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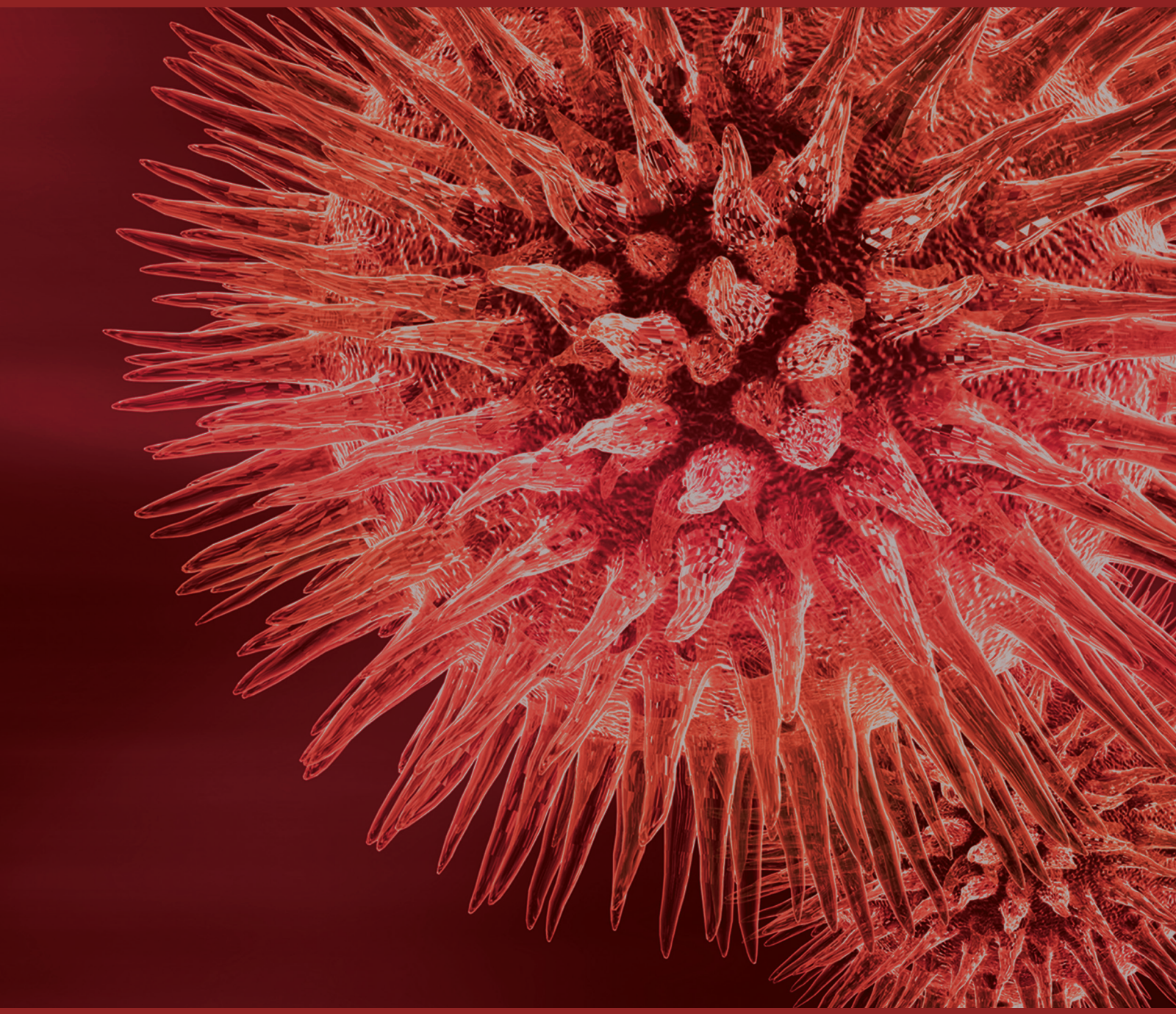
Biomarkers in Women's Cancers, Gynecology, and Obstetrics

Guest Editors: Peter A. Fasching, Gottfried E. Konecny, and Amanda B. Spurdle



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Editorial

Biomarkers in Women's Cancers, Gynecology, and Obstetrics

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More than 10 years after the publication of the reference sequence of the human genome several advances in biomarker discovery in obstetrics and gynecology and women's cancer research have been made. Genome-wide studies aiming at the identification of pathophysiologically relevant novel genes and pathways have discovered multiple previously unknown molecular mechanisms, and this has fostered a new phase of candidate gene and pathway research that is informed by complex genetic, biological, and bioinformatic data. Technological advances have permitted biomarker research on all levels of systems biology (Table 1). In breast and ovarian cancer large consortia have genotyped germ line DNA of more than 60,000 cancer patients and more than 60,000 controls for more than 200,000 single nucleotide polymorphisms [1, 2]. Moreover, several comprehensive reports have been published that decipher breast, ovarian, and endometrial cancer at all levels of gene regulation [3–5]. The ability to study circulating DNA through noninvasive sampling of blood is one of the most exciting and rapidly advancing fields in obstetrics and gynecology and cancer diagnostics. These advances now also enable the analysis of fetal DNA, which is circulating in the blood stream of the mother [6]. These developments have been driven not only by major technological advances, including the isolation of intact cancer cells and the analysis of cancer cell-derived DNA from blood samples, but also by the improvement of the sensitivity of analytic methods. Analysis of free circulating fetal DNA will change the diagnostic approach in prenatal medicine drastically. Moreover, in cancer research a "liquid biopsy" approach which has evolved most prominently in breast cancer will have a significant impact on early detection of cancer and treatment monitoring as well as understanding treatment failure in the near future [7]. Importantly, however, it is not only the discovery of biomarkers that counts, but also the replication and independent validation of results that allow their application in clinical practice. Last but not least, the increasing complexity of biomarker research and their potential clinical utility in the postgenomic era create a need for education of clinicians, clinical researchers, regulatory authorities, and patients alike. The amount of information requires new approaches, how biomarker use is to be understood and to be explained to those involved [8]. We believe that in this special issue we can provide fascinating reviews and original papers on the topic of biomarkers in obstetrics and gynecology and women's cancers in the postgenomic era. It is clear that this field of research will be of increasing importance to obstetrics and gynecology and women's cancers over the next years. We hope that this special issue with a focus on biomarker research in obstetrics and gynecology and women's cancers will provide all participants involved in the

Table 1: Different levels of biomarker research and application.

Analysis	Purpose
Methylation	Methylation analysis can reveal disease specific patterns of gene regulation.
Mutation analysis	Mutation analysis of affected tissues has been shown to be helpful in characterization of tumors and rare diseases.
Copy number variation	Copy number variation has been shown to be helpful in explaining tumor subtypes and identifying fetal chromosomal anomalies.
Chromosomal rearrangements	Chromosomal rearrangements are thought to explain new genomic effects seen in some tumors.
Genetic variation	Many diseases have been described to be partially caused by genetic variation. For many gynecologic diseases risk loci with high and low penetrance have been identified.
RNA expression	RNA expression of normal and diseased tissues has long been used to identify biomarkers for disease stage and prediction of therapies.
Proteomics	Modifications of proteins can be meaningful for their function and reveal additional functional information that supplements analysis of gene expression at the RNA level.
miRNA profiles	The number of small regulatory RNA sequences is growing and miRNAs are now understood as regulators for up to several hundreds of genes at once.

field of biomarker research with a comprehensive update on varied recent developments in this exciting field of research.

Peter A. Fasching
Gottfried E. Konecny
Amanda B. Spurdle

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Review Article

Expression of Stem Cell and Epithelial-Mesenchymal Transition Markers in Circulating Tumor Cells of Breast Cancer Patients

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Evaluation and characterization of circulating tumor cells (CTCs) have become a major focus of translational cancer research. Presence of CTCs predicts worse clinical outcome in early and metastatic breast cancer. Whether all cells from the primary tumor have potential to disseminate and form subsequent metastasis remains unclear. As part of the metastatic cascade, tumor cells lose their cell-to-cell adhesion and undergo epithelial-mesenchymal transition (EMT) in order to enter blood circulation. During EMT epithelial antigens are downregulated; thus, such tumor cells might elude classical epithelial marker-based detection. Several researchers postulated that some CTCs express stem cell-like phenotype; this might lead to chemoresistance and enhanced metastatic potential of such cells. In the present review, we discuss current data on EMT and stem cell markers in CTCs of breast cancer and their clinical significance.

1. Introduction

Presence of disseminated tumor cells (DTCs) in bone marrow and circulating tumor cells (CTCs) in peripheral blood of primary breast cancer patients was shown to be associated with impaired clinical outcome [1, 2]. Moreover, the persistence of CTCs/DTCs after completion of adjuvant treatment also represents a negative prognostic factor [3–5]. These cells are therefore assumed to be a surrogate marker of minimal residual disease and precursors of distant metastasis. Despite the prognostic relevance of tumor cell dissemination, detection of tumor cells in blood or bone marrow is not necessarily followed by relapse of disease. While most of these cells are already apoptotic or dead and others will successfully be eliminated by shear forces of the bloodstream, only a small group of CTCs possesses the ability to extravasate and migrate through the endothelial cell layer [6–10]. Merely a fraction of those is able to survive at secondary sites and cause tumor growth "metastatic inefficiency" [11, 12]. Although factors determining the fate of CTCs still remain to be elucidated, one presently discussed theory considers epithelial-mesenchymal transition (EMT) to be a crucial step in tumor cell dissemination.

EMT is a phenomenon hypothesized to contribute to cancer progression and metastasis [13]. In this process epithelial cells of the primary tumor undergo a series of phenotypic changes, such as reduction of cell-cell adhesion, increment in cell mobility and invasiveness, loss of epithelial markers, and acquisition of mesenchymal phenotype [14]. Moreover, it has been demonstrated that the process of EMT can generate cells with stem cell-like properties [15]. Cancer cells with stem cell-like, self-renewal capabilities (cancer stem cells: CSCs) are currently regarded to be the source of metastatic tumor spread [16]. Since CTCs have been shown to express mesenchymal and stem cell markers, it has been recently postulated that EMT plays a key role in the process of tumor cell dissemination [17–20]. In consequence, tumor cells undergoing EMT may migrate into peripheral blood as CTCs. Due to their mesenchymal stemness features, these cells might be able to reach distant sites of the body and initiate metastases. In the following review we will discuss current data on the EMT and stem cell markers in CTCs of breast cancer and their clinical relevance.

2. Tumor Cell Dissemination and Its Role in the Metastatic Cascade

Distant metastasis represents the major cause of morbidity and mortality in breast cancer patients [21, 22]. Tumor cell dissemination is a phenomenon that occurs in the very early stage of carcinogenesis and is thought to be a potential source of metastatic disease [23]. Disseminated tumor cells in bone marrow can be detected in up to 30–40% of primary breast cancer patients at the time of diagnosis and are strongly associated with impaired prognosis [1]. Depending on the sensitivity of the assay used and stage of disease, the detection rates of CTCs in peripheral blood range from 10 up to 80%; prognostic relevance of CTCs has been recently confirmed by several clinical trials both in the adjuvant and in the metastatic setting. However, data on CTC prevalence and their clinical significance, especially in early breast cancer, are to date incoherent [24–37]. Hematogenous spread of tumor cells into blood circulation of patients with solid malignancies has been a known phenomenon for a long time [35, 38, 39]. While numerous tumor cells daily reach peripheral blood, only a small fraction of these cells has the ability to survive and to arrive at secondary homing sites "metastatic inefficiency" [11, 12]. Moreover, their seeding at the secondary sites is not a random process. As suggested by Paget in the "seed and soil" hypothesis from 1889 and confirmed by several studies, the interactions between circulating tumor cells "seeds" and the microenvironment of their potential homing sites "soil" play a crucial role in the formation of metastasis [38, 40–42]. These findings are in accord with clinical data; a pooled analysis of nine studies involving 4703 primary breast cancer patients demonstrated that more than half of patients with disseminated tumor cells in bone marrow at the time of diagnosis do not develop metastatic disease [1]. CTCs seem to represent a highly heterogeneous cell population with regard to their morphology, molecular characteristics, implantation efficiency after dissemination and their metastatic potential [43–45].

3. EMT/MET

Epithelial-mesenchymal transition is a process well known from embryogenesis. In order to reach their final destination, embryonic epithelial cells acquire functional and phenotypic properties of migratory, invasive mesenchymal cells and thus become detached from the surface of the embryo [46, 47]. Interestingly, epithelial-mesenchymal transition represents a reversible mechanism; once the target localization has been reached, these cells undergo a reverse process of mesenchymal epithelial transition (MET) and recover their epithelial character to proliferate and form differentiated tissues [48]. This phenomenon, essential for embryonic development, has been recognized to represent a crucial step in tumor progression and metastasis [13].

The process of EMT involves the loss of cell-to-cell adhesions, loss of apicobasal cell polarity, and increment of migratory and invasive features of mesenchymal cells [48]. EMT can therefore compromise the mechanical integrity of the tissue [49]. EMT, once induced in tumor cells, may allow them to escape from primary tumor, migrate through the blood unaffected by therapeutic agents, and reach the site of future metastasis. Furthermore, it has been postulated that MET also represents the part of metastatic formation and that tumor cells regain their epithelial properties at their secondary homing sites [50, 51]. This hypothesis is in accord with the observation that metastatic lesions generally share epithelial features of the primary tumor (e.g., E-cadherin expression) [52, 53].

EMT process can be induced by extracellular factors like transforming growth factor β (TGFβ), Wnt, Notch, epidermal growth factor (EGF), hypoxia, and others [48]. Numerous transcription factors inducing EMT, like SNAIL, TWIST, SLUG, ZEB1, ZEB2, and FoxC2, have been evaluated [54]. Loss of E-cadherin, overexpression of N-cadherin, and cytoskeletal alterations (e.g., expression of vimentin) hallmark this process causing phenotypical and structural changes that lead to acquisition of motility and invasiveness of cells that have undergone EMT. Several studies have shown a correlation between EMT process and high aggressiveness of breast cancer. EMT markers seem to be associated with basal-like breast cancer phenotype and, therefore, with high invasiveness and metastatic potential [55, 56]. Table 1 summarizes markers used for detection and characterization of CTCs showing epithelial as well as mesenchymal phenotypes.

4. Detection of Tumor Cell Dissemination

The challenge in identifying and detecting CTCs is based on their rare number as well as the lack of a universal breast cancer marker. The majority of methods currently used are based on the detection of epithelial markers. The main disadvantage lies in the fact that cells undergoing EMT or with a mesenchymal phenotype might thus be missed. Only a few markers useful in the isolation of CTCs with a mesenchymal phenotype have been evaluated (Table 1). In the past ten years the number of assays to detect and characterize CTCs has increased steadily. All techniques have in common the fact that, due to the low frequency of the isolated tumor cells, they have to be extremely sensitive. In several cases the first step is the enrichment of tumor cells [57]. The choice of enrichment and characterization steps depending on the markers analyzed (especially EpCAM) is crucial to allow as well as to limit the detection of cells undergoing EMT or not. A short perception of enrichment and detection methods in regard to EMT and stem cell markers, some of them commercially available, will be given in the following. These methods are summarized in Table 2.

One way to enrich disseminated tumor cells is density gradient centrifugation. Mononuclear cells are isolated using Ficoll and are subsequently spun on glass slides. Visualization of the tumor cells beside the leukocytes is effected by means of immunocytochemistry. Due to the lack of a general marker, tumor cells are characterized as epithelial cells which are positive, among others, for EpCAM or cytokeratins [58]. Theodoropoulos et al. could identify CTCs with a putative stem cell-like phenotype in the blood of metastatic breast

Table 1: CTC detection and characterization markers.

Marker	Reference	CTC detection or enrichment marker	Epithelial marker	Mesenchymal marker	Stem cell marker
Akt2	[17, 19, 20]			x	
ALDH1	[17–20, 59, 64, 77, 78]				x
Bmi1	[19]				x
CD133	[18]				x
CD24	[18, 59, 77]				x
CD44	[18, 19, 59, 62, 77]				x
Cytokeratins 8, 18, 19	[24, 61]	x	x		
E-cadherin (Cadherin 1)	[69]		x		
EGFR	[69]	x	x		
EpCAM (GA733-2)	[24, 58, 60, 61, 65, 69]	x	x		
Fibronectin 1	[69, 78]			x	
FoxC2	[54, 74]			x	
HER2	[60, 65, 69]	x	x		
MUC1	[60, 65]	x	x		
N-cadherin (Cadherin 2)	[69, 75]			x	
pan-Cytokeratin	[59, 69]		x		
PI3K	[17, 19, 20]			x	
SERPINE1/PAI1	[69]			x	
SLUG	[54, 74]			x	
SNAIL 1	[18, 54, 74, 77]			x	
TG2	[18]			x	
TWIST 1	[17–20, 54, 74, 76, 77]			x	
Vimentin	[75, 76, 78]			x	
ZEB1	[18, 54, 74]			x	
ZEB2	[54]			x	

cancer patients using either cytokeratin, CD44, and CD24 or cytokeratin, ALDH1, and CD24 after density gradient centrifugation [59].

Another way to enrich CTCs is to label the cells with specific antibodies which are conjugated with magnetic particles. There are several tests commercially available which are based on the immunomagnetic enrichment of epithelial markers, especially EpCAM [24, 60], therefore limiting the possibilities to detect mesenchymal tumor cells which have undergone EMT. They differ in the subsequent characterization of the CTCs: commonly used techniques are the antibody-based detection of specific markers on the protein level and also on the RNA level using RT-PCR.

The semiautomatic CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) which has been approved by the FDA is based on an immunomagnetic enrichment of epithelial cells using EpCAM-specific antibodies coated with magnetic beads. CTCs are quantified and further characterized by immunofluorescence detecting cytokeratins 8, 18, and 19 and CD45 to exclude leucocytes as well as staining of the nuclei (DAPI) [24, 61]. Additional staining of CD44 could be shown by Lowes et al. [62]. Using the CellSearch Profile Kit which consists only of the immunomagnetic enrichment step of EpCAM+ cells without further characterization allows the individual subsequent characterization of the CTC, using among others ALDH1 [63, 64].

Additional assays are commercially available to detect CTCs based on the analysis of the expression levels of epithelial or tumor-specific genes, where applicable with a preceding enrichment step. In case of the AdnaTest Breast Cancer (AdnaGen GmbH, Langenhagen, Germany) this enrichment step is performed using magnetic beads which are coated with EpCAM- and MUC1-specific antibodies. Subsequent RT-PCR allows the quantitative analysis of the expression levels of MUC1, GA733-2, and HER2 [60, 65, 66]. The additional characterization of the CTCs is effected by means of detection of the EMT and stem cell markers TWIST, Akt2, PI3K, and ALDH1, respectively [17, 20].

There are several approaches to enrich CTCs using special chips combining microfluidics and immobilization of CTCs by binding of specific antibodies (e.g., CTC-chip, Herringbone Chip) [67, 68]. The latter chip was used by Yu et al. to establish an RNA in situ hybridization assay to detect and quantify CTCs with either an epithelial or mesenchymal phenotype or with a phenotype in between (partial EMT). The expression levels of seven pooled epithelial transcripts (EpCAM; cytokeratins 5, 7, 8, 18, and 19 and cadherin 1) and three pooled mesenchymal transcripts (SERPINE1/PAI1,

Table 2: Detection and characterization methods of CTCs.

Method	Reference	Based on	Detection marker	Characterization marker
AdnaTest Breast Cancer	[60, 65, 66]	PCR	EpCAM, MUC1	MUC1, GA733-2, HER2
AdnaTest EMT-1/stem cell	[17, 20]	PCR	EpCAM, MUC1	TWIST, Akt2, PI3K, ALDH1
CellSearch CTC Kit	[24, 61, 62]	Antibody	EpCAM	CK 8, CK 18, CK 19, CD45, DAPI, HER2, EGFR, CD44
CellSearch Profile Kit	[63, 64]	Antibody	EpCAM	To be determined; for example, ALDH1
CTC-Chip	[67]	Antibody	EpCAM	Cytokeratin, CD45, DAPI
Ficoll/immunocytochemistry	[58, 59, 73, 79]	Antibody	To be determined; for example, EpCAM, Cytokeratin	To be determined; for example, CD44, CD24, ALDH1
Filtration	[70, 71]	Filtration	To be determined; for example, CK 8, CK 18, CK 19, CD45	To be determined
Flow cytometry	[80]	Antibody	EpCAM, ALDH1	CD44, CD24
Herringbone-chip	[68, 69]	Antibody	EpCAM, HER2, EGFR	EpCAM, CK 5, CK 7, CK 8, CK 18, CK 19, cadherin 1, cadherin 2, SERPINE1/PAI1, fibronectin 1

cadherin 2, and fibronectin 1) were analyzed to characterize CTCs which were detected by binding at least one of the following antibodies on a herringbone chip: EpCAM, HER2, or EGFR [69].

Another technique to enrich CTC which is solely based on the size of the cells is filtration. Several systems are available, for example, the ISET filter using pores with a diameter of 8 μm [70]. The same pore size was used in another study combining Whatman Nuclepore track-etched membranes and immunofluorescent staining of cytokeratins 8, 18, and 19 as well as CD45 to exclude leucocytes [71].

Flow cytometry is another technique which allows an individual characterization of rare cells like CTCs. Using flow cytometry, Giordano et al. could detect a subpopulation of cancer stem cells expressing either ALDH1, CD44, and low amounts of CD24 or ALDH1 and CD133 [18].

Although the majority of assays use EpCAM as detection marker, different markers are currently used to detect and enrich CTC (Table 2). Due to the fact that CTCs change their phenotype during EMT and MET, false negative results can be obtained depending on which detection marker was used. EpCAM-based assays involve the risk that CTC showing a mesenchymal phenotype might be missed.

5. Can EMT Be Detected in CTCs?

To date, several methods have been developed to detect isolated tumor cells in peripheral blood and bone marrow of breast cancer patients. Since there is no breast cancer specific marker to identify these cells, most detection assays rely on their epithelial characteristics [72, 73]. Based on the assumption that the acquisition of a mesenchymal phenotype by a small fraction of tumor cells disseminated from primary tumor represents a crucial step in the metastatic cascade allowing these cells to migrate to their secondary homing sites and build metastasis, it is possible that EMT markers can be detected among the CTCs of breast cancer patients [13]. This hypothesis has been recently confirmed by various studies in both metastatic and early breast cancer [18–20, 20, 74–78]. Mego et al. demonstrated that EMT markers positive CTCs can be detected in up to 26% of metastatic breast cancer patients. Moreover, a high expression of EMT markers predicted shorter progression free survival in these patients [77]. Aktas et al. showed in their trial on 39 metastatic breast cancer patients that EMT markers, such as TWIST1, Akt2, and PI3Kα, can be codetected in up to 62% of CTC positive blood samples; EMT markers were more likely to be found in patients resistant to therapy, suggesting increased invasiveness of tumor cells undergoing this process. Interestingly, cells undergoing EMT have also been detected in the blood of 7% of patients negative for CTCs [20]. Similar findings in primary breast cancer were presented by Kasimir-Bauer et al.; EMT markers could be detected in 72% of CTC positive and 18% of CTC negative patients, respectively [17]. Raimondi et al. demonstrated the expression of EMT markers (e.g., vimentin, fibronectin) in up to 38% of breast cancer patients tested by the standard definition as CTC negative [78]. These findings suggest that, in addition to CTCs expressing epithelial antigens, a fraction of CTCs with exclusively mesenchymal phenotype could exist and thus remain undetectable for assays based on epithelial character of these cells. However, due to the methodology, morphological features of the cells were not evaluated in these trials and false positive results cannot be excluded [17, 20]. In this regard, CTCs coexpressing mesenchymal and epithelial markers have been visualized in three other studies in breast cancer patients confirming that both kinds of markers can be expressed in the same cell [69, 75, 76]. Additionally, in the analysis by Armstrong et al. vimentin-positive CTCs were detected in peripheral blood of metastatic breast cancer patients while paired metastases from the same patients were shown to be negative for this marker [75]. This suggests a reversibility of the EMT process once tumor cells reach their destination resembling the phenomenon of epithelial plasticity known from embryonic development [48]. Available literature on EMT in CTCs of breast cancer patients is summarized in Table 3.

Table 3: EMT markers in CTC of breast cancer patients.

Author	Year	N	Method	EMT marker	Expression rate in CTC
Kasimir-Bauer et al. [17]	2012	502[1]	RT-PCR	TWIST1, Akt2 PI3Kα	72%[3,*], 18%[4,*]
Giordano et al. [18]	2012	28[2]	RT-PCR	TWIST1 SNAIL1 ZEB1 TG2	88%[*]
Barriere et al. [19]	2012	24[1]	RT-PCR	TWIST1 Akt2 PI3Kα	13% 13% 67%
Mego et al. [77]	2012	21[2]	RT-PCR	TWIST1 SNAIL1	26% 21%
Armstrong et al. [75]	2011	16[2]	IFC	Vimentin N-cadherin	70% 67%
Kallergi et al. [76]	2011	50[1,2]	IFC	TWIST1 Vimentin	73%[1], 100%[2] 77%[1], 100%[2]
Mego et al. [74]	2011	52[1]	RT-PCR	TWIST1 SNAIL1 SLUG ZEB1 FoxC2	15,4%[*]
Raimondi et al. [78]	2011	92[1,2]	RT-PCR	Vimentin Fibronectin	28%[3], 38%[4] 18%[3], 35%[4]
Aktas et al. [20]	2009	39[2]	RT-PCR	TWIST1 Akt2 PI3Kα	62%[3,*], 7%[4,*]

[1]Primary breast cancer, [2]metastatic breast cancer, [3]CTC positive group, [4]CTC negative group; [*]at least one EMT marker was expressed, PFS: progression free survival.

6. Are CTCs Cancer Stem Cells?

One recently discussed hypothesis indicates that tumor progression and metastatic spread can be traced to a small fraction of tumor cells with stem cell-like characteristics [81, 82]. These cancer stem cells have been identified in breast cancer tissue and were shown to be associated with tumors of aggressive behavior [83]. Assuming that CSCs are responsible for tumor cell dissemination and further metastasis, it seems likely that putative stem cell-like features should be found among tumor cells disseminated from primary tumor. This hypothesis has been confirmed by several researchers [17–20, 77–79]. As reported by Balic et al., most disseminated tumor cells in bone marrow of breast cancer patients presented with $CD44^+/CD24^{-/low}$ phenotype [79]. Moreover, it has been shown that DTCs with $CD44^+/CD24^{-/low}$ phenotype are associated with increased prevalence of metastases and with tumors characterized by aggressive biology [80, 84].

According to recent data both stem cell and EMT markers are frequently coexpressed in CTCs of breast cancer patients [18, 77]. These findings support the theory that EMT generates a cell population with stem cell-like features, a phenomenon that has been confirmed by numerous experimental trials [15, 85]. CTCs presenting stem cell-like characteristics have been found in both primary and metastatic breast cancer. In a recent study by Kasimir-Bauer et al. on 502 primary breast cancer patients 46% of CTC positive and 5% of CTC negative blood samples were positive for ALDH1, a common stem cell marker [17]. Similar findings have been shown by Aktas et al. in the metastatic situation. Moreover, a presence of stem cell-like CTCs in peripheral blood of breast cancer patients was shown to be associated with therapy resistance; stem cell markers or EMT factors or both were detected in 74% (25/34) of nonresponders and in 10% (2/21) of patients who responded to systemic treatment [20]. In the trial by Raimondi et al. an overexpression of stem cell markers in CTCs was correlated with advanced stage of disease [78]. Cancer stem cells are currently believed to be the cause of therapy resistance and treatment failure in breast cancer [86]. Data on stem cells markers in CTC of breast cancer patients are summarized in Table 4.

7. Therapeutic Consequences

To date, systemic therapies target either highly proliferative tumor cells (cytotoxic therapy) or cells with a specific phenotype (e.g., HER2-targeted treatment). However, such therapies are not able to identify cells that act as a source for subsequent metastasis in a selective manner. Tumor cells with putative stem cell-like expression profile are assumed to enter the blood circulation early in the course of disease and might elude therapy precisely because of their stem cell character

Table 4: Stem cell markers in CTC of breast cancer patients.

Author	Year	N	Method	Stem cell marker	Expression rate in CTC
Kasimir-Bauer et al. [17]	2012	502[1]	RT-PCR	ALDH1	46%[3], 5%[4]
Giordano et al. [18]	2012	28[2]	Flow cytometry	ALDH $CD44^+/CD24^{low}$	0.1% 3%[3,*], 49%[4,*]
Barriere et al. [19]	2012	24[1]	RT-PCR	ALDH1 CD44 Bmi1	54% 67%[**] 33%[**]
Mego et al. [77]	2012	17[2]	Flow cytometry	ALDH $CD44^+/CD24^{low}$	n. d.
Raimondi et al. [78]	2011	61[1,2]	RT-PCR	ALDH1	46%
Aktas et al. [20]	2009	39[2]	RT-PCR	ALDH1	69%[3,*], 14%[4,*]
Theodoropoulos et al. [59]	2010	30[2]	IFC	ALDH1 $CD44^+/CD24^{low}$	18% 35%

[1]Primary breast cancer, [2]metastatic breast cancer, [3]CTC positive group, [4]CTC negative group; [*]among ALDH positive cells, [**]among EMT or ALDH1 positive cells, n. d.: not done, IFC: immunofluorescence.

[20, 75]. Hypothetically, specific elimination of these cells could prevent the colonization of secondary homing sites and metastasis formation. Thus, the potential existence of a stem cell-like cancer cell might lead to a paradigm shift in oncologic treatment.

Detection and characterization of CTCs have become an important focus of oncologic research; several clinical trials have been initiated during the last decade that evaluate not only CTCs within accessory translational projects, but also ones that focus exclusively on CTCs and stratify therapy according to CTC levels [87]. Most of these trials (e.g., SWOG0500, CirCe01, TREAT CTC, and DETECT III and IV) are based on immunocytochemical detection of CTCs using the FDA-approved CellSearch system (Veridex, Warren, NJ, USA), a semiautomated antibody-based quantitative technique [88]. Since CTCs are enriched by immunomagnetic beads linked with anti-EpCAM antibodies and detected using antibodies against epithelial antigens, loss of epithelial markers during EMT could make these cells "invisible" to the assay and possibly influence treatment decisions [78, 89]. Gorges et al. reported that use of EpCAM-based enrichment techniques may lead to failure in CTC detection; in an animal based model EpCAM-based AdnaTest failed to detect CTCs despite clinically apparent metastasis. However, CTCs could be detected by PCR without the enrichment step [89]. Recently, antibody-based therapies against tumor cells expressing epithelial markers have been introduced in the treatment of cancer of epithelial origin. Catumaxomab, a trifunctional antibody directed against EpCAM, is a potent therapeutic agent for malignant ascites in EpCAM-positive advanced cancer (e.g., ovarian cancer) [90, 91]. Since EMT involves at least temporary downregulation of EpCAM expression, it might influence the efficacy of EpCAM-directed therapy on tumor cells undergoing EMT.

Therefore, signaling pathways involved in EMT and responsible for the formation of CSCs represent potential targets for future treatment regimens, and drugs inhibiting these pathways are being tested in preclinical and clinical trials [92]. In this regard everolimus (RAD001), an oral inhibitor of PI3K/Akt/mTOR pathway, was shown to inhibit cancer stem cells in vitro and in vivo and demonstrated potential effectivity in treatment of breast cancer cells resistant to standard therapy possibly through this mechanism[93–95]. These data are in accordance with clinical results; in a phase II study RAD001 was shown to restore sensitivity to tamoxifen in metastatic breast cancer patients with endocrine resistance improving the clinical benefit rate at six months in these patients [96]. A phase III BOLERO-2 trial demonstrated a 6-month improvement in progression-free survival in patients with resistance to nonsteroidal aromatase inhibitor treated with everolimus in combination with exemestane versus exemestane alone [97]. Everolimus is currently being evaluated for its potential to overcome trastuzumab resistance as well. A phase III BOLERO-1 trial compares trastuzumab and paclitaxel with and without everolimus, while the phase III BOLERO-3 trial compares trastuzumab and vinorelbine with and without everolimus.

Hedgehog, Notch, and Wnt represent further signaling pathways involved in formation of breast cancer stem cells [98–100]. Since the expression of Notch ligands has been demonstrated to be significantly elevated in triple negative breast cancer, Notch has become a promising target in breast cancer treatment [101]. In this context blocking of Notch by γ-secretase inhibitors (GSIs) has been the most extensively used approach. GSIs were shown to induce apoptosis and decrease proliferation in breast cancer cell lines and to eliminate breast cancer stem cells in vitro [102, 103]. GSIs like MK-0752 or RO4929097 have been tested in phase I and II clinical trials in primary and metastatic breast cancer providing early clinical evidence of effectiveness for these agents in breast cancer therapy [104, 105]. A phase I study analyzes RO4929097 in combination with Hedgehog pathway antagonist vismodegib in metastatic breast cancer patients [106]. Vismodegib, established in the therapy of advanced basal cell carcinoma, was also shown to inhibit tumor cell growth in tamoxifen resistant breast cancer in vivo and in vitro [107]. Furthermore, PKF118-310 an inhibitor of Wnt signaling pathway was recently reported to eradicate breast

cancer stem cells in a mouse model overexpressing HER2, thus also representing a potential drug candidate for the treatment of breast cancer [108].

An additional agent that was demonstrated to be effective against breast cancer stem cells is all transretinoic acid (ATRA). In a recent experimental approach, ATRA was able to eliminate breast cancer cells that gained CSC properties, suggesting its effectiveness in cancer resistant to conventional oncologic therapies [109]. However, ATRA has to date performed poorly in clinical trials; in a pilot phase II study 17 metastatic or recurrent breast cancer patients were treated with ATRA in combination with paclitaxel showing time to progression and survival rates similar to those reported for paclitaxel alone [110].

Another promising drug candidate in this context is salinomycin, which was shown to inhibit tumor growth in mice by eradicating breast cancer stem cells [111]. Recent preclinical trials demonstrated that salinomycin is particularly effective against cancer growth in combination with conventional chemotherapeutics, supporting the postulation that targeting different cell populations is essential in cancer therapy [112].

8. Conclusions

Multiple studies have shown that single tumor cells undergo transdifferentiation which enables intravasation; this important step of metastatic cascade is termed epithelial-mesenchymal transition. Through EMT, circulating tumor cells downregulate epithelial antigens and cell-to-cell adhesion and thus enhance their motility and invasive potential. Cells that undergo EMT seem to gain stem cell-like properties; such cells represent a small fraction of tumor cells capable of self-renewal and highly resistant to cytotoxic treatment. Since the majority of CTC detection systems are based on the presence of epithelial markers, tumor cells that have undergone EMT might elude classical detection methods, which may lead to false-negative results.

Abbreviations

ALDH: Aldehyde dehydrogenase
CSC: Cancer stem cell
CTC: Circulating tumor cell
DAPI: 4′,6-Diamidino-2-phenylindole
DTC: Disseminated tumor cell
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EMT: Epithelial-mesenchymal transition
EpCAM: Epithelial cell adhesion molecule
GA733-2: Gastrointestinal tumor-associated antigen
HER2: Human epidermal growth receptor 2
MET: Mesenchymal epithelial transition
mTOR: Mammalian target of rapamycin
MUC1: Mucin 1
PCR: Polymerase chain reaction
PI3K: Phosphoinositide 3-kinase
TGFβ: Transforming growth factor beta.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Estimating Rate of Insulin Resistance in Patients with Preeclampsia Using HOMA-IR Index and Comparison with Nonpreeclampsia Pregnant Women

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Women with preeclampsia, independent of obesity and glucose intolerance, exhibit insulin resistance during pregnancy. The purpose of the present study is to determine whether early diagnosis of insulin resistance during pregnancy can predict preeclampsia. Through a case-control study, 675 pregnant women were selected and their first trimester blood was taken. Their fasting blood glucose and insulin were also measured after diagnosis of preeclampsia by 20 weeks of pregnancy. Based on the experiments conducted on 675 women who were 20 weeks past their pregnancy, 375 cases with preeclampsia were selected and assigned to the case group. 35 other pregnant women were put in the control group. Diagnosis criteria for the participants included blood pressure above 140/90 and proteinuria above 300 mg or above +1. Both groups were matched according to age, parity, gestational age, and BMI. Homa-Irand rate of insulin resistance was calculated by HOMA-IR and patients were followed up. Homeostatic model assessments (HOMA-IR) revealed that the average insulin resistance increased during pregnancy among both the case and control groups. There was a significant difference between insulin resistance of these two groups in both first trimester and third trimester and after developing preeclampsia ($P < 0.001$, $P = 0.021$). Insulin-resistance of the group with preeclampsia was higher in first trimester prior to diagnosis as well as the third trimester after diagnosis compared to natural pregnancy under similar conditions. Measurement of insulin resistance in first trimester may be useful in predicting the risk of preeclampsia.

1. Introduction

Insulin is a hormone that facilitates the transport of glucose from the bloodstream into cells. In response to increased blood sugar after a meal, pancreas secretes insulin into the bloodstream. When insulin resistance occurs, the normal amount of secreted insulin is not sufficient in order to deliver glucose into the cells. Pancreas subsequently increases its production of insulin to deliver blood sugar into the cells. Obesity and pregnancy are among the factors which can create insulin resistance. For these conditions there are theories that can explain etiology. Obesity is a cause of insulin resistance in modern societies. Obesity is often accompanied by an increase in fat cell size. This causes changes in adipokines, including a reduction in adiponectin and an increase in tumor necrosis factor alpha and free fatty acids which increase insulin resistance [1] (Figure 1). Many metabolic changes during pregnancy increase adipose tissue and subsequently insulin resistance. Various placental hormones, in addition, alter maternal physiology to supply embryonic requirements. There is also a 30-fold increase in human placental lactogen (hPL) which leads to the secretion of insulin from pancreas [2]. Studies show that hPL plays a role in insulin resistance [3]. 6-fold increase in human

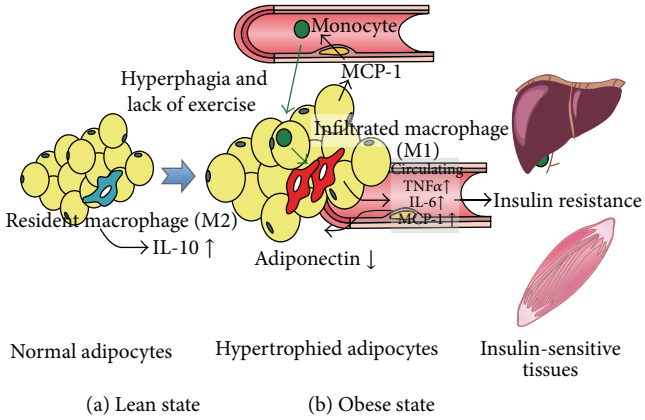

Figure 1: Effect of obesity on insulin resistance.

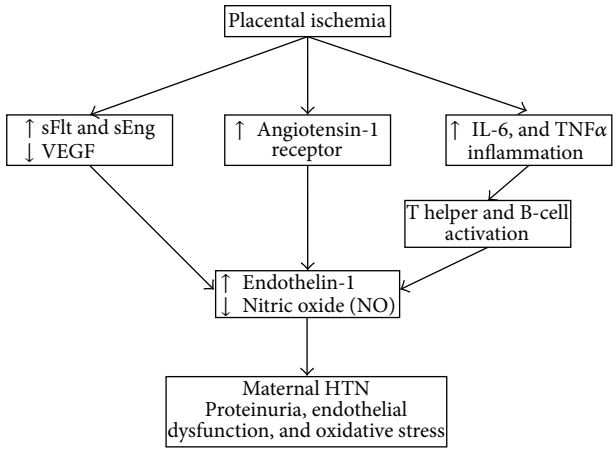

Figure 2: The effect of angiogenic and antiangiogenic factors on endothelial dysfunction.

chorionic growth hormone is another factor causing insulin resistance [4].

Preeclampsia is a condition unique to human pregnancy occurring after the twentieth week of pregnancy. Preeclampsia occurs in 2–8% of pregnancies [5, 6] and is associated with maternal and fetal mortality. Preeclampsia is defined as increased systolic blood pressure over 140 mmHg and diastolic blood pressure over 90 mmHg associated with proteinuria. Symptoms can be excessive edema of hands and feet, weight gain over 2 pounds a week, epigastric pain, severe nausea and vomiting, headaches, and vision and brain problems. Preeclampsia risk factors include previous history of preeclampsia, obesity, nulliparity, diabetes mellitus, age over 35 years at first pregnancy, and connective tissue disorders [6]. According to most theories of etiology, preeclampsia refers to maternal abnormal inflammatory response to endothelial damage and hemodynamic instability. Preeclampsia is characterized by placental hypoxia or ischemia, oxidative stress associated with endothelial dysfunction. Recent studies have shown that endothelial dysfunction is induced by antiangiogenic factors which are themselves induced by other factors [6] (Figure 2).

Table 1: Approval criteria for severe preeclampsia.

Persistent hypertension over 160/110 mmHg
Thrombocytopenia (platelets less than $103 \times 100\ \mu L$)
Nephrotic proteinuria (5 g in 24 hr)
Hemolysis (based on analysis of peripheral blood or increased bilirubin)
Resistant oliguria (less than 500 cc within 24 hours) (oliguria)
Liver dysfunction with an unknown cause
Renal failure (least criteria including increased serum creatinine mg/dL 1 above the base rate)
Persistent headache
Persistent pain in right upper quadrant or epigastrium
The estimated fetal weight below the fifth percentile for gestational age
Visual scotoma/blurred vision
Dyspnea with reduced oxygen saturation or pulmonary edema

All these changes lead to maternal hypertension and proteinuria which are main criteria for detecting preeclampsia.

Mild preeclampsia is associated with the lowest maternal and neonatal mortality and morbidity rate, while severe preeclampsia before 35 weeks into pregnancy is associated with significant maternal and prenatal complications [7]. Severe preeclampsia occurs when blood pressure reaches over 160/110 and proteinuria is above 5 g in 24-hour urine collection, as shown in Table 1.

In addition to maternal risk factors such as hypertension, type 2 diabetes, antiphospholipid antibody syndrome, obesity, and aging which have been proved to influence preeclampsia, recent studies have attested to the role of genetic factors and immune system in preeclampsia [8]. Reduced uterine-placental blood flow resulting from a combination of hypoxia and imbalances of angiogenic and antiangiogenic factors also exists in preeclampsia [6]. Some other factors in relation to preeclampsia are also under study [9, 10]. Studies show that women with preeclampsia have increased risk for developing diabetes later in life [11]. In another study, blood glucose and insulin levels were measured 2 hours after a 75 g oral glucose use in pregnant women; results showed that people with high blood insulin levels have higher risk for preeclampsia [12]. Additional research in Iran studying the relation between insulin levels and the risk for preeclampsia shows that fasting insulin levels are higher in women with preeclampsia during second trimester before the onset of clinical symptoms compared to normal pregnancy; fasting insulin level considerably increases when disease develops [13]. However, other studies found no relationship between elevated insulin and risk of preeclampsia [14, 15]. Criteria such as multiple pregnancy, prepregnancy fasting blood sugar, prepregnancy hypertension or before 20 weeks into pregnancy, systemic diseases (diabetes, hypertension, etc.), parity, prepregnancy body mass index (BMI), and drug use influence insulin resistance or susceptibility to preeclampsia which were considered in this study. A research on insulin resistance in the second trimester with subsequent preeclampsia claims that insulin resistance in

Table 2: Exclusion criteria.

1	Fasting blood sugar over 95 mg/dL
2	Hypertension over 140/90 mmHg before 20 weeks into pregnancy measured twice
3	History of renal disease, diabetes, hypertension, and heart disease
4	BMI over 25 kg/m^2
5	Receiving drugs other than routine medications which are used in pregnancy
6	History of diabetes in previous pregnancy

the second trimester of pregnancy is associated with later onset of preeclampsia [16]. There is evidence that the rate of insulin resistance in Asians is different from other races [17]. Considering the serious complications of preeclampsia on the mother and fetus and value of predicting insulin resistance in preeclampsia, the present study suggests a process for the prediction of preeclampsia before the onset of the condition and its prevention.

2. Materials and Methods

This case-control study was conducted between September 2010 and September 2011 during which 733 pregnant women who were referred to prenatal care in the time of study were examined during the first trimester. The project was approved by the local ethics committee. These women were selected from 12 clinics in Zanjan which provided prenatal care. The subjects were asked to test fasting blood sugar; their blood pressure was measured; they were examined for multiple pregnancy; they were evaluated for other systemic diseases including renal failure and cardiac ischemia; their BMI was calculated; and finally, they were examined for medication and a family history of diabetes. People with the following criteria were excluded from the study: fasting blood sugar over 95 mg/dL, hypertension over 140/90 mmHg before twenty weeks into pregnancy, history of diabetes, hypertension, and heart and renal disease, BMI over 25 kg/m^2, and receiving drugs other than routine medications which are used in pregnancy (Table 2).

Finally, 58 women met the exclusion criteria and were excluded from the study; 675 pregnant women did not meet the exclusion criteria and remained in the study. 12-hour fasting blood samples were taken from these 675 pregnant women during the second trimester of pregnancy. The samples were kept in −30°C for next examinations. During the followup, hypertension was measured among the participants who were past 20 weeks into pregnancy. Preeclampsia is defined as a twice-measured hypertension which is over 140/90 mmHg as well as a twice-measured proteinuria rated as above 1+ measured in random urine sample through dipstick or over 300 mg in a 24-hour urine collection (Table 3).

During this period, people were followed up for the risk of preeclampsia. Totally, 35 women were diagnosed with preeclampsia. They were classified in the case group and their 12-hour fasting blood samples were taken. Their fasting insulin and fasting glucose were also measured. The rest of pregnant women with normal blood pressure and previous blood samples were used for control group. The 35 women were assigned to the control group who were matched according to parity, BMI, maternal age, and gestational age after assessing their files in the centre and choosing most similar woman to from healthy group to preeclamptic group (Table 4). 12-hour fasting blood samples were taken from control group to determine blood insulin and glucose.

Table 3: Diagnosis criteria for preeclampsia.

Systolic hypertension over 140 mmHg or diastolic hypertension over 90 mmHg measured twice in an interval of at least 6 hours after 20 weeks of pregnancy
300 mg proteinuria in 24-hour urine collection or over 1+ on two random samples during an interval of at least 6 hours or less a week

Table 4: Case and control matching.

	Case	Control
Parity (mod)	2	2
BMI	21/15 kg/m^2	21/05 kg/m^2
Maternal age	33/6	32/1
Gestational age	23/2 w	22/8 w

3. Calculation of Insulin Resistance

Insulin levels were measured initially by radioimmunoassay from samples taken from mothers in the second trimester as well as after the twentieth week of pregnancy. Glucose was also measured by glucose oxidase. Insulin resistance was measured by hemostasis model assessment (HOMA-IR) formula [18]. Consider

$$\text{HOMA-IR} = \frac{\text{fasting insulin}\,(\mu\text{U/mL}) \times \text{fasting glucose}\,(\text{mg/dL})}{405}. \quad (1)$$

Data obtained from samples were analysed using the SPSS software, version 17. t-test and analysis of variance were used to compare mean values among the two groups of the study. P values below 0.05 were considered as significant.

4. Results

Of 675 pregnant women, 35 developed preeclampsia. Prevalence of preeclampsia was 5.18 among the population. The patients with preeclampsia were not significantly different from the control group in terms of average age, BMI, parity, and gestational age (Table 5).

Levels of fasting insulin and glucose were substituted into the formula and resistance to insulin was studied among the case and control groups both before and after the development of preeclampsia. The average fasting insulin increased for both case and control groups. Mean score for

Table 5: Characteristics of subjects.

	Control	Preeclampsia	P value
BMI (Kg/m^2)	23.70	23.79	0.788
Number of first pregnancies	23	23	
Age of pregnancy (in weeks)	33.9	34.24	0.922
Average age of mother	27.1	27.61	0.788

Table 6: The average HOMA-IR.

	Case group	Control group	P value
First trimester	3.09	1.9	0.021
Third trimester	4.76	2.93	0.001

the level of fasting insulin among people with preeclampsia was higher than that of control group ($P < 0.01$) (Figure 3).

According to results of t-test, changes in insulin level were significant for both groups during pregnancy ($P < 0.01$); therefore, insulin rate increased in both groups. No significant difference was found among fasting glucose among the two groups ($P = 0.724$) (Figure 4).

HOMA-IR was calculated for both groups during the first and the third trimesters; in both cases, there was a significant difference between insulin resistances (Table 6).

5. Discussion

Having matched case and control groups, this study calculated resistance to insulin using HOMA-IR index and found that the case group differed significantly in levels of insulin resistance both before and after the development of preeclampsia in comparison to the control group. It was thus concluded that insulin resistance could be an important risk factor predicting preeclampsia. Insulin resistance is described as inability of cells to respond to natural function of insulin hormone. Rate of insulin resistance naturally increases during pregnancy. It is believed placenta-derived hormones are the most important factors with the ability to change insulin resistance [19]. The golden standard for direct measurement of insulin resistance is euglycemic glucose clamp test [20]. However, this technique is time consuming and expensive requiring a skilled operator. The present study used indirect HOMA-IR which measures glucose and insulin after 12-hour fasting. Glucose homeostasis depends on its production by liver and insulin secretion from pancreatic beta cells. HOMA-IR shows this dependency in terms of an equation [18]. Our study suggested that insulin resistance during the first trimester among the members of the group which consequently developed preeclampsia was significantly different from the control group. This finding is consistent with previous findings [13, 16]. Other studies did not find such relationship [21]. Using three indices, Parretti et al. measured insulin sensitivity at the early and late stages of pregnancy. These indices were influential in predicting preeclampsia [22]. Using HOMA-IR, Sierra-laguado et al. identified insulin resistance in women with gestational hypertension in early pregnancy [23]; however,

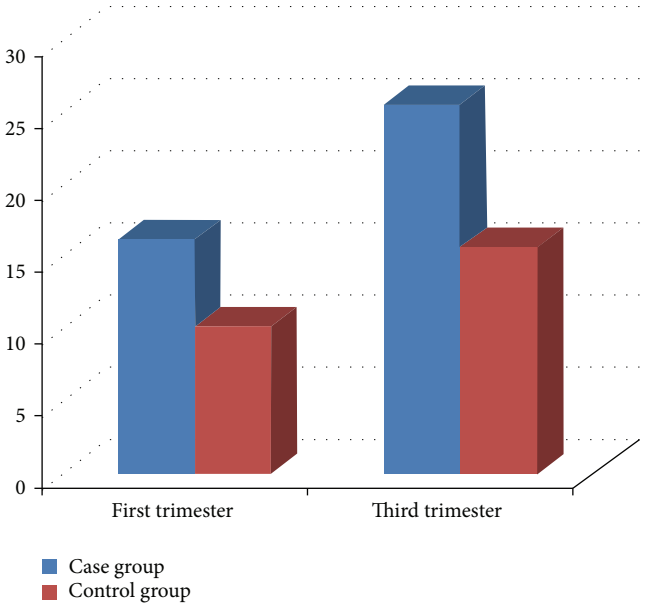

Figure 3: The average fasting insulin level of people with preeclampsia.

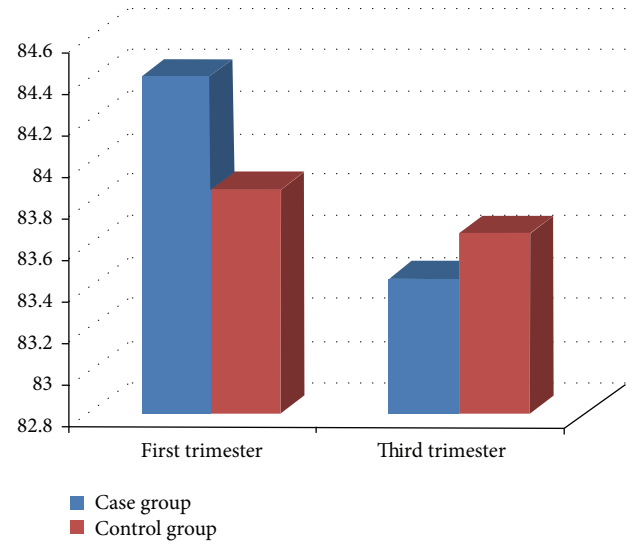

Figure 4: Fasting glucose levels in first and third trimester.

this study did not address preeclampsia. Lampinen et al. also found that early preeclampsia influenced later insulin resistance disorder [24]. Kaaja considered insulin resistance as a factor for preeclampsia pathogenesis [25]. According to this evidence, it seems that preeclampsia is associated with increased insulin resistance before the onset of the disease. Most of previous studies suffered problems which influenced the results. Our findings showed that insulin resistance was higher than control group prior to onset of preeclampsia and increased during pregnancy. Little is known on the relationship between insulin resistance and preeclampsia in terms of molecules; however, some studies have been conducted on this regard. Inositol phosphogly can obviously

increase by preeclampsia. As the secondary messenger of insulin, this molecule increases metabolic effects of insulin and it is related to insulin resistance [26]. Metabolic disorders causing insulin resistance syndrome can be seen in gestational hypertension disorders including increased levels of plasminogen activator inhibitor –1, leptin, and tumor necrosis factor alpha [27]. Insulin resistance may last for years increasing the likelihood of cardiovascular diseases in these people. These observations suggest that interventions to reduce insulin resistance may reduce increased risk of gestational hypertension and cardiovascular disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Evaluation of Two Different Analytical Methods for Circulating Tumor Cell Detection in Peripheral Blood of Patients with Primary Breast Cancer

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Background. Evidence is accumulating that circulating tumor cells (CTC) out of peripheral blood can serve as prognostic marker not only in metastatic but also in early breast cancer (BC). Various methods are available to detect CTC. Comparisons between the different techniques, however, are rare. *Material and Methods*. We evaluate two different methods for CTC enrichment and detection in primary BC patients: the FDA-approved CellSearch System (CSS; Veridex, Warren, USA) and a manual immunocytochemistry (MICC). The cut-off value for positivity was ≥1 CTC. *Results*. The two different nonoverlapping patient cohorts evaluated with one or the other method were well balanced regarding common clinical parameters. Before adjuvant CHT 21.1% (416 out of 1972) and 20.6% (247 out of 1198) of the patients were CTC-positive, while after CHT 22.5% (359 out of 1598) and 16.6% (177 out of 1066) of the patients were CTC-positive using CSS or MICC, respectively. CTC positivity rate before CHT was thus similar and not significantly different ($P = 0.749$), while CTC positivity rate immediately after CHT was significantly lower using MICC compared to CSS ($P < 0.001$). *Conclusion*. Using CSS or MICC for CTC detection, we found comparable prevalence of CTC before but not after adjuvant CHT.

1. Introduction

There is consistent data showing the prognostic relevance of CTC in metastatic BC. A CTC count of ≥5 CTCs per 7.5 mL was significantly associated with shortened overall survival (OS) and progression free survival (PFS) [1–4]. However, there is limited data on the prognostic value of CTC in early BC. Using the semi-automated detection method CSS (Veridex, Warren, USA), we could show that the count of CTC at the time of first diagnosis of an operable disease has an influence on OS and PFS of patients with BC [5]. Recent data proofed that CTC positivity predicted both decreased PFS and OS in early BC [6].

CTC with epithelial characteristics are a rare event in the peripheral blood of cancer patients both in terms of absolute numbers (<10 cells/mL) and in terms of relative numbers as compared to other blood cells (one CTC per 10^6–10^7 leukocytes) [7]. Various methods for isolation and characterization of CTC are available that differ with regard to enrichment, staining and detection [8] as well as sensitivity, specificity and reproducibility [9]. Currently used techniques rely on a first cellular enrichment step to isolate CTC from

other cell types such as red blood cells and leukocytes. Physical (filters and density gradients) and immunomagnetic approaches (magnetic affinity cell sorting, magnetic beads and ferrofluid-based systems) are common examples of cell enrichment methods [10]. Identification of CTC is based on either direct cytometric methods using antibodies such as immunocytochemistry (ICC), immunofluorescence (IF), or flow cytometry (FACS), or indirect nucleic acid-based methods which measure mRNA transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) [9, 10].

Further technologies aim at higher sensitivity and better phenotyping of CTC. Therefor evaluation and comparison of different approaches is needed. CSS is a semi-automated detection method based on an immunomagnetic enrichment using magnetic microbeads directed against the epithelial marker EpCAM. This step is followed by an IF staining for cytokeratin CK (CK8, CK18, CK19) and CD45 to distinguish epithelial cells from leukocytes. A staining for nucleus acid dye detects vital cells. After these automated preparation steps the detection of CTC is done visually. Until today it is the only CTC detection method which has been cleared by the U.S. Food and Drug Administration for use in patient care [11, 12].

Using MICC for CTC detection the enrichment is based on a density gradient centrifugation as in our study Onco-Quick (Greiner BioOne, Frickenhausen, Germany), which separates mononuclear cells from leukocytes and erythrocytes. It consists out of tubes with a porous barrier and a separation medium. It is efficient for the enrichment of CTC in the whole blood. A total of 1×10^6 mononuclear cells are poured on a microscopic slide which is air dried. Subsequently cells are stained for CK (CK8, CK18, CK19). No staining for the nucleus or for CD45 is performed. CTC are detected by conventional light microscopy.

In this study we detected CTC in a large patient cohort with early BC before and after adjuvant CHT treated in the SUCCESS A trial [13]. The aim of this analysis was to evaluate CTC positivity rate and CTC load using two different detection methods (CSS and MICC) in two different not overlapping but comparable patient groups in order to establish these methods for further patient care.

2. Patients, Material and Methods

2.1. Patients. Our patients' collective was treated within the SUCCESS A trial and defined as women with histologically confirmed, invasive primary BC. All patients had either node positive or high-risk node negative disease. Mastectomy or breast conservation leading to R0 resection in all cases was performed as primary surgery. 3754 patients were enrolled in this German trial in a time period from 2005 to 2007. All enrolled patients gave their informed written consent for study inclusion and the research project. As a translational research project CTC were assessed before and right after CHT. The SUCCESS A trial is a multicenter, randomized phase III study, which compared patients treated with 3 cycles of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) followed by 3 cycles of docetaxel (D) every three weeks (q3w) versus 3 cycles of FEC followed by 3 cycles of gemcitabine (G) and docetaxel (D) q3w as CHT. Parameters analyzed at primary diagnosis were the following: age, tumor stage, nodal stage, histological grading, histological type, estrogen (ER) and progesterone receptor (PR) status, HER2 status and menopausal status.

Two different methods to detect CTC were prospectively evaluated in two not overlapping but comparable patient cohorts of the whole study population. Due to lack of unlimited blood volume, samples were assigned to one or the other method. Out of the 3754 patients randomized for the clinical treatment study CTC detection was conducted in 3170 patients before CHT and in 2664 patients after CHT. Which method was used for CTC detection was prearranged: CSS was conducted for the first patients recruited (2000 patients planned). If CSS was not available (e.g., due to technical issues) and for the patients recruited later in the course of the clinical trial MICC was conducted for CTC detection. The patient selection process is illustrated in Figure 1. The corresponding blood sample of one patient after CHT was planned to be analyzed with the same method used for the sample before CHT (blue and red cohort in Figure 1). In order not to compromise statistical independency of the two groups to be compared (blood samples analyzed for the presence of CTC using either CSS or MICC), patients for whom CTC presence was investigated using both methods simultaneously were excluded from the analyses (22 cases before chemotherapy, 8 cases after chemotherapy).

Both methods were used according to the manufactures' instructions with minor modifications (as described below). The trial and the examination of blood samples were approved by the local ethic committees and conducted in accordance with the Declaration of Helsinki.

2.2. CTC Detection Using the CellSearch System. A total of 3570 samples (1972 samples before and 1598 after CHT) were analyzed using the CSS (Veridex, Warren, USA), which consists of the CellTracks AutoPrep System and the CellTracks Analyzer II. Prior to any therapy and right after CHT, about 30 mL of blood were collected into CellSave blood collection tubes (Immunicon, Inc., Huntingdon Valley, PA, USA) at the local site during routine blood draw by peripheral vain puncture. These tubes are evacuated blood drawtubes containing EDTA and a cellular preservative not described in details by the supplier. The CellSave tubes were used to maintain cell integrity and avoid cell degradation. The samples were then sent to the laboratory for tumor immunology at the Department of Obstetrics and Gynecology, Klinikum Innenstadt of the Ludwig-Maximilians-University (LMU), Munich, Germany for further investigation. There they were examined within a maximum of 96 h after blood drawing, which is the time period for which the vendor guarantees valid results after processing [14].

The samples were run with the Epithelial Cell Kit (Veridex, Warren, USA) and the CellTracks AutoPrep System as described before [15]. In brief, this system is based on immunomagnetic enrichment with an EpCAM-antibody, followed by labeling with monoclonal antibodies specific for CK (CK8, CK18, CK19) and leukocytes (CD45). For

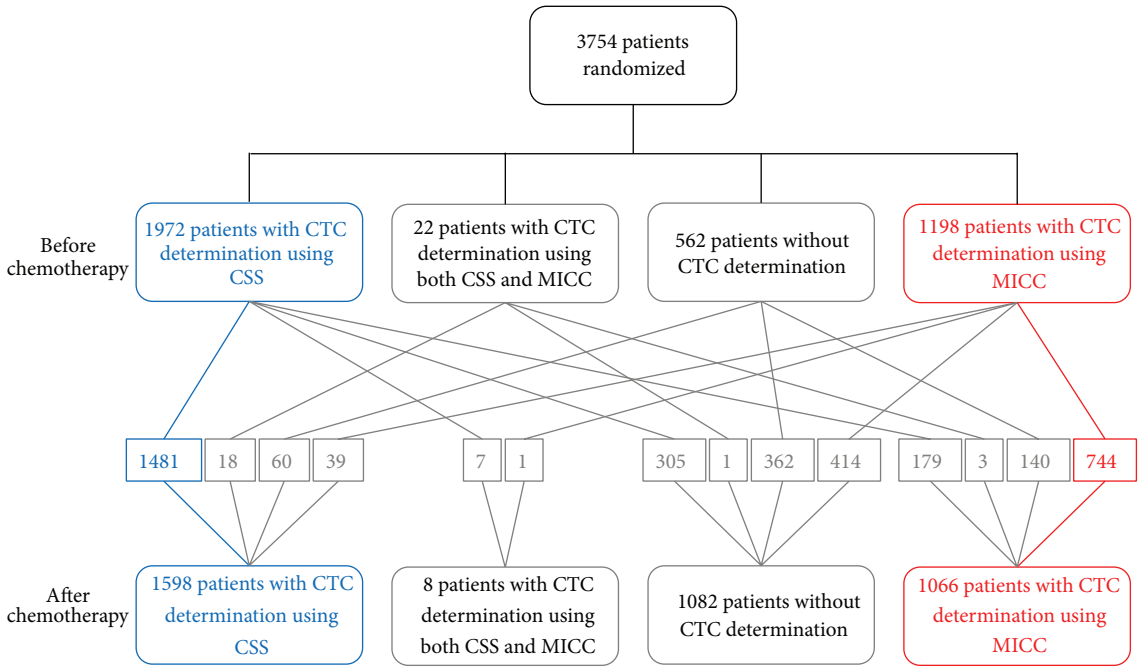
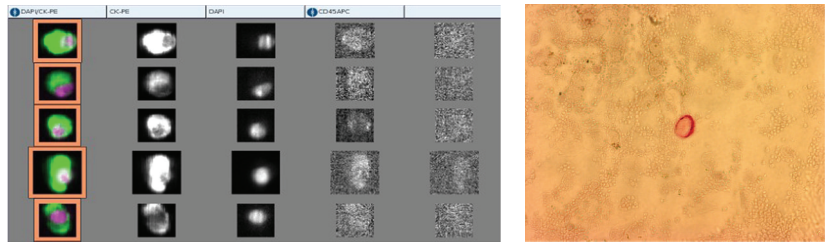


Figure 1: Flow chart illustrating the patient selection process.

that, the CellSearch Epithelial Cell kit contains all required reagents to conduct these steps: the ferrofluid particles coated with anti-EpCAM antibodies for the immunomagnetic enrichment, two phycoerythrin-conjugated antibodies directed against CK to specifically identify epithelial cells, a permeabilization buffer to allow CK antibodies entry into epithelial cells, a nuclear dye [4,6-diamidino-2-phenylindole (DAPI)] to fluorescently label the cell nuclei and an antibody against CD45 conjugated with allophycocyanin to identify leucocytes.

For the sample processing and evaluation the blood contained in three CellSave tubes were pooled using a ficoll density gradient. Then 7.5 mL of enriched blood were gently mixed with 6.5 mL of dilution buffer, centrifuged for 10 min at 800 g at room temperature, and transferred into the CellTracks AutoPrep System. The instrument does all remaining steps automatically. The final volume of 300 μL containing enriched CTC is then transferred automatically to a cartridge in a MagNest (magnetic device for incubation) and is placed inside the MagNest cell presentation chamber. After an incubation time of 20 min to a maximum of 24 h in the dark at room temperature, evaluation of the samples was done using the CellTracks Analyzer II, a semiautomated four-color fluorescence microscope. The captured images are presented in a picture gallery on a computer screen. Two independent readers classified the cells according to the following criteria: First, the staining patterns must be consistent with that of an epithelial cell (cytokeratinphycoerythrin positive/DAPI positive/CD45-allophycocyanin negative). Second, it has to be nearly round or oval with a visible nucleus within the cytoplasm. Third, CTC must have a minimum size of 4 μm, however present with a large heterogeneity regarding both CTC size and morphology [14].

2.3. CTC Detection Using a Manual Immunocytochemistry. The total number of samples analyzed using the MICC was 2264 (1198 samples before and 1066 samples after CHT). CTC were isolated from an EDTA evacuated blood draw tube and when available from the CellSave tube (total of 40 mL blood). The tumor cell enrichment was done by a density gradient centrifugation with the OncoQuick (Greiner BioOne, Frickenhausen, Germany) system. Compared to Ficoll-Hypaque (density of 1.077 g/mL), it uses a liquid separation medium optimized for the specific enrichment of CTC and an additional membrane. Density gradient centrifugation separates CTC and mononuclear cells from blood cells and granulocytes. However, CTC can easily be lost using this technique due to the presence of aggregates or to the migration of cells to the plasma layer. OncoQuick however reduces the cross-contamination of the different layers [10]. Cell separation was performed according to manufacturer's protocol [16]. In brief, precooled 50 mL OncoQuick vials were overlaid with the blood and centrifuged continuously for 25 min at 1105 g and 6°C. The entire volume of the compartment with interphase cells was poured into a fresh centrifugation tube. Cells were centrifuged twice with washing buffer (1 L PBS and 5 g of bovine albumin) at 209 g for 10 min at 6°C without break. If necessary red blood cells were lysed with lysis buffer (155 mM NH4Cl, 10 mM KHC03, 0.1 mM EDTA pH 7.2) according to the recommendations of the supplier R & D systems. 1×10^6 mononuclear cells were spun onto a glass slide (cytospin), using a cytocentrifuge (Hettich, Tuttlingen, Germany) [16]. 2 out of a maximum of 6 cytospins as well as one negative control (see below) were prepared from each blood sample. In addition to be sure that the staining worked, each time a positive control with cytospins containing MCF7 or SKBR3 cells (as available) were prepared. The slides were left to



(a) (b)

Figure 2: (a) shows images with CTC and artifacts from one patient presented in the gallery of the CSS. CTC show the characteristics defined before: round or oval shape, positive signal in the cytokeratin channel (second column), intact nucleus (third column), overlapping of nucleus and cytokeratin signal (first column), as well as no signal for CD45 as leucocytes marker (column 4), and in the negative control channel (fifth column). In comparison (b) shows a sample of a CTC detected by the MICC. Cells which are labeled with the anti-cytokeratin-antibody A45-B/B3 are then detected by the Z0259 antibody using the APAAP method and appear bright red.

air-dry overnight (12 to 24 h) at room temperature [17], followed by staining or storage at −80°C.

CTC detection was done by MICC, which is an open system with respect to the selected antibodies. Staining was performed using the anti-CK antibody A45-B/B3 (Micromet, Munich, Germany), which recognizes CK8, CK18, and CK19 [18–20]. For the detection of CTC, an ICC staining based on the alkaline phosphatase-antialkaline phosphatase (APAAP) technique was performed using the Z0259 antibody (Dako) as secondary antibody. Levamisole was taken for blocking endogenous alkaline phosphatase [16]. For the negative control the staining was done with the MOPC-21 antibody (Mouse IgG1, k; Sigma) instead of A45-B/B3 and the secondary antibody. Afterwards the slides were sealed with coverslips and stored at room temperature. Conventional light field microscopy (Axiophot; Zeiss, Oberkochen, Germany) was used for the detection of stained cells. The slides were analyzed by two independent observers. CTC were defined as bright red, round or oval shaped events with a minimum of 4 μm in size.

For both methods, the cut-off value for positivity was ≥1 CTC. Figure 2 shows sample pictures of both detection methods.

2.4. Statistical Analyses. All statistical analyses were conducted using the program IBM SPSS Statistics (Version 19). P-values below 0.05 were considered statistically significant. Descriptive statistics for all categorical data are summarized using frequency tables presenting absolute and relative frequencies. All tests regarding comparisons of patient or tumor characteristics between groups, associations between patient or tumor characteristics and the prevalence of CTC, or comparisons of CTC prevalence between methods were conducted using the Chi-Square test for all categorical data. Comparisons between groups regarding patient age were performed with the Mann-Whitney-U test.

3. Results

3.1. CTC Prevalence before Chemotherapy. Before CHT 1972 samples were analyzed using CSS and 1198 using MICC, in total 3170. The two patient cohorts were well balanced with respect to the common baseline patient and clinical parameters, which were listed in Table 1 (all $P > 0.15$). The majority of the patients showed pT1 or pT2 tumor stage, pN0 or pN1 nodal status and was postmenopausal. Concerning the histology most of the patients had a G2 or G3 histological grading and a ductal invasive BC, a positive ER and PR status as well as a negative HER2 status.

CTC positivity as assessed using CSS (21.1%) was significantly associated with positive lymph node status ($P < 0.001$), but not with any other of the clinico-pathological variables listed in Table 1 (all $P > 0.05$). There was no significant association between CTC positivity as assessed by MICC and any of the clinico-pathological variables (all $P > 0.2$).

In 1556 (78.9%) of the 1972 samples analyzed for the presence of CTC before CHT using CSS, no CTC were detected, while 416 (21.1%) were positive for CTC (median 1 CTC, range 1–827 CTC). 236 (12.0%) of the samples showed 1 CTC, and higher CTC loads were found in less than 10% of all samples with 80 (4.1%), 21 (1.1%) and 19 (1.0%) of the samples containing 2, 3 and 4 CTC respectively, while 5 CTC or more were detected in only 60 (3.0%) of the samples (Figure 3).

Out of the 1198 samples investigated for the presence of CTC before chemotherapy using MICC method, 951 (79.4%) were negative for CTC. In the majority of the 247 (20.6%) positive samples (median 1 CTC, range 1–256 CTC) only 1 CTC was detected ($n = 148$, 12.4%). Higher numbers occurred in less than 9% of all samples, with 45 (3.8%), 20 (1.7%), 10 (0.8%) and 24 (2.0%) of the samples containing 2, 3, 4, and 5 or more CTC, respectively (Figure 3).

The CTC positivity rate before CHT as assessed by the two methods did not differ significantly (CSS: 21.1% versus MICC: 20.6%, $P = 0.749$). The distributions of CTC numbers as detected before CHT using CSS or MICC are shown in Figure 3. The two distributions were very similar and not significantly different ($P = 0.351$).

3.2. CTC Prevalence after Chemotherapy. A total of 2664 blood samples were analyzed for the presence of CTC after adjuvant CHT, with 1598 samples being investigated using

Table 1: Baseline characteristics of patients for whom CTC detection before adjuvant chemotherapy was performed using the Cell Search System (CSS) or using manual immunocytochemistry (MICC).

Variable	CSS $N = 1972$	MICC $N = 1198$	P-value[1]
Age (years)			0.929[2]
Median	53.0	53.0	
Range	21–78	22–85	
Tumor stage			0.707[3]
pT1	818 (41.5%)	473 (39.5%)	
pT2	1021 (51.8%)	640 (53.4%)	
pT3	100 (5.1%)	66 (5.5%)	
pT4	27 (1.4%)	16 (1.3%)	
unknown	6 (0.3%)	3 (0.3%)	
Nodal stage			0.895[3]
pN0	664 (33.7%)	418 (34.9%)	
pN1	908 (46.0%)	545 (45.5%)	
pN2	277 (14.0%)	165 (13.8%)	
pN3	123 (6.2%)	70 (5.8%)	
Histological grading			0.457[3]
G1	97 (4.9%)	59 (4.9%)	
G2	931 (47.2%)	592 (49.4%)	
G3	940 (47.7%)	544 (45.4%)	
unknown	4 (0.2%)	3 (0.3%)	
Histological type			0.170[3]
ductal	1590 (80.6%)	997 (83.2%)	
lobular	238 (12.1%)	121 (10.1%)	
other	140 (7.1%)	78 (6.5%)	
unknown	4 (0.2%)	2 (0.2%)	
Estrogen receptor status			0.697[3]
negative	589 (29.9%)	365 (30.5%)	
positive	1380 (70.0%)	829 (69.2%)	
unknown	3 (0.2%)	4 (0.3%)	
Progesterone receptor status			0.174[3]
negative	678 (34.4%)	440 (36.7%)	
positive	1289 (65.4%)	754 (62.9%)	
unknown	5 (0.3%)	4 (0.3%)	
HER2 status			0.819[3]
negative	1452 (73.6%)	886 (74.0%)	
positive	483 (24.5%)	289 (24.1%)	
unknown	37 (1.9%)	23 (1.9%)	
Menopausal status			0.560[3]
premenopausal	822 (41.7%)	512 (42.7%)	
postmenopausal	1150 (58.3%)	686 (57.3%)	
Type of surgery			0.407[3]
breast conserving	1382 (70.1%)	856 (71.5%)	
mastectomy	587 (29.8%)	340 (28.4%)	
unknown	3 (0.2%)	2 (0.2%)	
Adjuvant chemotherapy			0.930[3]
FEC-DG	968 (49.1%)	590 (49.2%)	
FEC-DOC	1004 (50.9%)	608 (50.8%)	

[1] All tests without unknowns.
[2] Mann-Whitney U test.
[3] Chi-square test.
FEC-DG: 3 cycles of fluorouracil-epirubicin-cyclophosphamide followed by 3 cycles of docetaxel and gemcitabine; FEC-DOC: 3 cycles of fluorouracil-epirubicin-cyclophosphamide followed by 3 cycles of docetaxel.

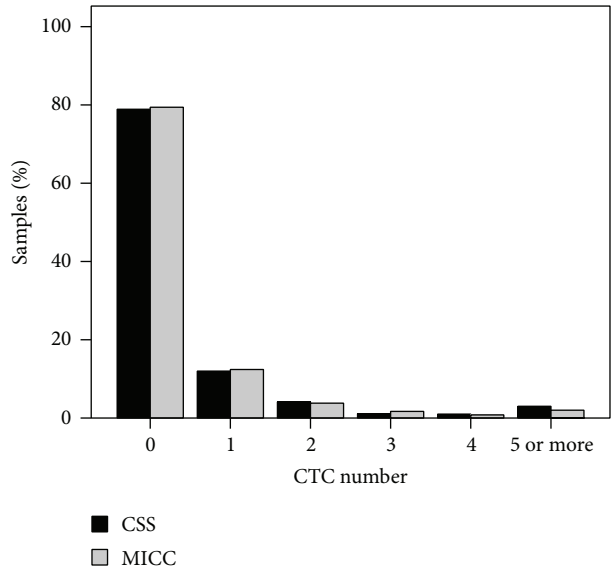


Figure 3: Distribution of the number of CTC detected before chemotherapy using the CellSearch System (CSS, black bars; n = 1972) or manual immunocytochemistry (MICC, gray bars; n = 1198).

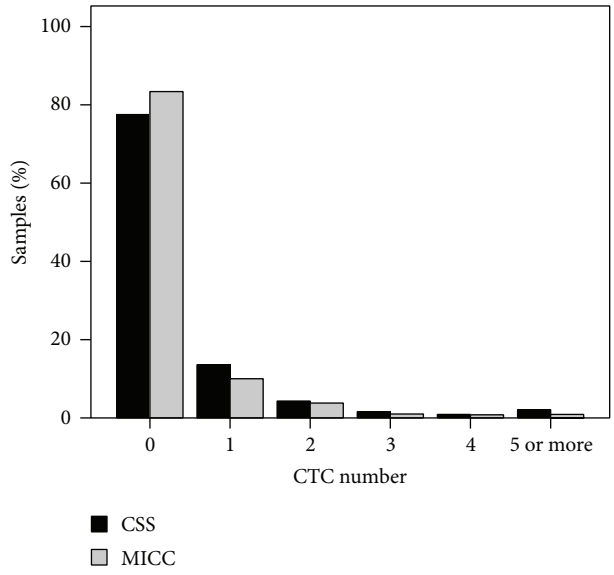


Figure 4: Distribution of the number of CTC detected after chemotherapy using the CellSearch System (CSS, black bars; n = 1598) or manual immunocytochemistry (MICC, gray bars; n = 1066).

CSS and 1066 samples being investigated using MICC. Again, the two groups were well balanced with respect to the common baseline patient and clinical parameters, which are listed in Table 2 (all $P > 0.05$).

No CTC were found in 1239 (77.5%) of the 1598 samples analyzed for the presence of CTC after CHT using the CSS. Of the 359 (22.5%) CTC positive samples (median 1 CTC, range 1–124 CTC), 217 (13.6%) samples had 1 CTC, 68 (4.3%) had 2 CTC, 26 (1.6%) had 3 CTC, 14 (0.9%) had 4 CTC, and 34 (2.1%) had 5 or more CTC (Figure 4).

889 (83.4%) of the 1066 samples investigated for the presence of CTC after CHT using MICC were negative for CTC and CTC were found in the remaining 177 (16.6%) samples (median 1 CTC, range 1–23 CTC). One CTC was detected in 107 (10.0%) samples, 2 CTC in 40 (3.8%) samples, 3 CTC in 11 (1.0%) samples, 4 CTC in 9 (0.8%) samples and 5 or more CTC in 10 (0.9%) samples (Figure 4).

The CTC positivity rate after CHT was significantly lower when assessed using MICC (16.6%) as compared to the CTC positivity rate after CHT assessed using CSS (22.5%, $P < 0.001$). Accordingly, the distributions of CTC numbers as detected after CHT using CSS or MICC were significantly different ($P = 0.005$; see Figure 4).

In contrast to CTC positivity before CHT, CTC positivity after CHT as assessed using CSS was not significantly associated with nodal stage ($P = 0.107$). However, contrary to the situation before CHT, a significant association was found between CTC positivity after CHT determined with CSS and HER2 status of the primary tumor ($P = 0.044$), with a higher proportion of HER2 positive tumors among the CTC positive samples (105 out of 252; 41.7%) as compared to the CTC negative samples (293 out of 921; 31.8%). There were no significant associations between CTC positivity after CHT as assessed by CSS and any of the other clinico-pathological parameters listed in Table 2 (all $P > 0.1$).

Similar to samples with CTC positivity being assessed using CSS, there was a significant association between CTC positivity after CHT as assessed using MICC and the HER2 status of the tumor ($P = 0.048$), with a higher proportion of HER2 positive tumors among the CTC positive samples (48 out of 123; 39.0%) as compared to the CTC negative samples (185 out of 688; 26.9%). No other significant associations between CTC positivity after CHT as assessed using MICC and clinico-pathological parameters listed in Table 2 were found (all $P > 0.1$).

3.3. CTC Prevalence before and after Chemotherapy. In 2225 patients blood samples were analyzed for the presence of CTC using the same method before and after CHT (CSS: 1481 patients; MICC: 744 patients). Regardless of the method used, most patients had no CTC in their blood at both time points (CSS: 62.3%; MICC: 66.0%) and in only a small proportion CTC were detected before and after CHT (CSS: 5.1%; MICC 3.2%). For samples analyzed using CSS the proportion of patients that were CTC negative before and CTC positive after CHT was higher as compared to samples analyzed with MICC (CSS: 17.4%; MICC: 13.0%). In contrast, the proportion of patients with CTC before but no CTC after CHT was lower in the CSS group (CSS: 15.3%; MICC: 17.7%). Figure 5 shows the proportions of patients in the four possible categories regarding CTC presence or absence before and after CHT (i.e., CTC negative both before and after CHT; CTC negative before and CTC positive after CHT, CTC positive before and CTC negative after CHT, CTC positive both before and after CHT) when the CTC analyses at both time points were performed with either CSS or MICC. The proportions of

Table 2: Baseline characteristics of patients for whom CTC detection after adjuvant chemotherapy was performed using the Cell Search System (CSS) or using manual immunocytochemistry (MICC).

Variable	CSS $N = 1598$	MICC $N = 1066$	P-value[1]
Age (years)			0.875[2]
Median	53.0	53.0	
Range	21–76	22–79	
Tumor stage			0.842[3]
pT1	660 (41.3%)	440 (41.3%)	
pT2	824 (51.6%)	549 (51.5%)	
pT3	86 (5.4%)	62 (5.8%)	
pT4	22 (1.4%)	11 (1.0%)	
unknown	6 (0.4%)	4 (0.4%)	
Nodal stage			0.561[3]
pN0	544 (34.0%)	383 (35.9%)	
pN1	731 (45.7%)	475 (44.6%)	
pN2	221 (13.8%)	151 (14.2%)	
pN3	102 (6.4%)	57 (5.3%)	
Histological grading			0.691[3]
G1	72 (4.5%)	48 (4.5%)	
G2	770 (48.2%)	531 (49.8%)	
G3	751 (47.0%)	483 (45.3%)	
unknown	5 (0.3%)	4 (0.4%)	
Histological type			0.919[3]
ductal	1290 (80.7%)	864 (81.1%)	
lobular	191 (12.0%)	122 (11.4%)	
other	113 (7.1%)	77 (7.2%)	
unknown	4 (0.3%)	3 (0.3%)	
Estrogen receptor status			0.968[3]
negative	482 (30.2%)	322 (30.2%)	
positive	1113 (69.6%)	741 (69.5%)	
unknown	3 (0.2%)	3 (0.3%)	
Progesterone receptor status			0.587[3]
negative	549 (34.4%)	377 (35.4%)	
positive	1045 (65.4%)	686 (64.4%)	
unknown	4 (0.3%)	3 (0.3%)	
HER2 status			0.077[3]
negative	1173 (73.4%)	811 (76.1%)	
positive	398 (24.9%)	233 (21.9%)	
unknown	27 (1.7%)	22 (2.1%)	
Menopausal status			0.775[3]
premenopausal	688 (43.1%)	453 (42.5%)	
postmenopausal	910 (56.9%)	613 (57.5%)	
Type of surgery			0.647[3]
breast conserving	1134 (71.0%)	747 (70.1%)	
mastectomy	461 (28.8%)	316 (29.6%)	
unknown	3 (0.2%)	3 (0.3%)	
Adjuvant chemotherapy			0.624[3]
FEC-DG	780 (48.8%)	510 (47.8%)	
FEC-DOC	818 (51.2%)	556 (52.2%)	

[1] All tests without unknowns.
[2] Mann-Whitney U test.
[3] Chi-square test.
FEC-DG: 3 cycles of fluorouracil-epirubicin-cyclophosphamide followed by 3 cycles of docetaxel and gemcitabine; FEC-DOC: 3 cycles of fluorouracil-epirubicin-cyclophosphamide followed by 3 cycles of docetaxel.

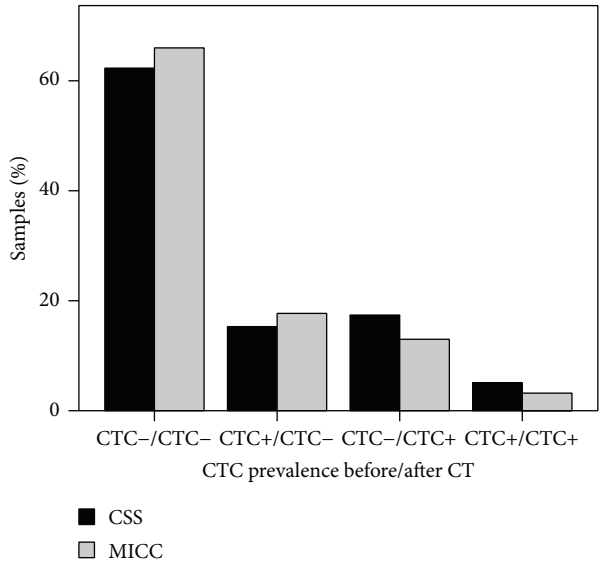


Figure 5: CTC prevalence before and after chemotherapy for patients whose blood samples were analyzed for the presence of CTC using either the CellSearch System (CSS, black bars; $n = 1481$) or manual immunocytochemistry (MICC, gray bars; $n = 744$) at both time points.

patients in the four categories differed significantly between the two methods ($P = 0.006$).

4. Discussion

We evaluated CTC prevalence in peripheral blood of patients with early BC treated within the SUCCESS A trial before and after CHT in two different but comparable and well-balanced patient cohorts of the entire study-population using two different methods for CTC detection. We found ≥1 CTC/30 mL blood in 416 (21.1%) patients before and in 359 (22.5%) patients after CHT using the semi-automated CSS. Using the MICC we detected ≥1 CTC/2×10^6 mononuclear blood cells (tow cytospins) in 247 (20.6%) patients before and in 177 (16.6%) patients after CHT. CTC positivity rate before CHT as assessed based on CSS was associated with a positive lymph-node status, while CTC positivity rate as assessed using MICC was not associated with any of the investigated clinical parameters. CTC positivity rate after CHT was associated with a positive HER2 status both for samples analyzed with CSS and MICC.

The CTC positivity rate as determined based on the two different methods was very similar before CHT (21.1% versus 20.6%). In contrast, the CTC positivity rate after CHT was considerably and significantly higher in samples analyzed using CSS as compared to samples analyzed using MICC (22.5% versus 16.6%).

Overall, detection rates for CTC in peripheral blood were reported in the range from 0.6% to 100% [19, 21–27]. This immense variability may be due to the broad diversity of detection methods which often lack a specific standardization and quality control. More recent data obtained from patients with metastatic BC using both immunocytochemical [1, 4, 22, 25, 28] and molecular techniques [4, 29] suggest a positivity range of 30% to 50% for a CTC positivity cut-off ≥5, and 65% to 85% for a CTC positivity cut-off ≥1 [30–33].

Only limited data exist on CTC prevalence in the adjuvant setting or during follow up period. However, compared to the metastatic situation these studies indicate an even lower prevalence of about 24% to 38% (cut-off ≥1 CTC with CSS) making the detection of CTC even more difficult [6, 34]. In patients with stage I or II BC only about 10% have ≥1 CTC/23 mL blood as stated by Wicha and Hayes [35]. Our results obtained with two different methods (CTC positivity rates of 21.1% by CSS and 20.6% by MICC before CHT, less than 10% of the patients having more than 1 CTC) are very similar to these reported values.

In our study we found comparable positivity rates before but not after CHT using CSS or MICC respectively. One possible explanation for the higher CTC positivity rate after CHT comparing CSS with MICC might be the detection of dormant cells which might not be affected by CHT. Furthermore, Aktas et al. propose that the persistence of CTC might be associated with stem cell like tumor cells and that these cells may undergo phenotypic changes, described as EMT, which enables them to escape conventional CHT [29]. This is however contradicting to the assumption, that these cells might not be detected by CSS (see below). Another hypothesis could be that CTC are somehow affected by CHT and as cell aggregates or smaller cells are lost using the OncoQuick enrichment method. Further investigations are needed to proof or neglect these theories.

A huge variety of different analytical systems for CTC isolation and detection has been developed in recent years, and attempts have been made to standardize preparation protocols and to increase assay efficiency. Two steps (isolation-enrichment and detection) are combined in most cases to identify CTC. Most of them include a separation step based on size (density gradient or filters) or biological characteristics (expression of epithelial- or cancer-specific markers), followed by the detection using ICC or molecular assays [36].

The most reliable and clinical significant results have been so far obtained using CSS as CTC detection method, which was one of the methods we evaluated. CSS is a semi-automated detection device, which importantly minimizes the source of technical failures and therefore reduces technical variability in comparison to a manual method. It is based on a combination of ICC and IF where specific markers for CTC, such as CK and EpCAM are linked to nuclear and leukocyte staining CSS has been validated in a broad clinical testing program. So far this technology has produced the largest amount of clinical data regarding CTC in BC. Different ring experiments with CSS showed very good and comparable results in the participating centers [14, 37]. In addition the supplier offers an online training every 4 months to evaluate its own results. In conclusion, CSS looks like a more reliable method compared to others because of the high standardization, the CD45 counterstaining and clearly defined selection criteria.

Nevertheless there are also some restrictions associated with the system: it is an enrichment and imaging method

only, non-offering the opportunity to further characterize the cells on a molecular level. Moreover, the number of markers available per run is limited to DAPI, CK, CD45, leaving only one additional channel free for one additional marker (usually HER2). Another main disadvantage is the cell enrichment based on EpCAM only. Not all CTC express the same cell-surface antigens (such as EpCAM) and a significant subpopulation of CTC shows epithelial-mesenchymal transition (EMT)/cell stem cell (CSC) traits. Therefore enrichment methods based on EpCAM may miss these cells and underestimate the number of CTC [38–40].

Concerning MICC, several alternative protocols, different in fixatives, buffers, incubation times and antibodies, have been proposed and used [16]. In our study in order to standardize the detection of tumor cells by ICC we followed the protocol published by Fehm et al. [41]. It is important to mention that in order to save time and reduce costs, only two slides per patient were analyzed. Therefore it might be that CTC have been missed and the total CTC load has been underestimated: this may explain the lower cell numbers found using MICC compared to CSS (range before CHT: 1–256 versus 1–827, range after CHT: 1–23 versus 1–124). These differences though were not significant, since the majority of the samples contain only a low CTC load (median 1 CTC) as detected with one or the other method. However it is also possible to get false positive results since the MICC is lacking a CD45 counterstaining for leucocytes. The detection itself is quite simple though and gives no room for doubtful results, since the stained cells appear bright red.

Further characterization of CTC by use of classic ICC techniques is possible according to the investigators preference. Visual observation of stained CK-positive epithelial CTC and quantification of the staining for every single cell for different markers can provide new insights into tumor biology. Thus, MICC opens a variety of possibilities to phenotype CTC and to correlate these results with the morphology of the cells. Detecting the expression of predictive markers such as HER2, ER and other markers simultaneously [42–44] as well as deregulated pathways such as the PI3K/AKT-kinase pathway or phosphorylated EGFR to phenotype CTC further by using MICC are promising research fields [45]. These steps are required as the utilization of biomarkers for BC treatment is evolving with a high pace [46–48]. In addition, the confirmation of EMT/stem cell marker expression such as CD133 or Twist and vimentin in CTC may proof the theory that a subpopulation of CTC shows stem cell characteristics and may play a key role in the metastatic process [49].

PCR based methods, image-based approaches, microfilter and microchip devices are new technical improvements in CTC detection and characterization. The AdnaTest Breast-Cancer (Adna-Gen AG, Langenhagen, Germany), which is a PCR based CTC analysis method, shows an equivalent sensitivity to CSS detecting ≥2 CTC [9]. Using AdnaTest it is possible to detect very low numbers of CTC by detecting the expression of tumor associated genes, which is one advantage compared to CSS [36]. Further AdnaTest allows the detection of different additional markers such as ER, PR and EMT- or CSC-markers [50, 51] offering the possibility to further characterize the biology and molecular abnormalities of CTC to better understand the metastasizing process as well as to conduct personalized CTC directed cancer treatment [9]. However, AdnaTest does not allow a correlation with the cell morphology or with the number of cells. Since an immediate proceeding of the samples is required, it is no approach suitable for a multicenter setting.

Limitations of our study are that we evaluated two very different CTC detection methods, which differ with respect to the enrichment, the identification and the blood volume used. Further we compared the two methods in two different non-overlapping patient populations of the entire patient collective treated within the SUCCESS A trial. However the analyzes was done with very large patient numbers (n = 3170 before CHT and n = 2664 after CHT) and the patient cohorts were well-balanced concerning the common clinicopathological parameters.

5. Conclusion

The detection of CTC in BC is a research field of high clinical interest and impact. The prognostic relevance in the metastatic setting is well demonstrated. Data in the adjuvant situation however is still limited. CTC detection in early BC is impaired by low positivity rate and low cell numbers. There exist a huge variety of detection methods with different advantages and disadvantages. In our study we evaluated the semi-automated CSS and MICC and found comparable CTC positivity rates before but not after adjuvant CHT in two different not overlapping but well-balanced cohorts of patient with early BC. Currently the CSS is regarded as the gold standard for CTC detection in studies aiming at investigating the prognostic value of CTC and should be further evaluated in clinical trials. However, characterization and phenotyping of CTC is crucial to deepen our understanding of metastases formation. Open systems as MICC used in our study offer the possibility to phenotype CTC and compare the marker expression with the cell morphology. In conclusion, the different approaches for CTC detection complement each other and may provide different insights in tumor biology. Highly standardized preparation protocols and high assay efficiency are strongly needed to reliably detect CTC [36].

Abbreviations

BC: Breast cancer
CK: Cytokeratin
CSC: Cancer stem cells
CTC: Circulating tumor cell
DAPI: 4,6-diamidino-2-phenylindole
DFS: Disease free survival
DTC: Disseminated tumor cell
EMT: Epithelial-mesenchymal-transition
EpCAM: Epithelial cell adhesion molecule-1
ER: Estrogen receptor
FACS: Flow cytometry
ICC: Immunocytochemistry
IF: Immunofluorescence
MBC: Metastatic breast cancer

MRD: Minimal residual disease
OS: Overall survival
PFS: Progression free survival
PR: Progesterone receptor
RT-PCR: Reverse transcriptase-polymerase chain reaction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Macrophage Capping Protein CapG Is a Putative Oncogene Involved in Migration and Invasiveness in Ovarian Carcinoma

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The actin binding protein CapG modulates cell motility by interacting with the cytoskeleton. CapG is associated with tumor progression in different nongynecologic tumor entities and overexpression in breast cancer cell lines correlates with a more invasive phenotype *in vitro*. Here, we report a significant CapG overexpression in 18/47 (38%) of ovarian carcinomas (OC) analyzed by qRealTime-PCR analyses. Functional analyses in OC cell lines through siRNA mediated CapG knockdown and CapG overexpression showed CapG-dependent cell migration and invasiveness. A single nucleotide polymorphism rs6886 inside the CapG gene was identified, affecting a CapG phosphorylation site and thus potentially modifying CapG function. The minor allele frequency (MAF) of SNP rs6886 (c.1004A/G) was higher and the homozygous (A/A, His335) genotype was significantly more prevalent in patients with fallopian tube carcinomas (50%) as in controls (10%). With OC being one of the most lethal cancer diseases, the detection of novel biomarkers such as CapG could reveal new diagnostic and therapeutic targets. Moreover, in-depth analyses of SNP rs6886 related to FTC and OC will contribute to a better understanding of carcinogenesis and progression of OC.

1. Introduction

Ovarian cancer globally represents the fifth leading cause of cancer-related death among women [1]. As no effective screening is available, it is mostly diagnosed in advanced and metastatic stages [2] and therefore it is associated with a poor overall prognosis. Despite multimodal treatment strategies including surgery, chemo-, and recently antiangiogenic therapy, the average five-year overall survival of advanced stages does not exceed 40% (American Cancer Society, 2013) and the ratio of new diagnosis and death is with 1.4 most unfavorable [3].

Ovarian carcinoma has traditionally been classified histologically into serous, mucinous, endometrioid, clear-cell, and Brenner tumors, but a new tumor progression model has just recently led to a dualistic classification based on cellular invasiveness and genetic alterations [4]. Type I tumors mostly develop from precursor lesions, are slow growing and in 65% show mutations in the *braf* and *kras* genes, which play a crucial role in the cell growth signaling cascade [5]. Their development is therefore thought to follow the adenoma-to-carcinoma sequence [6].

In contrast, type II or high-grade tumors evolve rapidly with early metastasis within the peritoneal cavity. No precancerous components allow for an early detection; the median survival averages 30 months only. In 50–80% they harbor various p53 mutations [7] and an overall genetic instability is found, while a certain sequence has not yet been identified.

Recognizing that migration and invasiveness play an important role, especially in high-grade ovarian carcinoma

development, we focused our study on CapG, an actin binding protein that promotes cellular motility and has previously been associated with increased invasiveness in breast cancer [8, 9].

CapG has been identified as an oncogene in various carcinomas [10–12]. In profiling array analyses, we have formerly shown an increased CapG expression in breast and ovarian cancer [13]. It is a member of the gelsolin protein family, which comprises cytoplasmic and nuclear proteins. These, among others, are involved in the shaping of the cytoskeleton by remodeling of actin filaments. This 39 kDa protein is found in both compartments, the cytoplasm and the nucleus [14], and contributes up to 1% to the entire protein amount in macrophages. It modulates actin length by capping its plus or so-called barbed ends in a Ca^{2+}- and PIP_2-dependent manner. Like all gelsolin-related proteins, it is structured in homologue domains and lacks, in contrast to other nuclear gelsolin-like proteins, a nuclear export sequence [15] while entering the nucleus is importin β dependent. However, it has no canonical nuclear localization signal (NLS) [16].

Previously, the unique role of CapG, independent of gelsolin, for macrophage motility, phagocytosis, and membrane ruffling has been shown in CapG knockdown mice [17]. Further, CapG and in particular its nuclear fraction has been postulated to promote cellular invasion in collagen *in vitro* [8, 16].

In the current study, we investigate CapG expression by qRT-PCR analyses. Moreover, we establish an examinable design to determine the impact of CapG on migration and invasiveness in OC cell lines.

Finally, by analyzing a single nucleotide polymorphism localized in exon 10 of the CapG gene, we suggest a novel link between fallopian tube and ovarian carcinomas.

2. Material and Methods

2.1. Patients and Tissue Samples. After receiving IRB approval, a total of 47 OC tissue samples and 21 normal adjacent tissues were obtained from a consecutive series of patients treated with surgery for ovarian cancer between 1994 and 2006 at the Department of Gynecology and Obstetrics, Heinrich-Heine-University Hospital, Duesseldorf, Germany. The median age of 47 patients with OC was 55 yrs (range: 18 to 83 yrs) and 54 yrs (24 to 71 yrs) among cases where normal tissue was obtained. FIGO classification was documented in 41/47 cases with 76% of these assigned to stages III and IV, while the majority displayed dedifferentiated tissues (grading ≥ 2). Samples were preserved in liquid nitrogen and stained with hematoxylin and eosin for tumor verification.

For SNP analyses, DNA samples (isolated from EDTA blood samples) from independent sets of 263 OC patients, 12 FTC patients, and 107 healthy controls from women aged older than 50 years (range: 52–74 yrs, mean age: 66 yrs) were collected. The age of OC patients ranged from 16 to 90 years with a mean of 61 yrs. Among fallopian tube carcinoma patients, the mean age was 70 yrs (range 44–78; $n = 12$). Samples from OC and FTC cases were obtained from patients treated with surgery and/or chemotherapy between 1997 and 2006 in our clinic. Control samples were collected from healthy women without any oncologic diseases participating in an endocrine-related study at the Department of Gynecology and Obstetrics, Heinrich-Heine-University Hospital, Duesseldorf, Germany.

2.2. Quantitative RealTime-PCR. Analyses were performed on 7500 Fast-RealTime Systems (Applied Biosystems, USA). RNA was isolated from samples using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. 1 μg RNA was processed for cDNA synthesis (Omniscript RT Kit, Qiagen, Germany) and specific primers (CapG Forward 5′-cga aca ctc agg tgg aga tt-3′; Reverse 5′-tcc agt cct tga aaa att gc-3′; GAPDH Forward 5′-cct gca cca act gct tag-3′; Reverse 5′-tgg cag tga tgg cat gga gtg-3′) were used in qRT-PCR. Relative gene expression was calculated by the ΔΔCT method using GAPDH as housekeeping gene.

2.3. Cell Culture. Ovarian cancer cell lines SK-OV-3 (ATCC number HTB-77) and Hey (ATCC number CLU-302) were maintained in RPMI1640 medium (GIBCO, Invitrogen, Life Technologies, USA) containing 2 mg/mL D-glucose, 1% L-glutamine (GIBCO), 10% fetal calf serum (FCS Gold Mycoplex, PAA, Austria), and 0.1% Gentamicin (GIBCO). Cultivation was carried out standardized (37°C; 5% CO_2; 95% humidity; incubator Heraeus, Germany).

2.4. Retroviral Expression of CapG. For stable CapG overexpression in Hey cells, a PCR-amplified fragment of human CapG (1047 bp) supplemented with restriction sites was digested following the manufacturer's instructions (New England Biolabs, UK) and cloned into the retroviral expression vector S11IN (kindly provided by H. Hanenberg) using bacteria strains DH5α, SURE, and XL-1 BLUE and the Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany) for plasmid extraction. Neomycin resistance was integrated for selection.

Retrovirus producer line EcoR-Phoenix was transfected with either expression vector S11-CapG-IN or S11IN for controls using the FUGENE-6 transfection reagent according to the manufacturer's instructions (Roche, Mannheim, Germany). 48 hours after transfection, supernatants were filtered through a 0.45 μm filter and 1 mL of virus solution was given to Hey cells for stable retroviral transfection. Cell selection was carried out over 7 days using Neomycin.

2.5. SiRNA Mediated CapG Knockdown. SK-OV-3 cells were disseminated in 6-well plates at a concentration of 5×10^4 cells per well containing 2 mL serum free medium (24 hrs). For transfection, 12.5 nM siRNA (sequence 5′-ggu ggu gug gag uca gca u-3′, binding within CapG coding region 335-373, Ambion, UK) was added using the HiPerfect transfection reagent (Qiagen, Germany) according to manufacturer's protocol. Optimal siRNA concentration was tested before and revealed best results after 5 days. siRNA effects were confirmed by qRT-PCR and Western blot analyses.

2.6. Wound Healing Assay (Scratch Assay). Cells were allowed to form confluent layers in standard 6-well plates. Then, cells

were rinsed and a horizontal reference line was drawn in the middle of each well. Three vertical scratches were made per well using a 200 μL pipette tip. For documentation purposes, each scratch was measured at baseline. Cells were cultured in medium (with halved serum concentration) for 24 and 72 hours, respectively, until first wound closure was apparent. Wells were washed again with PBS three times and cells were fixed in place by methanol, stained with toluidine blue (0.1%) and wound closure was measured. Experiments were carried out in triplicates and repeated three times.

2.7. Matrigel Transwell Invasion Assay. Cells were harvested and suspended in FBS-free RPMI1640 medium at a concentration of 1×10^5 cells for transwell invasion assays using BD Biocoat Matrigel invasion chambers (BD Biosciences, USA) following the manufacturer's instructions. Briefly, 500 μL of the cell suspension was added to the upper compartment, while the lower compartment contained 750 μL medium with EGF (15 ng/mL) additionally. After 22 hours of incubation, chambers were rinsed and the upper surface of the membrane was scrubbed with moistened cotton swabs to remove Matrigel matrix and noninvading cells. Afterwards, cells on the lower surface were fixed using methanol and stained with 0.1% toluidine blue. Membranes were cut out and placed on microscope slides for microscopic evaluation and documentation (AxioVision Software, Zeiss, Germany).

2.8. SDS PAGE and Western Blot. Each protein sample (20 μg protein) was processed using the Protein II apparatus (BioRad, Munich, Germany) and according to the Laemmli protocol [18] on 6–12% SDS-polyacrylamide gels. Proteins were then transferred on nitrocellulose membranes (Hybond, GE Healthcare, Freiburg, Germany) and unspecific bonding sites were blocked by Western Blocker Solution (Sigma-Aldrich, Germany). CapG antibody (rabbit IgG, polyclonal) was added and detected by anti-rabbit IgG-HRP (both Santa Cruz Biotech, USA), each in a 1 : 2500 dilution. α-Tubulin antibody (rabbit IgG; Sigma-Aldrich, Germany) and corresponding anti-rabbit-IgG-HRP were used at a 1 : 4000 dilution. Visualization was performed on films (Hyperfilm, GE Healthcare, Munich, Germany) in darkrooms.

2.9. HRM Analyses. After amplification of the target CapG sequence via qRT-PCR, HRM analyses were performed on Fast-RealTime-PCR Systems (Applied Biosystems; USA) detecting allele specific dsDNA dissociation curves. Previously, reference cell lines were analyzed by DNA sequencing and allele corresponding dissociation curves were identified (T47D A/A, Hec1A G/G, MDAMB231 G/A). For analyses, 2 μL DNA sample, 0.6 μL SYTO9 (Life Technologies, USA), and 0.2 μL AmpliTaq Gold DNA polymerase (Life Technologies, USA) were added to primers and buffer.

2.10. Statistical Analysis. Statistical analyses and graphic visualization of present data were performed with the R software (R Project for Statistical Computing; http://www.r-project.org/; Vienna, Austria) using the students t-test.

3. Results

3.1. Overexpression of CapG in OC Samples. CapG expression was determined in OC samples ($n = 47$ serous OC) and in adjacent tissues ($n = 21$). CapG results of each sample were normalized to the housekeeping gene GAPDH. Mean CapG expression level was calculated in normal tissue samples and CapG expression in each tumor sample was compared to mean expression in controls, consecutively (Figure 1).

Mean CapG expression in controls was complemented by standard deviation. Thus, the cutoff for CapG overexpression in tumor samples was set at a 2.0-fold increase of CapG expression compared to the mean expression level in the control probe set.

In 18/47 (38%) of the OC samples, CapG expression was determined above this cutoff.

3.2. CapG Expression in Ovarian Carcinoma Cell Lines. To establish a functional CapG expression *in vitro* model OC cell lines Hey, SK-OV-3, MDAH, and OvCa-3 were analyzed by qRT-PCR with respect to their CapG expression normalized against GAPDH gene expression (Figure 1) and compared to the mean expression level in normal tissues. Hey cells showed the lowest CapG expression (0.25-fold); SK-OV-3 and OvCa-3 cells exceeded normal expression by more than 2-fold. Additionally, CapG expression in OC cell lines was in the same range compared to the OC tissue samples.

3.3. CapG Expression Modulates OC Cell Migration and Correlates with Invasiveness. To estimate the impact of CapG expression on migration in ovarian tumor cell lines, the intrinsically low expressing cell line Hey was stably transfected with a retroviral expression vector (Sl1-CapG-IN), which resulted in 230-fold higher expression of CapG in Hey cells (confirmed at mRNA level by qRT-PCR and at protein level by Western Blot analysis). In contrast, SK-OV-3 cells (with high endogenous CapG expression) were transfected with CapG specific siRNA. A 4-fold reduction in CapG expression was verified by qRT-PCR and Western Blot, respectively (Figures 2(a) and 2(b)).

In wound healing assays, CapG overexpression led to a significantly increased migration rate within 72 hrs compared to untreated Hey cells (by 2.94-fold). Furthermore, CapG knockdown in SK-OV-3 cells decelerated time to complete wound closure (reduction by 0.56-fold). An overall difference was detected between the motility among both cell lines with SK-OV-3 displaying a 2-3-fold increase in migration potential (Figures 2(c) and 2(d)).

For further investigation of comparably small changes in migration rate in SK-OV-3 cells following CapG knockdown, invasiveness was assessed in this cell line by Matrigel transwell invasion assays. After 24 hours, SK-OV-3 cells revealed a significant decrease in invasiveness (to 0.25-fold) indicated by fewer cells transmigrating the BME matrix (Figures 2(e) and 2(f)).

Thus, cellular migration as well as invasiveness was positively correlated with CapG expression levels.

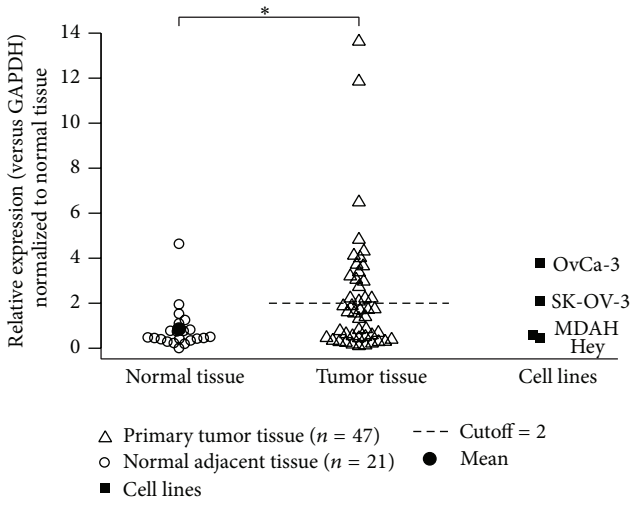

Figure 1: CapG expression in normal and OC tumor tissues. CapG expression in primary tumor tissue samples from 47 patients with OC was analyzed by qRT-PCR and compared to normal adjacent tissue samples ($n = 21$). Single probes were normalized to housekeeping gene GAPDH. Mean expression in normal tissue was calculated and standard deviation added for upper cutoff (dashed line). Thus, expression levels greater than 2.0-fold above mean CapG expression in controls was defined as overexpression and 38% of all tumor samples display such results. To the right, CapG expression of ovarian carcinoma cell lines OvCa-3, SK-OV-3, MDAH, and Hey. Each sample was analyzed in duplicates and repeated three times.

3.4. Single Nucleotide Polymorphism rs6886 is a Putative Link between Ovarian and Fallopian Carcinoma. SNP rs6886 (c.1004G > A) is located in exon 10 of the CapG gene (chromosome 2) affecting the first codon within a protein kinase C phosphorylation recognition motif [$R_{335}ES_{337}$]. This results in an amino acid exchange p.Arg335His destroying the PKC-recognition motif and consequently in a loss of Ser_{337} phosphorylation (unpublished results). This is the first time that a functional effect of rs6886 could be identified. So far, no association studies have been published with respect to oncologic aspects.

Here, we genotyped 263 DNA samples (isolated from EDTA blood samples) of OC patients and 107 DNA samples of healthy controls (age matched) by HRM analyses (Table 1). Results were compared to allele frequencies indicated in the HapMap database (http://www.hapmap.org/), where the homozygous A/A genotype (His) accounts for 8%, the homozygous G/G genotype (Arg) for 43%, and the heterozygous G/A genotype for 48% of all in a normal population.

Although results in OC and control cases were not significantly different ($P = 0.153$), a marked increase in minor A-allele frequency was detected in OC patients. The frequency of the homozygous A/A genotype was well above the own control sample set (16% versus 10%) and twice as high as in the HapMap dataset (8%) or in BC patients (6%, own unpublished data). Looking at the genotype of patients with fallopian tube tumors, half of all cases display the homozygous A/A genotype. Therefore, in this cohort, the homozygous histidine encoding variant (A/A) is significantly associated with fallopian tube carcinomas ($P < 0,0001$).

4. Discussion

In OC, diagnosis is usually late due to a lack of precursors and biomarkers. Just recently, a new classification has been established with type II tumors being aggressive with early invasive growth. Despite p53 mutations, no specific molecular alterations have been identified [2].

In a variety of tumors, overexpression of CapG has been demonstrated and recently the association of CapG overexpression and metastasis has been shown in colorectal cancer [19]. Performing an *in silico* expression profiling approach for identification of differentially regulated genes in gynecological cancer, CapG has been identified as a putative oncogene overexpressed in breast and ovarian cancer [13].

Moreover, recent microarray analyses have shown associations between CapG expression and tumor prognosis with CapG overexpression in deceased patients with stage III serous OC in comparison to normal CapG expression in tumors of a survivor cohort [20]. Underlining its crucial role in tumor progression, CapG expression was found elevated at the tumor invasion front, the so-called interface zone, in breast cancer particularly [21]. A correlation of CapG overexpression with cell motility and invasive phenotype of CapG overexpressing breast and pancreatic cancer cell lines was also shown previously [8, 12].

By capping actin polymers, CapG is a key player in cytoskeletal remodeling and membrane ruffling [17]. While cellular motility can be influenced through cytoplasmic CapG, the function of nuclear CapG remains unclear. *In vitro* experiments with epithelial kidney cells revealed that transfectants with modified CapG carrying a nuclear export sequence (NES) were less invasive [16].

Our study aims at investigating the effect of differential CapG expression in OC cells hypothesizing that CapG overexpression is involved in OC cell motility and invasiveness. In a sample set of 47 ovarian carcinomas and 21 normal tissue samples, CapG overexpression was determined in 38% of all tumor samples (18/47), which was concordant to previous results based on cancer profiling arrays [13]. The cutoff value was set at a standard deviation higher than the mean expression level determined in normal adjacent tissue samples (Figure 2). Mean CapG expression levels in tumor specimen and normal tissues differed significantly ($P < 0.027$) indicating the putative oncogenic function of CapG in ovarian carcinomas.

To establish an *in vitro* model for further functional characterization of CapG in ovarian carcinomas, four ovarian carcinoma cell lines were analyzed. CapG expression levels were comparable and within the range of the OC samples with a significant CapG overexpression in OvCa-3 and SK-OV-3 cells. The latter has been described as more aggressive with a higher intrinsic metastatic potential [22] and thus was chosen for in-depth analyses.

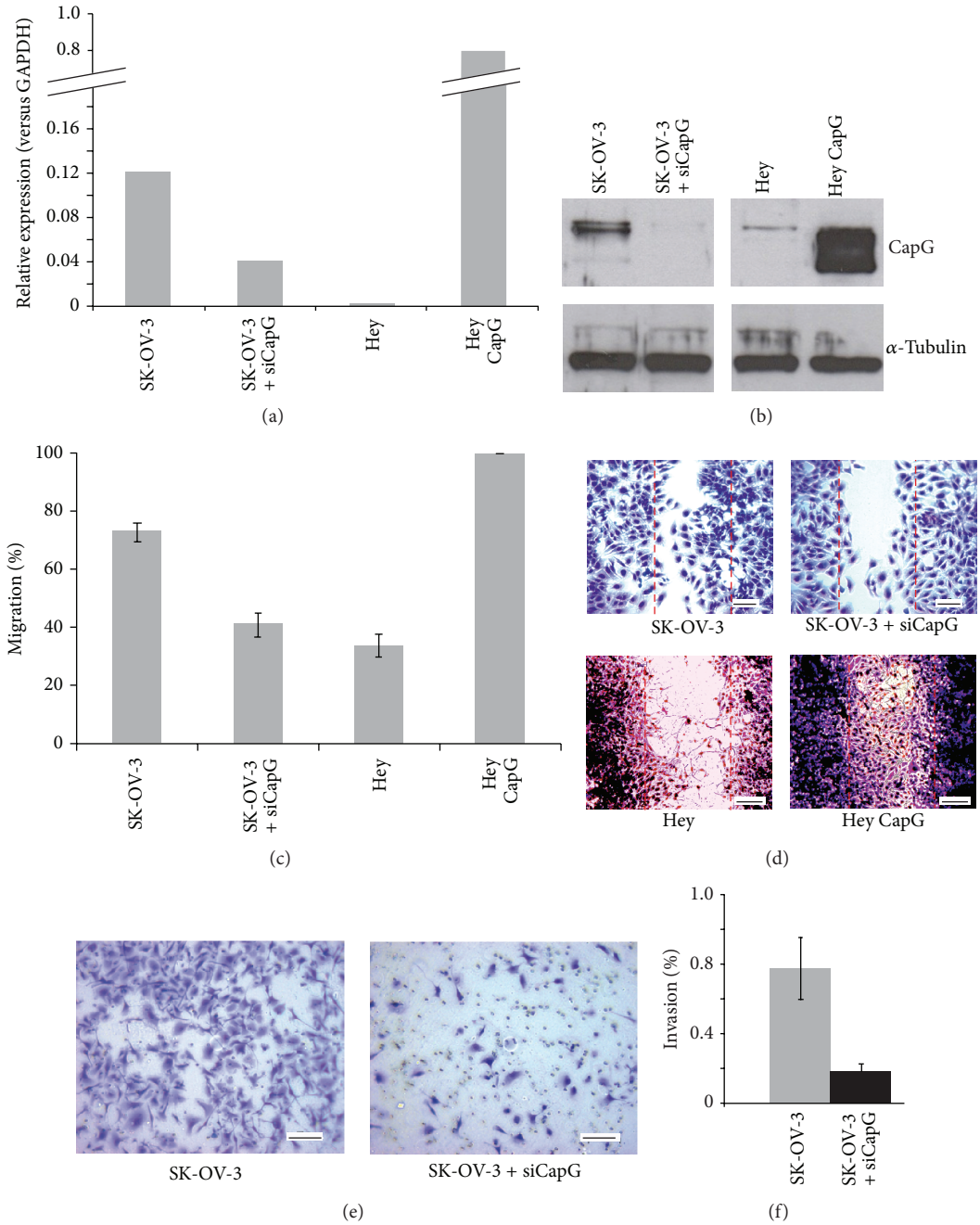

Figure 2: (a) Relative CapG expression of ovarian cancer cell lines SK-OV-3 and Hey, prior to and after alteration of CapG expression using CapG mediated siRNA (SK-OV-3 + siCapG) and stable retroviral transfection (Hey CapG), respectively. Values were calculated performing qRT-PCR in duplicates, repeated three times. Each probe was normalized to the housekeeping gene GAPDH. (b) Effects were confirmed by Western blot analyses. Equal detection of α-tubulin in samples verified equality of protein quantity. (c, d) Performing a migration assay (scratch assay), the motility in CapG siRNA treated cells was nearly halved in 24 hrs while overexpression in Hey cells led to a significantly earlier closure of the scratch after 72 hrs. (e, f) The invasiveness of siRNA treated cells was also significantly decreased in the transwell Matrigel invasion assay (by 4-fold) in SK-OV-3 cells.

To demonstrate the impact of CapG expression on cell migration we investigated the effects of CapG overexpression in Hey transfectants and CapG depletion in SK-OV-3 cells in comparison to the parental cell lines.

Transfected Hey cells displayed a significant increase in motility in wound healing assays compared to untreated Hey cells. Concordant to that, the migration rate in CapG knockdown SK-OV-3 cells was reduced by nearly 50% (Figure 2).

Table 1: Genotype frequencies of the single nucleotide polymorphism SNP rs6886.

SNP type	CTRL		OC		FTC		BC		HapMap
	n=	%	*n*=	%	*n*=	%	*n*=	%	%
homozygous-His (A/A)	11	10.28	42	15.96	6	50.00	7	6.14	8
homozygous-Arg (G/G)	51	47.66	107	40.68	5	41.66	55	48.24	43
heterozygous (G/A)	45	42.05	114	43.34	1	8.33	52	45.61	48
Total	**107**	**100**	**263**	**100**	**12**	**100**	**114**	**100**	**100**

SNP rs6886 (c.1004A>G, p.His335Arg) genotype frequencies were determined in healthy controls (CTRL), ovarian carcinoma (OC), fallopian tube carcinoma (FTC), and breast cancer (BC) cases compared to the published data of the International HapMap Project (http://www.hapmap.org/). CapG reference sequence (NM_001747.3) was obtained from the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq/).

Results suggested that CapG overexpression has a higher impact on migration than CapG reduction. But looking at a markedly stronger effect of overexpression on mRNA level in transfected Hey cells (230-fold) compared to relatively small changes following CapG knockdown (to 0.25-fold only), the altered migration rate appears to be proportional to the differential CapG expression in the investigated cell lines. With some CapG protein still being expressed, residual migration potential in treated SK-OV-3 cells may be expected.

However, to further evaluate the impact of CapG depletion on cell migration, we analyzed siRNA treated SK-OV-3 cells in a Matrigel transwell invasion assay. Cells invading a basement membrane extract (BME) *in vitro* are thought to transmigrate the basement membrane *in vivo*. Thus, we analyzed the role of CapG not only with regard to migration properties but also to proinvasive characteristics of an aggressive cell type [23]. Recent studies have shown that these CapG characteristics are most probably distinct from its major biological function [16] and possibly require nuclear CapG regulation.

The even more pronounced reduction (75%) of the invasive phenotype of SK-OV-3 cells after CapG depletion is in good agreement with the results of the scratch assay. It suggests that CapG might play an important role in migration as well as tumor invasion also in OC. Mechanistically, it remains to be explored how CapG influences the invasive behavior of cells. It is conceivable that tumor migration and proliferation rely on increased cancer cell motility through accelerated cytoplasmic actin turnover. On the basis of recent findings by that the nuclear fraction seems to be crucial for invasiveness, a key role of CapG in the nucleus is also possible [24]. By subcellular fractioning this matches with our findings (unpublished data) that SK-OV-3 cells, which display a highly invasive phenotype, contain a considerable amount of nuclear CapG protein. This becomes even more evident since nuclear complexes have been identified to carry nuclear actin and can be modified by actin-binding proteins such as CapG [25]. One of them is the BAF (Brg1/Brm-associated factor) complex which modulates DNA transcription in the nucleus [26, 27]. The molecular relationship of the subcellular localization of CapG and an invasive phenotype remains a challenging topic for in-depth investigations.

Since these results suggest that CapG modulates invasiveness in OC cell lines, we further strived to explore molecular mechanisms that contribute to CapG functioning.

Phosphorylation of CapG protein was already described as a potential posttranslational modification of functional relevance [28]. Using prediction tools like NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) and PhosphoMotif finder (http://www.hprd.org/PhosphoMotif_finder), we identified potential phosphorylation sites of the CapG protein sequence. Amino acid Ser_{337} was identified as a potential phosphorylation site within a protein kinase C phosphorylation site recognition motif. This motif including Arg_{335} and consequently phosphorylation of Ser_{337} is affected by SNP rs6886 (p.Arg335His). For further evaluation of SNP rs6886, we carried out an association study, analyzing allele frequencies in gynecological tumor entities in order to reveal indications for an oncologic impact of this polymorphism. So far, the SNP has been associated with increased intima media thickness in carotid ultrasounds [29] but has not been investigated on other backgrounds.

Comparing 263 OC cases and 107 controls by genotyping SNP rs6886 performing HRM analyses, the MAF (A allele) was higher in OC cases (37.5%) and FTC cases (54%) compared to controls (31%). Even more evident was the genotype distribution with the homozygous A/A genotype expressing the histidine variant in 50% of FTC cases compared to 10.3% in controls ($P < 0.0001$). Interestingly, no differences in allelic frequencies were found in BC samples. Despite the low number of cases/controls investigated in this study, these results suggest a functional relevance of SNP rs6886 in OC and FTC. Because of the rarity of FTC, only 12 samples were available and confirmation of these results in larger probe sets in future studies is needed.

Although its relevance is yet not understood, this polymorphism might hint to a possible link between OC and FTC. In case CapG phosphorylation at Ser_{337} affects cell motility and invasiveness, the CapG Arg_{335} variant with intact PKC phosphorylation recognition site may be relevant for migration of tumor cells from fallopian tubes into the ovaries. Recent findings have opened the discussion whether the fallopian tube might be point of origin of the serous OC. The development of ovarian cancer in Dicer-Pten (crucial for miRNA synthesis) double-knockout mice could be inhibited by removal of the fallopian tube *in vivo* [30]. Supporting these findings, the gene expression profiles of experimentally induced carcinomas in mice and serous high-grade OC in women resemble to a high degree. In a set of 41 serous high-grade tumors, 59% also displayed serous tubal intraepithelial carcinomas (STICs) [31].

5. Conclusion

In this study, CapG overexpression in 38% of OC was found and its functional relevance on cell migration as well as invasiveness has been clearly demonstrated. Although mechanisms are still to explore, the positive correlation of SNP rs6886 as a possible functional relevant CapG variant with FTC may introduce a novel interesting link between ovarian and fallopian tube carcinomas. However, further studies are needed to validate the association of SNP rs6886 with OC and FTC and to evaluate CapG as a prognostic biomarker.

Abbreviations

Arg:	Arginine
BC:	Breast cancer
BME:	Basement membrane extract
CTRL:	Control
EGF:	Epithelial growth factor
FTC:	Fallopian tube carcinoma
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
His:	Histidine
HRM:	High resolution melting
KDa:	Kilodalton
MAF:	Minor allele frequency
OC:	Ovarian carcinoma
PBS:	Phosphate buffered saline
PIP_2:	Phosphatidylinositol 4,5-bisphosphate
PKC:	Protein kinase C
Pten:	Phosphatase and tensin homolog
qRT-PCR:	Quantitative realtime-PCR
RefSeq:	Reference sequence
SiRNA:	Small interfering RNA
SNP:	Single nucleotide polymorphism
STIC:	Serous tubal intraepithelial carcinoma.

Conflict of Interests

The authors declare that there is no potential conflict of interests.

Authors' Contribution

D. Niederacher and M. C. Fleisch contributed equally to the direction of the work.

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Review Article

First Trimester Biomarkers in the Prediction of Later Pregnancy Complications

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Adverse obstetric outcomes, such as preeclampsia, preterm birth, gestational diabetes, and fetal growth restriction, are poorly predicted by maternal history and risk factors alone, especially in nulliparae. The ability to predict these outcomes from the first trimester would allow for the early initiation of prophylactic therapies, institution of an appropriate model and location of care, and recruitment of a truly "high risk" population to clinical trials of interventions to prevent or ameliorate these conditions. To this end, development of adequately sensitive and specific predictive tests for these outcomes has become a significant focus of perinatal research. This paper reviews the biomarkers involved in these multiparametric tests and also outlines the performance of these tests and issues regarding their introduction into clinical practice.

1. Introduction

It is common practice for pregnancies to be predictively categorised as "low" or "high" risk, based on the perceived likelihood of an adverse neonatal or maternal outcome. The dichotomous simplicity of such a categorisation is immediately appealing but fails to reflect adequately the spectrum of risk that exists for all pregnant patients and does not acknowledge the limited clinical utility of current strategies for the prediction of obstetric risk, especially among nulliparae. Routine antenatal investigations generally aim to identify concurrent conditions of obstetric relevance, such as thalassaemia, anaemia, and vertically transmissible maternal infections, such as syphilis [1]. First trimester screening for fetal aneuploidy represents the most commonly employed test in early gestation for the prediction of a later pregnancy complication, namely, the delivery of an infant with a chromosomal anomaly. The principles that underpin these multiparametric tests for aneuploidy have informed the recent development of screening strategies using multiple biomarkers in early gestation for the prediction of other later pregnancy complications [2], such as preeclampsia, spontaneous preterm birth, gestational diabetes, and fetal growth restriction. In addition to outlining the rationale for predictive testing in the first trimester, this paper aims to review the composition and performance of testing strategies for these four entities, which are cumulatively responsible for a significant proportion of adverse perinatal and maternal outcomes. For the purpose of this review, a biomarker is considered to be any objectively measured and evaluated characteristic that reflects normal or pathogenic biological processes [3].

2. The Rationale for Screening

The capacity in early gestation to predict later pregnancy complications allows for

(i) the early commencement of proven prophylactic therapies (pharmacological and otherwise) to reduce the risk of the adverse outcome in question;

(ii) institution of an appropriate model of antenatal care and level of clinical surveillance;

Table 1: Recurrence of pregnancy complications.

	Relative risk (RR)/odds ratio (OR)	95% Confidence interval (CI)
Preeclampsia [4]	RR 7.19	5.85–8.83
Preterm birth [5]	RR 13.56	11.5–16.0
Gestational diabetes [6]	RR 21.33	19.90–22.86
Fetal growth restriction [7]	OR 8.1	7.8–8.5

Table 2: Summary of first trimester multiparametric tests for later pregnancy complications.

Complication	Author	Biomarkers	Detection rate	False positive rate
Preeclampsia	Poon et al., 2009 [8]	MF, MAP, UtA PI, PlGF, and PAPP-A	93%	5%
	Park et al., 2013 [9]	MF, MAP, UtA PI, and PAPP-A	91.7%	10%
	Scazzocchio et al., 2013 [10]	MF, MAP, UtA PI, PAPP-A, and free βhCG	80.8%	10%
Preterm birth	Greco et al., 2012 [11]	MF, CRL, and cervical length	54.8%	10%
Gestational diabetes	Maged et al., 2013 [12]	SHBG and CRP	74%	24%
	Nanda et al., 2011 [13]	MF, adiponectin, and SHBG	74.1%	20%
Fetal growth restriction	Karagiannis et al., 2011 [14]	MF, NT, PAPP-A, free βhCG, MAP, UtA PI, PlGF, PP13, and ADAM12	73%	10%
	Poon et al., 2013 [15]	MF, UtA PI, MAP, PAPP-A, and PlGF	55.5%	10.9%

MF: maternal factors; MAP: mean arterial pressure: UtA PI: uterine artery Doppler pulsatility index; PlGF: placental growth factor; PAPP-A: pregnancy-associated plasma protein A; βhCG: beta human chorionic gonadotrophin; CRL: fetal crown-rump length; SHBG: sex hormone binding globulin; CRP: C-reactive protein; NT: fetal nuchal translucency; PP13: placental protein 13; ADAM12: A disintegrin and metalloprotease.

(iii) recruitment of a truly "high risk" population to trials of interventions intended to prevent or mitigate specific adverse outcomes.

Pregnancy is a domain in which past outcomes do, to a large extent, predict future risk. Among the strongest risk factors for preeclampsia, preterm birth, gestational diabetes, and fetal growth restriction is a history of these conditions in a prior gestation, as outlined in Table 1.

However, such information is of little benefit to a first-time mother's pregnancy complicated by these adverse outcomes, which may have resulted in significant maternal and perinatal morbidity, if not mortality. Trying to predict such adverse events on the basis of maternal factors alone is of limited utility in primigravidae. For example, for the prediction of preeclampsia in nulliparae, an algorithm incorporating maternal factors such as body mass index, mean arterial pressure, and family history yields only a 37% detection rate for a 10% false positive rate [16]. These limitations have prompted research into more sophisticated predictive tests for adverse pregnancy outcome.

Abnormal levels of maternal serum analytes assessed in conventional aneuploidy screening have long been acknowledged to have a statistically significant association with adverse obstetric outcome in euploid pregnancies. For instance, a pregnancy-associated plasma protein A (PAPP-A) of less than 0.42 multiples of the median (i.e., less than the 5th centile) in the first trimester has an adjusted odds ratio of 2.81 (95% CI 2.35–3.35) for low birth weight (less than the 5th centile) [17]. However, its sensitivity is only 12.23%, with a positive predictive value of 9.5%. Indeed, no later pregnancy complication can be predicted with sufficient specificity and sensitivity by any single biomarker. Multiparametric testing—the assessment of multiple parameters in combination (including baseline maternal characteristics)—is required to achieve adequate predictive performance, as is the case with first trimester combined screening for aneuploidy [18]. Table 2 summarises the best-performing multiparametric tests for the common pregnancy complications reviewed in this paper.

3. Preeclampsia

Preeclampsia is the commonest serious medical disorder of pregnancy, with a worldwide incidence of 2–8% [19]. The utility of a wide range of biomarkers in predicting this outcome has been investigated, including

(i) maternal mean arterial pressure [20, 21];

(ii) uterine artery Doppler indices [22, 23];

(iii) markers of placental function, such as PAPP-A and plasma protein 13 (PP13) [24];

(iv) other proteins of placental origin, including inhibin A [25] and activin A [26];

(v) angiogenic agents, such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) [27], and their inhibitors soluble fms-like tyrosine kinase-1 (sFlt-1) [28] and soluble endoglin (sEng) [29].

Only in combination do any of these biomarkers offer sufficient sensitivity and specificity to be of clinical utility in the prediction of preeclampsia. In 2009, researchers from the Fetal Medicine Foundation (UK) evaluated a multiparametric test incorporating maternal factors, mean arterial pressure, uterine artery Doppler pulsatility index, placental growth factor (PlGF), and PAPP-A in 7797 singleton pregnancies. It detected 93% of early-onset preeclampsia for a false positive rate (FPR) of 5% [8]. This approach has recently been validated in an Australian population ($n = 3066$), in which 91.7% of early-onset preeclampsia was detected for a FPR of 10% [9], and in Spain ($n = 5759$), with an 80.8% detection rate for early preeclampsia at a 10% FPR [10]. Some institutions have now introduced this testing strategy into clinical practice and are conducting it at the same time as conventional first trimester aneuploidy screening. Interestingly, just as cell-free fetal DNA in maternal serum is becoming the new "gold standard" screening test for aneuploidy, so too has it been found to have value in predicting preeclampsia, with a recent small retrospective case-control study ($n = 72$) demonstrating a sensitivity and specificity of 100% for the development of this condition [30].

The clinical utility of these predictive tests for preeclampsia is enhanced by the potential availability of prophylactic therapies to ameliorate the condition. Low-dose aspirin (75–100 mg daily) has been shown to reduce the risk of preeclampsia, especially if started in early pregnancy (<16 weeks) [31]. Its capacity to do so among those predicted to be at high risk of this condition on the basis of predictive tests is the subject of ongoing randomised trials [32]. The only other therapy proven to reduce the risk of preeclampsia is calcium [33], although many other agents are under investigation, including low-molecular-weight heparin [34], high-dose folate [35], vitamin D [36], and statins [37].

4. Preterm Birth

Spontaneous preterm labour accounts for around 60–70% of all preterm deliveries and thus makes a significant contribution to perinatal morbidity and mortality [38]. The disparate pathogenic processes that result in preterm labour are incompletely understood [39], and as such, its prediction remains challenging. Meta-analyses of 116 biomarkers studied over the last four decades [40], and 30 novel biomarkers investigated over the last ten years [41] have concluded that none perform sufficiently in predicting preterm birth to be clinically useful. Similarly, attempts to devise multiparametric models, incorporating biomarkers such as PAPP-A, PP13, and uterine artery Doppler indices in the first trimester, perform as well as or only marginally better than risk prediction based on maternal characteristics alone [42, 43]. Incorporating measurement of cervical length at 11–13 weeks may improve predictive performance, with one such model predicting 54.8% of all spontaneous preterm births earlier than 34 weeks (at a FPR of 10%) when evaluated in 9974 pregnancies [11], although others have not replicated these findings [44, 45]. This is in contrast to cervical assessment in the second trimester, which has consistently been shown to aid in the prediction of preterm birth [46, 47].

Similarly, current strategies to prevent early spontaneous delivery are limited in scope and effect [39]. Apart from modification of lifestyle factors, therapies with the most evidence include vaginal progesterone supplementation [48] and cervical cerclage [49]. These interventions have mostly been studied in women with a history of preterm birth and/or with a short cervix in the midtrimester. Until the pathogenic pathways for preterm labour are better understood, improved prediction and thus prevention of this outcome will remain an enigma [50], especially for nulliparae with no *a priori* risk factors, and may prove to be optimally instituted in the second rather than first trimester.

5. Gestational Diabetes

The worldwide incidence of gestational diabetes mellitus (GDM) is rising [51], and although controversy continues regarding optimal diagnostic thresholds [52], there is clear evidence that its identification and treatment optimise perinatal outcomes [53]. Numerous risk factors for gestational diabetes are well established, including maternal BMI, advancing age, cultural background, history of polycystic ovarian syndrome, and family history of diabetes [54], although some develop impaired glucose tolerance in the absence of any identifiable risk factor. Accurate early prediction of those destined to develop gestational diabetes would allow for the early initiation of measures that may prevent or ameliorate the effects of this condition, such as exercise programs [55] and dietary modifications [56], although further research is required to confirm the specific benefits of these early interventions.

Elevated fasting blood sugar levels (within the range of normoglycaemia) in the first trimester are independently associated with the later development of gestational diabetes, with fasting glucose cut-off levels of 80–85 mg/dL yielding sensitivities of 75–55% and specificities of 52–75% for GDM prediction in one study ($n = 4876$) [57]. Other biomarkers shown to be predictive for GDM include sex hormone binding globulin (SHBG), highly sensitive C-reactive protein (CRP), and adiponectin. A model incorporating SHBG and CRP assessed prior to 15 weeks ($n = 269$) predicted GDM with sensitivity and specificity of 74.07% and 75.62%, respectively, with an overall accuracy of 75.46% [12]. An alternative model, using adiponectin and SHBG in addition to maternal characteristics, demonstrated similar predictive performance in a case-control study of 380 women: 74.1% detection for a 20% false positive rate, compared with 61.6% detection using maternal characteristics alone [13].

6. Fetal Growth Restriction

A growth-restricted fetus is one that has failed to reach its growth potential. Many, but not all, of such fetuses will be small for gestational age: less than the 10th centile on population or customised growth charts, depending on local policy. Growth-restricted infants are overrepresented in perinatal morbidity and mortality statistics [58] and have increased lifetime risks of cardiovascular and metabolic disease [59]. Fetal growth restriction (FGR) can arise on account of maternal, placental, or fetal factors, many of which are unmodifiable (e.g., maternal age), or cannot be modified once pregnancy is established. As such, apart from lifestyle modifications such as smoking cessation, few interventions have been identified that can prevent or mitigate the effects of FGR in gravida predicted to be at high risk of the same. Low-dose aspirin (75–150 mg daily) has demonstrated benefit in preventing growth restriction in the absence of preeclampsia when commenced in early gestation: a meta-analysis comparing aspirin commenced at <16 weeks and >16 weeks found a significant difference in relative risk of FGR between the two groups (RR = 0.46 (95% CI 0.33–0.64) versus 0.98 (95% CI 0.88–1.08), $P < 0.001$) [60]. At present, the primary benefit arising from the early prediction of growth restriction is likely to be the adoption of a model of obstetric care that facilitates appropriate clinical and sonographic surveillance.

Given the putative similarities in the placental origins of some aspects of FGR and preeclampsia, it is not surprising that many biomarkers are common to the prediction of both. Doppler analysis of the uterine artery in the first trimester is significantly different in pregnancies destined to develop FGR, with a recent prospective study identifying a correlation between the lowest uterine artery pulsatility index value and subsequent birthweight in an unselected population [61]. Ultrasound can also be used to estimate placental volume in the first trimester, which is significantly smaller in FGR pregnancies [62].

As always, multiparametric testing strategies achieve the best predictive performance. Almost three-quarters (73%) of fetal growth restriction requiring delivery prior to 37/40 was identified in a study ($n = 32850$) using a first trimester screening algorithm incorporating maternal factors and numerous biomarkers, including fetal nuchal translucency (NT) thickness, serum pregnancy-associated plasma protein-A (PAPP-A), free beta-human chorionic gonadotrophin (beta-hCG), mean arterial pressure (MAP), uterine artery pulsatility index (PI), placental growth factor (PlGF), placental protein 13 (PP13), and A disintegrin and metalloprotease (ADAM12) [14]. A subsequently proposed model using a more pragmatic number of biomarkers (maternal characteristics, uterine artery pulsatility index, MAP, PAPP-A, and PlGF) achieved a detection rate for FGR requiring delivery <37 weeks of 55.5% for a 10.9% FPR in a study group of 62052 pregnant women [15].

7. Future Directions

The application of these predictive tests to the general population of pregnant women is an example of screening. It is crucial that the principles of screening [63], established in the 1960s and unchanged since, be applied to this domain. In particular, there must be interventions proven to improve outcomes for women and their babies who are screened to be at "high risk" of specific pregnancy complications. Patient and clinician acceptability would be enhanced through the delivery of these tests in an integrated model with well documented performance characteristics and would ideally involve screening for aneuploidy as well. The cost- effectiveness of these tests must be clearly established, and robust economic modelling will be required to justify the allocation of limited public healthcare resources to them. If the costs of these tests are to be borne privately, they run the risk of promoting further health inequity, particularly among those who live some distance from tertiary-level obstetric care, for whom these tests may be of significant potential benefit. Furthermore, the potential impact of commercial imperatives on the timing and clinical context of the introduction of these tests should be acknowledged, anticipated, and managed appropriately.

As with any screening program, participation should be voluntary, and clinicians must remain comfortable caring for patients who exercise their right "not to know" and decline any or all of these screening tests. Finally, the emotional and psychological impact of a "high risk" result can be substantial [64], whether the predicted outcome eventuates or not, and it is important that appropriate resources are in place to support those screened to be high risk.

Once these concerns are addressed, predictive testing strategies in early pregnancy could finally offer a clinically meaningful and accurate distinction between "low" and "high" risk pregnancies, with improved outcomes for both as a result.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Meconium Indicators of Maternal Alcohol Abuse during Pregnancy and Association with Patient Characteristics

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Aim. Identification of women with moderate alcohol abuse during pregnancy is difficult. We correlated self-reported alcohol consumption during pregnancy and patient characteristics with objective alcohol indicators measured in fetal meconium. *Methods*. A total of 557 women singleton births and available psychological tests, obstetric data and meconium samples were included in statistical analysis. Alcohol metabolites (fatty acid ethyl esters (FAEEs) and ethyl glucuronide (EtG)), were determined from meconium and correlated with patient characteristics. *Results*. We found that 21.2% of the 557 participants admitted low-to-moderate alcohol consumption during pregnancy. Of the parameters analyzed from meconium, only EtG showed an association with alcohol history ($P < 0.01$). This association was inverse in cases with EtG value above 120 ng/g. These values indicate women with most severe alcohol consumption, who obviously denied having consumed alcohol during pregnancy. No other associations between socioeconomic or psychological characteristics and the drinking status (via meconium alcohol metabolites) could be found. *Conclusion*. Women who drink higher doses of ethanol during pregnancy, according to metabolite measures in meconium, might be less likely to admit alcohol consumption. No profile of socioeconomic or psychological characteristics of those women positively tested via meconium could be established.

1. Introduction

Self-reported maternal alcohol abuse during pregnancy is not reliable and ethanol consumption is rarely admitted, if at all [1, 2]. However, alcohol consumption during pregnancy is a relevant problem, with its estimated prevalence ranging from 3.5 up to 53.9% in European countries [1–3]. Excessive prenatal alcohol exposure is reported to be associated with severe consequences for the fetus, such as premature birth, miscarriages, fetal alcohol syndrome (FAS) or fetal alcohol spectrum disease (FASD), and other physical and neuropsychological disorders [4–6].

Even low or moderate alcohol exposure may lead to a higher perinatal mortality [7], and it may cause congenital anomalies. In addition, the risk increases with higher dose [8, 9]. The role of moderate alcohol consumption in pregnant women is controversially discussed [3, 10–15], particularly because an exact dose-effect correlation between alcohol

intake and development of physical and neuropsychological problems could not be established. Therefore, a labeling for alcoholic drinks and a recommendation of complete abstinence during pregnancy have been established in many European countries, such as France.

Commonly performed laboratory tests for alcohol consumption such as carbohydrate deficient transferrin (CDT), liver enzymes such as gamma-glutamyl-transferase (GGT), and mean corpuscular volume (MCV) are indirect alcohol markers. They are difficult to interpret and insufficiently reliable during pregnancy [16, 17].

Other parameters for alcohol consumption exist as direct metabolites of ethanol degradation. They can be found in blood, urine, hair, and meconium. Fatty acid ethyl esters (FAEE) in meconium have been investigated and established in several studies as biomarkers of fetal ethanol exposure during the last 3 months of pregnancy [1, 18–20]. Additionally, the determination of ethylglucuronide (EtG) not only from the mothers' hair or urine but also from the children's meconium has been associated with the mother's drinking behavior during pregnancy [2, 20–23].

In our study, we aimed at the assessment of the association between patients' self-reported alcohol intake and meconium biomarkers for maternal alcohol consumption.

Furthermore, we tried to identify the characteristics of mothers, who, according to direct ethanol metabolites, presumably drank during their pregnancy, using epidemiological and medical history, and we standardized the psychological questionnaires.

2. Materials and Methods

The Franconian Maternal Health Evaluation Studies (FRAMES) were prospectively conducted from 2005 to 2007 [24–27]. A total of 1100 women were recruited as a consecutive cohort. The participating women had to be aged ≥ 18 years with at least 30 full weeks of gestational age, and were introduced as outpatients to the Department of Obstetrics and Gynecology of the University of Erlangen-Nuremberg. There was no preselection of the cases with respect to suspected alcohol abuse of the mother or any other parameter.

Only singleton births were allowed for this analysis, resulting in the exclusion of 114 births. Therefore, the final number of participants in this study was 986. Further, 247 newborns were transferred to the children's hospital because of perinatal problems and were excluded as well. Of the left 739 newborns, 137 had to be excluded because of missing consent of the mother or sampling was missed. From the remaining 602 samples, 45 could not be investigated due to technical issues (i.e., too little sample volume). There were no statistical differences between women with available meconium measurements (557) and those without.

This study was approved by the Local Ethics Committee of the Medical Faculty of the University of Erlangen-Nuremberg and was conducted in accordance with the Declaration of Helsinki and all patients gave informed consent.

All participating women were interviewed with standardized psychological questionnaires for the identification of comorbid psychiatric disorders at three occasions. The first was done prenatally from the 30th week of pregnancy onward (first contact with the pregnant women), the second was done 48–72 hours postpartum (when the meconium was collected), and the third was carried out 6–8 months postpartum. Furthermore, we collected diagnostic, obstetric, and medical history from the women.

Psychological questionnaires for other psychiatric disorders were comprised, including the Hamilton rating scale for depression (HAMD), the Edinburgh Postnatal Depression Scale (EPDS), and others—the results of these evaluations are published elsewhere. In addition, anamnestic data on partnership, sexual life, and social status as well as medical parameters of the mother and child were acquired.

About 1 g of meconium was collected from the newborns within the first 2–24 h after birth and frozen at −80°C for up to 30 months until analysis. The meconium samples were analyzed in the Department of Forensic Toxicology of the Institute of Legal Medicine, University Hospital Charité, and by the Lipidomix GmbH in Berlin. The procedure for determination of FAEE and EtG has been described in detail in a previous paper [20]. The FAEEs ethyl Myristate (E14), ethyl Palmitate (E16), ethyl Linoleate (E18:2), ethyl Oleate (E18:1), and ethyl Stearate (E18), as well as the corresponding deuterated standards D5-FAEE, were purchased or prepared as described previously [20, 28]. The quantification of FAEE in meconium was performed according to an optimized and validated method described previously [23].

EtG was determined in meconium according to a liquid-chromatography/tandem-mass-spectrometry (LC-MS-MS) procedure with D5-EtG as the internal standard in analogy to the measurement in hair [21]. The measurement was performed by LC-MS-MS as described in our previous paper [20].

2.1. Statistical Considerations. Univariant associations of alcohol history, meconium results, and socioeconomic parameters were analyzed with appropriate statistical tests. Wilcoxon rank-sum tests were used for ordinal parameters, and χ^2 tests were used for categorical (i.e., yes/no) parameters. All measures of meconium concentrations are reported in ng per g meconium.

In a preanalysis, boxplots were generated to get a first impression of the meconium data distributions in the two groups of reported alcohol consumption: yes (Y) and no (N). The distributions were asymmetric with many outliers in the group with no reported alcohol consumption as well as higher values for the majority of measurements in the group with reported alcohol consumption (Figure 1). We therefore hypothesized a cutoff point, which could divide the women into two groups: one with at least moderate measurements, which would have a positive association of the meconium results with alcohol history, and a second with higher meconium results, in which this association would be negative. To find an optimal cutoff, thresholds were run between the 10th and the 90th percentile of all

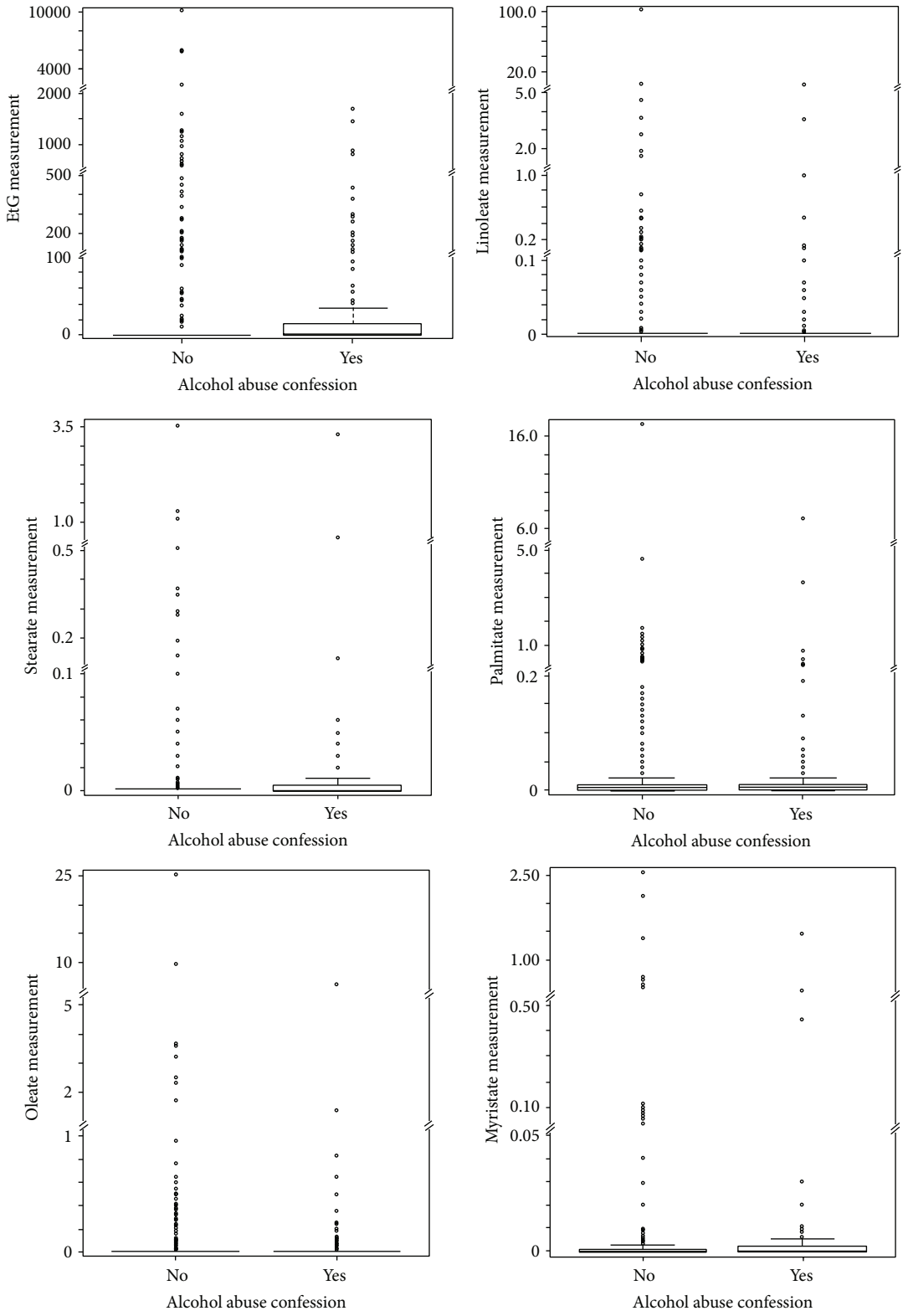

Figure 1: Boxplots of EtG and ester measures and alcohol abuse confession. Suitable ranges are displayed.

Table 1: Association of meconium results, report of alcohol consumption count and percentage, and P values of Wilcoxon rank-sum tests are shown (test based on raw values, classifications just for illustration).

Meconium results	ng/g	Total	No alcohol abuse reported	Alcohol abuse reported	P value
Stearate	0	411	80.8%	19.2%	0.08
	>0	139	72.7%	27.3%	
Linoleate	0	460	79.1%	20.9%	0.66
	>0	90	76.7%	23.3%	
Oleate	0	416	78.8%	21.2%	0.75
	>0	134	78.4%	21.6%	
Palmitate	0	121	80.2%	19.8%	0.55
	>0	423	78.5%	21.5%	
Myristate	0	367	79.3%	20.7%	0.76
	>0	183	77.6%	22.4%	
EtG	0	451	81.4%	18.6%	<0.01
	>0	92	66.3%	33.7%	

meconium measurements. For each choice of the threshold, both the subgroups (Group 1 < cutoff, Group 2 ≥ cutoff) were separately tested to determine the differences between the abusers and nonabusers with Wilcoxon rank-sum tests. The optimal cutoff point was defined as the minimum sum of P values from both tests.

Multiple logistic regression models with meconium measures (=0 versus >0, resp., <cutoff point versus ≥cutoff point) as target variables and socioeconomic parameters as predictive variables were performed to calculate the odds ratios (OR). The final models were obtained by the backward stepwise variable selection due to the Akaike information criterion. For each model, the area under the curve (AUC) of the receiver operation characteristic (ROC), ranging from 0.5 (random prediction) to 1 (perfect prediction), was calculated to summarize the strength of prediction.

All tests were two-sided, and a P value of <0.05 was considered to be statistically significant. The analyses were carried out using the R system for statistical computing (version 2.11.1; R Development Core Team, Vienna, Austria, 2010).

3. Results

3.1. Main Patient Characteristics. The final number of participating women with singleton birth in this study was 986. In average, participants were 32 years old and 557 gave birth to their first child in this study. Average pregnancy at the time of birth was 1.9; 514 were boys and 470 were girls. The average weight for the boys was 3497 g and the average weight for the girls was 3318 g.

A total of 204 women (20.8%) confessed to have drunk alcohol at some time point to some extent during pregnancy. Most women stated to have drunk moderately alcohol with a low frequency. None of the women admitted to having drunk alcohol extensively.

3.2. Main Measurement Characteristics. With regard to the meconium measures (Linoleate, Palmitate, Stearate, Oleate, Myristate, and EtG), most of the samples were free of fatty esters and EtG. The percentage of negative samples reached from 22% to 84% in the group of the fatty esters and was 83% for EtG. The maximum values were rather low for Myristate and Stearate with 2.6 and 3.6, respectively, and reached values of 103.2 with regard to Linoleate. Oleate and Palmitate maximum values were in between with 25.4 and 17.4. Maximum value for EtG was 10,235. The distribution of measurements is shown in Figure 1.

3.3. Association of Alcohol Abuse Confession with Meconium Measures. For the majority of meconium measures, the strong outliers seen in the boxplots of the meconium measures result in a higher mean in the group of alcohol abuse confessing women compared to the group of women who did not confess alcohol abuse. The mean EtG value in the group of alcohol abuse confessing women was 71 and it was 110 in the group of women who did not confess alcohol abuse. The corresponding values for Linoleate were 0.30 versus 0.05, for Oleate were 0.16 versus 0.11, and for Myristate were 0.023 versus 0.018.

Rank based testing confirmed only for EtG the observation that the group with alcohol abuse confession showed overall higher measurements ($P < 0.01$, Wilcoxon rank-sum test) (Table 1). Additionally, the five meconium measures Linoleate, Palmitate, Stearate, Oleate, and Myristate were analyzed with other statistical methods without any significant results (data not shown).

Only for EtG, an optimal cutoff with significant P values was found. In fact, this cutoff was at 120 ng/g for EtG. When looking only at the group of women with an EtG < 120 ng/g, those women denying alcohol abuse had mean EtG values of 3.0 ng/mL, while those women admitting alcohol abuse had mean EtG values of 6.6 ng/mL ($P < 0.001$). Above the cutoff, the mean EtG value was 482.2 for women with alcohol abuse confession and 1179.8 for women without alcohol abuse confession ($P = 0.055$). For all other meconium measures, the optimal cut point had no significant P values (data not shown). Due to small sample size, this cutoff could not be validated.

Table 2: Association of socioeconomic parameters and EtG measures. Count, percentage, and P value of nonparametric test are shown.

Patient characteristic	Total	EtG measurement <120 ng/g	EtG measurement ≥120 ng/g	P value
Age				
<20	3	66.7%	33.3%	
20–30	128	90.6%	9.4%	
30–40	382	90.1%	9.9%	
40+	32	90.6%	9.4%	
Total	**545**			0.92
Education				
No university-entrance diploma	245	91.8%	8.2%	
University-entrance diploma	296	88.5%	11.5%	
Total	**541**			0.25
Marital status				
Not married	110	84.5%	15.5%	
Married	434	91.5%	8.5%	
Total	**544**			0.05
Income				
<500€	1	0.0%	0.0%	
500–1000€	19	100.0%	0.0%	
1000–2000€	59	89.8%	10.2%	
2000–3000€	122	85.2%	14.8%	
3000–4000€	94	94.7%	5.3%	
4000–5000€	56	89.3%	10.7%	
>5000€	48	93.8%	6.3%	
Total	**399**			0.32
Smoking				
No	503	90.1%	9.9%	
Yes	40	90.0%	10.0%	
Total	**543**			1.00
EPDS				
No depression	428	90.0%	10.0%	
Slight depression	37	89.2%	10.8%	
Moderate-to-strong depression	36	91.7%	8.3%	
Total	**501**			0.88
HAMD				
No depression	466	91.8%	8.2%	
Slight-to-moderate depression	56	91.1%	8.9%	
Strong depression	11	90.9%	9.1%	
Total	**533**			0.77

Based on these results, further analyses with the alcohol abuse confession as target variable were conducted separately for the two subgroups defined by the EtG cutoff point.

3.4. Association of Meconium Measures with Socioeconomic Parameters. In order to understand whether there is a correlation between commonly known socioeconomic factors which might be correlated with alcohol abuse, both, the alcohol abuse confession variable and the meconium measures, were correlated with socioeconomic characteristics. Except for an association between smoking status and Oleate measures and marital status and Myristate measures, no associations were seen. Women who were smokers had more often an elevated Oleate measure (38.1% versus 23.2%; $P = 0.049$), and women who were married had less frequently elevated Myristate measures (30.2% versus 45.9%; $P < 0.01$). None of these tests were adjusted for multiple testing though, and none of the findings were consistent with other fatty ester measures or EtG. The associations between EtG and socioeconomic parameters for EtG with the determined cutoff of 120 ng/g are shown in Table 2.

3.5. Association between Alcohol Abuse Confession and Socioeconomic Factors. Comparing the women's statements about alcohol abuse confession revealed only an association

Table 3: Association of socioeconomic parameters and alcohol for Group 1 (EtG < cutoff of 120 ng/g). Count, percentage, and *P* value of nonparametric test are shown.

Patient characteristics	Total	Alcohol consumption not reported in Group 1	Alcohol consumption reported in Group 1	*P* value
Age				
<20	2	100.0%	0.0%	
20–30	116	81.9%	18.1%	
30–40	342	78.4%	21.6%	
40+	29	82.8%	17.2%	
Total	**489**			0.15
Education				
No university-entrance diploma	223	78.0%	22.0%	
University-entrance diploma	262	80.9%	19.1%	
Total	**485**			0.50
Marital status				
Not married	93	78.5%	21.5%	
Married	395	79.7%	20.3%	
Total	**488**			0.90
Income				
<500€	0	0.0%	0.0%	
500–1000€	19	84.2%	15.8%	
1000–2000€	53	83.0%	17.0%	
2000–3000€	104	83.7%	16.3%	
3000–4000€	89	79.8%	20.2%	
4000–5000€	50	78.0%	22.0%	
>5000€	45	77.8%	22.2%	
Total	**360**			0.27
Smoking				
No	453	79.0%	21.0%	
Yes	36	86.1%	13.9%	
Total	**489**			0.42
EPDS				
No depression	385	78.2%	21.8%	
Slight depression	33	84.8%	15.2%	
Moderate-to-strong depression	33	78.8%	21.2%	
Total	**451**			0.54
HAMD				
No depression	428	79.2%	20.8%	
Slight-to-moderate depression	51	80.4%	19.6%	
Strong depression	10	90.0%	10.0%	
Total	**489**			0.59

between alcohol abuse and age of women with an EtG measure above 120 ng/g (Tables 3 and 4).

3.6. Multivariate Models. Thinking of objective alcohol meconium measures as variables which might be helpful in clinical practice to predict, socioeconomic factors and alcohol abuse confession as independent variables, and meconium measures as dependent variables (six unique models) were analyzed with logistic regression models. Nontrivial final models (i.e., with at least one independent variable) are shown in Table 5. The selection procedure does not leave any independent variables in models for alcohol and Stearate.

4. Discussion

In this prospective study, we found EtG to be associated with self-reported alcohol abuse in women with low-to-moderate alcohol consumption. However, in women, whose meconium EtG indicated a severe alcohol abuse, there was an inverse correlation with self-reported alcohol abuse, indicating that women with severe alcohol abuse might most likely be the ones to deny the alcohol abuse.

Our results might be a hint that women who drink more heavily during their pregnancy are not likely to admit their drinking status truthfully compared to those who moderately consume alcohol.

Table 4: Association of socioeconomic parameters and reported alcohol abuse for Group 2 (EtG ≥ cutoff of 120 ng/g). Count, percentage, and *P* value of nonparametric test are shown.

Patient characteristic	Total	Alcohol consumption not reported in Group 2	Alcohol consumption reported in Group 2	*P* value
Age				
<20	1	100.0%	0.0%	
20–30	12	91.7%	8.3%	
30–40	38	68.4%	31.6%	
40+	3	33.3%	66.7%	
Total	**54**			0.03
Education				
No university-entrance diploma	20	80.0%	20.0%	
University-entrance diploma	34	67.6%	32.4%	
Total	**54**			0.51
Marital status				
Not married	17	82.4%	17.6%	
Married	37	67.6%	32.4%	
Total	**54**			0.34
Income				
<500€	1	100.0%	0.0%	
500–1000€	0	0.0%	0.0%	
1000–2000€	6	66.7%	33.3%	
2000–3000€	18	72.2%	27.8%	
3000–4000€	5	80.0%	20.0%	
4000–5000€	6	66.7%	33.3%	
>5000€	3	66.7%	33.3%	
Total	**39**			0.89
Smoking				
No	51	72.5%	27.5%	
Yes	3	66.7%	33.3%	
Total	**54**			0.31
EPDS				
No depression	43	67.4%	32.6%	
Slight depression	4	75.0%	25.0%	
Moderate-to-strong depression	3	100.0%	0.0%	
Total	**50**			0.32
HAMD				
No depression	48	72.9%	27.1%	
Slight-to-moderate depression	5	60.0%	40.0%	
Strong depression	1	100.0%	0.0%	
Total	**54**			0.79

The cutoff values for objective alcohol parameters, which are currently found and tested in science and research, are not yet established due to several reasons. Nutritional and other environmental factors can influence the amount of nonoxidative alcohol metabolites [29]. There are small amounts of FAEE in meconium of neonates without active maternal alcohol consumption, which may originate from endogenous ethanol or from ethanol traces contained in common foods [30–32]. In contrast to the analysis of EtG and FAEEs in the patients' hair, there are no established and scientifically tested cutoff values for differentiating the mothers' drinking behavior via meconium, and imagining a reliable, scientifically correct, and ethical way to test the cutoff values in pregnant women is hardly possible [33]. Taking this into consideration, a strict differentiation between teetotaler, low-to-moderate, and high-risk drinkers cannot be realized. The absolute values of FAEEs and EtG in meconium can only provide a hint about the degree of the mother's ethanol

Table 5: Various multiple logistic regression analyses (final models). The area under the curve (AUC) and odds ratios with 95% confidence interval and corresponding *P* values are shown.

Target variable	AUC	Predictive variables	Odds ratio	95% confidence interval	*P* value
Linoleate (0/>0)	0.58	Education			
		No university-entrance diploma	1		
		University-entrance diploma	1.74	[0.93, 3.31]	0.09
		Income			
		<500€	1		
		Per category	0.84	[0.67, 1.05]	0.13
Oleate (0/>0)	0.53	Smoking			
		No	1		
		Yes	2.05	[0.91, 4.42]	0.07
Palmitate (0/>0)	0.54	Marital status			
		Not married	1		
		Married	1.57	[0.86, 2.79]	0.13
Myristate (0/>0)	0.58	Age			
		<25	1		
		≥25	3.15	[1.06, 11.74]	0.05
		Marital status			
		Not married	1		
		Married	0.62	[0.35, 1.09]	0.09
		Income			
		<500€	1		
		Per category	0.85	[0.72, 1.01]	0.07
EtG (<120/≥120)	0.59	Education			
		No university-entrance diploma	1		
		University-entrance diploma	1.81	[0.86, 3.92]	0.12
		Income			
		<500€	1		
		Per category	0.79	[0.60, 1.04]	0.10

consumption. Using meconium as the material for analysis, we can overlook the last trimenon of pregnancy, as meconium is accumulated in the fetal gut from about the 20th week of gestation until birth, and the major amount of it is observed during the last weeks before birth. Therefore, at least 75% of the sample material originates from the last 8 weeks of pregnancy [34, 35].

Although we had a rather large sample with 986 participants and 557 analyzed meconium samples, the positive cases—according to the toxicological meconium measures—were only a small percentage of the participants. Consecutively, the statistical power was also limited and no cross-validation or adjustment for multiple testing was possible.

In other studies, FAEEs and EtG were already used as parameters to identify alcohol drinking mothers, and they showed partly drastic differences between the admitted drinking behavior and the one shown by using objective parameters [20–22, 36–38]. Our data are based on a large sample, and we use not only the mothers' statements, but also psychological questionnaires and objective alcohol parameters alongside each other.

Questionnaires and laboratory blood parameters used in the common routine of alcohol diagnostics are not reliable sources of information in pregnant women [2, 16, 17]. An objective evaluation of drinking status can be achieved using direct alcohol metabolites, such as EtG and FAEEs [18, 21, 28, 39], which have been shown to be parameters for the mothers' alcohol consumption [2, 19, 20, 40, 41].

Although there is no established cutoff value, Moore et al. [40] concluded that a total cumulative FAEE concentration of >10,000 ng/g may indicate that the newborn has been exposed to significant amounts of alcohol during gestation. Three of the meconium samples in our study showed higher values, with two of those women completely denying alcohol abuse during pregnancy.

Most of the women denied alcohol consumption and most had completely negative results concerning EtG; moreover, the mean value and rank-based tests showed higher values in the group of women admitting alcohol consumption during pregnancy. These findings seem logical if we act on the assumption that the women answered the question of drinking ethanol truthfully. Still, we found the characteristic cutoff to be at 120 ng/g of EtG, under which the women's admitting status correlated positively with the meconium measures, and above which the correlation was inverse. This paradox phenomenon invigorates the findings of many other

studies that suggest an unreliability of maternal statements concerning alcohol consumption during pregnancy [1, 2, 33].

5. Conclusion

There is an ongoing immense need for further investigations in the field of alcohol consumption during pregnancy, because in Germany alone, every year, about 4000 newborn children suffer from the FAS, and even more are those born with symptoms of the fetal alcohol spectrum disease. After validation, alcohol screening could be implemented systematically in prenatal care [42, 43] as well as the postnatal identification of women being at high risk for child neglect [44, 45].

We found that women with a high alcohol consumption are more likely to deny their alcohol abuse. This finding is of clinical and scientific importance. Identifying women with a severe alcohol problem as the ones who would most likely not admit their problem might indicate an even higher risk for the unborn child, as those pregnancies are more difficult to identify. Additionally, prediction models using self-reported alcohol abuse might be more complicated as there is a positive correlation with truth in women with low-to-moderate consumption and an inverse correlation with truth in women with severe alcohol abuse. More studies are needed especially confirming meconium measurements with clinical parameters concerning fetal and pediatric outcome to test their reliability concerning clinical and scientific use.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

The FRAMES project was initiated by Tamme W. Goecke, Johannes Kornhuber, and Peter A. Fasching, and the clinical data acquisition was conducted by Tamme W. Goecke, and Matthias W. Beckmann. The meconium study was initiated by Johannes Kornhuber as an add-on project to FRAMES. Peter A. Fasching, Anne Engel, and Lothar Häberle performed the quality control of the database and statistical analysis. Tamme W. Goecke, Florian Faschingbauer, and Franziska Voigt instructed and supervised the doctoral candidates. Pascal Burger conducted the meconium processing and was the psychiatric consultant and coordinator for the project. Abdulsallam Bakdash, Michael Rothe, and Fritz Pragst performed the biochemical measurements in the meconium samples. Tamme W. Goecke, Peter A. Fasching, Pascal Burger, Johannes Kornhuber, Nicolai Maass, Lothar Häberle, and Anne Engel wrote the paper. Tamme W. Goecke and Pascal Burger have equally contributed to this work.

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Research Article

Can Ki-67 Play a Role in Prediction of Breast Cancer Patients' Response to Neoadjuvant Chemotherapy?

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Background. Currently the choice of breast cancer therapy is based on prognostic factors. The proliferation marker Ki-67 is used increasingly to determine the method of therapy. The current study analyses the predictive value of Ki-67 in foreseeing breast cancer patients' responses to neoadjuvant chemotherapy. *Methods*. This study includes patients with invasive breast cancer treated between 2008 and 2013. The clinical response was assessed by correlating Ki-67 to histological examination, mammography, and ultrasonography findings. *Results*. The average Ki-67 value in our patients collectively ($n = 77$) is 34.9 ± 24.6%. The average Ki-67 value is the highest with 37.4 ± 24.0% in patients with a pCR. The Ki-67 values do not differ significantly among the 3 groups: pCR versus partial pathological response versus stable disease/progress ($P = 0.896$). However, Ki-67 values of patients with luminal, Her2 enriched, and basal-like cancers differed significantly from each other. Furthermore, within the group of luminal tumors Ki-67 values of patients with versus without pCR also differed significantly. *Conclusion*. Our data shows that the Ki-67 value predicts the response to neoadjuvant chemotherapy as a function of the molecular subtype, reflecting the daily routine concerning Ki-67 and its impressing potential and limitation as a predictive marker for neoadjuvant chemotherapy response.

1. Introduction

Breast cancer is the most diagnosed cancer in women. However, breast cancer mortality rate in industrialised western countries has decreased in the last decades [1, 2]. Early diagnosis and effective therapies contribute greatly to this decrease in mortality rate [3]. Currently the choice of therapy is based on prognostic factors. Different already known prognostic factors such as histological tumour type, tumour size, nodal status, grade, age, and estrogen receptor (ER) status and the proliferation marker Ki-67 influence the type of therapy decision [4]. The clinical use of these factors aims at identifying patients with an unfavourable prognosis and at improving the treatment according to the individual risk (recurrence and mortality). The use of this paradigm over the past three years has led to notable therapy improvement [5, 6].

Moreover, chemotherapy-indication is based on prognostic factors. All patients with an indication for adjuvant chemotherapy can be offered a neoadjuvant treatment [7–10]. A neoadjuvant chemotherapy regiment offers a lot of advantages compared to adjuvant treatment. The response to chemotherapy and therefore also its effectiveness can be better monitored, thus potentially increasing patient compliance. In addition, the use of neoadjuvant cytotoxic treatment may increase the rate of breast conserving therapy and reduce the extent of surgery [8].

Another potential prognostic marker is pathologic complete response (pCR). In many neoadjuvant studies, patients who achieve a pCR showed a better long-term outcome [11, 12]. A pooled analysis of seven randomized trials, including 6,377 patients, showed a significant difference in disease-free survival (DFS) between patients with pCR (ypT0/N0) and patients without pCR. The overall survival (OS) was also better for the former patients. Furthermore, this study shows that pCR is only in highly proliferating breast cancers, like triple negative breast cancer (TNBC), HER2 enriched (HER2

positive plus ER negative), or luminal B/HER2-negative tumours, a good prognostic value, whereas in luminal A and luminal B (ER plus HER2 positive) tumours the pCR is not able to discriminate between good and poor prognosis [13, 14]. In addition to the pCR after neoadjuvant chemotherapy, the proliferation marker Ki-67 is not only a prognostic but also a predictive value.

Ki-67 is a nuclear antigen identified in 1983 which is present in the nuclei of cells in all phases of the cell cycle as well as in mitosis, but quiescent cells in the G_0 phase do not express it [15–17]. In fact it is the most common marker used in clinical practice. Kwok et al. showed in 2010 that the proliferation marker Ki-67 in needle core biopsy showed better concordance with haematoxylin and eosin mitotic count in surgical excision specimen than routine haematoxylin and eosin mitotic count in needle core biopsy [18]. Relating to neoadjuvant chemotherapy and its response, it has been found that a high level of proliferation activity has predictive value [19]. Fasching et al. showed in 2011 that the response of neoadjuvant chemotherapy in patients with a high Ki-67 level (>30%) was better than in other tumours [20]. Moreover, after a neoadjuvant chemotherapy Ki-67 is still able to function as prognostic marker. Patients with high Ki-67 values in the residual tumour after chemotherapy had a poorer outcome regarding recurrence and mortality. These high risk patients may require further systemic therapy. However, despite these positive qualities Ki-67 is a regular topic of discussion due to its cut-off values and the intra- and interlaboratory reproducibility.

Therefore, the current study was performed in order to analyze retrospectively the predictive value of Ki-67 in prediction of responses of breast cancer patients to neoadjuvant chemotherapy treatment conducted in a German university hospital.

2. Patients, Material, and Methods

This retrospective single-center study is composed solely of patients treated by neoadjuvant chemotherapy for invasive breast cancer at a tertiary university center (Saarland University Hospital) between January 2008 and December 2013. The inclusion criteria are that the performance of initial core needle biopsy leading to histopathological diagnosis and surgery following neoadjuvant chemotherapy must be performed at the Department of Gynaecology and Obstetrics of Saarland University Hospital. The exclusion criteria are incomplete data, histopathologic diagnosis and surgery performed at a different institution, and patients with metastasis at the time of initial diagnosis.

Clinical data were obtained using medical records and original pathology reports and collected in an Excel database (Microsoft Corporation, Redmond, WA, USA). The following parameters were assessed: patient's age, tumor size (defined as sonographic diameter (mm) on diagnosis), initial tumor stage and nodal status according to TMN classification, histologic subtype, estrogen receptor status, progesterone status, HER2 status, grading and proliferation status as assessed by Ki-67 staining, neoadjuvant chemotherapy regime and neoadjuvant targeted therapy, posttherapeutic sonographic tumor diameter (mm), posttherapeutic histologic tumor diameter (mm), and posttherapeutic tumor stage and nodal status. Histopathological regression was classified using the semiquantitative scoring system according to Sinn from 0 to 4 (0 = no effect, 1 = resorption and tumor sclerosis, 2 = minimal residual invasive tumor [<0.5 cm], 3 = residual noninvasive tumor only, and 4 = no tumor detectable). A regression grade of four according to Sinn was defined as pathologic complete response (pCR) and a regression grade from two upwards was defined as pathologic partial remission (pPR).

Clinical response was assessed based on a physical examination, mammography, and ultrasonography according to the Response Evaluation Criteria in Solid Tumors (RECIST) [12]. A clinical complete response (cCR) was defined as the disappearance of all known lesions; a clinically partial response (cPR) was defined as a ≥30% reduction in the sum of the longest diameter (LD) of the primary lesion; progressive disease (PD) was defined as a ≥20% increase in the sum of the LD of the primary lesion; and stable disease (SD) was defined as neither sufficient shrinkage to qualify for cPR nor sufficient increase to qualify for PD. The study protocol was approved by the hospital's ethics board and informed consents were obtained from patients in the study.

All histopathological parameters included were derived from the original pathology reports. Tumor tissue was neutral-buffered, formalin-fixed, and paraffin-embedded. Staining of the pretreatment core biopsies was performed using monoclonal rabbit antibodies against estrogen receptor-alpha (clone SP1, DCS Hamburg, Germany), monoclonal rabbit antibody against the progesterone receptor (clone SP2, DCS Hamburg, Germany), and monoclonal antibody against Ki-67 (clone MIB-1, DAKO, Glostrup, Denmark), each according to the manufacturer's instructions using a slide stainer (BenchMark ULTRA, Ventana Medical Systems, Arizona, USA). For evaluation of Ki-67, areas with the highest Ki-67 labeling were investigated. Visualization of antigenic sites was performed using the DakoEnVision kit (Hamburg, Germany). For staining of Her2/neu rabbit antibody was used (A0485, DAKO, Glostrup, Denmark). Her2 status was given on a scale from 0 to 3+. A score of 0 or 1+ was regarded as Her2 negative and a score of 3+ as positive. In case of intermediate score (2+) samples were tested for gene amplification using a Her2 fluorescence in situ hybridization kit (Zytolight, SPEC HER2/CEN17 Dual Color Probe, Zyto Vision Ltd., Bremerhaven, Germany). Hereby gene copy numbers of HER2 and centromeres of the corresponding chromosome 17 were retrieved. A HER2/CEN17 ratio of > 2.2 was considered as amplification of HER2. Cases with a ratio between 1.8 and 2.2 were reevaluated by repeating the staining procedure [12]. Scoring was performed according to standardized protocols by specialized pathologist at the Department of Pathology, Saarland University Hospital.

We only analysed 77 patients in this study, since for the missing cases Ki-67 data was not available. Data was collected in an EXCEL database (Microsoft Corporation, Redmond, WA, USA) and statistical calculations were performed with SPSS (SPSS Inc. Chicago, IL, USA). One way analysis of

Table 1: Patient characteristics. Discrete data are given as numbers, continuous as the mean ± standard deviation.

Parameter	Value
Total number of patients	77
Age at first diagnosis (years)	57.8 ± 10.9
Tumor diameter (initial)	31.1 ± 13.6
Histotype	
Invasive ductal	70 (90.9%)
Invasive lobular	6 (7.7%)
Others	1 (1.3%)
Tumor stage (initial)	
1a	—
1b	1 (1.3%)
1c	6 (7.7%)
2	49 (63.6%)
3	5 (6.5%)
4	16 (20.7%)
Nodal status	
Negative	25 (32.4 %)
Positive	52 (67.5%)
Metastasis	
Negative	77 (100%)
Positive	—
Tumor grade	
1	1 (1.3%)
2	37 (48%)
3	39 (50.6%)
Ki-67	34.9 ± 24.6
Estrogen receptor (ER)	
Negative	35 (45.4%)
Positive	42 (54.5%)
Progesterone receptor (PR)	
Negative	40 (51.9%)
Positive	37 (48%)
Her2 receptor	
Negative	57 (74%)
Positive	20 (25.9%)
"Triple negative"	23 (29.8%)

variance (ANOVA) and paired samples t-test were used for analysis. A P value <0.05 was considered to indicate statistical significance. Data is reported as mean ± standard error.

3. Results

More than 1,000 patients with breast cancer were treated between 2008 and 2013 at the University Hospital of the Saarland. A total of 114 patients received neoadjuvant chemotherapy during this period. The complete medical records, including patient characteristics, tumor characteristics, treatment data, and epidemiological data, and furthermore a Ki-67 determination from 77 patients were analyzed retrospectively. The results of the analysis of these 77 patients are presented in this section.

Table 2: Therapeutic and postoperative characteristics. Discrete data are given as numbers, continuous as the mean ± standard deviation.

Parameter	Value
Total number of patients	77
Neoadjuvant chemotherapy	
EC/DOC	61 (79.2%)
TAC	11 (14.2%)
Others	5 (6.4%)
Endocrine therapy	—
Neoadjuvant targeted therapy	
Trastuzumab	15 (19.4%)
Lapatinib	3 (3.8%)
Trastuzumab + pertuzumab	2 (2.5%)
Bevacizumab	6 (7.7%)
None	51 (66.2%)
Posttherapeutic sonographic tumor diameter (mm)	12.6 ± 10.0
Postoperative histologic tumor diameter (mm)	15.7 ± 17.1
Postoperative tumor stage	
0	20 (25.9%)
1a	9 (11.6%)
1b	7 (9%)
1c	18 (23.3%)
2	15 (19.4%)
3	6 (7.7%)
4	2 (2.5%)
Grade of regression (Sinn)	
0	3 (3.8%)
1	28 (36.3%)
2	22 (28.5%)
3	2 (2.5%)
4	18 (23.3%)
Unknown	4 (5.1%)

The average patients' age was 57,8 years old when receiving the initial diagnosis of breast cancer. Tumor characteristics including tumor entity, the initial size of the tumor, the TNM status, the Ki-67 determination, the hormone receptor, and Her2 status were recorded, as shown in Table 1. All patients received neoadjuvant chemotherapy; none received a primary hormone therapy. Information about the administered chemotherapy and in some cases an additional targeted therapy depending on the receptor status is presented in Table 2. Table 2 also includes information about the average tumor size in ultrasound imaging after the neoadjuvant chemotherapy, the average size according to pathological assessment, the TNM status after the treatment, and the pathological response as in Sinn's assessment. The development of tumor size, before the start and after the completion of the neoadjuvant treatment is shown in Figure 1. The average Ki-67 value in our patients collectively was 34,9 ± 24,6% (range 1–90%). A correlation between the Ki-67 value and a response to the neoadjuvant chemotherapy is illustrated in Figure 2. Twenty patients showed a complete pathological response (pCR), thirty-eight patients showed a partial either

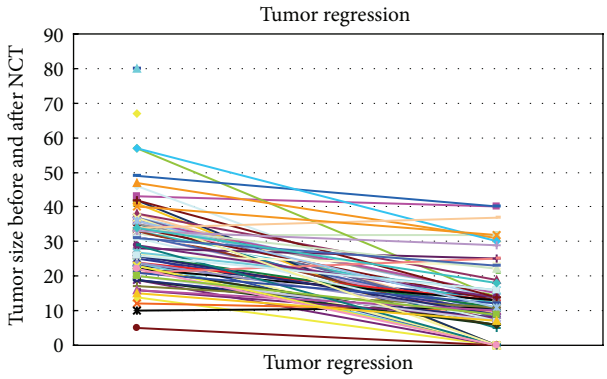

Figure 1: Tumor response, as measured from the maximum initial sonographic diameter and the maximum histological diameter after neoadjuvant chemotherapy and surgery. Data is presented for the values of $n = 77$ patients.

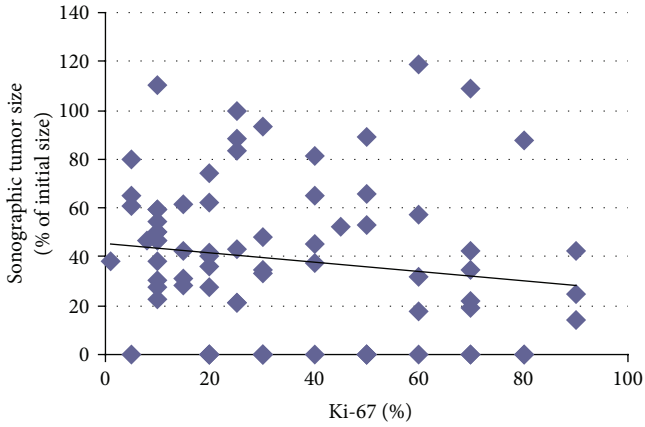

Figure 2: Ki-67 values and sonographic response after neoadjuvant chemotherapy. Tumor sizes are presented in % of the initial sizes.

clinical or pathological response, and seventeen patients had a stable disease or a progress of the disease after finishing the neoadjuvant chemotherapy (Figure 3). In the patient collective with a complete pathological response, the average Ki-67 value was the highest with 37.4 ± 24.0%. Patients with a partial pathological response showed an average Ki-67 value of 34.7 ± 25.5%. Patients with stable disease or rather progress had an average Ki-67 value of 33.8 ± 25.8%. The Ki-67 values do not differ significantly among the 3 groups ($P = 0.896$) as illustrated in Figure 3.

Additionally we divided our patients collectively into 3 groups depending on the cut-off values for Ki-67. We tried to find any differences in initial tumor size and tumor characteristics and investigated the predictive value of Ki-67 for the success of neoadjuvant chemotherapy by correlating it to the pathological response (Table 3). Group A ($n = 20$) represented a group of tumors showing low Ki-67 values (≤15%), group B ($n = 37$) included tumors with an average Ki-67 value between 15 and 50%, and group C ($n = 18$) consisted of tumors with high average Ki-67 values of more than 50%. There were no significant differences between the 3 groups

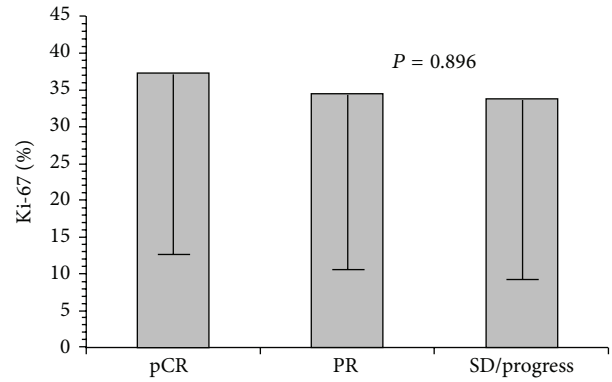

Figure 3: Ki-67 in dependence of the pathological response. pCR = complete pathological response, PR = partial either clinical or pathological response, and SD/progress = stable disease or a progress (mean ± standard deviation).

concerning initial tumor diameter, postoperative histologic tumor diameter, change from initial to posttreatment tumor size in ultrasound imaging, pathological assessment, grade of regression according to Sinn, and the number of patients with a complete pathological response (pCR).

However, subdividing the patients according to the molecular subtype of their cancer (Luminal, Her2 enriched, and basal-like), we detected significant differences of Ki-67 between those groups: Ki-67 values of triple negative cancers were 60.4 ± 18.3%, Her-2 positive cancers were 25.4 ± 12.6%, and luminal tumors 22 ± 19.5% ($P < 0.0001$). In addition, within the group of luminal tumors Ki-67 values of patients with versus without pCR differed significantly: patients receiving a pCR presented with Ki-67 values of 50 ± 36.5% versus 18.1 ± 12.9% ($P = 0.001$) (Table 4).

4. Discussion

In this retrospective study, 77 breast cancer patients receiving neoadjuvant chemotherapy were analysed concerning Ki-67 and its impact as predictive marker for chemotherapy response. We found a trend towards the highest Ki-67 values in patients achieving a pCR as compared to patients with partial response, stable disease, or progress. However, the observed differences are not significant. In addition, three different groups referring to low, medium, or high levels of Ki-67 have been formed and analysed by correlating the groups to the pathological response. Again no significant differences could be found.

The average Ki-67 value in our population was 34,9% ranging from 1 to 90%. Even in the pCR group with an average Ki-67 of 37,4% the values ranged ±24%. This average value showed a higher trend as compared to the group with partial response, stable disease, or progress. Although the St. Gallen Consensus 2013 recommended the use of Ki-67 as additional factor in order to distinguish the large group of receptor positive breast cancers in luminal A and B, there are several problems concerning the detection of Ki-67. One of the problems of Ki-67 use is the large inter- and intraobserver

Table 3: Therapeutical aspects as a function of Ki-67.

Parameter	Ki-67 ≤15%	Ki-67 16–50%	Ki-67 >50%	P
Number of patients	22	37	18	
Initial sonographic tumor diameter (mm)	29.3 ± 14.0	32.2 ± 15.0	31.1 ± 10.4	0.738
Postoperative histologic tumor diameter (mm)	14.6 ± 9.9	18.6 ± 21.7	11.0 ± 15.3	0.312
Change (% from initial tumor size)	57.9 ± 44.9	52.0 ± 66.2	39.4 ± 50.7	0.590
Sonographic change (% from initial tumor size)	47.6 ± 22.2	38.2 ± 32.8	34.4 ± 37.0	0.373
Grade of regression (Sinn)	1.7 ± 1.1	2.2 ± 1.2	2.1 ± 1.4	0.310
Number of patients with pCR	4 (18.1%)	10 (27%)	6 (33.3%)	

Table 4: Ki-67 as a function of molecular subtypes.

	Total	pCR yes	pCR no	P
Triple negative	$n = 23$	$n = 5$	$n = 19$	$P = 0.651$
	60.4 ± 18.3	57 ± 18.5	61 ± 18.7	
Her2 positive	$n = 20$	$n = 11$	$n = 9$	$P = 0.576$
	25.4 ± 12.6	23.9 ± 10.2	27.2 ± 15.6	
Luminal	$n = 33$	$n = 4$	$n = 29$	$P =$ **0.001**
	22 ± 19.5	50 ± 36.5	18.1 ± 12.9	
	$P <$ **0.0001**			

variation. In 2013 Polley et al. compared the Ki-67 levels in eight of the world's most experienced laboratories and observed a large variation among those laboratories [21]. The most commonly used assay to assess Ki-67 is immunohistochemical (IHC) staining with the MIB-1 antibody. Different groups used different antibodies on paraffin sections after antigen retrieval, such as MM-1, Ki-S5, SP-6, and MIB-1. This might be one reason for the considerable interlaboratory variability. Also, intraobserver variability is a highly discussed issue. The Ki-67 score is defined as the percentage of total number of tumor cells with nuclear staining. Some pathologists estimate the percentage of nuclei staining; others count several hundred nuclei in different areas of tumors to give an overall average index. Therefore more and more automated readers are used. Computer-assisted image analysis can raise the reproducibility of Ki-67 assessment [22], but it has a limited capacity of excluding normal stromal/inflammatory cells [23]. Also, tissue microarray technology has been introduced; its reliability and reproducibility were proven in studies [24]. A standardization of Ki-67 pathological assessment has not yet been accomplished [25]. This lack of consistency across laboratories has thus far limited Ki-67's value. The International Ki-67 in Breast Cancer Working Group was assembled to devise a strategy to harmonize Ki-67 analysis and increase scoring concordance [26].

The above discussed limitations concerning the determination of Ki-67 must be taken into account when discussing our results. However, although the detection and quantification of Ki-67 is difficult our observation of a higher average Ki-67 in the group of patients achieving pCR is in line with several findings of other groups. This concerns also a large number of studies with a neoadjuvant setting. The usefulness of Ki-67 in predicting response and outcome is explored by assessing pretreatment and posttreatment levels of tumor Ki-67 expression in neoadjuvant chemotherapy. Unfortunately, only few of those studies are randomized [27]. Most neoadjuvant chemotherapy studies only perform a univariate analysis looking at response as an outcome [28]. These four studies found Ki-67 to be a predictive marker for either clinical and/or pathological response but only few authors were able to demonstrate Ki-67 as an independent predictor for pCR and overall survival in multivariate models [20].

It has to be declared that a few studies report no correlation between Ki-67 and response to neoadjuvant chemotherapy [29], as we were also not able to find at least a significant correlation between Ki-67 and the response after neoadjuvant chemotherapy. However, since a trend towards response could be observed, it has to be assumed that the lack of significance is due to the relatively small number of patients and the retrospective character of our analysis. In addition, the explanatory power of our data is constricted since we do not provide a multicentric assessment.

In the next step, we subdivided our population into three groups depending on the cut-off levels for Ki-67 (≤15%, 15–50%, and >50%). Again the response after neoadjuvant chemotherapy has been analysed between those groups. However, we were not able to detect any significant differences. This issue addresses another major problem: the varying definition of cut-off values for Ki-67 across the different studies. Klintman et al., for example, uses a Ki-67 cut-off of ≤20%. The St. Gallen Consensus in 2009 classified tumors as low, intermediate, and highly proliferating according to the value of Ki-67 labeling index of ≤15%, 16–30%, and >30% [30]. Fasching et al. and Cheang et al. used a cut-off point for Ki-67 for more than 13% positively stained cells [20]. Denkert et al. in 2013 even pretends that Ki-67 is a significant predictive and prognostic marker over a wide range of cut points, suggesting that data-derived cut point optimisation might not be possible [31]. Nevertheless, Ki-67 may be an

important marker regarding the molecular cancer subtypes. We found that Ki-67 values of patients with Luminal, Her2 enriched and basal-like cancers differed significantly from each other. Furthermore, within the group of luminal tumors Ki-67 values of patients with versus without pCR differed significantly. This data is in line with Fasching et al. [20], who found that patients with luminal cancers and pCR have significantly higher Ki-67 values as compared to those without pCR. In our population, patients with triple negative cancer also had the highest levels of Ki-67. However, in contrast to the data of Fasching et al. we did not observe any differences between the group with and without pCR. This might be due to the relatively small subgroup consisting of only 23 patients.

In conclusion, our data shows that the Ki-67 value predicts the response to neoadjuvant chemotherapy in breast cancer patients as a function of the molecular subtype reflecting the daily routine concerning Ki-67 and its impressing chances and yet also its limitations as predictive marker for neoadjuvant chemotherapy response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Guideline Concordant Therapy Prolongs Survival in HER2-Positive Breast Cancer Patients: Results from a Large Population-Based Cohort of a Cancer Registry

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Even though randomized controlled clinical trials demonstrated improved survival by adjuvant trastuzumab treatment of HER2-positive breast cancer patients, data on its effect in clinical routine are scarce. This study evaluated the use and efficacy of trastuzumab in routine treatment of HER2-positive breast cancer patients. Data from the clinical cancer registry Regensburg (Germany) were analyzed. The present study investigated 6,991 female patients with primary invasive breast cancer. In premenopausal HER2-positive patients a considerable increase of trastuzumab therapy was observed from 58.1% in 2006 to 90.9% in 2011, whereas in postmenopausal patients trastuzumab was rather used on a constant rate of 49.1%. Best overall survival (OS) was found in HER2/steroid hormone receptor-positive patients receiving guideline concordant treatment with trastuzumab plus chemotherapy (CHT) plus antihormone therapy (AHT) with a 7-year OS rate of 96% compared to the non-trastuzumab group with a 7-year OS rate of 92%. In multivariable analysis, HER2-positive patients treated with CHT or AHT who did not get trastuzumab, had a worse 7-year OS (65%, $P = 0.006$ versus 79%, $P = 0.017$) than the control groups. This population-based study demonstrated that guideline concordant use of adjuvant trastuzumab improves OS for HER2-positive breast cancer patients treated in routine clinical care.

1. Introduction

One of the pivotal advancements in breast cancer research was the identification of HER2 overexpression as a significant predictor of both disease-free survival (DFS) and overall survival (OS) in breast cancer patients by Slamon et al. in 1987 [1]. HER2, a member of the epidermal growth factor receptor family of tyrosine kinases, is involved in cell growth and proliferation [2]. Overexpression and/or amplification of HER2 occurs in 15–25% of breast cancers and is associated with an unfavorable course of disease [1]. The development of trastuzumab has improved treatment results of HER2-positive breast cancer. Trastuzumab is a recombinant humanized monoclonal antibody directed against the extracellular domain of the transmembrane HER2 receptor [3]. Initially, the safety and efficacy of trastuzumab were evaluated in patients with HER2-positive metastatic breast cancer [4–8]. Trastuzumab was FDA-approved for treatment of metastatic breast cancer patients in 1998 [9] and in 2000 it was approved in Europe.

Later, five of six large phase III trials including more than 14,000 patients with HER2-positive early breast cancer demonstrated its efficacy in the adjuvant setting [10–15]. The joint analysis of the North American trials NSABP B-31 and NCCTG N9831 found that the addition of trastuzumab to chemotherapy resulted in a significant benefit in terms of DFS and OS for women with HER2-positive breast cancer [13]. After 3 years, the rate of DFS was 87.1% in

patients receiving trastuzumab plus chemotherapy compared with 75.4% in patients in the standard chemotherapy arm (absolute difference 11.8 percentage points, HR 0.48, 95% CI 0.39–0.59; $P < 0.0001$). Regarding OS, also a benefit of trastuzumab plus chemotherapy was shown (94.3% in the combination therapy group versus 91.7% in the standard therapy group, absolute difference 2.5 percentage points) [13]. In the Herceptin Adjuvant Trial (HERA), after a median follow-up of 2 years, a significant absolute advantage in OS of 2.7% in the trastuzumab group over the non-trastuzumab control group was shown (92.4% versus 89.7%, HR 0.66, 95% CI 0.47–0.91; $P = 0.0115$) [11]. A second interim analysis of the BCIRG 006 study demonstrated superior DFS and OS in the trastuzumab arms after a median follow-up of 36 months [16]. The DFS was 83% and the OS 92% in patients receiving doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab, in comparison with 77% and 86% in the control patients (HR 0.61 for DFS; $P < 0.0001$) [16]. At a median follow-up of 38 months, the DFS in the FinHER trial was longer with trastuzumab plus chemotherapy than with chemotherapy alone (89% versus 78%, HR 0.42, 95% CI 0.21–0.83; $P < 0.01$) [12]. Also OS was better in the trastuzumab group (96.3% versus 89.7%), with a reduction of the risk of dying (HR 0.41, 95% CI 0.16–1.08; $P = 0.07$) [12].

In summary, these studies showed that one year of trastuzumab treatment in combination with or sequentially after chemotherapy improved the relative risk for DFS by approximately 50% and OS by 30% irrespective of tumor size, nodal status, and type of chemotherapy. As a consequence, in 2006 trastuzumab was approved for adjuvant treatment of breast cancer in Europe.

Trastuzumab was the first FDA-approved monoclonal antibody targeting solid tumors [17]. Commonly, trastuzumab is well tolerated. Cardiac toxicity is considered to be the most serious adverse effect, but in the large trials it was almost always reversible [18, 19]. Meanwhile novel anti-HER2 strategies are under development like the combination of two HER2-targeted agents with nonoverlapping mechanisms of action to optimize HER2-directed therapy [20–22]. One potential approach is the combination of trastuzumab with the HER2 dimerization inhibitor pertuzumab [23, 24].

The positive results of controlled clinical trials notwithstanding, data on the effect of trastuzumab in clinical routine are scarce [25–29]. Thus, it is essential to ensure that treatment strategies, which are recommended in current national and international guidelines, are implemented in routine clinical care. As early as 1993 a systematic review suggested a positive impact of clinical guidelines on both the process and outcomes of care for several health conditions [30]. Nevertheless, it is difficult to demonstrate effects of treatment improvements on survival due to the lack of high quality clinical cancer registries with long term follow-up. Therefore, the intention of this population-based study was to evaluate guideline concordant treatment of HER2-positive breast cancer patients in the routine clinical adjuvant setting in a large cohort of more than 6,000 patients by analyzing data from a population-based regional cancer registry.

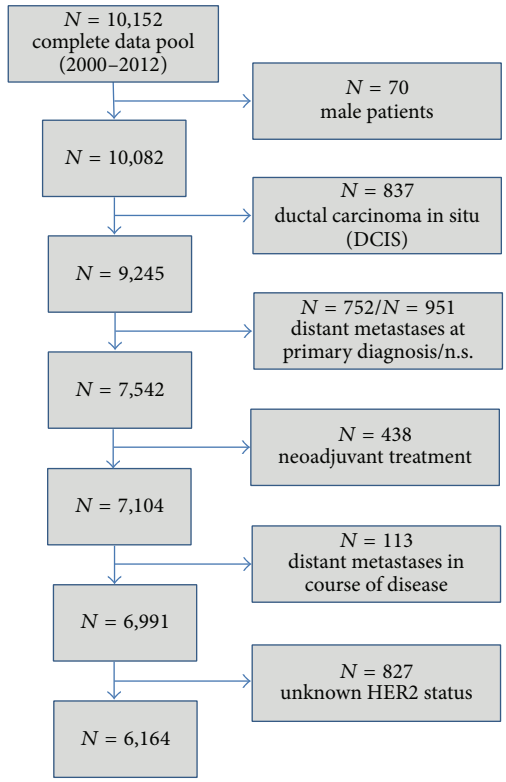

Figure 1: Scheme of data extraction.

2. Material and Methods

2.1. Database. Data from the Tumor Centre Regensburg (Bavaria, Germany), a high quality population-based regional cancer registry covering a population of more than 2.2 million people of the districts of Upper Palatinate and Lower Bavaria, were analyzed. The clinical cancer registry Regensburg was founded in 1991 and currently disposes the follow-up of 240,655 patients. This cancer registry achieves a cross-sectorial documentation of all breast cancer patients in the area, following a rigorous prospective protocol. Documentation includes diagnosis, course of disease, therapies, and long term follow-up. Patient data stem from 53 regional hospitals, the University Hospital Regensburg, and more than 1,000 practicing doctors. The population-based data were routinely analyzed in each case and documented in the cancer registry on the basis of medical reports, pathology, and follow-up records. Mortality data were obtained from all regional registry offices.

2.2. Inclusion and Exclusion Criteria. The present analysis includes all female patients of the registry with primary, non-metastatic (M0) invasive breast cancer diagnosed between January 2000 and December 2012 (13 years). Patients were followed up until May 2013. Exclusion criteria were male patients, ductal carcinoma in situ (DCIS), neoadjuvant treatment, and distant metastases at primary diagnosis or during the course of disease (Figure 1).

Table 1: Time dependent rates of HER2 analyses.

Year of diagnosis	Number of patients (N)	HER2 status unknown (N, %)	HER2 status analyzed (N, %)	HER2 negative (N, %)	HER2 positive (N, %)
2000	430	360 (83.7%)	70 (16.3%)	49 (70.0%)	21 (30.0%)
2001	456	137 (30.0%)	319 (70.0%)	236 (74.0%)	83 (26.0%)
2002	455	72 (15.8%)	383 (84.2%)	304 (79.4%)	79 (20.6%)
2003	518	68 (13.1%)	450 (86.9%)	351 (78.0%)	99 (22.0%)
2004	568	52 (9.2%)	516 (90.8%)	421 (81.6%)	95 (18.4%)
2005	569	34 (6.0%)	535 (94.0%)	438 (81.9%)	97 (18.1%)
2006	527	8 (1.5%)	519 (98.5%)	409 (78.8%)	110 (21.2%)
2007	570	34 (6.0%)	536 (94.0%)	439 (81.9%)	97 (18.1%)
2008	560	47 (8.4%)	513 (91.6%)	423 (82.5%)	90 (17.5%)
2009	666	5 (0.8%)	661 (99.2%)	544 (82.3%)	117 (17.7%)
2010	589	6 (1.0%)	583 (99.0%)	469 (80.4%)	114 (19.6%)
2011	543	2 (0.4%)	541 (99.6%)	476 (88.0%)	65 (12.0%)
2012	540	2 (0.4%)	538 (99.6%)	471 (87.5%)	67 (12.5%)
Total	6991	827 (11.8%)	6164 (88.2%)	5030 (81.6%)	1134 (18.4%)

2.3. Analysis of HER2 Expression. HER2 testing was performed according to the German interdisciplinary S3 Guidelines for Diagnosis, Treatment and Follow-up Care of Breast Cancer (registry number 032-045OL of Association of the Scientific Medical Societies, AWMF) [31, 32] and to ASCO/CAP recommendations [33]. HER2-protein overexpression was analyzed by means of DAKO HercepTest, a semiquantitative immunohistochemical assay.

A total of six institutes of pathology were involved in these assessments. Consistency and quality control are ensured through biannual quality assurance conferences and the participation in round robin tests [34].

2.4. Primary Trastuzumab Therapy. In the trastuzumab group (1 year of trastuzumab with 3 weeks intervals of application) either concurrent or sequential administration relative to chemotherapy was performed. Different types of anthracycline- and taxane-based chemotherapy regimens were used according to the previously reported large controlled clinical trials [18].

2.5. Statistical Methods. Continuous data are expressed as means ± standard deviation (SD) and categorical data as frequency counts (percentages). Baseline characteristics of patients were compared between HER2 status by Student's t-test for continuous variables and by Pearson's chi-square tests for categorical variables. OS was calculated from the date of cancer diagnosis to the date of death from any cause. Patients who are not dead or patients without follow-up were classified as censored. To assess the influence of trastuzumab, chemotherapy (CHT), and antihormone therapy (AHT) on OS in HER2-positive patients and of CHT and AHT in HER2-negative patients, two multivariable Cox regression models were calculated. Both models were adjusted for the known confounding variables age, tumor size, nodal status, grading, receptor status, and Ki-67. Kaplan-Meier plots were used for graphical illustrations. For all combinations of trastuzumab, CHT, and AHT, odds ratios and corresponding 95% confidence intervals (95% CI) were calculated. All reported P values are two-sided, and a P value of 0.05 was considered the threshold of statistical significance. Calculations were made with the software packages SPSS 21.0.0.1 (Chicago, EUA) and R (version 2.14.2).

3. Results

3.1. Patient Characteristics and Histopathological Parameters. From the total data pool of breast tumor patients, 6,991 female patients with invasive early breast cancer according to the ICD-10 classification (C50) were extracted for further analysis (Figure 1). In 88.2% (6,164 patients) the HER2 status was available. In 11.8% (827 patients) the HER2 status was not available owing to missing information in the medical reports or no assessment. In the year 2000, the HER2 status was analyzed only in 16.3% of patients (70/430). This number increased to 84.2% of patients (383/455) in 2002 right up to 99.6% of patients (538/540) in 2012 (Table 1).

Only patients with known HER status were considered for further evaluation. Thus, a total of 6,164 patients with invasive breast cancer were taken into consideration for subsequent statistical analyses. Of these, 1,387 patients (22.5%) were premenopausal and 4,777 patients (77.5%) were postmenopausal. The mean age was 61 years (median: 62 years, range: 21–97 years). 5,030 patients (81.6%) were HER2 negative, whereas 1,134 patients (18.4%) were HER2 positive (overexpression and/or amplification). High grade tumors were more likely to be HER2 positive than low grade tumors. 44.1% ($n = 500$) of HER2-positive patients had poorly differentiated tumors, whereas only 6.2% ($n = 70$) were low grade. Estrogen receptor (ER) and progesterone receptor (PR) negative tumors were more likely to be HER2-positive than ER-positive and PR-positive tumors (23.9% versus 58.7%). Vice versa, in HER2-negative tumors 79.2% were ER and PR positive whereas only 10.7% were ER and PR

Table 2: Rate of adjuvant trastuzumab therapy in HER2-positive patients.

Year of diagnosis	Total number of HER2-positive patients (N)	Trastuzumab received		
		Total	Premenopausal	Postmenopausal
2000	21	0/21 (0%)	0/10 (0%)	0/11 (0%)
2001	83	1/83 (1%)	0/19 (0%)	1/64 (2%)
2002	79	2/79 (3%)	2/22 (9%)	0/57 (0%)
2003	99	2/99 (2%)	1/28 (4%)	1/71 (1%)
2004	95	2/95 (2%)	0/26 (0%)	2/69 (3%)
2005	97	34/97 (35%)	11/23 (48%)	23/74 (31%)
2006	110	42/110 (38%)	18/31 (58%)	24/79 (30%)
2007	97	57/97 (59%)	15/19 (79%)	42/78 (54%)
2008	90	53/90 (59%)	15/19 (79%)	38/71 (54%)
2009	117	64/117 (55%)	21/33 (64%)	43/84 (51%)
2010	114	65/114 (57%)	19/29 (66%)	46/85 (54%)
2011	65	43/65 (66%)	20/22 (91%)	23/43 (54%)
2012	67	24/67 (36%)	11/24 (46%)	13/43 (30%)
Total	1134	389/1134 (34%)	133/305 (44%)	256/829 (31%)

Table 3: Concomitant diseases in HER2-positive patients without adjuvant trastuzumab treatment.

	Premenopausal	Postmenopausal	All patients
Concomitant diseases, n (%)	**35 (14.8%)**	**202 (85.2%)**	**237 (100.0%)**
Cardiopulmonary	12/35 (34.3%)	126/202 (62.4%)	138/237 (58.2%)
Gastrointestinal/hepatic/renal	—	10/202 (5.0%)	10/237 (4.2%)
Metabolic	7/35 (20.0%)	22/202 (10.9%)	29/237 (12.2%)
Mental	6/35 (17.1%)	15/202 (7.4%)	21/237 (8.9%)
Others	10/35 (28.6%)	29/202 (14.4%)	39/237 (16.5%)

negative. In HER2-positive tumors, lymphatic and vascular invasion was more frequent than in HER2-negative tumors (37.9% and 8.5% versus 26.6% and 5.2%). Low Ki-67 values ≤ 15% were predominant in HER2-negative tumors (45.8%) whereas higher Ki-67 categories (Ki-67 >25%) were found in HER2-positive patients.

3.2. HER2 Overexpression and Trastuzumab Therapy. Up to 2004, trastuzumab therapy was hardly administered in adjuvant treatment. Only about 2% of HER2-positive patients received trastuzumab from 2000 to 2004. Since trastuzumab approval for adjuvant therapy, the number of HER2-positive patients receiving the antibody continuously increased from 38.2% in 2006 to 66.2% in 2011. The analyses of age-related subcategories (pre- and postmenopausal patients) showed that nonuse of trastuzumab was found predominantly in postmenopausal women. Differences in trastuzumab therapy between premenopausal and postmenopausal patients were further analyzed (Table 2). There was a considerable increase of trastuzumab therapy in premenopausal patients from 58.1% in 2006 to 90.9% in 2011. The decrease of trastuzumab use in 2012 can be explained by incomplete documentation. In contrast, in postmenopausal patients trastuzumab was rather used on a constant rate of 49.1% from 2006 to 2011 after a first augmentation from 30.4% in 2006 to 53.8% in 2007. In the total population of HER2-positive patients, who were diagnosed between 2006 and 2012 ($n = 660$), only 52.7% (348 patients) received the appropriate treatment with trastuzumab. Among these, more premenopausal HER2-positive patients received trastuzumab in comparison to postmenopausal patients (67.2% ($n = 119$) premenopausal patients versus 47.4% ($n = 229$) postmenopausal patients).

To elucidate reasons for the insufficient application of trastuzumab, we further analyzed HER2-positive patients who did not receive trastuzumab between 2006 and 2012 with respect to their concomitant diseases. 312 HER2-positive patients did not receive trastuzumab. Out of these, 237 patients (76.0%) had at least one serious concomitant disease. 21 patients (6.7%) had no comorbidity and in 54 patients (17.3%) concomitant diseases were not documented. The majority of patients (58.2%) suffered from cardiopulmonary disease. Others had metabolic (12.2%), mental (8.9%), and gastrointestinal/hepatic/renal (4.2%) disorders (Table 3).

Furthermore, combinations of systemic therapies were analyzed since 2006, also contemplating CHT and AHT (Table 4). 34.3% ($n = 221$) of HER2-positive patients were treated with trastuzumab plus CHT plus AHT. 17.4% ($n = 112$) received trastuzumab plus CHT; 21.4% ($n = 138$) received only AHT. A considerable number of HER2-positive patients (15.0%, $n = 97$) received neither trastuzumab nor CHT nor AHT.

3.3. Survival Analyses. Since patients received a number of different systemic treatments, analyses of trastuzumab effects

Table 4: Different systemic therapies in HER2-positive patients.

	HER2-positive patients (year of diagnosis: 2006–2012)						
	Trastuzumab + CHT + AHT	Trastuzumab + CHT	CHT + AHT	CHT	AHT	No adjuvant therapy	Total
2006	27 (24.8%)	14 (12.8%)	9 (8.3%)	9 (8.3%)	40 (36.7%)	10 (9.2%)	109 (100%)
2007	30 (32.6%)	22 (23.9%)	6 (6.5%)	1 (1.1%)	21 (22.8%)	12 (13.0%)	92 (100%)
2008	31 (34.8%)	21 (23.6%)	6 (6.7%)	4 (4.5%)	16 (18.0%)	11 (12.4%)	89 (100%)
2009	45 (40.2%)	14 (12.5%)	9 (8.0%)	4 (3.6%)	24 (21.4%)	16 (14.3%)	112 (100%)
2010	44 (39.3%)	19 (17.0%)	9 (8.0%)	3 (2.7%)	18 (16.1%)	19 (17.0%)	112 (100%)
2011	29 (45.3%)	13 (20.3%)	4 (6.2%)	3 (4.7%)	10 (15.6%)	5 (7.8%)	64 (100%)
2012	15 (22.4%)	9 (13.4%)	1 (1.5%)	9 (13.4%)	9 (13.4%)	24 (35.8%)	67 (100%)
Total	221 (34.3%)	112 (17.4%)	44 (6.8%)	33 (5.1%)	138 (21.4%)	97 (15.0%)	645 (100%)

Table 5: Overall survival rates categorized by HER2 status and adjuvant therapy.

	3-year OS	5-year OS	6-year OS	7-year OS
HER2 positive				
Trastuzumab + CHT+ AHT	97%	96%	96%	96%
CHT + AHT	98%	98%	92%	92%
Trastuzumab + CHT	95%	92%	92%	86%
CHT	82%	65%	65%	65%
AHT	91%	83%	79%	79%
No adjuvant therapy	75%	63%	55%	55%
HER2 negative				
CHT + AHT	98%	95%	93%	92%
CHT	89%	86%	83%	83%
AHT	95%	90%	88%	88%
No adjuvant therapy	81%	75%	73%	64%

were performed by comparing survival data obtained from these treatment groups (Table 5). HER2-positive patients who did not receive trastuzumab had a worse OS in all of the compared groups. Kaplan-Meier curves (Figures 2 and 3) show that guideline concordant therapy—that is, trastuzumab in HER2-positive, CHT in risk factor positive (high grade, large, nodal positive tumors, etc.), and AHT in ER/PR-positive patients—resulted in best survival rates. Deviation from guideline concordance reduced OS significantly. Best OS was found in HER2/ER/PR-positive patients receiving trastuzumab plus CHT plus AHT with a 7-year OS rate of 96%. Depriving HER2/ER/PR-positive patients of trastuzumab, 7-year OS rate deteriorated to 92%. Similarly, the nonuse of trastuzumab in HER2-positive/ER/PR-negative patients decreased 7-year OS rates from 86% to 65%. The worst OS of all patients was found in HER2-positive patients who received neither trastuzumab nor CHT nor AHT with a 7-year OS rate of 55%. Similarly, the impact of guideline concordant therapy was seen in HER2-negative patients whereas the effects were not that distinct. Consequently, an appreciable benefit of following guidelines was demonstrated. Remarkably, the addition of trastuzumab overcame the primarily worse outcome of HER2-positive patients indicating its efficacy. A particularly striking effect was found when comparing HER2-positive patients receiving no adjuvant therapy with triple-negative breast cancer patients receiving no adjuvant therapy at all. HER2-positive patients had an even worse survival (55%) than triple-negative breast cancer patients (64%).

By using Cox regression models (Tables 6(a) and 6(b)), it was demonstrated that HER2-positive patients ($n = 516$, 53 events) without trastuzumab/CHT/AHT therapy ($n = 80$, 21 events) showed the worst OS of all patients (HR = 10.44, 95% CI 3.02–36.06, $P < 0.001$) compared to patients receiving trastuzumab/CHT/AHT therapy (reference group). Also patients without trastuzumab therapy but only CHT (HR = 9.50, 95%-CI 1.90–47.43, $P = 0.006$) or AHT (HR = 4.28, 95%-CI 1.30–14.05, $P = 0.017$), respectively, had worse survival than the control groups (Table 6(a)). In the HER2-negative group ($n = 2{,}727$, 219 events) patients receiving only AHT (HR = 2.33, 95%-CI 1.40–3.87, $P = 0.001$) or only CHT (HR = 3.15, 95%-CI 1.61–6.16, $P = 0.001$) or no adjuvant therapy at all (HR = 4.91, 95% CI 2.81–8.59, $P < 0.001$) showed a highly significant decrease in OS compared to patients with both CHT and AHT (reference group) (Table 6(b)).

4. Discussion

National oncological guidelines are essential for optimal treatment of cancer patients. In Germany, the majority of breast cancer patients are treated in specialized breast cancer centers which have to follow current guidelines for diagnosis and treatment of breast cancer [32, 35]. Determination of

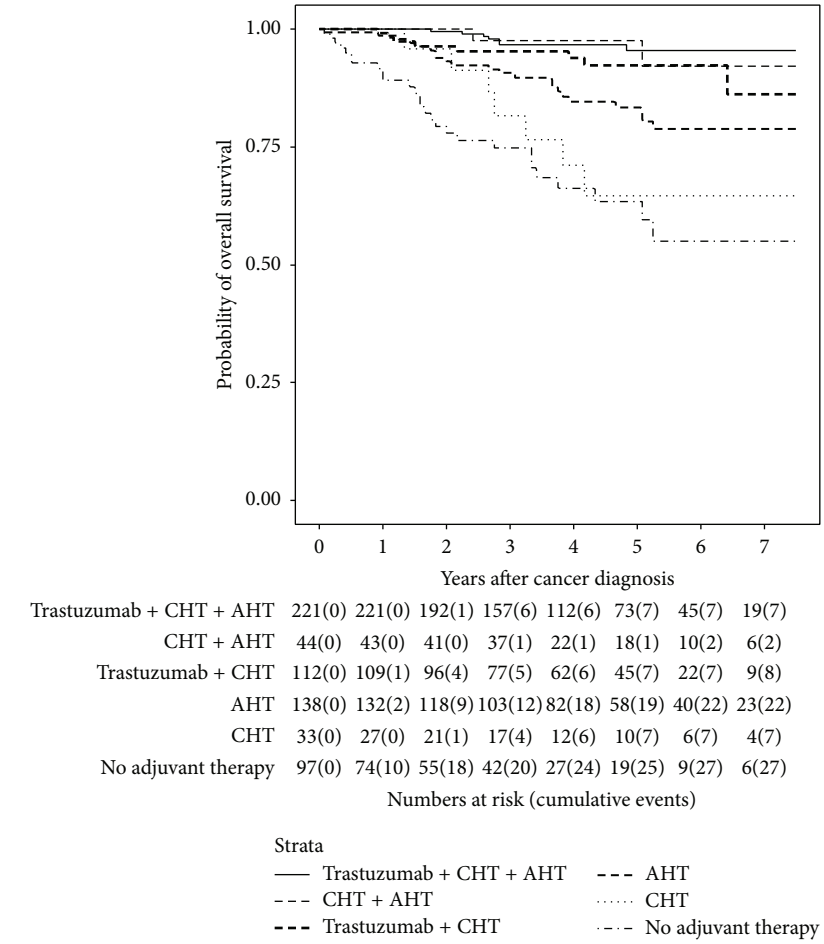

Figure 2: Kaplan-Meier plot of overall survival in years of HER2-positive patients based on adjuvant therapy.

HER2 status is required in all patients with invasive breast cancer according to national [32] and international guidelines [36]. In HER2-positive early breast cancer, adjuvant CHT in combination with trastuzumab is indicated. Using data from a high-quality population-based regional cancer registry we were able to analyze guideline concordant patient care.

The steady increase in HER2 determination from 2000 (16.3%) up to 2012 (99.6%) illustrates the implementation of guidelines in routine clinical practice. The first German interdisciplinary S3 Guidelines for Diagnosis, Treatment and Follow-up Care of Breast Cancer were published in 2004 and first updated in 2008 [31]. Concerning HER2 status, the number of HER2-positive tumors declined over time. In 2000, 30.0% of tumors were HER2 positive, whereas over the years, the percentage of HER2-positive tumors continuously decreased to 12.5% in 2012. The decrease of HER2 positivity might be due to false positivity because of short fixation time and revised cutoff definition in CISH and FISH in course of the investigated period [33, 37].

All common histopathological parameters showed highly statistically significant differences between HER2-negative and HER2-positive patients as shown in previous studies [38, 39]. For instance, HER2 overexpression has been found to correlate with several adverse prognostic parameters such as large tumor size, high grade, and steroid hormone receptor negativity [39, 40].

As expected, up to 2004, trastuzumab therapy was hardly administered in primary therapy. In Europe, trastuzumab was approved for adjuvant therapy in 2006. Consequently, since 2006, the number of HER2-positive patients receiving trastuzumab continuously increased from 38.2% to 66.2% in 2011. In a cohort study from the National Comprehensive Cancer Network (NCCN) overall 44% of HER2-positive breast cancer patients received neoadjuvant or adjuvant trastuzumab with increasing proportions over time (8% of patients diagnosed in 2000, 66% of patients diagnosed in 2005, and 77% of patients diagnosed in 2007) [25]. Another study that investigated the management of HER2-positive breast cancer patients in routine clinical setting reported a rate of 54.5% of patients receiving adjuvant trastuzumab [26].

Besides analyzing the fact of guideline conform diagnostics and therapies, we demonstrated the consequences of guideline adherence on survival of patients. Guideline-conform treatment led to similar OS of HER2-positive patients compared to HER2-negative patients. This has also been shown in previous studies [27, 41, 42]. A retrospective population-based analysis of adjuvant trastuzumab use among 703 HER2-positive Canadian women, of whom 480

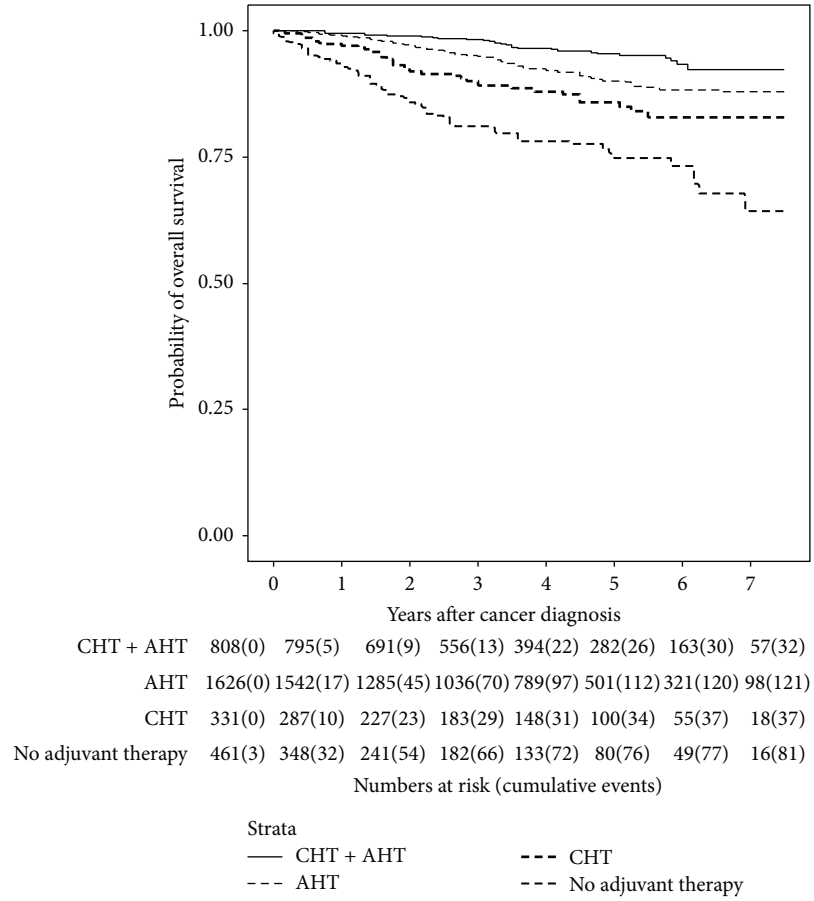

Figure 3: Kaplan-Meier plot of overall survival in years of HER2-negative patients based on adjuvant therapy.

(68%) received trastuzumab, demonstrated highly favorable outcomes at a 2-year follow-up [28]. In patients receiving trastuzumab, the 2-year DFS was 96.1% (95% CI: 93.6% to 97.7%) and the OS was 99.3% (95% CI 97.9% to 99.8%). Among node-negative and node-positive patients, the 2-year DFS was 97.8% and 94.8% ($P = 0.09$) for the trastuzumab-treated group and 90.9% and 77.3% ($P = 0.01$) for the group not receiving trastuzumab ($n = 223$) [28]. Similar to our results, their population had better survival outcomes than those reported in the large adjuvant clinical trials. So far, only a few healthcare research studies have analyzed the impact of guideline-adherent therapies on clinical outcomes in breast cancer patients [43–45]. Overall, several prior studies confirmed that there appears to be a strong association between guideline-adherent treatment and improved survival [45–48]. Nevertheless, a study from the Netherlands Cancer Registry showed that adherence to treatment guidelines was affected by age at diagnosis but was not associated with OS in either age group [49]. Remarkably, a considerable number of patients did not receive guideline concordant therapy both in our study and previous studies, which demonstrates the difficulty of implementing guideline recommendations in routine clinical care. The reasons for this are multifactorial. Indicated therapy either never had been started or was discontinued prematurely [50]. A potential explanation for noninitiation is the preference of patients who are not willing to receive indicated therapy from the start or discontinue in the course of treatment [51]. In particular older patients may be less willing to trade current quality of life for survival [52]. Nevertheless, an observational retrospective multicenter Italian study showed that trastuzumab treatment was feasible and well tolerated in routine clinical practice [29].

Moreover, there might be a percentage of patients with small HER2-positive tumors with no CHT indication. The large international, adjuvant, randomized clinical trials have demonstrated significant improvements in DFS and OS with trastuzumab-based CHT in node-positive and/or greater than 1 cm HER2-positive tumors [14, 15, 53, 54]. Node-negative patients with smaller tumors were generally excluded [55]. Current guidelines from the NCCN recommend trastuzumab-based CHT for women with node-positive breast cancer, and women with node-negative tumors that are ≥0.6 cm.

Several other factors were assumed to influence guideline adherence in breast cancer patients in prior studies such as education, access to medical resources, health care services themselves, and an urban versus rural location [56]. However, these effects could not be replicated in the present analyses since these variables are not part of the data set.

Table 6: Multivariable Cox regression models.

(a) Multivariable Cox regression models in HER2-positive patients since 2006

Characteristic	Overall survival (n = 516, events = 53)		
	HR	95% CI	P value
Trastuzumab + CHT + AHT (n = 180, events = 4)	Reference	—	—
CHT + AHT (n = 35, events = 2)	2.34	0.43; 13.38	0.32
Trastuzumab + CHT (n = 91, events = 6)	3.80	0.87; 16.59	0.08
AHT (n = 106, events = 17)	4.28	1.30; 14.05	**0.017**
CHT (n = 24, events = 3)	9.50	1.90; 47.43	**0.006**
No adjuvant therapy (n = 80, events = 21)	10.44	3.02; 36.06	<**0.001**

Model is controlled for age, Ki67 categories, tumor size, nodal status, grading, and receptor status; HR: hazard ratio; 95% CI: 95% confidence interval.

(b) Multivariable Cox regression model in HER2-negative patients since 2006

Characteristic	Overall survival (n = 2727, events = 219)		
	HR	95% CI	P value
CHT + AHT (n = 671, events = 23)	Reference	—	—
AHT (n = 1392, events = 104)	2.33	1.40; 3.87	**0.001**
CHT (n = 279, events = 28)	3.15	1.61; 6.16	**0.001**
No adjuvant therapy (n = 385, events = 64)	4.91	2.81; 8.59	<**0.001**

Model is controlled for age, Ki67 categories, tumor size, nodal status, grading, and receptor status; HR: hazard ratio; 95% CI: 95% confidence interval.

A main cause for nonadherence to guideline recommendations may be the existence of comorbidities of which elderly patients are more often affected than younger ones. Comorbidities, especially cardiovascular diseases, may also be the cause of reduction of OS. These data have to be included in a systematic way in the update of documentation of cancer registries and will then allow systematic analyses. A retrospective study on diagnosis and treatment according to national guidelines in the Netherlands demonstrated that deviation from guidelines in elderly breast cancer patients mainly occurs due to a deliberate adjustment to patients' comorbidity and preference. Similar to our results cardiovascular disease was the most frequently observed comorbidity (53% versus 58% in our study) [44]. Moreover, a 5-year multicenter cohort study of 3,976 patients by Wöckel et al. also concluded that advanced age at initial diagnosis was associated with a reduction in guideline adherence [43]. An observational study from Schwentner et al. found lower guideline adherence in triple-negative breast cancer (TNBC) patients compared to non-triple-negative subtypes. These lower rates of guideline adherence were observed in all age groups and were most pronounced in the >65-year-old subgroup (<50 (20.9% versus 42.0%), 50–64 (25.1% versus 51.1%), and >65 (38.4% versus 74.6%)). 22.9% of their TNBC subgroup did not receive any CHT which is comparable to our results [51]. Thus, several studies have shown that patients with comorbidities are less likely to be treated according to guidelines than patients without comorbidities [52, 57, 58].

5. Conclusions

In conclusion, this population-based study was able to demonstrate that guideline adherent use of adjuvant trastuzumab improves OS for patients with HER2-positive breast cancer treated in routine clinical care. This extends our knowledge on the efficacy of such treatment. However, a considerable proportion of HER2-positive postmenopausal breast cancer patients did not get the appropriate therapy. It is therefore mandatory to analyze the reasons for nonadherence and to develop means to improve guideline adherence.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

E. C. Inwald and O. Ortmann contributed equally to this paper.

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Research Article

Genetic Variants in the Genes of the Stress Hormone Signalling Pathway and Depressive Symptoms during and after Pregnancy

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Purpose. The aim of this study was to investigate whether single nucleotide polymorphisms (SNPs) in genes of the stress hormone signaling pathway, specifically *FKBP5*, *NR3C1*, and *CRHR1*, are associated with depressive symptoms during and after pregnancy. *Methods*. The Franconian Maternal Health Evaluation Study (FRAMES) recruited healthy pregnant women prospectively for the assessment of maternal and fetal health including the assessment of depressiveness. The German version of the 10-item Edinburgh Postnatal Depression Scale (EPDS) was completed at three time points in this prospective cohort study. Visit 1 was at study entry in the third trimester of the pregnancy, visit 2 was shortly after birth, and visit 3 was 6–8 months after birth. Germline DNA was collected from 361 pregnant women. Nine SNPs in the above mentioned genes were genotyped. After construction of haplotypes for each gene, a multifactorial linear mixed model was performed to analyse the depression values over time. *Results*. EPDS values were within expected ranges and comparable to previously published studies. Neither did the depression scores differ for comparisons among haplotypes at fixed time points nor did the change over time differ among haplotypes for the examined genes. No haplotype showed significant associations with depressive symptoms severity during pregnancy or the postpartum period. *Conclusion*. The analysed candidate haplotypes in *FKBP5*, *NR3C1*, and *CRHR1* did not show an association with depression scores as assessed by EPDS in this cohort of healthy unselected pregnant women.

1. Introduction

During pregnancy the overall prevalence of depressive episodes is about 10–20% [1, 2] and about 6–15% in childbed [3, 4]. A total of about 70,000–90,000 women in Germany suffer from this disorder every year, with 5-6% developing major depression [2, 4, 5]. The prevalence compares to that of non-pregnancy-associated depression; however, new depression occurs more often during pregnancy and in the postpartum period [6].

There is a correlation of pregnancy-associated depression with poorer obstetric outcome measures, with fetal and neonatal complications [7, 8], with the length of the mother's hospital stay at the time of delivery [9], and with a negative impact on the child's development [10–13]. Information about the pathogenesis for pregnancy-associated depression may

therefore be helpful for planning early interventions and understanding the pathogenesis of this disease, as it is not a part of the early intervention program in Germany yet [14]. In the general population, it is thought that between 33% and 77% of major depression can be attributed to genetic susceptibility [15, 16]. Several genome-wide association studies have been conducted [17–24] with some evidence for genetic susceptibility variants. Some studies described an association between perinatal depression and a family history of depression or perinatal depression [25–27]; however, only few studies have investigated specific genetic risk factors for perinatal depression.

One signalling pathway that is of specific interest in this context is the stress hormone system [28]. This signalling system is thought to be the key regulator of the response to environmental stressors. Its dysregulation is found consistently in stress related psychiatric disorders like major depression or posttraumatic stress disorder [29–31] and might play a relevant role in pregnant women [32]. With regard to pregnancy-associated depression, stress appeared to be one of the most stable factors in multivariate models for the prediction of depression during pregnancy [33], making this topic interesting for further research concerning this phenotype. Furthermore in utero exposure to stress and its subsequent exposure to glucocorticoids are discussed to have an influence on the development of behavioural stress response in the offspring [34, 35].

This study focuses on three genes, for which it is has been shown that genetic variants are associated with depressive symptomatology, especially in the context of stressful or adverse life events: the genes encoding the corticotropin releasing factor receptor 1 (*CRHR1*), the glucocorticoid receptor (*NR3C1*), and FK506 binding protein 51 (*FKBP5*).

CRHR1 function has been reported to be specifically associated with increased fear, alertness, depression, and anxiety [36–39]. Genetic variants in *CRHR1* have been associated with anxiety disorders, major depression, and alcoholism, especially in the context of early life adverse events [28, 40].

NR3C1 encodes the glucocorticoid receptor (GR). GR signalling has been reported to be disrupted in both depression and anxiety disorders [30, 41]. Several genetic variants have been described to result in functional changes of the GR [42, 43].

FKBP5 is known to bind to and alter the function of steroid hormone receptors, including the GR [44] and is a negative feedback regulator of GR function [31, 45]. Functional genetic variants in FKBP5 have been described to alter stress hormone response regulation as well as the risk to suffer from depression and other psychiatry disorders when exposed to childhood trauma [28, 46].

The aim of the present study was to test whether genetic variants in *FKBP5*, *GR (NR3C1)*, and *CRHR1*, previously described to increase the risk for depression, are associated with longitudinal measures of depressive symptoms in a cohort of pregnant women assessed in the third trimester of pregnancy, 2-3 days and 6 months after delivery.

2. Patients and Methods

2.1. Patient Selection and Biomaterial Retrieval. The Franconian Maternal Health Evaluation Study (FRAMES) is a prospective study, which recruited pregnant women from 2005 to 2007. Aim was the investigation of risk factors for pregnancy-associated depression [4, 47–49]. Previously we presented the influence of variants in *TPH2* on depression measurement scores during the pregnancy [47] and that of variants in the serotonin transporter *5-HTTLPR* on different depression levels after childbirth with regard to lifetime and current psychological stressors [50].

Inclusion criteria were age of 18 years or older with an intact pregnancy and at a gestational age of at least 31 weeks. They were invited to participate when they presented to register for the upcoming birth. A total of 1100 women were prospectively included. Assessment of genetic risk factors for postpartum depression was included as a study aim after the recruitment of women was completed in 2008. Blood samples for genetic analysis were therefore not taken prospectively, and the women had to be recalled for this purpose. This took place between January 2008 and July 2008. The patients were contacted by phone and invited to undergo blood sampling and take part in the genetic association study. From the primary study population ($n = 1100$) current phone numbers could be determined from 780 patients and 705 could be reached. 130 women declined to take part; the rest was appointed for a blood draw. Women, who did not show up, were contacted again and offered another appointment. A total of 431 women presented for blood sampling (final study population). DNA extraction was successful in 423 cases. DNA was considered unsuitable for the study if the DNA concentration was below 30 ng/μL according to the PicoGreen DNA concentration measurements. In addition, 62 women had to be excluded from the analysis because the depression measurement was lacking for at least one time point in the study, resulting in a final sample size for this study of 361 patients. The study was approved by the Ethics Committee of the Medical Faculty of Friedrich-Alexander University of Erlangen-Nuremberg and all of the patients provided written informed consent.

2.2. Questionnaire. The participants were interviewed using standardised 10-item Edinburgh Postnatal Depression Scale (EPDS) questionnaires, in the German version [51], at three time points: prepartal, from the 31st week of pregnancy onwards (Q1); 48–72 hours postpartum (Q2) to capture the initial phase of the maternity blues; and 6–8 months after birth (Q3). Additionally a structured questionnaire was used to document common epidemiological parameters and medical history that was not documented in the patients' files. This questionnaire included the question about preexisting psychiatric disorders, which was an exclusion criteria for this study. The first two questionnaires (Q1, Q2) were structured as personal interviews using standardised manuals, which were conducted by trained and medically qualified staff. The third questionnaire (Q3) was carried out by phone interview. The reliability of phone questionnaires in this setting can be regarded as confirmed [52].

Table 1: Genotype and allele distribution for each single nucleotide polymorphism (SNP). Absolute frequencies and percentages (in brackets) are shown.

SNP	Chrom.[1]	Position	Alleles[2]	MAF[3] (%)	Homozygous, common[4]	Heterozygous[4]	Homozygous rare[4]
rs1360780 (FKBP5)	6	35607571	C/T	32.0	168 (46.5)	155 (43.2)	38 (10.5)
rs9296158 (FKBP5)	6	35567082	G/A	32.0	166 (46.1)	156 (43.3)	37 (10.2)
rs3800373 (FKBP5)	6	35542476	T/G	28.4	185 (51.8)	147 (40.7)	29 (8.0)
rs9470080 (FKBP5)	6	35646435	C/T	34.7	155 (42.9)	160 (44.6)	45 (12.5)
rs41423247 (NR3C1)	5	142778575	G/C	34.7	156 (43.3)	158 (43.9)	46 (12.7)
rs6195 (NR3C1)	5	142779317	A/G	4.7	327 (91.6)	34 (9.4)	0 (0.0)
rs10482605 (NR3C1)	5	142783521	T/C	18.3	242 (67.0)	99 (27.6)	16 (4.4)
rs110402 (CRHR1)	17	43880047	C/T	45.3	110 (30.6)	175 (48.6)	76 (21.1)
rs7209436 (CRHR1)	17	43870142	C/T	44.0	115 (32.2)	174 (48.2)	72 (19.9)

[1]Chromosome; [2]major/minor allele, based on the forward strand and minor allele frequency; [3]minor allele frequency; [4]frequency, percentage in brackets.

2.3. SNP Selection. SNPs in the genes *FKBP5*, *NR3C1*, and *CRHR1* have been selected for genotyping based on published positive association studies with depression or depressive symptoms for the respective SNPs and haplotypes (see Section 1). The SNPs with the strongest gene environment interaction effects, which mean depressive symptoms, were selected (*CRHR1* SNPs: rs7209436 and rs110402 [28, 40]; *NR3C1* SNPs: rs41423247, rs6195, and rs10482605 [42, 43]; *FKBP5* SNPs: rs1360780, rs9296158, rs3800373, and rs9470080 [28, 46]). SNP IDs and their minor allele frequency (MAF) are reported in Table 1.

2.4. DNA Preparation and Genotyping. DNA was extracted from 10 mL of ethylenediaminetetraacetic acid (EDTA) blood using the Puregene whole-blood DNA extraction kit (Gentra Systems, Minneapolis, MN, USA). *FKBP5*, *NR3C1*, and *CRHR1* SNPs were analysed on a Sequenom platform using the iPlex technology (Sequenom, San Diego, CA, USA) in a multiplex assay using 10 ng of DNA. For quality control, duplicate DNAs as well as negative controls were included in the genotyping plates. Genotype calls were made using the ArrayTyper 3.4 software (Sequenom, San Diego, CA, USA).

2.5. Statistical Considerations. Genotypes were analysed as haplotypes. The reconstruction of haplotypes was carried out with an expectation-maximisation (EM) algorithm [53]. For the haplotype reconstruction, all SNPs were grouped by gene. Genotype distributions were tested for Hardy-Weinberg Equilibrium. Haplotypes were examined rather than single SNPs because haplotypes may provide more genetic information. Associations between SNPs and outcome measure are expected to be reflected in associations between haplotypes and outcome measure, but not necessarily vice versa.

The EPDS value was regarded as continuous measurement with a range from 0 to 26. Depression values from the three different time points Q1, Q2, and Q3 were compared. For each haplotype, a categorical variable with levels according to the frequency of 0, 1, or 2 copies per patient was generated. Small groups with fewer than five carriers of two copies of a haplotype were joined with the carriers of one copy. Extremely rare haplotypes with an overall haplotype frequency of fewer than 10 occurrences were excluded from the analysis. Consideration of the haplotypes as ordinal variables was rejected due to nonlinear coherence with EPDS.

The association between haplotypes and the course of depression was analysed using linear mixed models with EPDS as target variable. For each haplotype block, a linear mixed model was fitted with patient as random effect and haplotypes, time (Q1, Q2, Q3), and the interactions of haplotypes by time as fixed effects. These linear models were each compared with a basic linear mixed model with patient as random effect and time as the only fixed effect, using the likelihood ratio test. A significant test result means that the haplotypes are associated with EPDS. In that case the linear model was further analysed using F-tests of fixed effects. The P values of the likelihood ratio tests were adjusted for multiple testing according to the method of Bonferroni-Holm.

The model requirements (e.g., normal distribution of the standardised residuals) were tested graphically. No replacement of missing data took place. The random effect "patient" takes into account the fact that each patient had repeated EPDS measures. The models were fitted by maximum likelihood (ML) instead of restricted maximum likelihood (REML) in order to apply likelihood ratio tests to models with different fixed effects. A sensitivity analyses showed that both estimation methods gave almost identical results.

All of the tests were two-sided, and a P value of <0.05 was regarded as statistically significant. The statistical analyses were carried out using the R system for statistical computing (version 2.13.1; R Development Core Team, Vienna, Austria, 2011) and the SAS software package (version 9.2, SAS Institute, Inc., Cary, NC, USA).

3. Results

The genotype frequency and allele distributions are shown in Table 1. The genotype distribution for all SNPs was consistent with the Hardy-Weinberg equilibrium ($P = 0.16$ for rs10482605; P between 0.56 and 1.00 for the other SNPs). The distributions and frequencies for each haplotype block are presented in Table 2. For the most frequent haplotype of each gene the mean EPDS values of Q1, Q2, and Q3 are shown in Figures 1, 2, and 3 for carriers of 0, 1, or 2 copies of the respective haplotypes.

Table 2: Reconstructed haplotypes for each gene and absolute frequencies and percentages (in brackets).

No	Gene	SNP	Haplotype	Haplotype frequency		
				0	1	2
1	FKBP5	1–4	CGTC	46 (12.74%)	163 (45.15%)	152 (42.11%)
2			CGTT	341 (94.46%)	19 (5.26%)	1 (0.28%)
3			CATC	360 (99.72%)	1 (0.28%)	0 (0.00%)
4			CATT	360 (99.72%)	1 (0.28%)	0 (0.00%)
5			CAGC	360 (99.72%)	1 (0.28%)	0 (0.00%)
6			TGTT	358 (99.17%)	3 (0.38%)	0 (0.00%)
7			TATC	359 (99.45%)	2 (0.55%)	0 (0.00%)
8			TATT	339 (93.91%)	22 (6.09%)	0 (0.00%)
9			TAGC	360 (99.72%)	1 (0.28%)	0 (0.00%)
10			TAGT	187 (51.80%)	145 (40.17%)	29 (8.03%)
11	GR-NR3C1	5–7	GAT	118 (32.69%)	178 (49.31%)	65 (18.01%)
12			GAC	247 (68.42%)	99 (27.42%)	15 (4.16%)
13			GGT	327 (90.58%)	33 (9.14%)	1 (0.28%)
14			CAT	158 (43.77%)	158 (43.77%)	45 (12.47%)
15			CAC	359 (99.45%)	2 (0.55%)	0 (0.00%)
16	CRHR1	8+9	CC	76 (21.05%)	175 (48.48%)	110 (30.47%)
17			TC	352 (97.51%)	9 (2.49%)	0 (0.00%)
18			TT	115 (31.86%)	174 (48.20%)	72 (19.94%)

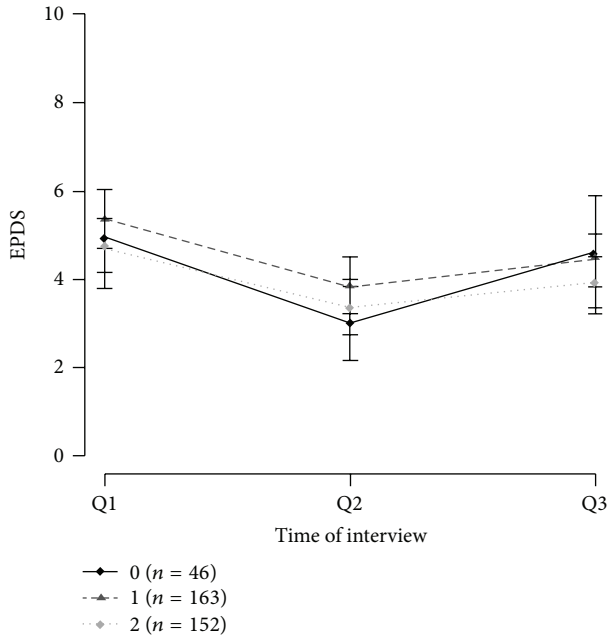

Figure 1: Mean EPDS values of FKBP5 haplotype CGTC with 95% confidence intervals.

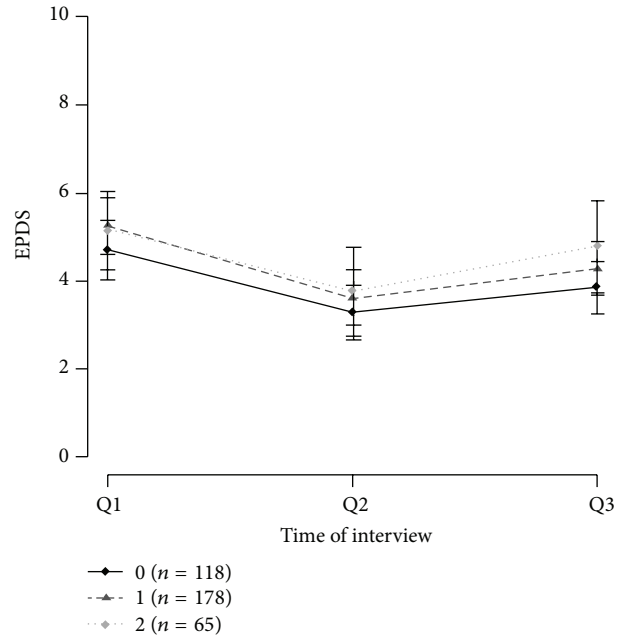

Figure 2: Mean EPDS values of GRNR3C1 haplotype GAT with 95% confidence intervals.

The SNPs within gene *FKBP5* formed ten haplotypes, but only four of them, CGTC, CGTT, TATT, and TAGT, occurred with a frequency usable for analysis. For haplotype CGTT the group of carriers of two copies (0.28%) was joined with the carriers of one copy (5.26%).

Haplotype reconstruction with the *NR3C1* SNPs resulted in five haplotypes. The most common haplotype was GAT, with 49.31% of patients carrying two copies and 32.69% carrying one copy. For haplotype GGT the group of carriers of two copies (0.28%) was joined with the carriers of one copy (9.14%). The haplotype CAC had to be discarded because of only two occurrences.

The haplotype reconstruction within gene *CRHR1* resulted in three haplotypes where haplotype CC was the

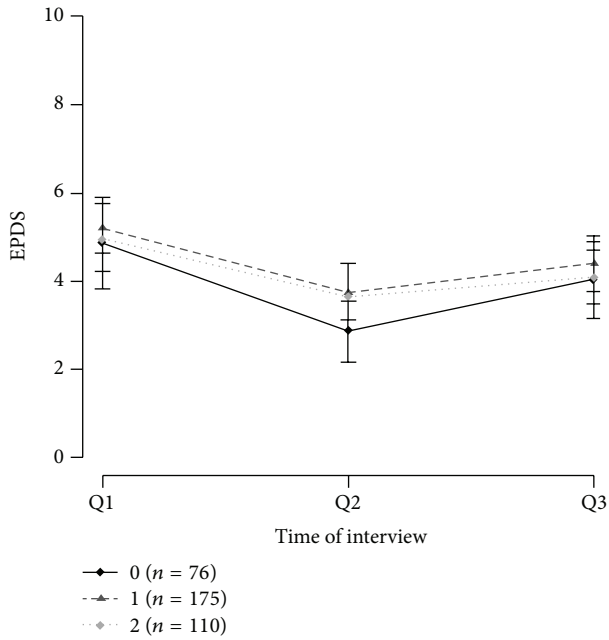

Figure 3: Mean EPDS values of CRHR1 haplotype CC with 95% confidence intervals.

most common with 30.47% carrying two copies and 48.48% carrying one copy.

None of the haplotypes showed a significant result for the likelihood ratio test (unadjusted P values: FKBP5, $P = 0.45$; NR3C1, $P = 0.78$; *CRHR1*, $P = 0.61$). Therefore no further analysis was performed, as differences between genotype groups at one time point or over different time points cannot be assumed.

4. Discussion

With our association study in a cohort of pregnant women without further risk factors for depressive or anxiety disorders, we could not show that candidate single nucleotide polymorphisms within the genes *FKBP5*, *NR3C1*, and *CRHR1* are associated with EPDS values during or after pregnancy.

The candidate genes were selected because of their role within stress hormone signalling system which is one of the possible mediators between environmental stressors and the development of a depressive reaction. Several genetic factors have been discovered that explain individual responses to stressful events [28, 29, 54–56].

However, with our study design and the examined genetic variants, no effect on EPDS values could be seen, neither between haplotypes at specific time points, nor in comparing the changes of EPDS over time according to haplotypes. Several factors specific to this study will be discussed below.

As we were studying a cohort of women with an uncomplicated pregnancy and no prior history of psychiatric disease, there might be a different genotype distribution in our cohort than in cohorts of women without the inclusion criterion of pregnancy. It was reported that variants in NR3C1 may have an influence on gonadotropin levels in women with anovulatory polycystic ovary syndrome (PCOS) [57]. Another study reported variants in NR3C1 to be associated with recurrent miscarriages [58]. There is further preclinical evidence that exposure to glucocorticoids leads to the apoptosis of fetal ovary germline cells, having possible impact on fertility [59]. Preselecting of women with an uncomplicated pregnancy could therefore result in a population with slightly different genotype distribution.

Furthermore during pregnancy many signalling pathways adapt with regard to ensuring the function of the pregnancy, with one of them being the hypothalamic-pituitary-adrenal axis or stress hormone system [60]. Therefore genetic associations that are observed in women without a pregnancy might not be found in a population of pregnant women. In particular during the third trimester progressively increasing circulating levels of placental CRH are seen [61] as well as gradually decreasing levels of CRH binding protein [62]. Maternal distress during pregnancy increases plasma levels of cortisol and CRH in addition to the already physiologically increased levels [63]. After delivery a central suppression of hypothalamic CRH secretion might explain a generally increased vulnerability to the affective disorders observed during this period [64]. For these reasons associations between genotypes and phenotypes might be different in pregnant and nonpregnant populations.

Several limitations of this study have to be taken into consideration. One might be the use of the EPDS questionnaire a few days after childbirth. The EPDS reflects the experience and mood state of women during the week before completing the questionnaire, intentionally skipping somatic symptoms that are associated with depression but appear quite often after delivery in healthy women, such as sleep disturbances or fatigue, and it has been validated for administration during pregnancy and a few weeks into the postpartum period [65–69]. The rating within the first days after delivery might thus also reflect the mood during the last days of pregnancy. Another limitation might be that patients were recontacted for blood sampling for DNA extraction after the end of the study. However, there were no differences with regard to prepartum or postpartum EPDS scores in women participating or not participating in the genetic substudy (data not shown). Women were screened for preexisting psychiatric disorders only by a questionnaire. This self-reported depression is not as accurate as the assessment by a formal psychiatric diagnostic interview. However, in contrast to other studies, a classic case/control design was not used for the analysis, and the prevalence of clinical depression was rather low in this cohort (6% as measured with the EPDS). Continuous EPDS values were therefore selected as the outcome variable in order to maximize the power of the study. Finally, due to the limited sample size our study might not show smaller effect of the examined genetic variants.

In conclusion we could not show an association between depression measurements as assessed by EPDS values during or after pregnancy and candidate haplotypes in the genes *FKBP5*, *NR3C1*, and *CRHR1*. As other studies have shown some association between genetic variants in these genes and depressive symptomatology, our null results could be explained by a small sample size or a generally different role

of genetic variants in genes of the stress hormone signalling pathway in pregnant women.

Conflict of Interests

The authors have no conflict of interests to report.

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Research Article

Decreasing Quality of the New Generations of Anti-Müllerian Hormone Assays

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Anti-Müllerian hormone (AMH) measurements are widely used to optimize the stimulation protocols. First generation AMH kits correlated well with ovarian reserve and response to stimulation. In the present study we aimed to asses if the new generation kits share the same accurate correlations. Retrospective data were collected from 8323 blood samples. For comparison we used Immunotech I generation kit (ImI 4035 samples), Beckman Coulter II generation kit RUO (BCII RUO 3449, samples) and Beckman Coulter II generation kit with IVD certificate (BCII IVD 839 samples). We compared average AMH concentrations measured with different kits, as well as correlation between kits. We also compared average AMH concentrations in sera collected on different cycle days and samples of different quality of preservation. AMH serum concentrations differed for each kit, ranging 4.4 ± 4.12 (mean ± SD) for the ImI, 2.68 ± 3.15 for the BCII RUO, and 1.64 ± 2.85 for BCII IVD. The mean differences from an adjusted regression model were −48.7%, −40%, and −69.2%, respectively. In conclusion, the changes of the BC AMH kits are unpredictable; however, the improvement of them is still possible. It would be very dangerous to use elaborated stimulation protocol (based on the Ist generation AMH results) with the results from the IInd generation assays.

1. Introduction

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β superfamily. In nature it exists as a glycoprotein dimer and this chemical structure affects its function and detection method [1, 2]. It is primarily produced by granulosa cells in primordial, preantral, and antral follicles [3, 4]. Although serum level of AMH is about 10–100 times lower than in the place of its production (antral follicle fluid) and far from the level of potential biological activity, it is well correlated with the pool of antral follicles and this correlation implicates its clinical usefulness [5, 6]. That is why it is well established as the best ovarian reserve marker [7–12]. There is good correlation between the amount of antral follicles and the response of patients to stimulation protocols [13–16]. However, the correlation between AMH and IVF main outcome (live births) has still not been confirmed [15, 17–19].

It is well known that AMH measurements were very useful for different reasons and for different investigated populations. As a predictor of diminished ovarian reserve it was less useful in young patients as screening test but it was a powerful tool for middle aged and older patients (higher

risk populations) as test to establish diagnosis because of high levels of both sensitivity and specificity. But mostly, AMH measurements were widely used to optimize the stimulation protocols. It was well correlated with ovarian response to stimulation. There were few publications emphasizing the decision-making process based on AMH serum concentrations [20].

The importance of the AMH measurements caused the need for commercialization of the kits. Beckman Coulter Company consolidated the available systems from Immunotech Ltd. and Diagnostic Systems Ltd (DSL). They were based on different monoclonal antibodies and also different standards used. They were integrated into the Beckman Coulter II generation kit. According to information from the manufacturer, the AMH Gen II ELISA kit uses the same antibody as in the DSL kit, but with the standards of the Immunotech assay kit [21]. Unfortunately, the results began to correlate poorly in clinical situations. These signals were reported by different clinicians (but also patients) and caused doubts among them. Taking above concerns into consideration, we decided to revise our quality management data. Therefore, this paper represents a retrospective study of our results.

2. Materials and Methods

A total of 8323 blood samples from different women were taken into consideration. Samples were obtained from women of 12–62 years of age being seen for investigation of infertility or fertility preservation reasons requiring AMH assessment. Most of the samples were collected at the Fertility Clinic Invicta—6259 patients in Warsaw and Gdansk from the beginning of 2007 till December 2012. Additionally, a total of 2064 samples were collected from different clinics during this period. The tests were done sequentially and each patient was tested only once using one kit. The AMH concentration in the blood plasma was measured from 2007 till April 2011 with an Immunotech I generation kit (4035 samples) and from August 2010 till August 2012 with a Beckman Coulter II generation kit RUO (3449 samples). From August 2010 till April 2011, both tests (Immunotech I generation and Beckman Coulter II generation RUO) were used in parallel depending on the availability from the supplier. From August 2012 till December 2012, we were supplied with the same Beckman Coulter II generation kit but with an IVD certificate (839 samples).

Blood samples were taken between first and fifth day of the menstrual cycle in 47% of cases, when patients attended the clinic for the routine first visit. They were collected aseptically into tubes with clotting activator, vacuum blood collection system Vacutainer Becton Dickinson. The blood collection was on different days of the cycle in 28% of the samples. The results from 25% of the samples from different fertility clinics were sent to us without the information about the women's cycle days. The serum for AMH assay was separated within 2 hours from venipuncture and frozen in aliquots at −80°C until it could be analyzed in batches. Samples that were lipaemic or haemolysed and samples not frozen within 2 hours of venipuncture were excluded from the study.

AMH was measured using ELISA kits according to the manufacturer's instructions. EIA AMH/MIS (catalogue number A16507) (Immunotech, Marseille, France) has sensitivity of 1 pmol/L and reported intra- and interassay coefficients of variation of less than 12.3% and 14.2%, respectively, according to the product insert. The Beckman Coulter Gen II RUO assay (catalogue number A73818) (Beckman Coulter Inc. Brea, CZ 92821 USA) and The Beckman Coulter Gen II IVD assay (catalogue number A79765) (Beckman Coulter Inc. Brea, CZ 92821 USA) both have sensitivity of 0.57 pmol/L and reported intra- and interassay coefficients of variation of less than 5.4% and 5.6%, respectively, according to the products' inserts. We were informed by the manufacturer that there was no difference between the BCII RUO and BCII IVD kits, except for the label.

3. Sample Processing

Collection and handling of all AMH samples were conducted according to the standards set by the manufacturers and did not vary between different assays. Serums samples were transported immediately to the Invicta Routine Laboratory and separated within 2 hours. Samples were frozen in aliquots at −80°C until analysis, normally within 3-4 days of receipt. The laboratory participates in the External Quality Assurance Schemes for Reproductive Medicine (from 2010 till 2012), which confirms its satisfactory performance.

Clinical data were collected retrospectively using an electronic database (Invictus ver. 3.3.3, Invicta Ltd., Poland). We took into consideration age, menstrual cycles (duration, regularity, and bleeding), the interval between blood collection for AMH, and the beginning of stimulation.

We acquired laboratory data from laboratory software (Invictus Laboratory ver. 2.1.3, Invicta Ltd., Poland). We obtained the exact day of the cycle when blood was collected, the time of collection, and the history of sample trip from the blood collection point to the result (the duration and temperature of the sample transportation, time and temperature of the centrifugation, the period between serum collection and freezing, the duration of freezing, and time from thawing till getting results).

The study was approved by the Local Research Ethics Committee (the Varmia and Masuria Chamber of Physicians).

4. Statistical Analysis

Data analysis was performed using StatSoft, Inc. (2011) STATISTICA (data analysis software system), Version 10. http://www.statsoft.com/.

Quality control was shown as mean concentration against the expected one with standard deviation, coefficient of variation, and biases.

The characteristics of the investigated assay groups were compared using Mann-Whitney U test.

Table 1: Quality control of each analyzed AMH test.

Control	Expected conc. (ng/mL)	Imprecision between run					Trueness	
		Mean Conc. (ng/mL)	Min	Max	SD	CV%	Bias	Bias%
AMH/MIS ELISA Immunotech REF A16507								
Controls 1	0.42	0.4	0.24	0.49	0.06	13.4	−0.05	−0.3
Controls 2	11.3	11.4	10.5	12.3	0.48	4.2	0.01	0.6
AMH Gen II ELISA Beckman Coulter REF A73818								
Controls 1	3.0	3.09	2.4	3.6	0.26	8.3	0.03	3.0
Controls 2	9.0	9.05	7.6	10.6	0.75	8.3	0.01	0.5
AMH Gen II ELISA Beckman Coulter REF A79765								
Controls 1	2.9	2.86	2.51	3.11	0.22	7.7	−0.01	−1.4
Controls 2	8.2	8.47	7.44	9.65	0.58	6.8	0.03	3.3

CV: coefficient of variation, AMH: anti-Müllerian Hormone, and SD: standard deviation.

Table 2: The characteristics of the investigated groups and AMH results in each assay.

	AMH (ng/mL)			Age (year)		
	Imm. gen. I	BC gen II RUO	BC gen II IVD	Imm. gen. I	BC gen II RUO	BC gen II IVD
N	4035	3449	839	4035	3449	839
Mean	4.4	2.68	1.65	34.33	32.29	33.32
Std. dev.	4.12	3.15	2.85	4.95	5.09	5.63
Median	3.4	1.8	1.2	34	33	32
Upper quartile	5.9	3.8	2.1	37	36	36
Lower quartile	1.7	1.0	0.52	31	30	29

The age-related relationship of the three assays to AMH was visualized using scatter plots and quadratic fit on a logarithmic scale. The age-adjusted regression analysis was used to estimate the difference in AMH serum concentrations between investigated assays. Influence of the blood collection cycle day on AMH serum concentration was analyzed using Mann-Whitney *U* test. *P* value of <0.05 was considered statistically significant.

5. Results

The analytical characteristics of AMH tests according to manufacturer show the linearity of the ImI test from 0.1–21.0 ng/mL for ImI to 0.08–22.5 ng/mL for both of BC II tests. They are comparable but 6.8% lower in range for Immunotech 1st.

Table 1 compares the quality controls of all tests performed in our laboratory. We did not find any problems using tests with the manufacturers' controls. All of them were marked as good by external quality control company.

5.1. The Characteristics of the Results Received on Three Different Kits. There are statistically significant differences between each group in AMH serum concentrations, as well as age (Table 2). The average ages of the groups differ significantly. The groups measured by II generation assays were younger and should be effective in higher AMH results but AMH results were much lower comparing to those received with the I generation kit. The difference between both II generation assays results was also very big which is not possible to be explained by the patients' small age difference only. Figure 1 shows the correlation of AMH with age for the unselected groups.

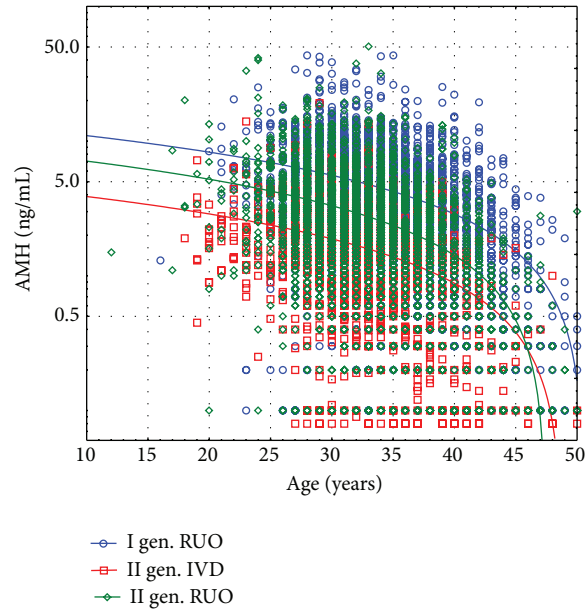

Figure 1: Unselected AMH values from Immunotech I (blue line), Beckman Coulter gen. II RUO (green line), and Beckman Coulter II IVD (red line) assays as a function of age. Lines show the regression fits of AMH serum concentration against a quadratic function of age.

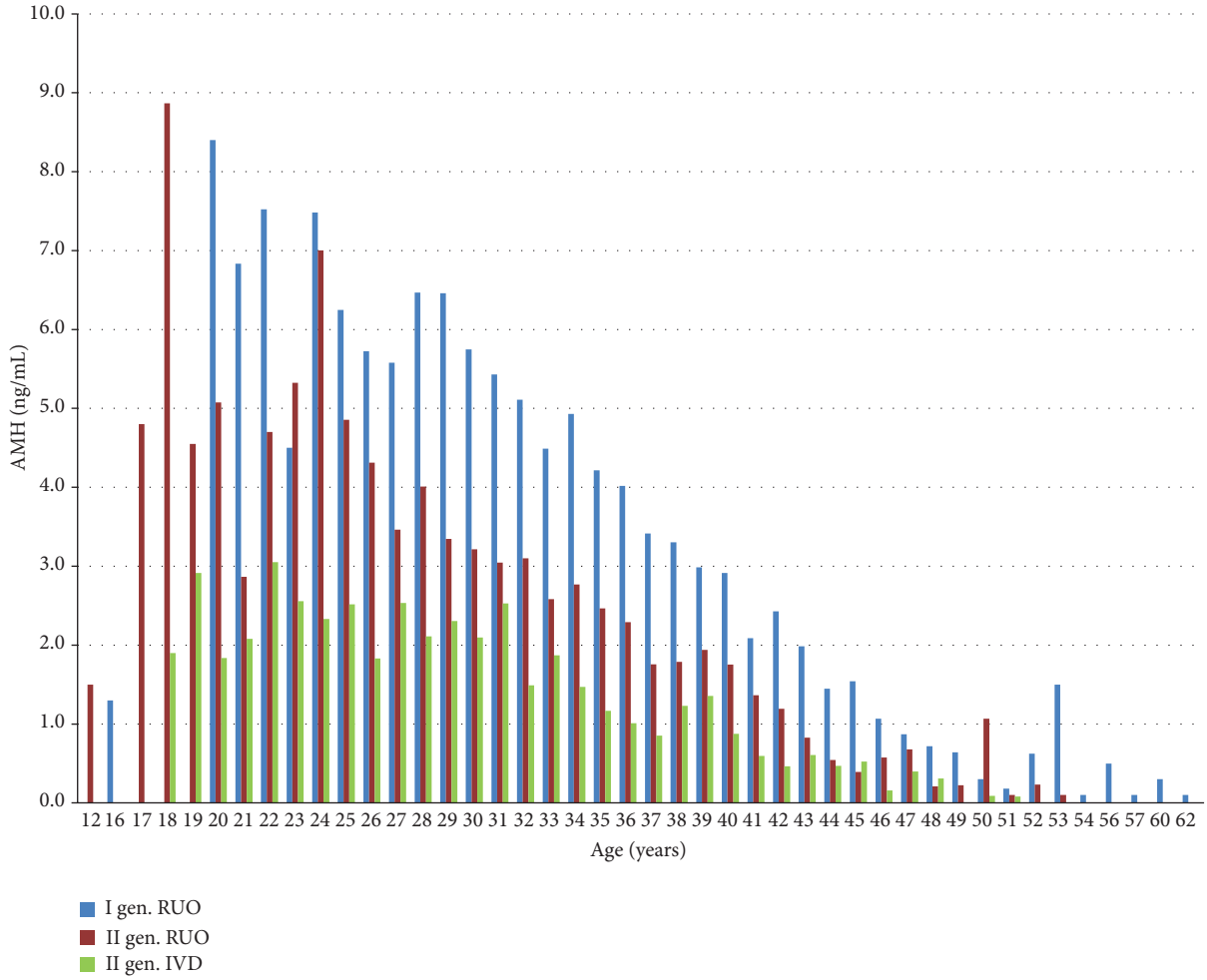

Figure 2: Mean values of AMH of three different tests as a function of age. We can see the decrease in the mean values of each consecutive test placed on the market.

For the estimation of the difference between the expected results among the assays, we adjusted the ages of the investigated groups (Table 3). We found an approximately 50% difference between 1st and 2nd generation assays and an approximately 70% difference between the 1st generation and the last version of the 2nd generation Beckman Coulter kits. The difference between both 2nd generation kits was 40%, which contradicts the manufacturer's information about the identity of both assays. These differences are visible in each investigated age group as shown in Figure 2. The amounts of the analysed samples performed in each women ages were proportional for all analyzed kits—data not shown.

Figure 1 demonstrates unselected AMH values from Immunotech I, Beckman Coulter gen. II RUO, and Beckman Coulter II IVD assays as a function of age. Lines show the regression fits of AMH.

Figure 2 presents mean values of AMH of three different tests as a function of age. We can see the decrease in the mean values of each consecutive test placed on the market.

We found no differences in average AMH results in days 1–5 and day 6 through the end of the cycle (Table 4). We found lower results from the 1st generation tests' results from the other clinics where the quality of the samples protection and transportation were unknown.

Table 3: Mean difference from an age-adjusted regression model expressed as a percentage difference (%).

Immunotech gen. I		
	−48.7 (−52.4 to −45.5)	
BC gen. II RUO		−69.2 (−76.2 to −68.2)
	−40.0 (−50.0 to −41.7)	
BC gen. II IVD		

6. Discussion

The aim of our work was to compare different AMH measurement kits.

AMH is a very useful marker for estimating the ovarian reserve in women. As we demonstrated before, the results obtained using I generation kits correlated well with the stimulation effects (own data submitted to

Table 4: The mean (±standard deviation) of the different kits depending from the day of the cycle, from day 1 to day 5 of the cycle, from day 6 to the end of the cycle, and all known cycle days together and results from unknown cycle day.

	Cycle day	*N*	Mean ± SD	Median	95% CI	*P*
Immunotech I gen RUO	1–5	1845	4.56 ± 3.86	3.7	2.0–6.2	0.07
	>5	856	4.51 ± 4.51	3.3	1.6–5.9	
	Known	2701	4.55 ± 4.08	3.6	1.9–6.1	<0.001
	Unknown	1334	4.11 ± 4.21	3.0	1.3–5.5	
BC II gen RUO	1–5	1446	2.6 ± 2.91	1.8	0.9–3.3	0.69
	>5	1097	2.67 ± 2.9	1.9	0.8–3.4	
	Known	2543	2.63 ± 2.91	1.8	0.8–3.4	0.91
	Unknown	906	2.84 ± 3.74	1.9	0.8–3.7	
BC II gen IVD	1–5	358	1.62 ± 1.65	1.3	0.6–2.0	0.55
	>5	287	1.62 ± 1.69	1.1	0.5–2.2	
	Known	645	1.62 ± 1.67	1.2	0.5–2.0	0.71
	Unknown	194	1.76 ± 2.07	1.2	0.5–2.2	

Reproductive Biology). The clinical decisions of stimulation protocols were simple and safe. In the previous study we found positive correlation between AMH serum concentrations and life-birth rates assessed in multivariate regression analysis [22]. Moreover, other authors also reported good correlation between single AMH measurements and stimulation results using generation I assays [20].

There are lots of proposals of AMH serum concentrations use in clinical practice. Up to now, our clinical decisions were routinely based on AMH serum concentrations. However, we noticed that the correlation between the AMH serum concentration measurements and clinical results in our clinic decreased in subsequent years. Moreover, we received signals of problems from our laboratory, where AMH is the accredited measurement with all necessary additional quality controls.

That is why we decided to compare our results from different generations of kits. We started to look more carefully at the correlation between AMH serum concentrations and clinical practice. The introduction of the second generation of AMH kits changed our clinical practice. We were informed by the manufacturer about the changes in results that we should expect. The AMH results should have been lower by approximately 30% on average. The compared groups were of different ages, but the group with BCII RUO was younger than the first group, which should have even increased received results comparing to ImI (Table 3). The comparison was made after adjustment of the age. The generation II RUO AMH results were totally different than before and had lost their correlation with clinical situation (unpublished data). We found the results of AMH 48.7% lower on average (Table 4). The next kit, which was introduced by Beckman Coulter in August 2012, was introduced as being the same with the changes of the label only. It ceased being labelled RUO and was dedicated for clinical use (IVD). The results were diminished once again. We found another 40.0% decrease of the mean results that gave us a 69.2% decrease compared to the average results of the 1st generation kits. We have to remember that we were informed by Beckman Coulter about constant results from the both generation II kits.

Rustamov et al. [23] suggested that the decrease in AMH serum concentrations obtained using Gen II assays could have been caused by degradation of the specimens in one or both assays. We could not have confirmed this assertion because we had been using the same procedure, the same equipment, and the same staff. All samples in this retrospective study were subjected to the same handling procedures and analysed by the same laboratory. We, as the clinicians, use AMH measurements as a routine first step in the infertility investigation procedure. No other alterations in our practice have happened contemporaneous with assay change. We are working on one system where each bias in the stimulation protocols and the results are directly reported to board and corrected immediately. We reported our problems to Beckman Coulter, but we did not receive any reliable information.

Some authors have reported the variability of the results between assays [23]. They concluded that they were the results of marked degree of sample instability seen in the laboratory. They excluded biological variation, which was confirmed to be small [24, 25], and the intra-and interassay variation (which they found <5%) [23].

We found good intra- and interassay variations in all kind of kits. We conclude that most of biases that we found were probably connected with the manufacturer's technological problems.

We also wanted to find out whether the AMH results are dependent on the cycle day when the blood is collected and on the quality of the samples protection and transportation. We did not find the differences between the average results from different cycle days of blood collection from our lab independently from the assays type. For the generation II kits, we also did not find the difference between the results of our optimized laboratory procedure and the results of the unattended samples of unknown quality. The results from the 1st generation kit were lower in the unattended samples, even

after age adjustment. This can be explained by degradation of the sample that could have happened in the higher rate during the unattended transportation. On the other hand, Rustamov et al. found the increase in the AMH concentration after the room temperature storage [23]. Those authors performed all analyses using the Gen II systems. The increase in the AMH concentration can be explained by the second binding site on the antigen being exposed by the dissociation which could be undetected by the II generation kits. This is the first study to report the decrease in sensitivity and the diminished average level of AMH when using II generation kits. We found our results to be very important and urgent for clinicians. It would be very dangerous to use elaborated stimulation protocol relaying on the results from the II generation kits. Moreover, we presume that relaying on those results would lead to the increase of patients suffering from OHSS as the reason for the application of the inappropriate high doses of gonadotropins for stimulation. Finally, relaying on the lower levels of AMH while making decisions about the stimulation method can lead to higher percentage of patients suffering from OHSS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Does Loop Electrosurgical Excision Procedure of the Uterine Cervix Affect Anti-Müllerian Hormone Levels?

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Background. A delayed time to pregnancy was recently reported for women who had a loop electrosurgical excision procedure (LEEP) to remove cervical intraepithelial neoplasia (CIN) grade 2 or 3. The objective of the current study was to determine if treatment of CIN with LEEP is associated with decreased levels of anti-Müllerian hormone (AMH), a marker of ovarian reserve. *Methods*. AMH levels were measured in 18 women treated with LEEP and 18 age-matched controls, who had colposcopy only and did not require LEEP. Cases and controls had their blood drawn at study entry time zero and again 6 months later. *Results*. The mean AMH level decreased significantly from baseline to follow-up; however, no significant differences were observed when stratifying by LEEP status, suggesting that both groups experienced a similar decrease in AMH levels during the follow-up period. Although women treated with LEEP had lower overall AMH levels than controls at both baseline and follow-up, these differences were not statistically significant. *Conclusion*. Overall, the delayed time to pregnancy observed in women treated with LEEP is likely not due to a LEEP-associated decrease in ovarian reserve as measured by AMH; thus, other mechanism are responsible for the delayed time to pregnancy associated with LEEP.

1. Introduction

In the United States (US), pap smears to detect precancerous cervical intraepithelial neoplasia (CIN) are an integral part of a woman's health care regimen. As a result of screening, women with biopsy confirmed CIN2 or CIN3 routinely undergo loop electrosurgical excision procedure (LEEP) to remove cervical dysplasia, equating to approximately half a million LEEP procedures in the United States each year (American Cancer Society/[1]). Because most women requiring LEEP are of child bearing age, preserving fertility is paramount. However, the impact of LEEP on fertility is not established. Although a few small studies from the early 1990s concluded that LEEP had no effect on future fertility, these studies were not designed to directly address this question and lacked information on potentially confounding factors such as a history of infertility [2–4].

In a recent study of time to pregnancy following LEEP and other cervical surgical procedures, Spracklen et al. reported that women with a history of LEEP were significantly more likely to require more than 12 months to conceive a pregnancy resulting in a live birth (odds ratio 2.47, 95% Confidence Interval 1.10–5.55) when compared to similar women with no history of cervical surgery [5]. While this finding suggests that LEEP is associated with reduced fertility, the underlying mechanisms responsible for this link have not yet been identified. Because LEEP is a common procedure, investigating underlying biological causes of a delayed time to conception of a viable pregnancy for women with a history of LEEP is of interest.

Anti-Müllerian hormone (AMH) is an established marker of ovarian reserve, which is tightly linked to female fertility. Therefore, measuring AMH levels before and after LEEP could determine if the procedure affects this vital component

of female fertility. AMH is a small peptide hormone within the TGF-beta family that is currently used to diagnose subfertility/infertility, primary ovarian insufficiency (POI), and polycystic ovarian syndrome (PCOS), among other disorders. In addition, AMH is now the leading predictor of in vitro fertilization (IVF) success [3, 4, 6–8]. Recent literature has demonstrated that AMH is a more reliable measure of fertility than follicle-stimulating hormone (FSH) [4, 7–11]. Normal ranges of AMH are broad and the normal range defined specifically for the AMH assay used in this study is 1–8 ng/mL [12]. For healthy women of reproductive age, an AMH level less than 1 ng/mL signifies impaired fertility due to an inadequate ovarian reserve [12]. AMH can be measured at any point in the menstrual cycle as it does not appear to fluctuate significantly throughout the cycle, unlike FSH, which must be measured on day 3 of the menstrual cycle [6–8]. Because the only cells that produce AMH in women are the granulosa cells in the ovaries, AMH is not subject to the feedback mechanisms of the hypothalamic-pituitary-gonadal axis [6, 8]. In women, AMH levels rise just prior to puberty, remain elevated throughout a woman's peak reproductive years, then significantly decline to undetectable levels in the years prior to menopause, indicative of a loss of fertility [13, 14].

Elevated levels of cytokines resulting from endometriosis, sexually transmitted infections, pelvic inflammatory disease, and other gynecologic disorders have been reported to negatively influence fertility. We hypothesize that the nonspecific inflammation and associated cytokine production resulting from LEEP may cause indirect damage to the urogenital microenvironment, thereby impairing ovarian reserve [12, 15]. Inflammatory cytokines are capable of negatively affecting ovulation, hormones required for reproduction, sperm and egg quality, and implantation [12, 15, 16]. To investigate the potential causes for the described decrease in fertility after LEEP [5], we designed a study to compare levels of the fertility marker, AMH, in women of reproductive age who had LEEP (CIN2/3) and a similar group of women who did not require LEEP (<CIN2) following colposcopy.

2. Materials and Methods

2.1. Subjects. The subjects for this analysis participated in a longitudinal study to assess the impact of LEEP on the immunologic properties of cervical mucus. Of these subjects, only a subset was used for our current study assessing the effect of LEEP on AMH levels (Supplemental Figure 1 in Supplementary materials available online at http://dx.doi.org/10.1155/2014/875438). This study recruited women of reproductive age who had underwent colposcopy at the University of Iowa from 2009-2010. Written informed consent was obtained to permit collection and analysis of demographic data, clinical data, cervical secretions and blood samples. All protocols and informed consent procedures were approved by the University of Iowa Institutional Review Board. Women eligible to participate were 18–38 years old, were not pregnant, had no prior history of cervical surgery, had regular menstrual cycles of 21–35 days, and had no history of D&C, induced abortion, cervical dysplasia, cancer, HIV/AIDS, or autoimmune disease (e.g., rheumatoid arthritis, lupus, and multiple sclerosis). Samples were not collected from women who used oral steroids within the past 2 weeks or inhaled steroids within the past 24 hours; used emergency contraception in the past 30 days; douched within the past 48 hours; engaged in vaginal intercourse within the past 48 hours; currently had a vaginal or sexually transmitted infection other than HPV; became pregnant during the study; or were currently menstruating.

A total of 63 qualifying subjects who required a LEEP procedure (cases: CIN2/3) and 49 subjects who did not require cervical surgery (controls: <CIN2) enrolled in the study. A subset of the enrolled women (19 LEEP and 28 No LEEP controls) also consented to provide blood samples at baseline and at follow-up for future analysis. Cases and controls had their blood drawn at study entry time zero (baseline), which was just prior to the L EEP procedure for cases, and again 6 months later (follow-up). Blood was collected during the same phase of the menstrual cycle for each individual woman at both time points.

For this study, we selected all 19 available cases, defined as women who were treated with LEEP after colposcopy and 19 age-matched controls, who did not receive LEEP after colposcopy. Cases and controls were age-matched within 2 years because AMH levels are known to decrease with age following peak fertility [13, 17–19]. Five of 19 cases and 2 of 19 controls were smokers at study entry and through the follow-up period. Additionally, 18/19 cases and 15/19 controls were using hormonal contraception at study entry and continued to do so throughout the duration of the study. One case-control pair was eliminated because of an approximately 300% increase in AMH levels from baseline to follow-up measurements. All other subjects had less than a 60% change in AMH levels from baseline to follow-up. Reported analyses were performed on the remaining 18 cases and 18 age-matched controls.

2.2. AMH ELISA Assay. AMH was measured in the serum of all subjects using the sensitive (LOD = 0.09 ng/mL) Gen II AMH ELISA from Beckman Coulter, Inc. (United States) at Frederick National Laboratory for Cancer Research (FNLCR) (Frederick, MD) according to the manufacture's protocol. This assay is used clinically in Europe and in the US. Excellent intra- and interplate reproducibility has been reported in the literature for this assay [20]. The assay reproducibility was confirmed in the FNLCR lab where the assay consistently performed well with intra- and interplate variability of <10%. For this study, case and control samples were randomized and run in duplicate in three separate AMH assays.

2.3. Statistical Analysis. Univariate, bivariate, and stratified analyses were conducted to assess the change in AMH levels within the LEEP and No LEEP groups over the 6-month follow-up period. Paired *t*-tests were used to compare mean AMH levels from baseline to follow-up. Analysis of variance was performed to assess for differences in the mean change in AMH levels between case and control groups. Percent change in AMH levels between baseline and follow-up was calculated for all subjects. Although the estimated percent change in AMH in a healthy population of reproductive age over

Table 1: AMH descriptive statistics.

	Baseline					Follow-up					P^a	% mean decrease (BL − FU)[c]
	N	Mean (SD)	Med.	Min.	Max.	*N*	Mean (SD)	Med.	Min.	Max.		
AMH levels: all subjects												
	36	3.56 (2.0)	3.19	0.73	7.73	36	3.07 (1.9)	2.66	0.66	8.82	0.009	13.76
AMH levels by LEEP status												
LEEP	18	3.02 (1.6)	2.81	1.05	6.55	18	2.66 (1.6)	2.19	0.82	5.76	0.07	11.92
No LEEP	18	4.09 (2.3)	3.32	0.73	7.73	18	3.48 (2.1)	3.22	0.66	8.82	0.06	14.91
P^b				0.11					0.2			

Abbreviations: AMH: anti-Müllerian hormone; LEEP: loop electrosurgical procedure.
[a]Paired *t*-test for baseline versus follow-up AMH levels (difference between the means).
[b]Analysis of variance to test for difference between mean at baseline or at follow-up for LEEP versus No LEEP.
[c]Baseline mean AMH value minus follow-up mean AMH value.
Med.: median; Min.: minimum; Max.: maximum.

Table 2: Percent change in AMH levels associated with crude and adjusted odds of LEEP, Iowa.

	LEEP *n* (%)	No LEEP *n* (%)
Continuous AMH	18 (100)	18 (100)
Percent change		
No change[a]	3 (16.7)	4 (22.2)
Increase	3 (16.7)	5 (27.8)
Decrease	12 (66.7)	9 (50.0)

Abbreviations: AMH: anti-Müllerian hormone; LEEP: loop electrosurgical excision procedure.
[a]No change is equivalent to an AMH change of ±9.9%.

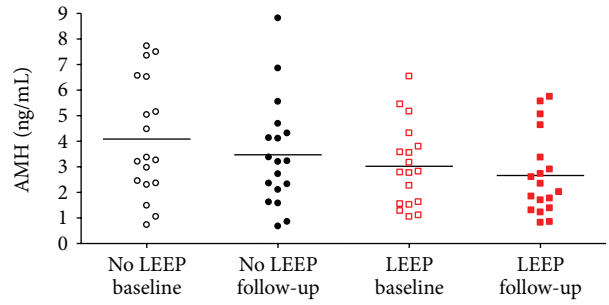

Figure 1: AMH levels at baseline and follow-up grouped by treatment status. Each data point on the graph represents a single AMH measurement. Each individual (No LEEP: $n = 18$; LEEP: $n = 18$) is represented by two data points, one at baseline and one 6 months later at follow-up. The black horizontal lines represent the mean value for each group. ○ No LEEP baseline, ● No LEEP follow-up, □ LEEP baseline, and ■ LEEP follow-up.

a 6-month period is 7.5% [21], the variability of the assay can range to almost 10%. For this reason, we defined ±9.9% change in AMH levels over the follow-up period as being equivalent to no significant percent change in AMH. All data analyses were conducted using SAS software, version 9.3 for Microsoft, SAS Institute Inc., Cary, NC, USA. Graphs were generated using GraphPad Prism 4.

3. Results and Discussion

3.1. Results. The mean AMH levels for all subjects by LEEP status are shown in Table 1. Mean levels of AMH declined significantly from baseline to the 6-month follow-up among all 36 subjects (3.56 versus 3.07 ng/mL, $P = 0.009$). When women were stratified based on LEEP status, both groups had a similar decrease in AMH, although the decrease was not statistically significant at the $P < 0.05$ level (LEEP, $P = 0.07$; No LEEP, $P = 0.06$). Women from the LEEP group had lower levels of AMH compared to the No LEEP group at both baseline (LEEP: 3.02 ng/mL versus No LEEP: 4.09 ng/mL) and follow-up (LEEP: 2.66 ng/mL versus No LEEP: 3.48 ng/mL), though these differences were not statistically significant. At the 6-month follow-up visit, two women in each group had AMH levels indicative of an impaired ovarian reserve (<1 ng/mL) (data not shown). Figure 1 shows the AMH measurements for each subject at baseline and follow-up stratified by LEEP status.

Figure 2(a) displays the baseline and follow-up AMH levels for each subject. Overall, AMH levels decreased over the 6-month follow-up period for most women. Although a reduction in AMH over time is expected, several subjects' levels decreased in excess of the 9.9% expected based on age and assay variability [21]. On average, AMH levels in LEEP and No LEEP subjects decreased similarly (−10.47% versus −9.38%, resp.) (Figure 2(b)). There was not a significant difference in the overall decrease in AMH levels in women

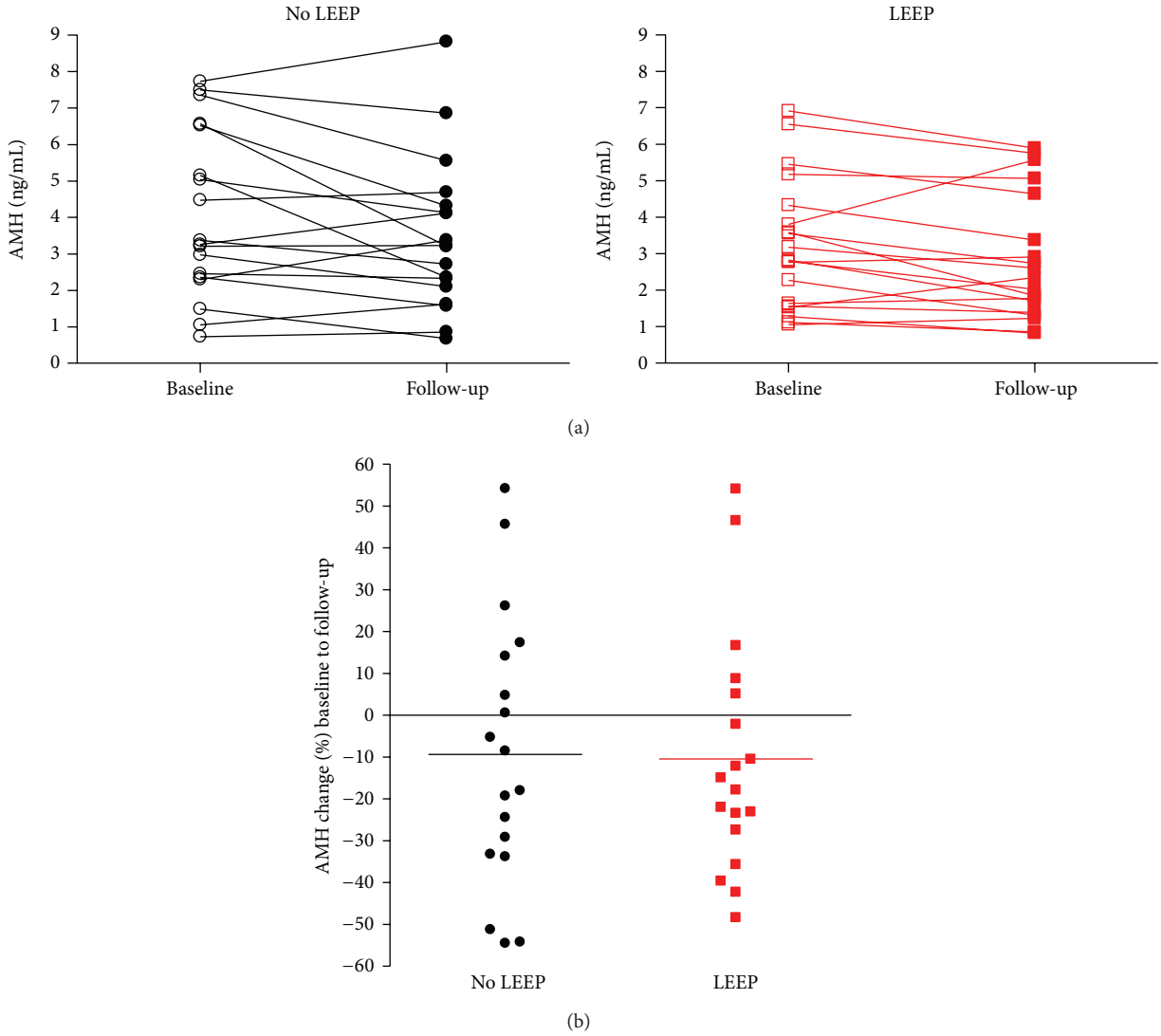

FIGURE 2: Change in AMH levels from baseline to follow-up. In (a), the two graphs, one graph for No LEEP subjects ($n = 18$) and another for LEEP subjects ($n = 18$), represent the two AMH measurements (baseline and follow-up) from each individual. Baseline and follow-up measurements are connected linearly to show the increase or decrease in AMH over the 6-month follow-up period. ○ No LEEP baseline, ● No LEEP follow-up, □ LEEP baseline, and ■ LEEP follow-up. In (b), the percent change in AMH levels between baseline and follow-up AMH measurements is plotted by treatment status for each subject. Each data point represents the percent change in AMH from baseline to follow-up for each subject, and the horizontal line represents the mean percent change for each treatment group. ● No LEEP and ■ LEEP.

who had tissue removed by LEEP versus their age-matched controls who had no tissue removed; however, more subjects from the LEEP group had a notable decrease in AMH levels (>9.9%) compared to No LEEP subjects (12 versus 9) (Table 2). Similar numbers of LEEP and No LEEP subjects had no significant change in AMH levels (< ±9.9%) (3 versus 4), though almost twice as many control subjects had an increase in AMH levels (>9.9%) when compared to case subjects (5 versus 3).

3.2. Discussion. In the first study to investigate underlying mechanisms of reduced fertility after LEEP, our findings suggest that AMH is not affected by LEEP and, therefore, another mechanism is responsible for the delayed time to pregnancy observed in women treated with LEEP. Furthermore, innate and environmental factors can cause the levels of AMH to vary broadly from woman to woman, even of the same age, so the fairly large range of AMH levels observed in our study is not surprising [17]. Over the 6-month follow-up period, the percent decrease in AMH levels in women who had LEEP versus those observed in age-matched controls was quite comparable and not statistically significant. These data suggest that LEEP does not significantly affect ovarian reserve.

An interesting observation from this small study is that the mean and median AMH levels for LEEP subjects, all of whom had been diagnosed with CIN2/3 cervical lesions, tended to be lower than those among control women who had less than CIN2 pathology and did not require LEEP. This is in agreement with our finding that women whose AMH

levels increased over the 6-month follow-up period were less likely to be in the LEEP group (CIN2/3). There may be an increased risk of cervical disease for women with lower AMH levels since mounting evidence demonstrates the anticancer effects of AMH in vitro and in vivo [13]. It is possible that AMH may play a role in cancer control in humans [13]. In recent years, AMH has been investigated as an anti-cancer agent, in addition to its better known role as a predictor of female fertility.

Because pathology reports and HPV DNA information were unavailable, further studies are needed to investigate the possibility of a direct association between HPV-associated cervical disease and AMH. Small sample size is another limitation of the current study. To address these limitations we are conducting a larger study to more directly investigate if lower AMH levels can serve as a risk factor for cervical disease.

Factors known to negatively affect AMH levels include smoking, chemotherapy, radiation, and any surgery removing or disturbing the ovaries [22–25]. It is largely reported that hormonal contraceptives do not have a significant effect on AMH levels and the levels can be measured at any time during the menstrual cycle; however, these issues are still debated in the literature.

Our study was controlled for any potential AMH flux as a result of cycling as AMH was measured during the same phase of the menstrual cycle for each subject's baseline and follow-up visits. However, AMH was not measured during the same phase of the menstrual cycle for all case and control subjects on the whole. Factors that may lead to an increase in AMH production include PCOS, granulosa cell ovarian tumors, smoking cessation, and increased sun exposure/Vitamin D levels [26–30]. It is likely that there are also unidentified intrinsic and environmental factors that affect AMH levels. These unknown factors deserve further investigation as they could be determinants for the wide ranges of AMH in women of similar age and may contribute to unexpectedly high AMH variability across the 6-month period for some subjects.

Despite the small sample size and information of yet unknown factors that affect AMH levels, this study has several strengths, namely, the careful screening process designed to exclude women with confounding factors including history of infertility, vaginal infections (other than HPV), and irregular menstrual cycles, which could bias case and control populations.

4. Conclusions

In summary, this study suggests that the recently reported increased time to pregnancy among women who have had cervical surgery is not the result of a LEEP-induced decrease in AMH, though the reason why there is delayed fertility in women treated with LEEP remains unknown [5]. Direct damage to the ovaries and ovarian reserve resulting from LEEP is doubtful. LEEP-induced endometriosis may inhibit joining of sperm and egg as would the destruction of mucus-producing cervical glands, which aid in sperm capitulation and subsequent fertilization [16, 31]. Additionally, direct or indirect physical damage to cervical tissue during LEEP could cause cervical stenosis, impair embryonic implantation, or result in an unfavorable microenvironment for pregnancy [15, 16, 32, 33]. Because half a million women of reproductive age are treated with LEEP each year in the United States alone, future research is needed to investigate the underlying causes for delayed time to pregnancy with LEEP [5].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Expression of Neuroendocrine Markers in Different Molecular Subtypes of Breast Carcinoma

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Background. Carcinomas of the breast with neuroendocrine features are incorporated in the World Health Organization classification since 2003 and include well-differentiated neuroendocrine tumors, poorly differentiated neuroendocrine carcinomas/small cell carcinomas, and invasive breast carcinomas with neuroendocrine differentiation. Neuroendocrine differentiation is known to be more common in certain low-grade histologic special types and has been shown to mainly cluster to the molecular (intrinsic) luminal A subtype. *Methods*. We analyzed the frequency of neuroendocrine differentiation in different molecular subtypes of breast carcinomas of no histologic special type using immunohistochemical stains with specific neuroendocrine markers (chromogranin A and synaptophysin). *Results*. We found neuroendocrine differentiation in 20% of luminal B-like carcinomas using current WHO criteria (at least 50% of tumor cells positive for synaptophysin or chromogranin A). In contrast, no neuroendocrine differentiation was seen in luminal A-like, HER2 amplified and triple-negative carcinomas. Breast carcinomas with neuroendocrine differentiation presented with advanced stage disease and showed aggressive behavior. *Conclusions*. We conclude that neuroendocrine differentiation is more common than assumed in poorly differentiated luminal B-like carcinomas. Use of specific neuroendocrine markers is thus encouraged in this subtype to enhance detection of neuroendocrine differentiation and hence characterize the biological and therapeutic relevance of this finding in future studies.

1. Introduction

Breast carcinoma is the most common cancer of women and generally exhibits a favorable overall 5-year prognosis [1]. However it is becoming increasingly evident that carcinoma of the breast represents a heterogeneous disease with different prognostic subtypes. Invasive mammary carcinomas with neuroendocrine differentiation were first described in 1963 [2] and recognized as a subtype of mammary carcinoma and included in the World Health Organization (WHO) classification in 2003 [3]. According to the current WHO they are defined as carcinomas exhibiting expression of synaptophysin and/or chromogranin A with the exception of small cell carcinoma which is defined morphologically and usually only exhibits limited or less frequent expression of specific neuroendocrine markers but expresses NSE more frequently [3]. The current WHO classification of tumors subdivides carcinomas of the breast with neuroendocrine features into well-differentiated neuroendocrine tumors (NET), poorly differentiated neuroendocrine carcinomas/small cell carcinomas, and invasive breast carcinomas with neuroendocrine differentiation, stating an overall incidence of <1% of all breast carcinomas [3]. However, the true incidence of mammary neuroendocrine neoplasms is difficult to assess because neuroendocrine markers are not routinely used in the diagnostic immunohistochemical panel of breast cancer. It is assumed that neuroendocrine differentiation can be detected more frequently in invasive carcinoma of no special type (NST) and certain morphological special types, particularly cellular invasive mucinous carcinoma and solid papillary carcinoma [3].

Apart from the general histological classification of invasive breast carcinomas, Perou et al. defined molecular subtypes: luminal A, luminal B, HER2 enriched, and basal-like carcinomas using DNA microarray technology in a set of invasive breast carcinomas of no histologic special type [4]. This classification has since then been shown to be of prognostic [5] and predictive [6] value with luminal A carcinomas showing the best prognosis. It is now known that some histologic special types of invasive breast carcinoma cluster to mainly one molecular subtype: Weigelt et al. showed that cellular invasive mucinous carcinoma and neuroendocrine carcinomas mostly represent the luminal A molecular subtype [7].

In a case-control study Wei et al. described a worse prognosis of breast carcinomas with neuroendocrine differentiation when compared to carcinomas of no histologic special type [8]. We recently encountered several metastatic breast carcinomas which exhibited diffuse neuroendocrine differentiation associated with high-grade histologic features (falling short of criteria used to diagnose small cell carcinoma of the breast [9]) but at the same time retaining estrogen receptor expression. These data can result in considerable confusion regarding treatment options and prognostic impact when clinicians are confronted with the diagnosis of invasive breast cancer with neuroendocrine differentiation. In particular, it is still unclear whether to treat poorly differentiated breast carcinoma with neuroendocrine differentiation analogous to poorly differentiated invasive breast cancer or similar to poorly differentiated neuroendocrine carcinomas of other sites. The aim of the current study is to examine the frequency and extent of neuroendocrine differentiation in immunohistochemically characterized different molecular subtypes of breast carcinomas using a wide array of immunohistochemical markers.

2. Material and Methods

Invasive breast carcinomas of different molecular subtypes diagnosed between 2002 and 2009 at the Institute of Pathology of the University Hospital Erlangen, Germany, were retrieved from routine surgical pathology files by a computer assisted search. Molecular subtypes were determined using immunohistochemical surrogate markers, which were stained during routine surgical pathology practice. Luminal carcinomas were defined by nuclear estrogen receptor expression in more than 10% of tumor cells. Furthermore luminal carcinomas were divided in luminal A and luminal B carcinomas using a Ki67 index cut-off value of 13% as proposed by Cheang et al. [10] To enhance segregation of luminal A and B carcinomas for the purpose of this study, we included in the luminal A group only grade 1 carcinomas and in the luminal B group only grade 3 carcinomas. Since differentiation of luminal A and luminal B carcinomas is not entirely reliable on morphologic and immunohistochemical grounds, we use the terms luminal A-like and luminal B-like in this study. The HER2 amplified group was defined by lacking expression of the estrogen and progesterone receptors and strong circular membranous staining for HER2 in more than 10% of tumor cells (DAKO score 3+). The basal-like subtype was defined as triple negative.

Using these criteria, we arbitrarily selected 30 luminal A-like carcinomas (including 10 cases of invasive lobular carcinomas, ILC), 20 luminal B-like carcinomas, 20 HER2 amplified carcinomas and 30 basal-like carcinomas without further knowledge of the histological features of the carcinomas (apart from the ILC no other histologic special types were included). The different study groups were filled consecutively from a large cohort of breast carcinomas at our institution until at least 20 unselected cases were present in every subgroup. To identify possible precursor lesions of invasive neuroendocrine carcinoma, we also included 10 cases of low-grade and 10 cases of high-grade ductal carcinoma in situ (DCIS) and 80 normal breast tissue specimens. From these cases a tissue microarray (TMA) was constructed: representative areas of the lesions (or the normal tissue) were marked on the glass slides and a tissue core with a diameter of 2 mm was punched out of the donor block and transferred onto the recipient block. The recipient block was then cut (3 μm) and the sections mounted on SuperFrost slides (Menzel Gläser, Braunschweig, Germany). All slides were stained with haematoxylin and eosin (H&E).

To determine neuroendocrine differentiation we used antibodies against chromogranin A (monoclonal mouse anti-human antibody, clone LK2H10, Beckman Coulter Inc., Diagnostics Division Headquarters, 250 South Kraemer Boulevard, Brea CA 92821-6232, USA, dilution 1 : 500) and synaptophysin (monoclonal mouse anti-human antibody, clone Snp88, Biogenex Laboratories Inc., 4600 Norris Canyon Road, San Ramon, CA 94583, USA, dilution 1 : 50). Further, we assessed the expression of some other non-specific markers known to be frequently expressed in neuroendocrine neoplasms including CD56 (monoclonal mouse anti-human antibody, clone 1B6, Novocastra Laboratories Inc., Balliol Business Park West, Benton Lane, NE12 8EW Newcastle Upon Tyne, UK, dilution 1 : 50), CD117 (polyclonal rabbit anti-human, code A4502, Dako Denmark, Produktionsvej 42, DK-2600 Glostrup Denmark, dilution 1 : 100) and NSE (monoclonal mouse anti-human antibody, clone BBS/NC/VI-H14, Dako, dilution 1 : 300). Immunohistochemical stainings were performed on 1 μm slides using the fully automated slide preparation system "Benchmark XT System" (Ventana Medical Systems Inc., 1910 Innovation Park Drive, Tucson, Arizona, USA). H&E and immunohistochemical stainings were evaluated by two of the authors (DLW, AA) and the extent of positive staining (%) and staining intensity (negative, weak, moderate, and strong) were noted. Only moderate to strong immunohistochemical staining was considered positive. Unfortunately the WHO does not provide a clear cut-off to define neuroendocrine differentiation in breast carcinomas. Since the WHO cites a study by Sapino et al. who used a cut-off of 50% [11] and Wei et al. confirmed prognostic impact of neuroendocrine differentiation using the same cut-off, we also regarded a minimum of 50% of tumor cells with moderate to strong expression of chromogranin A or synaptophysin as indicative of neuroendocrine differentiation. After identification of the carcinomas with neuroendocrine

Table 1: Expression of specific (chromogranin A and synaptophysin) and non-specific (CD56, CD117, NSE) neuroendocrine markers in different molecular subtypes of breast carcinoma, DCIS, and normal tissue.

Molecular subtype	Chromogranin A	Synaptophysin	CD56	CD117	NSE
Luminal A					
Focal (<50%)	0/25 (0%)	1/24 (4.2%)	1/24 (4.2%)	0/22 (0%)	1/23 (4.3%)
Diffuse (>50%)	0/25 (0%)	0/24 (0%)	3/24 (12.5%)	1/22 (4.5%)	17/23 (73.9%)
Luminal B					
Focal (<50%)	1/18 (5.6%)	3/18 (16.7%)	0/18 (0%)	1/18 (5.6%)	6/17 (35.3%)
Diffuse (>50%)	**2/18 (11.1%)**	**3/18 (16.7%)**	0/18 (0%)	1/18 (5.6%)	6/17 (35.3%)
HER2					
Focal (<50%)	1/15 (6.7%)	2/15 (13.3%)	0/14 (0%)	0/15 (0%)	4/15 (26.7%)
Diffuse (>50%)	0/15 (0%)	0/15 (0%)	0/14 (0%)	2/15 (13.3%)	2/15 (13.3%)
Basal-like					
Focal (<50%)	0/29 (0%)	1/29 (3.4%)	4/29 (13.8%)	0/29 (0%)	0/29 (0%)
Diffuse (>50%)	0/29 (0%)	0/29 (0%)	4/29 (13.8%)	12/29 (41.4%)	13/29 (44.8%)
High-grade DCIS					
Focal (<50%)	1/10 (10%)	2/10 (20%)	1/10 (10%)	2/10 (20%)	3/10 (30%)
Diffuse (>50%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	3/10 (30%)
Low-grade DCIS					
Focal (<50%)	0/6 (0%)	0/6 (0%)	0/7 (0%)	1/5 (20%)	1/5 (20%)
Diffuse (>50%)	0/6 (0%)	0/6 (0%)	0/7 (0%)	0/5 (0%)	4/5 (80%)
Normal tissue					
Focal (<50%)	0/69 (0%)	5/69 (7.2%)	44/62 (70.9%)	10/67 (14.9%)	25/64 (39.1%)
Diffuse (>50%)	0/69 (0%)	0/69 (0%)	1/62 (1.6%)	55/67 (82.1%)	25/64 (39.1%)

differentiation using the TMA slides the whole sections of these cases were examined for assessment of morphological features of the carcinomas and the adjacent tissue.

3. Results (Table 1)

3.1. Normal Breast Tissue. Of the 69 evaluable normal tissue cores none showed chromogranin A positive cells and only 5 (7.2%) cases revealed moderate to strong apical expression of synaptophysin in isolated mostly luminal cells (Figure 1(a)). Some of these luminal cells showed probable apocrine differentiation (Figure 1(b)). One case showed a single positive basally located cell (Figure 1(b) inset), possibly representing a true neuroendocrine cell, but no chromogranin A expression was seen. Regarding the non-specific neuroendocrine markers, 44 of 62 normal tissues (70.9%) showed moderate to strong expression of CD56 in up to 70% (usually around 30%) of luminal cells (Figure 1(c)) and the vast majority of the normal breast tissues (55 of 67; 82.1%) showed moderate to strong diffuse expression of CD117 in the luminal cells (usually more than 80% of luminal cells, Figure 1(d)). 25 of the 64 (39.1%) normal tissues showed positivity for NSE in the luminal cells.

3.2. Low-Grade DCIS. All of the 6 available sections were negative for chromogranin A, synaptophysin and all of 7 available cases were negative for CD56. One of 5 samples showed focal expression of CD117 and 4 of 5 cases revealed diffuse expression of NSE.

3.3. High-Grade DCIS. One of 10 cases showed strong but only focal expression of chromogranin A (Figure 2(a)) and 2 of 10 cases revealed focal expression of synaptophysin (Figure 2(a) inset). One of 10 cases showed moderate focal (<10% of tumor cells) expression of CD56 (Figure 2(b)) and 1 of 10 cases showed moderate diffuse (>80%) expression of CD117 (Figure 2(c)) with another 2 cases showing moderate focal (<10%) expression of CD117. 3 of 10 cases showed diffuse moderate to strong expression of NSE and another 3 of 10 cases showed moderate to strong focal expression of NSE.

3.4. Luminal A-Like Carcinomas. Chromogranin A was negative in all of the 25 cases (including 9 invasive lobular carcinomas). Synaptophysin was focally expressed in 1/24 carcinomas, notably this positive case was an ILC with moderate staining in <10% of tumor cells. CD56 was diffusely (>70% of tumor cells) expressed in 3/24 (12.5%) cases, including 2 of 8 (25%) ILC. One additional case showed focal strong expression in 30% of tumor cells. CD117 was diffusely positive in 1 of 22 (4.5%) carcinomas. This case was an ILC (1/7, 14.3%) with moderate staining in more than 80% of tumor cells. NSE was diffusely expressed in 17 of 23 (73.9%) of cases with moderate to strong staining in at least 60% of the tumor cells and moderate focal expression in <10% of tumor cells in 1 case. According to WHO criteria, no carcinomas with neuroendocrine differentiation were found.

3.5. Luminal B-Like Carcinomas. Of 18 stained cases 2 (11.1%) showed moderate to strong diffuse expression of

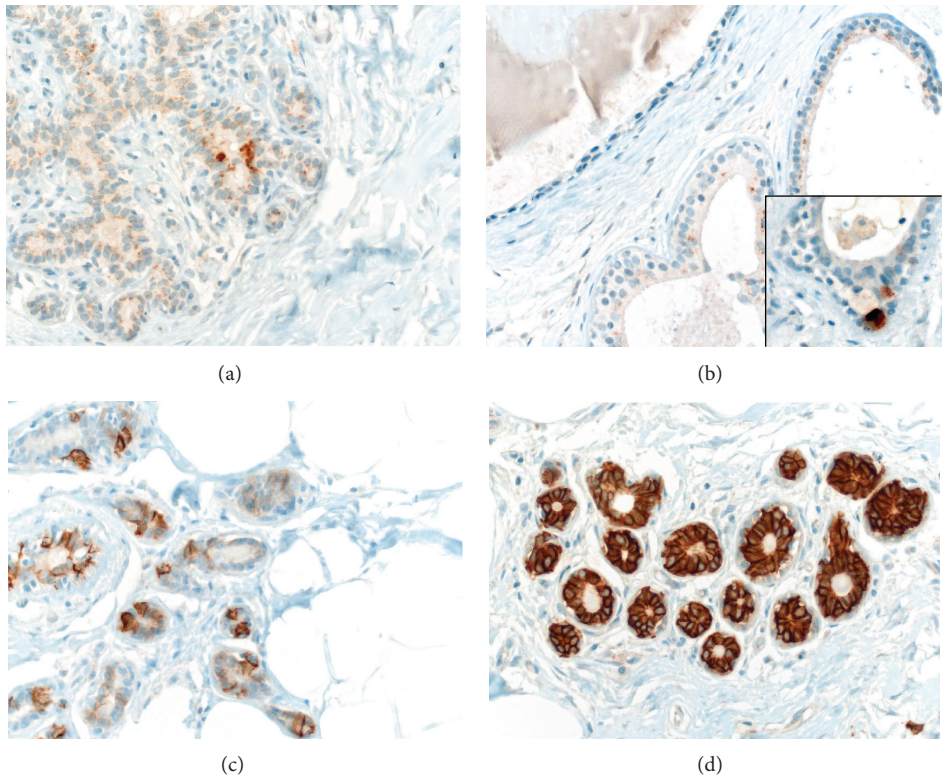

Figure 1: Expression of specific (synaptophysin & chromogranin A) and non-specific (CD56 & CD117) neuroendocrine markers in normal breast tissue. (a) Expression of synaptophysin in luminal cells of normal breast tissue. (b) Synaptophysin expression in probably apocrine differentiated luminal cells (inset: strongly synaptophysin positive basal cell, consistent with a true neuroendocrine cell; however, no chromogranin A expression was seen in this area). (c) CD56 expression in a subset of normal luminal cells. (d) CD117 expression in the majority of luminal cells in normal breast tissue.

chromogranin A in over 80% of tumor cells (Figure 2(d)). One additional case showed focal moderate granular cytoplasmic expression in 20% of tumor cells. One of the chromogranin A positive tumors was negative for synaptophysin (Figure 2(e)). 3 cases (16.7%) showed diffuse staining (one case with staining in 70% of tumor cells, the other two with staining in more than 90% of tumor cells) for synaptophysin. Another 3 of 18 cases showed moderate to strong but only focal staining. One of the diffusely synaptophysin positive tumors was negative for chromogranin A. According to WHO criteria, neuroendocrine differentiation was found in 4 cases (22.2%).

All of the 18 tested cases were negative for CD56. One of 18 (5.6%) cases showed strong diffuse CD117 staining in >80% of tumor cells and another one revealed moderate focal CD117 staining in <10% of tumor cells. Of 17 tested carcinomas, 6 showed moderate to strong diffuse NSE positivity in more than 80% of tumor cells, another 6 cases showed only focal expression in less than 50% of tumor cells.

3.6. HER2 Amplified Carcinomas. One of 15 stained HER2 amplified carcinomas showed strong focal chromogranin A expression in only <1% of tumor cells and two of the 15 stained cases showed moderate to strong focal synaptophysin staining in <1% and <10% of tumor cells respectively. All of the 14 tested carcinomas were negative for CD56 and 2 of 15 cases (13.3%) showed moderate diffuse CD117 expression in >80% of tumor cells. 2 of 15 (13.3%) cases showed diffuse moderate NSE expression and another 4 cases only showed focal expression. No carcinomas with specific neuroendocrine differentiation were found.

3.7. Basal-Like Carcinomas. All of the 29 tested carcinomas were negative for chromogranin A and one of the 29 cases stained moderately and focally (<10% of tumor cells) for synaptophysin. 4/29 (13.8%) cases showed moderate to strong diffuse CD56 expression in >80% of tumor cells (Figure 2(f)) and another 4 cases showed moderate to strong focal expression in less than 50% of tumor cells. 12 of the 29 (41.4%) carcinomas showed moderate to strong diffuse expression of CD117 in >80% of tumor cells. 13 of 29 (44.8%) cases revealed moderate to strong diffuse NSE expression in >80% of tumor cells. No carcinomas with specific neuroendocrine differentiation were found.

Taken together, 4 of 18 (22.2%) stained cases of luminal B-like carcinomas exhibited neuroendocrine differentiation using current WHO criteria. The luminal A-like, HER2 amplified and basal-like carcinoma subgroups as well as all the DCIS cases did not reveal diffuse neuroendocrine

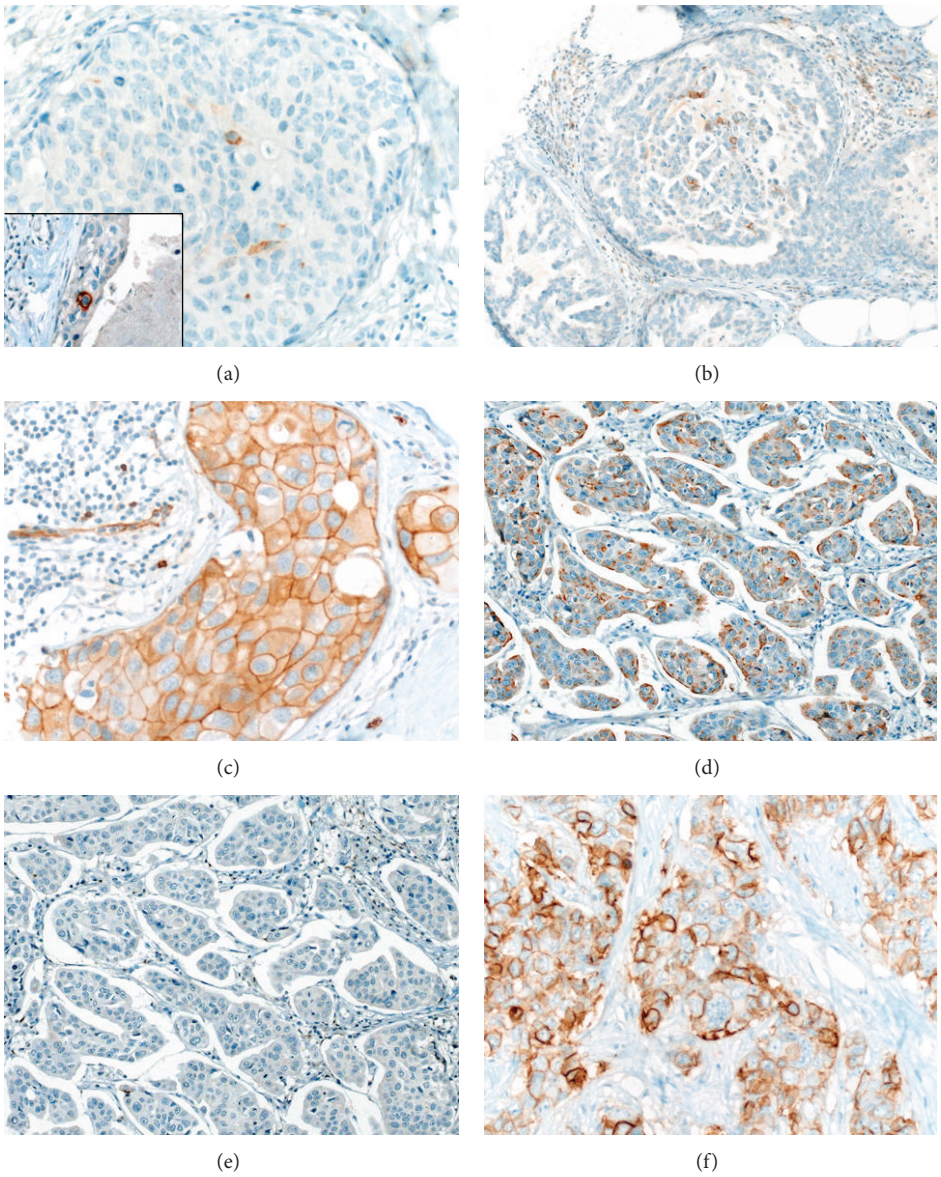

Figure 2: Expression of specific (synaptophysin and chromogranin A) and non-specific (CD56, CD117) neuroendocrine markers in DCIS and invasive carcinoma. (a) Isolated chromogranin A positive cells in high-grade DCIS (inset: isolated synaptophysin positive cells in the same case). (b) High-grade DCIS with isolated CD56 positive cells. (c) High-grade DCIS diffusely expressing CD117. (d) Luminal B carcinoma NST diffusely expressing chromogranin A. (e) The same luminal B carcinoma is negative for synaptophysin. (f) Diffuse expression of CD56 in a triple-negative carcinoma.

differentiation. No clear-cut neuroendocrine cell progenitor lesion was seen in the normal tissues.

3.8. Clinicopathological Features of Breast Carcinomas with Neuroendocrine Differentiation (Table 2). Three of the cases affected patients around 80 years of age and showed lymphovascular invasion and/or lymph node metastases at presentation. The fourth case without lymph node metastases presumably originated in ectopic axillary mammary tissue and relapsed locally after 2 years. Two of the four patients died of disease after 15 and 36 months, respectively; the other two were lost to follow-up.

Morphologically the four carcinomas revealed a wide spectrum of architectural and cytological features: the first case showed solid (insular) infiltrates of medium-sized tumor cells with bright cytoplasm, coarse nuclear chromatin, and multiple prominent nucleoli (Figure 3(a)) and in other areas pseudocribriform DCIS-like structures (Figure 3(b)). Another case was composed of solidly arranged medium-sized tumor cells with sometimes plasmacytoid morphology and perinuclear condensation of eosinophilic cytoplasm. The nuclei displayed fine chromatin and prominent nucleoli. At the periphery of the small insular solid structures some of the tumor cells showed subnuclear eosinophilic cytoplasmic

Table 2: Clinicopathologic features at presentation and follow-up data.

Case	Age	Tumor size (mm)	Multifocality (number of tumors)	Lymphovascular invasion	Metastases	Follow-up
1	59	30	No	No	Bone	Local relapse after 2 years; died of disease after 36 months
2	77	35; 5	Yes (2)	Yes	Axillary lymph nodes	Disease free after 3 years and then lost to follow-up
3	83	27	No	No	Axillary lymph nodes	Disease free after 3 months and then lost to follow-up
4	85	35; 7; 5	Yes (3)	Yes	Axillary lymph nodes	Skin metastases after 4 months; died of disease after 15 months

granules (Figure 3(c)). Of note small areas comprising <10% of the tumor area showed extracellular mucin pools (Figure 3(d)). The third case showed a solid trabecular growth pattern with isolated scattered tumor cells at the infiltrative border (Figure 3(e)). Focally poorly formed small rosettes were seen (Figure 3(f)). In one slide a minute focus of high-grade DCIS (solid type with necrosis) was appreciated but was not available on deeper sections for immunohistochemical stainings. The fourth case revealed a carcinoma composed of medium-sized tumor cells with eosinophilic cytoplasm and vesicular nuclei with fine chromatin and prominent nuclei. The tumor cells grew in small solid formations with prominent shrinking artifacts imparting an invasive micropapillary-like pattern (Figure 3(g)). Perifocal high-grade DCIS with solid, cribriform and micropapillary architecture, and central necrosis was seen. Immunohistochemically >80% of the invasive tumor cells showed moderate granular chromogranin A expression and showed moderate membranous expression of CD117, whereas synaptophysin, NSE, and CD56 were negative. Only <10% of the cells of the high-grade DCIS showed moderate granular chromogranin A expression (Figure 3(h)) and >80% of the DCIS cells membraneously expressed CD117.

The adjacent normal tissue in all of these cases showed no signs (morphologically or immunohistochemically) of neuroendocrine cell hyperplasia.

4. Discussion

In our experience, tumors resembling NET of the gastrointestinal tract or small cell carcinomas of the breast are extremely rare. On the other hand, identifying carcinomas with neuroendocrine differentiation lacking the features of NET, small cell carcinoma, cellular mucinous carcinoma, or solid papillary carcinoma using morphology alone can be very challenging or even impossible. We have recently encountered several cases of aggressive metastatic breast cancers, which showed features of neuroendocrine differentiation with preserved nuclear estrogen receptor expression. These cases were not recognized as having neuroendocrine differentiation in the primary breast tumors during routine workup. The lacking histomorphological features of neuroendocrine differentiation in many such cases and the lack of standardized immunohistochemical testing for neuroendocrine markers in invasive breast carcinomas suggest underestimation of neuroendocrine differentiation in invasive breast carcinomas, especially in tumors of the molecular luminal B subgroup.

Given that the neuroendocrine differentiation often cannot be diagnosed on morphologic grounds alone, routine testing for neuroendocrine markers should be considered in the future. In this context, it is of note that two of the four carcinomas with neuroendocrine differentiation in the present study only expressed chromogranin A or synaptophysin and not both of these markers, arguing for a routine immunohistochemical panel including both chromogranin A and synaptophysin.

Additionally our findings show that CD56, CD117, and NSE, although relatively sensitive in neuroendocrine neoplasms at other sites, should not be used as markers of neuroendocrine differentiation in the breast as they have a very low specificity and are also expressed in a high frequency in normal breast tissue. Interestingly CD56 is expressed in a considerable subset of luminal A-like and basal-like carcinomas although the significance (if any) of this finding remains unknown. CD117 was expressed in about 40% of the basal-like carcinomas, in around 13% of HER2 amplified carcinomas and in 5% of luminal carcinomas making this marker of limited or no value in segregating neuroendocrine subtypes of breast carcinoma.

In the present study, we were not able to identify clear-cut neuroendocrine cells in normal breast tissue adjacent to carcinomas with neuroendocrine differentiation and tissues unrelated to these cases. Recently, Kawasaki and colleagues demonstrated isolated and increased (hyperplastic) neuroendocrine cells in normal appearing breast tissue adjacent to DCIS with neuroendocrine differentiation [12]. In the 3 reported cases, the DCIS showed diffuse neuroendocrine differentiation and the patients were 28 to 38 years old. The authors suggested that the DCIS arose from these hyperplastic neuroendocrine cells. If true, this points to at least two differing pathways of development of neuroendocrine

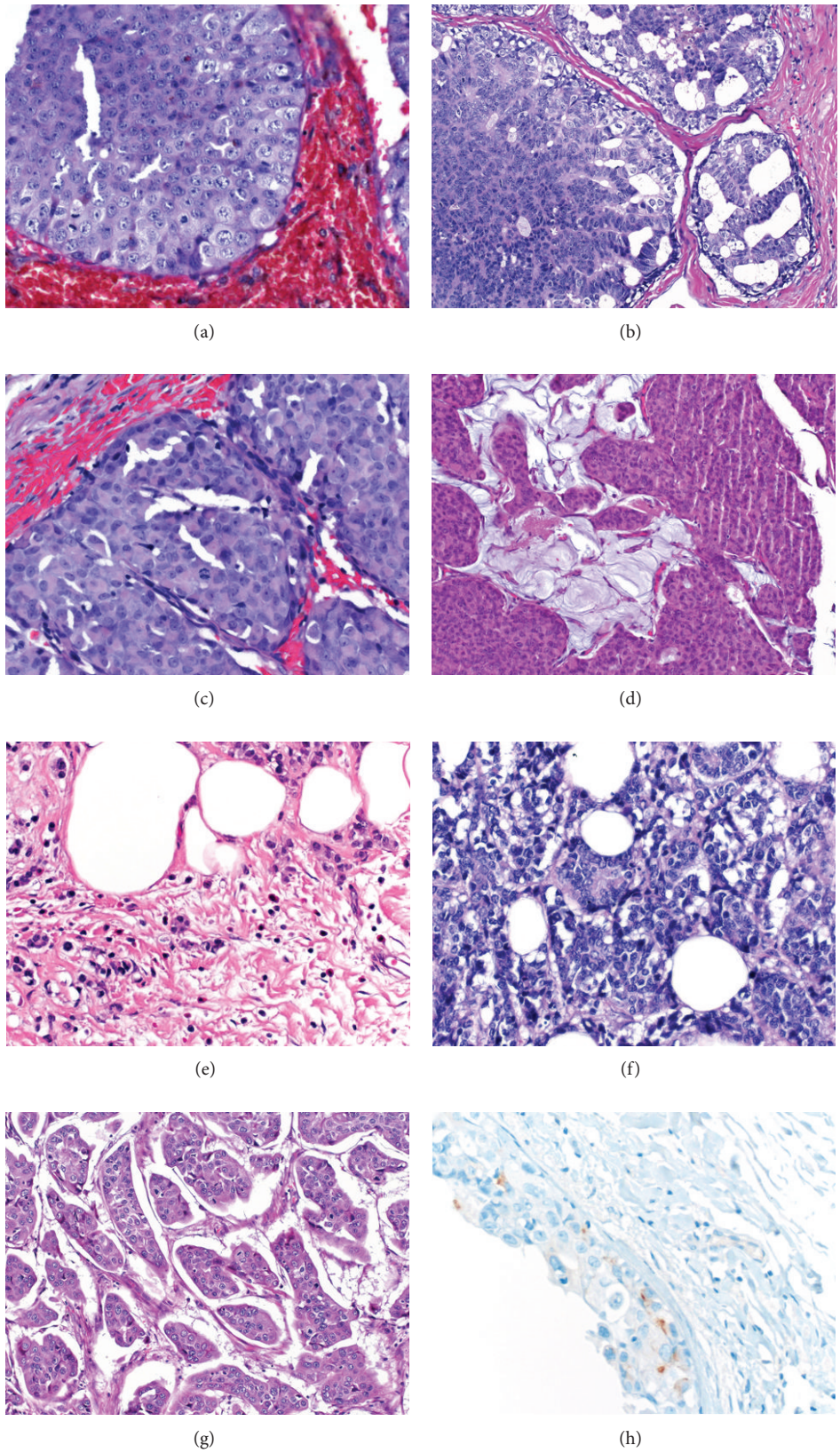

Figure 3: Morphological features of luminal B carcinomas with neuroendocrine differentiation. (a) Solid (insular) formations of tumor cells. (b) Cribriform, DCIS-like areas of the same case. (c) Solid infiltrates of plasmacytoid tumor cells with subnuclear eosinophilic cytoplasm. (d) Same case with focal mucinous differentiation. (e) Infiltrative border of a carcinoma with scattered tumor cells. (f) The same case with focal rosette-like structures. (g) Prominent peritumoral clefts, imparting a micropapillary-like morphology, (h) The same case with perifocal high-grade DCIS with only focal expression of chromogranin A.

neoplasia in the breast. In the first model, neoplasia could develop in a hyperplasia-neoplasia sequence similar to some types of pulmonary and gastrointestinal counterparts [12]. In the second model, neuroendocrine differentiation could represent a secondary phenomenon by tumor cell evolution through ongoing mutations and subsequent clonal overgrowth of the neuroendocrine tumor cells in established carcinoma [13, 14]. Given that the accompanying DCIS of the luminal B-like carcinomas NST with neuroendocrine differentiation in this study only showed focal neuroendocrine differentiation and the adjacent normal tissue did not show neuroendocrine cell hyperplasia, we hypothesize that in these cases the neuroendocrine cell population developed secondarily. The large size of the tumors, lack of progenitor lesions, and sometimes patchy staining pattern of neuroendocrine markers in whole-section slides support this hypothesis. This mode of development would seem analogous to the well-studied mixed-adenoneuroendocrine carcinomas (MANEC) of the gastrointestinal tract [15] and other sites which also show only partial neuroendocrine differentiation, potentially posing diagnostic difficulties in limited biopsy material. However molecular data regarding these types of tumors of the breast are lacking.

Confrontation with a diagnosis of invasive breast cancer with neuroendocrine differentiation often results in confusion of the clinician and the patient regarding prognosis or therapeutic options. This underlines the need to specify the subtype and grade of neuroendocrine tumor as accurately as possible. As cellular invasive mucinous carcinomas (with low-grade morphology) clusters in the luminal A molecular subgroup [7] and solid papillary carcinoma is considered to mostly represent an intraductal carcinoma with possible associated invasive carcinoma [16], these tumors obviously represent low-grade carcinomas with a very good prognosis. On the other hand small cell carcinoma of the breast is a neuroendocrine carcinoma of high-grade, although in a recent study it has been shown to be less aggressive than, for example, small cell carcinoma of the lung [9]. Carcinomas NST (as in our study) with neuroendocrine differentiation probably represent the most frequent invasive breast carcinomas with neuroendocrine differentiation. In a recent study Wei and colleagues showed that if WHO 2003 criteria are applied (leaving out solid papillary carcinoma and only including 3 cellular invasive mucinous carcinomas) and cases of carcinomas with neuroendocrine differentiation are matched with control patients (carcinomas NST without neuroendocrine differentiation), the carcinomas with neuroendocrine differentiation carry a worse prognosis than those without [8].

As the vast majority of carcinomas with neuroendocrine differentiation seem to cluster in the intrinsic luminal subgroups with preserved nuclear estrogen receptor expression antihormonal treatment is a reasonable treatment option. In the present study all of the carcinomas with neuroendocrine differentiation were found in the luminal B-like subgroup. These carcinomas have been shown to be more aggressive with poorer prognosis and better response to chemotherapy compared to luminal A carcinomas so that chemotherapy is to be considered in the appropriate clinical setting. However, although modern prognostic and predictive biomarkers are emerging [17], it is known that currently only a subgroup of patients benefits from chemotherapy and that response to therapy cannot be reliably predicted in individual patients [18]. Additional biomarkers for prediction are therefore necessary and diagnosis of neuroendocrine differentiation might become a therapeutic option in the future. Thus, larger studies are needed to assess the predictive value of routine testing for neuroendocrine differentiation in breast carcinomas. In the future even special targeted therapies might be an option for this patient subgroup as isolated case reports have described promising results using octreotide in this setting [19].

In conclusion, we showed that neuroendocrine (trans)-differentiation is a frequent event in luminal B-like breast carcinomas. The routine search for neuroendocrine differentiation in invasive breast carcinoma apart from cellular invasive mucinous carcinoma, solid papillary carcinoma, and small cell carcinoma should focus on luminal B-like carcinomas in order to understand the significance of this phenomenon in the future. As these carcinomas often present with advanced stage disease, studies exploiting the neuroendocrine differentiation with targeted therapies might be of benefit.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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