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DISSERTATION

Analysis of human breast tumors (Mammacarcinomas) using molecular and genetic high-throughput methods

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der technischen Wissenschaften unter der Leitung von

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Kurzfassung der Dissertation

Das Mammakarzinom ist die häufigste Krebserkrankung bei Frauen und ca. 12% aller Frauen in Österreich erkranken im Laufe ihres Lebens an Brustkrebs. In Industrienationen ist Brustkrebs für beinahe 22% aller weiblichen Krebsfälle verantwortlich; in Österreich sind das ca. 4350 neue Fälle und etwa 1800 Todesfälle jedes Jahr. Wenngleich die Inzidenz des Mammakarzinoms mit dem Alter kontinuierlich ansteigt, so führt der Brustkrebs mittlerweile auch bei Frauen zwischen dem 35. und 55. Lebensjahr die Einzeltodesstatistik an.
Um auf molekularer und genetischer Ebene Untersuchen durchführen zu können, wurde eine DNA- und Plasma-Bank etabliert. Während der Dissertation wurden 433 Patientinnen für die DNA-Isolierung und 254 Patientinnen für die Plasma-Isolierung rekrutiert, was im Rahmen von routinemäßigen Blutabnahmen erfolgte. Diese Patientinnen gaben ihr schriftliches Einverständnis für die Verwendung ihres Blutes für wissenschaftliche Untersuchungen. Zur Eruierung einer schnellen und billigen DNA-Isolierungsmethode wurden mehrere Produkte von verschiedenen Firmen getestet. Durchgeführt wurden dann einerseits der Extract-N-Amp™ Blood PCR Kit (Sigma), um eine einfache und äußerst schnelle Methode der DNA Isolierung, andererseits der QIAamp® DNA Blood Midi Kit (QIAGEN), um hochwertige DNA für die Analysen zur Verfügung zu haben.
Um vielversprechende Ausgangspunkte für Polymorphismus-Analysen zu ermitteln, wurde PathwayAssist verwendet, ein Programm, welches aus der publizierten Literatur Zusammenhänge findet und anschaulich darstellt. Aufgrund dieser Analysen wurden der Androgen Rezeptor (AR – Androgen gilt als Antagonist vom Östrogen), die Aromatase (CYP19 – als eines der wichtigsten Enzyme im Östrogenmetabolismus) und Cyclooxygenase-2 (COX-2 – ein Enzym, welches in der Prostaglandinsynthese eine wichtige Rolle spielt) selektiert. Nach Studium der Literatur wurden die folgenden vier Polymorphismen ausgewählt.

1. Der $(CAG)_n$ Längenpolymorphismus des Androgen Rezeptor Gens (*AR*) im Exon 1, weil in Studien gezeigt werden konnte, dass proportional mit steigender Anzahl an CAGs die Transaktivierungsaktivität des AR abnimmt. Bei den „langen Allelen" wurden signifikant weniger CAG Triplets in der Gruppe der Brustkrebspatientinnen als in der Kontrollgruppe gefunden. Die größte odds ratio (OR) war bei ≤ 21 Wiederholungen gegeben, das Ergebnis blieb aber auch bei ≤ 22 noch signifikant. Das Resultat, dass eine homozygote Frau eher in die Gruppe mit malignem Brustkrebs fällt, war ebenfalls statistisch signifikant. Der von Rebbeck et al. [4] bei premenopausalen *BRCA1* Mutationsträgerinnen festgestellte Zusammenhang, dass bei diesen Patientinnen mit steigender Anzahl an CAG Wiederholungen das Diagnosealter sinkt, konnte auch bei Patientinnen mit Tumoren gefunden werden, die negativ/schwach positiv für Östrogen Rezeptor (ER) und Progesteron Rezeptor (PgR) waren. Dies wurde zum ersten Mal von mir gezeigt und könnte durch die Tatsache erklärt werden, dass *BRCA1* Mutationsträgerinnen zum Großteil Tumore mit negativem ER und PgR Status haben.
2. Der *CYP19* Exon 10 C → T single nucleotide polymorphism (SNP), weil in einer skandinavischen Fall-Kontroll-Studie ein direkter Zusammenhang des T-Allels mit hohem Grading und großer Tumorgröße gefunden wurde [5]. Für premenopausale Patientinnen, die homozygot für das Wildtyp-Allel (C) sind, zeigte sich eine starke, aber nicht statistisch signifikante Tendenz für einen protektiven Effekt gegenüber heterozygoten oder für das T-Allel homozygoten Frauen mit Brustkrebs. Die publizierten Resultate konnten statistisch nicht bestätigt werden, obwohl manchmal eine übereinstimmende Tendenz feststellbar war.
3. Der *CYP19* Codon 39 Tyr → Arg SNP, weil eine japanische Studie zeigte, dass die Frauen, die ein Allel mit Arg haben, ein signifikant reduziertes Brustkrebsrisiko aufweisen. In dem von mir untersuchten Patientinnenkollektiv fand sich eine einzige Patientin, welche ein Allel mit Arg hatte (diese war homozygot für Arg), womit die

Annahme nahe liegt, dass dieser SNP in der kaukasischen Population nur sehr selten vorkommt. Diese Tatsache, dass sich die Häufigkeit von polymorphen Allelen in verschiedenen Regionen stark unterscheiden kann, wurde schon in vielen Fällen gezeigt.

4. Der -765 G → C SNP im COX-2 Gen, weil beim C-Allel durch das Fehlen einer Sp1 Stelle die Promoteraktivität um 30% reduziert ist. Ristimäki et al. [6] fanden, dass eine COX-2-Überproduktion mit größeren Tumoren, mit positiven Lymphknoten-Status, mit negativem Hormonrezeptor-Status, mit p53-Expression und mit HER2-Amplifikation einhergeht. Per logistischer Regression konnte ein signifikanter Zusammenhang der Krebsfälle nur mit dem heterozygoten Genotyp, nicht aber bei für das C-Allel homozygoten Patientinnen, festgestellt werden, was sich nicht mit einer reduzierten Promoteraktivität des C-Allels erklären lässt. Bezüglich der anderen publizierten Zusammenhänge konnten keine statistisch signifikante Bestätigung gefunden werden.

Im klinischen Alltag besteht ein dringender Bedarf an neuen Serum-/Plasma-Biomarkern, um Patientinnen (mit malignem Brustkrebs und mit benignen Läsionen) und gesunde Frauen anhand von Serumanalysen unterscheiden zu können. Die vorhandenen Marker lassen in Bezug auf Sensitivität und Spezifität zu wünschen übrig und werden deshalb in der Routine nur mehr selten angewandt. Mit dem Ziel der Entdeckung von brustkrebsspezifischen Serum-Biomarkern per 2D Gelelektrophorese, wurden in Kooperation mit der Gruppe von Lukas A. Huber in Innsbruck die im Rahmen der Dissertation gesammelten Plasmaproben analysiert. Nach Abtrennung von Albumin und IgG unter reduzierenden Bedingungen und Aufkonzentration der nieder-molekulargewichtigen Proteine, konnte eine 2D DIGE Analyse mit fluoreszenzmarkierten Proteinen durchgeführt werden. Anschließend wurden die Gele digitalisiert und mittels biologischer Variationsanalyse (BVA) ausgewertet. Es wurden acht hoch- und neun hinunterregulierte Proteine bei Brustkrebspatientinnen gefunden (diese waren zum Großteil statistisch hochsignifikant), die zurzeit am Massenspektrometer (SELDI-TOF mit IDA-Cu^{2+}-Cellulose als Oberfläche) identifiziert werden.

Mit einem seit kurzem kommerziell erhältlichen Serum Biomarker Chip, der Antikörper für 120 krebsspezifische Serum-Biomarker enthält, sollte die klinische Anwendbarkeit vorhandener Krebsmarker eruiert werden. Zu diesem Zweck wurden jeweils 10 verschiedene Plasmaproben von malignen Brustkrebspatientinnen, von Frauen mit benignen Läsionen und von gesunden Kontrollen gepoolt, um mögliche patientenabhängige Störfaktoren zu mitteln. Am ersten Chip wurde Plasma der malignen Brustkrebspatientinnen mit dem Pool der gesunden Kontrollen und am zweiten Chip Plasma der Frauen mit benignen Läsionen mit dem Pool der gesunden Kontrollen verglichen. Die fünf am stärksten hinauf- bzw. hinunterregulierten Marker könnten nach genauerer Analyse in mehreren Proben (auch mit anderen Methoden, z.B. ELISA) interessante Biomarker für Brustkrebs darstellen. Vor allem die Kombination von PAI-1 (plasminogen activator inhibitor) und uPA (urokinase plasminogen activator), die bei Patientinnen mit positiven Lymphknoten manchmal bereits als prognostischer Faktor für das Ansprechen einer adjuvanten Chemotherapie herangezogen werden, scheint viel versprechend zu sein.

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There are at least three major challenges that will occupy most cancer researchers' time over the next 10 years. The first is the discovery of new genes that have a causal role in neoplasia, particularly those that initiate and conclude the process. The second is the delineation of the pathways through which these genes act and the basis for the varying actions in specific cell types. The third is the development of new ways to exploit this knowledge for the benefit of patients.

Vogelstein B & Kinzler KW [7]

1. Synopsis

A DNA- and a plasma-bank were established to analyze human breast cancer using molecular and genetic methods, and to identify new and improved diagnostic molecular markers of breast cancer. Venous blood samples were taken from 433 patients for DNA isolation and from 254 patients for human plasma isolation during this dissertation at the Department of Obstetrics and Gynecology. These women provided written informed consent for research. Five different products to isolate DNA from whole blood were tested to determine a quick and cost-effective method. For a quick DNA isolation, the Extract-N-Amp™ Blood PCR Kit (Sigma) was used and to obtain high-quality DNA the QIAamp® DNA Blood Midi Kit (QIAGEN) was used.

The following genes were found to be interesting for polymorphism analysis in breast cancer with PathwayAssist, a program which identifies and visualizes published connections between genes of interest. The androgen receptor (AR), because androgen is an antagonist of estrogen, aromatase (CYP19), as one of the most important enzymes for estrogen metabolism, and cyclooxygenase-2 (COX-2), because it is an important enzyme in the synthesis of prostaglandin, were chosen. After extensive literature research, the following four polymorphisms were chosen from these three genes:

1. The $(CAG)_n$ repeat length polymorphism in exon 1 of the AR gene (*AR*), because it was shown in different studies that the transactivational activity of the AR decreases with increasing numbers of CAG repeats. My findings showed a significant difference in the number of CAG repeats of the "long allele" between breast cancer patients and the control group. The largest difference was obtained by a cut-off ≤ 21 CAG repeats, but the results remained significant when alleles with ≤ 22 CAG repeats were compared with alleles with more CAG repeats. The finding that a homozygous woman is more likely to belong to the group of breast cancer patients, was also statistically significant. Rebbeck et al. [4] have shown that – mostly premenopausal – *BRCA1* mutation carriers who carried one long *AR* CAG allele were diagnosed with breast cancer earlier than women who did not carry at least one such allele. This could also be found in premenopausal patients with tumors negative/weakly positive for ER and PgR of the collective of this dissertation. This was shown for the first time and the reason for this accordance may be that *BRCA1* mutation carriers mostly have tumors negative or weakly positive for ER and PgR.

2. The *CYP19* Exon 10 C → T single nucleotide polymorphism (SNP), because a Scandinavian case-control study showed that the T-allele is associated with higher grading and bigger tumor size [5]. For premenopausal women homozygous for the C-allele, a strong protective trend against breast cancer could be shown compared to women who are heterozygous or homozygous for the T-allele. The recently published findings from Kristensen et al. [5] could not be statistically confirmed, although a corresponding trend was sometimes found.
3. The *CYP19* Codon 39 Tyr → Arg SNP, because it was shown in a Japanese study, that women with one Arg-allele have a significantly reduced breast cancer risk. In all patients of this dissertation, only one woman with malignant breast cancer carried an Arg-allele (interestingly, she was homozygous for Arg), indicating that this SNP is rare in the Caucasian population. This finding supports the hypothesis that the frequency of polymorphic alleles can be very different in different populations.
4. The G-765C SNP of the COX-2 gene, because a missing Sp1 site in the C-allele causes a 30% reduced promoter activity. Ristimäki et al. [6] showed that a COX-2 overproduction is associated with bigger tumors, with positive lymph node status, with negative hormone receptor status, with p53 expression, and with HER2 amplification. In my study, I could only find a significant association of breast cancer risk with the heterozygous genotype. An equal association could not be shown for the homozygous CC-genotype, arguing against the hypothesis that a reduced COX-2 promoter activity explains the findings of Ristimäki et al. [6]. No significant correlation could be found between the other published histological parameters [6] and breast cancer risk.

There is an urgent need for new serum biomarkers of breast cancer, because it was found in daily routine that the sensitivity and specificity of the serum biomarkers used today is not sufficient. Therefore, 2D gel electrophoretic analysis was performed in cooperation with the group of Lukas A. Huber in Innsbruck to find new breast cancer specific biomarkers in the collection of plasma of this dissertation. After depletion of human albumin and IgG under reducing conditions and concentration of the low molecular weight proteins, a 2D DIGE analysis with fluorescently labelled proteins was done. The gels were digitalized and analyzed by biological variation analysis (BVA), resulting in the identification of eight up-regulated and nine down-regulated proteins. To identify these 17 proteins, mass spectrometry analyses (SELDI-TOF with IDA-Cu^{2+}-Cellulose as the surface) are currently underway.

The newly developed Serum Biomarker Chip® (Whatman, Schleicher & Schuell) contains 120 antibodies and is designed to measure serum or plasma proteins with known involvement in different cancer types, with a sensitive two-color labelling and fluorescent detection method. The aim of this experiment was to identify one or several different biomarkers with the Serum Biomarker Chip® which can then be analyzed by ELISA in a comprehensive plasma bank to evaluate the findings. Plasma pools of ten different malignant breast cancer patients, ten women with benign lesions, and ten healthy controls, respectively, were analyzed to eliminate the patient specific differences because only one experiment with two chips was done. With the first chip, the pooled plasma samples of the malignant breast cancer patients were compared with the pool of the healthy controls, and with the second chip the pool of women with benign lesions was compared with the pool of the healthy controls. The five most up- and down-regulated plasma biomarkers ranked by the fold change may be interesting candidates for novel biomarkers for breast cancer, which should be validated in a comprehensive plasma bank, e.g. be ELISA. Particularly the combination of PAI-1 (plasminogen activator inhibitor) and uPA (urokinase plasminogen activator), which are suggested as prognostic factors for the response to adjuvant chemotherapy, seems to be a promising biomarker.

2. Introduction

Breast cancer (BC) is the most frequent female cancer in the industrialized world with a life-time disease risk of 12% [8]. In industrial nations BC is responsible for nearly 22% of all female cancer cases; in Austria, that are 4350 new cases and about 1800 deaths each year. Although the incidence of mammacarcinoma increases with the age, breast cancer also is the leading cause of death for women between 35 and 55 years of age. Breast cancer is being actively studied and is among the human cancers best understood at the molecular level [9].

Though, of all the current research and reasonably advanced understanding of breast cancer biology, many critical targets for onset and progression of breast cancer remain to be identified. Thus, there is considerable interest in the scientific as well as in the medical community to implement biological therapies (like ones that aim to intervene with estrogen receptor signalling) to the clinics or in clinical trials.

Within the general population, breast cancer risk is modified by a large number of environmental and genetic factors. Within sporadic or inherited forms of BC such factors may affect susceptibility and / or progression of the disease. Because hormonal factors play a role in the development and maturation of the breast, variation within genes involved in hormone production and regulation is hypothesized to be particularly important.

Mammary carcinomas are unusually heterogeneous. Overexpression of a functional estrogen receptor (ER), which occurs in about 50% of all cases, is a strong prognostic and predictive marker, estrogen being a potent mitogen and survival factor of mammary epithelial cells. Because of that important role of estrogen in the carcinogenesis and progression of breast cancer [10, 11], the enzymes involved in the biosynthesis and metabolism of estrogens (*CYP17*, *CYP19*, *CYP2D6*, *COMT*, *CYP1A1* etc.) have been main targets in the study on identification of genetic polymorphisms associated with breast cancer risk [12]. Also other endogenous steroid hormones play an important role in the development and progression of breast cancer, which is strongly supported by epidemiological and experimental evidence [13, 14]. Polymorphisms in genes involved in the biosynthesis and metabolism of steroid hormones may alter steroid hormone levels and partially explain ethnic variation in breast cancer risk [15, 16]. Identifying these variant alleles and characterizing their biological function may enhance the understanding of individual susceptibility to hormonally related

cancer as well as potentially better define who may benefit from specific therapy and chemoprevention.

Therefore, the Cytochrome P450 family of these enzymes, which catalyses many reactions of the estrogen biosynthesis (Fig.1) could be a fine working basis to start searching for (some) polymorphisms which are connected with breast cancer risk.

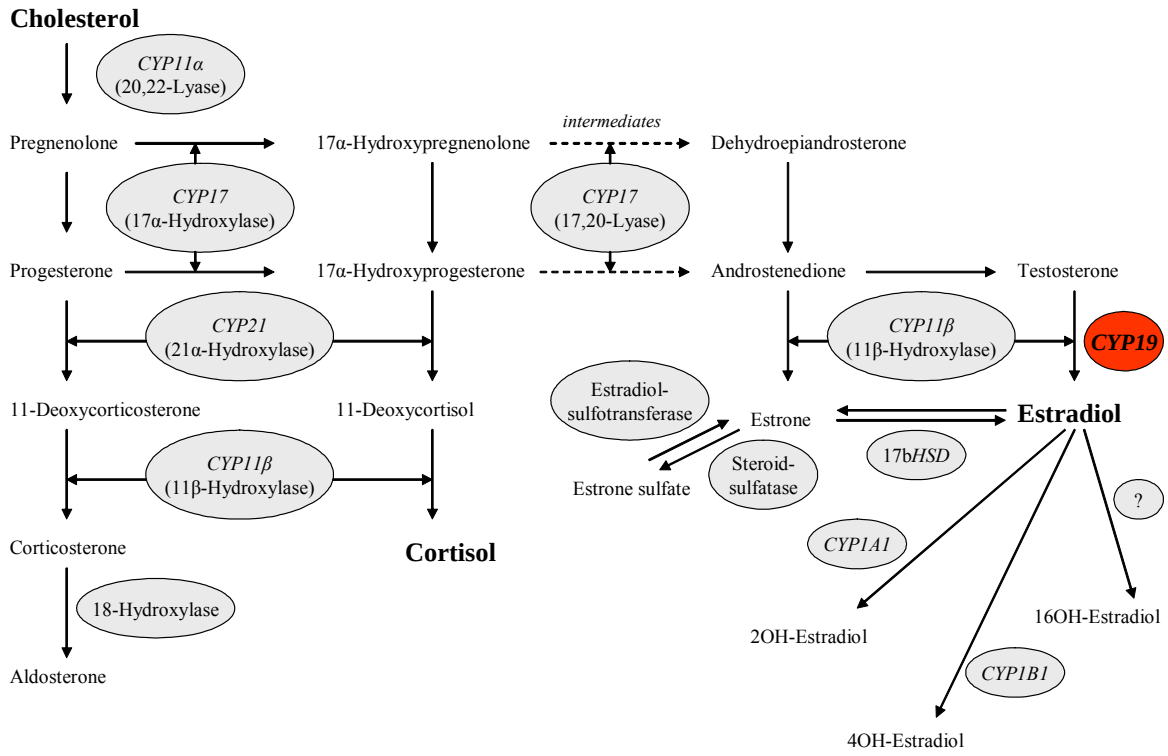
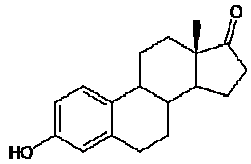
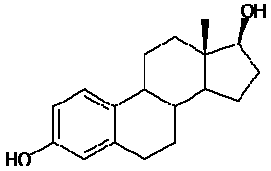
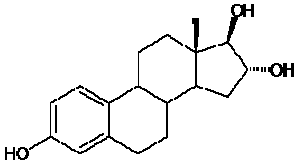


Fig. 1. The metabolic pathways of synthesis, conjugation and oxidation of estradiol [2].

2.1. *Estrogen*

Estrogen is not one hormone; it is the name of a group of hormones. There are three major forms of estrogen found in the human body: estrone, estradiol and estriol, also known as E1, E2 and E3, respectively (see Tab. 1. for chemical characteristics; Fig. 2). There is also a group of compounds called phytoestrogens, generally found in food, which can have "estrogen like" effects in the body. Estradiol (E2) is the primary estrogen produced by the ovaries. Estrone (E1) is formed from estradiol. It is a weak estrogen and is the most abundant estrogen found in the body after menopause. Estriol (E3) is produced in large amounts during pregnancy and

	E1	E2	E3
Chemical structure			
Common name	Estrone	17β-Estradiol	Estriol
Chemical name	3β-Hydroxyesta-1,3,5(10)-trien-17-one	Estra-1,3,5(10)-triene-3,17β-diol	Estra-1,3,5(10)-triene-3,16α,17β-triol
Molecular formula	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_2$	$C_{18}H_{24}O_3$
Molecular weight	270.3706 g/mol	272.3864 g/mol	288.3858 g/mol

Tab. 1. Chemical characteristics of the three principle forms of estrogen found in the human body.

is a breakdown product of estradiol. Estriol is also a weak estrogen and may have anti-cancer effects. Before menopause estradiol is the predominant estrogen. After menopause estradiol levels drop more than estrone so that estrone becomes the predominant estrogen.

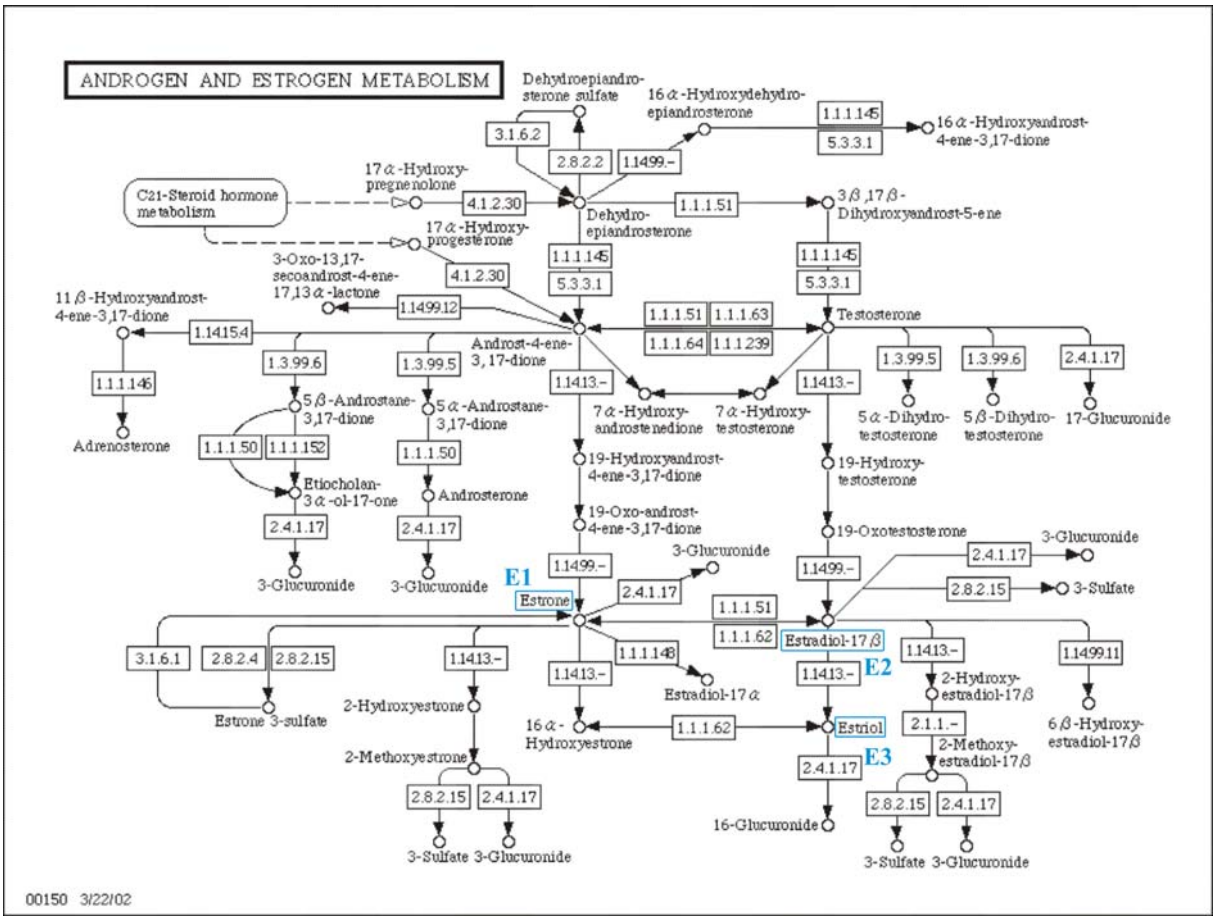
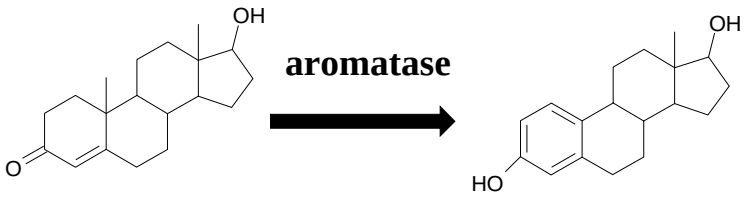


Fig. 2. Androgen and estrogen Metabolism. From http://www.genome.jp/kegg/pathway.html.

Estrogens are primarily responsible for female sexual maturation (development of breasts, further development of the uterus and vagina, broadening of the pelvis, growth of pubic and axillary hair, and increase in adipose tissue) but they also participate in the monthly preparation of the body for a possible pregnancy and in pregnancy if it occurs. Furthermore, estrogens have non-reproductive effects such as promoting blood clotting and they antagonize the effects of the parathyroid hormone, minimizing the loss of calcium from bones and thus helping to keep bones strong.

2.2. *CYP19* (Aromatase)

Aromatase is expressed in the stromal cell compartments of adipose tissue [17]. *CYP19*, which converts androgens into estrogens (Fig. 3), is one of the key enzymes involved in estrogen biosynthesis in the ovaries [18]. The activity of aromatase is induced by cortisol. This reaction is inhibited by progesterone in a dose-dependent manner. Thus, progesterone seems to be a suppressor of aromatase-induction within the adipose tissue of pre-menopausal women [19]. Some studies have demonstrated the expression of *CYP19* in breast cancer tissues and its importance in the intra-tumoral biosynthesis of estrogens [20, 21].



Testosterone 17β-Oestradiol

Fig. 3. Steroid metabolism in target tissues
[*http://matweb.hcuge.ch/endo/Reproductive_health/Steroid_hormone_metabolism_Fig3.html*]

Although the plasma concentration of estradiol of postmenopausal women is very low, the level in the breast tissue is equal to the level of the plasma concentration of premenopausal women. Immunohistochemical studies traced back these high estradiol levels to the high activity of aromatase which was detected by *in-situ* hybridisations in mammary glands and mammary tumors [22, 23]. Brueggemeier et al. suggested a relationship between CYP19 gene expression and the expression of COX genes [24]. Their results suggest that prostaglandin E2 induces aromatase activity and expression through at least two prostanoid receptor subtypes [25].

2.3. Androgen receptor (AR)

In normal mammary glands, estrogen promotes and maintains development, and inhibits cell death [26]. Ovarian granulosa cell apoptosis is inhibited by estrogen, and androgens counteract this effect [27]. In breast cancer cell lines it has also been documented repeatedly that androgen directly is anti-estrogenic [28-30]. The past and above all the recently administered anti-estrogenic therapies of (advanced) BC demonstrate the clinical efficacy of androgen [31].

The action of androgens is mediated through the androgen receptor (AR) which is a protein of 910 amino acids and a ligand-dependent, zinc-finger type transcription factor. The AR is involved in the differentiation, development and regulation of breast cell growth [32, 33] although it remains unclear if the androgens play a role in breast cancer development and carcinogenesis [34, 35]. Androgens may influence breast cancer risk directly by binding to the AR and promoting or opposing breast cancer cell growth, or indirectly through their conversion to estradiol or by competing for steroid binding proteins [36].

2.4. COX-2

Over a long period, the proposed mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin-like drugs was the inhibition of prostaglandin biosynthesis via the enzyme cyclooxygenase (COX or prostaglandin G/H synthase 2) [37]. In 1982, Sir John Vane's efforts were recognized with a Nobel Prize in Medicine. The first NSAIDs were used in therapy for rheumatic diseases, in spite of there associated gastrointestinal toxicity and their ability to cause renal damage in susceptible patients. The COX molecule consists of three independent folding units; an EGF-like domain, a membrane binding motif and an enzymatic domain. Within the enzymatic domain, the two functional sites (cyclooxygenase and peroxidase) are adjacent but spatially distinct sites. From 1990 on, after the discovery of COX-2, we know that there are at least two COX isoforms [38] and some COX-splice variants. The COX-2 gene structure was discovered in 1992 by several groups [39, 40]. Both COX enzymes have a molecular weight of around 70 kDa and similar K_m and V_{max} for arachidonic acid with 60% homology at the protein level [41]. COX-1 is a constitutive enzyme which is responsible for the production of prostanoids necessary for platelet aggregation and protection of the endothelium, the gastric mucosa and the kidney. The

inducible enzyme (COX-2) is newly synthesized at sites of tissue damage and produces prostaglandins that exert pathological effects (Fig. 4).

From the early beginnings of therapy for rheumatic diseases with aspirin in 1897 to nowadays, the focus on COX enzymes has shifted to other therapeutic targets for COX-2 inhibitors, which have relatively few gastric side-effects. Selective COX-2 inhibitors are efficacious in the prophylaxis and treatment of colon cancer (and other types of cancer with solid tumor formation), in preventing pre-term delivery and in treating Alzheimer's disease [42].

It is now well established that COX-2 is commonly overexpressed in many solid tumors [43, 44]. Epidemiological studies as well as clinical trials employing selective and non-selective COX-2 inhibitors indicate that COX-2 is mechanistically involved not only in colorectal carcinogenesis, but also in other types of cancer [45, 46]. In addition to early cancer development, evidence is beginning to accumulate that COX-2 may also contribute to late-stage progression (i.e. tumor metastasis) [47]. Early reports in breast cancer suggest a linkage between prostaglandin production and aggressive disease (larger tumor size, axillary lymph node involvement, poor postoperative survival, and HER2 amplification) [6], but less is known regarding the specific contribution of the COX-2 isoform to behaviour.

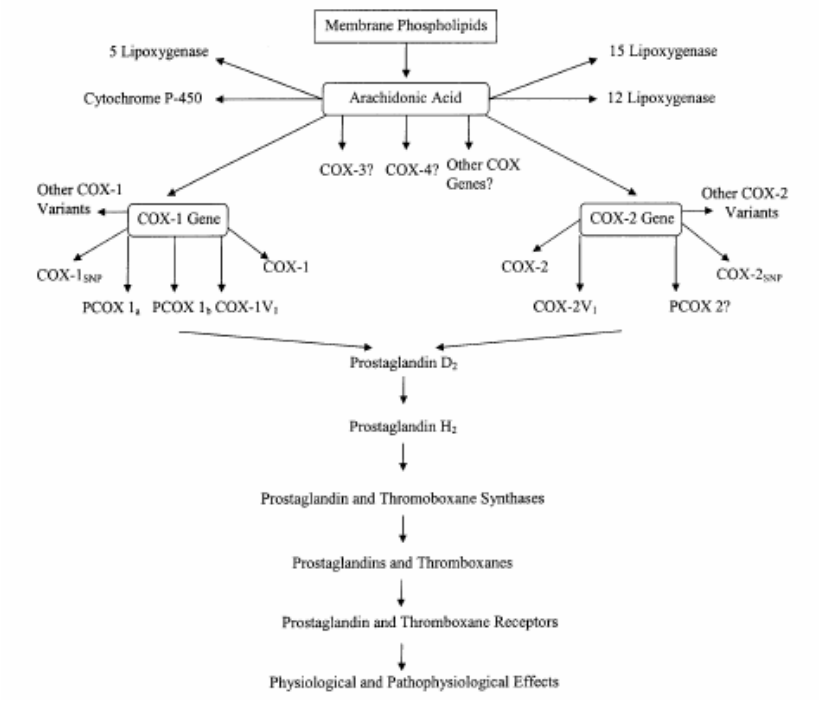


Fig. 4. Pathways of Prostaglandin and Thromboxane Formation and Action. [48]

Studies of mammary tumors in mice suggested that tumors of the breast are associated with high prostaglandin levels and induction of aromatase [25]. Mechanistically, lack of apoptosis and increased angiogenesis and invasiveness have been implicated as mechanisms of tumor growth in COX-2-dependent mammary tumors.

In September 2004, COX-2 inhibiting NSAIDs came back to the center of interest of physicians and the pharmacological industry because Merck & Co voluntarily withdraw their product Vioxx (Rofecoxib) due to an increased risk of cardiovascular events. The decision was made after a clinical study testing the prolonged use of the drug for prevention against colorectal polyps, the so-called APPROVe (Adenomatous Polyp Prevention on Vioxx) study, suggested that, overall, the risk of myocardial infarction is doubled in patients on Rofecoxib compared with those taking placebo and also the risk for stroke is increased.

If this problem only concerns Vioxx is not clear yet (it is possible that it is a class effect of also other COX-2 inhibitors – e.g. Celebrex and Bextra; Pfizer), but it is heavily discussed within the community and the results of other clinical studies are urgently awaited.

2.5. Single nucleotide polymorphism (SNP) and microsatellites

Microsatellites have been the workhorse of human genetic analysis since the late 1980s [49]. Their polymorphism is due to variations in the number of tandem repeats of short sequence units typically ranging from two to four nucleotides in size. Many have 5 – 10 alleles and heterozygosity levels of 0.75 or greater. More than 10000 microsatellites have been identified. Their alleles are easily and rapidly distinguished on the basis of variations in the electrophoretic movement of fluorescently labelled PCR products amplified with primers complementary to conserved sequences flanking the repeats.

SNPs came into use a decade after microsatellites [50, 51], and as the scientific community anticipated the final completion of the DNA sequence of an entire representative human genome, even more attention focused on SNPs. The detailed map of the human genome can potentially transform future cancer therapy by merging genomics with pharmacology, thereby identifying which patients will benefit from specific therapeutic agents. Each SNP represents a nucleotide variation at a single nucleotide site in the genome and most are biallelic. A site is generally considered a SNP if the minor allele frequency reaches 1% in a population [52]. The major advantage of single nucleotide polymorphisms is their abundance (SNPs are the most prevalent form of genetic variation), theoretically allowing detection of tighter linkages and

associations. Millions of SNPs have already been identified (about 10 millions in January 2005; SNP database of the National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), corresponding to a frequency of about one per 1000 bp. Although found throughout the genome, as expected, they are more abundant in non-coding sequences. In addition to frequency, SNPs have the benefit of being more stable and easily amenable to automation for assessment in large scale experiments [53] and the assay price is expected to decrease as the scale of the experiment increases, as experienced with microsatellites genotyping in the last decade. Due to their frequency and distribution, SNPs may serve as superior genetic markers for assembly of a high-resolution map, aiding in the identification of disease-related loci [54, 55]. Single nucleotide polymorphisms provide a valuable tool for the pharmacogenetic approach (to identify patients who will benefit from specific medication) to cancer therapy. The discovery of SNPs as disease markers may facilitate identification of populations at increased risk for certain cancers. In addition, genetic screening of SNPs may facilitate administration of appropriate treatment modalities or reveal specific genetic profiles that have importance in drug efficacy and toxicity.

Because of their mean density, stability and high-throughput genotyping capabilities, SNPs have recently emerged as genetic markers of choice for disease gene discovery and mapping. SNPs may be located within coding regions (cSNPs) or map outside of coding regions (associated SNPs). The cSNPs may result in mutations affecting protein function (these can be enriched in particular disease populations compared to controls) or result in neutral mutations that do not affect the protein function. It has been estimated that individuals are heterozygous for 24000 – 40000 polymorphisms (i.e., less than 1% of all known SNPs) that alter amino acid composition [56]. However, it is thought that single disease-related SNP alleles can increase or modify risk for disease, but are not sufficient to cause disease [57]. Associated SNPs map outside the coding region, but may serve as useful markers because of their proximity to disease loci or loci associated with drug metabolism, or because effects on the regulation of transcription. These SNPs, while not the causative agent of the phenotype, can be used for detailed mapping of the chromosome to pinpoint the mutation(s) that are actually responsible for the disease phenotype.

2.6. Proteomics

Proteomics is the systematic study of proteins in a cell. Proteins in plasma have been studied since the time before we knew genes existed.

Human plasma is not only the primary clinical sample, but also represents the largest and deepest version of the human proteome present in any sample: in addition to the classical "plasma proteins", it contains all secreted tissue proteins (as leakage markers) plus very large numbers of distinct immunoglobulin sequences. It has an extraordinary dynamic range in that more than 10 orders of magnitude in concentration separate the high abundant serum albumin (normal concentration range 35-50 mg/ml, or 35-50 x 10^9 pg/ml) and the rarest low abundant proteins (e.g. interleukin 6; normal range 0-5 pg/ml) now measured clinically (Fig. 5).

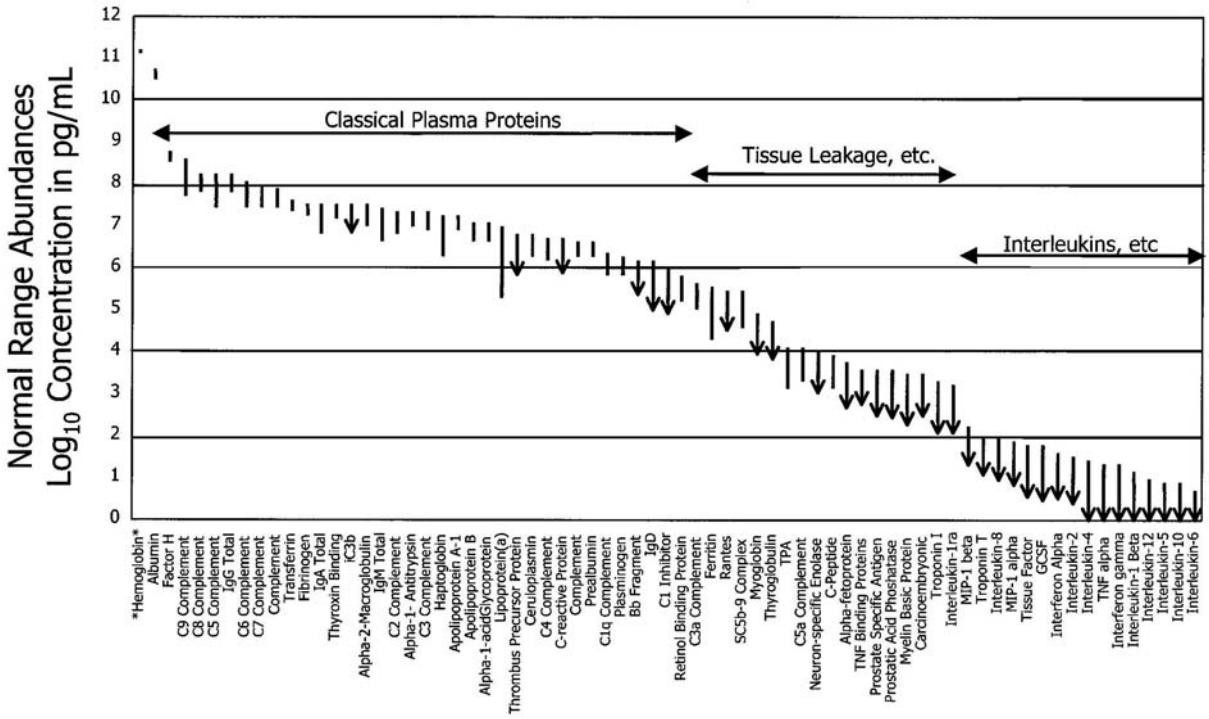


Fig. 5. Reference intervals for 70 protein analytes in plasma. Abundance is plotted on a log scale spanning 12 orders of magnitude. Where only an upper limit is quoted, the *lower end* of the interval line shows an *arrowhead*. The classical plasma proteins are clustered to the *left* (high abundance), the tissue leakage markers (*e.g.* enzymes and troponins) are clustered in the *center*, and cytokines are clustered to the *right* (low abundance). Hemoglobin is included (*far left*) for comparison.
Data were obtained from the Specialty Laboratories publications [1].
TPA, tissue plasminogen activator; *GCSF*, granulocyte colony-stimulating factor; *TNF*, tumor necrosis factor.

The attraction of plasma for disease diagnosis lies in two characteristics: the ease by which it can be safely obtained and the fact that it comprehensively samples the human phenotype, the state of the body at a particular time point. There is abundant scientific evidence, from

proteomics and other disciplines, that abundance and structure changes of plasma proteins refer to many human diseases.

There are a lot of methods to analyze the human plasma nowadays; three of them shall be introduced briefly:

2.6.1. Protein Microarrays

The development of microarray-based assay systems depended on new developments in technology, newly established detection systems and improvements in computer technology and bioinformatics. Microarrays (for genomics and proteomics; see Fig. 6), as diagnostic tools, have a big impact on medical, biological and pharmaceutical research (e.g. analysis of SNPs and expression profiling). In medical research, protein microarrays will accelerate immune diagnostics significantly by analyzing in parallel all diagnostic parameters of interest. The reduction of sample volume is of great importance for all applications in which only minimal amounts of samples are available (e.g. the analysis of multiple tumor markers from a minimum amount of biopsy material). Furthermore, new possibilities for patient monitoring during treatment and therapy can be done and the upcoming methods will help the physicians to give their patients a better, individualized treatment.

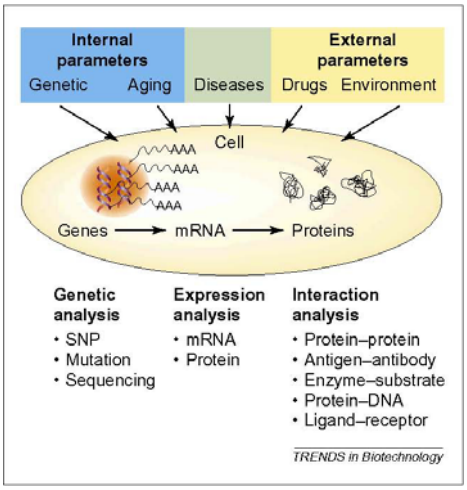


Fig. 6. Microarrays for genomics and proteomics. The physiological state of a cell is influenced by external and internal parameters. Microarray technology can be applied to monitor intracellular mRNA and protein expression mechanisms. DNA microarrays are used for genetic analysis as well as expression analysis at the mRNA level. Protein microarrays are used for expression analysis at the protein level and in the expansive field of interaction analysis.
Taken from Templin, M.F., et al. [3]

The analysis of the proteome of a cell (not only the quantification of all proteins but also the determination of their post-translational modifications and their influence on cell-state) needs high-throughput protein analysis methods which allow a fast, direct and quantitative detection, otherwise no detailed information about any complex biological system can be obtained.

Some gene expression studies revealed that mRNA level and protein expression do not necessarily correlate [58, 59] and therefore DNA- and protein microarray data should be compared to obtain the best results (Fig. 7).

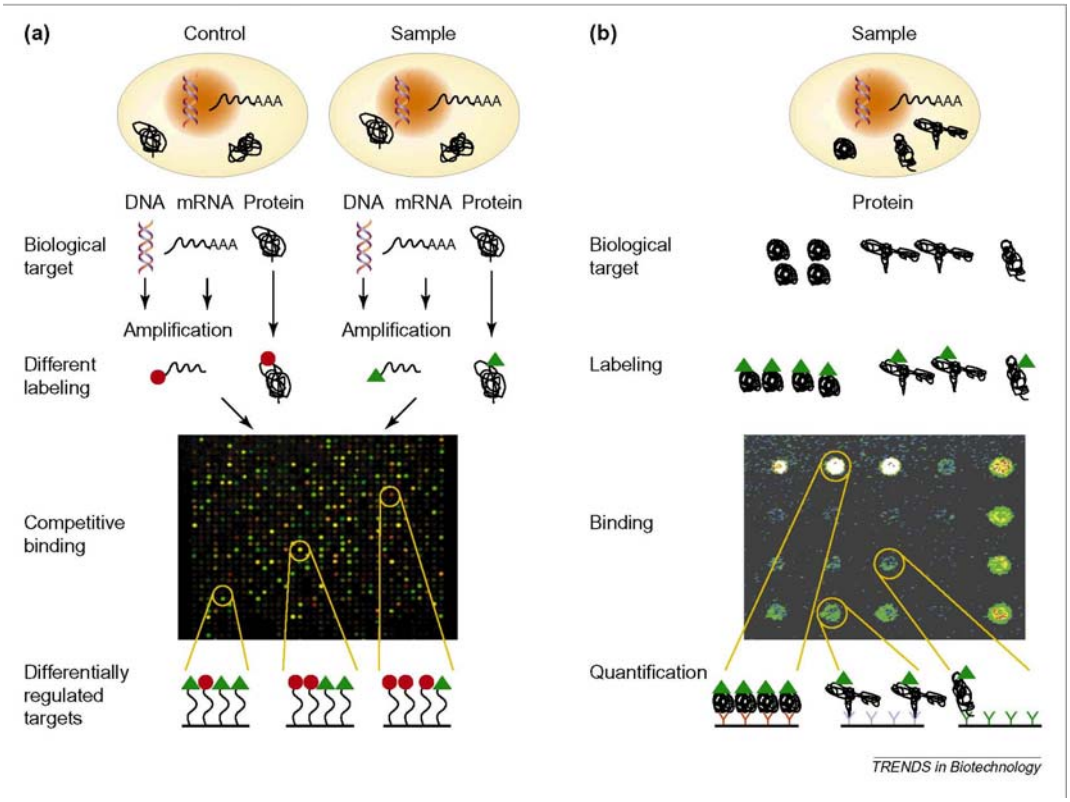


Fig. 7. Comparative and quantitative proteome analysis. (a) *Comparative genome and proteome analysis*. Equivalents of the proteome of phenotypically different sources are isolated, are labelled with different radioisotopes or fluorescent dyes and are cross-matched analyzed. The scans of both fluorescent dyes are matched and the resulting color of each spot is visualized. For an up-regulation a red spot, for a down-regulation a green spot and for an unchanged expression level, a yellow spot (as an intermediate color) is visible in the array picture. (b) *Parallel quantification of proteins.* Different proteins labelled with fluorochromes can be detected in parallel with a protein-microarray. Specific capture antibodies immobilized in an array interact with their respective target proteins present in the solution. The resulting signal intensity correlates with the amount of captured target. Taken from Templin, M.F., et al. [3]

When switching from DNA to proteins, one has to be aware that DNA and proteins are different classes of molecules with different chemical and physical properties. DNA is built out of four nucleotides, generates a uniform molecule with a well-defined structure and a negatively charged, hydrophobic sugar backbone, whereas proteins are made from 20 different amino acids, highly diverse molecules with different abilities. Proteins can be hydrophobic or hydrophilic, acidic or basic and post-translationally modified (acetylation, phosphorylation, glycosylation). They are often assembled as complexes and as a result, a strong signal on a microspot of a protein microarray can result from a large amount of target

or from the capture of a huge complex; therefore, it is very important to keep the captured protein in a well defined, functional state, when immobilized onto a microarray.
Most protein microarrays are made of glass with polymer membranes to immobilize proteins. The immobilization is often done using non-covalent protein surface interaction with hydrophobic (nitrocellulose, polystyrene) or positively charged (polylysine, aminosilane) surfaces. Covalent attachment can be done using a variety of chemically activated surfaces (e.g. epoxy, aldehyde, active esters) or by specific biomolecular interactions (e.g. streptavidin-biotin, His-tag-nickel-chelates). The production of tiny microspots on such surfaces is either performed using contact printing arrayers or non-contact deposition technologies (capillaries or ink jet technology).

2.6.2. 2-dimensional gel electrophoresis

This technique was applied to the plasma proteins soon after the introduction of high resolution two-dimensional gel electrophoresis (2D GE) in 1975 by Klose [60] and O'Farrell [61]. The practical utility of 2D GE for studies of the high abundance plasma proteome has been substantial. Because the first dimension of the procedure (isoelectric focusing) is exquisitely sensitive to molecular charge and the second dimension (SDS electrophoresis) is sensitive to polypeptide length, 2D GE is very effective at revealing genetic variants (about one-third of which differ in net charge from wild type), proteolytic cleavages, and variations in sialic acid content. Several genetic variants have been discovered by 2D GE [62, 63], and a broad survey concluded that heterozygosity estimates obtained by this method were significantly higher than those obtained in cellular protein samples [64], possible reflecting a smaller selection pressure against structural change in the highly soluble plasma proteins. The first 2D GE map of human plasma looked identical to those produced later by many investigators: in contrast to cellular protein patterns, the plasma 2D GE pattern appears basically the same in everyone's hand perhaps due to the very high solubility of the proteins involved and the ease with which the distinctive glycosylation trains of specific proteins can be recognized. Until now, the very high abundance of a few proteins in plasma (albumin, transferrin, immunoglobulins, etc.) and the extreme heterogeneity of plasma glycoproteins and immunoglobulins, limited the identification of 2D GE of unfractionated plasma to 69 proteins (626 spots identified, a majority by immunodetection; Swiss Institute of Bioinformatics, SWISS 2D-PAGE, ExPASy Molecular Biology Server at http://us.expasy.org/ch2d/). By combining 2D GE with additional separation steps (e.g.

recycling depletion of high abundant proteins, classical chromatographic separations, such as size exclusion, ion exchange, and hydrophobic interaction) this limit can be overcome. With these plasma fractions one can obtain more than 250 different proteins with unique accession numbers which can be identified by mass spectrometry (MS) relative to a non-redundant protein database. It is estimated that these 250 different proteins correspond to 1000-1500 distinct post-translationally modified protein spots.

2.6.3. Mass Spectrometry (MS)

MS has solved the problem of identifying proteins resolved by 2D GE and other methods and appears poised to provide general solutions to the analysis of complex protein mixtures as well. The power of mass spectrometry techniques to discover proteins in complex samples relies upon the existence of large protein sequence databases generally derived from DNA sequencing. Whole proteins can be analyzed by an approach termed SELDI-TOF (for surface-enhanced laser desorption ionization time-of-flight) mass spectrometry, a variant of MALDI-TOF (for matrix-assisted laser desorption ionization time-of-flight), in which chemical fractionation based on protein affinity for derivatized MS targets is used to reduce sample complexity to a level at which whole protein MS can resolve a series of fairly reproducible features. A significant disadvantage of this approach is that MS analysis of whole proteins does not directly provide a sequence-based identification (there being many proteins with a very similar mass), and hence the protein peaks discovered as markers have to be identified with peptide mass fingerprinting. Peptide mass fingerprinting is a more general approach, involving digestion of proteins (e.g. with trypsin) into peptides that can be further fragmented (MS/MS) in a mass spectrometer to generate a sequence-based identification and mostly electrospray ionization (ESI) or MALDI are used. Adkins et al. [65] have used two chromatographic separations with MS to identify a total of 490 different proteins in human plasma and the technical development of better instruments and of new analysing methods is steadily pushed by different groups and commercial companies.

2.6.4. Biomarkers for breast cancer

Early detection and therapy of BC is the ultimate goal of all breast cancer researchers (clinicians & biochemists) and could have an important effect on public health. To achieve this goal, specific and sensitive molecular markers are essential. These two points are the real problems of nowadays biomarkers in clinical practice for either early diagnosis, treatment

response, or survival of breast cancer (e.g. CA 15-3[1], CEA[2], cytokeratins[3] etc.) [66]. Therefore most of them have been abandoned due to their poor reliability. However, biomarkers can not only be used in diagnosing cancer in patients but also in therapy monitoring. The first biological biomarkers that have been recommended in routine use are the presence of estrogen and progesterone receptor [67]. Also some attempts to identify relevant oncogenes (e.g. *erb*B2) [68] or tumor-suppressor genes (e.g. mutations in *BRCA1* and *BRCA2*) [69] were successful and are very helpful markers in current clinical practice. From the clinical point of view, there is a critical need for better molecular markers that could help in early diagnosis, as well as for staging of the pathology. Plasma proteins or the discovery of patterns of disease-related protein features from cancer patients might reflect the pathological state of their organs and aid in the early detection of cancer [70].

[1] Cancer antigen 15-3
[2] Carcinoembryonic antigen
[3] i.e. TPA (tumor polypeptide antigen), TPS (serum tissue polypeptide specific antigen) and Cyfra 21.1 (cytokeratin 19 fragment)

3. Materials and methods

3.1. Subjects

Venous blood samples were taken from 433 patients for DNA isolation and from 254 patients for human plasma isolation at the Medical University of Vienna, Department of Obstetrics and Gynecology, Division of Senology. These women provided written informed consent for research (under protocols approved by the institutional review board). All patients were of Caucasian race. Blood was always taken from patients in fasting conditions on the day of surgery before 10 a.m. Of the 433 women, 247 (53.0%) were affected by breast cancer (mean age 58.6 years, range 25.2 – 92.5 years) and 186 (47.0%) had benign breast lesions (mean age 45.0 years, range 14.6 – 87.8 years) (Tab. 2).

	DNA		plasma	
Characteristic	**No. of patients**	**%**	**No. of patients**	**%**
All patients	**433**	**100**	**254**	**100**
Benign lesions[4]	**186**	**43.0**	**109**	**42.9**
Premenopausal	81	43.5	44	40.4
Postmenopausal	34	18.3	14	12.8
Malignant breast cancer[4]	**247**	**57.0**	**145**	**57.1**
Premenopausal	60	24.3	35	24.1
Postmenopausal	161	65.2	96	66.2
Estrogen receptor (ER) status[4]				
Negative	54	21.9	32	22.1
Weakly positive	21	8.5	11	7.6
Moderately positive	62	25.1	38	26.2
Highly positive	96	38.9	57	39.3
Progesterone receptor (PgR) status[4]				
Negative	107	43.3	63	43.4
Weakly positive	17	6.9	12	8.3
Moderately positive	73	29.6	43	29.7
Highly positive	36	14.6	20	13.8
Histologic type				
Ductal carcinoma	191	77.3	103	71.0
Lobular carcinoma	38	15.4	26	17.9
Other carcinoma	18	7.3	16	11.1
Histologic grade[4]				
Grade 1	42	17.0	30	20.7
Grade 2	105	42.5	56	38.6
Grade 3	80	32.4	51	35.2
Tumor size[4] [mm]				
≤ 20	139	56.3	48	33.1
> 20	76	30.8	80	55.2

Tab. 2. Characteristics of all patients.

[4] For some patients, not all information was available.

Of the 254 women of whom blood was taken to isolate plasma, 145 (57.1%) were affected by breast cancer (mean age 59.2 years, range 29.5 – 92.5 years) and 109 (42.9%) had benign breast lesions (mean age 43.7 years, range 15.9 – 87.8 years).
For all parameters (age, menopausal status, and histological parameters) a Microsoft Access database was established. Of course all information was coded thus the results will be totally anonymous. Another important advantage of a database is that it allows to easily group and regroup the patients. Furthermore, it is comfortable to work with an electronic database because of the possibility to index different parameters.

3.2. Plasma isolation from whole blood

The plasma-tubes (Vacuette®, #455071; greiner bio-one; Kremsmuenster, Austria) were centrifuged at 3000 rpm (Hettich; Rotanta/TRC; Tuttlingen, Germany) at 4°C for 10 minutes and afterwards the plasma was transferred to 1.8 ml cryo-tubes (#377267; Nalge Nunc International Corp.; Rochester, USA). These cryo-tubes were shock-frozen in liquid nitrogen and stored at -80°C.

3.3. DNA isolation from whole blood

On the day the blood samples were taken, the EDTA-tubes (Vacuette®, #454217; greiner bio-one; Kremsmuenster, Austria) were frozen at -20°C until the isolation of DNA of whole blood.
In general two different methods for DNA isolation of whole blood were used. One method is to just break up the blood cells and chemically stabilize the DNA until use without purifying the DNA (Extract-N-Amp™ Blood PCR Kit, #XNABE2; Sigma-Aldrich; St.Louis, USA). The other method mostly uses columns (all the other kits except the one from Fermentas) after lysis and chloroform extraction to purify the DNA.
For a quick, cheap and effective isolation at first four different products with two different methods were evaluated (in the end a fifth product had to be used for purified DNA).

The **E.Z.N.A.® Blood DNA Kit II** (Omega Bio-Tek, #D3481-00; Doraville, USA) uses HiBind® centrifuge columns (with silica membrane) and requires 750 µl blood. It is done in 2 ml Eppendorf tubes and the yield is about 30 – 40 µg DNA; a good value. One big problem with this kit is that at the centrifugal steps the blood forms excessive amounts of foam and the centrifuge has to be cleaned after each step.

- Pipette 750 µl blood into a 2 ml Eppendorf tube.
- Add 75 µl OB™ Protease and 750 µl BL-Puffer and vortex for 10 seconds.
- Incubate at 70°C for 10 minutes (vortex occasionally).
- Split the volume (1575 µl) into 2 fractions in 1.5 ml Eppendorf tubes.
- Add 390 µl isopropanol (Sigma-Aldrich, #I9516) into each Eppendorf tube.
- Put the HiBind®-DNA-column into a 2 ml tube.
- Load one third of the total volume onto the column and centrifuge for 1 minute at 8000 g at room temperature.
- Discard the flow through and repeat the loading and centrifuging step two more times.
- Transfer the column into a new 2 ml tube and add 750 µl completed DNA-wash-Puffer (to the concentrated Puffer add the 1.5x volume of absolute Ethanol (Merck KGaA, #1.00983.1011; Darmstadt, Germany)).
- Centrifuge the column at 8000 g for 1 minute and discard the flow through.
- Repeat the washing step once.
- To dry the column completely the column is centrifuged for 2 minutes at max. speed.
- Transfer the column into a clean 1.5 ml Eppendorf tube and pipette 200 ml of the preheated (70°C) elution-Puffer onto the HiBind®-column.
- Incubate at room temperature for 2 minutes.
- Centrifuge for 1 minute at 8000 g.
- Repeat the elution process once again with 200 µl preheated elution-Puffer.

The **Genomic DNA Purification Kit** (Fermentas, #K0519; Lithuania) requires 200 µl blood. It is done in Eppendorf tubes and the yield is about 0.8 – 1.1 µg DNA, which is not sufficient.

- Mix 200 µl of blood with 400 µl of lysis solution in a 1.5 ml Eppendorf tube and incubate at 65°C for 5 minutes.
- Immediately add 600 µl of chloroform (Sigma-Aldrich, #C2432), gently emulsify by inversion (3 – 5 times) and centrifuge at 10000 rpm for 2 minutes.

- Prepare precipitation solution by mixing 720 µl of deionized water (Mayrhofer Pharmazeutika Ges.m.b.H., #15.533; Linz, Austria) with 80 µl of supplied 10x concentrated solution.
- Transfer the upper aqueous phase (containing the DNA) to a new tube and add 800 µl of the freshly prepared precipitation solution.
- Mix gently by several inversions at room temperature for 1 – 2 minutes and centrifuge at 10000 rpm for 2 minutes.
- Remove supernatant completely and dissolve DNA pellet in 100 µl of 1.2 M NaCl solution by gentle vortexing.
- Add 300 µl of prechilled ethanol, let the DNA precipitate for 10 minutes at -20°C and spin down at 10000 rpm for 3 – 4 minutes.
- Pour off the ethanol, wash the pellet once with 70% prechilled ethanol and dissolve DNA in 100 µl deionized water by gentle vortexing.

The **GenElute™ Blood Genomic DNA Kit** (Sigma-Aldrich, #NA2000) requires also 200 µl blood and uses silica-based columns to purify the DNA. The procedure takes a long time and it also foams, although in a much milder form than the kit from Omega Bio-Tek does. The yield is an excellent 2.76 – 6.23 µg. Purified DNA is ready for downstream applications such as restriction endonuclease digestions, PCR, Southern blots and sequencing reactions.

- Place 20 µl of the Proteinase K solution into a 1.5 ml Eppendorf tube.
- Add 200 µl of the blood sample and vortex to ensure thorough mixing of the enzyme.
- Add 200 µl of Lysis Solution C (a chaotropic salt-containing solution), vortex thoroughly (15 seconds) and incubate at 55°C for 10 minutes.
- To prepare the column add 500 µl of the Column Preparation Solution to each pre-assembled GenElute™ Miniprep Binding Column, centrifuge at 12000 g for 1 minute, and discard the flow through.
- Add 200 µl ethanol to the lysate and mix thoroughly by vortexing for 5 – 10 seconds (a homogenous solution is essential).
- Transfer the entire lysate onto the column using a wide bore pipette to reduce shearing of the DNA.
- Centrifuge at more than 6500 g for 1 minute.
- Place the column into a new 2 ml collection tube.

- Add 500 µl of the Prewash Solution (to ensure the removal of all contaminants associated with older blood samples) to the column, centrifuge for 1 minute at more than 6500 g, and discard the flow through.
- Add 500 µl of the Wash Solution to the column and centrifuge for 3 minutes at max. speed to dry the column (before eluting the DNA the column has to be free of ethanol).
- Transfer the column into a new 2 ml collection tube.
- To elute the DNA pipette 200 µl of the Elution Solution directly into the center of the column and incubate for 5 minutes at room temperature.
- Centrifuge at more than 6500 g for 1 minute to elute the DNA.
- Repeat the elution process once with 200 µl of the Elution Solution.

The **Extract-N-Amp™ Blood PCR Kit** is the only kit which does not use any cleaning step. It is done in a 96-well plate and is the quickest method tested (96 samples can be done in about 20 minutes) to extract host genomic DNA from whole blood and amplify targets of interest by PCR. This extraction system eliminates the need for any type of purification, organic extraction, centrifugation, heating, filtration or alcohol precipitation.
The DNA obtained can be used in a PCR but the concentration cannot be determined by photometric measurement. The second disadvantage is that the PCR (4 µl of the neutralized blood extract are used instead of the 2 µl indicated by the manufacture's protocol) has to be done with an expensive PCR ready mix from Sigma-Aldrich, which is especially formulated for amplification directly from the extract. This formulation uses an antibody based Hot Start, for specific amplification.

- 20 µl of the Lysis Solution are placed into each well of a 96-well plate.
- Add 10 µl of blood and thoroughly mix by pipetting.
- Incubate at room temperature for 5 minutes without shaking.
- Add 180 µl of the Neutralization Solution and again mix thoroughly by pipetting.
- The neutralized blood extract should be stored at 4°C (for short time storage), but can also be frozen at -20°C for long time storage (two years were possible).

The **QIAamp® DNA Blood Midi Kit** (QIAGEN, #51185; Venlo, Netherlands) uses 2 ml blood and columns to purify the DNA. The yield is an excellent 2.76 – 6.23 µg.

- Pipet 200 µl QIAGEN Protease into the bottom of a 15 ml centrifuge tube (Corning®, #430791; Corning Incorporated; Corning, USA).
- Add 2 ml blood and mix briefly.
- Add 2.4 ml Buffer AL and thoroughly vortex the solution 3 times (for more than 5 seconds each time).
- Incubate at 70°C in a water bath for about 10 minutes.
- Add 2 ml 100% Ethanol and mix again by vortexing.
- Carefully centrifuge (Hettich Rotanta/TRC) the solution in two steps through the provided QIAamp Midi column (placed in a 15 ml centrifugation tube) at 3000 rpm (1850 x g) for 3 minutes.
- Discard the filtrate after each centrifugation and wash the column once with 2 ml Buffer AW1 (carefully, without moistening the rim) at 4500 rpm (4500 x g) for 1 minute.
- Wash the column once with 2 ml Buffer AW2 (carefully, without moistening the rim) at 4500 rpm for 15 minutes.
- Place the QIAamp Midi column in a 15 ml centrifuge tube (provided).
- Add 300 µl of (room temperature) Buffer AE directly onto the membrane of the QIAamp Midi column to elute the DNA.
- Incubate for 5 minutes at room temperature and centrifuge at 4500 rpm (4500 x g) for 5 minutes.
- To obtain highly concentrated DNA the filtrate is reloaded onto the membrane of the QIAamp Midi column, incubated and filtrate as mentioned above.

3.4. Photometric determination of DNA concentration

- Dilute the DNA in an appropriate manner (e.g. 1:25)
- Measurement of the adsorption in the UV-range (260 nm and 280 nm)
- Calculation: **($A_{260\ nm}$ * 50 * dilution factor) : 1000 = conc. [µg/µl]**
- Quality control: ($A_{260\ nm}$ / $A_{280\ nm}$) should be ≥ 1.5

3.5. DNA fragment length analysis with the ABI sequencer

3.5.1. PCR amplification of the AR $(CAG)_n$ repeat

PCR reactions were carried out using primers that flank the polymorphic $(CAG)_n$ repeat: forward, 5'-TCCAGAATCTGTTCCAGAGCGTGC-3', coupled with the fluorescence marker 6-FAM (PE / Applied Biosystems, Foster City, CA); reverse, 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'. The total volume of the reaction was 20 µl, using 1.0 µM of each primer, 4 µl of the DNA extract and 10 µl of the PCR ReadyMix (Extract-N-Amp™ Blood PCR Kit). Samples were amplified for an initial 5 minutes denaturation cycle at 95°C followed by 35 cycles of amplification (95°C for 30 s, 65°C for 30 s and 72°C for 1 min) and a final extension cycle at 72°C for 25 min. As another speciality the PCR was held at room temperature until loaded onto the ABI Prism™ 3100.

3.5.2. Analysis of PCR products by agarose gel electrophoresis

Successful amplification was verified by agarose gel electrophoresis using a NuSieve 3:1 agarose mixture (BioWhittaker Molecular Applications, #50094; Rockland, ME) and ethidium bromide (Sigma-Aldrich; #E1510) staining.

3.5.3. AR CAG repeat length analysis

The number of CAG repeats was determined by an analysis of the fragment length with an automated ABI Prism™ 3100 genetic analyzer (Applied Biosystems, #3100-01; Foster City, USA). To a mix of 12 µl Hi-Di™ Formamide (Applied Biosystems, #4311320) and 0.3 µl GeneScan®-400HD [ROX]™ (internal length standard; Applied Biosystems, #402985) in a 96-well plate 1 µl of the PCR product was added. After 2 minutes of a denaturation cycle at 92°C the plate was cooled on ice at least for 5 minutes. For device specific introductions refer to the GeneScan® Reference Guide (Applied Biosystems). The filter-set D, a 50 cm long capillary and performance optimized polymer 6 (POP-6) were used. Results were analyzed with GeneScan® Analysis software (version 3.1.2). Each peak in a sample file corresponds to PCR products of a given length. Fragments up to 400 nucleotides that differ by a single nucleotide can be easily resolved by this method. For each peak, the height of the peak (peak intensity) and size of the corresponding fragment were determined by the GeneScan® software.

3.5.4. Sequencing of a few AR CAG repeats to determine the exact repeat number

To correlate the fragment length with the number of CAG repeats, five samples with different length were sequenced on the ABI Prism™ 3100. The PCR was done as described above, but with a non-fluorescence-labelled forward primer. To clean the products, they were precipitated with an Ethanol/$MgCl_2$-method. The sequence reactions were performed with the non-fluorescence marked primers and a premix of the BigDye® Terminator Kit (Applied Biosystems, #4303152) under the following conditions: initial denaturation at 94°C for 1 minute, followed by 30 cycles of amplification (96°C for 10 s, 55°C for 2 s and 60°C for 4 min). Afterwards the sequence products were again cleaned up, this time with the QIAquick PCR Purification Kit (QIAGEN, #28106). The pellets were dissolved in 10 µl Hi-Di™ Formamide and directly loaded onto the sequencer.

By manually controlling the sequencing results it was possible to analyse the number of the CAG repeats of the short alleles. Until the end of the short allele the sequences of the long and the short allele were identical. For an optimal "ladder" samples with different CAG repeats were sequenced. From these results it was possible to interpolate the numbers of the long repeats and for all non-sequenced PCR products.

3.6. Custom TaqMan® SNP Genotyping Assay

To design the assay, the amino acid sequence of the CYP19 (aromatase) codon 39 (paper: Miyoshi Y et al., 2000, Int.J.Cancer, 89: 325-328) was controlled in the Single Nucleotide Polymorphism Database of the NCBI (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2236722) and the database of Japanese Single Nucleotide Polymorphisms of the University of Tokyo and Japan Science and Technology Agency (JST; http://snp.ims.u-tokyo.ac.jp/cgi-bin/SnpInfo.cgi?SNP_ID=IMS-JST061460). An order file is automatically created by loading the sequence to the Assays-by-Design™ File Builder (version 2.0; Applied Biosystems) which can be electronically submitted.

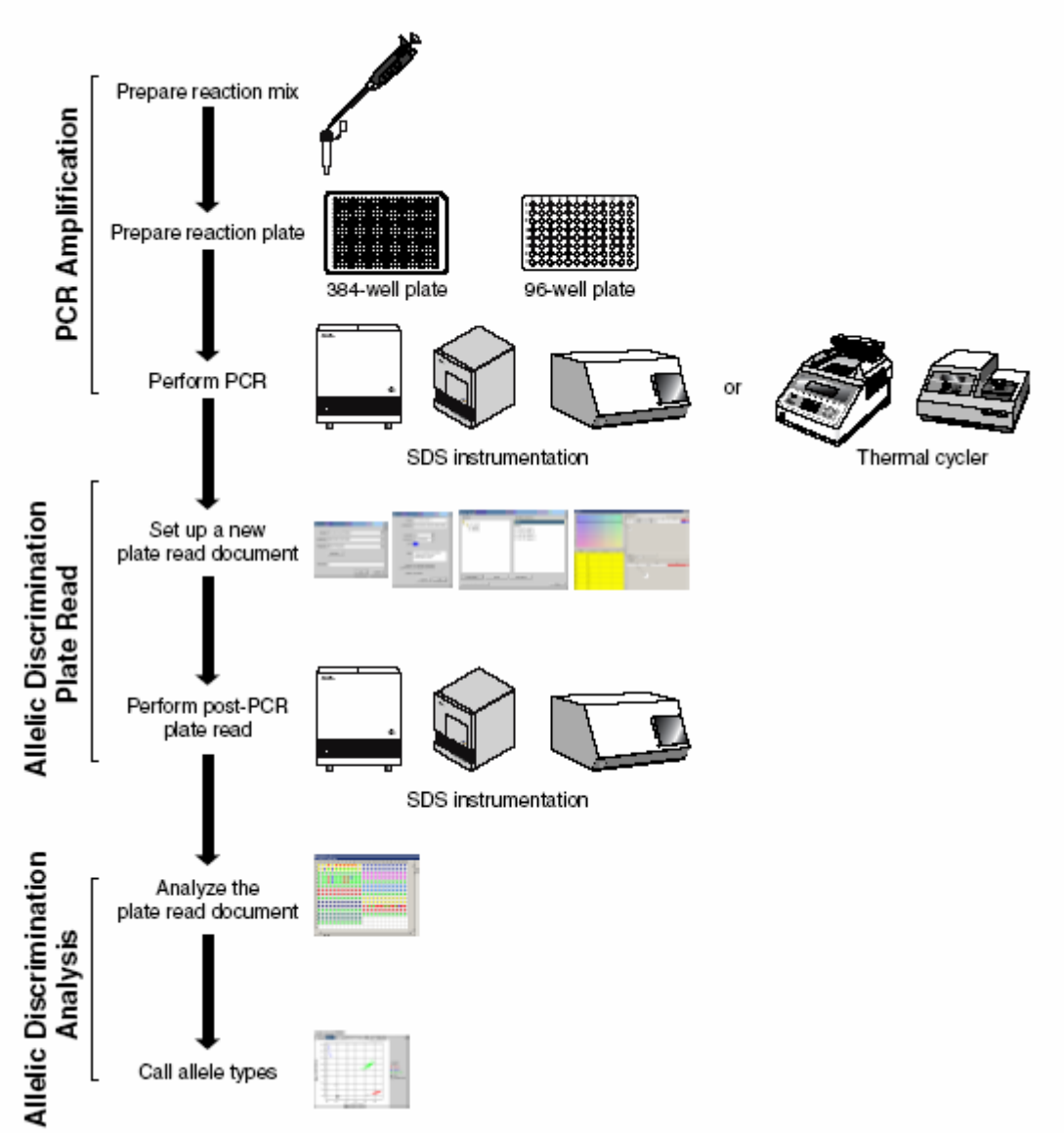


Fig. 8. Procedure Flowchart (SNP Genotyping Products Protocol)

The 40x Assay Mix (Applied Biosystems, #4332072) contains two PCR primers (36 µM each; CYP19_CD39_TNCF: 5'-CTGCTCCTCACTGGCCTTT and CYP19_CD39_TNCR: 5'-CAGGACCTGGTATTGAGGATGTG) and two fluorescently labelled TaqMan® MGB (minor grove binding) probes (8 µM each; CYP19_CD39_TNCV2: 5'-CCTCATAATTCC**A**CACCAA, VIC® dye, NFQ (non-fluorescent quencher) and CYP19_CD39_TNCM2: 5'-CCTCATAATTCC**G**CACCAA, FAM™ dye, NFQ). The reactions were done in 96-well Optical Reaction Plates (Applied Biosystems, #4306737) and the total reaction volume was modified to 20 µl. According to the manufacture's protocol 20 ng of genomic DNA were diluted in dH_2O to a volume of 9.5 µl, then 0.5 µl of the 40x Assay mix and 10.0 µl of the TaqMan® Universal PCR Master Mix (No AmpErase® UNG (2x); Applied Biosystems, #4324018) were added. The plate was sealed with an Optical Adhesive

Cover (Applied Biosystem, #4311971) and both the PCR reaction and the allelic discrimination were done on an ABI PRISM™ 7000 Sequence Detection System (SDS; Applied Biosystems, #4339940). The results were evaluated with the ABI PRISM™ 7000 SDS Software (version 1.1; Applied Biosystems). The thermal cycler conditions were an initial holding step at 95°C for 10 minutes and 40 cycles of a denature step at 92°C for 15 seconds and an annealing / extending step at 60°C for one minute. Afterwards the allelic discrimination was done in just one step: the plate was kept at 60°C for one minute and measured at the same time (Fig. 8).

3.7. Proteomics - 2D gel electrophoresis analysis

Protein Assay Dye (Bradford) Reagent Concentrate (5X) (Bio-Rad Labratories, #500-0006; Hercules, USA)

The Bradford Reagent can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present.

3.7.1. Bradford determination of plasma protein concentration

- 1:100 dilution of the concentrated Bradford reagent with PBS (Phosphate-Buffered Saline (PBS) 7.4 (1X), liquid; Invitrogen Corporation, #10010-015; Carlsbad, USA)
- addition of 50 µl plasma to 1 ml of diluted Bradford solution
- Measurement of the adsorption at 595 nm with the BioPhotometer (Eppendorf AG; #6131000.012; Hamburg, Germany)
- Calculation: **($A_{595\ nm}$ / slope of a calibration curve) * dilution factor = conc. [µg/µl]**

3.7.2. Depletion of Albumin and IgG

Two POROS® Affinity Depletion cartridges (Applied Biosystems; Protein G (0.2 ml), #4337331; Anti-Human Serum Albumin (0.2 ml), #4337344) were directly coupled onto a BioCAD 700E Perfusion Chromatography™ (Applied Biosystems). Because of the loading capacity of each cartridge, which is 200 µg, the plasma sample had to be diluted. The total

loading volume of the diluted plasma sample in PBS was 100 µl. The wavelength of the UV detector was set to 280 nm and 260 nm and the UV detector lamp was turned on. After a special equilibration procedure with different salt concentrations (hydrochloric acid; Sigma-Aldrich, #30720; sodium chloride; Sigma-Aldrich, #71378; in PBS) 30 µl of the sample was injected into the block and the UV detector was turned off. Both the washing and the elution fractions (three times 1 ml each) were collected. To store the cartridges and the block for further analysis another equilibration step was necessary and with the UV detector the progress of the cleaning could be followed.

12% SDS gel (10 ml)

3.3 ml dH_2O
4.0 ml 30% Acrylamide solution (Sigma-Aldrich, #A3553)
2.5 ml 1.5 M Trizma® buffer (Tris; pH 8.8) (Sigma-Aldrich, #93392)
0.1 ml 10% Sodium dodecyl sulfate solution (SDS; Sigma-Aldrich, #71736)
0.1 ml 10% Ammonium persulfate (APS; Sigma-Aldrich, #09920)
4 µl N,N,N',N'-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, #T9281)

4% stock gel

2.95 ml	dH_2O
0.25 ml	30% Acrylamide / Bisacrylamide solution (Sigma-Aldrich, #A3449)
1.25 ml	0.5 M Tris (pH 6.8) (Sigma-Aldrich, #42861)
0.05 ml	10% SDS
0.05 ml	10% APS
5 µl	TEMED

1x loading buffer

25 mM	Tris-Base (Sigma-Aldrich, #T1503)
192 mM	Glycine (Sigma-Aldrich, #G8898)
0.1%	SDS (pH 8.3) (Sigma-Aldrich, #93389)

Rehydration buffer

8M	Urea (Sigma-Aldrich, #U6504)
2%	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma-Aldrich, #C9426)
18 mM	(±)-*threo*-1,4-Dimercapto-2,3-butanediol **(**DTT; Sigma-Aldrich, #D9163)
0.5%	IPG buffer (pH 3 – 10 NL) (Amersham Biosciences, #17-6000-88; Little Chalfont, U.K.)
0.01%	Bromphenol blue (Sigma-Aldrich, #B0126)

3.7.3. Protein precipitation with TCA / DOC (Bensadoun & Weinstein, [71])

- 1 ml sample + 8.3 µl (2%) 7-Deoxycholic acid sodium salt (DOC; Sigma-Aldrich, #D6750)
- vortex
- incubate at room temperature for 15 minutes
- add 330 µl of a 25% Trichloroacetic acid (TCA; Sigma-Aldrich, #T8657) solution
- vortex
- centrifuge at 3000 rpm (Eppendorf AG; Centrifuge 5415 R (refrigerated); #5426000.018) at 4°C for 30 minutes
- carefully discard the supernatant with big pipette tip
- wash once with pre-chilled (-20°C) Acetone (Sigma-Aldrich, #00585) to remove excess of TCA
- dissolve in 5 µl loading buffer + 15 µl rehydration buffer

3.7.4. SDS PAGE

Load 20 µl onto the gel and run it at 80 V for 20 minutes and at 180 V for about an hour.

Farmer's reducer (has to be freshly prepared!!!)

30 mM	$K_3Fe(CN)_6$ (Sigma-Aldrich, #12639)
30 mM	$Na_2S_2O_3$ (Sigma-Aldrich, #13481)

Fixing solution

10% acetic acid (Merck KGaA, #1.00058)
40% EtOH

3.7.5. Silver staining of protein gels (under reducing conditions)

- Shake the gel in fixing solution for 15 minutes
- Transfer gel into Farmer's reducer and shake it for 2 minutes
- Wash several times with dH_2O until the yellow color completely disappears (takes about 30 minutes)
- Transfer gel into a freshly prepared 0.1% silver nitrate solution (0.1 g / 100 ml dH_2O; Sigma-Aldrich, #85229) and shake for at least 15 minutes
- Wash with dH_2O for 30 seconds
- Transfer gel into a 2.5% Na_2CO_3 (Sigma-Aldrich, #71351) solution and shake for 30 seconds
- Develop silver stain by placing the gel into a freshly prepared 0.1% formaldehyde (Sigma-Aldrich, #F1635) – 2.5% Na_2CO_3 solution and shake it under monitoring: stop the staining when the background is getting too strong
- Add an excess of 10% acetic acid in order to stop the staining reaction and shake it for 10 minutes
- Wash several times with dH_2O
- Dry the gel between two transparent folia in a frame

3.7.6. Non-linear isoelectronic focusing

Immobiline DryStrip 18 cm pH 3-10 NL (Amersham Biosciences, #17-1235-01)

- Pool all wash and all elution fractions (45 µl each) and add 315 µl rehydration buffer and 1.25 µl IPG buffer (pH 3-10 NL; Amersham Biosciences, #17-6000-88)
- Centrifuge at 13000 rpm for 1 minute (Eppendorf Centrifuge 5415 R)
- Pipette the solution into a clean 18 cm Strip Holder (Amersham Biosciences, # 80-6417-44) and avoid air bubbles!
- Add a top layer of paraffin (Sigma-Aldrich, #76235) so that the gel will not dry

Amersham IPG phor (Amersham Biosciences)

Running conditions are: 20°C and 50 µA / strip

(1)	30 V	10 hours, linear
(2)	500 V	2 hours, linear
(3)	1 kV	1 hour, linear
(4)	1 – 8 kV	2 hours, gradient
(5)	8 kV	9 hours, linear
(6)	300 V	∞

3.7.7. 9% – 16% SDS gradient gels

Gels (dimensions 182 × 190 × 1.0 mm) are poured from the bottom with the help of a gradient former.

		9%	16%
Resolving gel:	Acrylamide / PDA[†] 30.8%	45 ml	87 ml
Leading buffer:	1.5 M Tris HCl (pH 8.8) [††]	38 ml	38 ml
	dH_2O	66.25 ml	24.25 ml
Additive:	5% Sodium thiosulfate[‡]	0.75 ml	0.75 ml
	Degas		
Polymerization agents:	10% APS	0.725 ml	0.725 ml
	TEMED	0.0725 ml	0.0725 ml

[†] Piperazine diacrylyl (PDA, 1,4-Bis(acryloyl)piperazine; Sigma-Aldrich, #D1538)

[††] (Sigma-Aldrich, #T2819)

[‡] Sodium thiosulfate in dH_2O solution (Sigma-Aldrich, #13481)

Equilibration buffer

50 mM	Tris HCl (pH 6.8) (Sigma-Aldrich, #T1819)
6 M	Urea
30%	Gycerol (Sigma-Aldrich, #G8773)
2%	SDS

Running the second dimension (gradient SDS gel)

- clean Immobiline DryStrip from paraffin with dH_2O
- Put the Immobiline DryStrip together with 3 ml equilibration buffer and 30 mg DTT onto an orbital shaker for 15 minutes
- Incubate the Immobiline DryStrip in 3 ml equilibration buffer and 75 mg iodoacetamide (IAA; Sigma-Aldrich, #I6125) at the orbital shaker for 15 minutes
- Put the Immobiline DryStrip onto the SDS gel
- Seal the gel with a running buffer – Comassieblue (Sigma-Aldrich, #27815) solution
- Running conditions: 20 mA / gel for about 15 minutes
  40 mA / gel for at least 6 hours

*3.7.8. **Ammonium silver staining** (very sensitive, non-MS compatible)*

This procedure is calculated for 2 gels. All steps are performed on an orbital shaker (about 50 rpm; neo-Lab, #7-0030; Heidelberg, Germany) under continuous shaking.

- Transfer gels into two Pyrex glass dishes (Merck KGaA, #25-380-07)
- Wash with cold dH_2O for 5 minutes
- Soak water and fill in 500 ml glutaraldehyde solution for 30 minutes

1% glutaraldehyde (Sigma-Aldrich, #34,085-5)	10 ml
0.5 M sodium acetate trihydrate (Sigma-Aldrich, #71193)	32.02 g
fill up with cold dH_2O to	500 ml

- Soak and wash two times with cold dH_2O for 15 minutes
- Treat the gels in a cold 0.05% (v/w) NDS (Naphthalendisulfonic acid disodium salt; Aldon Corporation, #EKN05257; Avon, USA) – dH_2O solution for 15 minutes, make a fresh NDS solution and treat the gels for a further 15 minutes
- Wash 6 times in dH_2O for 5 minutes
- Stain the gels for 30 minutes in a silver nitrate solution
  (1) Dissolve 3 g silver nitrate in dH_2O to a final volume of 15 ml
  (2) In a 500 ml cylinder under agitation:

80 ml	dH_2O
5 ml	25% NH_3 solution (Sigma-Aldrich, #09860)
0.75 ml	10 N NaOH (Sigma-Aldrich, #71692)

  (3) Gently pour the concentrated silver solution into the cylinder under constant stirring. A transient brown precipitate forms and after it is cleared …
  (4) … fill up with dH_2O to a final volume of 375 ml

- Wash four times with dH_2O for 4 minutes

  Decrease the speed of the orbital shaker and add a large volume of water to remove the maximum free silver. Do not stop the shaker during sucking, as the gels may stick to the glass.
- Take new glass dishes and fill them with the developing solution

  Develop the gels until the ampholine front becomes visible.

  Developing solution: 0.05g citric acid (Sigma-Aldrich, #C0759)
  1 ml formaldehyde
  fill up with dH_2O to 1000 ml
- Stop developing by transferring gels into the blocking solution:

  25 g Tris-Base
  25 ml acetic acid
  fill up with dH_2O to 500 ml
- Avoid contact between developing and blocking solution!
- Seal gels in plastic foil when finished and scan them

3.8. High throughput comparative analysis of known serum / plasma biomarkers

3.8.1. Materials

- S&S® Serum Biomarker Chip (Schleicher & Schuell, #10486077; Dassel, Germany)
- Chip Clip™ Slide Holder (Schleicher & Schuell, #10486081)
- ULS® Protein Labeling System (Schleicher & Schuell, #10486085)
- HPLC grade H_2O (Sigma-Aldrich, #95304)
- PBS (pH 7.4)
- ScanArray 4000 Scanner (Version 3; Packard BioChip Technologies / Perkin Elmer; Billerica, USA)

3.8.2. Protein Labelling

- Prepare four solutions in Eppendorf tubes labelled as reactions A, B, C, and D using the following solutions:
  - **A:** 10 µl 10x Protein Labeling Buffer + 5µl Biotin-ULS + 81 µl of HPLC grade water + 4 µl of plasma A
  - **B:** 10 µl 10x Protein Labeling Buffer + 5µl Fluorescein-ULS + 81 µl of HPLC grade water + 4 µl of plasma A
  - **C:** 10 µl 10x Protein Labeling Buffer + 5µl Biotin-ULS + 81 µl of HPLC grade water + 4 µl of plasma B
  - **D:** 10 µl 10x Protein Labeling Buffer + 5µl Fluorescein-ULS + 81 µl of HPLC grade water + 4 µl of plasma B
- Incubate for about 6 hours at 42°C with 300 rpm
- Carefully place the dual-well incubation chamber and the two-pad slide into the Chip Clip™ Slide Holder
- Add 500 µl of Protein Array Blocking Buffer to each pad
- Put the chamber with a wet towel into a sealable plastic bag and lock it
- Agitate the slide for 30 minutes on an orbital shaker
- After the labelling reaction is complete, add 5 µl of 10x KREA*stop* to each reaction tube (A, B, C, and D) and incubate for 5 minutes
- Vigorously invert the supplied spin columns (these are needed to remove free ULS-reagent from all four reactions) several times to remove any bubbles and to resuspend the gel matrix
- Remove the tip and the top of the column to allow the packing buffer to flow out until it reaches the top of the gel bed
- Place the spin column into a clean 2 ml Eppendorf tube and spin it at 1000 g for 2 minutes to remove any residual packing buffer and to pack the gel bed
- Carefully place the 100 µl ULS reaction mix onto the gel bed
- Place the column into a tube and centrifuge at 1000 g for 4 minutes
- Transfer and combine 90 µl of each eluate containing the labelled plasma proteins from “Reaction A” and “Reaction D” into a clean tube (labelled “Mix 2”) and add 120 µl of 1x Protein Array Wash Buffer (final volume: 300 µl) and mix well
- Transfer and combine 90 µl of each eluate containing the labelled plasma proteins from “Reaction B” and “Reaction C” into a clean tube (labelled “Mix 1”) and add 120 µl of 1x Protein Array Wash Buffer (final volume: 300 µl) and mix well

*3.8.3. **Processing the Chip** (all steps at room temperature)*

- Remove blocking buffer by flicking the Chip Clip™ Slide Holder over a sink
- Add the entire 300 µl of "Mix 2" to the pad which is closer to the barcode and the entire 300 µl of "Mix 1" to the other pad of the slide
- Put the chamber with a wet towel into a sealable plastic bag, lock it and incubate slide overnight with gentle agitation
- Remove chamber out of the plastic bag after incubation (be careful: the pads shall not be dry and look white!) and wash each pad 6x with 1 ml of 1x Protein Array Wash Buffer for about 1 minute
- In a clean Eppendorf tube prepare "two-color fluorescent detection solution" and mix well:

  60 µl streptavidin-DY647 conjugate solution + 150 µl anti-Fluorescein Antibody-DY547 conjugate solution + 390 µl 1x Protein Array Wash Buffer
- Add 300 µl of the "two-color fluorescent detection solution" to each pad
- Put the chamber with a wet towel into a sealable plastic bag, lock it, protect from light and incubate for 1 hour with agitation
- Remove chamber out of the plastic bag and 6x wash each pad with 1 ml of 1x Protein Array Wash Buffer for 1 minute
- Remove Protein Wash Array Buffer by flicking the Chip Clip™ Slide Holder over a sink
- Remove the slide from the Chip Clip™ Slide Holder and air dry it until the membrane appears white
- Store FAST® slide in a dark, dust-free environment until scanned (slides should be scanned within 24 hours after drying)

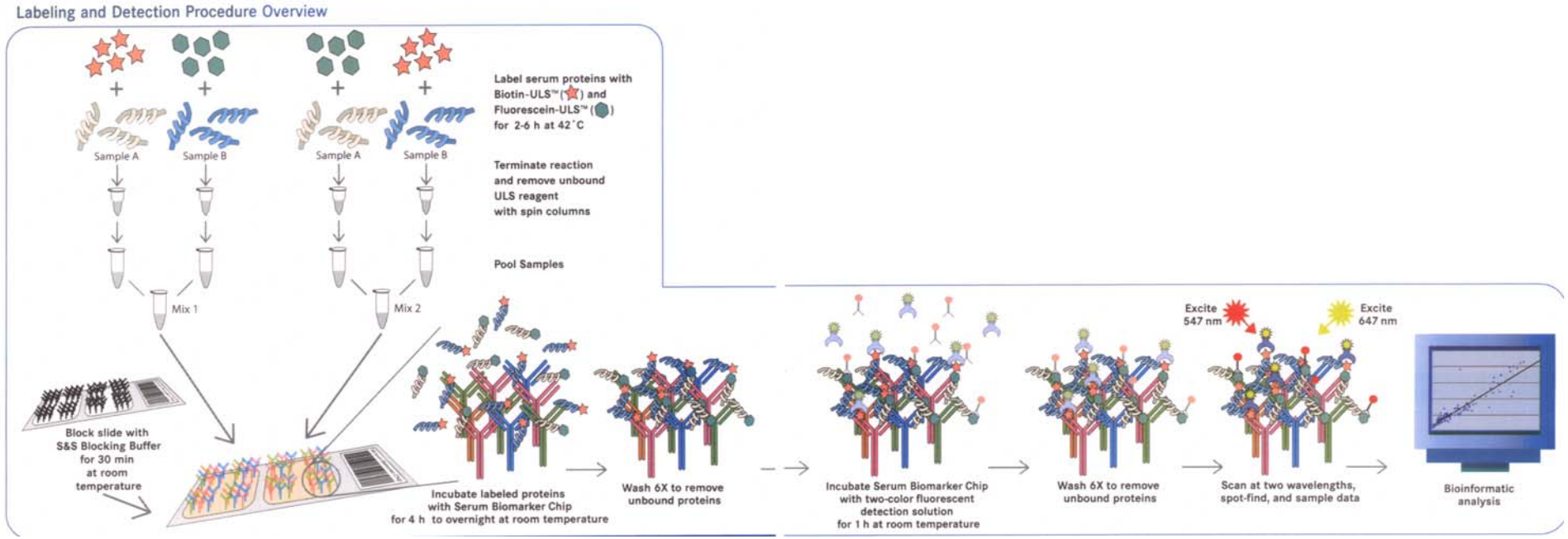


Fig. 9. Labelling and detection procedure overview of the S&S® Serum Biomarker Chip. High throughput comparative analysis of known serum biomarkers. For a more detailed description of the protocol see text.

3.8.4. Scanning FAST® Slides

Additional equipment used was ScanArray Express (Perkin Elmer; Boston, USA; 5 wavelength, 5 µm resolution) and GenePix Pro software (Version 4.1.1.40; Axon Instruments / Molecular Devices Corporation; Union City, USA).

Fig. 10. ScanArray 4000 and ScanArray Express at the "Chip Facility" of the Medical University of Vienna.

- After turning on the computer and the scanner the appropriate lasers are selected (the warm up time is about 20 minutes).
- Press "eject slide" and insert the slide with the non-barcode side first until only the barcode is visible and one can feel a slight resistance.
- At the acquire image site the position of the slide is set to the total possible area, the fluorophore selection is done, the settings for laser power and the PMT gain can be adjusted and a quick scan (preview) at 30 µm can be done.
- Settings used: Cy5 at 632 nm for Streptavidin-DY647 (red signal)
  Power = 90%; PMT = 40%
  Cy3 at 532 nm for anti-fluorescein antibody-DY547 (green signal)
  Power = 90%; PMT = 60%
- After quick scan it was clear that the scanner did an auto-focus of the focal length to adjust for the thickness of the nitrocellulose polymer and also the PMT can be analyzed in GenePix and if necessary (if the intensity distribution does not look the same) adjusted for the scan of interest.

- For the scan of interest the resolution was set to 10 µm; all the other parameters remained unchanged.
- To start scanning the acquire button is pressed.
- After the scan the pictures are saved as 16 bit TIFF-images.

3.8.5. Analysis of the Results

To further analyze the pictures the software ArrayVision Eval. 8.0 (Rev. 4; Imaging Research Incorporation; St. Catharines, Canada) was used. After loading a special protocol provided by Schleicher & Schuell for the Serum Biomarker Chip the pictures (Cy5 as control on top and Cy3 as data on bottom) were loaded into the software. Afterwards many parameters can be changed. The template (grid, layout) organisation is defined by levels, rows, columns and spot size and spot shape. There are many different forms of background subtraction and data normalization and the range of the spots can be varied. The most important thing at the ArrayVision software is the spot alignment which always has to be done individually because each scanner has different scan areas. Therefore the spot-to-spot alignment has to be done manually and exact. After these steps the data can be analysed and automatically transferred into an Excel file for further analysis.

Schleicher & Schuell provided an Excel macro which statistically transforms the raw data exported from ArrayVision into much more descriptive and meaningful tables and builds some graphs automatically. In the summary table one can find the most important data sets in a compact form and the most important figure is the ‘sorted log2 trimmed chart’ where the log2 ratio of both fluorescent dyes of one spot are sorted.

3.9. Statistical analysis

Statistical analysis was performed using SPSS Version 10.0 and STATA 8. Apart from descriptive statistics univariate analysis was performed using T-Tests and χ^2-Tests as appropriate. To calculate the odds ratios (ORs) a multiple logistic regression was performed adjusting the OR for age of the patients or otherwise indicated. For linear regression the Pearson correlation was used. To compare statistical models the method of generalized linear models with Newton-Raphson optimization was done. All p-values are 2-sided or otherwise indicated. A p-value <0.05 was considered to be statistically significant.

4. Results and discussion

4.1. Development of a Database

To perform the desired statistical analysis after completion of the molecular work, and to correlate the experimental findings with clinical and histological parameters, a database with all required data was developed. First, the personal data of the patients had to be coded (according to the Austrian guidelines for data security) and a cheap (already available) and widely accessible system was desired. Another important point was that it had to be easily possible to perform inquiries and to rearrange the data. Accordingly, the database was developed in Microsoft Access (Microsoft Corporation, Redmont, USA).

All data of each patient ever admitted to the "AKH Wien" are stored in a large, hospital-wide database termed KIS ("Krankenhaus-Informations-System"; hospital information system). Only authorized personnel is allowed to query data. Because KIS is an old system it was not possible to simply 'copy – paste' the required data for our Access-database and therefore an additional query of the patient data had to be done. The histological results and "Onkokonsilium" reports (a summary of all test results of a patient with malignant breast cancer) had to be searched on the KIS-PC and the relevant data was printed and then entered into our database. The problem was that there are only a few KIS-PCs, which are heavily used by physicians and nurses for their routine work. Therefore the waiting period to access a KIS-PC often was a week or even longer. Another difficulty was that each patient result had to be examined and released by a physician before it became accessible for others and this can last about one month from the examination.

As shown in Fig. 11, our database includes the age, the menopausal status (premenopausal, unknown and postmenopausal), and histological data (estrogen receptor status, progesterone receptor status, grading, tumor size, lymph node status, p53 status, HER2 status). The most important parameter was the division into patients with malignant breast cancer (group 2) and into patients with benign lesions (group 1).

number	age	group	menopausal status	ER status	PgR status	grading	pT	pN	tumor size {cm}
35	66,12	2	post	3	2	1	1a	0	0,3
36	40,52	1	pre						
37	54,29	2		0	0	3	2	0	3
38	32,5	2	pre	0	1				
39	49,31	2		2	0	2	4b	1biii	14
40	44,38	2	pre	2	2	2	1c		1,5

number	p53	HER2	MIB-1 {%}	disease
35	0	3		invasiv, ductal, breast carcinoma
36				intracanalicular fibroadenoma
37	2	0	70	invasiv, low differentiated, ductal, breast carcínoma
38	2	0	10	intraductal, high differentiated, breast carcinoma
39	2	1	30	expanded, invasiv, middle differentiated, lobular, breast carcinoma
40	0	3	10	intraductal, high differentiated, breast carcinoma with micro chalk

Fig. 11. Layout of the database. Data for 6 patients (coded ID number 35-40) are shown. The abbreviations used are: estrogen receptor (ER), progesterone receptor (PgR), pT (primary tumor) and pN (regional lymph nodes) of the TNM clinical classification, MIB-1 (Ki67).

Whole blood to isolate genomic DNA was collected from 1st May 2002 until 22nd December 2004, resulting in 433 samples. Beginning on 23rd January 2003 tubes for the isolation of human plasma were also collected, and by 22nd December 2004, 254 patients could be included in our collective. All participating women provided written informed consent for research.

4.2. DNA isolation

First, the different DNA isolation methods were tested to find a cheap, quick, and easy to handle way to isolate DNA from whole blood (Tab. 3).

It is not possible to quantify or to control the DNA obtained with the Extract-N-Amp™ Blood PCR Kit (Sigma), because there is no separation of the DNA from the other blood components. Therefore, the solution is deeply red-colored and a photometric measurement of the concentration is not possible. This does not represent a problem as long as the purity and the clearness (e.g. for measurement with fluorescent dyes) of the solution are not important for further analysis.

Because the DNA solution has to be totally clear when using with TaqMan® SNP genotyping assays, all DNAs had to be isolated using the QIAamp® DNA Blood Midi Kit (QIAGEN). When using 2 ml whole blood, the average yield was about 40 µg (range, 7.3 µg – 121.3 µg), as determined by photometric measurement, and because of the column separation steps the

quality of the received DNA was very good (the average ratio A_{260}/A_{280} was about 1.6; Tab. 3).

	E.Z.N.A.® Blood DNA Kit II (Omega Bio-Tek)	Genomic DNA Purification Kit (Fermentas)	GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich)	Extract-N-Amp™ Blood PCR Kit (Sigma-Aldrich)	QIAamp® DNA Blood Midi Kit (QIAGEN)
Starting blood volume	750 µl	200 µl	200 µl	10 µl	2000 µl
hands-on time required	60 min for 10 samples	75 min for 10 samples	80 min for 10 samples	15 min for 96 samples	120 min for 10 samples
Tube / plate	2 ml tube	1.5 ml tube	1.5 ml tube	96-well plate	15 ml tube
Yield	30 – 40 µg	0.8 – 1.1 µg	2.7 – 6.2 µg	not measurable	7.3 – 121.3 µg
Purity (A_{260}/A_{280})	1.5	1.5	1.5	not measurable	1.6
Advantages	➢ yield ➢ purity	➢ purity	➢ yield ➢ purity	➢ duration ➢ price	➢ purity ➢ yield
Disadvantages	➢ excessive foaming	➢ yield ➢ time required	➢ foams ➢ time required	➢ purity ➢ narrowed applicability of the DNA	➢ time required ➢ price

Tab. 3. Comparison of the DNA isolation methods.

The biggest problem of the E.Z.N.A.® Blood DNA Kit II was that during the centrifugation steps it excessively foamed. The consequence was that the centrifuge had to be cleaned after each centrifugal step and therefore this method was rejected. The foaming during centrifugal steps was the only problem why the GenElute™ Blood Genomic DNA Kit was not suitable. The obtained yield and purity was very good, and the time required was similar to the QIAGEN kit. The handling of the Fermentas Kit was quite well but the obtained yield was not sufficient for the following analysis. Therefore the Extract-N-Amp™ Blood PCR Kit was chosen for analysis where the purity of the obtained DNA and the clearness of the DNA solution were not important; otherwise the very pure DNA from the QIAGEN kit was used.

4.3. Pathway analysis

Information about protein function and cellular pathways is central to the system-level understanding of living organism. This knowledge is scattered throughout numerous scientific publications. The need to bring the relevant information together calls for software systems to organize and study pathway data. The pathway analysis was performed with the software PathwayAssist (v2.53, Ariadne Genomics Inc.; Rockville, USA), a recently developed bioinformatic program that automatically searches the biological literature to predict pathways of interacting genes. PathwayAssist comes with a database of molecular networks

automatically assembled from scientific abstracts that are retrieved from the PubMed. The program enables researchers to create their own pathways and produce publication-quality pathway diagrams (for some publication examples refer to http://www.ariadnegenomics.com/company/publications.html).

The PathwayAssist analysis was intended both to obtain information about breast cancer, and to illustrate and explore the increasing potential of bioinformatic approaches to complement linkage analysis. It was started from two different nodes (nodes can be e.g. proteins, complexes, or small molecules). The starting nodes used were aromatase (CYP19A1) and the androgen receptor (AR), and I searched for (breast cancer relevant) protein or small molecule connections directly between these two key breast cancer regulators. This analysis revealed some interesting proteins interacting both with aromatase and the androgen receptor, above all, cyclooxygenase-2 (COX-2; Fig. 12). Based on these findings, aromatase, the androgen receptor, and COX-2 were selected for single nucleotide polymorphism (SNP) analysis.

4.4. Selection of breast cancer related SNPs

Next, the SNP database from the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) was searched for SNPs which are located in the promoter region or which influence the transcriptional activity of the interesting protein identified in the pathway analysis. Special attention was given to those polymorphisms with a high degree of heterozygosity in Caucasians. This will simplify the subsequent analysis (significant p-values can be obtained with fewer study participants). After selection of the SNPs, the literature was examined to find already published connections between breast or other types of cancer and the chosen polymorphisms.

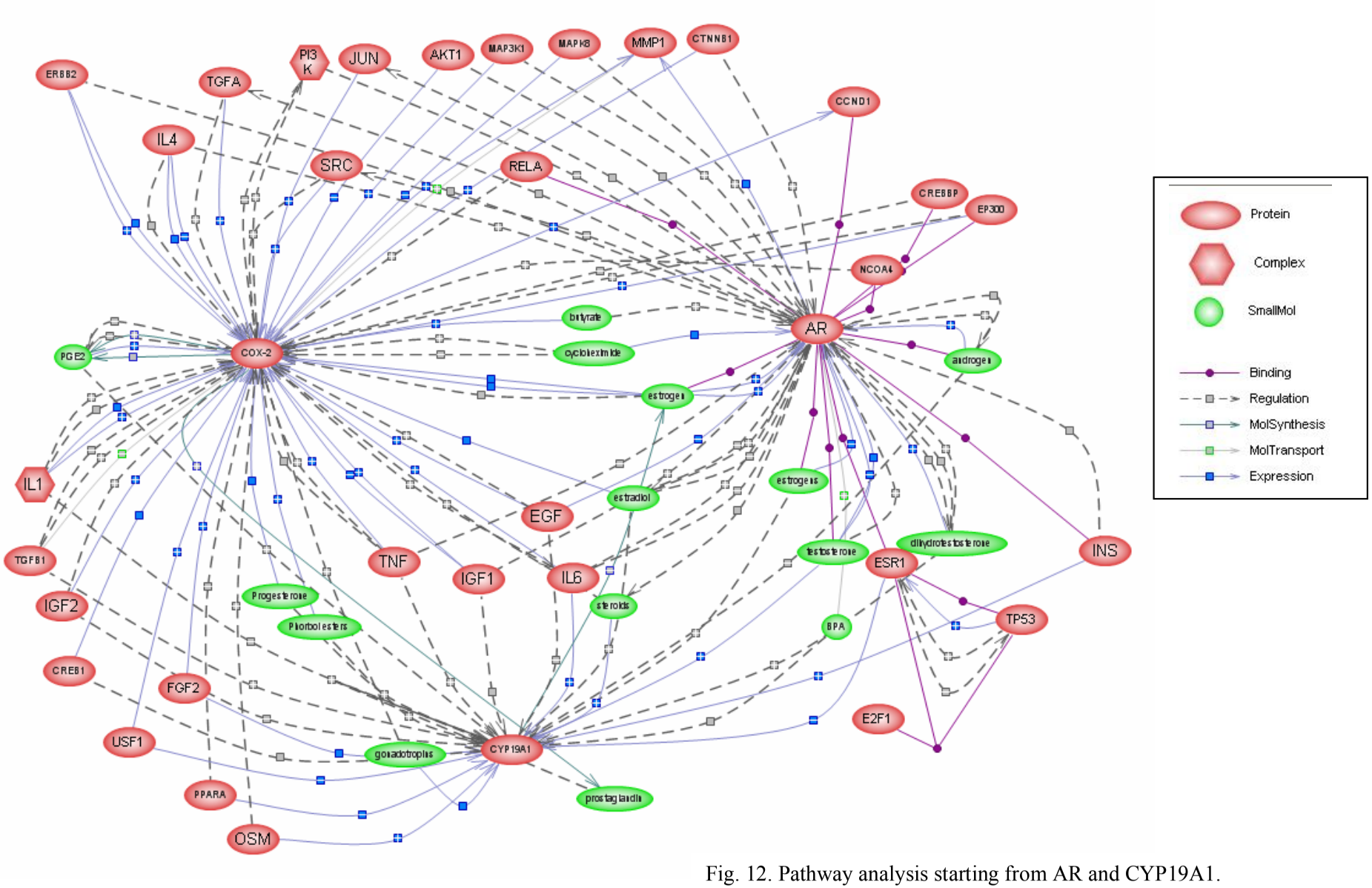


Fig. 12. Pathway analysis starting from AR and CYP19A1.

4.5. *AR* Exon 1 CAG repeat length polymorphism

The X-linked androgen receptor gene (*AR*) is more than 90 kb long and codes for a protein that has 3 major functional domains: the N-terminal domain, the DNA-binding domain, and the androgen-binding domain (Fig. 13). One transcriptional activation domain of the AR encoded by exon 1, contains a polymorphic glycine repeat and a polymorphic glutamine repeat that modulates transactivation [72-74]. In representative human populations, the length distribution of the CAG-coded glutamine repeat varies from 11 to 33 repeat units [75-78]. It was shown in transfection studies that the number of *AR* CAG-repeats correlates inversely with the transactivational activity of the AR [36, 72, 73].

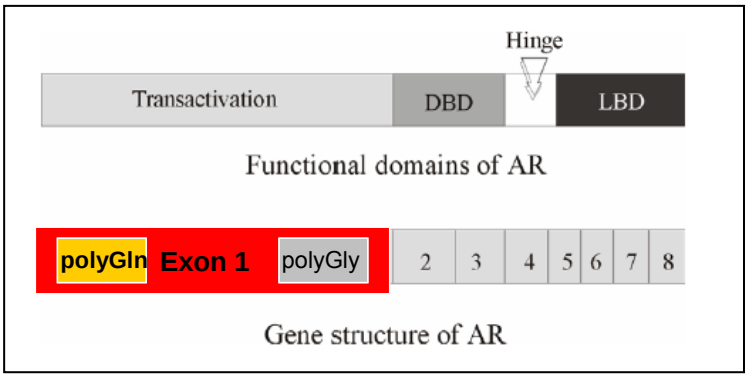


Fig. 13. Schematic structure of the androgen receptor.
There are eight exons encoding the receptor with a large exon 1 required for transactivation and exons 2-8 encoding a DNA-binding domain (DBD), a hinge region, and a hormone/ligand binding domain (LBD). There are two polymorphic trinucleotide repeat segments that encode polyglutamine (CAG) and polyglycine (GGC) tracts.

In the development of prostate cancer there has been evidence that the androgen receptor plays an important role. Stanford et al. [78] showed a direct correlation of each additional CAG repeat with a 3-14% decrease in prostate cancer risk.

Among sporadic breast cancer patients there are a lot of contradictory studies and by now only in the group of hereditary breast cancer (mostly *BRCA1*) some association which were found by a study could be confirmed by others. A few published studies from all over the world showed that most *BRCA1* mutation carrier are estrogen receptor (ER), progesterone receptor (PgR) and c-*erb*B-2 negative or weakly expressed [79-82].

According to Rebbeck et al. [4] in their *BRCA1* associated breast cancer study women who carried an *AR* CAG allele of ≥ 28, ≥ 29, or ≥ 30 repeats were given a diagnosis 0.8, 1.8, or 6.3 years earlier than women who did not carry at least one such allele. They concluded that because of the rare frequency of the long *AR* CAG repeats this polymorphism may only be relevant to some *BRCA1* mutation carriers. Park et al. [83] found that *BRCA1* directly interacts with the AR as a co-activator. These correlations were only found in the group of *BRCA1* mutation carriers and have not been studied in sporadic breast cancer patients.

Characteristic	No. of patients	%
All patients	229	100
Benign lesions[5]	107	46.7
Premenopausal	43	40.2
Postmenopausal	22	20.6
Malignant breast cancer[5]	122	53.3
Premenopausal	34	27.9
Postmenopausal	77	63.1
Estrogen receptor (ER) status[5]		
Negative	25	20.5
Weakly positive	12	9.8
Moderately positive	35	28.7
Highly positive	43	35.2
Progesterone receptor (PgR) status[5]		
Negative	50	40.9
Weakly positive	7	5.8
Moderately positive	42	34.4
Highly positive	16	13.1
Histological type		
Ductal carcinoma	96	78.7
Lobular carcinoma	15	12.3
Other carcinoma	11	9.0
Histological grade[5]		
Grade 1	16	13.1
Grade 2	57	46.7
Grade 3	39	32.0
Tumor size[5] [mm]		
≤ 20	77	63.1
> 20	29	23.8

Tab. 4. Characteristics of the patients and controls used in the *AR* CAG repeat length analysis.

In my study, a set of 122 female patients with breast cancer and 107 women with benign breast lesions were analyzed (Tab. 4). The CAG repeat polymorphism present in the first exon of *AR* was scored using fragment length analysis on an ABI Prism™ 3100 sequencer. Representative results of this analysis are shown in Fig. 14. To correlate the fragment length with the number of CAG repeats, five samples with different length were sequenced on the ABI Prism™ 3100 (data not shown). All analyzed alleles could be evaluated, and the zygosity analysis revealed that 213 women were hetero- and 16 were homozygote (93.0% and 7.0%, respectively; of 229 patients in total; Tab. 5).

[5] For some patients, not all information was available.

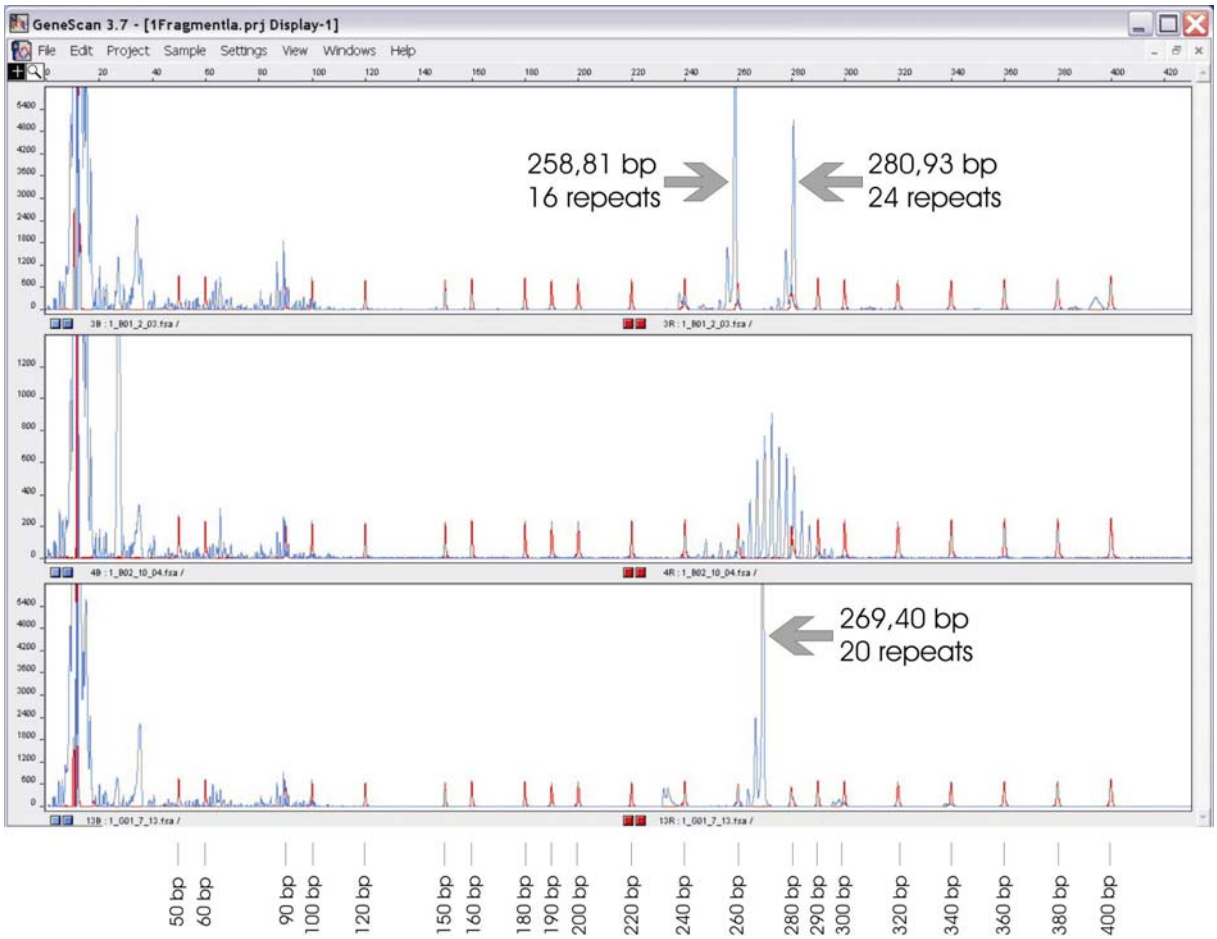


Fig. 14. DNA fragment length analysis. Graphs of *AR* CAG repeat fragment lengths of three different genomic DNA samples are shown. The red peaks are the internal length standard (length in bp indicated below the graph), and blue peaks are the PCR products containing the CAG repeat. In the upper lane there is a heterozygous patient with 16 and 24 CAG repeats (259 bp and 281 bp respectively), in the middle lane there is a DNA pool as positive control, and in the bottom lane, the result of a homozygous women with 20 repeats (269 bp) is shown.

43 of the women with benign breast lesions (46.7%) were pre- and 22 women (20.6%) were postmenopausal (Tab. 4). On the other hand 34 patients (27.9%) with breast cancer were pre- and 77 patients (63.1%) were postmenopausal. Of the 122 breast cancer patients, 96 women had a ductal (78.7%), 15 women had a lobular (12.3%), and only 9% (i.e. 11 patients) had an other type of carcinoma. While the PgR status was nearly equally divided between the group of negative/weakly positive (57 patients; 46.7%) and moderately/highly positive (58 patients; 47.5%), the picture at the ER status was different. In 30.3% the ER status was negative/weakly positive compared to 63.9% where the ER status was moderately/highly positive (37 and 78 patients, respectively). Furthermore, all patients were of Caucasian race.

Importantly, the human AR gene is located on the X-chromosome. Thus, males have only one *AR* allele, and in females only one *AR* allele is expressed in any given cell, because the other one is silenced by X chromosome inactivation. Accordingly, all statistical analysis was

conducted separately on the shorter allele, the longer allele, and on the sum of repeat numbers of both alleles. The distribution of *AR* CAG polymorphic alleles among the patients is shown in Fig. 15.

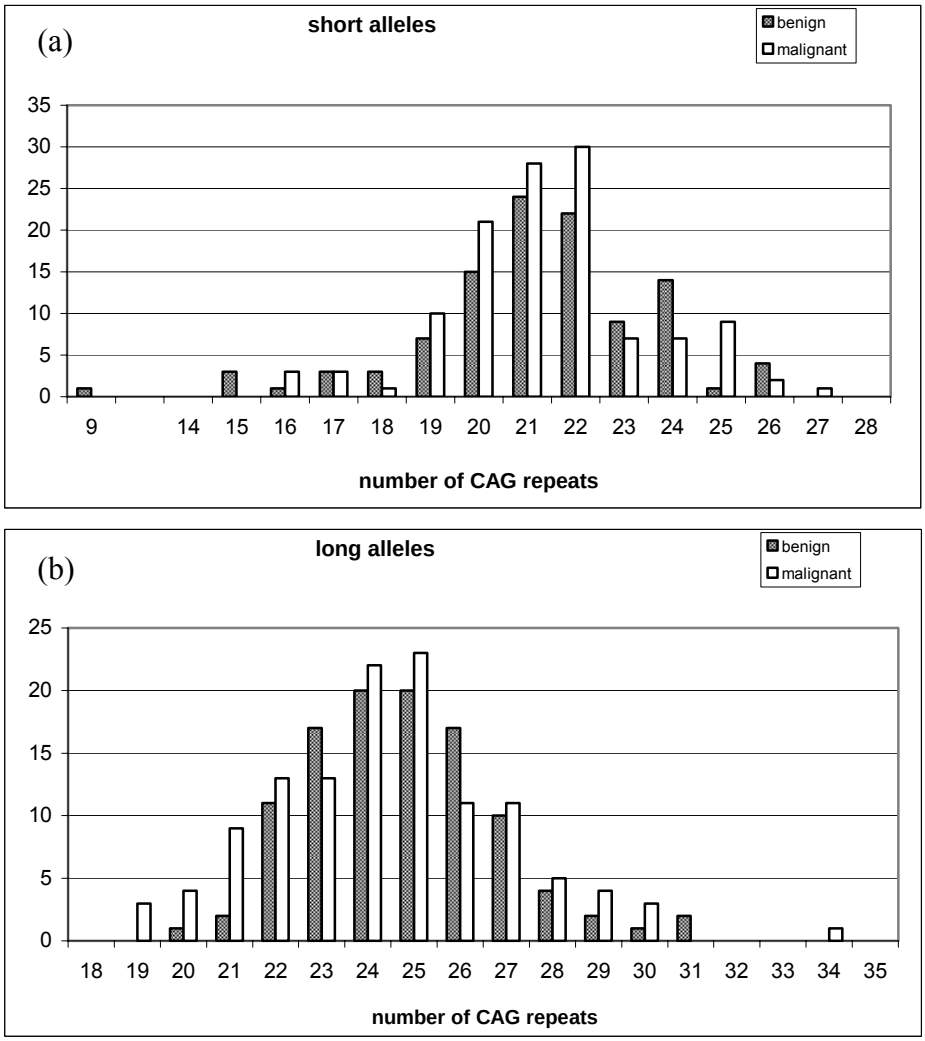


Fig. 15. The distribution of androgen receptor CAG repeat alleles among breast cancer patients and women with benign lesions. The number of patients and controls with the indicated number of CAG repeats of the short alleles (a) and of the long alleles (b) is shown.

13 patients with malignant breast cancer and only 3 of the patients with benign breast lesions were homozygous for the *AR* CAG repeat (OR 0.24; 95% CI 0.05–0.82; Tab. 5).

	heterozygote	homozygote	OR	95% CI	p	Total
Malignant BC	109	13	1.00			122
Benign lesions	104	3	0.24	0.05 – 0.82	0.02	107
Total	213	16				229

Tab. 5. Comparison of homo- and heterozygote subjects of the different groups.

			Benign lesions					Malignant					Total				
			Mean	SEM	Max	Min	n	Mean	SEM	Max	Min	n	Mean	SEM	Max	Min	n
Menopause	Pre	Short CAG repeats	21	0.33	26	15	43	22	0.35	26	19	34	22	0.24	26	15	77
		Long CAG repeats	24	0.26	28	20	43	24	0.38	30	21	34	24	0.22	30	20	77
		Sum of both alleles	45.67	0.52	53	35	43	46.06	0.68	56	40	34	45.84	0.41	56	35	77
		Age	34.49	1.39	68.28	16.81	43	39.83	1.09	48.84	25.17	34	36.85	0.96	68.28	16.81	77
	Post	Short CAG repeats	22	0.49	26	15	22	21	0.25	27	16	77	21	0.22	27	15	99
		Long CAG repeats	25	0.56	31	22	22	24	0.32	34	19	77	25	0.28	34	19	99
		Sum of both alleles	47.32	0.92	57	38	22	45.64	0.48	55	35	77	46.01	0.43	57	35	99
		Age	66.77	1.51	81.04	56.50	22	66.04	1.08	90.29	50.07	77	66.20	0.90	90.29	50.07	99
	Total	Short CAG repeats	21	0.25	26	9	107	21	0.19	27	16	122	21	0.15	27	9	229
		Long CAG repeats	25	0.20	31	20	107	24	0.24	34	19	122	25	0.16	34	19	229
		Sum of both alleles	45.95	0.38	57	34	107	45.78	0.37	56	35	122	45.86	0.26	57	34	229
		Age	46.24	1.42	81.04	16.81	107	57.31	1.31	90.29	25.17	122	52.14	1.03	90.29	16.81	229

Tab. 6. Summary of the distribution of CAG repeat numbers and age according to the menopausal status, and between patients with breast cancer and benign breast disease. SEM: standard error of mean, n: number of subjects included.

For all of the subjects, the numbers of the shorter *AR* CAG repeats ranged from 9 to 27 (mean, 21), the longer *AR* CAG repeats varied from 19 to 34 (mean, 25) and the sum of both alleles ranged from 34 to 57 (mean, 45.86) (Tab. 6).

First, the repeat length of the longer CAG allele was categorized in tertiles (Tab. 7), defined as short alleles (16-23 repeats), intermediate alleles (24-25 repeats), and long alleles (26-34 repeats), respectively. In this analysis, no evidence for a significant association between breast cancer and CAG repeat length was found. Relative to the lowest tertile of CAG repeat length, women in the intermediate and highest tertiles had an odds ratio (OR) of 0.95 (95% CI, 0.48-1.86) and of 0.67 (95% CI, 0.33-1.35), respectively.

The odds ratio (OR) is a statistical method of comparing whether the probability of a certain event is the same for two groups. In Tab. 7, the numbers of controls are compared with the numbers of cases. An OR below one (e.g. OR = 0.67 for the highest tertile) means that the event is more likely in the group of patients with benign lesions (first group). An odds ratio greater than one (e.g. OR = 4.37 for the > 52 years) implies that the event is more likely in the second (malignant breast cancer patients) group and an odds ratio of one implies that the event is equally likely in both groups. The OR is calculated from a 2 x 2 matrix. It is important to state which group is compared with which other group, because if rows and columns are exchanged within the matrix the results are reciprocal ORs.

		Benign lesions		Malignant diseases		OR[6]	95% CI	p
		Count	Col%	Count	Col%			
Age (years)	≤ 52	73	68.2	41	33.6	1.00		
	> 52	34	31.8	81	66.4	4.37	2.49 – 7.66	< 0.0001
Menopausal status	Pre	43	66.2	34	30.6	1.00		
	Post	22	33.8	77	69.4	4.50	2.33 – 8.68	< 0.0001
Long CAG repeats	16 – 23	31	29.0	42	34.4	1.00		
(tertiles)	24 – 25	40	37.4	45	36.9	0.95	0.48 – 1.86	
	26 – 34	36	33.6	35	28.7	0.67	0.33 – 1.35	n.s.[7]
Long CAG repeats	16 – 21	3	2.8	16	13.1	1.00		
(cut off point: ≤21)	≥ 22	104	97.2	106	86.9	0.18	0.05 – 0.67	0.005
Long CAG repeats	16 – 22	14	13.1	29	23.8	1.00		
(cut off point: ≤22)	≥ 23	93	86.9	93	76.2	0.48	0.23 – 0.97	0.039
ER	≤ 1			37	32.2			
	≥ 2			78	67.8			
PgR	≤ 1			57	49.6			
	≥ 2			58	50.4			

Tab. 7. ORs of women with benign lesions compared to patients with malignant disease. Col% = percentage for one variable results in 100%; 95% CI = 95% confidence interval of the OR; p = p-value from Fisher's exact test for the OR.

[6] adjusted for age and long CAG repeats respectively

[7] n.s. not significant; p = 0.472

One big problem of this study was that the difference in age (≤ 52 vs. > 52), and therefore also in the menopausal status (pre- or postmenopausal), between patients with malignant breast cancer and those with benign breast disease was rather large (OR 4.37, $p < 0.0001$ and OR 4.50, $p < 0.0001$ respectively; Tab. 7). It was not possible to better align the groups because of the limited number of study subjects.

CAG repeat cut-off	No. of cases	No. of controls	ORs[8]	95% CI	p
AR short allele					
≤ 19	17	18	0.80	0.39 – 1.64	0.545
≤ 20	38	33	1.01	0.58 – 1.77	0.960
≤ 21	66	57	1.03	0.61 – 1.73	0.900
≤ 22	96	79	1.31	0.71 – 2.41	0.388
≤ 23	103	88	1.17	0.58 – 2.35	0.658
≤ 24	110	102	0.45	0.15 – 1.32	0.137
≤ 25	119	103	1.54	0.34 – 7.04	0.575
AR long allele					
≤ 21	16	3	5.23	1.48 – 18.48	**0.005**
≤ 22	29	14	2.07	1.03 – 4.17	**0.039**
≤ 23	42	31	1.29	0.74 – 2.26	0.377
≤ 24	64	51	1.21	0.72 – 2.03	0.469
≤ 25	87	71	1.26	0.72 – 2.21	0.418
≤ 26	98	88	0.88	0.45 – 1.71	0.711
≤ 27	109	98	0.77	0.32 – 1.88	0.565
AR mean of alleles					
≤ 20	11	8	1.23	0.48 – 3.18	0.673
≤ 21	27	16	1.62	0.82 – 3.20	0.165
≤ 22	45	35	1.20	0.69 – 2.07	0.509
≤ 23	72	55	1.63	0.81 – 2.30	0.247
≤ 24	94	86	0.82	0.43 – 1.55	0.540
≤ 25	104	95	0.73	0.33 – 1.60	0.428
≤ 26	116	100	1.35	0.44 – 4.15	0.596
≤ 27	118	106	0.28	0.03 – 2.54	0.226

Tab. 8. ORs for breast cancer associated with each cut off point of *AR* allele length from 19 to 27 repeats.

We also estimated the ORs for breast cancer associated with each cut off point of *AR* allele length from 19 to 27 repeat units (Tab. 8). We found an increased risk of breast cancer associated with a low CAG repeat number within the group of the long alleles (Tab. 7). For both cut offs, ≤ 21 and ≤ 22 CAG repeats of the long allele, the ORs are significantly elevated: OR 5.23 (95% CI, 1.48-18.48; p=0.005) and 2.07 (95% CI, 1.03-4.17; p=0.039), respectively. This is in contrast to the findings reported by Giguère et al. [84] who found an OR 0.5 (95% CI, 0.3-0.83; p=0.007) for women with a low number of CAG repeats (two

[8] The odds ratios presented are those for breast cancer risk for women falling in the category below the cut-off value, compared to women above the cut-off point.

alleles ≤ 20 repeats each) and to the results of Liede et al. [85] in a population study in the Philippines. Due to the specific distribution of allele lengths in our study population, the largest statistical significance was observed with a cut off of ≤ 21 repeat units.

In Tab. 8, the numbers of cases are compared with the numbers of controls. An OR below one (e.g. OR = 0.77 for long allele and CAG repeat cut-off ≤ 27) means that the event is more likely in the group of controls (patients with benign breast disease). An odds ratio greater than one (e.g. OR = 5.23 for long allele and CAG repeat cut-off ≤ 21) implies that the event is more likely in the malignant breast cancer group.

Interestingly, some other studies which have also grouped their patients by allele length [86-88], have used the same cut off which showed the largest difference (i.e., highest OR) between breast cancer patients and women with benign breast lesions in our population (< 22 *versus* ≥ 22).

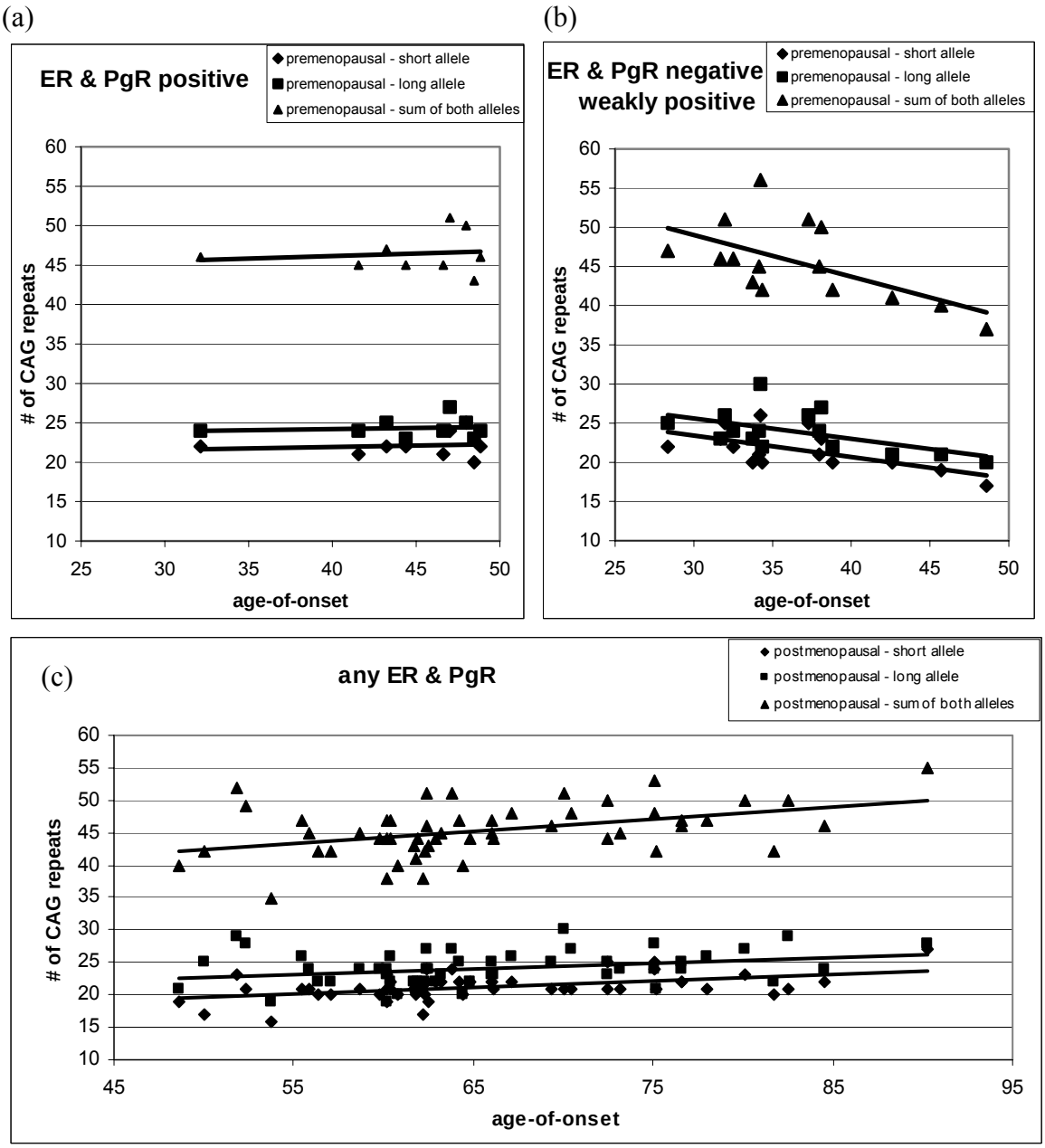
The results published by Rebbeck et al. [4] of mostly young *BRCA1* mutation carriers revealed that women who carried one long *AR* CAG allele were diagnosed with breast cancer earlier than women who did not carry at least one such allele. Other studies [79-82] have extensively documented that the breast tumors of most *BRCA1* mutation carriers are ER and PgR negative, or that the receptors express only weakly. Thus, we reasoned that the findings of Rebbeck et al. [4] in *BRCA1* mutation carriers might also be observed in sporadic breast cancer patients with a weak or no ER and PgR expression.

The findings (Fig. 16b) of premenopausal breast cancer patients with a low or negative ER and PgR expression are very similar to the results from *BRCA1* mutation carriers [4]. The more *AR* CAG repeats (it does not matter on which allele) a woman of this group (premenopausal and ER and PgR negative or weakly positive) has, the earlier she is diagnosed with breast cancer. The Pearson Correlation of the numbers of CAG repeats with the age – of onset of this subgroup (Fig. 16b) showed a negative number (1-tailed $p < 0.018$) compared to the other two subgroups (positive ER and PgR status or any given ER and PgR status, respectively; Fig. 16a and 16c), which showed a positive number (1-tailed $p < 0.382$ (not significant) and $p < 0.029$, respectively), meaning a slight increase in the slope. A positive value of the Pearson Correlation means a positive slope and a negative Pearson Correlation value describes a negative slope.

My results demonstrate that the fact that women with longer CAG repeats are diagnosed with breast cancer earlier, can not exclusively be seen in the group of *BRCA1* mutation carriers [4], but also in the group of sporadic breast cancer who have ER and PgR negative or weakly positive tumors (Fig. 16).

It is known that *AR* CAG repeat length modulates the transcriptional activity of *AR* in vitro and inversely correlates with the androgen sensitivity [72, 73]. Breast epithelial cells in women express only one of the two *AR* alleles because of the mapping of the AR gene to the X chromosome. Thus, each cell is under influence of only a single *AR* allele. The findings of Rebbeck et al. [4] and Giguère et al. [84] led them to hypothesize that, considering the role of *AR* activity in breast cell proliferation, decreased androgen activity in breast cells expressing a very long CAG repeat result in increased breast cancer risk in their study group. In contrast to their hypothesis, my findings show that in the longer of the two alleles, fewer CAG repeats are found in patients compared to women with benign breast lesions (Fig. 15b). According to the X inactivation this would mean that the critical allele for developing breast cancer in the subgroup of patients with ER and PgR negative or weakly positive tumors is the longer one.

Additional work is necessary to elucidate the question whether the short or the long allele is inactivated by X inactivation and if the active allele is important in the development of breast cancer.



Correlations[9]

	premenopausal						postmenopausal		
	ER & PgR positive			ER & PgR negative[10]			any ER & PgR		
	short	long	sum	short	long	sum	short	Long	sum
Pearson Correlation AGE	0.130	0.117	0.134	-0.591	-0.544	-0.610	0.478	0.265	0.411
Sig (1-tailed)	0.370	0.382	0.366	0.010	0.018	0.008	0.000	0.029	0.001
N	9	9	9	15	15	15	52	52	52

Fig. 16. Correlation of the age – of onset with the *AR* CAG repeat lengths in different breast cancer. Results for patients with (a) ER and PgR positive tumors, (b) ER and PgR negative or weakly positive tumors, and (c) tumors with of any ER and PgR status are shown. To statistically correlate these findings, linear regression using Pearson Correlation was done. Sig (significance) is the 1-tailed p-value from Fisher's exact test.

[9] Only patients were included where both receptor statuses were known.
[10] ER & PgR negative and weakly-positive were included here.

4.6. CYP19 (Aromatase)

The human CYP19 gene maps to chromosome 15q21.1, spans at least about 70 kb of genomic DNA, and comprises 10 exons [89]. The translational initiation site is located in exon 2 and the termination site is found in exon 10. Sebastian and Bulun [90] showed that the entire gene spans more than 123 kb of DNA by analysis of overlapping BAC clones. Only the 30 kb 3-prime region contains the protein-coding exons, whereas a large 93 kb 5-prime flanking region serves as the regulatory unit of the gene.
The CYP19 gene contains 5 alternative promoters. Transcripts in breast adipose tissue contain 5-prime termini corresponding to expression derived from use of promoters I.4 (distal promoter) predominantly as well as II (proximal promoter) and I.3 (distal promoter). Promoter I.4 contains a glucocorticoid response element and an interferon-gamma activation site, and is responsible for expression of aromatase in the presence of glucocorticoids and members of the class I cytokine family [17].

4.7. *CYP19* Exon 10 C → T

Since the Exon 10 C → T polymorphism is in the vicinity (19 nucleotides downstream) of the UAG stop-codon for termination of translation, one may speculate that a possible change in the secondary structure of the transcript might influence both the stability of the transcript and the regulation of translation termination. There are some contradicting results about the C → T substitution in exon 10 of the CYP19 gene. Kristensen et al. [5] found an association of the T-allele with a 'high activity' phenotype in their Scandinavian case-control study, whereas Haiman et al. [91] could not confirm any correlation of breast cancer risk with the *CYP19* genotype. In the Scandinavian study a strong association of the T-allele with mRNA levels, large tumor size (tumors larger than 5 cm) and staging III or IV was observed. Carriers of the C-allele who are postmenopausal and receive hormone replacement therapy frequently have a higher breast density, as found by mammography [92].

To genotype the Exon 10 → T polymorphism in a collective of breast cancer patients and controls, two separate PCRs were performed (after a long testing period I was not able to establish a working multiplex PCR) with the following primer pairs. With primers CYP19-E10-C→T fwd (5' – ATA TTC TGG CAA CTG TCT G – 3') and CYP19-E10-in rev (5' – GAG AAA TGC TCC AGA GTG – 3'), a 217 bp long PCR product was produced only if the

C-allele was present. Primers CYP19-E10-in(mut) fwd (5' – AAG GCT GGT CAG TAC CT – 3') and CYP19-E10-C→T rev (5' – GAG GAT GAC ACT ATT GGC – 3') resulted in a 90 bp long PCR product if the T-allele was present, and both bands were detected on agarose gels if the patient was heterozygous (Fig. 17). The thermal cycler conditions were an initial 5 minutes denaturation cycle at 95°C followed by 35 cycles of amplification (95°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute) and a final extension cycle at 72°C for 7 min.

Sample No.	C-allele		T-allele		
291		yes		no	homozygous (CC)
303		no		yes	homozygous (TT)
315		yes		no	homozygous (CC)
327		yes		yes	heterozygous (CT)
339		yes		yes	heterozygous (CT)
351		yes		no	homozygous (CC)
363		no		yes	homozygous (TT)
375		yes		yes	heterozygous (CT)

Fig. 17. Agarose gel after PCR with primers CYP19-E10-C→T fwd and CYP19-E10-in rev for the C-allele and primers CYP19-E10-in(mut) fwd and CYP19-E10-C→T rev for the T-allele.

Characteristic	*CYP19* Exon 10 No. of patients	%	*CYP19* Codon 39 No. of patients	%
All patients	395	100	330	100
Benign lesions[11]	162	41.0	135	40.9
Premenopausal	73	45.1	63	46.7
Postmenopausal	33	20.4	27	20.0
Malignant breast cancer[11]	214	54.2	177	53.6
Premenopausal	49	22.9	41	23.2
Postmenopausal	142	66.4	118	66.7
Estrogen receptor (ER) status[11]				
Negative	45	21.0	39	22.0
Weakly positive	17	7.8	15	8.5
Moderately positive	57	26.6	44	24.9
Highly positive	82	38.3	67	37.9
Progesterone receptor (PgR) status[11]				
Negative	91	42.5	74	41.8
Weakly positive	14	6.5	10	5.6
Moderately positive	65	30.4	58	32.8
Highly positive	31	14.5	23	13.0
Histologic type				
Ductal carcinoma	172	80.4	145	81.9
Lobular carcinoma	34	15.9	26	14.7
Other carcinoma	8	3.7	6	3.4
Histologic grade[11]				
Grade 1	37	17.3	27	15.3
Grade 2	89	41.6	75	42.4
Grade 3	70	32.7	59	33.3
Tumor size[11] [mm]				
≤ 20	112	52.3	98	55.4
> 20	66	30.8	56	31.6
Healthy control[11]	19	4.8	18	5.5
Premenopausal	6	31.6	6	33.3
Postmenopausal	5	26.3	5	27.8

Tab. 9. Characteristics of the patients and controls used in the *CYP19* Exon 10 und Codon 39 study.

395 women were analyzed for the *CYP19* Exon 10 C → T polymorphism (Tab. 9). Of these 395 women, 214 (54.2%) were affected with breast cancer (mean age 58.6 years, range 25.2 – 90.3 years), 162 (41.0%) had a benign breast lesions (mean age 45.4 years, range 14.6 – 87.8 years) and 19 (4.8%) were healthy controls (mean age 43.0 years, range 15.7 – 60.1 years).

The study population was in Hardy-Weinberg equilibrium with respect to the polymorphism (χ^2=0.677, p-value 0.410). The unbiased estimation for heterozygosity gave an excellent value of 0.5007 (standard deviation: 0.0009) with 95% confidence limits of 0.4988 to 0.5025.

[11] For some patients, not all information was available.

	control group								breast cancer group							
	all		premenopausal		postmenopausal		not known		all		premenopausal		postmenpausal		not known	
homozygous (CC)	38	21.0%	16	20.3%	8	21.1%	14	21.9%	57	26.6%	17	34.7%	35	24.7%	5	21.8%
homozygous (TT)	39	21.5%	18	22.8%	8	21.1%	13	20.3%	52	24.3%	11	22.4%	32	22.5%	9	39.1%
heterozygous (CT)	104	57.5%	45	54.9%	22	57.8%	37	57.8%	105	49.1%	21	42.9%	75	52.8%	9	39.1%
	181		79		38		64		214		49		142		23	

Tab. 10. Genotype distribution (number and frequency) of the polymorphism in exon 10 of *CYP19*, and its relation to menopausal status.

	breast cancer group versus control group								
	all			premenopausal			postmenopausal		
	OR	CI (95%)	p-value	OR	CI (95%)	p-value	OR	CI (95%)	p-value
homozygous (CC)	1.3662	0.8549 – 2.1835	0.196437	**2.0918**	**0.9358 – 4.6760**	**0.095800**	1.2266	0.5148 – 2.9228	0.830630
homozygous (TT)	1.1687	0.7287 – 1.8744	0.550125	0.9810	0.4183 – 2.3009	1.000000	1.0909	0.4554 – 2.6134	1.000000
heterozygous (CT)	0.7132	0.4789 – 1.0622	1.000000	0.5667	0.2758 – 1.1643	0.146653	0.8141	0.3949 – 1.6782	0.589162

Tab. 11. Odds ratios (ORs) with 95% confidence intervals (CI) and p-values (Fisher's exact test) of developing breast cancer for the different genotypes in the C>T polymorphism in the 3' untranslated region of exon 10 of *CYP19*, and its relation to menopausal status.

Because of the very small number of healthy controls available, these subjects were combined with the women with benign breast lesions to obtain a control group for the subsequent analysis (Tab. 10).

Tab. 11 shows the ORs with 95% CI and the p-value from Fisher's exact test of developing breast cancer for the different genotypes. For premenopausal women who are homozygous for wildtype allele (CC genotype), the statistical analysis shows a trend for protection from breast cancer (OR 2.0918; 95% CI 0.9358 – 4.6760; p-value 0.0958). This is in agreement with the findings of Kristensen et al. [5], who showed an association of the T-allele with a 'high activity' phenotype in their Scandinavian case-control study. The strong correlation of the T-allele with tumor size (tumors larger than 5 centimeters) found by these authors [5] could not be verified due to the small numbers of patients with tumors larger than 5 centimeters in our collective.

A higher proportion of the breast cancer patients than controls (26.6% vs. 21.0%) were carriers of the CC genotype, but this difference was not significant. A similar distribution was found for the TT genotype (24.3% vs. 21.5%; Tab. 10).

When looking at the association of cases or controls with the genotype (Tab. 12), logistic regression showed a strong trend (OR 0.661; CI 0.396 – 1.103; p-value 0.113) for the group of heterozygous patients (CT) compared to the patients homozygous for the wildtype allele (CC). Thus, a heterozygous woman has only a 0.661 fold chance to develop malignant breast cancer than a woman who is homozygous for the C-allele. The significance could be enhanced if the menopausal status (OR 5.153; CI 3.304 – 8.037; p-value <0.001) was included into the model, then showing an OR of 0.607 (95% CI 0.349 – 1.058; p-value 0.079). A postmenopausal woman has a 5.153 fold chance to fall into the malignant breast cancer group than a premenopausal woman. In this model 69.58% of all data were correctly classified. Interestingly, the inclusion of age reduced the model's significance (data not shown). Surprisingly, the same analysis for women homozygous for the T-allele, who should be protected from breast cancer even more than the heterozygous group [5], showed a poorer trend (Tab. 12).

Cases versus controls						
	OR	Std. Err.	p-value	95% CI		Coef.
heterozygous (CT)	0.607	0.172	0.079	0.349	1.058	-0.498
homozygous (TT)	0.778	0.256	0.446	0.408	1.484	-0.251
menopausal status	5.153	1.169	<0.001	3.304	8.037	1.640

Tab. 12. Influence of the genotype (relative to the homozygous CC-genotype) and menopausal status on the distribution of cases versus controls.

The influence of the genotype and various other factors on different clinical parameters was analyzed using the generalized linear model with Newton-Raphson optimization. The genotype was not significantly correlated with grading (data not shown). Not surprisingly, the tumor size (≤ 2 cm vs. > 2 cm) enhanced the trend of the CT genotypes for protection, and the best trend was obtained when including the menopausal status in the model (Tab. 13).

Grading					
	Coef.	Std. Err.	p-value	95% CI	
heterozygous (CT)	0.104	0.125	0.403	-0.140	0.349
homozygous (TT)	-0.073	0.144	0.614	-0.354	0.209
tumor size (≤2 cm vs. >2 cm)	0.158	0.047	0.001	0.066	0.250
menopausal status	-0.007	0.004	0.064	-0.014	<0.001

Tab. 13. Influence of the genotype (relative to the homozygous CC-genotype), tumor size, and menopausal status on the distribution of grading.

In postmenopausal breast cancer patients, a slight trend for heterozygous women (Coef. -0.320; p-value 0.173) and a strong trend for women homozygous for the T-allele (Coef. -0.482; p-value 0.082) was found in connection with the tumor size (≤ 2 cm vs. > 2 cm; Tab. 14). Interestingly, when comparing these two genotypes (TT and CT) with the wildtype (CC), a protective effect (negative Coef. values) was seen. This is contrary to the findings of Kristensen et al. [5], who found an association of the T-allele with larger tumor size. However, these authors chose a cutt-off of 5 centimeters for the tumor size, which could not be done within the population of this dissertation due to a very small number of tumors larger than 5 centimeters. Likewise, due to a very small number of lobular breast cancers in the analyzed patient group, any differences between ductal and lobular tumors could not be analyzed.

tumor size (≤ 2 cm vs. > 2 cm) in postmenopausal breast cancer patients					
	Coef.	Std. Err.	p-value	95% CI	
heterozygous (CT)	-0.320	0.234	0.173	-0.779	0.140
homozygous (TT)	-0.482	0.277	0.082	-1.024	0.061

Tab. 14. Influence of the genotype (relative to the homozygous CC-genotype) on the distribution of the tumor size (≤ 2 cm vs. > 2 cm) in postmenopausal breast cancer patients.

No significant correlation was found between tumor size (≤ 2 cm vs. > 2 cm) and age, menopausal status, ER status, PgR status, p53 expression, and HER2 amplification, although the combination of all these parameters marginally improved the model (data not shown). Nevertheless, the best model is the one where only the genotype is included (Tab. 14).

For ER status and p53 expression no correlation was found with genotype, age, menopausal status, PgR status, HER2 amplification, tumor size, or any combination of these parameters (data not shown).

The TT-genotype showed a statistically significant (OR 0.344; p-value 0.022), protective effect on the distribution of PgR negative or PgR positive tumors, when ER status, HER2 amplification, and p53 expression was included into the model (data not shown). This means that a TT homozygous patient more likely has a negative PgR tumor. An improvement of this model could be achieved by including the age (Tab. 15), resulting in an OR of 0.333 and a p-value of 0.019. In this model, 68.53% of all data were correctly classified.

PgR status						
	OR	Std. Err.	p-value	95% CI		Coef.
heterozygous (CT)	0.984	0.386	0.968	0.456	2.125	-0.157
homozygous (TT)	0.333	0.156	0.019	0.133	0.834	-1.101
ER	2.008	0.322	<0.001	1.466	2.751	0.697
HER2	0.826	0.119	0.185	0.622	1.096	-0.191
p53	0.499	0.117	0.003	0.315	0.791	-0.695
Age	0.992	0.014	0.548	0.965	1.019	-0.008

Tab. 15. Influence of the genotype (relative to the homozygous CC-genotype), age, ER status, HER2 amplification, and p53 expression on the distribution of PgR negative versus PgR positive tumors.

When this correlation between TT-genotype and PgR status was analyzed only in patients with ductal tumors, this model was highly significant (OR 0.196; p-value 0.004). Thus, a patient with a ductal tumor and homozygous for the T-allele is more likely to have a PgR negative tumor (Tab. 16). 71.34% of the data were correctly classified in this model in women with ductal tumors.

PgR status in breast cancer patients with ductal tumors						
	OR	Std. Err.	p-value	95% CI		Coef.
heterozygous (CT)	0.732	0.342	0.504	0.294	1.827	-0.311
homozygous (TT)	0.196	0.110	0.004	0.065	0.591	-1.628
ER	2.266	0.401	<0.001	1.602	3.205	0.818
HER2	0.812	0.128	0.185	0.596	1.105	-0.209
p53	0.502	0.125	0.006	0.308	0.817	-0.690
menopausal status	0.715	0.340	0.481	0.282	1.814	-0.335

Tab. 16. Influence of the genotype (relative to the homozygous CC-genotype), menopausal status, ER status, HER2 amplification, and p53 expression on the distribution of PgR negative or PgR positive tumors within the ductal tumor group.

Within the group of patients with ductal tumors, a similar correlation to HER2 amplification was seen for homozygous TT patients, with an OR of 0.171 and a p-value of 0.044 (Tab. 17). The best result for the HER2 amplification model was achieved by including the tumor size (in centimeter). An excellent 80.30% of all data were correctly classified in this model.

HER2 amplification in breast cancer patients with ductal tumors						
	OR	Std. Err.	p-value	95% CI		Coef.
heterozygous (CT)	1.407	0.737	0.515	0.504	3.928	0.341
homozygous (TT)	0.169	0.149	0.043	0.030	0.946	-1.776
ER	0.728	0.151	0.126	0.485	1.093	-0.317
PgR	0.670	0.161	0.096	0.419	1.073	-0.400
p53	0.861	0.184	0.482	0.566	1.308	-0.150
menopausal status	0.641	0.324	0.378	0.238	1.725	-0.445
tumor size (in centimeter)	1.224	0.213	0.246	0.870	1.720	0.202

Tab. 17. Influence of the genotype (relative to the homozygous CC-genotype), menopausal status, ER status, PgR status, and p53 expression on the distribution of negative or positive HER2 amplification within the ductal tumor group.

To briefly summarize these findings, I did not observe an association between the genotype of the C → T SNP in exon 10 of *CYP19* and breast cancer risk among Caucasian women in the study population of this dissertation. These findings are in accordance with the results recently published by Haiman et al. [91]. My results do not provide support for the previous observation that the T-allele is associated with larger, more advanced tumors [5]. However, Haiman et al. [91] showed a decrease in androgen levels and a larger estrogen-to-androgen ratio, which supports the hypothesis that the T-allele may have elevated aromatase activity.

4.8. The *CYP19* codon 39 Tyr → Arg polymorphism

Miyoshi et al. [16] recently found a single nucleotide polymorphism in codon 39 (Trp to Arg) in their study of Japanese patients. It is a rare polymorphism, with 4% and 10% of the C-allele (Arg) in breast cancer patients and in controls, respectively, found by Miyoshi et al. [16]. Preliminary studies have shown that allele frequencies of the CYP19 gene are different among Caucasian and Japanese women [93-95]. The relationship between this SNP and the CYP19 enzyme activity and its role in breast cancer still remains to be studied. Interestingly, the presence of the variant C-allele (Arg) was significantly associated with a reduced risk of breast cancer, but also correlated with a significantly higher frequency of lymph node metastasis positive cases [16]. It is speculated that carcinogenesis of biologically less aggressive breast cancer is inhibited in carriers of the variant Arg allele due to low estrogen conditions, and that, once cancer arises, these tumors progress to a biologically more aggressive phenotype because they arise without the help of estrogens.

Genotyping of the *CYP19* codon 39 Typ → Arg polymorphism was performed in 330 subjects using the Taqman Allelic Discrimination method, but only one person who carried the rare C-allele was found (interestingly, this woman was homozygous) (data shown in the Appendix, Fig. 24 – 25). Therefore, any further analysis was impossible.

Thus, in contrast to the Japanese population, the variant Arg allele in codon 39 of *CYP19* appears to be very rare in the Caucasian population. Interestingly, this correlates with the fact that Caucasian women have a higher breast cancer incidence than Japanese women, and that higher serum estrogen levels are reported in Caucasian women than in Japanese women [96].

4.9. A -765 G → C SNP IN THE COX-2 GENE

The human COX-2 gene maps to chromosome 1q25.2-q25.3, is about 8.3 kb in size, and contains 10 exons [97]. The 1.8 kb promoter region contains a TATA box and a number of potential regulatory elements including CRE, NF-κB, NF-IL-6, GRE, PEA-3, AP2, C/EBP, TGF-β, and multiple Sp1 response elements [98]. In particular, Sp1 is considered a positive activator of COX-2 transcription and acts through G-rich elements [99]. Deletion and mutation experiments altering this sequence have identified critical regions involved in inducing COX-2 gene transcription [100].

COX-1 and -2 play key physiological roles in blood clotting, renal function, and maintenance of gastrointestinal integrity, and also participate in pathophysiological processes like inflammation, arthritis, Alzheimer's disease, and cancer [101] (Fig. 18).

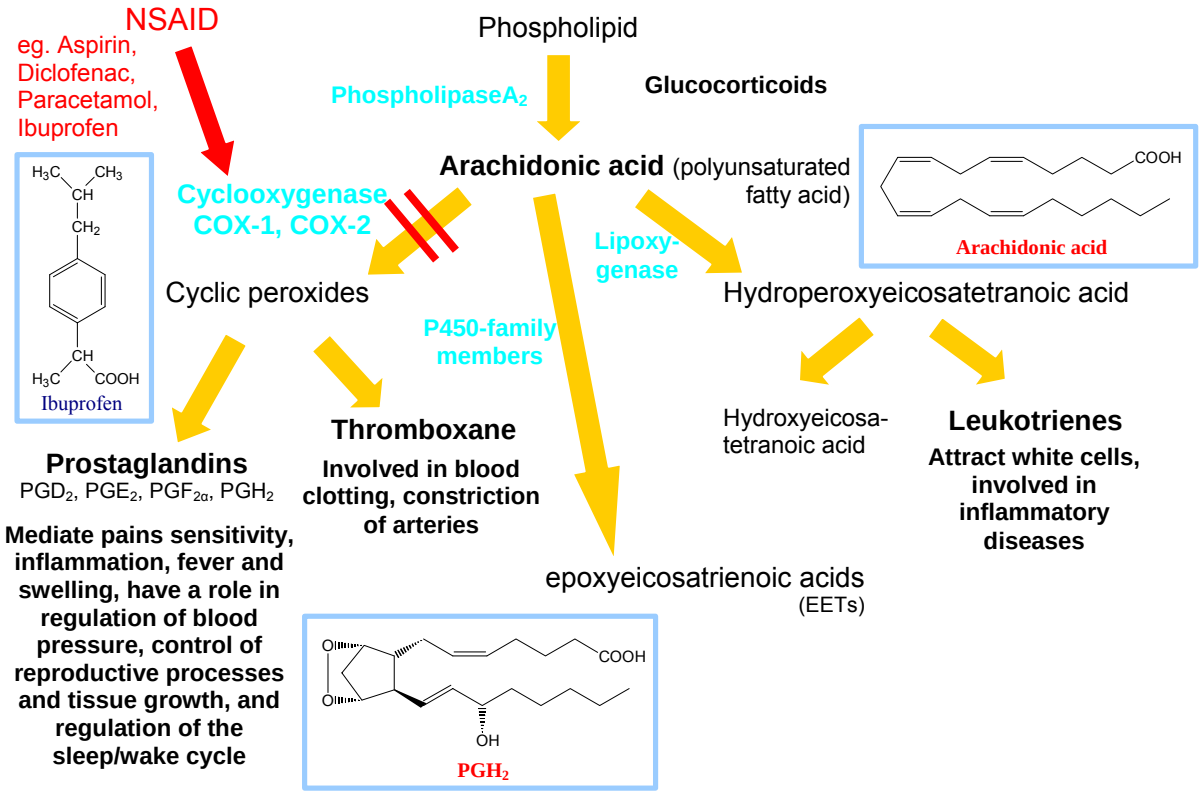


Fig. 18. Metabolic pathway of prostaglandin biosynthesis from arachidonic acid, which critically involves cyclooxygenase. Non steroidal anti-inflammatory drugs (NSAIDs) inhibit the effect of cyclooxygenase.

Rare polymorphisms have been identified in both the COX-1 and the COX-2 gene [102]. The demonstration of the low numbers of and rare allelic variations in functionally important polymorphisms of both COX genes suggest that because of critical roles of these enzymes, SNPs are not likely to develop. In the case of COX-2 (GeneBank accession number U04636),

most polymorphisms are intronic, or synonymous changes where functional effects are not likely. Far less has been done to functionally characterize polymorphisms in COX-2 coding regions.

The C-variant of the G-765C single nucleotide polymorphism in the promoter region does not contain a Sp1 site and therefore has an about 30% reduced promoter activity [103]. Ristimäki et al. [6] showed a higher COX-2 expression in ductal tumors when compared to lobular or any other carcinoma, in tumors with high grade, in large tumors, if the hormone receptor status is negative, and if p53 expression and HER2 amplification is high. If there is an overproduction of COX-2, the prostaglandin production also increases and this leads to a more aggressive form of the disease (bigger tumors, positive lymph nodes, positive HER2 amplification etc.). Elevated COX-2 levels favour the formation of metastasis [47] and are particularly found in solid tumors [43, 44].

Characteristic	**No. of patients**	**%**
All patients	275	100
Benign lesions[12]	127	46.2
Premenopausal	52	40.9
Postmenopausal	25	19.7
Malignant breast cancer[12]	147	53.5
Premenopausal	36	24.5
Postmenopausal	95	64.6
Estrogen receptor (ER) status[12]		
Negative	32	21.8
Weakly positive	14	9.5
Moderately positive	41	27.9
Highly positive	52	35.4
Progesterone receptor (PgR) status[12]		
Negative	63	42.9
Weakly positive	7	4.8
Moderately positive	50	34.0
Highly positive	19	12.9
Histologic type		
Ductal carcinoma	121	82.3
Lobular carcinoma	22	15.0
Other carcinoma	4	2.7
Histologic grade[12]		
Grade 1	23	15.6
Grade 2	62	42.2
Grade 3	52	35.4
Tumor size[12] [mm]		
≤ 20	81	55.1
> 20	46	31.3
Healthy control	1	0.3
Premenopausal		

Tab. 18. Characteristics of the patients and controls used in the COX-2 -765 G → C study.

[12] For some patients, not all information was available.

The primers (CF8 and CR7 of Papafili et al. [103]) gave a 306-bp band that was digested by *Aci*I (New England Biolabs; #R0551L; Beverly, USA) in the -765G allele into two fragments of 188 bp and 118 bp, respectively (Fig. 19). The thermal cycler conditions were an initial 5 minutes denaturation cycle at 95°C followed by 35 cycles of amplification (95°C for 30 s, 47°C for 30 s and 72°C for 1 min) and a final extension cycle at 72°C for 7 min.

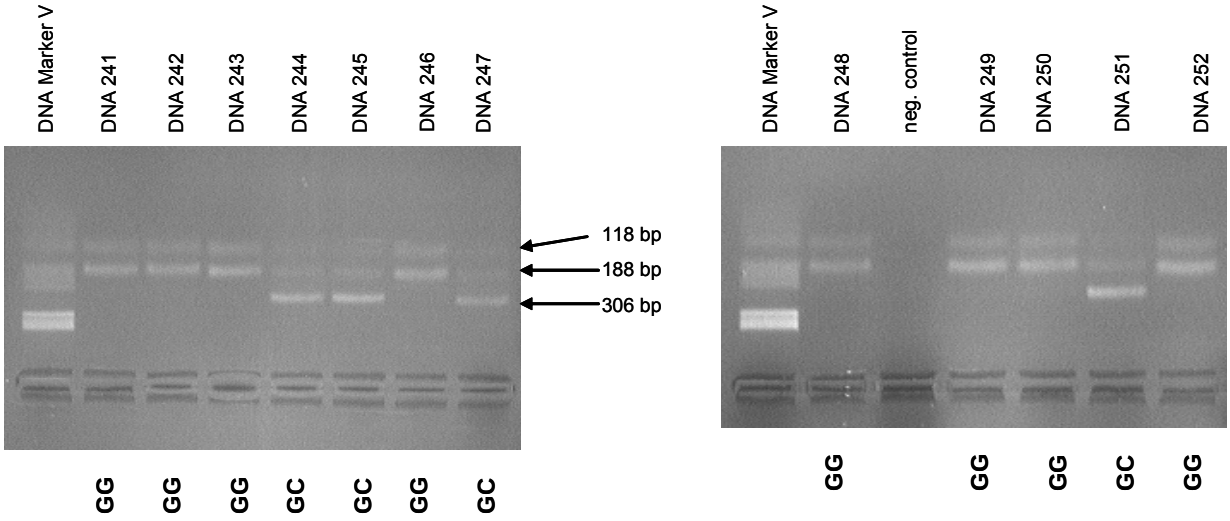


Fig. 19. Agarose gel after *Aci*I digest for PCR fragments from 12 different subjects. The genotypes of each subject are indicated (homozygous GG and heterozygous GC genotypes are shown).

Of the 275 women analyzed (Tab. 18), 147 (53.5%) were affected with breast cancer (mean age 58.2 years, range 25.2 – 90.3 years), 127 (46.2%) had a benign breast lesions (mean age 45.4 years, range 14.6 – 81.0 years) and one (0.3%) was a healthy control (age 35.0).

The study population was in Hardy-Weinberg equilibrium with respect to the polymorphism (χ^2=1.915, p-value 0.166). The unbiased estimation for heterozygosity gave a value of 0.3766 (standard deviation: 0.0184) with 95% confidence limits of 0.3404 to 0.4127.

When looking at the association of cases or controls with the genotype, logistic regression showed a statistically significant effect (OR 0.589; 95% CI 0.359 – 0.965; p-value 0.036) for the group of heterozygous patients (GC) compared to patients homozygous for the wildtype allele (GG), in agreement with the findings published by Ristimäki et al. [6]. Thus, a heterozygous woman has only a 0.589 fold chance to develop malignant breast cancer than a woman who is homozygous for the G-allele. This significance could be further enhanced if the age (OR 1.062; CI 1.042 – 1.083; p-value <0.001) was included into the model, then showing an OR of 0.557 (95% CI 0.325 – 0.957; p-value 0.034). Each additional year of age increases the risk of being in the breast cancer group by a factor of 1.062. In this model, 70.18% of all data were correctly classified (Tab. 19). Interestingly, inclusion of the

menopausal status reduced the model's significance although the OR remained significant (data not shown). Surprisingly, the same analysis in women homozygous for the C-allele, who should be protected from breast cancer even more than the heterozygous group [103], showed no significant correlations.

Cases versus controls						
	OR	Std. Err.	p-value	95% CI		Coef.
heterozygous (GC)	0.557	0.154	0.034	0.325	0.957	-0.584
homozygous (CC)	1.102	0.723	0.882	0.305	3.983	0.097
Age	1.062	0.010	<0.001	1.042	1.083	0.060

Tab. 19. Influence of the genotype (relative to the homozygous GG-genotype) and age on the distribution of cases versus controls.

The influence of the genotype and various other factors on different clinical parameters was analyzed using the generalized linear model with Newton-Raphson optimization. The genotype was not significantly correlated with grading (data not shown). Not surprisingly, the tumor size (pT) enhanced the trend of the homozygous CC-genotype and the heterozygous GC-genotype for protection, and the best trend was obtained when including the age into the model (Tab. 20).

Grading					
	Coef.	Std. Err.	p-value	95% CI	
heterozygous (GC)	-0.085	0.135	0.529	-0.349	0.179
homozygous (CC)	-0.272	0.280	0.331	-0.820	0.277
tumor size (pT)	0.249	0.096	0.009	0.062	0.437
age	-0.007	0.004	0.138	-0.015	0.002

Tab. 20. Influence of the genotype (relative to the homozygous GG-genotype), tumor size, and age on the distribution of grading.

Although the status of the ER, p53, HER2, and the age (p-values of 0.015, 0.002, 0.047, and 0.044, respectively) significantly influenced the trend of the genotype on the grading, this model was inferior to the model without these additional variables. The progesterone receptor and the menopausal status had no significant effect (Tab. 21). Overall, this again suggests that the genotype has no significant influence on the grading, which is in contrast to the findings of Ristimäki et al. [6].

Grading					
	Coef.	Std. Err.	p-value	95% CI	
heterozygous (GC)	-0.048	0.121	0.688	-0.285	0.188
homozygous (CC)	0.073	0.251	0.772	-0.420	0.566
ER	-0.150	0.062	0.015	-0.271	-0.029
PgR	-0.012	0.059	0.845	-0.127	0.104
p53	0.176	0.056	0.002	0.067	0.286
HER2	0.093	0.047	0.047	0.001	0.185
menopausal status	-0.348	0.189	0.066	-0.719	0.023
age	0.013	0.007	0.044	0.000	0.026

Tab. 21. Influence of the genotype (relative to the homozygous GG-genotype), ER status, PgR status, p53 expression, HER2 amplification, menopausal status, and age on the distribution of the grading.

No analysis of differences between the ductal and the lobular cancer group could be performed due to a very small number of lobular breast cancers in the analyzed patient group.

The influence of larger tumor size as published by Ristimäki et al. [6] could not be verified. No significant correlation was found between tumor size (pT or tumor size in centimeters) and any combination of the other parameters, except, of course, of grading. Although grading positively influences the correlation of the genotype with the tumor size, the best model is the one where only the genotype is included (data not shown).

The associations of this SNP in the COX-2 gene with ER, p53, HER2 (data not shown), and PgR, as published by Ristimäki et al. [6], could not be confirmed, although a protective trend could be observed for the homozygous CC-genotype with PgR negativity (Tab. 22). The OR (0.379) indicates that a patient homozygous for the C-allele will rather be in the PgR negative group. For each patient belonging to the PgR negative group, 0.379 patients will belong to the PgR positive group.

PgR status					
	OR	Std. Err.	p-value	95% CI	
heterozygous (GC)	1.280	0.623	0.612	0.493	3.322
homozygous (CC)	0.379	0.387	0.342	0.051	2.808
ER	2.468	0.560	<0.001	1.582	3.849
HER2	0.974	0.212	0.903	0.635	1.492
p53	0.649	0.167	0.094	0.392	1.076
age	1.008	0.018	0.674	0.972	1.044
tumor size	0.740	0.118	0.058	0.542	1.011

Tab. 22. Influence of the genotype (relative to the homozygous GG-genotype), ER status, HER2 amplification, p53 expression, age, and tumor size on the distribution of PgR negative or PgR positive tumors.

In summary, within the study group a significant association of breast cancer risk with the genotype was found for the group of heterozygous patients (GC) compared to patients homozygous for the wildtype allele (GG), which verified the findings from Ristimäki et al [6]. In contrast, no statistically significant correlation between the genotype in the COX-2 G → C SNP and larger tumor size, higher histological grading, negative hormone receptor status, p53 expression, HER2 amplification, or ductal type of histology could be found, although, sometimes an agreeing trend was shown. This may be due to a very small number of cases in one group (e.g. in the group of lobular carcinomas), or may be the effect of regional differences, which is often seen in polymorphism analysis. The results of this study suggest that the heterozygous GC-genotype has a protective effect on breast cancer risk. However, this effect can not be explained by the missing Sp1-site at the C-allele, which results in a 30% decreased promoter activity, because the analysis of the homozygous CC-genotype, which should protect from breast cancer even more than the heterozygous genotype [103], revealed no significant correlations.

4.10. 2D GE analysis for the discovery of new serum biomarkers

There is an urgent need for new serum biomarkers of breast cancer because it was found in daily routine that the sensitivity (nearly 30% in breast cancer) and specificity (at low concentrations, e.g. 1 – 10 ng/ml) of the serum biomarkers used today is not sufficient (Tab. 23), and that different research groups found conflicting results. Therefore, representative samples from the serum bank collected in this dissertation was used to find new biomarkers for diagnosis and treatment of breast cancer, in cooperation with the group of Lukas A. Huber, especially with Hong-Lei Huang (Biocenter, Division of Cell Biology, Innsbruck Medical University, Innsbruck.

Cancer specific serum marker	**Sensitivity**	**Sensitivity in metastatic patients**	**Note**
Carcinoembrogenic antigen (CEA)	16.7%	43.1%	[104]
Cancer antigen (CA) 15.3	33.0%	80.8%	[104]; marker for determining response to chemotherapy treatment and to evaluate the recurrences
Serum tissue polypeptide specific antigen (TPS)	30.0%	66.3%	[105-107]

Tab. 23. Sensitivity of breast cancer serum biomarkers in metastatic and non-metastatic patients.

Because of the low specificity and sensitivity serum biomarkers know to date for breast cancer (Tab. 24) are rarely used to monitor the treatment with chemotherapeutica at the Division of Senology at the General Hospital in Vienna. For a clinically useful biomarker (or a set of biomarkers) not only the specificity and sensitivity is important, but also that this biomarker is easily accessible. Therefore, serum is the first choice, since serum constantly perfuses tissues. It is conceivable that the presence of disease can be detected by measuring the altered levels or the total abundance of the proteins in the patient's serum.

CA 15-3	Assays for MUC1 (polymorphic epithelial mucin); considered the "gold" standard; MUC1 overexpression could allow the neoplastic cell to escape detection by the immune system
CA 125	Mostly used in patients with epithelial ovarian cancer; moderate rates of detection in metastatic BC; elevated levels are more common in visceral or pleural-based diseases than with bone or soft tissue involvement; huge protein (> 1 mio daltons)
CEA	Domain structure of CEA proteins and the heavy chain of IgG are very similar → CEA proteins involved in the intercellular / cellular-matrix recognition mechanisms
CA 19-9	May be helpful in establishing the nature of pancreatic masses; devoted levels in gastrointestinal tumors / mammary & bronchial carcinomas
TPS	Different epithelial cells express characteristic, differentiation-dependent combinations of two or more cytokeratins (CKs); measures CK8 & CK18

Tab. 24. Serum biomarkers for breast cancer.

Because the presence of the abundant serum proteins Albumin and IgG influence the resolution of 2D gel electrophoresis, the separation of these two proteins is necessary. Therefore, a new strategy for breast cancer serum proteome research using 2-D DIGE (two-dimensional difference gel electrophoresis) was developed and contains the following steps (manuscript submitted [108]):

1. Group the serum samples according to age.
2. Add 4 protease inhibitors.
3. Albumin/IgG depletion at reducing condition (5% Acetonitrile).
4. Concentrate the sample using a centrifugal filter (Centriplus® YM30 Centrifugal Filter Units; Millipore, #4412; Billerica, USA).
5. Fluorescence labelling with Cy3 and Cy5 and 2D electrophoresis.
6. Image acquisition and biological variation analysis (BVA).
7. Spot picking and protein identification by fingerprinting on MS (mass spectrometry).

Fig. 20. The BioCAT 700E work station in process with two coupled POROS® affinity depletion columns. The two columns, with a yellow mark, are visible in the center of the picture.

After building four age groups of the serum samples for healthy controls and for breast cancer patients, four protease inhibitors were added. 200 µg of one group were used for the Albumin

& IgG depletion and the separation was performed on a BioCAD 700E work station (Fig. 20). The affinity depletion was controlled on a 12% SDS gel (Fig. 21), which showed that nearly all Albumin and IgG was separated and collected in the wash fraction (Fig. 23). Since most of Albumin and IgG was depleted, the low molecular weight proteins which bind to Albumin and IgG (they serve as carrier proteins) were also depleted and lost for further analysis. To solve this problem and to harvest more low molecular weight proteins, reducing conditions (5% ACN in PBS) were used to release these proteins from Albumin and IgG prior to depletion (Fig. 22).

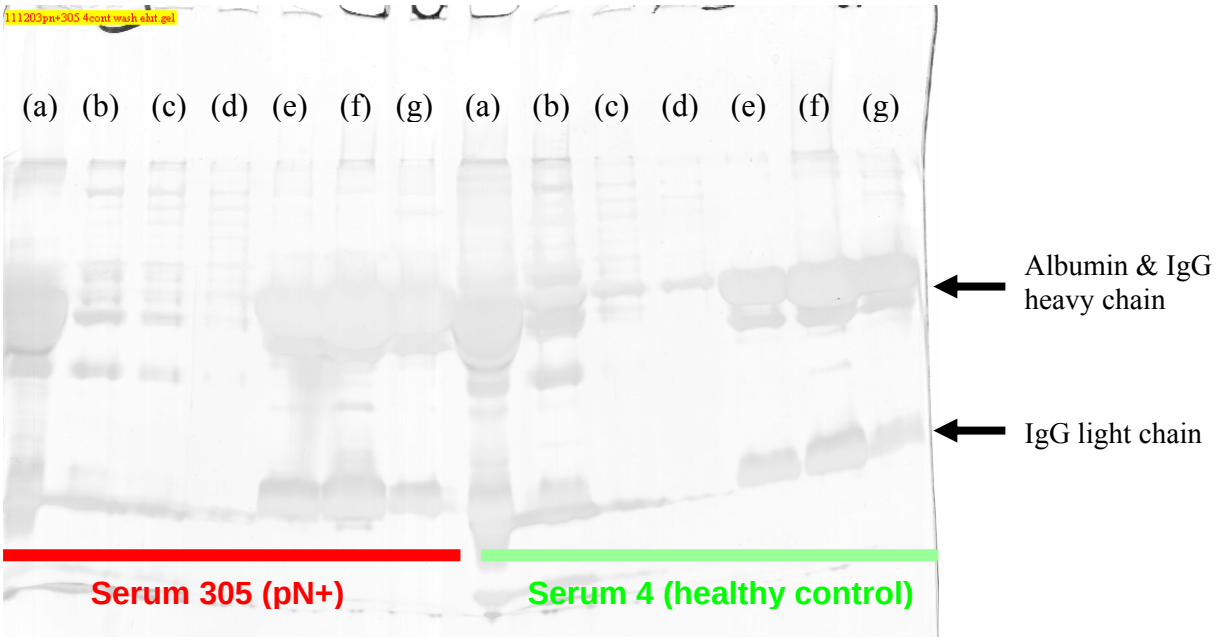


Fig. 21. SDS gel to control successful affinity depletion. (a) pure serum sample, (b-d) elute fraction 1-3, (e-g) wash fraction 1-3.

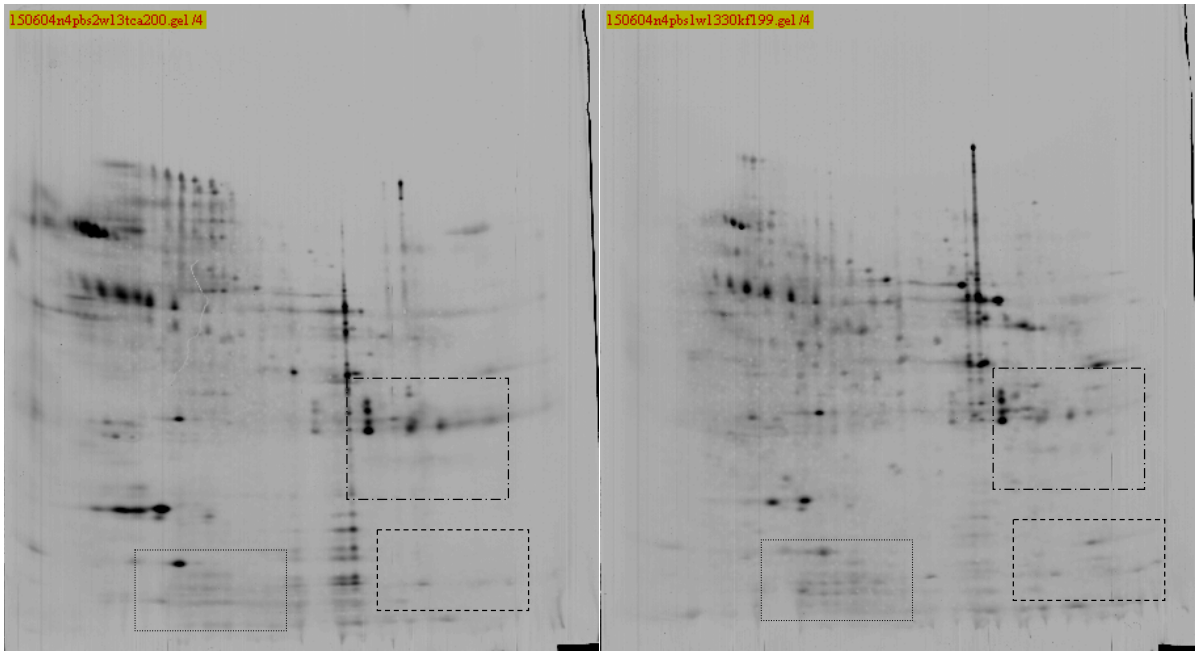
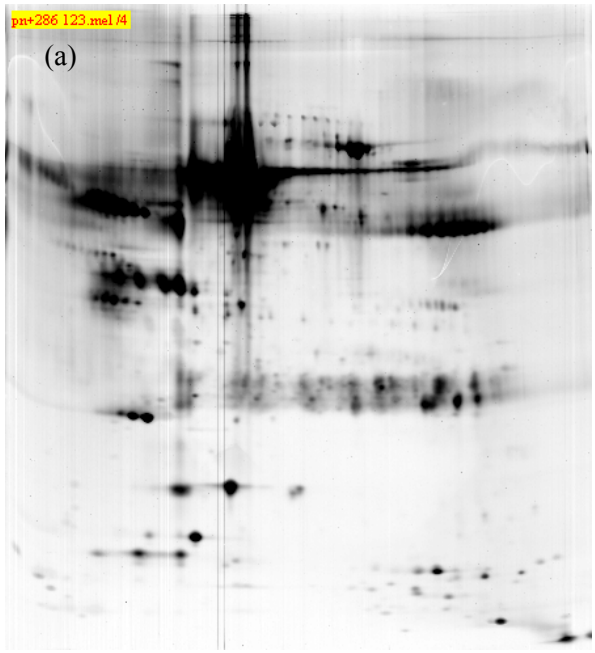
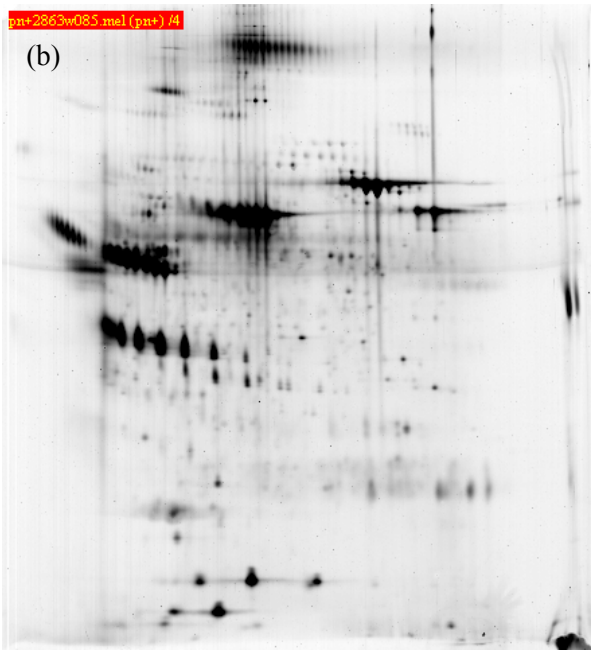


Fig. 22. Albumin and IgG depletion with different conditions: (a) non-reducing (PBS) and (b) reducing (5% ACN in PBS). Boxes mark regions were the differences can be seen clearly.





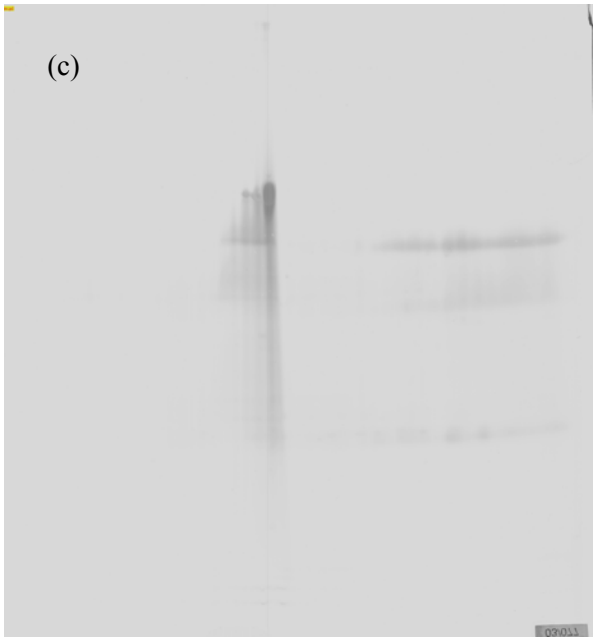


Fig. 23. 2D gels, (a) sample without Albumin and IgG depletion, (b) sample with Albumin and IgG depletion (without reducing conditions), and (c) separated Albumin and IgG fraction.

From this point on the practical work was done by Hong-Lei Huang in Innsbruck and therefore will be summarized only briefly. During the analysis we were in contact constantly. A further step to concentrate the proteins was done by centrifugal filtration using Centriplus® Centrifugal Filter Units YM30. Afterwards the different samples were labelled with fluorescent dyes (healthy control group with Cy3, breast cancer patient group with Cy5, and standard samples with Cy2). A 2D DIGE analysis was done with the fluorescently labelled proteins and after the image acquisition, a biological variation analysis (BVA) was performed (Appendix; Fig. 26 – 30). The results of the BVA showed eight protein candidates that are up-regulated in patients and nine protein candidates that are down-regulated (average ratio from 1.36 to 1.60 and from -1.45 to -2.43, respectively) (Tab. 25).

The 17 spots correlating to the recently found proteins were picked and digested with trypsin. To identify these proteins, mass spectrometry analysis (SELDI-TOF with IDA-Cu^{2+}-Cellulose as the surface) are currently underway by Isabel Feuerstein (laboratory of Günther Bonn, Institute for Analytical Chemistry and Radiochemistry, University of Innsbruck), therefore no results can be presented here. After successful SELDI-TOF analysis, another round of 2D DIGE with other serum samples will be performed to check if the same proteins will be found and if the results can be reproduced.

(a) Serum proteins up-regulated in the patient group.

Protein Table		patients / controls (all ages)		patients / controls (> 60 years)		patients / controls (50 – 60 years)		patients / controls (40 – 50 years)		patients / controls (< 40 years)	
Master No.	Appearance	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio
1595	24 (24) A, M	0.12	1.45	0.021	-2.53	0.0036	1.93	0.018	1.41	0.047	7.87
1802	18 (24) A, M	0.087	1.43	0.015	2.69	-	1.25	0.49	-1.17	-	1.75
2020	24 (24) A, M	0.045	1.46	0.25	1.49	0.51	-1.25	0.53	1.19	0.074	2.88
2058	24 (24) A, M	0.014	1.47	0.0048	1.84	0.95	-1.06	0.39	1.26	0.0047	2.26
2064	24 (24) A, M	0.024	1.36	0.021	1.52	0.0032	-1.31	0.0086	1.19	0.0031	2.53
2128	15 (24) A, M	0.021	1.52	0.4	1.42	-	1.51	-	1.45	-	1.74
2153	9 (24) A, M	0.0032	1.60	0.019	1.67	-	-	-	1.48	-	-
2264	18 (24) A, M	0.22	1.41	-	1	-	-1.05	0.88	1.19	0.028	2.26

(b) Serum proteins down-regulated in the patient group.

Protein Table		patients / controls (all ages)		patients / controls (> 60 years)		patients / controls (50 – 60 years)		patients / controls (40 – 50 years)		patients / controls (< 40 years)	
Master No.	Appearance	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio
1887	18 (24) A, M	0.00021	-1.62	0.12	-1.28	-	-2.21	0.042	-1.59	-	-2.02
1901	24 (24) A, M	5.00E-08	-2.41	0.039	-1.93	0.0024	-3.12	0.044	-2.33	0.062	-2.45
1910	24 (24) A, M	7.80E-09	-1.99	6.70E-05	-1.81	0.0026	-2.43	0.056	-1.64	0.011	-2.16
2024	24 (24) A, M	2.00E-05	-2.43	0.0098	-4.05	0.097	-1.53	0.077	-1.77	0.0032	-4.01
2033	24 (24) A, M	0.01	-2.24	0.0092	-3.50	0.65	1.06	0.39	-1.17	0.00058	-18.62
2055	21 (24) A, M	0.00027	-2.30	0.01	-4.46	-	-1.55	0.11	-1.40	0.043	-3.01
2087	24 (24) A, M	0.032	-1.52	0.06	-2.67	0.59	-1.33	0.73	-1.16	0.41	-1.61
2101	24 (24) A, M	0.00012	-1.90	0.038	-3.10	0.29	-1.35	0.19	-1.51	0.045	-2.25
2129	24 (24) A, M	0.00047	-1.45	0.015	-1.77	0.032	-1.73	0.59	-1.08	-	-1.34

Tab. 25. Serum proteins (a) up-regulated and (b) down-regulated in breast cancer patients relative to healthy controls and the distribution in each age group. The Master No. is the protein spot number on the master spot map assigned by the DeCyder program. Appearance indicates the number of Spot Maps in which the selected spot appears, and the function these Spot Maps have. (Example: "24 (24) A, M" means that the spot is present in 24 out of 24 Spot Maps. Furthermore, the Spot Maps are all assigned to be used in statistical analysis. The spot is also present on the master image. The value from the T-test is the p-value. A positive average ratio means that this protein is up-regulated and a negative average ratio means that the protein is down-regulated in the patient group.

4.11. Serum Biomarker Chip results

The Serum Biomarker Chip contains 120 antibodies and is designed to measure serum or plasma proteins, with known involvement in different cancer forms, with a sensitive two-color labelling and fluorescent detection method. The aim of this experiment was to identify one or several different biomarkers with the Serum Biomarker Chip which can then be analyzed by ELISA in a comprehensive plasma bank to evaluate the findings. To analyze this experiment a statistical test, the Grubb's Z-test was performed in Excel. The Grubb's Z-test (or extreme studentized deviate), is a modified Z-test, which is implemented for the detection of outliers in replicate slide batches and is recommended by the US Environmental Protection Agency (EPA) as a statistical test for outliers (US EPA, 1992).

Serum pools of ten different malignant breast cancer patients, ten women with benign lesions, and ten healthy controls, respectively were done to eliminate the patient specific differences because only one experiment with two chips was done (Tab. 26). With the first chip, the pooled plasma samples of the malignant breast cancer patients were compared with the pool of the healthy controls, and with the second chip the pool of women with benign lesions was compared with the pool of the healthy controls.

Concerning the statistical analysis, which was done in Excel with a macro provided from Schleicher & Schuell, the following points should be mentioned and they should explain which data is more accurate – raw or trimmed. The used Grubb's Z-Test is applied to triplicate values from a single channel at a time to remove outliers from the data set. Data points with zero signals are also removed after this test. Collectively, this is referred to as 'Trimmed data'. 'Raw data' includes all data points regardless of there values. The Grubb's Z-Test assumes that information is collected from a normally distributed data set. Within an experiment, the sample size is limited to three (three spots on a pad). One must be aware that in particular cases artefacts may arise due to the assumption of a normal distribution during the Grubb's Z-Test. These artefacts typically occur when a large standard deviation does not allow suspect data points to be omitted from down stream calculations automatically. Statistically, it is valid to include these data points in down stream calculations since one can not determine which it the 'true' value. Scientifically these data points may warrant further investigation as to what caused the large deviation in the first place. Potential causes of a large standard deviation for triplicates are, but not limited to: non-uniform background

surrounding spots, dust contamination, antigens present below detectable levels, inaccurate spot detection during analysis, or damage to slide surface. After controlling the raw data set it was decided to use the 'Trimmed data' for the following discussion of the results.

Table 27 shows the five most up- and down-regulated plasma biomarkers ranked by the fold change (chip one: ratio of malignant breast cancer group versus healthy control group; chip two: ratio of benign lesion group versus healthy control group). The calculated ratios and the inferred from these two experiments are also shown.

On the first chip there were some really interesting results. In the up-regulated group, the plasminogen activator inhibitor (PAI-1; ratio 2.65) and the urokinase plasminogen activator (uPA; ratio 1.22) were found to be the most promising results of the Serum Biomarker Chip which should be analyzed by ELISA in the complete plasma bank to evaluate the findings in the near future. They are already known for their critical role in cancer invasion and metastasis [109]. The prognostic value of uPA/PAI-1 in patients with axillary lymph node-negative breast cancer had suggested that high levels of uPA and PAI-1 in breast cancer are associated with a preferential response to adjuvant chemotherapy but relative resistance to hormone therapy [110]. Therefore these two factors have predictive value for therapy success in advanced BC [110]. Parker et al. [111] examined the molecular signature that defines the tumor microvasculature. The up-regulation of vascular epithelial-cadherin (VE-cadherin), as found on the Serum Biomarker Chip (ratio 1.33), was validated in breast tumor vasculature and is essential for proliferating and migrating endothelial cells in invasive breast cancer [112]. The Von Willebrand Factor (vWF; ratio 1.28) is an important factor at the angiogenesis of breast cancer and supports the tumor vascular invasion process. An antibody against vWF is already in use for microvessel density (MVD) staining analysis in immunohistochemistry [147].

To find the chorionic gonadotropin-α (ratio 1.26) in the dataset of up-regulated proteins was surprising and opposite to published findings. It was shown that chorionic gonadotropin-α inhibits DMBA-induced (7,12-dimethylbenz[a]anthracene) rat mammary carcinogenesis through inactivation of programmed cell death, a p53-dependent process which is modulated by c-myc expression [113]. Srivastava et al. [113] hypothesized that the mechanism of tumor inhibition could make hCG treatment a useful approach for the prevention and therapy of breast cancer. Rao et al. [114] investigated the anti-proliferative and anti-invasive effects of human chorionic gonadotropin (hCG) in MCF7 breast cancer cells and found that the activation of the transcription factors NF-κB and AP-1 was inhibited.

No.	Age	Group	Menopausal status	ER[13]	PgR[13]	Grading[14]	pT[14]	pN[14]	Tumor size [cm]	p53[13]	HER2/neu[13]	MIB-1 [%]	
G1	37.30	healthy											
193	34.14	malignant	premenop.	0	0	3	1b	1a	0.7	3	3	40	ductal
360	37.31	benign	premenop.										
G2	40.20	healthy											
262	40.30	malignant	premenop.	2	0	3	2	0	5	0	3	10	ductal
294	40.73	benign											
G4	57.40	healthy	postmenop.										
297	60.89	malignant	postmenop.	3	2	1	1b	1	0.8	0	0	5	ductal
210	58.20	benign	postmenop.										
G6	54.30	healthy	postmenop.										
223	54.98	malignant	postmenop.	2	0	1	2	0	3	2	0	30	ductal
332	54.72	benign											
G8	34.50	healthy	premenop.										
313	33.68	malignant	premenop.	0	2	3	2	1	3	0	3	40	ductal
329	33.79	benign	premenop.										
G9	45.80	healthy											
321	45.54	malignant	premenop.	1	1	2	2	0	3	0	0	20	ductal
208	46.11	benign	premenop.										
G10	50.80	healthy	premenop.										
232	47.98	malignant	premenop.	3	2	3	2	3a	3.8	2	0	40	ductal
324	48.68	benign											
G11	52.50	healthy	postmenop.										
298	50.26	malignant	postmenop.	0	0	3	1c	0	2	0	3	40	ductal
211	50.25	benign											
G16	56.40	healthy	postmenop.										
209	60.23	malignant	postmenop.	0	0	3	2	1a	4.5	3	3	40	ductal
257	56.50	benign	postmenop.										
G17	60.10	healthy	postmenop.										
307	62.37	malignant	postmenop.	0	0	3	1c	0	1.5	3	0	90	ductal
325	62.12	benign	postmenop.										

Tab. 26. Characteristics of 10 plasma probes each pooled to the healthy control group, the malignant breast cancer group, and the group with benign breast lesions, respectively.

[13] 0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = highly positive
[14] on the basis of the international TNM classification

	up-regulated			down-regulated		
		Ratio	Biomarker name		Ratio	Biomarker name
malignant / healthy	1	2.65	plasminogen activator inhibitor	1	0.65	GM-CSF
	2	1.33	VE-Cadherin	2	0.65	C-reactive protein
	3	1.28	Von Willebrand Factor	3	0.68	IgM
	4	1.26	Chorionic gonadotropin-α	4	0.69	Fibroblast growth factor-basic
	5	1.22	Urokinase Plasminogen Activator	5	0.69	IL-13
					0.76	Neuron specific enolase
					0.76	TNF α
benign / healthy	1	1.61	Laminin	1	0.40	Tumor-associated Trypsin Inhibitor
	2	1.50	Fas ligand	2	0.57	Sialyl Lewis X
	3	1.44	TNF α	3	0.61	VCAM-1
	4	1.25	IgM	4	0.62	CYFRA 21-1
	5	1.23	IL-13	5	0.64	E-Selectin
					0.65	CA 125
malignant / benign[15]	1	3.89	plasminogen activator inhibitor	1	0.53	TNF α
	2	2.62	Tumor-associated Trypsin Inhibitor	2	0.53	Fas ligand
	3	1.86	Chorionic gonadotropin-α	3	0.55	IgM
	4	1.83	Von Willebrand Factor	4	0.56	IL-13
	5	1.82	VCAM-1	5	0.72	Neuron specific enolase
		1.64	CYFRA 21-1			Laminin
						C-reactive protein

Tab. 27. The five most up- and down-regulated plasma biomarkers ranked by the calculated ratio for both experiments (experiment 1: malignant breast cancer group vs. healthy control group; experiment 2: group with benign breast lesions vs. healthy control group) and the inferred ratios and ranking of malignant breast cancer group / benign breast lesions group.

[15] Calculated Ratios of the two experiments (malignant breast cancer group / healthy control group & benign lesions group / healthy control group).

There are two possible explanations why a protein is down-regulated in breast cancer patients compared to healthy controls. On the one hand it is possible that the decreased levels are promoting the angiogenesis or the tumor formation or that survival factors are reduced. On the other hand it is possible that the down-regulation of proteins in the human plasma of a breast cancer patient is due to the fact that the tumor needed almost everything of these specific proteins to promote the tumor progression.

The down-regulation of GM-CFS (granulocyte-macrophage colony-stimulating factor; ratio 0.65) is in accordance with some studies that have shown that a vector expressing murine GM-CSF was efficacious to delay tumor growth or, in rare cases, to complete regression of visible tumor in murine colorectal [115] and renal tumor models [116]. After showing a good safety profile in preclinical studies [117] this vector has entered clinical trials for metastatic melanoma using direct injection into surface tumors in patients with unresectable disease. In Balb/c mice (4T1 breast cancer cell line was originally isolated from a spontaneous mammary tumor of these mice) it was shown that direct intratumoral injection of this vector led to a delay in tumor growth and that the efficacy of chemotherapeutics given at the same time was unaltered, therefore indicating the possibility of combinig chemo- and immunotherapy [118]. In clinical trials, Alters et al. [119] found that dentritic cells immunized with GM-CSF and IL-13 (ratio 0.69) can be used to stimulate the immunity of cancer patients by protective and therapeutic antitumoral activities. The down-regulation of IgM (ratio 0.68) seems logical in the context of the fact that the IgM expression is crucial for B cell development [120], and moreover, because specific B-cell responses occur during breast cancer angiogenesis.

Unexpected was the down-regulation of C-reactive protein (ratio 0.65), basic fibroblast growth factor (ratio 0.69) and the weak down-regulation of neuron specific enolase (NSE; ratio 0.76). C-reactive protein is a plasma inflammatory protein and is expected to be elevated in tumor patients. I cannot explain why decreased levels of fibroblast growth factor are found, a growth factor that induces the cells to progress from G_0/G_1 to S-phase [121]. NSE is an already used breast cancer biomarker to monitor patients for lung metastasis. It is possible that none of the examined patients of the breast cancer group will develop a metastatic lung tumor; nevertheless, it was confusing to find this protein to be down regulated.

Most surprising was the finding that tumor necrosis factor α (TNF α; ratio 0.76) was reduced in breast cancer patients. This is in contrast with published data showing that TNF α may be directly involved in vivo in the progression of metastatic breast cancer [122]. Presumably, this finding has to be a fault of the Serum Biomarker Chip experiment. Berberoglu et al. [123] recently showed that the plasma concentration of tumor necrosis factor α can be an indicator

of response to neoadjuvant chemotherapy, and that this factor could be used in clinical decision-making for patients with locally advanced breast cancer. There are many more publications showing elevated plasma levels of TNF α and therefore the results of the Serum Biomarker Chip were rechecked for faulty values or analysis mistakes. But no mistake could be found and I cannot explain this result. Surprisingly, on the second chip, which compared the benign lesion group with the healthy control group, TNF α (ratio 1.44) was correctly found in the up-regulated group.

The Fas/Fas ligand system plays an important role in cellular apoptosis and is involved in cancer cell death induced by the immune system and anticancer drugs [124]. This may explain why an immune system of a patient with a benign lesion, which tries to eliminate these abnormally transformed cells, up-regulates the Fas ligand (ratio 1.50). Fas is a trans-membrane receptor that belongs to the TNF receptor family and plays a key role in apoptosis signalling [125]. The interaction of Fas and Fas ligand plays an important role in tumor progression and drug-induced apoptosis during chemotherapy [126, 127]. When both Fas and Fas ligand are expressed, prevention of Fas – Fas ligand interactions or inhibition of Fas signals may lead to resistance against Fas-mediated apoptosis [128, 129]. The up-regulation of IL-13 (ratio 1.23) is in contrast to the down-regulation observed in the first experiment, but can be easily explained by the fact that IL-13 stimulates the immune system of patients with abnormally transformed cells [119]. Likewise, the increased level of IgM (ratio 1.25) could be a response of the immune system towards an increased development of B cells [120].

Laminin is an adhesive and structural glycoprotein and a non-collagenous constituent of the extracellular matrix (basement membrane). The up-regulation of laminin (ratio 1.61) in women with benign lesions was unexpected because extracellular laminin is considered a product of malignant epithelial tumor cells [130]. Sidholm and Imam [131] evaluated plasma laminin as a tumor marker in breast cancer and they found that the laminin level was significantly higher in breast cancer patients than in healthy controls (sensitivity: 75%, specificity: 97%, with a 98% positive predictive value, 66% negative predictive value, and 82% diagnostic efficiency). There was also a significant association between plasma laminin and metastasis (sensitivity: 77%, specificity: 100%, with a 100% positive predictive value, 81% negative predictive value, and 88% diagnostic efficiency).

Tumor-associated trypsin inhibitor (TATI; ratio 0.40), the most down-regulated factor in patients with benign breast disease, will not be a good prognostic factor for discriminating between breast cancer and benign lesions, because TATI levels overlap in these two groups [132]. TATI was initially detected in the urine of patients with ovarian cancer [133, 134]. This

peptide is also produced by the mucosa of the gastrointestinal tract, where it may protect the mucosal cells from proteolytic breakdown. Elevated serum and urine levels occur particularly in patients with mucinous ovarian cancer and may also occur in nonmalignant diseases, e.g., pancreatitis, severe infections, and tissue destruction [135]. A function of vascular cell adhesion molecule-1 (VCAM-1; ratio 0.61), a cell surface glycoprotein expressed by cytokine-activated endothelium, in breast cancer is not described. VCAM-1 mediates the adhesion of monocytes and lymphocytes, and in inflammatory conditions it is up-regulated in endothelium of postcapillary venules.

The adhesion of circulating cancer cells to the vascular endothelium is an important step in the hematogenous metastasis of cancer [136]. E-selectin (ratio 0.64) expressed on endothelial cells and carbohydrate ligands expressed on cancer cells mediate this adhesion. The sialyl Lewis x (ratio 0.57) oligosaccharide determinant is an essential component of leukocyte counter-receptors for E-selectin- and P-selectin-mediated adhesions of leukocytes. This oligosaccharide molecule is displayed on the surfaces of granulocytes, monocytes, and natural killer cells. The formation of leukocyte adhesions to these selectins is an early and important step in the process that ultimately allows leukocytes to leave the vascular tree and become recruited into lymphoid tissues and sites of inflammation. Thus, sialyl Lewis x and E-selectin should be up-regulated in the benign lesion group rather than down-regulated, as found with the Serum Biomarker Chip.

Serveral breast cancer specific biomarkers which are sometimes measured in clinical practice are also contained on the Serum Biomarker chip (Tab. 28). Interestingly, all these biomarkers are moderately down-regulated rather than up-regulated in patients with breast cancer compared to controls (results of the first chip), which would disqualify all these biomarkers except for insulin-like growth factor binding protein 3 (IGFBP3), where the findings are in accordance with published data. Several studies have demonstrated elevated IGF1 and reduced IGFBP3 plasma concentrations in breast cancer patients, and some have suggested their potential use as prognostic factors [137, 138]. During my master's thesis, I could show that IGF1 plasma levels are about 30% elevated in breast cancer patients compared to women with benign lesions or to healthy controls [139]. Unfortunately, IGF1 could not be measured due to poor protein – antibody binding on the chip. Because this happened on both chips, it appears that the IGF1 specific antibody is not capable of binding the fluorescently labeled protein for whatever reason. Two other breast cancer serum biomarkers, which are routinely measured in Austria, were found in the down-regulated group of the second Serum Biomarker Chip: CYFRA 21-1 (ratio 0.62) and CA 125 (ratio 0.65).

The ErbB2 oncogene (ratio 0.94) encodes a tumor antigen, p185, which is related to the epidermal growth factor receptor (EGFR) [141]. Di Fiore et al. [142] demonstrated that overexpression of ErbB2 alone is sufficient for oncogenic transformation, and Qui et al. [143] showed that erbB2 is a critical component of signalling through the MAP kinase pathway. There are many publications showing that the HER2/neu gene (the human form of erbB2) is amplified and HER2/neu is overexpressed in 25-30% of breast cancers, increasing the aggressiveness of the tumor. Slamon et al. [144] found that herceptin, a humanized monoclonal antibody, appears to block growth signals transmitted by HER2/neu to the nucleus, enhances the response to chemotherapeutic agents, and increases the clinical benefit of first-line chemotherapy in metastatic breast tumors that overexpress HER2/neu [144, 145]. In breast cancer the overexpression of erbB2 confers Taxol resistance by transcriptionally up-regulating CDKN1A which associates with CDC2, inhibits Taxol-mediated CDC2 (p34) activation, delays cell entrance to G2/M phase, and thereby inhibits Taxol-induced apoptosis [146]. Of the ten women included in the pool of breast cancer patients, five had a HER2 amplification, but none of these five patients had metastases. Schulze [140] showed that only metastatic breast cancer patients with positive HER2 amplification have elevated HER2 plasma levels. Therefore, the results of the Serum Biomarker Chip experiment are in agreement with published data.

A final conlusion concerning the Serum Biomarker Chip is that the findings were partially contradicting published results (Tab. 28), as mentioned above. No other group has published any data determined with the Serum Biomarker Chip yet, because it is a new product, therefore the results should be interpreted with great caution. To judge the reliability of the results of the Serum Biomarker Chip, more experiments have to be done, and the results have to be validated with independent methods such as ELISA.

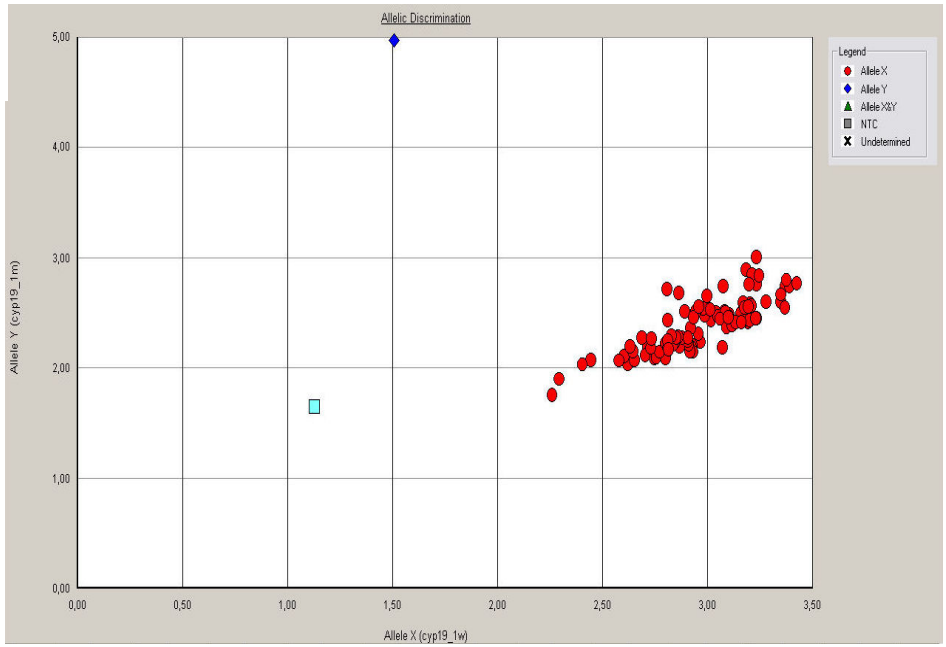
human plasma biomarker	limit values	Ratio malignant BC / healthy controls	Ratio benign breast lesions / healthy controls	published data
α-fetoprotein	0 – 7 kU/l	0.85	1.08	Marker for liver metastasis
CA 15-3	0 – 30 kU/l	0.85	0.84	Prognostic predictor in recurrence BC
CA 19-9	0 – 37 kU/l	0.88	0.68	Gastrointestinal marker
CA 125	0 – 35 kU/l	0.90	0.65	Detection of metastatic BC
CEA (group 2 specific)	0 – 5 µg/l	0.82	-	Elevated in patients with higher-grade tumors
CYFRA 21-1	0 – 3.3 µg/l	1.01	0.62	Marker for disease relapse & assessing traetment efficacy
Neuron specific enolase	0 – 12 µg/l	0.76	1.06	Marker for lung metastasis
erbB2	0 – 27 kU/l	0.94	0.93	Elevated plasma levels in metastatic BC patients!
ER		0.93	-	Levels in BC unchanged
IGF1		-	-	30% elevated plasma levels in BC!
IGFBP3		**0.74**	0.97	Decreased plasma levels in BC!

Tab. 28. Comparison of the resulting ratios of both experiments of some breast cancer plasma markers and of some additional proteins. kU, kilo-Units; BC, breast cancer

5. Appendix

5.1. Allelic discrimination analysis for the CYP19 Codon 39 Tyr → Arg SNP

(a)

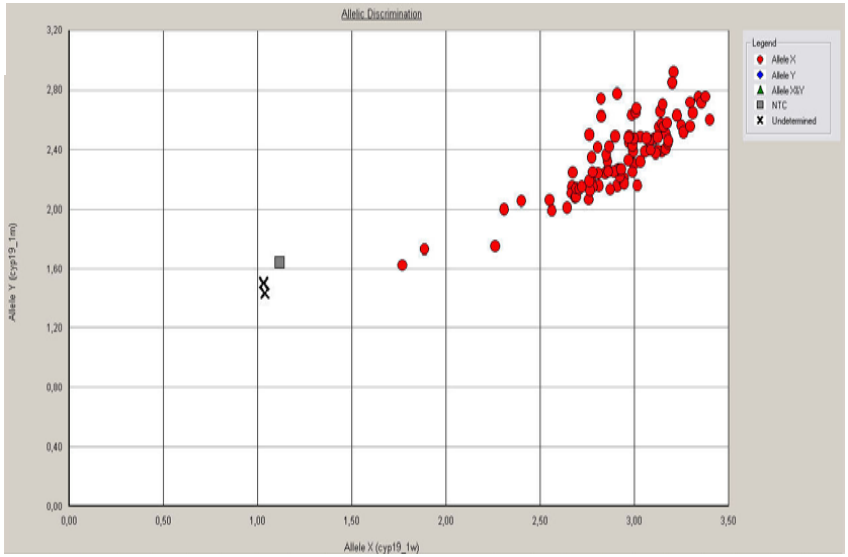


(b)

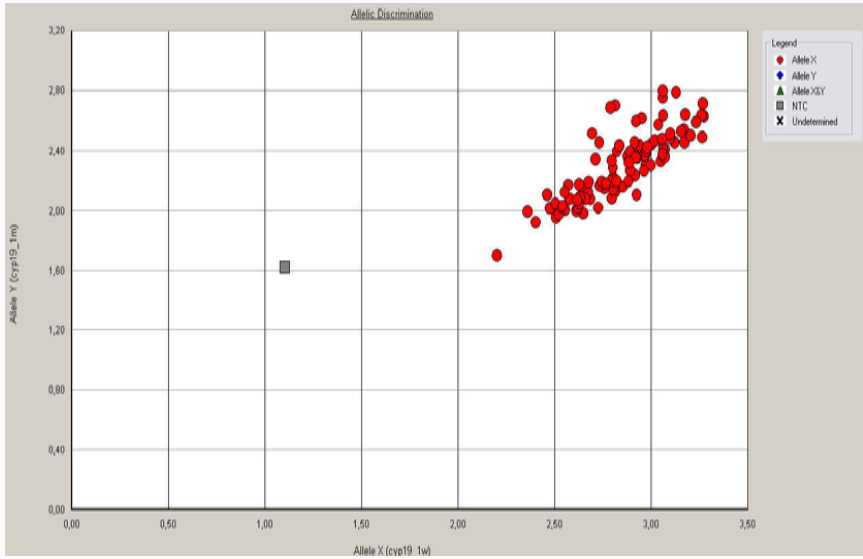
B10		cyp19	2.259	1.752	cyp19_1w
B11		cyp19	3.103	2.484	cyp19_1w
B12		cyp19	3.168	2.592	cyp19_1w
C1		cyp19	3.212	2.652	cyp19_1w
C2		cyp19	3.015	2.433	cyp19_1w
C3		cyp19	3.424	2.767	cyp19_1w
C4		cyp19	3.348	2.602	cyp19_1w
C5		cyp19	3.115	2.385	cyp19_1w
C6		cyp19	3.133	2.415	cyp19_1w
C7		cyp19	2.798	2.089	cyp19_1w
C8		cyp19	3.160	2.414	cyp19_1w
C9		cyp19	3.069	2.190	cyp19_1w
C10		cyp19	3.098	2.474	cyp19_1w
C11		cyp19	3.089	2.483	cyp19_1w
C12		cyp19	3.080	2.511	cyp19_1w
D1		cyp19	3.231	3.010	cyp19_1w
D2		cyp19	1.505	4.972	cyp19_1m
D3		cyp19	3.206	2.565	cyp19_1w
D4		cyp19	3.277	2.599	cyp19_1w
D5		cyp19	3.048	2.467	cyp19_1w
D6		cyp19	2.811	2.437	cyp19_1w
D7		cyp19	2.864	2.197	cyp19_1w
D8		cyp19	2.745	2.092	cyp19_1w
D9		cyp19	2.905	2.247	cyp19_1w
D10		cyp19	3.176	2.548	cyp19_1w

Fig. 24. (a) Allelic discrimination of the PCR fragment of the CYP19 Codon 39 Tyr → Arg SNP (samples 1-95).
(b) Excerpt of the data table of the allelic discrimination; the indicated line (D2) is the one for the Arg-allele. All subjects are homozygous and only one woman has an Arg-allele.
Allele X = Tyr, Allele Y = Arg, NTC = negative control

(a)



(b)



(c)

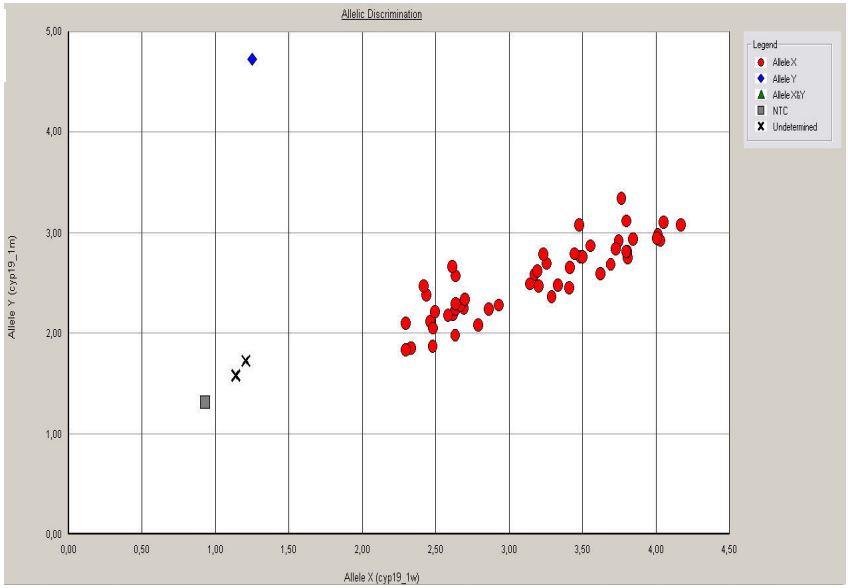


Fig. 25. Allelic discrimination of PCR fragment of the CYP19 Codon 39 Tyr → Arg SNP of samples (a) 96 – 190, (b) 191 – 285, and (c) 286 – 337 & 38, 166, and 185. In (a) two PCR fragments (166 & 185) which did not work are indicated with an "x". The same two fragments also failed in (c). In (c) the one Arg-allele shown is the same as in Fig. 24 (sample 38).

5.2. 2D DIGE analysis & biological variant analysis

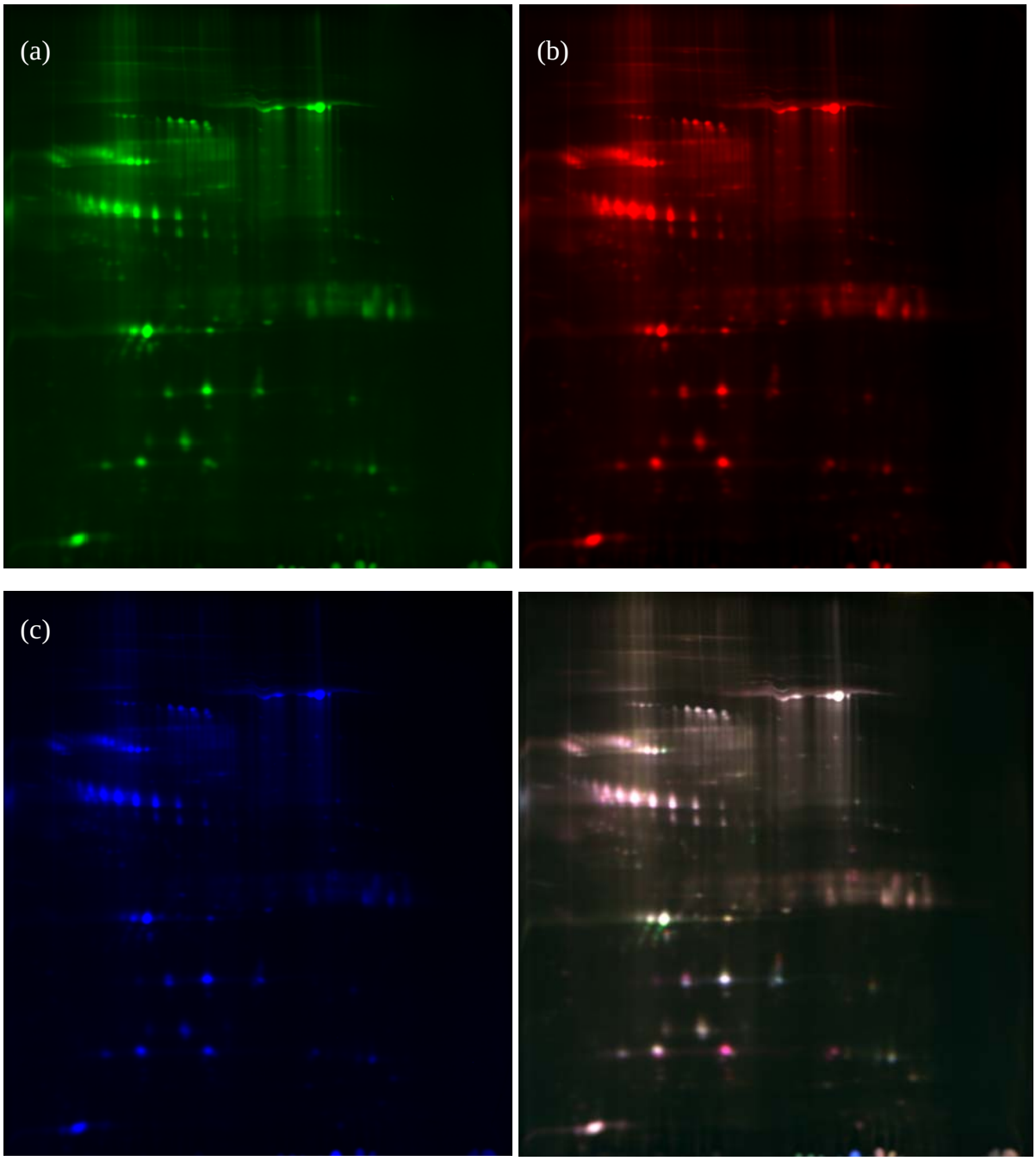


Fig. 26. 2D DIGE image acquisition of serum samples. (a) healthy control group labelled with Cy3, (b) breast cancer patient group labelled with Cy5, (c) standard samples labelled with Cy2, and (d) merged image.

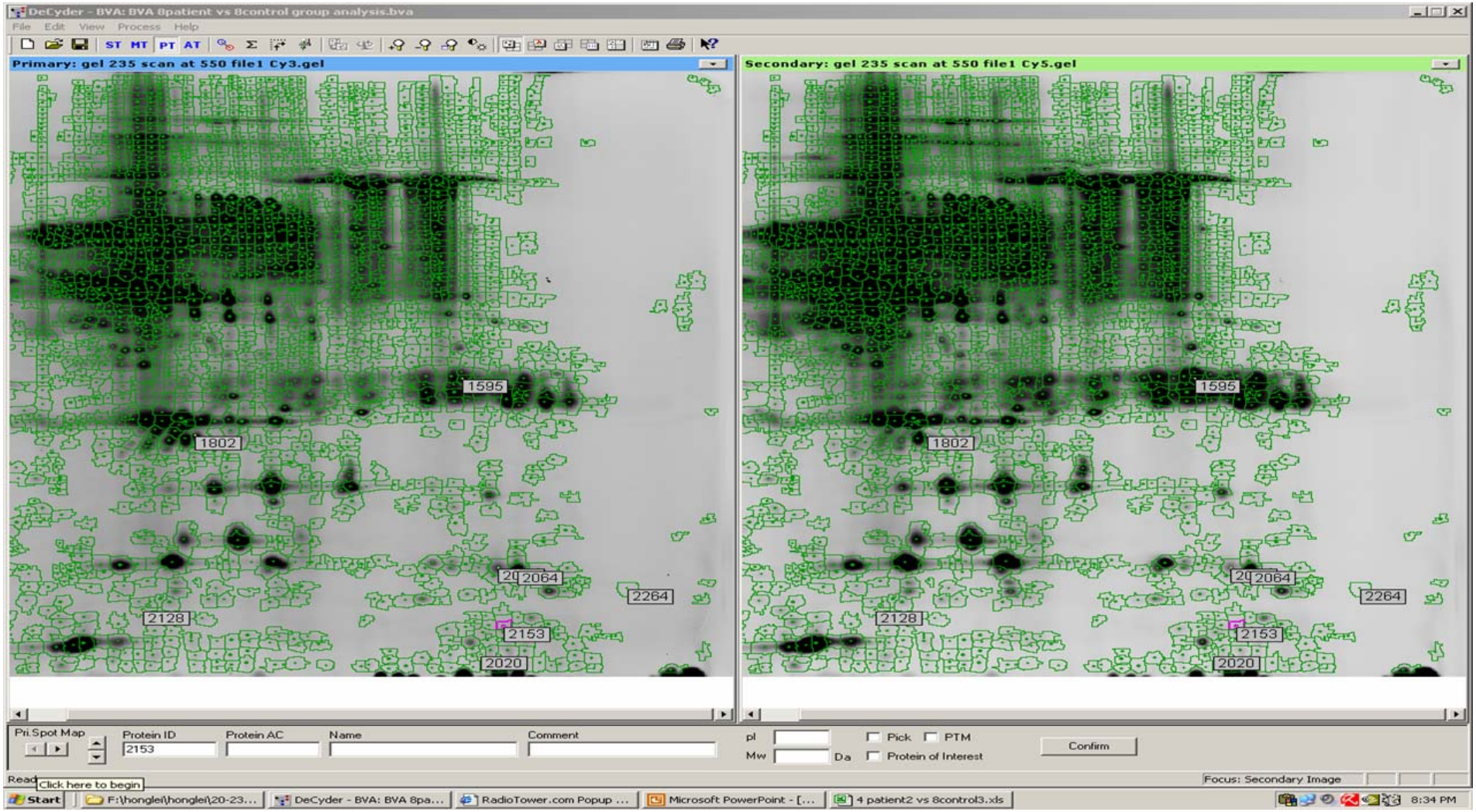


Fig. 27. Biological variation analysis (BVA) between the healthy control group (left side; labelled with Cy3) and the patient group (right side; labelled with Cy5). The marked spots (1595, 1802, 2020, 2058, 2064, 2128, 2153, and 2264) are up-regulated in patients.

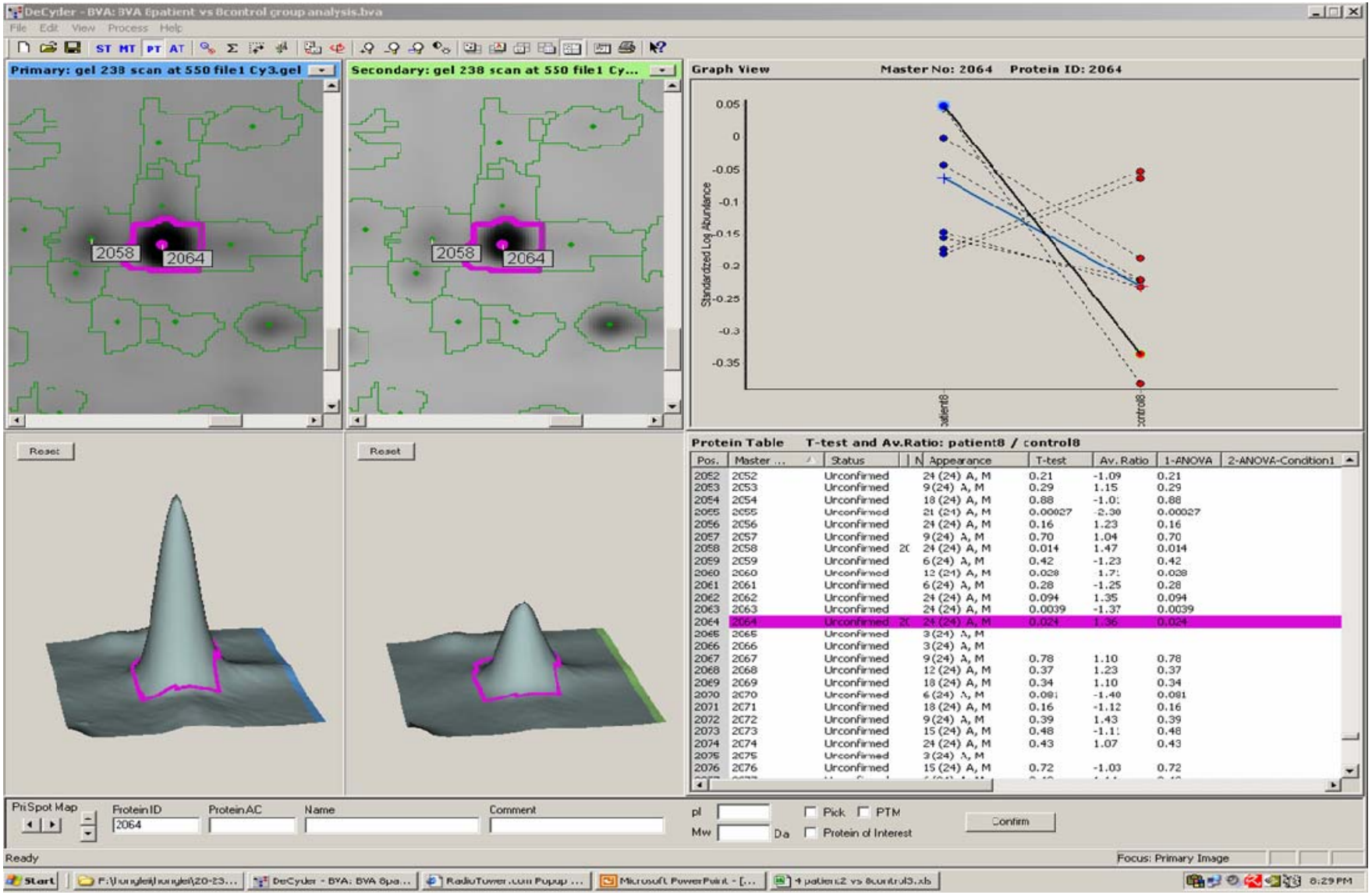


Fig. 28. BVA of spot 2064 which is up-regulated in the patient group.

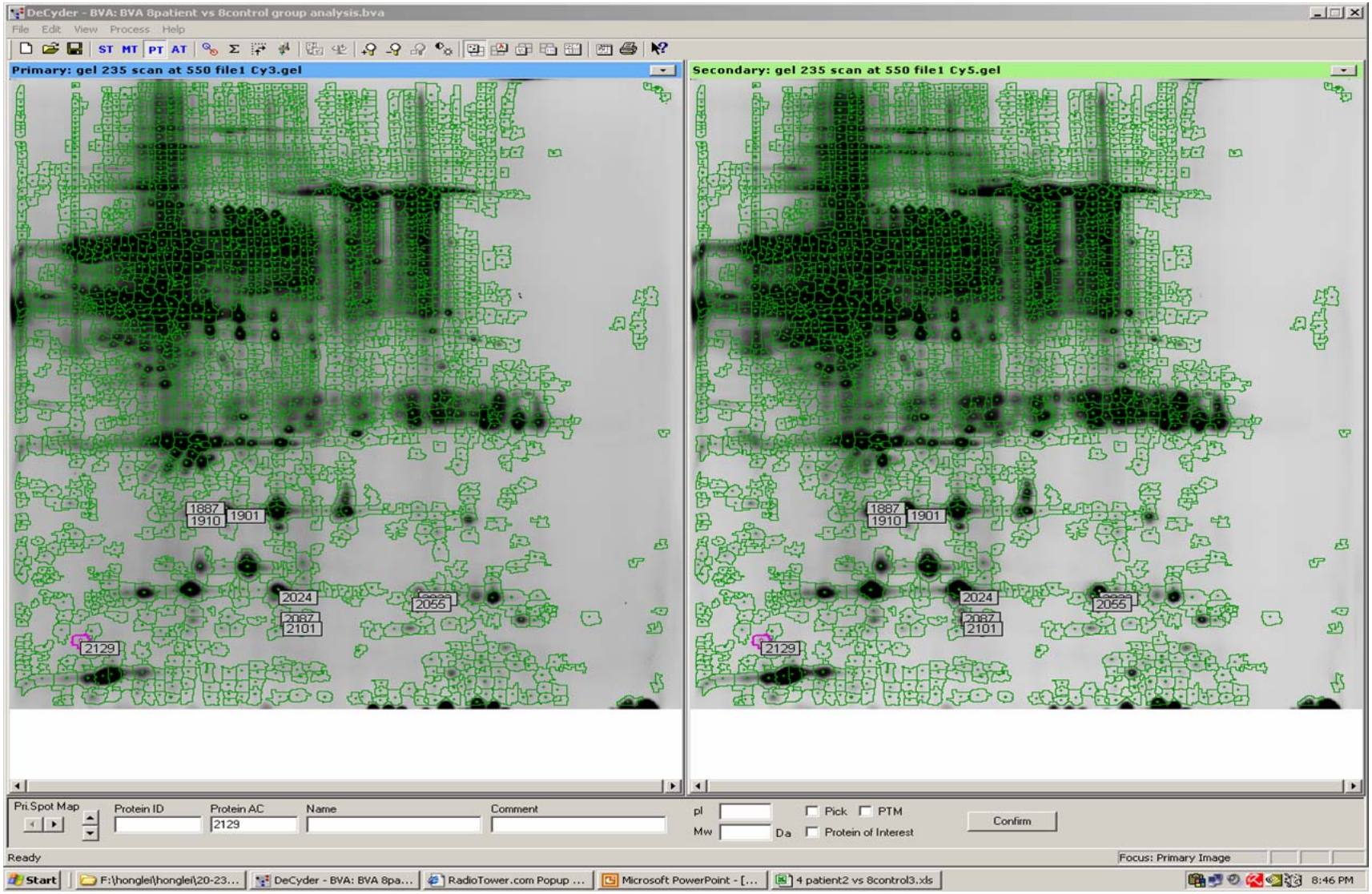


Fig. 29. Biological variation analysis (BVA) between the healthy control group (left side; labelled with Cy3) and the patient group (right side; labelled with Cy5). The marked spots (1887, 1901, 1910, 2024, 2033, 2055, 2087, 2101 and 2129) are down-regulated in patients.

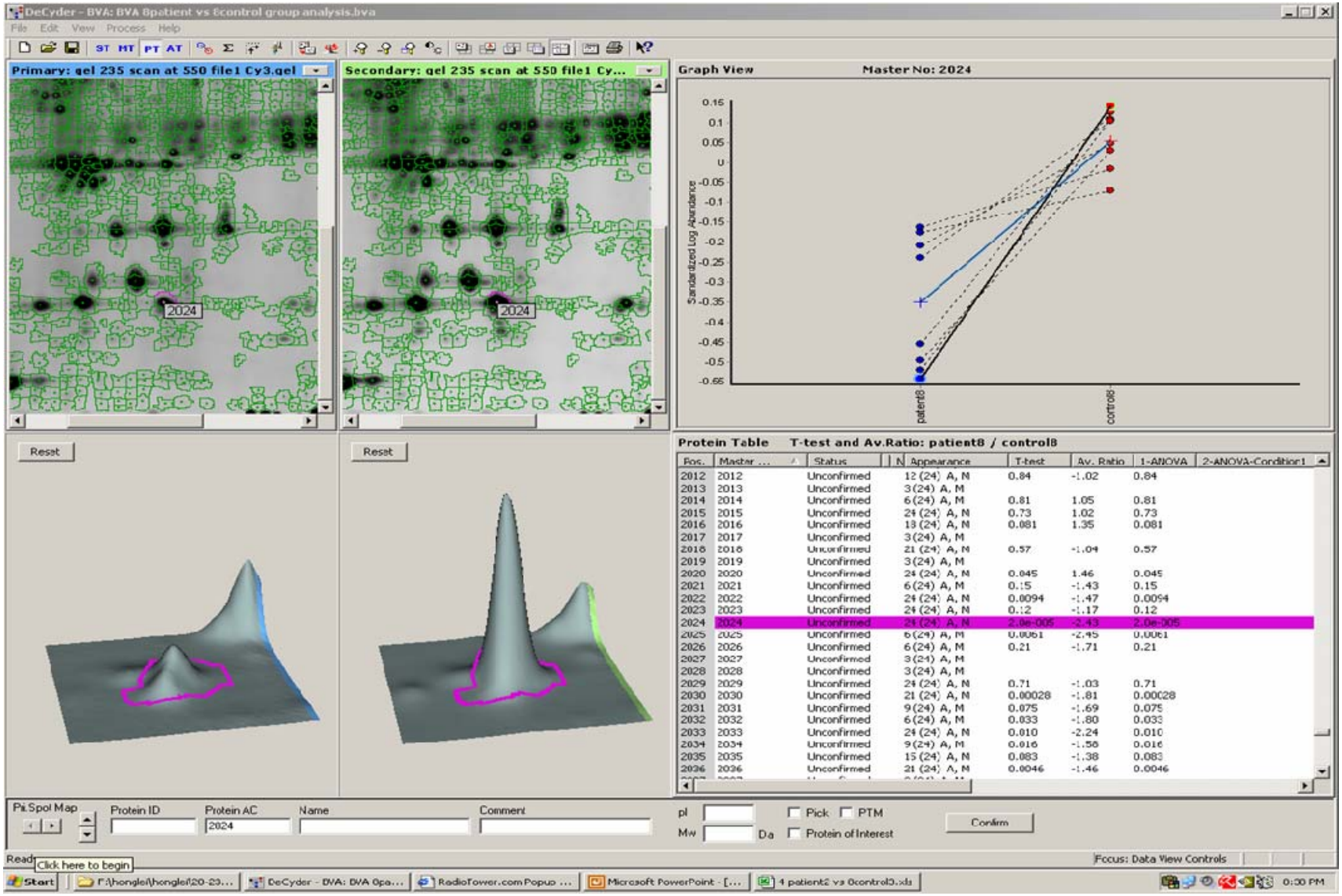


Fig. 30. BVA of spot 2024 which is down-regulated in the patient group.

5.3. Results of the two Serum Biomarker Chip® experiments

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
Alpha fetoprotein	2.133	1.805	2.133	1.805	1,18	1,18	0,85	**0,85**
alpha1 anthchymotrypsin	899	705	899	680	1,28	1,32	0,78	**0,76**
alpha2 macroglobulin	11.995	9.457	11.995	9.457	1,27	1,27	0,79	**0,79**
Angiogenin	2.103	1.840	1.884	1.840	1,14	1,02	0,87	**0,98**
Angiopoietin-2	1.863	1.760	1.863	1.595	1,06	1,17	0,95	**0,86**
Angiostatin	35.288	41.936	35.288	41.936	0,84	0,84	1,19	**1,19**
Apolipoprotein	2.249	1.967	2.249	1.967	1,14	1,14	0,87	**0,87**
Apolipoprotein J	7.701	6.450	7.701	7.250	1,19	1,06	0,84	**0,94**
beta2 microglobulin	5.146	5.084	5.146	5.084	1,01	1,01	0,99	**0,99**
Bone sialoprotein	818	1.032	736	935	0,79	0,79	1,26	**1,27**
CA125	3.214	2.905	3.214	2.905	1,11	1,11	0,90	**0,90**
CA15-3	3.483	3.338	3.483	2.951	1,04	1,18	0,96	**0,85**
CA19-9	3.159	3.113	3.539	3.113	1,01	1,14	0,99	**0,88**
CA50	1.243	1.146	1.243	1.146	1,09	1,09	0,92	**0,92**
Carcinoembryoinc antigen (group 4 specific)	3.910	3.055	3.853	3.055	1,28	1,26	0,78	**0,79**
Carcinoembryoinc antigen (group 2 specific)	3.564	2.915	3.564	2.915	1,22	1,22	0,82	**0,82**
Cathepsin B	2.879	3.141	2.879	3.141	0,92	0,92	1,09	**1,09**
Ceruloplasmin	2.708	2.830	2.708	2.830	0,96	0,96	1,05	**1,05**
Chondroitin Sulftate	3.018	2.993	3.018	2.993	1,01	1,01	0,99	**0,99**
Chorionic gonadotropin-alpha	1.748	2.208	1.748	2.208	0,79	0,79	1,26	**1,26**
Chorionic gonadotropin-beta	1.959	1.714	1.959	1.714	1,14	1,14	0,87	**0,87**
Chromogranin	4.608	3.991	4.608	3.991	1,15	1,15	0,87	**0,87**
Collagen Type I	14.319	13.327	14.319	13.327	1,07	1,07	0,93	**0,93**
complement C4	12.563	9.920	12.563	11.610	1,27	1,08	0,79	**0,92**
C-reactive protein	4.252	2.809	4.821	3.121	1,51	1,54	0,66	**0,65**
Cyclin-dependent kinase inhibitor 2A	1.102	1.044	1.102	1.044	1,06	1,06	0,95	**0,95**
Cytokeratin Fragment 21-1 (CYFRA 21-1)	5.933	6.014	5.933	6.014	0,99	0,99	1,01	**1,01**
Eotaxin	2.476	1.941	2.476	1.941	1,28	1,28	0,78	**0,78**
Epidermal growth factor	1.092	832	1.092	880	1,31	1,24	0,76	**0,81**
Epidermal growth factor receptor	2.170	1.781	1.872	1.781	1,22	1,05	0,82	**0,95**
ErbB2	4.766	4.176	4.766	4.487	1,14	1,06	0,88	**0,94**
E-Selectin	2.103	2.574	2.355	2.574	0,82	0,91	1,22	**1,09**
Estrogen Receptor	2.979	2.782	2.979	2.782	1,07	1,07	0,93	**0,93**
Fas	5.565	5.742	6.539	5.742	0,97	1,14	1,03	**0,88**
Fas ligand	3.227	2.809	3.227	2.571	1,15	1,26	0,87	**0,80**
Ferritin	2.291	2.028	2.018	2.028	1,13	1,00	0,88	**1,00**
Fibroblast Growth factor-7	1.902	1.573	1.654	1.429	1,21	1,16	0,83	**0,86**
Fibroblast Growth factor-basic	4.568	3.606	5.249	3.606	1,27	1,46	0,79	**0,69**
G-CSF	2.219	2.054	2.455	1.893	1,08	1,30	0,93	**0,77**
GM-CSF	4.340	3.687	4.938	3.190	1,18	1,55	0,85	**0,65**
haptoglobulin	1.445	1.081	1.301	1.203	1,34	1,08	0,75	**0,92**
Hemoglobin	28.806	6.160	28.806	6.160	4,68	4,68	0,21	**0,21**
Hepatocyte Growth Factor	1.565	1.174	1.393	1.167	1,33	1,19	0,75	**0,84**
ICAM-1	2.299	2.206	2.594	2.206	1,04	1,18	0,96	**0,85**

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
IgA	31.927	30.820	31.927	30.820	1,04	1,04	0,97	**0,97**
IgG	11.282	10.665	10.834	10.500	1,06	1,03	0,95	**0,97**
IgM	13.124	8.969	13.124	8.969	1,46	1,46	0,68	**0,68**
IL-10	3.786	3.430	3.786	3.019	1,10	1,25	0,91	**0,80**
IL-12p40	2.934	2.323	2.934	2.323	1,26	1,26	0,79	**0,79**
IL12-p70	1.854	1.700	1.621	1.913	1,09	0,85	0,92	**1,18**
IL-13	2.404	1.899	2.754	1.899	1,27	1,45	0,79	**0,69**
IL-17	5.398	4.355	5.398	4.355	1,24	1,24	0,81	**0,81**
IL-1-alpha	4.902	4.093	4.295	4.093	1,20	1,05	0,83	**0,95**
IL1-beta	2.197	1.774	2.197	1.763	1,24	1,25	0,81	**0,80**
IL-2	2.886	2.659	2.886	2.409	1,09	1,20	0,92	**0,83**
IL-2 receptor-alpha	1.817	1.442	1.817	1.442	1,26	1,26	0,79	**0,79**
IL-2 receptor-beta	2.013	1.649	2.013	1.837	1,22	1,10	0,82	**0,91**
IL-3	2.458	2.068	2.458	2.068	1,19	1,19	0,84	**0,84**
IL-4	3.035	2.470	3.035	2.470	1,23	1,23	0,81	**0,81**
IL-5	1.602	1.315	1.602	1.467	1,22	1,09	0,82	**0,92**
IL-6	1.283	1.022	1.283	1.022	1,26	1,26	0,80	**0,80**
IL-7	4.164	3.508	4.164	3.508	1,19	1,19	0,84	**0,84**
IL-8	1.032	908	1.032	908	1,14	1,14	0,88	**0,88**
Insulin	2.236	1.982	2.236	1.982	1,13	1,13	0,89	**0,89**
Insulin-like growth factor binding protein 3	2.637	2.004	2.716	2.004	1,32	1,36	0,76	**0,74**
insulin-like growth factor 1	282	275	282	275	1,03	1,03	0,97	**0,97**
Interferon-gamma	3.623	3.149	3.623	3.149	1,15	1,15	0,87	**0,87**
IP-10	4.747	3.981	4.042	3.981	1,19	1,02	0,84	**0,98**
Kallikrein-12	2.254	2.095	2.254	2.095	1,08	1,08	0,93	**0,93**
Kallikrein-14	2.950	2.729	2.950	2.729	1,08	1,08	0,93	**0,93**
Kallikrein-5	3.309	3.090	3.309	3.090	1,07	1,07	0,93	**0,93**
Kallikrein-9	2.776	2.344	2.494	2.344	1,18	1,06	0,84	**0,94**
Laminin	4.589	4.426	4.144	4.792	1,04	0,86	0,96	**1,16**
low-density lipoprotein	10.518	8.384	10.518	8.384	1,25	1,25	0,80	**0,80**
MCP-1	3.059	2.870	2.709	2.870	1,07	0,94	0,94	**1,06**
MCP-2	5.873	5.686	5.873	6.522	1,03	0,90	0,97	**1,11**
MCP-3	3.447	3.055	3.447	2.639	1,13	1,31	0,89	**0,77**
MCP-4	6.081	4.773	6.081	4.773	1,27	1,27	0,78	**0,78**
M-CSF	2.781	2.535	2.781	2.535	1,10	1,10	0,91	**0,91**
MIP-1-alpha	3.209	2.955	3.209	2.955	1,09	1,09	0,92	**0,92**
MMP-2	3.262	2.780	3.262	2.780	1,17	1,17	0,85	**0,85**
MMP-3	2.626	2.427	2.469	2.427	1,08	1,02	0,92	**0,98**
MMP-9	3.675	3.886	3.675	3.886	0,95	0,95	1,06	**1,06**
Myeloperoxidase	3.051	2.726	3.051	2.480	1,12	1,23	0,89	**0,81**
Myoglobin	2.615	2.293	2.615	2.292	1,14	1,14	0,88	**0,88**
Neuron Specific Enolase	2.796	2.124	2.796	2.124	1,32	1,32	0,76	**0,76**
Osteopontin	7.929	8.002	7.929	8.002	0,99	0,99	1,01	**1,01**
PDGF (all isoforms)	2.124	2.497	2.124	2.497	0,85	0,85	1,18	**1,18**
PDGF (BB isoform only)	8.062	5.954	8.062	5.954	1,35	1,35	0,74	**0,74**
Placental Alkaline Phosphatase	1.548	1.367	1.548	1.254	1,13	1,23	0,88	**0,81**

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
Plasminogen	5.915	4.999	5.915	4.499	1,18	1,31	0,85	**0,76**
plasminogen activator inhibitor	1.968	2.367	895	2.367	0,83	0,38	1,20	**2,65**
Prostatic acid phosphatase	2.561	2.158	2.561	2.158	1,19	1,19	0,84	**0,84**
PSA (Free)	5.644	5.556	5.016	5.556	1,02	0,90	0,98	**1,11**
PSA (Total)	2.601	2.276	2.601	2.276	1,14	1,14	0,87	**0,87**
PSA-ACT Complex	4.913	4.149	4.913	4.773	1,18	1,03	0,84	**0,97**
RANTES	2.210	2.071	2.210	2.275	1,07	0,97	0,94	**1,03**
S100	3.039	2.832	3.039	2.832	1,07	1,07	0,93	**0,93**
Serum Albumin	18.716	18.402	18.741	16.441	1,02	1,14	0,98	**0,88**
Sialyl Lewis X	4.896	5.667	4.896	5.712	0,86	0,86	1,16	**1,17**
TAG-72	1.812	1.605	1.812	1.605	1,13	1,13	0,89	**0,89**
Tetranectin	2.214	1.986	1.958	1.986	1,11	0,99	0,90	**1,01**
TGF-alpha	1.819	2.137	1.819	2.137	0,85	0,85	1,17	**1,17**
TGF-beta	3.251	2.878	3.251	2.878	1,13	1,13	0,89	**0,89**
Thrombopoietin	2.264	1.940	2.264	1.940	1,17	1,17	0,86	**0,86**
Thrombospondin-1	2.814	2.324	2.814	2.324	1,21	1,21	0,83	**0,83**
Thyroglobulin	3.718	3.729	4.142	3.729	1,00	1,11	1,00	**0,90**
TIMP1	1.577	1.402	1.481	1.353	1,12	1,09	0,89	**0,91**
TIMP2	1.211	1.027	1.211	1.161	1,18	1,04	0,85	**0,96**
TNF-alpha	821	571	821	625	1,44	1,31	0,69	**0,76**
TNF-beta	1.885	1.646	1.885	1.646	1,14	1,14	0,87	**0,87**
Transferrin	6.516	5.812	7.388	5.812	1,12	1,27	0,89	**0,79**
Tumor-Associated Trypsin Inhibitor	2.562	2.667	2.562	2.667	0,96	0,96	1,04	**1,04**
Tyrosinase	2.012	1.791	2.012	1.791	1,12	1,12	0,89	**0,89**
Urokinase Plasminogen Activator	1.744	2.132	1.744	2.132	0,82	0,82	1,22	**1,22**
VCAM-1	2.452	2.726	2.452	2.726	0,90	0,90	1,11	**1,11**
VE-Cadherin	3.673	4.242	3.189	4.242	0,87	0,75	1,15	**1,33**
VEGF	1.701	2.119	1.701	1.856	0,80	0,92	1,25	**1,09**
VEGF-D	1.627	1.367	1.627	1.309	1,19	1,24	0,84	**0,80**
Von Willebrand Factor	2.763	3.119	2.763	3.546	0,89	0,78	1,13	**1,28**

Tab. 29. Data of the first experiment (malignant breast cancer group / healthy control group) of the Serum Biomarker Chip.

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
Alpha fetoprotein	1.352	1.471	1.356	1.471	0,92	0,92	1,09	**1,08**
alpha1 anthchymotrypsin	325	290	649	871	1,12	0,75	0,89	**1,34**
alpha2 macroglobulin	11.263	10.900	11.263	10.900	1,03	1,03	0,97	**0,97**
Angiogenin	2.647	2.208	2.647	2.208	1,20	1,20	0,83	**0,83**
Angiopoietin-2	3.154	2.941	3.466	3.068	1,07	1,13	0,93	**0,89**
Angiostatin	33.878	28.842	33.130	31.532	1,17	1,05	0,85	**0,95**
Apolipoprotein	1.477	1.502	1.492	1.502	0,98	0,99	1,02	**1,01**
Apolipoprotein J	12.207	9.413	12.207	9.413	1,30	1,30	0,77	**0,77**
beta2 microglobulin	7.929	5.503	7.005	5.503	1,44	1,27	0,69	**0,79**
Bone sialoprotein	34	101	103	288	0,34	0,36	2,92	**2,79**
CA125	2.809	1.815	2.809	1.815	1,55	1,55	0,65	**0,65**
CA15-3	2.880	2.366	2.880	2.423	1,22	1,19	0,82	**0,84**
CA19-9	3.235	2.142	3.235	2.203	1,51	1,47	0,66	**0,68**
CA50	663	698	1.325	1.396	0,95	0,95	1,05	**1,05**
Carcinoembryoinc antigen (group 4 specific)	3.053	2.324	3.053	2.324	1,31	1,31	0,76	**0,76**
Carcinoembryoinc antigen (group 2 specific)	857	960	1.715	1.919	0,89	0,89	1,12	**1,12**
Cathepsin B	4.605	3.452	4.118	3.162	1,33	1,30	0,75	**0,77**
Ceruloplasmin	3.767	2.535	3.767	2.535	1,49	1,49	0,67	**0,67**
Chondroitin Sulftate	4.427	3.941	4.427	3.941	1,12	1,12	0,89	**0,89**
Chorionic gonadotropin-alpha	2.101	1.545	2.275	1.545	1,36	1,47	0,74	**0,68**
Chorionic gonadotropin-beta	1.788	1.423	1.788	1.423	1,26	1,26	0,80	**0,80**
Chromogranin	4.260	2.692	4.260	2.814	1,58	1,51	0,63	**0,66**
Collagen Type I	17.140	12.896	17.140	12.896	1,33	1,33	0,75	**0,75**
complement C4	12.821	11.370	12.821	11.370	1,13	1,13	0,89	**0,89**
C-reactive protein	3.227	2.610	2.913	2.610	1,24	1,12	0,81	**0,90**
Cyclin-dependent kinase inhibitor 2A	27	285	82	570	0,10	0,14	10,37	**6,92**
Cytokeratin Fragment 21-1 (CYFRA 21-1)	8.070	4.975	8.070	4.975	1,62	1,62	0,62	**0,62**
Eotaxin	1.898	1.316	1.713	1.316	1,44	1,30	0,69	**0,77**
Epidermal growth factor	363	631	715	631	0,57	1,13	1,74	**0,88**
Epidermal growth factor receptor	1.980	1.941	1.781	2.018	1,02	0,88	0,98	**1,13**
ErbB2	3.947	3.333	3.947	3.671	1,18	1,08	0,84	**0,93**
E-Selectin	2.818	1.814	2.818	1.814	1,55	1,55	0,64	**0,64**
Estrogen Receptor	1.284	1.377	1.284	2.753	0,93	0,47	1,07	**2,14**
Fas	4.152	3.534	3.690	3.192	1,17	1,16	0,85	**0,86**
Fas ligand	2.965	4.435	2.965	4.435	0,67	0,67	1,50	**1,50**
Ferritin	2.157	1.913	2.157	1.913	1,13	1,13	0,89	**0,89**
Fibroblast Growth factor-7	1.615	1.384	1.615	1.384	1,17	1,17	0,86	**0,86**
Fibroblast Growth factor-basic	4.005	3.418	4.005	3.437	1,17	1,17	0,85	**0,86**
G-CSF	2.466	2.036	2.502	2.036	1,21	1,23	0,83	**0,81**
GM-CSF	4.542	3.430	4.542	3.577	1,32	1,27	0,76	**0,79**
haptoglobulin	1.008	1.000	995	1.000	1,01	0,99	0,99	**1,01**
Hemoglobin	3.016	21.062	2.840	17.783	0,14	0,16	6,98	**6,26**
Hepatocyte Growth Factor	1.357	1.299	1.357	1.299	1,05	1,05	0,96	**0,96**
ICAM-1	1.319	1.646	1.527	1.646	0,80	0,93	1,25	**1,08**
IgA	17.657	20.369	17.657	20.369	0,87	0,87	1,15	**1,15**
IgG	12.181	12.640	11.973	12.098	0,96	0,99	1,04	**1,01**

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
IgM	6.634	8.275	6.634	8.275	0,80	0,80	1,25	**1,25**
IL-10	5.118	4.292	4.554	4.292	1,19	1,06	0,84	**0,94**
IL-12p40	1.797	1.787	1.797	1.787	1,01	1,01	0,99	**0,99**
IL12-p70	1.515	1.443	1.515	1.443	1,05	1,05	0,95	**0,95**
IL-13	1.998	2.451	1.998	2.451	0,82	0,82	1,23	**1,23**
IL-17	4.025	4.182	4.025	4.182	0,96	0,96	1,04	**1,04**
IL-1-alpha	6.314	5.700	6.314	5.700	1,11	1,11	0,90	**0,90**
IL1-beta	1.610	1.465	1.610	1.457	1,10	1,11	0,91	**0,90**
IL-2	4.860	4.747	4.860	4.747	1,02	1,02	0,98	**0,98**
IL-2 receptor-alpha	2.013	1.627	2.013	1.627	1,24	1,24	0,81	**0,81**
IL-2 receptor-beta	1.713	1.296	1.754	1.296	1,32	1,35	0,76	**0,74**
IL-3	1.801	1.574	1.801	1.574	1,14	1,14	0,87	**0,87**
IL-4	2.486	2.091	2.486	2.091	1,19	1,19	0,84	**0,84**
IL-5	1.153	1.224	1.153	1.182	0,94	0,97	1,06	**1,03**
IL-6	1.194	898	1.194	917	1,33	1,30	0,75	**0,77**
IL-7	2.489	2.455	2.489	2.455	1,01	1,01	0,99	**0,99**
IL-8	755	734	695	798	1,03	0,87	0,97	**1,15**
Insulin	1.179	1.200	1.179	1.259	0,98	0,94	1,02	**1,07**
Insulin-like growth factor binding protein 3	3.080	2.739	2.821	2.739	1,12	1,03	0,89	**0,97**
insulin-like growth factor 1	250	269	500	539	0,93	0,93	1,08	**1,08**
Interferon-gamma	4.850	5.184	4.850	5.184	0,94	0,94	1,07	**1,07**
IP-10	2.513	2.533	2.513	2.458	0,99	1,02	1,01	**0,98**
Kallikrein-12	1.447	1.750	1.737	1.750	0,83	0,99	1,21	**1,01**
Kallikrein-14	5.666	2.606	3.150	2.606	2,17	1,21	0,46	**0,83**
Kallikrein-5	4.019	3.247	3.697	3.102	1,24	1,19	0,81	**0,84**
Kallikrein-9	3.150	3.490	3.358	3.186	0,90	1,05	1,11	**0,95**
Laminin	3.084	2.518	3.230	5.205	1,22	0,62	0,82	**1,61**
low-density lipoprotein	9.672	8.067	9.672	8.067	1,20	1,20	0,83	**0,83**
MCP-1	4.681	3.738	4.681	3.813	1,25	1,23	0,80	**0,81**
MCP-2	7.371	5.116	6.386	5.541	1,44	1,15	0,69	**0,87**
MCP-3	4.963	3.347	4.554	3.347	1,48	1,36	0,67	**0,74**
MCP-4	4.157	3.041	4.157	3.294	1,37	1,26	0,73	**0,79**
M-CSF	3.879	2.751	3.879	2.751	1,41	1,41	0,71	**0,71**
MIP-1-alpha	4.875	4.643	4.439	4.643	1,05	0,96	0,95	**1,05**
MMP-2	2.374	2.362	2.266	2.362	1,01	0,96	0,99	**1,04**
MMP-3	6.090	1.964	2.188	1.960	3,10	1,12	0,32	**0,90**
MMP-9	3.908	3.471	3.908	3.471	1,13	1,13	0,89	**0,89**
Myeloperoxidase	4.061	3.987	4.061	3.987	1,02	1,02	0,98	**0,98**
Myoglobin	2.890	2.299	2.735	2.282	1,26	1,20	0,80	**0,83**
Neuron Specific Enolase	2.746	2.679	2.615	2.776	1,02	0,94	0,98	**1,06**
Osteopontin	4.850	4.121	4.850	4.121	1,18	1,18	0,85	**0,85**
PDGF (all isoforms)	3.266	2.484	3.266	2.484	1,31	1,31	0,76	**0,76**
PDGF (BB isoform only)	10.100	10.004	10.100	10.004	1,01	1,01	0,99	**0,99**
Placental Alkaline Phosphatase	1.072	1.105	1.072	1.105	0,97	0,97	1,03	**1,03**
Plasminogen	8.439	8.247	8.439	8.247	1,02	1,02	0,98	**0,98**
plasminogen activator inhibitor	2.523	1.627	2.394	1.627	1,55	1,47	0,64	**0,68**

	Raw Average Sample A	*Raw Average Sample B*	*Trimmed Average Sample A*	*Trimmed Average Sample B*	*Raw Ratio (A/B)*	*Trimmed Ratio (A/B)*	*Raw Ratio (B/A)*	***Trimmed Ratio (B/A)***
Prostatic acid phosphatase	1.881	1.541	1.726	1.541	1,22	1,12	0,82	**0,89**
PSA (Free)	10.070	7.929	10.070	7.929	1,27	1,27	0,79	**0,79**
PSA (Total)	3.113	2.924	2.826	2.924	1,06	0,97	0,94	**1,03**
PSA-ACT Complex	4.777	3.855	4.777	3.459	1,24	1,38	0,81	**0,72**
RANTES	2.138	1.661	2.210	1.591	1,29	1,39	0,78	**0,72**
S100	3.390	2.884	3.157	2.884	1,18	1,09	0,85	**0,91**
Serum Albumin	12.433	13.114	12.596	12.965	0,95	0,97	1,05	**1,03**
Sialyl Lewis X	5.689	3.082	5.689	3.220	1,85	1,77	0,54	**0,57**
TAG-72	592	496	1.185	992	1,19	1,19	0,84	**0,84**
Tetranectin	2.358	2.270	2.358	2.270	1,04	1,04	0,96	**0,96**
TGF-alpha	3.171	2.354	3.171	2.354	1,35	1,35	0,74	**0,74**
TGF-beta	1.976	2.182	2.310	2.042	0,91	1,13	1,10	**0,88**
Thrombopoietin	2.367	2.037	2.367	2.037	1,16	1,16	0,86	**0,86**
Thrombospondin-1	3.429	2.164	2.990	2.087	1,59	1,43	0,63	**0,70**
Thyroglobulin	4.143	3.375	4.143	3.375	1,23	1,23	0,81	**0,81**
TIMP1	809	956	1.619	1.901	0,85	0,85	1,18	**1,17**
TIMP2	860	992	963	992	0,87	0,97	1,15	**1,03**
TNF-alpha	599	861	599	861	0,70	0,70	1,44	**1,44**
TNF-beta	2.086	2.252	2.086	2.335	0,93	0,89	1,08	**1,12**
Transferrin	6.141	4.818	5.520	4.818	1,27	1,15	0,78	**0,87**
Tumor-Associated Trypsin Inhibitor	3.590	1.359	3.590	1.429	2,64	2,51	0,38	**0,40**
Tyrosinase	2.731	2.439	2.731	2.439	1,12	1,12	0,89	**0,89**
Urokinase Plasminogen Activator	2.241	1.741	2.241	1.741	1,29	1,29	0,78	**0,78**
VCAM-1	3.905	2.391	3.905	2.391	1,63	1,63	0,61	**0,61**
VE-Cadherin	6.620	5.215	6.620	5.215	1,27	1,27	0,79	**0,79**
VEGF	1.796	1.154	1.796	1.154	1,56	1,56	0,64	**0,64**
VEGF-D	1.337	1.463	1.379	1.414	0,91	0,98	1,09	**1,03**
Von Willebrand Factor	5.507	3.869	5.507	3.869	1,42	1,42	0,70	**0,70**

Tab. 30. Data of the second experiment (benign lesions group / healthy control group) of the Serum Biomarker Chip.

6. Abbreviations

2D GE	2-dimensional gel electrophoresis
2D DIGE	2-dimensional difference gel electrophoresis
A	Adenin
ACN	acetonitrile
APS	ammonium persulfate
AR	androgen receptor
Arg	Arginin
bp	basepair
C	Cytosin
CI	confidence interval
COX-2	cyclooxygenase-2
CYP19	aromatase
dH_2O	distilled water
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
EtOH	ethanol
FCS	fetal calf serum
G	Guanin
IgG	immunoglobulin G
$MgCl_2$	magnesium chloride
OR	odds ratio
o/n	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PgR	progesterone receptor
SDS	sodium dodecyl sulfate
SELDI-TOF	surface enhanced laser desorption ionization time-of-flight
SNP	single nucleotide polymorphism
T	Thymidin
TEMED	N, N, N', N' -Tetramethylethylenediamine
Tris	Trizma® base
Tyr	Tyrosin
rpm	rounds per minute

7. Literature

1. Laboratories, S., *Directory of Services, and Use and Interpretation of Tests.* 2001.
2. Kristensen, V.N., et al., *Genetic susceptibility and environmental estrogen-like compounds.* Mutat Res, 2001. **482**(1-2): p. 77-82.
3. Templin, M.F., et al., *Protein microarray technology.* Trends Biotechnol, 2002. **20**(4): p. 160-6.
4. Rebbeck, T.R., et al., *Modification of BRCA1-associated breast cancer risk by the polymorphic androgen-receptor CAG repeat.* Am J Hum Genet, 1999. **64**(5): p. 1371-7.
5. Kristensen, V.N., et al., *Genetic variants of CYP19 (aromatase) and breast cancer risk.* Oncogene, 2000. **19**(10): p. 1329-33.
6. Ristimaki, A., et al., *Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer.* Cancer Res, 2002. **62**(3): p. 632-5.
7. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. **10**(8): p. 789-99.
8. Abellof, M.D., *The breast: editorial overview.* Curr. Opin. Oncol., 1999. **11**: p. 427-428.
9. Schultz, L.B. and B.L. Weber, *Recent advances in breast cancer biology.* Curr Opin Oncol, 1999. **11**(6): p. 429-34.
10. Hsieh, C.C., et al., *Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: associations and interactions in an international case-control study.* Int J Cancer, 1990. **46**(5): p. 796-800.
11. Kelsey, J.L., *Breast cancer epidemiology: summary and future directions.* Epidemiol Rev, 1993. **15**(1): p. 256-63.
12. Dunning, A.M., et al., *A systematic review of genetic polymorphisms and breast cancer risk.* Cancer Epidemiol Biomarkers Prev, 1999. **8**(10): p. 843-54.
13. Hankinson, S.E., et al., *Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women.* J Natl Cancer Inst, 1998. **90**(17): p. 1292-9.
14. Yue, W., et al., *In situ aromatization enhances breast tumor estradiol levels and cellular proliferation.* Cancer Res, 1998. **58**(5): p. 927-32.
15. Ingelman-Sundberg, M., M. Oscarson, and R.A. McLellan, *Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment.* Trends Pharmacol Sci, 1999. **20**(8): p. 342-9.
16. Miyoshi, Y., et al., *Breast cancer risk associated with polymorphism in CYP19 in Japanese women.* Int J Cancer, 2000. **89**(4): p. 325-8.
17. Agarwal, V.R., et al., *Alternatively spliced transcripts of the aromatase cytochrome P450 (CYP19) gene in adipose tissue of women.* J Clin Endocrinol Metab, 1997. **82**(1): p. 70-4.
18. Mendelson, C.R., et al., *Use of molecular probes to study regulation of aromatase cytochrome P-450.* Biol Reprod, 1990. **42**(1): p. 1-10.
19. Schmidt, M., C. Renner, and G. Loffler, *Progesterone inhibits glucocorticoid-dependent aromatase induction in human adipose fibroblasts.* J Endocrinol, 1998. **158**(3): p. 401-7.
20. Blankenstein, M.A., et al., *Intratumoral levels of estrogens in breast cancer.* J Steroid Biochem Mol Biol, 1999. **69**(1-6): p. 293-7.
21. Brodie, A., Q. Lu, and J. Nakamura, *Aromatase in the normal breast and breast cancer.* J Steroid Biochem Mol Biol, 1997. **61**(3-6): p. 281-6.
22. Brodie, A., B. Long, and Q. Lu, *Aromatase expression in the human breast.* Breast Cancer Res Treat, 1998. **49 Suppl 1**: p. S85-91; discussion S109-19.

23. Sasano, H. and H. Murakami, *Immunolocalization of aromatase in human breast disorders using different antibodies.* Breast Cancer Res Treat, 1998. **49 Suppl 1**: p. S79-84; discussion S109-19.
24. Brueggemeier, R.W., et al., *Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens.* Cancer Lett, 1999. **140**(1-2): p. 27-35.
25. Brueggemeier, R.W., J.A. Richards, and T.A. Petrel, *Aromatase and cyclooxygenases: enzymes in breast cancer.* J Steroid Biochem Mol Biol, 2003. **86**(3-5): p. 501-7.
26. Cho-Chung, Y.S., *Endocrine Control of Neoplasia: Interaction of cyclic AMP and estrogen in tumor growth control.* 1978: p. 335-346.
27. Billig, H., I. Furuta, and A.J. Hsueh, *Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis.* Endocrinology, 1993. **133**(5): p. 2204-12.
28. Boccuzzi, G., et al., *Dehydroepiandrosterone antiestrogenic action through androgen receptor in MCF-7 human breast cancer cell line.* Anticancer Res, 1993. **13**(6A): p. 2267-72.
29. Couture, P., et al., *Androgen receptor-mediated stimulation of 17 beta-hydroxysteroid dehydrogenase activity by dihydrotestosterone and medroxyprogesterone acetate in ZR-75-1 human breast cancer cells.* Endocrinology, 1993. **132**(1): p. 179-85.
30. Szelei, J., et al., *Androgen-induced inhibition of proliferation in human breast cancer MCF7 cells transfected with androgen receptor.* Endocrinology, 1997. **138**(4): p. 1406-12.
31. Ingle, J.N., et al., *Combination hormonal therapy with tamoxifen plus fluoxymesterone versus tamoxifen alone in postmenopausal women with metastatic breast cancer. An updated analysis.* Cancer, 1991. **67**(4): p. 886-91.
32. Birrell, S.N., R.E. Hall, and W.D. Tilley, *Role of the androgen receptor in human breast cancer.* J Mammary Gland Biol Neoplasia, 1998. **3**(1): p. 95-103.
33. Zhu, X., et al., *Identification of an exon 3 deletion splice variant androgen receptor mRNA in human breast cancer.* Int J Cancer, 1997. **72**(4): p. 574-80.
34. Adams, J.B., *Adrenal androgens and human breast cancer: a new appraisal.* Breast Cancer Res Treat, 1998. **51**(2): p. 183-8.
35. Bernstein, L. and R.K. Ross, *Endogenous hormones and breast cancer risk.* Epidemiol Rev, 1993. **15**(1): p. 48-65.
36. Maggiolini, M., et al., *Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha.* Cancer Res, 1999. **59**(19): p. 4864-9.
37. Vane, J.R., *Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs.* Nat New Biol, 1971. **231**(25): p. 232-5.
38. Fu, J.Y., et al., *The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes.* J Biol Chem, 1990. **265**(28): p. 16737-40.
39. Kujubu, D.A., et al., *TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue.* J Biol Chem, 1991. **266**(20): p. 12866-72.
40. Xie, W.L., et al., *Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing.* Proc Natl Acad Sci U S A, 1991. **88**(7): p. 2692-6.
41. Vane, J.R. and R.M. Botting, *New insights into the mode of action of anti-inflammatory drugs.* Inflamm Res, 1995. **44**(1): p. 1-10.
42. Vane, J.R. and R.M. Botting, *Anti-inflammatory drugs and their mechanism of action.* Inflamm Res, 1998. **47 Suppl 2**: p. S78-87.
43. Taketo, M.M., *Cyclooxygenase-2 inhibitors in tumorigenesis (part I).* J Natl Cancer Inst, 1998. **90**(20): p. 1529-36.

44. Taketo, M.M., *Cyclooxygenase-2 inhibitors in tumorigenesis (Part II).* J Natl Cancer Inst, 1998. **90**(21): p. 1609-20.
45. Harris, R.E., et al., *Breast cancer and NSAID use: heterogeneity of effect in a case-control study.* Prev Med, 1995. **24**(2): p. 119-20.
46. Harris, R.E., K.K. Namboodiri, and W.B. Farrar, *Nonsteroidal antiinflammatory drugs and breast cancer.* Epidemiology, 1996. **7**(2): p. 203-5.
47. Fujita, T., et al., *Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas.* Cancer Res, 1998. **58**(21): p. 4823-6.
48. Davies, N.M., et al., *Cyclooxygenase-3: axiom, dogma, anomaly, enigma or splice error?--Not as easy as 1, 2, 3.* J Pharm Pharm Sci, 2004. **7**(2): p. 217-26.
49. Weber, J.L. and P.E. May, *Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction.* Am J Hum Genet, 1989. **44**(3): p. 388-96.
50. Sachidanandam, R., et al., *A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms.* Nature, 2001. **409**(6822): p. 928-33.
51. Venter, J.C., et al., *The sequence of the human genome.* Science, 2001. **291**(5507): p. 1304-51.
52. Botstein, D. and N. Risch, *Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease.* Nat Genet, 2003. **33 Suppl**: p. 228-37.
53. Goddard, K.A., et al., *Linkage disequilibrium and allele-frequency distributions for 114 single-nucleotide polymorphisms in five populations.* Am J Hum Genet, 2000. **66**(1): p. 216-34.
54. Lai, E., *Application of SNP technologies in medicine: lessons learned and future challenges.* Genome Res, 2001. **11**(6): p. 927-9.
55. Riley, J.H., et al., *The use of single nucleotide polymorphisms in the isolation of common disease genes.* Pharmacogenomics, 2000. **1**(1): p. 39-47.
56. Cargill, M., et al., *Characterization of single-nucleotide polymorphisms in coding regions of human genes.* Nat Genet, 1999. **22**(3): p. 231-8.
57. Pritchard, J.K., *Are rare variants responsible for susceptibility to complex diseases?* Am J Hum Genet, 2001. **69**(1): p. 124-37.
58. Anderson, N.L. and N.G. Anderson, *Proteome and proteomics: new technologies, new concepts, and new words.* Electrophoresis, 1998. **19**(11): p. 1853-61.
59. Gygi, S.P., et al., *Correlation between protein and mRNA abundance in yeast.* Mol Cell Biol, 1999. **19**(3): p. 1720-30.
60. Klose, J., *Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals.* Humangenetik, 1975. **26**(3): p. 231-43.
61. O'Farrell, P.H., *High resolution two-dimensional electrophoresis of proteins.* J Biol Chem, 1975. **250**(10): p. 4007-21.
62. Harrison, H., et al., *Identification of a serum protein polymorphism via two-dimensional electrophoresis. Family and population studies in two genetically isolated groups: North American Hutterites and Australian aborigines.* Am J Hum Genet, 1991. **48**(2): p. 362-9.
63. Harrison, H.H., et al., *Biochemical and clinical characterization of prealbuminCHICAGO: an apparently benign variant of serum prealbumin (transthyretin) discovered with high-resolution two-dimensional electrophoresis.* Am J Med Genet, 1991. **39**(4): p. 442-52.
64. Rosenblum, B.B., J.V. Neel, and S.M. Hanash, *Two-dimensional electrophoresis of plasma polypeptides reveals "high" heterozygosity indices.* Proc Natl Acad Sci U S A, 1983. **80**(16): p. 5002-6.

65. Adkins, J.N., et al., *Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry.* Mol Cell Proteomics, 2002. **1**(12): p. 947-55.
66. Cheung, K.L., C.R. Graves, and J.F. Robertson, *Tumour marker measurements in the diagnosis and monitoring of breast cancer.* Cancer Treat Rev, 2000. **26**(2): p. 91-102.
67. Ciocca, D.R. and R. Elledge, *Molecular markers for predicting response to tamoxifen in breast cancer patients.* Endocrine, 2000. **13**(1): p. 1-10.
68. Colomer, R., et al., *Herceptin: from the bench to the clinic.* Cancer Invest, 2001. **19**(1): p. 49-56.
69. Hamann, U., *Hereditary breast cancer: high risk genes, genetic testing and clinical implications.* Clin Lab, 2000. **46**(9-10): p. 447-61.
70. Petricoin, E.F., et al., *Use of proteomic patterns in serum to identify ovarian cancer.* Lancet, 2002. **359**(9306): p. 572-7.
71. Bensadoun, A. and D. Weinstein, *Assay of proteins in the presence of interfering materials.* Anal Biochem, 1976. **70**(1): p. 241-50.
72. Chamberlain, N.L., E.D. Driver, and R.L. Miesfeld, *The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function.* Nucleic Acids Res, 1994. **22**(15): p. 3181-6.
73. Kazemi-Esfarjani, P., M.A. Trifiro, and L. Pinsky, *Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG)n-expanded neuronopathies.* Hum Mol Genet, 1995. **4**(4): p. 523-7.
74. McPhaul, M.J., H.U. Schweikert, and D.R. Allman, *Assessment of androgen receptor function in genital skin fibroblasts using a recombinant adenovirus to deliver an androgen-responsive reporter gene.* J Clin Endocrinol Metab, 1997. **82**(6): p. 1944-8.
75. Edwards, A., et al., *Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups.* Genomics, 1992. **12**(2): p. 241-53.
76. Giovannucci, E., et al., *The CAG repeat within the androgen receptor gene and its relationship to prostate cancer.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3320-3.
77. Irvine, R.A., et al., *The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer.* Cancer Res, 1995. **55**(9): p. 1937-40.
78. Stanford, J.L., et al., *Polymorphic repeats in the androgen receptor gene: molecular markers of prostate cancer risk.* Cancer Res, 1997. **57**(6): p. 1194-8.
79. Johannsson, O.T., et al., *Tumour biological features of BRCA1-induced breast and ovarian cancer.* Eur J Cancer, 1997. **33**(3): p. 362-71.
80. Karp, S.E., et al., *Influence of BRCA1 mutations on nuclear grade and estrogen receptor status of breast carcinoma in Ashkenazi Jewish women.* Cancer, 1997. **80**(3): p. 435-41.
81. Loman, N., et al., *Steroid receptors in hereditary breast carcinomas associated with BRCA1 or BRCA2 mutations or unknown susceptibility genes.* Cancer, 1998. **83**(2): p. 310-9.
82. Noguchi, S., et al., *Clinicopathologic analysis of BRCA1- or BRCA2-associated hereditary breast carcinoma in Japanese women.* Cancer, 1999. **85**(10): p. 2200-5.
83. Park, J.J., et al., *Breast cancer susceptibility gene 1 (BRCAI) is a coactivator of the androgen receptor.* Cancer Res, 2000. **60**(21): p. 5946-9.
84. Giguere, Y., et al., *Short polyglutamine tracts in the androgen receptor are protective against breast cancer in the general population.* Cancer Res, 2001. **61**(15): p. 5869-74.

85. Liede, A., et al., *Androgen receptor gene polymorphism and breast cancer susceptibility in The Philippines.* Cancer Epidemiol Biomarkers Prev, 2003. **12**(9): p. 848-52.
86. Dunning, A.M., et al., *No association between androgen or vitamin D receptor gene polymorphisms and risk of breast cancer.* Carcinogenesis, 1999. **20**(11): p. 2131-5.
87. Spurdle, A.B., et al., *Androgen receptor exon 1 CAG repeat length and breast cancer in women before age forty years.* J Natl Cancer Inst, 1999. **91**(11): p. 961-6.
88. Suter, N.M., et al., *Androgen receptor (CAG)n and (GGC)n polymorphisms and breast cancer risk in a population-based case-control study of young women.* Cancer Epidemiol Biomarkers Prev, 2003. **12**(2): p. 127-35.
89. Toda, K., et al., *Structural and functional characterization of human aromatase P-450 gene.* Eur J Biochem, 1990. **193**(2): p. 559-65.
90. Sebastian, S. and S.E. Bulun, *A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project.* J Clin Endocrinol Metab, 2001. **86**(10): p. 4600-2.
91. Haiman, C.A., et al., *No association between a single nucleotide polymorphism in CYP19 and breast cancer risk.* Cancer Epidemiol Biomarkers Prev, 2002. **11**(2): p. 215-6.
92. Haiman, C.A., et al., *Polymorphisms in steroid hormone pathway genes and mammographic density.* Breast Cancer Res Treat, 2003. **77**(1): p. 27-36.
93. Kurosaki, K., et al., *Combined polymorphism associated with a 3-bp deletion in the 5'-flanking region of a tetrameric short tandem repeat at the CYP19 locus.* Nippon Hoigaku Zasshi, 1997. **51**(3): p. 191-5.
94. Siegelmann-Danieli, N. and K.H. Buetow, *Constitutional genetic variation at the human aromatase gene (Cyp19) and breast cancer risk.* Br J Cancer, 1999. **79**(3-4): p. 456-63.
95. Takahashi, M., et al., *Allele detection and population study in Japanese using two STR loci (CYP19 and HUMTH01).* Int J Legal Med, 1996. **108**(6): p. 321-2.
96. Shimizu, H., et al., *Serum oestrogen levels in postmenopausal women: comparison of American whites and Japanese in Japan.* Br J Cancer, 1990. **62**(3): p. 451-3.
97. Kosaka, T., et al., *Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2.* Eur J Biochem, 1994. **221**(3): p. 889-97.
98. Tazawa, R., et al., *Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene.* Biochem Biophys Res Commun, 1994. **203**(1): p. 190-9.
99. Suske, G., *The Sp-family of transcription factors.* Gene, 1999. **238**(2): p. 291-300.
100. Xu, Q., Y.S. Ji, and J.F. Schmedtje, Jr., *Sp1 increases expression of cyclooxygenase-2 in hypoxic vascular endothelium. Implications for the mechanisms of aortic aneurysm and heart failure.* J Biol Chem, 2000. **275**(32): p. 24583-9.
101. Dubois, R.N., et al., *Cyclooxygenase in biology and disease.* Faseb J, 1998. **12**(12): p. 1063-73.
102. Fritsche, E., et al., *Functional characterization of cyclooxygenase-2 polymorphisms.* J Pharmacol Exp Ther, 2001. **299**(2): p. 468-76.
103. Papafili, A., et al., *Common promoter variant in cyclooxygenase-2 represses gene expression: evidence of role in acute-phase inflammatory response.* Arterioscler Thromb Vasc Biol, 2002. **22**(10): p. 1631-6.
104. Guadagni, F., et al., *A re-evaluation of carcinoembryonic antigen (CEA) as a serum marker for breast cancer: a prospective longitudinal study.* Clin Cancer Res, 2001. **7**(8): p. 2357-62.

105. D'Alessandro, R., et al., *Serum tissue polypeptide specific antigen (TPS): a complementary tumor marker to CA 15-3 in the management of breast cancer.* Breast Cancer Res Treat, 2001. **68**(1): p. 9-19.
106. Hou, M.F., et al., *Evaluation of serum CA27.29, CA15-3 and CEA in patients with breast cancer.* Kaohsiung J Med Sci, 1999. **15**(9): p. 520-8.
107. Martoni, A., et al., *CEA, MCA, CA 15.3 and CA 549 and their combinations in expressing and monitoring metastatic breast cancer: a prospective comparative study.* Eur J Cancer, 1995. **31A**(10): p. 1615-21.
108. Huang, H.L., et al., *Enrichment of low abundant serum proteins by Albumin/IgG immunoaffinity depletion under reducing conditions.* Electrophoresis, submitted Jan., 2005.
109. Duffy, M.J. and C. Duggan, *The urokinase plasminogen activator system: a rich source of tumour markers for the individualised management of patients with cancer.* Clin Biochem, 2004. **37**(7): p. 541-8.
110. Manders, P., et al., *The complex between urokinase-type plasminogen activator (uPA) and its type-1 inhibitor (PAI-I) independently predicts response to first-line endocrine therapy in advanced breast cancer.* Thromb Haemost, 2004. **91**(3): p. 514-21.
111. Parker, B.S., et al., *Alterations in vascular gene expression in invasive breast carcinoma.* Cancer Res, 2004. **64**(21): p. 7857-66.
112. Lewalle, J.M., et al., *Alteration of interendothelial adherens junctions following tumor cell-endothelial cell interaction in vitro.* Exp Cell Res, 1997. **237**(2): p. 347-56.
113. Srivastava, P., J. Russo, and I.H. Russo, *Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death.* Carcinogenesis, 1997. **18**(9): p. 1799-808.
114. Rao Ch, V., et al., *Human chorionic gonadotropin decreases proliferation and invasion of breast cancer MCF-7 cells by inhibiting NF-kappaB and AP-1 activation.* J Biol Chem, 2004. **279**(24): p. 25503-10.
115. Todryk, S., et al., *Disabled infectious single-cycle herpes simplex virus as an oncolytic vector for immunotherapy of colorectal cancer.* Hum Gene Ther, 1999. **10**(17): p. 2757-68.
116. Ali, S.A., et al., *Preclinical evaluation of "whole" cell vaccines for prophylaxis and therapy using a disabled infectious single cycle-herpes simplex virus vector to transduce cytokine genes.* Cancer Res, 2000. **60**(6): p. 1663-70.
117. Loudon, P.T., et al., *Preclinical safety testing of DISC-hGMCSF to support phase I clinical trials in cancer patients.* J Gene Med, 2001. **3**(5): p. 458-67.
118. Loudon, P.T., et al., *Preclinical evaluation of DISC-GMCSF for the treatment of breast carcinoma.* J Gene Med, 2003. **5**(5): p. 407-16.
119. Alters, S.E., et al., *IL-13 can substitute for IL-4 in the generation of dendritic cells for the induction of cytotoxic T lymphocytes and gene therapy.* J Immunother, 1999. **22**(3): p. 229-36.
120. Lutz, C., et al., *IgD can largely substitute for loss of IgM function in B cells.* Nature, 1998. **393**(6687): p. 797-801.
121. Bhalla, V., et al., *Effect of growth factors on proliferation of normal, borderline, and malignant breast epithelial cells.* Exp Mol Pathol, 2000. **68**(2): p. 124-32.
122. Bozcuk, H., et al., *Tumour necrosis factor-alpha, interleukin-6, and fasting serum insulin correlate with clinical outcome in metastatic breast cancer patients treated with chemotherapy.* Cytokine, 2004. **27**(2-3): p. 58-65.
123. Berberoglu, U., E. Yildirim, and O. Celen, *Serum levels of tumor necrosis factor alpha correlate with response to neoadjuvant chemotherapy in locally advanced breast cancer.* Int J Biol Markers, 2004. **19**(2): p. 130-4.

124. Bewick, M., et al., *Soluble Fas (CD95) is a prognostic factor in patients with metastatic breast cancer undergoing high-dose chemotherapy and autologous stem cell transplantation.* J Hematother Stem Cell Res, 2001. **10**(6): p. 759-68.
125. Evan, G.I. and K.H. Vousden, *Proliferation, cell cycle and apoptosis in cancer.* Nature, 2001. **411**(6835): p. 342-8.
126. Muller, M., et al., *Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53.* J Clin Invest, 1997. **99**(3): p. 403-13.
127. Poulaki, V., C.S. Mitsiades, and N. Mitsiades, *The role of Fas and FasL as mediators of anticancer chemotherapy.* Drug Resist Updat, 2001. **4**(4): p. 233-42.
128. Mitsiades, N., et al., *Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity.* Cancer Res, 2001. **61**(2): p. 577-81.
129. O'Connell, J., et al., *Expression of Fas (CD95/APO-1) ligand by human breast cancers: significance for tumor immune privilege.* Clin Diagn Lab Immunol, 1999. **6**(4): p. 457-63.
130. Christensen, L., *The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix.* APMIS Suppl, 1992. **26**: p. 1-39.
131. Sidhom, G. and M. Imam, *Evaluation of serum laminin as a tumor marker in breast cancer.* Int J Clin Lab Res, 1999. **29**(1): p. 26-9.
132. Rapellino, M., et al., *Tumor-associated trypsin inhibitor (TATI) in pleural effusions.* Scand J Clin Lab Invest Suppl, 1991. **207**: p. 47-9.
133. Huhtala, M.L., et al., *Purification and characterization of a tumor-associated trypsin inhibitor from the urine of a patient with ovarian cancer.* J Biol Chem, 1982. **257**(22): p. 13713-6.
134. Stenman, U.H., et al., *Immunochemical demonstration of an ovarian cancer-associated urinary peptide.* Int J Cancer, 1982. **30**(1): p. 53-7.
135. Stenman, U.H., E. Koivunen, and O. Itkonen, *Biology and function of tumor-associated trypsin inhibitor, TATI.* Scand J Clin Lab Invest Suppl, 1991. **207**: p. 5-9.
136. Matsuura, N., et al., *Increased level of circulating adhesion molecules in the sera of breast cancer patients with distant metastases.* Jpn J Clin Oncol, 1997. **27**(3): p. 135-9.
137. Bruning, P.F., et al., *Insulin-like growth-factor-binding protein 3 is decreased in early-stage operable pre-menopausal breast cancer.* Int J Cancer, 1995. **62**(3): p. 266-70.
138. Rocha, R.L., et al., *Insulin-like growth factor binding protein-3 and insulin receptor substrate-1 in breast cancer: correlation with clinical parameters and disease-free survival.* Clin Cancer Res, 1997. **3**(1): p. 103-9.
139. Singer, C.F., et al., *Insulin-like growth factor (IGF)-I and IGF-II serum concentrations in patients with benign and malignant breast lesions: free IGF-II is correlated with breast cancer size.* Clin Cancer Res, 2004. **10**(12 Pt 1): p. 4003-9.
140. Schulz, G., *Her-2/neu gene product in serum -- an oncoprotein in the diagnosis and therapy of breast carcinoma*. Anticancer Res., 2003. **23**(2A): p. 1007-10
141. Coussens, L., et al., *Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene.* Science, 1985. **230**(4730): p. 1132-9.
142. Di Fiore, P.P., et al., *erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells.* Science, 1987. **237**(4811): p. 178-82.
143. Qiu, Y., L. Ravi, and H.J. Kung, *Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells.* Nature, 1998. **393**(6680): p. 83-5.

144. Slamon, D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.
145. Pietras, R.J., et al., *Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs.* Oncogene, 1998. **17**(17): p. 2235-49.
146. Yu, D., et al., *Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase.* Mol Cell, 1998. **2**(5): p. 581-91.
147. Offersen, B.V., et al., *Immunohistochemical determination of tumor angiogenesis measured by the maximal microvessel density in human prostate cancer.* Apmis, 1998. 106(4): p. 463-9.

PAPERS

Singer CF[1], **Mogg M[1]**, Koestler W, Marton E, Pacher M, Kubista E, and Schreiber M
IGF1 and IGF2 Serum Concentrations in Patients with Benign and Malignant Breast Lesions: Free IGF2 is Correlated with Breast Cancer Size
Clinical Cancer Research, 2004, 10, 4003-4009

Huang HL, Stasyk T, Morandell S, **Mogg M**, Schreiber M, Feuerstein I, Huck CW, Bonn GK, and Huber LA
Enrichment of low abundant serum proteins by Albumin/IgG immunoaffinity depletion under reducing conditions
Manuscript submitted to *Electrophoresis*, 2005

Mogg M, Vogl S, Zidek T, Kubista E, and Schreiber M
A CAG repeat polymorphism in Exon1 of the androgen receptor gene in breast cancer patients
Manuscript in preparation

Mogg M, Vogl S, Esterbauer H, Kubista E, and Schreiber M
Two single nucleotide polymorphisms in the aromatase gene (*CYP19*) in breast cancer patients
Manuscript in preparation

Mogg M, Vogl S, Esterbauer H, Kubista E, and Schreiber M
A G -765 C single nucleotide polymorphism in the promoter of the cyclooxygenase-2 (COX-2) gene in breast cancer patients
Manuscript in preparation

Pacher M, Seewald M, Oehler S, Mikula M, **Mogg M**, Vinatzer U, Eger A, Schweifer N, Varecka R, Mikulits W, and Schreiber M
Impact of IGF1 and IGF2 stimulation on the Expression Profiles of Human Breast Cancer Cells
Manuscript in preparation

ABSTRACTS

Schreiber M, **Mogg M**, Pacher M, Singer C, and Kubista E
Induction of cell proliferation and invasiveness by IGF1/2 overexpression in human breast cancer cells revealed by expression profiling with DNA microarrays
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[1] Note: Singer CF and Mogg M contributed equally to this work.

POSTER PRESENTATIONS

Mogg M, Kubista E, Vogl S, and Schreiber M
A CAG repeat polymorphism in Exon 1 of the androgen receptor in breast cancer patients
Joint Annual Meeting of the ÖGBM/ÖGGGT/ÖGBT, September 2003, Graz

Pacher M, **Mogg M**, Kubista E, and Schreiber M
IGF1/2 effects on Proliferation, chemoresistance, invasiveness, and expression profiles in human breast cancer
EMBL/Salk/EMBO Conference on Oncogenes & Growth Control "Signalling and Cancer", 20.-23. April 2002, Heidelberg

Pacher M, **Mogg M**, Kubista E, and Schreiber M
Enhanced Cell Proliferation, Chemoresistance, Invasiveness, and Altered Gene Expression Profiles by IGF1/2 Overexpression in Human Breast Cancer Cells
12th IMP Spring Conference "Epigenetic Programming of the Genome", 23.-25. Mai 2002, Hofburg, Wien

Pacher M, **Mogg M**, Mikula M, Kubista E, and Schreiber M
Enhanced Cell Proliferation, Invasiveness, and Altered Gene Expression Profiles by IGF1/2 Overexpression in Human Breast Cancer Cell
EMBO Lecture Course on New Developments in Genomics for Biomedicine, 8.-13. April 2003, Slowenien

Pacher M, **Mogg M**, Schweifer N, Mikula M, Kubista E, Mikulits W, and Schreiber M
Enhanced Cell Proliferation, Invasiveness, and Altered Gene Expression Profiles by IGF1/2 Overexpression in Human Breast Cancer Cell
Joint Annual Meeting of the ÖGBM/ÖGGGT/ÖGBT, September 2003, Graz

Pacher M, **Mogg M**, Schweifer N, Mikula M, Eger A, Mikulits W, Kubista E, and Schreiber M
Characterization of the Biological Effects of IGF1/2 Overexpression in Human Breast Cancer Cells: a Link from Transcription Profiles to Functional Biology
First International Symposium of the Austrian Proteomics Platform, 26.-29. Jänner 2004, Seefeld / Tirol

Pacher M, Oehler S, Seewald M, Schweifer N, Mikula M, **Mogg M**, Eger A, Kubista E, and Schreiber M
IGF1/2 Mediated Deregulation of Translational Initiation Leads to Enhanced Malignancy
EMBL/Salk/EMBO Conference on Oncogenes & Growth Control "Signalling and Cancer", 17.-20. April 2004, Heidelberg

Lebenslauf

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Zivildienst

Feb. 2002 – Jän. 2003	Ableistung des Zivildienstes im Wr. Krankenanstaltenverbund

Besondere Kenntnisse

EDV	Microsoft Windows & Linux Betriebssysteme Microsoft Office Paket Hardwarekenntnisse
Sprachen	Englisch – Perfekt in Word und Schrift Französisch – Gut in Word und Schrift
Führerschein	B