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1 **Relevance of Circulating Tumor Cells, Extracellular Nucleic Acids and Exosomes in**  
2 **Breast Cancer**

3

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5

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**22 Abstract**

23 Early detection of cancer is vital to improved overall survival rates. At present, evidence is  
24 accumulating for the clinical value of detecting occult tumor cells in peripheral blood, plasma  
25 and serum specimens from cancer patients. Both molecular and cellular approaches, which  
26 differ in sensitivity and specificity, have been used for such means. Circulating tumor cells  
27 and extracellular nucleic acids have been detected within blood, plasma and sera of cancer  
28 patients. As the presence of malignant tumors are clinically determined and/or confirmed  
29 upon biopsy procurement -which in itself may have detrimental effects in terms of  
30 stimulating cancer progression/metastases- minimally-invasive methods would be highly  
31 advantageous to the diagnosis and prognosis of breast cancer and the subsequent tailoring of  
32 targeted treatments for individuals, if reliable panels of biomarkers suitable for such an  
33 approach exist. Herein, we review the current advances made in the detection of such  
34 circulating tumor cells and nucleic acids, with particular emphasis on extracellular nucleic  
35 acids, specifically extracellular mRNAs and discuss their clinical relevance.

36

**37 Keywords**

38 Breast cancer, extracellular nucleic acids, circulating tumor cells, exosomes, cancer stem  
39 cells

40

41

## 42 **1.0 Introduction**

43 Breast cancer is the second most frequently diagnosed cancer after skin cancer in the US,  
44 with only lung cancer responsible for more female cancer-related deaths [1]. In recent times  
45 gene expression studies and immunohistochemical analyzes have classified breast cancer into  
46 five major subtypes depending on the presence or absence of hormone receptor expression  
47 and certain other markers.

48  
49 Perou and colleagues' [2] intrinsic classification defines these subtypes as basal-like breast  
50 cancers which largely correspond to estrogen receptor (ER) negative, progesterone receptor  
51 (PR) negative and HER2 negative tumors (ie triple negative); luminal A (mostly ER positive  
52 and low-grade histologically); luminal B (also mostly ER positive, can express low levels of  
53 hormone receptors and often are high grade); HER2 (also referred to as ErbB2) positive  
54 (show amplification and high expression of Her2 gene) and triple negative tumors. Some  
55 basal-like cancers have high expression of cytokeratin (CK) 5, but as of yet,  
56 immunohistochemical methods of defining basal-like cancers are not well established due to  
57 logistical difficulties of performing many IHC markers in a clinical setting [3]. A final  
58 subtype is defined as normal breast-like tumors.

59

## 60 **1.1 Breast cancer recurrence**

61 The majority of breast cancer deaths are as a result of recurrent metastatic disease. Metastatic  
62 disease occurs as a result of a series of steps involving multiple host-tumor interactions [4]  
63 (reviewed by Gibley *et al.*, [5]). Primary tumor cells, whether they possess genetic mutations  
64 or have certain stem-like properties, grow aberrantly and may eventually detach, intravasate  
65 into the lymphatic and/or blood systems, before developing into secondary disease [4]. In  
66 breast cancer when tumor cells enter the lymphatic system, they travel to the sentinel nodes in  
67 the axilla and intercostal spaces before entering the bloodstream and subsequent progression  
68 to other organs [4]. In addition, tumor cells can disseminate directly through the blood to  
69 distant organs [6]. The detection and characterization of such cells would be an advantage in  
70 the identification of patients who are at risk of disease recurrence and the subsequent tailoring  
71 of individualized treatments.

72

## 73 **1.2 The need and settings for Breast Cancer Biomarkers**

74 Breast cancer diagnosis is often merely confirmatory when a growth/lump is detected in the  
75 breast by the patient. Although mammography has greatly aided diagnosis for some patients

76 (unfortunately it is dependent on mammography availability and also on the individual's  
77 age/breast tissue density, etc.), currently breast cancer is typically diagnosed on tissue biopsy.  
78 Individuals referred to a breast clinic (generally by their primary care physician) include  
79 those who will be diagnosed with breast cancer and those with a benign/harmless lump *e.g.*  
80 fibroma. However, due to the lack of a blood/serum diagnostic test, biopsies must be assessed  
81 for all referred individuals. This often results in unnecessary delays for those with breast  
82 cancer, who need prioritising; unnecessary worry for those who have a benign condition; and  
83 an overall strain on clinics and health care systems. As outlined above, gene expression  
84 analysis of tumor tissue has enabled breast cancer sub-classification into 5 major sub-types.  
85 This information is relevant, as treatment options (*e.g.* hormone, Trastuzumab,  
86 chemotherapy) depends on the tumor sub-type. However, panels of biomarkers are urgently  
87 needed for earliest possible detection of breast cancer, for prognosing outcome and for  
88 predicting response to therapy, so as to determine those who require treatment versus over-  
89 treating those who do not, and to ensure that those who would benefit from neo-  
90 adjuvant/adjuvant therapy receive the treatment to which they are most likely to respond.  
91 Undoubtedly, minimally-invasive blood/serum tests that could aid in breast diagnosis (to  
92 triage individuals) and in tumor sub-typing (ensure optimal treatment selection) would  
93 contribute to timely, personalized management of those referred to the breast clinic;  
94 ultimately contributing to improved quality of life and overall survival.

95 **No single biological biomarker can accurately detect breast cancer.** The heterogeneity of  
96 breast cancer tumors is well documented. This is evident by the detection of tumor-initiating  
97 capabilities of certain cell populations, based on expression levels of specific surface markers  
98 (CD44<sup>+</sup>, CD24<sup>-/low</sup>, EpCAM<sup>+</sup>, Lineage<sup>-</sup>)[7]. These cells -often termed cancer stem cells, due  
99 to certain stem-like characteristics they share with normal stem cells- are capable of  
100 reconstituting tumors *in vivo*. Such cancer stem cells have also been documented in a variety  
101 of different tumors including ovary (CD44<sup>+</sup>/CD117<sup>+</sup>) [8], (CD133<sup>+</sup>) [9-10] and endometrium  
102 [11].

103

## 104 **2.0 Circulating tumor cells**

105 Circulating tumor cells (CTCs) are defined as tumor cells circulating in the peripheral blood  
106 of patients, shed from either the primary tumor or from its metastases. The presence of solid  
107 tumor-derived cells in peripheral blood was first reported in 1869 [12]. CTC detection in  
108 breast cancer, based on expression of specific cell surface markers, is a fast emerging area of

109 research with efforts focusing on detection of early metastatic disease. The development of a  
110 suitable assay has proven difficult due to the heterogeneous nature of CTCs. In general, there  
111 are two main methods for their detection. These are based on cytometric and nucleic acid  
112 manipulation. Both methods generally require an enrichment step to increase sensitivity of  
113 the assay. This step is based on either detection of specific surface markers using immuno-  
114 selection and/or on morphological features, such as cell size or density. Each of the several  
115 methods available to detect CTCs have distinct advantages and disadvantages. We present a  
116 brief description of the most clinically relevant assays used, including their associated  
117 advantages/disadvantages. Readers are directed towards a recent article for an in-depth  
118 review on technical and statistical considerations for CTC analyzes in the clinic [13].

119

## 120 **2.1 Detection methods of circulating tumor cells**

### 121 **2.1.1 CellSearch<sup>®</sup>**

122 The most established application of CTC analysis so far is based on CTC counts  
123 (CellSearch<sup>®</sup> System) and has been FDA approved for monitoring metastatic breast cancer  
124 [14]. This semi-automated technology combines both positive (epithelial cell adhesion  
125 molecule: EpCAM; using ferrofluid nanoparticles coated with anti-EpCAM antibodies) and  
126 negative (anti-CD45) selection. The enriched CTC population is then immunostained for  
127 specific markers and counted using a semi-automated fluorescence microscope *i.e.* the  
128 CellSpotter analyzer (Veridex; Johnson & Johnson). Thus, isolated CTC are defined as  
129 EpCAM<sup>+</sup>, CK<sup>+</sup>, DAPI<sup>+</sup> and CD45<sup>-</sup>. In metastatic breast cancer an initial and first follow up  
130 CTC count of  $\geq 5$  per 7.5ml blood is predictive of shorter progression free survival and  
131 overall survival [15-16]. While a CTC count  $\geq 5$  per 7.5ml blood at any time during therapy  
132 is indicative of a rapid disease progression and mortality [17]. A further study highlights the  
133 importance of use of the CellSearch<sup>®</sup> system as an independent prognostic factor for early  
134 breast cancer relapse [18]. [However, at present \(based on the most recent publication\) the  
135 American Society of Clinical Oncology \(ASCO\) does not recommend the use of CTC  
136 detection for diagnosis or clinical management decisions in breast cancer \[19\]. Similarly even  
137 though the CellSearch<sup>®</sup> test is FDA approved, ASCO does not recommend its use for clinical  
138 decisions in metastatic breast cancer until further validation proves otherwise. While it is  
139 recognised that this method is highly sensitive, reproducible, commercially available, FDA  
140 approved, and has the capability of distinguishing between viable/non-viable cells, limitations  
141 include: usage of EpCAM to capture CTCs may miss some EpCAM<sup>-</sup> tumor cells, result in  
142 false positives \(due to expression of the same antigens on non-tumor cells\), the multiple](#)

143 enrichment/processing steps may also result in loss of CTC detection and no further  
144 processing of the CTC samples is possible.

145

### 146 **2.1.2 CTC-microchip**

147 Other immuno-selection approaches for CTC capture include the CTC-microchip. The CTC-  
148 chip is a microfluidics platform based on the interaction of target CTCs with anti-EpCAM-  
149 coated microposts under precisely controlled laminar flow conditions, without requiring any  
150 pre-labelling or processing of specimens [20]. This gives the opportunity for further analysis  
151 of the captured, unfixed CTCs. Furthermore, good enrichment (98% cell viability) has been  
152 reported with this approach [21]. Limitations, similar to CellSearch<sup>®</sup>, include the fact that  
153 only EpCAM-positive CTCs are captured in this way, the technology is not available  
154 commercially and clinical validation has yet to be achieved.

155

### 156 **2.1.3 EPISPOT**

157 An alternative antibody-based immunological approach is the Epithelial Immunospot  
158 (EPISPOT), , It detects cells that secrete proteins such as cathepsin-D, mucin-1 (MUC1) [22]  
159 and CK19; thus avoiding assessment of non-viable CTCs which would be unable to progress  
160 to form metastasis [23]. However, as the CTCs are not captured *per se*, further analysis is not  
161 possible, isolated CTCs require 48 hour culturing before analysis – delaying analysis and -as  
162 for CTC-microchip- clinical validation has yet to be achieved.

163

164 The application of AdnaTest BreastCancer<sup>™</sup> test (AdnaGen AG) has generated interesting  
165 data thus far [24]. This “combination-of-combination” approach include immuno-selection  
166 for cell surface markers including EpCAM and MUC1 (AdnaGen BreastCancerSelect),  
167 followed by enhanced specificity through RT-PCR analysis for selected markers, including  
168 HER-2 and GA73.3-2 ((AdnaGen BreastCancerDetect). Such assays have demonstrated that  
169 a change in the phenotype of tumor derived cells can occur after initial diagnosis [21,25].  
170 Whilst the AdnaTest approach has been described as potentially having the same limitations  
171 (as for CellSearch<sup>®</sup> and CTC-Chip) of false-positives and/or false negatives due to loss of  
172 antigens on CTCs, AdnaTest has also been proposed as suitable for characterising events  
173 from stem cells to epithelial-mesenchymal-transition (EMT) [26].

174

### 175 **2.1.4 Size and density approaches to CTC analyzes**

176 Size- or density-based approaches for CTC analysis include Isolation by Size of Epithelial  
177 Tumor cells (ISET) [27]. This filtering (though 8  $\mu$ m-pore) approach is based on size  
178 differential between larger epithelial cells/CTCs and smaller leucocytes, followed by fixing  
179 and staining of the retained larger cells. Limitations of this approach, which deem it to be of  
180 limited sensitivity and specificity, include loss of small CTC cells and retention of large  
181 leucocytes/clumps of leucocytes. Density gradient approaches reported for CTC selection  
182 include OncoQuick<sup>®</sup> [28-29] and RosetteSep<sup>®</sup> [30-31]. In brief, the basic Ficoll-Hypaque  
183 differential density gradient centrifugation approach has been improved upon with  
184 OncoQuick<sup>®</sup> (includes special membranes to prevent cell cross contamination) and  
185 RosetteSep<sup>®</sup> (includes an antibody cocktail which binds to unwanted hematopoietic cells,  
186 thus forming tetrameric complexes; which are subsequently avoided when selecting CTCs  
187 from the density gradient). *Despite these advances on the basic density gradient approach,*  
188 *limitations described for OncoQuick<sup>®</sup> and RosetteSep<sup>®</sup>, respectively, include low specificity*  
189 *and possible cross contamination of cells.* As a comprehensive review of the application of  
190 these –and other approaches for CTC analysis is beyond the scope of this review, we suggest  
191 readers with a particular interest in CTC analysis in breast cancer see three reviews [26,32-  
192 33]. Readers are also directed towards a recent article for an in-depth review on technical and  
193 statistical considerations for CTC analyzes in the clinic [13].

194

195

196 *So far CellSearch<sup>®</sup> is the only standardised and validated test approved by the FDA. While*  
197 *this is likely to remain a very important approach to CTC analysis in breast cancer, studies*  
198 *from the Erasmus Medical Center, Rotterdam, indicate that not all subtypes of breast cancer*  
199 *(particularly normal-like breast tumor cells) are detectable by this method, emphasising the*  
200 *need to increase the capabilities of this approach possibly by combining with some of the*  
201 *other technologies for CTC analysis outlined above. Furthermore, as CellSearch<sup>®</sup> only counts*  
202 *CTCs, undoubtedly combining this approach with additional technologies to capture more*  
203 *cells, different populations of cells, and the gene expression of cells, is necessary before CTC*  
204 *analysis is of more extensive use in the clinical setting. To date only one such multiparameter*  
205 *gene expression assay has gained approval by ASCO. The *Oncotype DX<sup>®</sup>* test, used on FFPE  
206 *tumor tissue specimens, can be used to predict the risk of recurrence in patients treated with*  
207 *tamoxifen. The clinical usefulness of other multiparameter assays such as MammaPrint,**



208 “Rotterdam Signature” and the Breast Cancer Gene Expression Ratio are under investigation  
209 by ASCO.

210

## 211 **2.2 Characterisation of circulating tumor cells**

212 Several studies have published on the characterization of CTCs and subsequent criteria used  
213 in the classification of CTCs [34]. Austrup *et al.*, [35] reported on the prognostic significance  
214 of genomic alterations (Her-2 amplification) in CTCs derived from breast cancer patients.  
215 This study investigated amplification, loss of heterogeneity (LOH) and mutations. The  
216 presence and higher number of genomic imbalances in these CTCs were significantly  
217 associated with poorer prognosis. Genomic imbalances (present in CTCs from breast cancer  
218 patients) such as aneusomy are documented as originating from the primary tumor [36].  
219 Meng *et al.*, demonstrated that CTCs can recapitulate the HER-2 status of the primary tumor  
220 [37]. In a separate study the authors reported that a fraction of patients with HER-2 negative  
221 primary tumors had detectable Her-2 gene amplification in their CTCs, suggesting an  
222 acquired phenotype during cancer progression (9/24 patients; 37.5%). [38].

223

224 So, while many of these formats of CTC analysis have great potential, it is likely that they  
225 routine incorporation into clinical laboratories is some way of and that their future application  
226 will be as a component of a panel of assays; rather than the definitive method.

227

## 228 **3.0 Disseminated tumor cells**

229 Minimal residual disease (presence of tumor cells not detectable by routine diagnostic  
230 procedures used in tumor staging after surgery) cannot be monitored by conventional clinical  
231 methods. Evidence suggests that tumor cells, of epithelial origin, can home to the bone  
232 marrow (BM). Such tumor cells found in the BM are named disseminated tumor cells (DTCs)  
233 (reviewed by Pantel *et al.*, [39]). Several studies have assessed and reported on the prognostic  
234 values of disseminated tumor cells in BM of breast cancer patients [40-42]. Braun *et al.*, [41]  
235 analyzed data from 9 clinical studies on the long term outcome of 4703 breast cancer patients  
236 presenting with and without DTCs. Strong evidence was presented for the adverse prognostic  
237 significance of initial BM DTC presence at the diagnosis of operable cancer. A similar study  
238 investigated BM DTCs from early stage breast cancer patients in recurrence free follow-up  
239 spanning a period of 29 months [42]. This study associated the positive presence of DTCs, at  
240 follow-up, with disease recurrence and highlighted the presence of persistent DTCs as being  
241 indicative of failed primary therapy, while suggesting that secondary adjuvant therapy may

242 be of benefit in these patients. With regards to characterisation of DTCs following therapy,  
243 HER-2 positive cells have been detected in breast cancer DTCs even though the  
244 corresponding primary tumor was HER-2 negative, implying that targeted therapy may be  
245 beneficial for such patients [43]. These studies provide compelling evidence of the  
246 importance of the initial detection and any subsequent detection and characterisation  
247 following therapy of DTCs in BM of breast cancer patients. At present ASCO does not  
248 recommend the assessment of BM for DTC in the management of patients with breast cancer.  
249 [In reality, the sampling of BM DTCs for monitoring purposes may not generally be feasible](#)  
250 [in a typical breast clinic and so DTC analysis is unlikely to become routine in the timeframe](#)  
251 [of some of the less-invasive analyzes \(which involve more straightforward blood sampling\)](#)  
252 [outlined in this review.](#)

253

#### 254 **4.0 Circulating nucleic acids and cancer**

255 Circulating nucleic acids are defined as extracellular nucleic acids (DNA or RNA) found  
256 circulating in the blood throughout the body. Such extracellular nucleic acids are thought to  
257 arise as a result of being secreted from cells; be it from normal cells, or malignant cells of  
258 primary or metastatic origin. The presence of circulating nucleic acids in plasma and serum  
259 has implications for minimally-invasive diagnostic and predictive applications in benign and  
260 malignant conditions. Current terminology in literature for such nucleic acids include:  
261 circulating nucleic acids, cell-free nucleic acids and extracellular nucleic acids. For the  
262 purpose of this review we will refer to such nucleic acids as extracellular nucleic acids.

263

#### 264 **4.1 Evidence of extracellular nucleic acids in cancer**

265 Extracellular nucleic acids, specifically extracellular DNA (exDNA), were first described in  
266 human plasma/serum by Mandel and Metais in 1948 [44]. Early studies reported increased  
267 levels of exDNA in sera from patients with lupus erythematosus [45-46], rheumatoid arthritis  
268 [47] and glomerulonephritis [46]. Additionally, the presence of exDNA was reported in  
269 leukemia patients [46]. In 1977, with the discovery of elevated exDNA levels in patients with  
270 various cancers *versus* healthy individuals, the implications of extracellular nucleic acid  
271 detection for diagnostic purposes became evident [48].

272

273 Stroun *et al.* first suggested that malignant tumors may be a source of exDNA [49], but it  
274 was only with the detection and verification of tumor-derived mutations of K-ras in plasma  
275 from pancreatic adenocarcinoma patients [50] and point mutations of N-ras in plasma of

276 patients with myelodysplastic syndrome and acute myelogenous leukaemia [51] that an  
277 association between tumor and exDNA was convincing. Numerous studies since have  
278 reported on the presence of exDNA in sera/ plasma from patients presenting with several  
279 different cancers, the most recent being studies on breast [52-54], lung [55-56] and metastatic  
280 colon [57] cancers. Several authors have documented correlations between the methylation  
281 status of genes present in the primary tumor with that found in patient serum [58], mutation  
282 detection [57,59-60] and LOH [61]. Similarly the presence of exRNA is well documented in  
283 sera/plasma from patients presenting with breast [62-64] and colon cancers [65-66].

284

#### 285 **4.2 Origin of extracellular nucleic acids**

286 Questions remain as to the origin of extracellular nucleic acids, which can be found as free  
287 nucleic acids [67], bound to a cell's surface *via* proteins with specialised nucleic acid binding  
288 properties [68-70], complexed with proteolipids [71-72] and within apoptotic bodies [73].  
289 Studies have shown that they (DNA and RNA) can be released spontaneously from cells [74-  
290 75]. However the exact mechanism of release from normal cells and tumor cells remains to  
291 be fully elucidated.

292

293 Anker *et al.* [76] and Jahr *et al.* [77] proposed tumor cell apoptosis/necrosis, apoptosis or  
294 lysis of CTCs, and/or active release from tumor cells as possible release mechanisms. RNA-  
295 proteolipid complexes/microvesicles/exosomes have been isolated from sera of cancer  
296 patients [78-79] and from cancer cell lines [80-81]. The importance of communication  
297 between tumor cells and their environment through fusion of shed membrane microvesicles  
298 to neighbouring cells was discussed by Ratajczak *et al.* [82]. These microvesicles, 30-100 nm  
299 in diameter [83], can be shed from the cell surface or *via* inward budding of endosome  
300 membranes [84]. Once released into extracellular space these microvesicles are termed  
301 exosomes [85] and may be involved in trans-cellular signalling [86], transfer of membrane  
302 receptors, proteins, mRNA/microRNA [87], and organelles (*e.g.* mitochondria) between  
303 cells. Another role may be in the delivery of infectious and toxic agents (*e.g.*  
304 chemotherapeutic drugs) into cells [88](reviewed by [82]).

305

306 It is thought the contents of exosomes depend on their cell of origin [78]; those derived from  
307 immune cells having immunostimulatory and anti-tumor effects *in vivo* [89], while those  
308 derived from tumor cells accelerate tumor growth [78,90] and invasiveness [91-92].  
309 Additionally, the RNA contained within exosomes is amplifiable; suggesting protection from

310 RNase degradation by the exosome membrane [78]. Other groups have also demonstrated  
311 that serum/plasma RNAs in cancer patients are contained and protected in membrane bound  
312 structures [73,93], (reviewed by [94]). [Although circulating exosomes have been identified](#)  
313 [as having potential diagnostic relevance in some cancer types \(including ovarian cancer \[95\];](#)  
314 [glioblastomas \[78\]; and lung cancer \[95\], the prevalence and clinical relevance of exosomes](#)  
315 [in sera from breast cancer patients has yet to be elucidated.](#)

316

### 317 **4.3 Extracellular RNAs and CTC associated RNAs in breast cancer**

318 More recent efforts have focused on the identification of possible biomarkers for diagnostic  
319 purposes in autoimmune and malignant diseases. In breast cancer exRNAs have been  
320 documented by a number of research groups [63,96-98]. We present the most documented  
321 studies on cell free exRNAs and CTC associated RNAs.

322

#### 323 **4.3.1 Cytokeratin 19**

324 Cytokeratins belong to a family of over 20 related polypeptides located in the cytoskeleton of  
325 various epithelial cells. Their main role is to provide cellular structure and integrity. They are  
326 classified into two types: acidic type I (CK9-CK20) and basic type II (CK1-CK8) [99].  
327 Epithelial cells are targets of caspase-mediated proteolysis during apoptosis [100]. CK19 is  
328 cleaved by caspase 3 and soluble fragments (CYFRA 21-1), released into extracellular space,  
329 have been detected in sera of cancer patients [62,101] and reportedly do not correlate with  
330 that of full-length CK19 mRNA [62].

331

332 Initially, Silva *et al.* [96] identified CK19 mRNA in plasma of breast cancer patients (49%) in  
333 comparison to healthy controls (20%). On evaluation of clinicopathological parameters  
334 (including patient age, tumor size, histopathological type and stage, lymph node metastases,  
335 ER and PR status, proliferative index) with molecular data, two parameters (tumor size and  
336 proliferative index as determined by Ki-67 IHC) were associated with the presence of plasma  
337 CK19 mRNA. As the recurrence rate in stage I and stage II breast cancer is between 10-20%  
338 and 40-65%, respectively [102], it was proposed that the high proportion of patients in the  
339 study with early stage cancer (stage II) and showing positivity for CK19 mRNA expression,  
340 might be an indicator of patient clinical outcome and recurrence. Ninety percent of patients  
341 with detectable plasma CK19 mRNA were also positive for CK19-positive CTCs. Although  
342 the authors are unclear as to the significance of this finding, they suggest that shedding rates

343 of nucleic acids from CTCs with metastatic potential may be higher than from tumor cells *i.e.*  
344 that the exRNA identified in their study may have originated from CTCs.

345

346 More recent reports have demonstrated the prognostic significance of CK19 mRNA positive  
347 CTCs in patients with early-stage breast cancer [103-105]. Studies that investigated the  
348 prognostic value of CTCs/DTCs at the time of completion of adjuvant therapy [42,106-107]  
349 showed that the detection of DTCs [42,108] and CTCs [106] after the completion of  
350 chemotherapy is associated with an unfavourable clinical outcome [103,105]; further  
351 reviewed by Ignatiadis *et al.* [109]. In keeping with this work suggesting diagnostic and  
352 prognostic relevance for CK19, more recently full-length CK19 has been detected in bone  
353 marrow specimens from breast cancer patients and is associated with the presence of overt  
354 metastases and poor survival rates [110].

355

#### 356 **4.3.2 Human telomerase reverse transcriptase**

357 Telomeres are a sequence of repetitive DNA located at the end of linear chromosomes where  
358 they serve to prevent the chromosome end from being recognized as DNA double-stranded  
359 breaks. Telomerase is the ribonucleoprotein reverse transcriptase complex that maintains  
360 telomere length and is reactivated in 90% of cancers [111]. It is composed of two subunits: an  
361 RNA component (TERC) and a telomerase reverse transcriptase (TERT). TERT is generally  
362 not expressed in most differentiated somatic cells; therefore, telomerase activity is absent.  
363 TERT is the major determinant for activation of telomerase. Absence of telomerase activity  
364 leads to a progressive shortening of telomeres and cellular senescence. However, reactivation  
365 of telomerase is considered a crucial step for malignant cells to become immortal.

366

367 Detection of hTERT expression either in plasma [65,112], peripheral blood mononuclear  
368 cells [113] or by IHC in primary tumors [114] has been identified as diagnostic for prostate,  
369 colon, esophageal cancers and paragangliomas, respectively. In terms of breast cancer, Chen  
370 *et al.* [97] identified hTERC and hTERT mRNA in primary tumors and sera of breast cancer  
371 patients presenting with invasive lobular carcinoma and invasive ductal carcinoma. Of the  
372 specimens analyzed, hTERT was identified in 25% of sera and 94% of primary tumors, while  
373 hTERC was identified in 28% of sera and 94% of primary tumors. hTERC or hTERT was not  
374 detected in sera from control subjects or patients presenting with benign disease. No  
375 correlation was found between patient age, tumor size, tumor grade or the presence of nodal  
376 metastases.

377

378 A more recent study identified hTERT mRNA-positive CTCs [115]. ChangXin *et al.* [115]  
379 2009 reported hTERT expression in 59.6% of patients presenting with breast cancer in  
380 comparison to healthy volunteers (0%) and benign controls (0%). Importantly, detection of  
381 hTERT mRNA significantly correlated with the TNM (internationally used tumor, node,  
382 metastases staging system) stage and lymph node metastasis, with hTERT present in 24.2%,  
383 67.9%, 80.0% and 100% of patients with a TNM stage of I, II, III and IV, respectively. In  
384 conjunction with hTERT, this group also investigated expression of survivin and hMAM  
385 (human mammaglobin), and reported improved sensitivity and specificity with all three  
386 biomarkers correlating to TNM stage. The authors conclude that the use of peripheral blood  
387 would be advantageous in clinical monitoring of early stage hematogenous spreading that  
388 may further develop into breast cancer metastasis or recurrence.

389

### 390 **4.3.3 Bmi-1**

391 Polycomb group (PcG) proteins are implicated in embryonic development and oncogenesis  
392 [116-118]. PcG protein expression is often dysregulated in several types of cancer and  
393 strongly correlates with an invasive and/or metastatic phenotype [119-120]. Bmi-1 (PcG  
394 protein B lymphoma Mo-MLV insertion region 1 homolog), was initially identified as an  
395 oncogene cooperating with c-myc in the development of murine pre-B-cell lymphomas [121].  
396 Bmi-1 plays an essential role in maintaining the self-renewal of normal and malignant human  
397 mammary stem cells [122]. Over-expression of Bmi-1 is reported and correlated to poor  
398 clinical prognosis in solid tumors of breast [123], lung [124] and colon [125] cancers; to  
399 name but a few. Silva *et al.* [98] investigated Bmi-1 mRNA expression in plasma of a cohort  
400 of patients presenting with various subtypes and stages of breast cancer. Bmi-1 mRNA was  
401 detected in 43.2% of breast cancer specimens and also in 55% of healthy controls. However,  
402 expression levels were significantly higher in patients presenting with cancer *versus* the  
403 healthy controls. Upon correlation of gene expression with clinical-pathological features of  
404 the primary tumor (including vascular invasion, tumor subtype/stage, ER and PR receptor  
405 status, p53 status, HER-2 status, proliferative status), higher Bmi-1 levels were associated  
406 with p53-positive immunostaining and PR-negative status. Importantly, the presence of  
407 plasma Bmi-1 mRNA was found to be associated with poorer survival at a more advanced  
408 clinical stage suggesting plasma Bmi-1 to be a potential surrogate biomarker of poor  
409 prognosis that could be analyzed minimally-invasively.

410

#### 411 **4.3.4 Human Mammaglobin**

412 Human mammaglobin (MGB) belongs to the uteroglobin/Clara cell protein family of small  
413 epithelial secretory proteins [126-127] and was discovered by Watson and Fleming [128] in  
414 their isolation of sequence fragments that were abundantly expressed in breast tumors relative  
415 to normal breast tissue. The MGB1 promoter contains a polyoma enhancer-related motif  
416 which is associated with over-expression of HER-2 in breast cancer [129]. Two isoforms of  
417 MGB have been reported. These are mammaglobin 1 (also referred to as MGB1,  
418 mammaglobin A, SCG2A2) and mammaglobin 2 (MGB2, mammaglobin B, SCGB2A1).  
419 Most research on the use of MGB as a potential diagnostic biomarker in cancer has focused  
420 on MGB1, although there are reports of high levels of MBG2 mRNA and protein being  
421 associated with well (Grade 1) and moderately (Grade 2) differentiated endometrioid  
422 endometrial tumors [130].

423

424 Initially, Watson and Fleming [131] detected MGB1 expression in several breast carcinoma  
425 cell lines but not in primary breast stromal cells nor in immortalised luminal ductal breast cell  
426 lines. They concluded that MGB1 expression is mammary-specific, as MGB1 was expressed  
427 exclusively in mammary epithelial cells. It was since reported however, that MGB1 is  
428 expressed at the mRNA and protein level in tumors of the endometrium, ovary and cervix  
429 [132]. Watson and Fleming also reported that primary breast carcinomas over-expressed  
430 MGB1 relative to normal breast tissue specimens, with approximately 50% of breast  
431 carcinoma cell lines and metastatic breast tumors analyzed exhibiting high levels of MGB1  
432 mRNA [131]. This over-expression did not appear to correlate with histology, tumor grade,  
433 tumor stage, or ER/PR status. A more recent study reported high MGB1 levels in primary  
434 breast cancers to be indicative of a less aggressive tumor phenotype and correlating with the  
435 expression of ER and PR, low Ki-67 labeling and absence of nodal invasion [133]. There has,  
436 so far, been no reported documentation of circulating transcripts of MGB1, however, MGB1  
437 protein has been detected in sera of breast cancer patients [134-135].

438

439 Recent efforts have focused on the detection of MGB1 in peripheral blood [136-138] as a  
440 diagnostic biomarker of metastatic disease. Mikhitarian *et al.* [139] used a panel of  
441 biomarkers (CK19, MUC1, MGB1, EpCAM, HER-2, prolactin inducible protein) in their  
442 investigation of a possible correlation between mRNA expression in peripheral blood CTCs,  
443 bone marrow DTCs and clinicopathological indicators (tumor type, TNM, ER/PR status,  
444 HER-2 status, age and race). Their main finding was the lack of correlation between

445 biomarker positivity in CTCs and tumor type and/or biomarker status of axillary lymph  
446 nodes. However, a significant association was reported between biomarker positivity in CTCs  
447 and tumor grade (grade II-III *versus* grade I), with this association holding true for expression  
448 of MGB1 mRNA alone. As this was an interim report of an ongoing study, the clinical  
449 significance in terms of overall survival and disease free survival remain to be seen.

450

#### 451 **4.3.5 Cyclin D1**

452 Cyclin D1 is a cell cycle regulator. It is induced by a variety of factors, such as epithelial  
453 growth factor, hormones and oncogenic signals and its over-expression is one of the most  
454 commonly observed alterations in cancer [140]. In breast cancers, cyclin D1 is over-  
455 expressed by 30–50% of tumors [140] and is documented as being predictive of poor clinical  
456 outcome [141]. Garcia *et al.* [63] investigated whether plasma mRNA from breast cancer  
457 patients is related to disease-free survival and overall survival. Presence of cyclin D1 plasma  
458 mRNA was significantly associated with non-responsive patients following treatment after  
459 relapse. Additionally the authors reported a trend towards a significant relation between  
460 reduced overall survival rates and cyclin D1 presence. No association was detected between  
461 cyclin D1 plasma mRNA presence and disease free survival.

462

#### 463 **4.3.6 HER-2**

464 Approximately 25% of breast cancers over-express HER-2. Trastuzumab, a monoclonal  
465 antibody against the HER-2 receptor is the only approved targeted agent for treatment of  
466 HER-2 over-expressing cancers. However, not all patients respond to treatment and some  
467 develop resistance. The most frequent approach to measure HER-2 levels is by FISH, IHC  
468 and ELISA. The extracellular domain of HER-2 can be proteolytically cleaved from the cell  
469 membrane and this domain can be measured in peripheral blood. Patients with HER-2  
470 positive breast cancer have high serum HER-2 levels, which correlate positively with the  
471 number of metastatic sites and are indicative of disease recurrence [142]. While the presence  
472 and thus relevance of Her2 mRNA in serum or plasma has yet to be investigated, detection of  
473 Her2 mRNA-positive CTCs in blood specimens from patients with operable breast cancer  
474 have been reported as prognostic of unfavourable outcome, in terms of both relapse-free  
475 survival and overall survival [143].

476

477



478 As detailed above, research to date indicates that mRNAs (including CK19, TERT, Bmi-1,  
479 MGB, cyclin D1 and HER-2, to name but a few) exist in the systemic circulation- either  
480 freely or sequestered in microvesicles or exosomes. Undoubtedly, such mRNAs have  
481 potential as breast cancer biomarkers. However, while these studies have “proven-the-  
482 principle” and opened up an opportunity for biomarker searches that was initially considered  
483 to be irrelevant due to the high levels of RNase enzymes in this environment, we believe that  
484 more extensive studies are now needed to identify and validate the most relevant panels of  
485 mRNAs in large cohorts of relevant cases.

486

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## 489 **5.0 MicroRNAs in breast cancer**

490 MicroRNAs (miRNAs) are a family of endogenous small noncoding RNA molecules, of 19-  
491 28 nucleotides in length (reviewed:[144-146]. Over the past two years, since the first report  
492 of circulating microRNAs (miRNAs) associated with diffuse large B-cell lymphoma [147],  
493 circulating miRNAs have been reported in studies of a range of other cancer types.  
494 Specifically, in prostate cancer, serum *miR-141* level was found to distinguish metastatic  
495 prostate patients (n=25) from age-matched controls [148]. Similarly, *miR-25* and *miR-223*  
496 were reported as non-small cell lung cancer specific following their analysis in sera from 152  
497 patients compared to 75 healthy volunteers [149]. Circulating miRNAs, *albeit* in exosomes,  
498 have also been reported to have potential as biomarkers for ovarian cancer [150] and for  
499 glioblastoma [78]. Studies of circulating miRNAs associated with breast cancer have been  
500 limited to date. In our pilot study, in addition to circulating mRNAs [151], we detected  
501 circulating miRNAs (including *miR-141* and *miRNA-195*) to be present at significantly higher  
502 levels (p<0.001) in serum from patients recently diagnosed with breast cancer compared to  
503 age- and gender- matched healthy volunteers (as yet unpublished). In agreement with our  
504 findings, *mi-195* and *let-7a* have recently been reported as at significantly higher levels in  
505 blood from breast cancer patients, compared to healthy volunteers [152].

506

507 Therefore, while studies of circulating miRNAs are still in their infancy, analyzes performed  
508 thus far suggested that these miRNAs –whether freely existing or enclosed in  
509 exosomes/microvesicles- may prove to have great potential as biomarkers for many cancers,  
510 including breast cancer. Due to their short sequences and stability (*e.g.* can be routinely  
511 assessed in formalin-fixed paraffin-embedded tissue and apparently can withstand many

512 rounds of freeze-thawing when in solution) it is possible that miRNAs may have certain  
513 advantages over mRNAs as circulating biomarkers. Alternatively, a biomarker panel could  
514 conceivable include single or multiplex co-analysis of both types of RNA molecules; possibly  
515 offering some advantages over protein biomarker analysis. Now that their potential  
516 usefulness in this setting has been established, more extensive studies are essential in order to  
517 establish how best to exploit our understanding of circulating RNA molecules in the interest  
518 of breast cancer patients.

519

## 520 **6.0 Limitations of blood-based biomarkers & future directions**

521 Although research into potential cancer biomarkers has been ongoing for many years, the  
522 actual number of markers used in clinical settings remains limited [153-155]. The main  
523 reason being the discovery and development of useful biomarkers pose many challenges,  
524 with several factors contributing to the slow rate of biomarker development [156-157]. The  
525 ASCO published a report on recommendations for the use of tumor markers in breast cancers.  
526 They report a lack of validation of potential biomarkers as being a major obstacle in the  
527 development of new biologics or treatment strategies for patients. Areas that contribute to this  
528 problem include non-uniform procurement, processing and storage of specimens, differences  
529 in technologies used, non-standardised assays/non-validated equipment- all contributing to  
530 lack of study reproducibility, inappropriate statistical analyzes and insufficient patient  
531 specimen numbers. In addition, while some studies may show promising results, the overall  
532 lack of sufficient high-quality studies coupled with a lack of consistent data results in their  
533 omission from use as clinical markers for breast cancers [156].

534

535

536 To date, ASCO has recommended 8 tumor biomarkers for breast cancer – all of which are  
537 proteins *i.e.* CA 15-3 and CA 27.29, carcinoembryonic antigen (CEA), ER, PR, HER2,  
538 urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1. CA 15-  
539 3, CA 27.29 and CEA are biomarkers for monitoring; ER, PR and HER2 are biomarkers for  
540 treatment planning; and uPA and PAI-1 are biomarkers for recurrent risk prediction[19].  
541 While these are the only markers ASCO approved for clinical use, ASCO does not  
542 discourage the use of other novel or innovative approaches in the context of clinical trials  
543 (*e.g.* I-SPY 2 trial[158]). For future advancement in clinically useful biomarkers, well-  
544 designed, collaborative studies involving the inclusion of specimens –procured, processed  
545 and analyzed using standard operating procedures - from large cohorts of consenting patients

546 and healthy volunteers (for example, in Ireland such studies are possible, as facilitated by the  
547 All Ireland Irish Clinical Oncology Research Group (ICORG), as well as through  
548 international collaborations) should help in identifying and validating biomarkers panels  
549 which can be translated to the clinic, in the interest of cancer patients.

550

551 In 2001, Pepe *et al.*, [159] defined 5 phases of biomarker development for early detection of  
552 cancer *i.e.* (1) preclinical exploratory studies; (2) clinical assay development; (3)  
553 retrospective longitudinal repository studies; (4) prospective screening studies; and (5) cancer  
554 control studies assessing impact of screening on reducing the burden of disease. While all  
555 breast cancer biomarker studies are not aimed at diagnosis, the same overall strategy is very  
556 relevant to consider. An open mind should be kept as to what the final portfolio of any given  
557 biomarker panel may include. So far, there are no proven advantages of one molecule type  
558 over another with regards to their potential as biomarkers. Although, historically, focus has  
559 mainly been on proteins and more recently CTCs, results emerging from exploratory studies  
560 suggest that RNAs -which are proving to be reproducibly detected and can be amplified using  
561 a multiplex approach (thus minimizing the amount of specimen required for analysis) will  
562 form members of useful breast cancer biomarker panels. To date, what could possibly be best  
563 described as proof-of-concept studies have been reported on RNAs (mRNAs and miRNAs) as  
564 biomarkers. Relative to protein research, however, we believe that this field of research is  
565 still in its infancy and should now be much more extensively explored. While this has yet to  
566 be established, it is probably that optimal sensitivity and specificity will include a  
567 combination of mRNA, miRNA, protein -and possible also CTC- analysis from a patient's  
568 blood specimen. It is our view that it is only through carefully designed research efforts and  
569 systems that are patient-orientated and collaborative, that the development of robust panels of  
570 such biomarkers and associated reliable assays will translate to the clinic to contribute to  
571 improvements in personalized treatment, quality of life and survival for breast cancer  
572 patients.

573

## 574 **7.0 Conclusion**

575 Breast cancer is the second leading cause of cancer related deaths in the US and the most  
576 common cancer in women in the western world. The need for rapid methods for **early**  
577 **detection of breast cancer**, **prognosing outcome**, and **predicting response to treatment** –by  
578 analysing CTCs or specific circulating protein and/or nucleic acids- would greatly aid current  
579 treatment modalities. Additionally, such biomarkers could potentially be used for **continual**

580 monitoring of tumor response to treatment allowing for a change in treatment regime if  
581 needed *i.e.* tailoring treatment for the individual.

582

583 In the past, the development of suitable assays to detect cancer cells has proven difficult due  
584 to the heterogeneous nature of breast cancer tumors. Current advances in molecular profiling  
585 have enabled certain surface biomarkers and gene expression patterns to be used for  
586 identification of breast tumor cells and their associated genes. As the majority of breast  
587 cancer deaths are as a result of recurrent metastatic disease, which is may be caused by  
588 spread of CTCs/DTCs resulting from tumor resection, a minimally-invasive method for  
589 cancer detection and monitoring would be clinically highly advantageous. In addition such a  
590 system has the potential to allow for early detection screening of people at increased risk of  
591 cancer. For the most part, biomarkers are not yet ready for routine use due to challenges in  
592 their clinical validation for breast cancer detection/diagnosis, prognosis and response to  
593 treatment prediction. However, advancing on the recent exciting studies reviewed here,  
594 current and future research focussing on the identification of diagnostic, prognostic and/or  
595 predictive circulating nucleic acids/CTCs has immense importance clinically to the  
596 development and improvement of current diagnostic and therapeutic options for breast cancer  
597 patients.

598

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