. Especially the hypothalamic pathway plays an important role mediating, for example, food intake. Leptin is actively transported through the blood brain barrier to act on the hypothalamic system [Kershaw and Flier, 2004 / Housa et al., 2006]. Leptin receptors are expressed in brain and in some peripheral tissues like ovaries, testis and prostate. In the peripheral tissue, including muscle and pancreatic β-cells, effects are mediated via direct action [Kershaw and Flier, 2004].

Leptin and cancer

Although it was detected that the leptin receptor (ObR) is over-expressed in many colorectal, breast and endometrial cancers, the definitive link between leptin and cancer is still not clear. Moreover, experimental studies have shown that depending on the cell line, leptin has a mitogenic effect. Leptin is stimulating the growth of breast, esophagus and prostate cells whereas it inhibits the growth of pancreatic cells. Leptin also has an anti-apoptotic effect on breast, esophagus and prostate cells [Van Kruijsdijk et al., 2009].

Leptin and breast cancer

Different experiments on breast cancer cells have shown that leptin brings the cells to proliferate, to survive and encourage them to grow independently [Surmacz, 2007]. Since in human breast cancer cells both leptin and leptin-receptors are frequently elevated, an autocrine pathway was suggested. Patients with overexpression of leptin and leptin receptor positive tumors have a worse outcome than patients without overexpression of leptin [Ray and Cleary, 2010].

Leptin has not only effects by itself, it also interacts with other signalling systems in breast cancer cells. An activated leptin receptor can for example transactivate HER2/neu. Moreover, leptin can elevate the expression of vascular endothelial

growth factor and increases the aromatase activity [Surmacz, 2007]. By activating the mitogen-activated protein (MAP) kinase pathway leptin has an impact on oestrogen receptor positive human breast cancer cell lines in such a way that it increases their growth.
Results of the studies dealing with leptin expression in breast cancer tissue and physiological breast tissue are not homogeneous. While some studies found an increased serum leptin in breast cancer patients other reports do not see such a relationship [Ray and Cleary, 2010]. Therefore it is important to bear in mind that the study designs were not the same. For example, there is a study including 300 biopsies supporting the supposition that leptin is over expressed in breast cancer. But according to this study leptin is decreased or not detectable in healthy epithelium or benign tumors [Surmacz, 2007].
Beyond this, another aspect and important issue is leptin and breast cancer therapy. Studies have shown that anti oestrogen drugs (Tamoxifen and Toremifene), used to treat oestrogen receptor positive breast cancer, increases serum leptin levels in postmenopausal breast cancer patients [Housa et al., 2006]. Therefore it is supposed that increased leptin levels compete with different breast cancer therapies [Surmacz, 2007].

Leptin and female genital tract cancer

Obesity is also a risk factor for developing endometrial cancer. It is suggested that leptin could have an impact on endometrial cancer too. Studies have shown that endometrial cancer patients have a higher leptin level, but by losing weight there was no significant difference in the plasma leptin values compared to healthy controls. This would point out that the elevated leptin level is rather associated with the obesity than with the endometrial carcinoma [Housa et al., 2006].

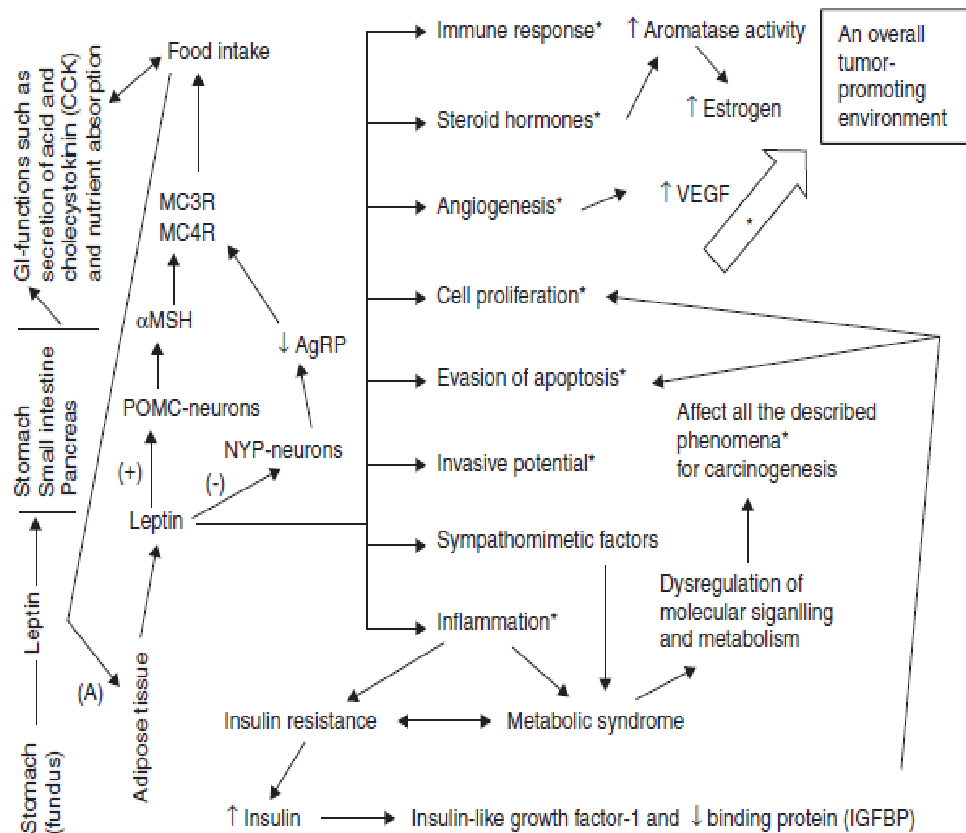

Figure 4: **Modified schematic diagram of pathophysiological leptin pathways.** The scheme was adapted from [Ray and Cleary, 2010].

Tumor necrosis factor-α (TNF-α)

The cytokine tumor necrosis factor-α (TNF-α) is mainly produced by macrophages which are amongst others infiltrating adipose tissue. TNF-α has several immunological functions and it was thought to cause, as the name says, necrosis of tumors [Vona-Davis and Rose, 2007]. Nowadays it is known that chronically increased TNF-α is doing exactly the opposite, namely increasing carcinogenesis and cancer progression [Van Kruijsdijk et al., 2009]. As a cytokine, TNF-α is involved in systemic inflammation and it stimulates release of interleukin-6 [Neilson, Brockton, Friedenreich, et al., 2009].

TNF-α is not only produced by macrophages but also by different tumor cells. Furthermore, adipocytes and stromavascular cells express TNF-α too. Moreover, adipocytes also express membrane bound and soluble types of TNF-α receptors [Kershaw and Flier, 2004].

Nowadays it is known that not only the inflammatory markers in general but also specifically TNF-α is elevated in obese humans. This increased level is accompanied by an increased risk of cancer related death [Van Kruijsdijk et al., 2009 / Neilson et al., 2009]. Furthermore, there are several studies dealing with the influence of TNF-α on insulin resistance and it seems that high TNF-α levels lead to insulin resistance. Moreover, there might be a link between TNF-α and the high levels of oestradiol in breast cancer. The dehydrogenase which stimulates biosynthesis of oestrogen is induced by tumor derived growth factor, IL-6 and TNF-α [Vona-Davis and Rose, 2007].
Pursuant to a cohort study including 2438 black and white women and men from the age of 70 to 79 followed for around 5.5 years, no correlation between elevated TNF-α levels and increased postmenopausal breast cancer risk was found [Il'yasova, Bauer, Colbert, et al., 2005].

Interleukin-6 (IL-6)

The cytokine Interleukin-6, mainly produced by leucocytes, is involved in the acute inflammatory response. Nevertheless, leucocytes are not the only cells secreting IL-6. Macrophages, fibroblasts, endothelial cells, skeletal muscle cells, adipose tissue and tumor cells are producing IL-6 or IL-6 receptors too [Ronti, Lupattelli, Mannarino, 2006 / Van Kruijsdijk et al., 2009 / Gnacińska et al., 2009]. Breast, prostate, renal, myeloma and ovarian cancer cells, for instance, are also expressing IL-6 receptors [Salgado et al., 2003]. Even so the adipose tissue is secreting only around 10%-30% of the total amount of IL-6, obese people have elevated IL-6 concentration, whereas intra abdominal fat respectively visceral fat secretes two to three times more IL-6 than subcutaneous fat. Increased IL-6 level is partially associated with a higher risk developing diabetes mellitus type 2, a myocardial infarct and cancer in general. Weight loss decreases IL-6 concentration and expression in adipose tissue and its concentration in serum.
IL-6 inhibits adipogenesis and adiponectin secretion [Ronti et al., 2006 / Van Kruijsdijk et al., 2009 / Gnacińska et al., 2009]. Further functions are elevating expression of anti-apoptotic and angiogenic proteins [Salgado et al., 2003]. Moreover, by influencing the aromatase activity, IL-6 is, so as some other

adipocytokines (e.g. TNF-α and leptin), affecting the oestrogen biosynthesis [Rose, Komninou, Stephenson, 2004].

Salgado et al. started a study with the question if the serum IL-6 has prognostic significance for breast cancer patients with metastasis. According to this prospective study, including 96 unselected and untreated female patients with progressive metastatic breast cancer, patients having circulating IL-6 have a worse outcome than those without. Serum IL-6 level was associated with the amount of detected metastasis; the more metastasis the higher serum IL-6 level. No correlation between serum IL-6 levels and the common breast cancer risk factors was found [Salgado et al., 2003].

Adiponectin

The 30 kDa heavy adiponectin is exclusively expressed in white adipose tissue. Adiponectin has, in contrast to leptin, anti-diabetic, anti-inflammatory and anti-atherogenetic effects. It is suggested that this adipocytokin has autocrine and paracrine functions. Normal weight people have a physiologically increased serum adiponectin level, whereby expression of adiponectin is higher in subcutaneous fat than in visceral adipose tissue. In other words adiponectin level and body mass index are inversely correlated. In obese individuals, as well as in diabetics, adiponectin biosynthesis by adipocytes is downregulated, which leads to hypoadiponectinemia. There are two adiponectin receptors; adiponectin receptor 1 is mainly expressed in the muscles and adiponectin receptor 2 is expressed in the liver. There is a direct correlation between elevated adiponectin levels and high insulin sensitivity [Kershaw and Flier, 2004; Brochu-Gaudreau et al., 2010].

In general, a low plasma adiponectin level is associated with the occurrence of metabolic syndrome, type 2 diabetes mellitus, hypertension, dyslipidemia, fatty liver disease and ischemic heart disease [Gnacińska et al., 2009].

Not only the BMI, but also the risk for breast-, endometrial-, prostate-, colorectal- and kidney cancer are inversely correlated with adiponectin level in serum. In vitro studies with breast cancer cell lines have shown that adiponectin prevents growth of cancer cells [Van Kruijsdijk et al., 2009]. It is assumed that adiponectin can lead to apoptosis by inhibiting angiogenesis, which in turn stops tumor growth [Brochu-Gaudreau et al., 2010].

Adiponectin is impeding cell proliferation, which contrasts once more with leptin. For this reason the two adipocytokines are combined to calculate the leptin:adiponectin ratio. It was observed that people with an increased leptin:adiponectin ratio have a higher risk developing breast cancer [Ray and Cleary, 2010]. The higher the leptin:adiponectin ratio the greater is the tumor size, this indicating a positive correlation [Vona-Davis and Rose, 2007].

In order to show the impact of high adiponectin levels on breast cancer Miyoshi et al. made a retrospective case control study including 102 histological proofed breast cancer patients (cases) and 100 healthy women (controls). The results show that the risk of developing breast cancer, for premenopausal as well as postmenopausal women, is inversely correlated with serum adiponectin level. The lower the serum adiponectin level the higher is the risk for developing breast cancer and vice versa. Above all, that results have shown that hypoadiponectinemia is associated with histological high grade and large (>2 cm) tumors in other words with more aggressive phenotypes [Miyoshi et al., 2003].

Mantzoros et al. also made a case control study with 174 pre- and postmenopausal female breast cancer patients and 167 control women. They wanted to evaluate whether low serum adiponectin levels are associated with increased breast cancer risk for both pre- and postmenopausal women. Mantzoros et al. as well as Miyoshi et al. have shown that postmenopausal women have an elevated risk developing breast cancer if they have decreased serum adiponectin values. However, in contrast to Miyoshi et al. they have shown that this does not apply for premenopausal women [Mantzoros et al., 2004].

Resistin

The 12 kDa heavy polypeptide resistin, which is the abbreviation for *resistance to insulin*, was firstly described in 2001. This cystein-rich protein belongs to the group called resistin-like molecules [Kershaw and Flier, 2004] and is very different from the other adipocytokines. Resistin is also well known under the synonym FIZZ3 (found in inflammatory zones) and ADSF. It is still not well described which human cells are producing resistin, but it is prefigured that resistin is produced by different immunocompetent cells such as monocytes [Gnacińska et al., 2009].

Since IL-6 and resistin levels correlate, it is supposed that resistin plays a role in inflammation. It is suggested that resistin could be one more piece in the puzzle explaining the insulin resistance in obese subjects. It is assumed that resistin elevates blood glucose as well as insulin concentration. Nevertheless it should be said that increased resistin expression in obese, insulin resistant and type two diabetic patients has not been found in some studies [Ronti et al., 2006]. So far known, resistin is exerting its effects on following target organs: the liver, adipose tissue and skeletal muscle [Gnacińska et al., 2009]. To conclude, resistin is still not very well explored. Irrespective of the tissue type, in our study resistin was expressed at a very low, hardly detectable level and was for this reason eliminated from the data set.

Serum amyloid A1 (SAA)

The acute phase proteins serum amyloid A belongs to the family of apolipoproteins and is responsible for the transportation of the high density lipoprotein (HDL) in plasma. The other tasks of SAA are degradation of extracellular matrix and recruitment of inflammatory cells. SAA is secreted in response to the cytokines (IL-1, IL-6, TNF-α) [Pierce et al., 2009]. It is mainly produced in the liver but also in extra hepatic tissues, e.g. in human carcinoma metastases and cancer cell lines [Malle, Kovacevic, Sodin-Semrl, 2009].

Obese people, who consequently have increased adipose tissue expressing proinflammatory cytokines, have elevated serum amyloid A, IL-6 and TNF-α levels. It is suggested that multiplied expression of proinflammatory cytokines stops fat stores from expanding further. In other words, IL-6, TNF-α and SAA lead to local as well as to peripheral reactions. This also includes low-grade systemic inflammation which leads to the dreaded metabolic obese co-morbidities.

Salas-Salvadó et al. tried to explain whether weight loss can decrease systemic inflammation and serum amyloid. He observed 19 obese non diabetic patients and 20 lean control subjects. As supposed, the results showed that obese people had a higher SAA serum level, which was decreased after weight loss [Sala-Salvadó et al., 2006]. It is speculated that SAA and the inflammatory status in general could be used as a marker for cancer risk and/or survival. SAA might be a prognostic factor for breast cancer which is maybe promoted by chronic inflammation.

In their study Pierce et al. have shown a correlation between elevated plasma serum amyloid levels and reduced overall survival. Their conclusion was that SAA could be a marker for long-term prediction of breast cancer survival independent of race, tumor stage and body mass index [Pierce et al., 2009].

Angiotensinogen

Angiotensinogen, which is mainly produced in the liver, takes part in the renin-angiotensinogen-aldosterone system (RAAS). The greatest extra hepatic source of angiotensinogen is the adipose tissue. Not only angiotensinogen is secreted in the adipose tissue but also some other proteins from the RAA-system (RAAS) as for example renin, angiotensin I and II, angiotensin receptor type I and II and angiotensin-converting enzyme. ACE inhibitors are used in hypertension treatment. The RAAS plays an important role in the regulation of blood pressure. Therefore it is assumed that angiotensinogen could be the link between obesity and hypertension. However, the RAAS not only affects the blood pressure but also has influences on the adipose tissue. Angiotensinogen II is promoting lipogenesis and therefore it increases mass of adipose tissue. Moreover it inhibits lipolysis, decreases insulin dependent glucose uptake, increases hepatic gluconeogenesis and glycogenolysis and shows angiogenic activity, which is mediated by the angiotensin type 1 receptor. Angiotensinogen on the other hand is believed to be antiproliferative. The relationship between angiotensinogen and angiotensin II and breast cancer risk is still unclear. In higher levels the antiproliferative angiotensinogen is transformed into angiotensin II and angiotensin II is promoted by the tumor. In general, proteins participating in the RAAS are regulating and influencing other adipocytokines such as PAI-1 (SERPIN) and leptin. Angiotensinogen as well as the other proteins from the RAAS has been detected in normal and in cancerous breast tissues [Kershaw and Flier, 2004 / Gonzáles-Zuloeta et al., 2007].

Retinol Binding Protein

Vitamin A (also called retinol) is ingested with food, absorbed by intestine and delivered by chylomicrons to the liver, where retinol is stored. Retinol binding

protein as the name already suggests is carrying, in association with transport protein transthyretin, retinol from the liver to the target tissues. In other words, retinol binding protein is a transport protein which is protecting retinol from oxidative destruction. Moreover, retinol is a fat soluble vitamin that is why RBP serves to solubilise it in the aqueous serum. Uptake of retinol into the target tissues is mediated by RBP specific receptors. Target tissues are every tissue which needs retinol for their function. To give an example: retinol is needed in the epithelial cells in the retina [Wolf, 2007]. RBP is mainly expressed in epithelial tissues.

Kuppumbatti et al. found out that in some breast cancer patients expression of retinol binding protein is decreased in cancer tissue [Kuppumbatti et al.,2001]. RBP also influences other receptors such as the retinoic acid receptor. Activation of this receptor causes cell differentiation. Depending on the retinol storage, RAR is activated. As mentioned before RBP is mediating cellular uptake of retinol via receptors with high affinity to RBP. Downregulation of RBP also decreases RAR activity. This consequently induces loss of cell differentiation, which in turn leads to tumor progression and autonomous growth. Moreover, Farias et al. and Kuppumbatti et al. have shown that retinol binding protein is expressed in normal breast epithelium, whereas it is downregulated in 24% of human breast cancer and this in turn results in a local deficit in vitamin A storage. Other investigators have seen similar results as well and are arguing that downregulation of RBP in human cancers is an epigenetic event [Farias et al., 2005].

Janke et al. compared 74 lean, overweight and obese menopausal women in a cross sectional study. They found out that serum RBP4 levels were the same in all women, independent of their BMI. That is why weight loss influences only the RBP4 level in adipose tissue by downregulating it but does not influence serum RBP4. Because of that reason, Janke et al. supposed adipose tissue to be a less important source for circulating RBP [Janke et al., 2006].

Hypoxia inducible factor-1α (HIF-1α)

As the name already suggests, the transcription factor HIF-1 is induced by hypoxia and therefore it plays a very important role in the cellular response to hypoxia. As a transcription factor, HIF-1 can control flow or transcription of specific oxygen

regulated genes. For example, genes induced by HIF-1 are genes involved in energy metabolism, neovascularisation, survival, cell migration and pH balance. Such target genes are, for example, vascular endothelial growth factor and plasminogen activator inhibitor.

HIF-1 is a heterodimeric protein subdivided into HIF-1β, which is constitutively expressed, and a HIF-1α subunit underlying rapid up- and down-regulation in dependence on the oxygen concentration [Cascio et al., 2008]. Hypoxia occurs during wound healing, ischemic heart disease as well as in tumors. Malignant tumors have the characteristic of fast neoplastic cell growth and high metabolic demands. Fast growth leads to a nutrition and oxygen supply bottleneck which consequently leads to increased HIF-1α levels in the solid tumor. Furthermore, as mentioned before, HIF-1α is stimulating VEGF, which on its part induces angiogenesis and this in turn promotes cell survival and metastatic processes. To sum up, it is thought that HIF-1α plays an important role in tumor progression [Trayhurn and Wood, 2004; Wang, Trayhurn, Wood, 2007; Van Kruijsdijk et al., 2009].

Tan et al. made a study with tissue microarray from 295 invasive breast carcinomas. Immunohistochemistry was used to detect FIH-1 (=factor inhibiting hypoxia-inducible factor 1), HIF-1α and carbonic anhydrase 9. It was shown that in invasive breast cancer the higher HIF-1α level, the lower cell differentiation and oestrogen-receptor expression. Moreover, the risk for early widespread metastasis is increased and therefore the higher the HIF-1α level, the lower the clinical outcome and overall survival. In other words it seems that HIF-1α is an inverse prognostic indicator for breast cancer [Tan et al., 2007].

HIF-1α and VEGF are not the only adipocytokines which are stimulated by hypoxia; IL-6 and leptin are also over-expressed under hypoxic conditions, whereas adiponectin is inhibited. Nowadays it is known that HIF-1α can activate overexpression of leptin under hypoxic conditions. It was observed that hypoxia in adipose tissue also leads to high levels of HIF-1α and VEGF. This correlates with clinic data which shows increased HIF-1α and VEGF levels in obese people [Trayhurn and Wood, 2004 / Wang et al., 2007 / Van Kruijsdijk et al., 2009].

Hepatocyte growth factor (HGF)

One of the first detected functions of the scatter factor HGF was to induce hepatocyte growth, which gives this cytokine its name: hepatocyte growth factor (HGF). Later on different tasks promoted by HGF were found such as cell proliferation, movement and invasiveness of cells, morphogenesis and angiogenesis for endothelial cells as well as for tumor cells [Sheen-Chen et al., 2005]. HGF is produced in mesenchymal cells. HGF acts by binding to the Met receptor which is a product of the proto oncogenic c-met. The receptor is located and therefore HGF is acting on epithelial and endothelial cell types. By binding to the receptor, a tyrosin kinase cascade is activated [Rose et al., 2004].
HGF are positively correlated with BMI; therefore obese people have elevated serum HGF levels. Weight loss not only reduces serum HGF levels but also HGF secretion in adipocytes [Vona-Davis and Rose, 2007]. Sheen-Chen et al. made a prospective study including 124 invasive breast cancer patients undergoing surgery. The control group included 35 patients with benign breast tumor. Venous blood was taken before surgery and HGF was measured in serum by quantitative sandwich enzyme immunoassay technique. Serum HGF level was increased in the invasive breast cancer patients compared to the patients with the benign breast tumor. In addition, the higher serum HGF, the lower differentiated the tumors. Moreover, high serum HGF levels were found in patients with oestrogen receptor negative breast cancers. But HGF was not only found in breast tissue, it was also detected in the lung, kidney and in the liver [Sheen-Chen et al., 2005]. Taniguchi et al. have also examined serum concentration of HGF in 134 breast cancer patients using ELISA. 49 of the 134 patients had significant elevated HGF levels in contrast to the healthy control group. However, serum HGF level and intratumoral detected HGF did not correlate. After removing the tumour, surgical serum HGF decreased. This led to the assumption that HGF must be expressed in the tumor too. Research with cultured human breast cancer cells have shown that although the cells expressed c-met receptors they did not segregate HGF by themselves. It is therefore assumed that there must be paracrine signals [Taniguchi et al., 1995].

Serine protease inhibitor - Plasminogen activator inhibitor (PAI-1)

The glycoprotein PAI-1 belongs to the serine protease inhibitor (serpin) family. The main function of PAI-1 is the inhibition of fibrinolysis and regulation of thrombus formation by inactivating the tissue plasminogen activator and urokinase plasminogen activator. Those are two anticlotting factors. By binding of PAI-1 to the active side of uPA, plasminogen cannot be converted into its active form plasmin. uPA is responsible to cleave plasminogen into plasmin. Nowadays more functions have been attributed to PAI-1 as angiogenesis, atherogenesis and by binding to uPA it has affects on cell migration and adhesion. Furthermore, the plasminogen activator system has also been suggested to play a role in tumor cell invasion. Thus, for example, an increased uPA level indicates a bad prognosis for many types of cancer including breast cancer. Furthermore, the risk for obese women with increased serum PAI-1 levels to develop a more aggressive breast cancer with a higher risk developing metastasis is most likely [Kershaw and Flier, 2004; Carter and Church, 2009]. PAI-1 is mainly secreted by the endothelium but it is also synthesized in the liver and adipose tissue. PAI-1 is also thought to be expressed by macrophages, which are mainly in the visceral adipose tissue. This could be an explanation why increased amounts of PAI-1 are measured in visceral relative to subcutaneous adipose tissue. It is a fact that elevated serum PAI-1 levels are associated with visceral adiposity independently of total adipose tissue mass, insulin sensitivity, sex or age. It is generally observed that in contrast to lean people the concentrations of PAI-1 are increased in obese. It is not clear yet if increased PAI-1 levels induce obesity and insulin resistance [Kershaw and Flier, 2004 / Ronti et al., 2006]. However, increased PAI-1 levels constitute an independent risk factor for diabetes mellitus type 2 or cardiovascular diseases such as a myocardial infarction. Compared to healthy people, a three times higher PAI-1 concentration was measure in type two diabetics. Serum PAI-1 and PAI-1 mRNA expression in adipose tissue are directly correlating. BMI reduction due to weight loss and metformin treatment let the PAI-1 level decrease [Ronti et al., 2006 / Gnacińska et al., 2009].

1.3 Description and aim of the experiments

Studies done so far are mainly dealing with the plasma or serum levels of the adipocytokines. So the question is what is happening directly in the tumor and in the fat tissue cells surrounding the tumor. Are the adipocytokines measured in the tumor reflecting the values in the literature? Beyond that there is another question unanswered. The breast cancer bordering fat tissue has in contrast to the distant fat tissue a darker and more intensive yellow colour.

Figure 5: Breast cancer tissue with surrounding darker fat tissue. Provided by Univ.Prof.Dr.med.Univ. Farid Moinfar (Institute of pathology, MEDUNI Graz).

The question rises up if there are expression differences of adipocytokines in the tumor tissue, in the adjacent fat and the distant fat. An unanswered question is also whether this special colour of adjacent fat tissue can be explained by differences in adipocytokine expression. Answers to these questions might help to understand the pathophysiology of breast cancer better.

For this retrospective study breast fat tissue (adjacent fat tissue and distant fat tissue, which is defined as more than 2 cm away from the breast cancer tissue) and breast cancer tissue (different histological grades; necrotic regions were avoided) were used. The tissue was gained after planned mastectomies from five coincidentally selected postmenopausal women of different ages. These 5 patients underwent surgery at the Landeskrankenhaus-Univ. Klinikum Graz. Patients' names were obtained in coded numeric form. Age, BMI and tumor grade were known. In total there were 15 different tissue samples.

Calculations and graphics were edited in Excel.

2 Methods

2.1 Tissue homogenization

Breast cancer, distant fat and fat adjacent to the breast cancer biopsies were put into a tube and immediately frozen at minus 80° C for short term storage. To win the RNA from the tissues (tumor, adjacent fat and distant fat) approximately 100 mg of tissue was homogenized. For this purpose tissue pieces have been put into tubes with stainless steel beads and 1 ml QIAzol Lyses Reagent (for fat tissue) or peqGOLD°TriFast™ (for tumor) per tube, to stabilize the RNA. To prevent thawing of tissue it was necessary to work very fast. The tissue was homogenized at 6500 rpm for 20 seconds. For the tumor tissue 5 cycles and for the fat tissue 4 cycles were necessary. Incomplete homogenization could lead to significantly reduced RNA yields. Samples were frozen over night at minus 20°C in order to improve the amount of isolated RNA.

2.2 RNA isolation from adjacent and distant fat tissue with the RNeasy® Lipid Tissue Mini Kit

Tubes containing the homogenate were removed from the fridge and samples were gently thawed on ice. Afterwards the samples were incubated at room temperature (15-25°C) for 5 minutes.

In the meantime the DNase digestion solution was prepared by mixing 10 µl DNase I stock solution and 70 µl RDD buffer per sample. This solution was gently mixed by inverting the tube followed by centrifugation step. Prepared DNase mix was stored on ice until further use.

Per sample, 200 µl chloroform was added; the sample has been shaken for 15 seconds and incubated at room temperature for 2-3 minutes. Thereupon the samples were centrifuged at 12.000 g for 15 minutes at 4°C. As a result of this step the sample separates into 3 phases: an upper colourless aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.

The upper colourless phase was transferred into a new tube without contamination from lower phase. One volume of 70% ethanol was added to each supernatant and samples were mixed by vortexing. Precipitates might be visible after adding ethanol. 700 µl of the sample was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube. The lid was closed and samples were centrifuged for 15 seconds at 13.000 g at room temperature (15-25°C). The flow-through was discarded and the collection tube was reused. This step was repeated with the rest of the sample. After a further centrifugation step the flow-through was discarded again. Afterwards 350 µl RW1 buffer were added to RNeasy column, the lid was closed and the sample was centrifuged for 15 seconds at 13.000 g. Once more the flow-through was discarded.

The DNase I incubation mix, which had been prepared before, was added to the sample. 80 µl DNAse I incubation mix were directly added to the RNeasy column membrane, placed on bench top and incubated at room temperature for 15 minutes. If the DNase mix is not placed directly on the membrane there will be no digestion of contaminating DNA.

350 µl RW1 buffer were added to the RNeasy column, the lid was closed and the sample was centrifuged for 15 seconds at 13.000 g. The flow-through was discarded. To wash the membrane 500 µl RPE buffer were added to the RNeasy spin column and the sample was centrifuged for 2 minutes at 13.000 g. During this step it is important to dry the column so that no ethanol is carried over during RNA elution. Now the collection tube with the flow-through was thrown away and replaced with a new collection tube. The sample was centrifuged for 1 minute at 13.000 g.

The RNeasy spin column was placed in a new tube and 30 µl of pre-warmed (60°C) RNase-free water were added directly to the spin column membrane. The sample was finally centrifuged for 1 minute at 13.000 g and flow-through containing RNA was collected and stored at minus 20°C.

2.3 RNA isolation from tumor tissue with the peqGOLD HP total RNA Kit™

Table 1: Samples and working protocol.

Patient Nr.	Tissue weight (mg)	Homogenisation cycles	Transparent phase (µl)	Ratio 260/280 nm	Nucleic acid concentration (ng/µl)
1					
adjacent fat	77 mg	5x	-	2.04	53.5 ng/ µl
distant fat	105 mg	4x	500 µl	1.92	89.6 ng/ µl
tumor	47.9 mg	5x	-	2.02	889.1 ng/ µl
2					
adjacent fat	89 mg	4x	600 µl	2.02	89.9 ng/ µl
distant fat	100 mg	4x	530 µl	1.81	63.4 ng/ µl
tumor	90 mg	5x	590 µl	2.05	840.2 ng/ µl
3					
adjacent fat	90 mg	4x	560 µl	2.03	163.5 ng/ µl
distant fat	90 mg	4x	560 µl	2.02	87.2 ng/ µl
tumor	52 mg	5x	590 µl	2.02	415 ng/ µl
4					
adjacent fat	92 mg	4x	560 µl	2.0	362.4 ng/ µl
distant fat	92 mg	4x	530 µl	2.03	307.4 ng/ µl
tumor	92 mg	5x	580 µl	2.05	1283.4 ng/ µl
5					
adjacent fat	60 mg	3x	590 µl	2.03	58.5 ng/ µl
distant fat	90 mg	3x	560 µl	2.03	95.4 ng/ µl
tumor	66 mg	5x	530 µl	2.05	1559.2 ng/ µl

To win RNA from tumor the peqGOLD HP total RNA Kit was used. For homogenization see point 2.1. Homogenate samples were gently thawed on ice and incubated at room temperature (15-25°C) for 5 minutes. The DNase digestion solution was prepared as described before. Per sample 200 µl chloroform were added, the sample was shaken for 15 seconds and incubated at room temperature (15-25°C) for 2-3 minutes. Afterwards the samples were centrifuged for 5 minutes at 12.000 g leading to separation into 3 phases: an upper colourless aqueous phase containing RNA, a white interphase containing DNA and proteins and a lower, red, organic phase. The aqueous phase was approximately 60% of the total sample volume.

The upper colourless phase was transferred into a new tube without contamination from lower phase and mixed 1:1 with 70% ethanol by vortexing. Precipitates might be visible after addition of ethanol. The perfectBind RNA column was placed into a 2 ml collection tube and up to 700 µl of the sample was transferred to the perfectBind RNA column. The sample was centrifuged for 15 seconds at 13.000 g at room temperature (15-25°C). The flow-through was discarded and the step was repeated with the remaining sample.

500 µl RNA Wash Buffer I were added to the PerfectBind RNA column and the sample was centrifuged for 15 seconds at 13.000 g. The collection tube containing the flow-through was discarded and a new collection tube was used for DNase digestion step. 80 µl DNase I incubation mix was directly added to the RNeasy column membrane and incubated at (20-30°C) for 15 minutes. The collection tube was changed, 400 µl RNA Wash Buffer were added, and the sample was incubated at room temperature for 5 minutes and finally centrifuged for 5 minutes at 10.000 g. Once more the flow-through was discarded and the collection tube was reused for washing step with 600 µl RNA Wash Buffer II followed by centrifugation for 15 seconds at 13.000 g. The flow-through was discarded and the washing step was repeated. The PerfectBind RNA column was plugged in the empty collection tube. To dry the column the sample was centrifuged for 1 minute at 10.000 g. The RNeasy spin column was placed in a new collection tube and RNA was eluted with 30 µl of pre-warmed (60°C) RNase-free water added directly to the spin column membrane and final centrifugation for 1 minute at 13.000 g. Collected RNA was stored at minus 20°C till usage as a template for reverse PCR.

2.4 Polymerase chain reaction (PCR)

The method of the polymerase chain reaction (PCR) was developed 1986 and is nowadays widely used in different fields and for diagnostic purposes. This technique allows the multiplication of a particular DNA sequence up to high amount. The DNA-Polymerase is binding to the DNA (or RNA) matrix. Oligonucleotides act as primers and are binding at the beginning and at the end of the target DNA sequence. These primers, which are constructed to build the starting point for the replication, lead the DNA polymerase to the sequence. So the DNA polymerase can replicate the DNA template and create millions of copies.
At the beginning of each reaction cycle double-stranded DNA has to be denatured by temperature. The hydrogen bonds have to be melted by high temperatures up to 95°C. That is why a special heat resistant DNA polymerase, which was isolated from the bacterium *Thermus aquaticus*, is used. This denaturation step is followed by annealing. During this step the temperature decreases so that the primers can bind to the single stranded DNA. This is usually done at 45-55°C but the annealing temperature is highly dependent on the primer sequences. Afterwards the elongation step (usually at 72°C) follows. The DNA polymerase synthesises a new DNA strand complementary to the template DNA. The four nucleotide phosphates are needed: adenosintriphosphat, cytidintriphosphat, guanosintriphosphat and thymidintriphosphat. PCR is an exponential reaction, doubling the amount of target DNA after each cycle. By heating up again to denature the double-stranded DNA a new cycle is started. Usually 30 to 40 cycles are done [Alberts et al., 2001, p.354-355]. In our study the RNA had to be transcribed into DNA first. For this purpose reverse transcriptase reaction was used.

Reverse transcription of RNA into cDNA (RT-PCR)

The steps were carried out according to the supplier's manual. For PCR, a template (in our case the sample RNA), a primer pair and water were needed. The amount of water and RNA was calculated (see table 2). The mix has to be homogenous so vortexing and centrifugation are important. The samples were incubated in the PCR machine at 70°C for 5 min.

For the master mix 8 µl buffer, 4 µl dNTP's and 2 µl reverse transcriptase were mixed per sample. To each sample 14 µl master mix was added and samples were heated to 25°C for 5 minutes. Afterwards 2 µl reverse transcriptase was added to each sample and samples were incubated in the PCR machine under following conditions: 70°C for 5 minutes, 25°C for 5 minutes, 25°C for 10 minutes and 42°C for 60 minutes. The amplified cDNA was stored at minus 20°C.

Table 2: Example for calculation and preparation of RT-PCR samples.
(aF= adjacent fat /dF=distant fat/T=tumor).

nr.	sample	RNA conc. (ng/µl)	for 1000 ng (µl)	H_2O to 22 µl (µl)
1	P1aF	51.4	19.46	2.54
2	P1dF	92.6	10.80	11.20
3	P1T	853.4	1.17	20.83
4	P2aF	86.9	11.51	10.49
5	P2dF	63.0	15.87	6.13
6	P2T	827.4	1.21	20.79
7	P3aF	161.5	6.19	15.81
8	P3dF	87.9	11.38	10.62
9	P3T	407.9	2.45	19.55
10	P4aF	364.2	2.75	19.25
11	P4dF	309.7	3.23	18.77
12	P4T	1328.0	0.75	21.25
13	P5aF	57.4	17.42	4.58
14	P5dF	97.5	10.26	11.74
15	P5T	1385.3	0.72	21.28

2.5 Quantitative Real-time-PCR (qPCR)

The quantitative real time PCR works similar as the PCR which is described in 2.4. However, unlike the conventional PCR, where the detection and quantification is done at the end of the PCR by for example gel electrophoresis, the real time PCR is quantified in real time. This is possible by using fluorescence dye (e.g. SYBR Green fast). It is assumed that this dye is absorbed by the newly built double-stranded DNA and not by the primer-dimers or at the end of the PCR by overproduced non specific products. This problem was excluded by using pre-designed, commercially available primers (see table 3). The amount of DNA doubles at the end of every cycle and the fluorescence signal increases proportionally. For that reason quantification is possible [Holzapfel and Wickert L., 2007]. “Upon binding and excitation it emits a fluorescent signal that is detected by the real-time cycler during the extension step of the PCR.” [Qiagen GmbH – Germany, 28.03.2011].

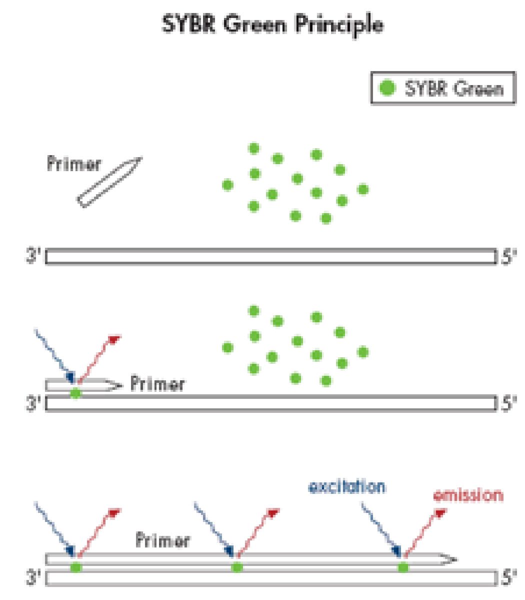

Figure 6: Principle of SYBR Green based detection of PCR products in real-time PCR. The scheme was adapted from [Qiagen GmbH – Germany. Available on:http://www.qiagen.com/literature/qiagennews/weeklyarticle/06_09/e14/popup.aspx [28.03.2011]].

SYBR Green is very sensitive, whereas the specificity is quite low. Therefore the analysis of the melting curve at the end of the PCR is important. Because of the high temperature the DNA is denatured (melted). The melting curve shows the melting point of the target gene which is expressed by a peak in the curve. Dimers, which would be detected by SYBR Green too, would have lower melting points and consequently there would be more and smaller peaks in the melting curve. The standard curve, which is used to evaluate the starting concentration of an unknown sample, is calculated by software with the C_t data and the standard concentration [Holzapfel and Wickert L., 2007].

qPCR conditions

With qPCR reaction the cDNA was amplified by using gene specific primers in order to quantify the expression of different adipocytokines in different tissues (tumor, adjacent fat and distant fat). To be able to compare expression data from different tissues, these data have to be normalized to the housekeeping gene, in our case β_2-microglobulin (β_2M). In our preliminary experiments we tested three housekeeping genes frequently used in qPCR analyses: Glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH), ß-actin and ß2-microglobulin. In our study-setup β_2M was detected as the most stable housekeeping gene and was therefore used for further analyses and data-normalization. The primers were diluted in a 1.1 ml TE buffer pH8 (10 mM Tris-HCl, 1 mM EDTA) and stored in 50 µl aliquots at minus 20°C. Primers used are listed in table 3. It was checked out that β_2M has its working optimum at a dilution of 1:100. For this reason the cDNA was diluted 1:100 too, so 1 µl cDNA was mixed with 99 µl RNase-free water. In our format (384-well plate) 6 µl of 1:100 diluted cDNA were amplified using QuantiFast™ SYBR® Green PCR master mix supplemented with QuantiTect® Primer specific for 11 different adipocytokines. 4 µl per well of these mixture, including all components, was pipetted into three wells of a 384 well plate and amplified in a LightCycler® 480 Real-time PCR device (Roche Diagnostics GmbH, Vienna, Austria). The thermal profile initiates with a 5 min step at 95° C followed by 40 cycles of 10 sec at 95° C and 30 sec at 60° C. The amplification period was followed by a melting curve analysis with a temperature gradient of 0.1° C/sec

from 70°C to 95°C to exclude amplification of unspecific products. A non-template control and a negative control were included in each experiment.

To analyze the data from the quantitative real time PCR, a relative quantification was used. Thereby the expression of a target gene (in our case the 11 adipocytokines) was normalized to a not regulated, constantly expressed reference gene (housekeeping gene). Housekeeping genes are found in every human individual in all human cells and are expressed at relatively constant levels. Therefore housekeeping genes serve as internal control genes for such type of studies. Our calculations for tumor and adjacent fat tissue samples were further normalized to the distant fat tissue which has been defined as healthy tissue.

The C_t for a given amplification curve occurs at the point where the fluorescent signal falls beyond the value of the threshold setting. The threshold value must be set within the phase where the data points increase linearly [meduni-graz.at/zmf, 26.03.2011]. The C_t datas correlate with the numbers of PCR cycles which are needed to reach a constant defined fluorescence level. If the efficiency is 100%, as supposed, the amount of DNA and the corresponding fluorescence signal doubles at the end of every cycle. To calculate the relative gene expressions (ratio) the following formula was used [Livak and Schmittgen, 2001].

$\Delta C_{T(\text{tumor/fat nearby})} = C_{T,\ \text{Time adipokcytocin}} - C_{T,\ \text{Time reference housekeeping gene } (\beta 2M)}$

$\Delta\Delta C_T = \Delta C_{T\ \text{tumor/fat nearby}} - \Delta C_{T\ \text{fat farther}}$

$\text{Ratio} = 2^{-\Delta\Delta C_T}$

Table 3: Primers used in our study.

Primer assay	Short description	Gene name	Gene ID
B2-Mikroglobulin		B2-MG	
LEP_1 QT00030261	leptin, 105 bp,amplified exons 2/3, length of transcript 3444 bp.	leptin	NM_000230
TNF_3 QT01079561	tumor necrosis factor, 104 bp, amplified exons 1/2/3, length of trascript 1669 bp.	tumor necrosis factor	NM_000564
IL_6 QT00083720	interleukin 6, 107 bp, amplified exons 2/3, length of transcript 1201 bp.	interleukin 6	NM_000600
ADIPOQ_1 QT00014091	adiponectin, 89 bp, amplified exons 1/2, length of transcript 4592 bp.	adiponectin	NM_004797
RETN_1 QT00210483	resistin, 177 bp, amplified exons??, length of transcript 379 bp.	resistin	NM_020415
SAA1_1 QT00045206	serum amyloid A1, 98 bp, amplified exons 1/2, length of transcript 716 bp.	serum amyloid A1	NM_000331
AGT_1 QT00005614	angiotensinogen, 115 bp, amplified exons 2/3, length of transcript 2587 bp.	angiotensinogen	NM_000029
RBP4_1 QT00010255	retinol binding protein 4, 76 bp, amplified exons 4/5, length of transcript 941 bp.	retinol binding protein 4	NM_006744
HIF1A_1 QT00083664	hypoxia inducible factor 1, 104 bp, amplified exons 4/5, length of transcript 4082 bp.	hypoxia inducible factor 1	NM_001530 NM_181054
HGF_2 QT01758988	hepatocyte growth factor (HGF), 133 bp, amplified exons 1/2, length of transcript 2820 bp.	hepatocyte growth factor	NM_000601
SERPINE1_1 QT00062496	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), 105 bp, amplified exons 3/4, length of transcript 3198 bp.	serpin peptidase inhibitor	NM_000602

3 Results

3.1 Clinical information from the patients

Table 4: Data from patients from whom the tissue was collected.

Patient nr.	1	2	3	4	5
Age (years)	80	66	79	51	71
BMI	26.6	36.3	22.5	21.1	27.4
Diagnose	Invasive ductal carcinoma	Invasive ductal and lobular carcinoma	Clear cell carcinoma	Invasive ductal carcinoma	Invasive ductal carcinoma
Stage	G-3	G-1	G-3	G-1	G-3
HER2/neu	neg.	neg.	neg.	neg.	neg.
Oestrogen/ progesterone receptor	ER pos. PR pos.	ER pos. PR pos.	ER pos. PR pos.	ER pos. PR pos.	ER pos. PR pos.

3.2 Expression of adipocytokines in all patients

Leptin

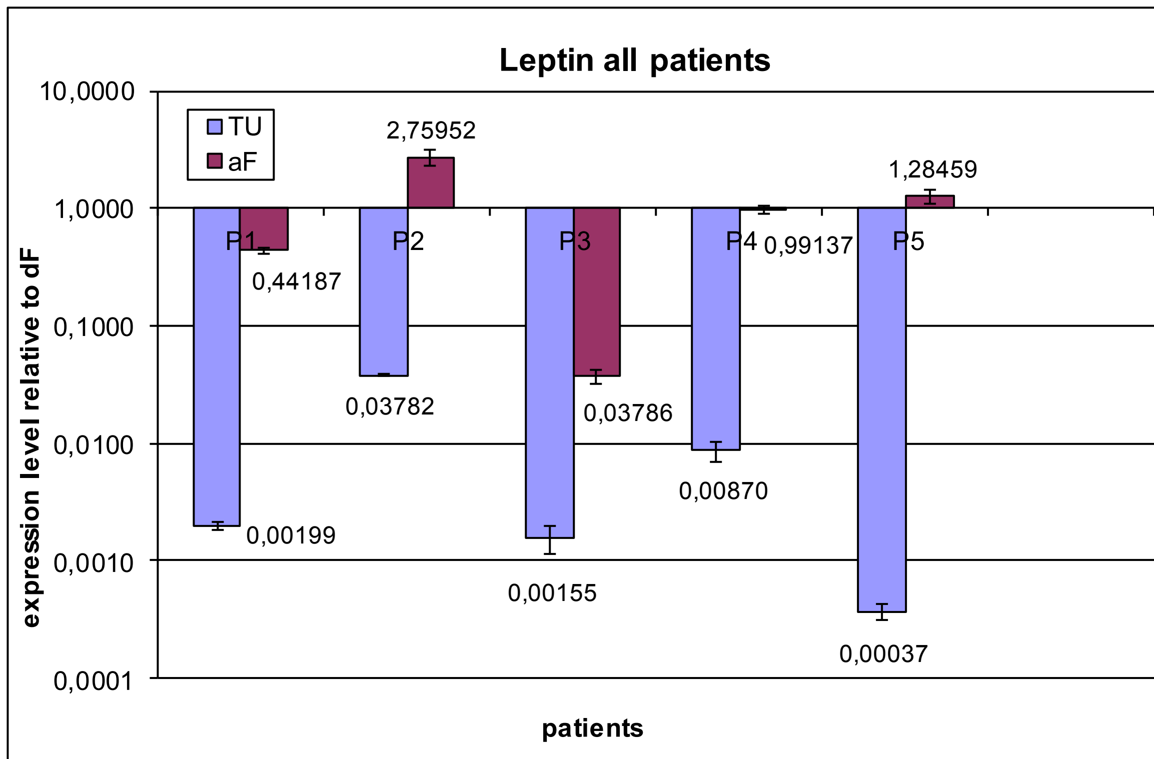

Figure 7: Expression of leptin in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Expression of adipocytokines in tumor tissue and adjacent fat is compared to distant fat, which is assumed to be a “healthy” tissue expressing adipocytokines at physiological levels. On Figure 7 expression differences compared to the distant fat tissue can be seen. As expected it is obvious that each patient has an individual expression level of adipocytokines.

The graphic shows that leptin in the tumor is down-regulated in all 5 patients. The greatest downregulation of leptin are detected in the tumor tissue from patients #1, #3 and #5.

Results in the adjacent fat tissue are not so consistent. Only patient #3 shows a clear downregulation, whereas other patients show minimal expression differences to the distant fat tissue.

Tumor necrosis factor α (TNF-α)

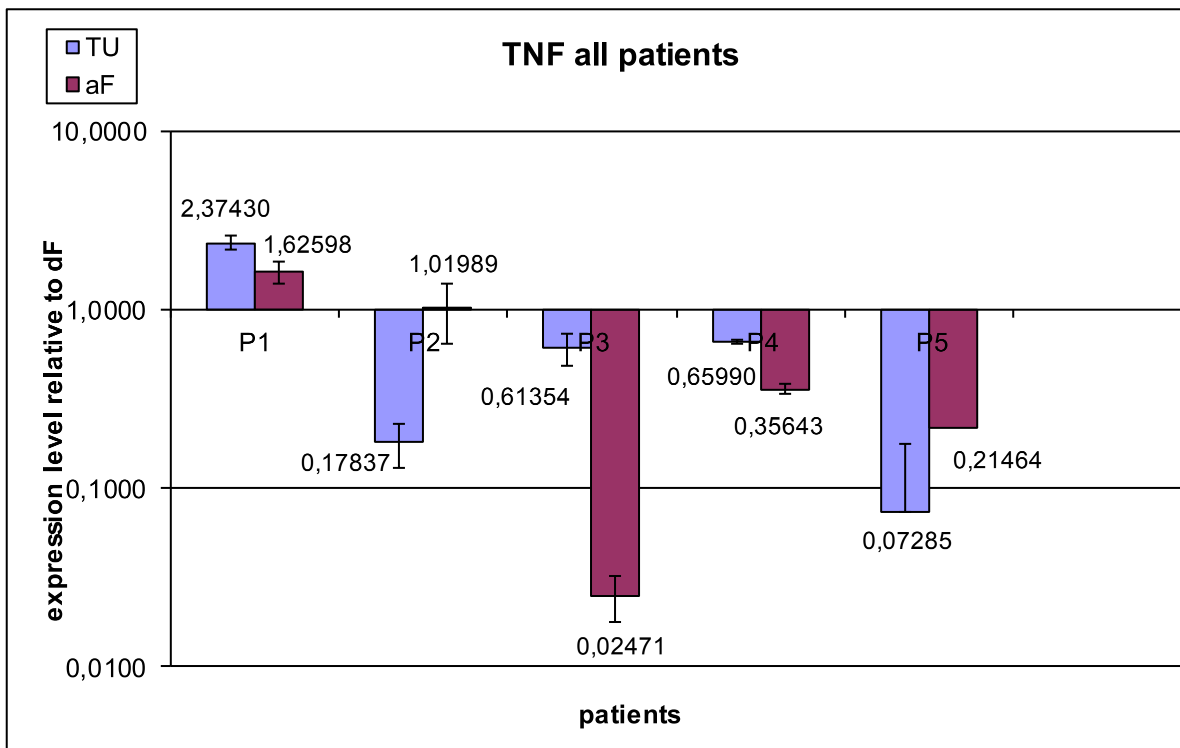

Figure 8: Expression of TNF-α in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

TNF-α expression in the tumor and adjacent fat is compared to distant fat tissue. Results for TNF-α expression are not very consistent. However the trend in tumor tissue and adjacent fat tissue is a downregulation of TNF-α expression in most patients. Patient #1 is an exception concerning the expression of TNF-α, because TNF-α expression is up-regulated both in adjacent fat and in tumor tissue, though the differences in the expression level are minimal. Downregulation of TNF-α in the adjacent fat from patient #3 seems to be an exception. The greatest downregulation in tumor was detected in patient #5.

Adiponectin

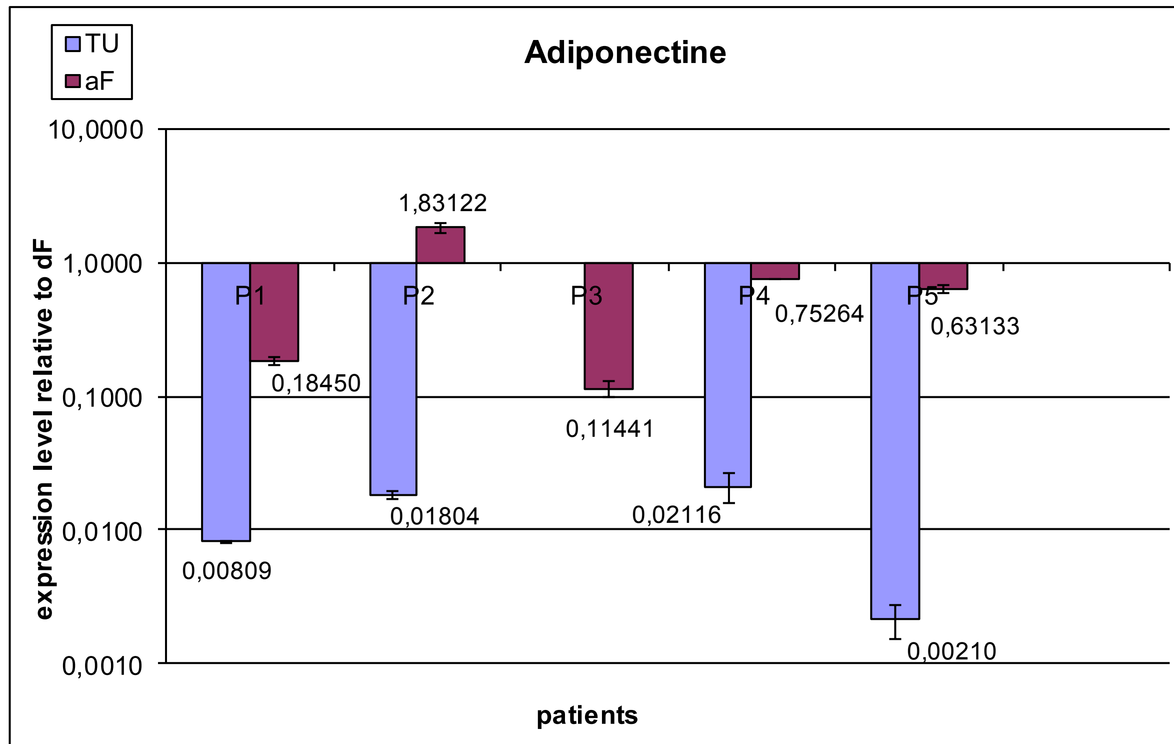

Figure 9: Expression of adiponectin in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Adiponectin expression in the tumor and adjacent fat the tumor is compared to distant fat tissue. Results show that adiponectin in the tumor is down-regulated in all 5 patients. The greatest downregulation of adiponectin is detected in the tumor tissue from patient #5 followed by patient #1. Adiponectin expression in the tumor in patient #3 is not detectable.

Downregulation of adiponectin in adjacent fat is not as clear as in the tumor. However a downregulation can be seen in patients #1, #3 and #5. For the patient #4 no clear statement can be given because results are very close to the expression from adiponectin of the healthy tissue. Patient #2 shows an upregulation of adiponectin in adjacent fat. To conclude, the trend is towards a downregulation.

Interleukin 6 (IL-6)

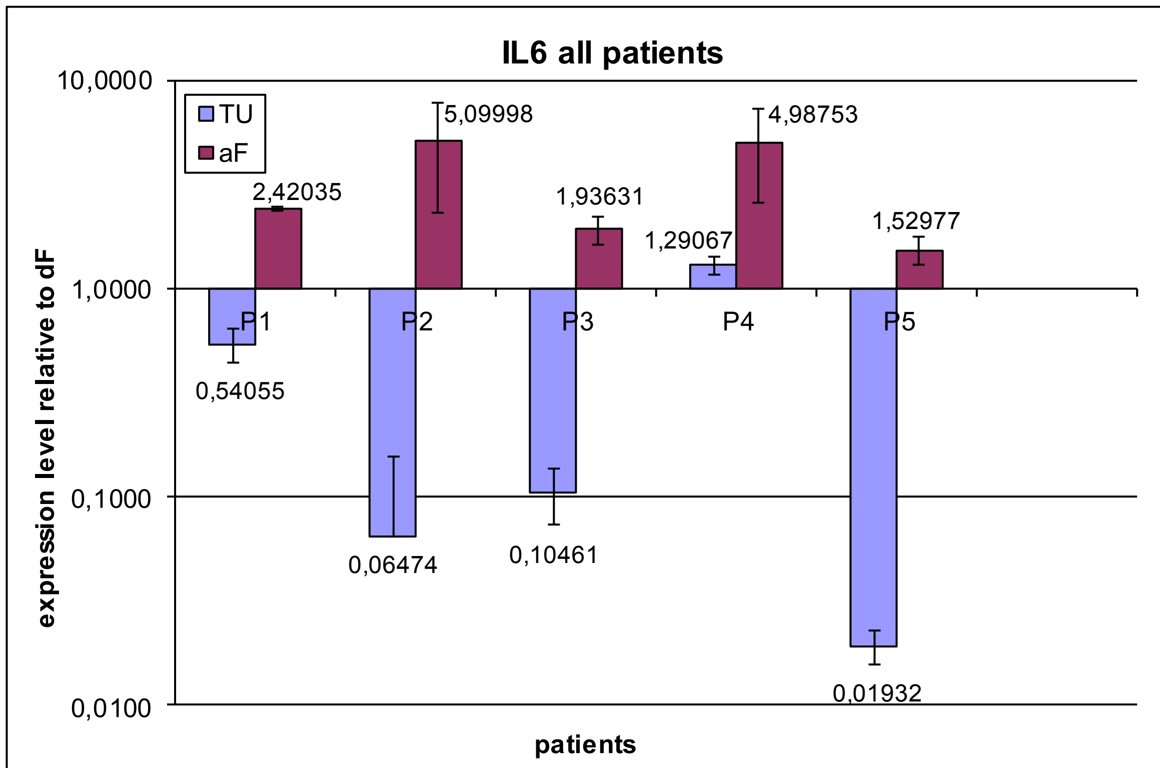

Figure 10: Expression of IL-6 in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Results for IL-6 expression in tumor differ from results in the adjacent fat. However the trend in tumor tissue is a downregulation of IL-6 expression in 5 patients. For patient #4 no statement can be made, though the difference in expression level is minimal. The greatest downregulation in tumor is detected in patient #5.

Results in the adjacent fat tissue show an upregulation of IL-6 in all patients, whereas expression differences, to distant fat tissue, in patients #3 and #5 are minimal. The greatest upregulation in adjacent fat is detected in patients #2 and #4.

Angiotensinogen

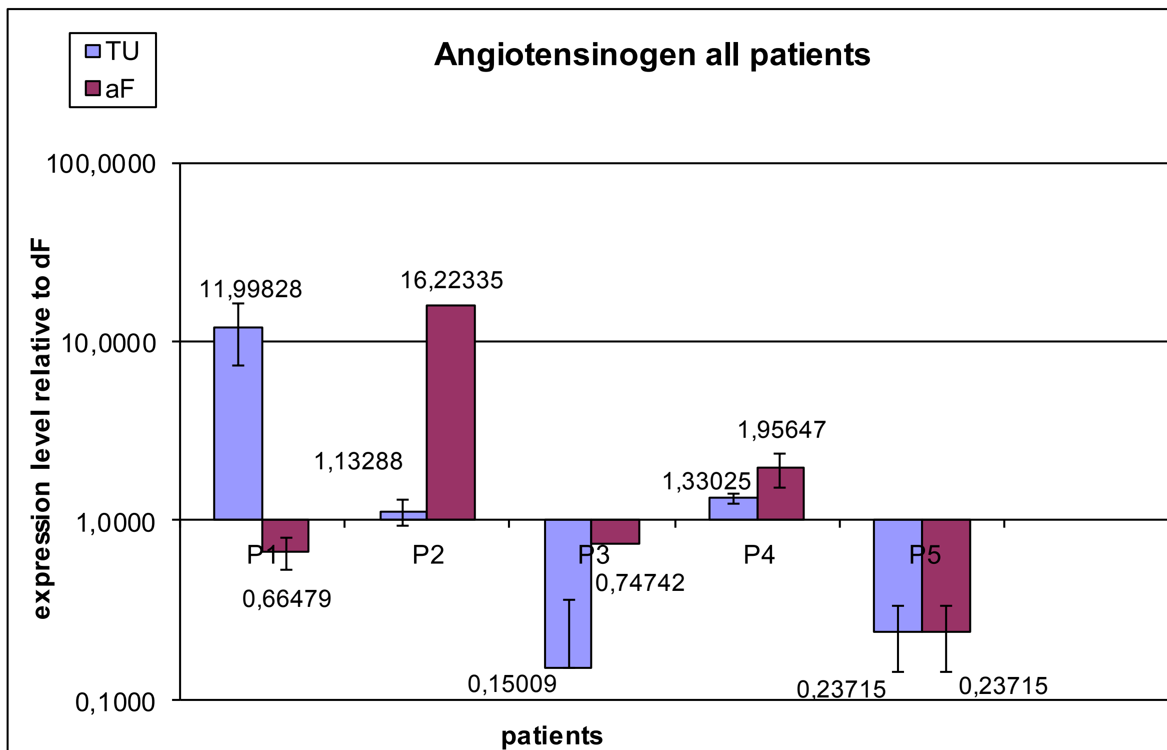

Figure 11: Expression of angiotensinogen in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Results for angiotensinogen expression are very inconsistent. However a significant upregulation of angiotensinogen in tumor tissue is detected in patient #1 whereas a clear downregulation is detected in patients #3 and #5. Results for angiotensinogen expression in adjacent fat are as well not consistent. Patient #2 shows a very clear upregulation whereas in patient #5 a clear downregulation of angiotensinogen in adjacent fat is detected. Patient #4 shows minimal expression differences in tumor as well in the adjacent fat tissue as in distant fat tissue.

Serum amyloid A1

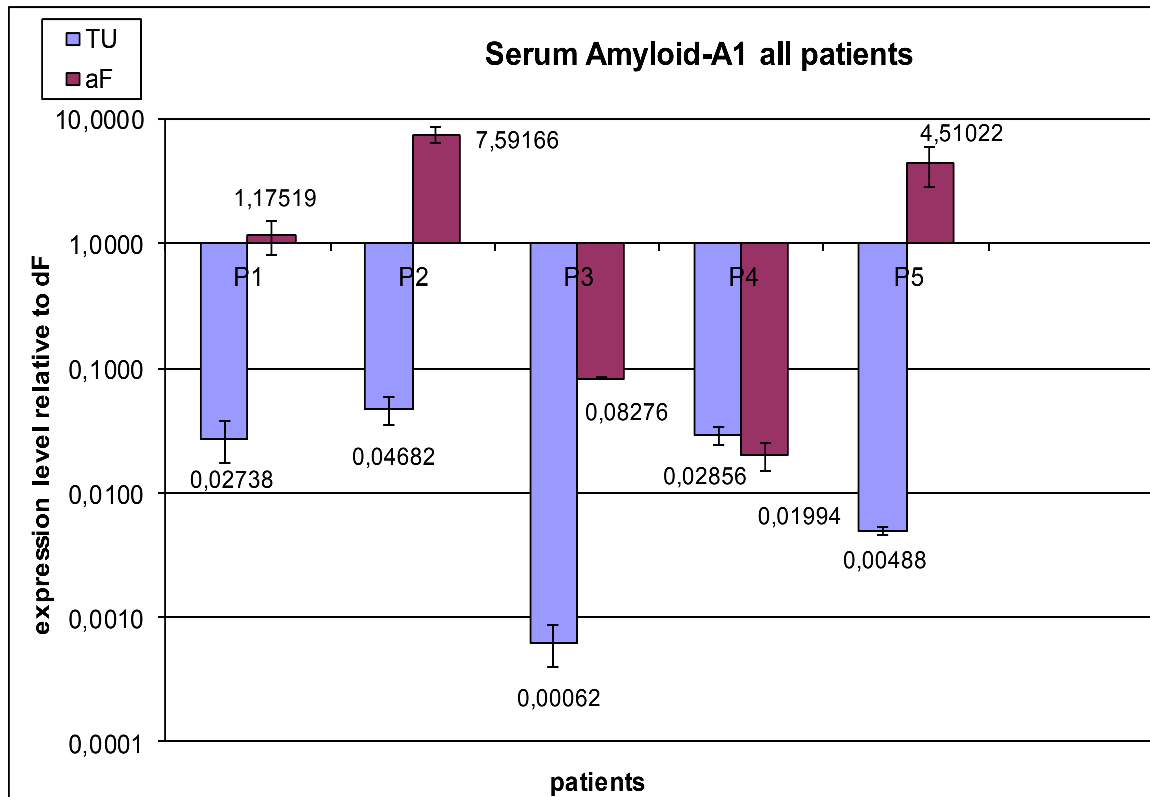

Figure 12: Expression of serum amyloid A 1 in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

The graphic shows that serum amyloid A in the tumor is down-regulated in all 5 patients. The greatest downregulation of serum amyloid A is detected in tumor tissue from patient #3.

Results in the adjacent fat tissue are not so consistent. Results for serum amyloid A expression in adjacent fat of patient #1 is negligible because their expression level is minimal to the distant fat tissue. An upregulation of serum amyloid A was detected in patients #2 and #5, whereby a downregulation can be seen in patients #3 and #4.

Retinol binding protein 4 (RBP4)

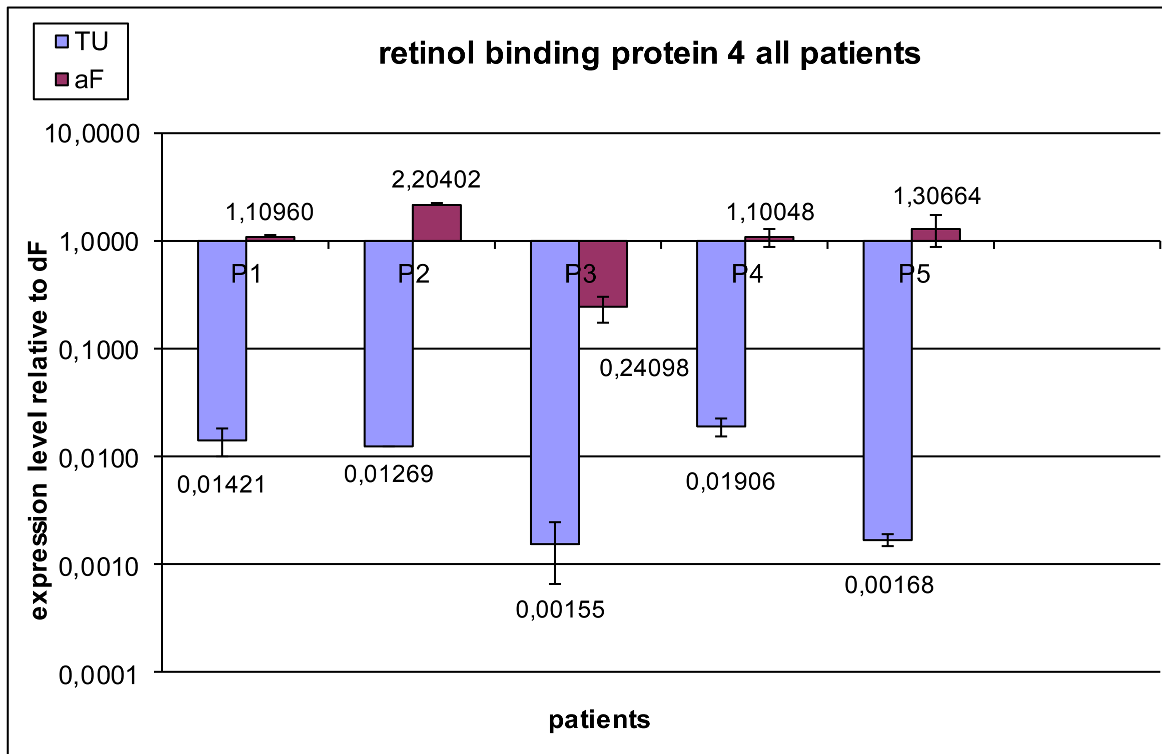

Figure 13: Expression of RBP-4 in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

The trend in tumor tissue is a clear downregulation of RBP4 expression in all patients. The greatest downregulation in tumor was detected in patient #3 followed by patient #5.

There are no clear results of RBP4 expressions in the adjacent fat tissue. Only patient #3 show a downregulation. Other patient’s samples show minimal expression differences to the distant fat tissue.

Hypoxia inducible factor-1α (HIF-1α)

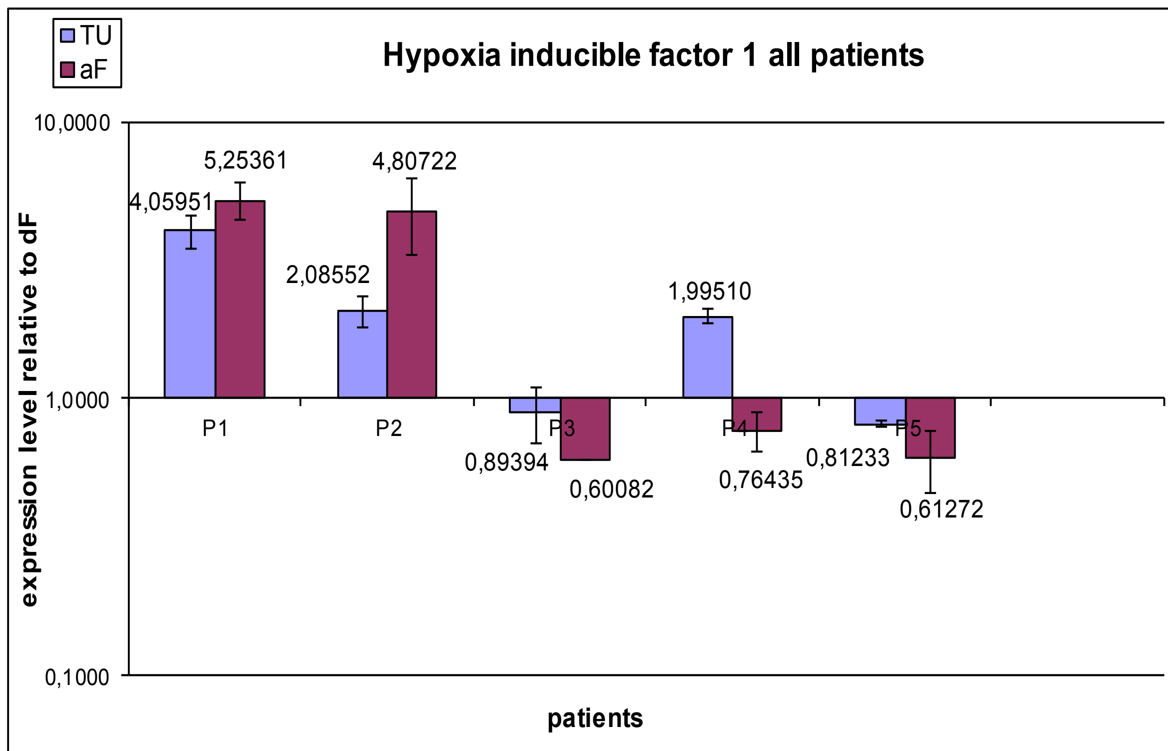

Figure 14: Expression of HIF-1 α in tumor tissue and fat tissue near tumor in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Results for HIF-1α expressions in tumor and in adjacent fat for all patients are not consistent. Thus for example, HIF-1 α in tumor tissue is up-regulated in patients #1, #2 and #4, whereas in patients #3 and #5 HIF-1 α are slightly down-regulated and therefore cannot be evaluated. Results of HIF-1 α expressions in adjacent fat are also inconsistent. The graphic shows that HIF-1 α in adjacent fat is up-regulated in patients #1 and #2. Patients #3, #4 and #5 show a downregulation but the expression differences are minimal compared to the distant fat tissue.

Hepatocyte growth factor (HGF)

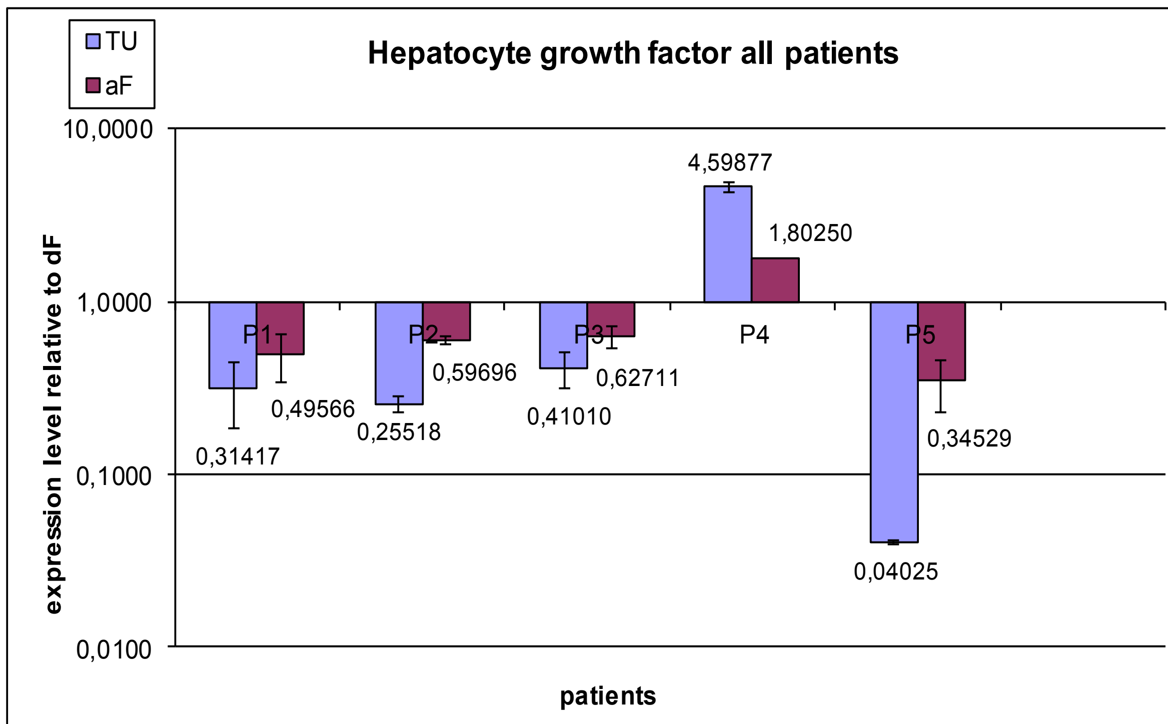

Figure 15: Expression of HGF in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

The trend in tumor tissue and adjacent fat tissue is a downregulation of HGF expression in most patients. Patient #4 is an exception. In general, the differences in expression level are minimal, only patient #5 shows a clear downregulation in tumor tissue.

Results in the adjacent fat tissue show a slight downregulation in patients #1, #2, #3 and #5, though the differences in expression level are minimal. The slight upregulation of HGF in the adjacent fat from patient #4 seems to be an exception. The greatest downregulation in adjacent fat is detected in patient #5. To sum up no clear expression differences are detected.

Serpin

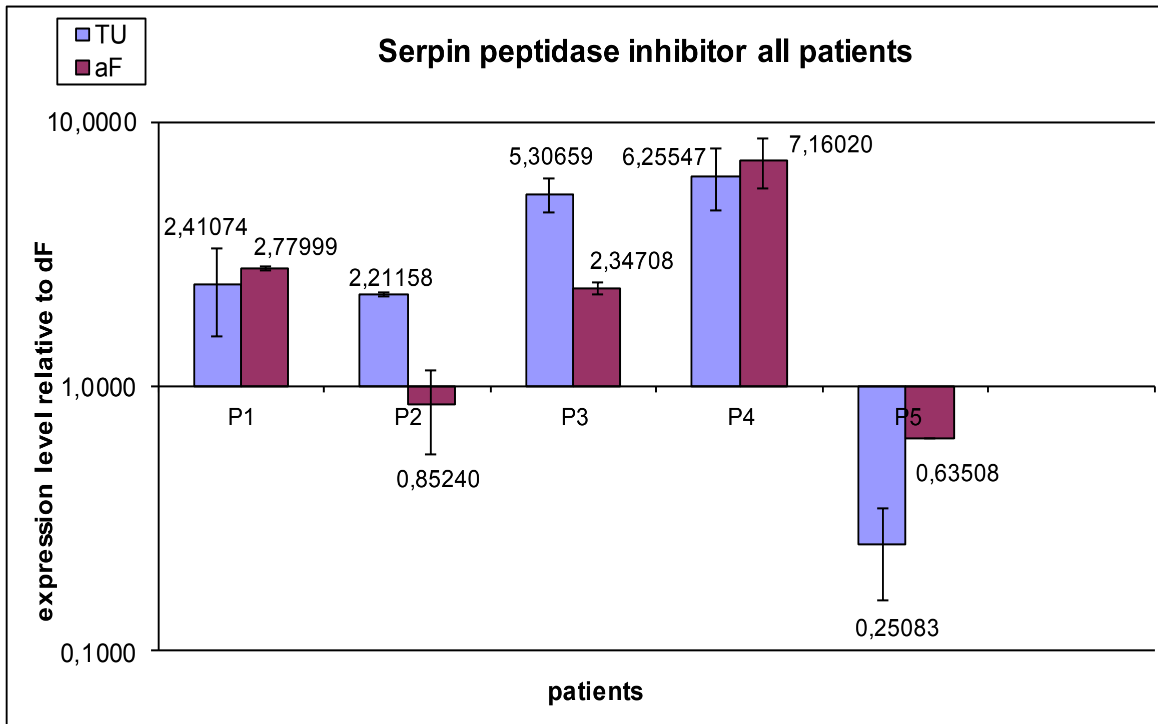

Figure 16: Expression of serpin in tumor tissue and adjacent fat tissue in comparison to distant fat. Data for all patients are shown (dF= distant fat, aF= adjacent fat, TU=tumor tissue).

Serpin expression is up-regulated both in adjacent fat and in tumor tissue in all patients, except patient #5. For the patient #2 no clear statement can be given because results are very close to the expression from serpin of the healthy tissue. The greatest upregulation of serpin is detected in tumor tissue from patient #3 and #4. Only patient #5 shows a clear downregulation of serpin in tumor tissue as well as in adjacent fat. The graphic shows that serpin in the adjacent fat is up-regulated in 3 patients except patients #2 and #5. Patient #4 shows the greatest upregulation in adjacent fat.

3.3 Distant fat (all patients)

Figure 17 shows the expressions of all adipocytokines in all patients (presented by different colours and types of lines) in the fat distant to the breast cancer tissue.
It was made as an internal proof for the quality of the tissue, because it was not very clear if the tissue might have been affected by the freezing by minus 80 centigrade. The diagram shows a very similar expression pattern in all patients. Of course there are expression differences in the different patients but regularity of a pattern can be seen. This let us conclude that the tissue quality was comparable.

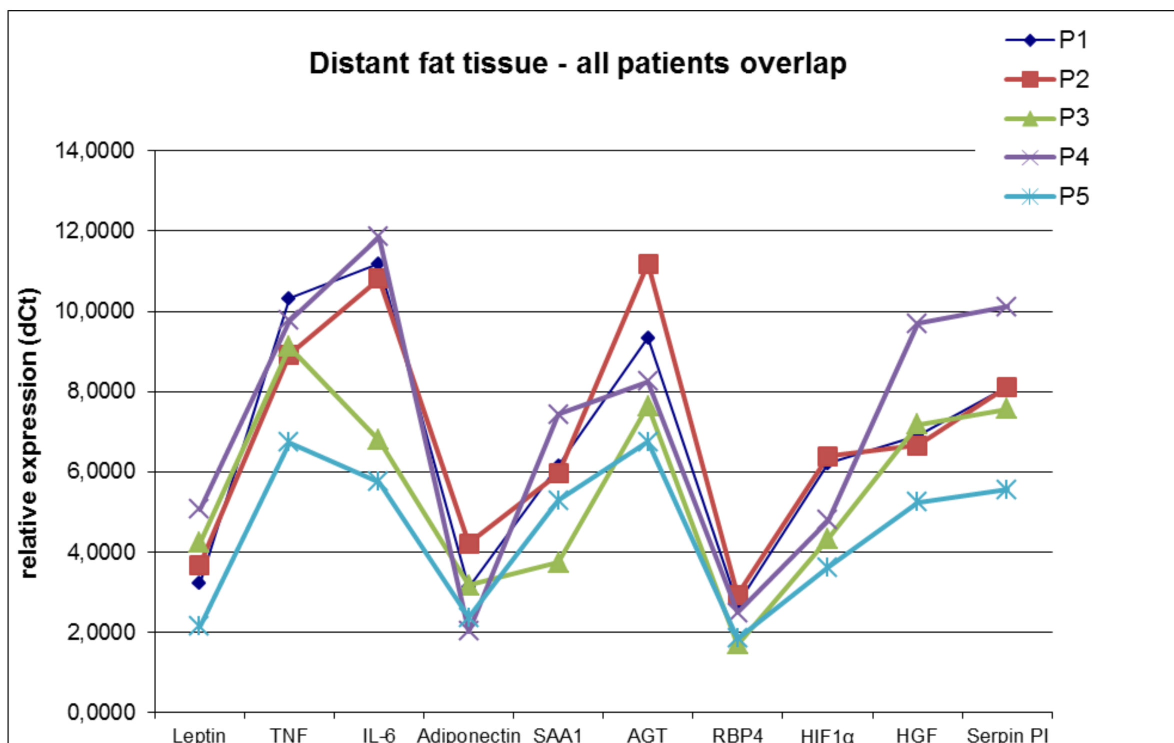

Figure 17: Expression levels of all 10 adipocytokines in distant fat tissue in all 5 patients. (x=adipocytokine/y=relative expression dCt).

4 Discussion

The adipose tissue produce bioactive peptides (proteins) known as adipocytokines (e.g., TNF-α, Interleukin-6 or amyloid A, etc.) which have different metabolic roles. The positive correlation between obesity and postmenopausal breast cancer risk is known for a long time. However, the reason behind correlation between elevated BMI and risk for breast cancer is still not resolved entirely. Expression profile of different adipocytokines in adipose- and tumor tissue might shed more light on this. Therefore, we wanted to see whether there are differences in the expressions of adipocytokines in tumor tissue and in fat tissue adjacent to the tumor in comparison to distant fat tissue.
Although this retrospective pilot study includes only 5 patients, to our knowledge it is one of very few studies dealing exclusively with expressions of adipocytokines in the tumor tissue, tumor surrounding adipose tissue and adipose tissue away from tumor. In our study we wanted to analyze differences in expression of various adipocytokines in tumor-tissue and adjacent fat tissue in comparison to "healthy" distant fat tissue (≥2 cm away from tumor). It should be noted that exclusively HER2/neu negative and ER/PR positive cases were included in the study. In most cases we observed a down regulation of adipocytokines in tumor tissue in comparison to the distant fat tissues. We also observed that adipocytokine expressions in adjacent fat tissues are very inconsistent.
This study was not designed to evaluate the clinical usefulness of adipocytokines as predicting factors in breast cancer. We rather wanted to measure expression differences in mentioned tissue-samples. Our results do not perfectly reflect data of some previous studies (see below for details). However, we cannot definitely exclude the possibility that adipocytokine expression pattern in tissue could have been influenced by micro invasion of neoplastic cells and, therefore, do not represent the true physiological level. The question of the intensive yellow colour of the breast cancer bordering fat tissue still remains unanswered. Neither previous reports, nor our results, can explain those colour differences.
The limitation of this study is the small number of patients. More patient's samples also involving tissues from a healthy control group and serum samples should be

tested to see a clear trend. Furthermore, more patient-related clinical data such as if they are suffering from diabetes mellitus or if they underwent a neoadjuvant therapy might be of interest to evaluate interactions of adipocytokines.
Data for six most strongly deregulated adipocytokines are discussed here in more details.

4.1 Leptin

Leptin is described as an anti-apoptotic and mitogenic factor and, therefore, supports tumor growth and survival. Previous studies found out that leptin as well as the leptin receptors are elevated above others in breast cancer cells. Moreover if leptin is overexpressed survival prediction is worse. [Ray and Cleary, 2010].
We observed that in tumor tissue from patients #1-5 leptin mRNA is down-regulated. This stands in contrast to some other reports. The greatest down regulation of leptin mRNA is detected in the tumor tissues from patient #1, #3 and #5. These three patients have tumor grade G3 in common. However, comparing BMI (#1 = 26,6; #2 = 36,3; #3 = 22,5; #4 = 21,1; #5 = 30,4) does not explain why those patients stand out.
Results in the adjacent fat tissue are not consistent and expression differences are minimal compared to the distant fat tissue. Therefore, no clear statement can be given. But it can be speculated that the distant fat tissue is not expressing leptin on a physiological level and therefore is not useful as a reference for the other tissues. As mentioned before leptin also effects and interacts with other signalling systems and can, therefore, indirectly affect the tumor growth. For example an activated leptin receptor can transactivate HER2/neu [Surmacz, 2007].

4.2 Adiponectin

We have found a significant downregulation of adiponectin mRNA in the tumor as well as in the adjacent fat in patient #1, #2, #4 and #5. For patient #3 adiponectin was expressed at a very late cycle and therefore not measurable by our technique. The greatest downregulation of adiponectin was detected in the tumor tissue from

patient #5, followed by patient #1. These patients neither stick out because of their age nor because of their BMI or tumor grade. Results for adjacent fat are not as consistent as for the tumor tissue. However, a downregulation can be seen in patients #1 and #3.

Our results clearly demonstrate that the trend is a downregulation, which reflects data from previous publications. Adiponectin has, in contrast to leptin, anti-diabetic, anti-inflammatory and anti-atherogenic effects. [Kershaw and Flier, 2004; Brochu-Gaudreau et al., 2010]. Moreover, in vitro studies with breast cancer cell lines have shown that adiponectin prevents growth of cancer cells [Van Kruijsdijk et al., 2009]. It is assumed that adiponectin can lead to apoptosis by inhibiting angiogenesis which in turn stops tumor growth [Brochu-Gaudreau et al., 2010]. Our data showing that adiponectin is down-regulated in fat tissue nearby and in tumor tissue are in line with these statements.

In order to show the impact of high adiponectin levels on breast cancer Miyoshi et al. made a retrospective case control study including 102 histological proofed breast cancer patients (cases) and 100 healthy women (controls). Their results showed that the risk, both for premenopausal and for postmenopausal women, for developing breast cancer is inversely correlated with serum adiponectin level. In other words the lower the serum adiponectin level, the higher is the risk for developing breast cancer and vice versa. Moreover, all those results have shown that hypoadiponectinemia is associated with histological high grade and large (>2cm) tumors, in other words more aggressive phenotypes [Miyoshi et al., 2003].

Mantzoros et al. also made a case control study with 174 pre and postmenopausal female breast cancer patients and 167 control women. They wanted to evaluate whether low serum adiponectin levels are associated with increased breast cancer risk for both pre- and postmenopausal women [Mantzoros et al., 2004]. Both research groups have shown that postmenopausal women have an elevated risk developing breast cancer if they have decreased serum adiponectin values. In contrast to Miyoshi et al. Mantzoros et al. have shown that this does not apply for premenopausal women. However, the design of these two studies does not allow us to compare them directly with our study. Miyoshi and Mantzoros used serum adiponectin levels whereas we measured adiponectin expression in the tissues.

4.3 IL-6

Expressions of IL-6 mRNA in tumor markedly differ from expressions in the adjacent fat. The trend in tumor tissue is a downregulation of IL-6 expression in all 5 patients. The greatest downregulation in tumor tissue was detected in patient #5. Results in the adjacent fat tissue show a clear upregulation of IL-6 in all patients. This correlates with the assumption that IL-6 elevates expression of anti-apoptotic and angiogenic proteins [Salgado et al., 2003]. This consequently helps the tumor cells to survive and to grow. Breast cancer cells express IL-6 receptors whereas an autocrine and paracrine mediated tumor growth has been reported. [Salgado et al., 2003]. It could be speculated that, in our case, IL-6 receptors in breast cancer tissue are overexpressed and the upregulation of IL-6 in adjacent fat effects via a paracrine pathway tumor progression.
The greatest upregulation in adjacent fat was detected in patient #2 and which possibly can be explained by the high BMI. However, the line of argument is inconsistent because in patient #4, who has the lowest BMI, an overexpression was also detected.
Obese people have elevated IL-6 concentration [Ronti et al., 2006; Van Kruijsdijk et al., 2009; Gnaciǹska et al., 2009]. Moreover, by influencing the aromatase activity IL-6 is like some other adipocytokines e.g. TNF-α and leptin, affecting the oestrogen biosynthesis [Rose, Komninou, Stephenson, 2004]. Salgado et al. performed a study with the question about whether the serum IL-6 has prognostic significance. According to this prospective study, including 96 unselected and untreated female patients with progressive metastatic breast cancer, those patients having circulating IL-6 have a worse outcome than those without. In 90% of study participants' very high IL-6 serum levels were detectable. The more metastasis, the higher serum IL-6 levels have been measured. However, no correlation between serum IL-6 levels and the common breast cancer risk factors was found [Salgado et al., 2003]. This study cannot be directly compared with our but it gives reason for speculations. As mentioned before, in our study IL-6 mRNA is up-regulated in all patients, which means that more IL-6 is probably produced and might be released in the serum. This would correspond with the study reporting that breast cancer patients have an elevated IL-6 serum level.

4.4 Serum amyloid A1 (SAA)

We found a significant downregulation of SAA in the tumor in all 5 patients. The greatest downregulation of SAA was detected in tumor tissue from patient #3. Results in the adjacent fat tissue are not so consistent.
Obese people, who consequently have increased adipose tissue expressing proinflammatory cytokines, have an elevated serum SAA level. Salas-Salvadó et al. made a study observing 19 obese non-diabetic patients and 20 lean control subjects. These results have shown that obese people have higher SAA plasma levels and that weight loss decreases the SAA level [Sala-Salvadó et al., 2006]. Pierce and co-workers have shown with their study that there is a correlation between elevated plasma SAA levels and reduced overall survival. Their conclusion was that SAA could be a marker for long-term prediction of breast cancer survival independent of race, tumor stage and body mass index [Pierce et al., 2009]. Results of our study are quite different but because of the study design both studies cannot be compared to our study.

4.5 RBP4

The trend in tumor tissue is a clear downregulation of RBP4 mRNA expression in all patients.
Kuppumbatti et al. found out that in some breast cancer patients the expression of retinol binding protein is decreased in breast cancer cells. Physiologically, RBP appears to inhibit a survival pathway and suppresses anchorage-independent growth. [Kuppumbatti et al., 2001]. RBP also influences other receptors such as the retinoic acid receptor. Activation of this receptor causes cell differentiation. Depending on the retinol storage, RAR is activated. RBP is mediating cellular uptake of retinol via receptors with high affinity to RBP. Downregulation of RBP decreases RAR activity. This consequently induces loss of cell differentiation, which in turn leads to tumor progression and autonomous growth.
Moreover, Farias et al. and Kuppumbatti et al. have shown in different studies, that retinol binding protein is expressed in normal breast epithelium whereas it is down-regulated in 24% of human breast cancer and this in turn results in a local deficit in

vitamin A storage. Some other investigators have seen such results too and they argue that downregulation of RBP in human cancers is an epigenetic event [Farias et al., 2005].
Our results clearly demonstrate decreased expression of RBP4 mRNA in breast cancer tissue. Taken together it could be that a turn off of RBP4 in the tumor leads to unregulated cell growth and tumor progression. With only slight downregulation of RBP4 mRNA in patient #3, results in the adjacent fat tissue are inconsistent.

4.6 Serpin

Nowadays different functions have been attributed to serpin PAI-1 as e.g.: angiogenesis, atherogenesis and influence of cell migration and adhesion by binding to urokinase plasminogen activator. Furthermore, the plasminogen activator system has also been suggested to play a role in tumor cell invasion. Increased levels of PAI-1 lead to a destruction of the cell-cell adhesion which in turn leads to an enhanced cell motility and increased risk for metastases [Kershaw and Flier, 2004 / Carter and Church, 2009].
We have demonstrated that serpin mRNA expression is up-regulated both in adjacent fat and in tumor tissue in all patients, except patient #5. Only patient #5 shows a clear downregulation of serpin in tumor tissue as well as in adjacent fat. Patient #4 shows the greatest upregulation in adjacent fat. Upregulation of serpin supports tumor growth and progression which would correspond with our results.

To sum up, because of the limited number of samples and the heterogeneity of our data it is not possible to make a clear statement about expression of adipocytokines in tumor and adjacent fat tissue. However, in context with literature data, we propose that cancer cells may effect adipocytokin expression not only in the tumor but also in the adjacent fat tissue. Adipose tissue and adipocytokines seem to play a role in pathophysiology of breast tumors. Further studies are necessary to elucidate this interesting topic in more details.

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