1 **Cytokine profiling of tumour interstitial fluid of the breast and its relationship with**
2 **lymphocyte infiltration and clinicopathological characteristics**

3 Jaime A. Espinoza [a*], Shakila Jabeen [b*], Richa Batra [c], Elena Papaleo [c], Vilde Haakensen [d], Vera
4 Timmermans Wielenga [e] Maj-Lis Møller Talman [e], Nils Brunner [f], Anne-Lise Børresen-Dale [d,g],
5 Pavel Gromov [h], Åslaug Helland [b,i], Vessela N. Kristensen [b*], Irina Gromova [h*#]

6 [a] SciLifeLab, Division of Translational Medicine and Chemical Biology, Department of
7 Medical Biochemistry and Biophysics, Karolinska Institutet, Solna, Stockholm, Sweden
8 [b] Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital,
9 The Norwegian Radium Hospital, Oslo, Norway; K.G. Jebsen Center for Breast Cancer
10 Research, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo,
11 Norway; Department of Clinical Molecular Biology (EpiGen), Akershus University Hospital,
12 University of Oslo (UiO), Oslo, Norway
13 [c] Danish Cancer Society Research Center, Computational Biology Laboratory, Unit of
14 Statistics, Bioinformatics and Registry, Copenhagen, Denmark
15 [d] Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital,
16 The Norwegian Radium Hospital, Oslo, Norway
17 [e] Department of Pathology, Center of Diagnostic Investigations, Copenhagen University
18 Hospital, Copenhagen, Denmark
19 [f] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences,
20 University of Copenhagen, Strandboulevarden 49, Copenhagen, Denmark
21 [g] Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Norway
22 [h] Danish Cancer Society Research Center, Genome Integrity Unit, Cancer Proteomics
23 Group, Copenhagen, Denmark
24 [i] Department of Oncology, Oslo University Hospital, The Norwegian Radium Hospital, Oslo,
25 Norway
26

27 * These authors equally contributed to this work;

28 # corresponding author: Irina Gromova;

29 Mailing address: Gromova Irina, Danish Cancer Society Research Center, Genome Integrity Unit,
30 Cancer Proteomics Group, Copenhagen, Denmark

31 E-mail: iig@cancer.dk

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34 **Abstract**

35 The tumour microenvironment is composed of many immune cell subpopulations and is an
36 important factor in the malignant progression of neoplasms, particularly breast cancer
37 (BC). However, the cytokine networks that coordinate various regulatory events within the
38 BC interstitium remain largely uncharacterised. Moreover, the data obtained regarding the
39 origin of cytokine secretions, the levels of secretion associated with tumour development,
40 and the possible clinical relevance of cytokines remain controversial. Therefore, we
41 profiled 27 cytokines in 78 breast tumour interstitial fluid (TIF) samples, 43 normal
42 interstitial fluid (NIF) samples, and 25 matched serum samples obtained from BC patients
43 with Luminex xMAP multiplex technology. Eleven cytokines exhibited significantly higher
44 levels in the TIF samples compared with the NIF samples: interleukin (IL)-7, IL-10,
45 fibroblast growth factor-2, IL-13, interferon-γ-inducible protein (IP-10), IL-1 receptor
46 antagonist (IL-1RA), platelet-derived growth factor (PDGF)-β, IL-1β, chemokine ligand 5
47 (RANTES), vascular endothelial growth factor, and IL-12. An immunohistochemical
48 analysis further demonstrated that IL-1RA, IP-10, IL-10, PDGF-β, RANTES, and VEGF are
49 widely expressed by both cancer cells and tumour infiltrating lymphocytes (TILs), while IP-
50 10 and RANTES were preferentially abundant in triple-negative breast cancers (TNBCs)
51 compared to Luminal A subtype cancers. The latter observation corresponds with the high
52 level of TILs in the TNBC samples. IL-1β, IL-7, IL-10, and PDGFβ also exhibited a
53 correlation between the TIF samples and matched sera. In a survival analysis, high levels
54 of IL-5, a hallmark T_H2 cytokine, in the TIF samples was associated with a worse
55 prognosis. These findings have important implications for BC immunotherapy research.

56

57 **Keywords**: Breast cancer, cytokine, growth factor, interleukin, interstitial fluid, tumour-
58 infiltrating lymphocyte, T_H2, array.

59 **Abbreviations**: ANOVA, analysis of variance; BC, breast cancer; DFS, disease-free
60 survival; FFPE, formalin-fixed, paraffin embedded; ER, estrogen receptor; FGF: fibroblast
61 growth factor; FISH, fluorescence *in situ* hybridization; GM-CSF: granulocyte-macrophage
62 colony-stimulating factor; HER2, human epidermal growth factor receptor 2; IFN:
63 interferon; IHC, immunohistochemistry; IL, interleukin; IP: inducible protein; MCP-1,
64 monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; NIF, normal
65 interstitial fluid; PDGF: platelet-derived growth factor; PgR, progesterone receptor; TAM:
66 tumour-associated macrophage; TIF, tumour interstitial fluid; TILs, tumour-infiltrating
67 lymphocytes; TNBC, triple-negative breast cancer; TNF, tumour-necrosis factor; VEGF,
68 vascular endothelial growth factor.

69

70 ## 1. Introduction

71 Breast cancer (BC) is currently the most commonly diagnosed form of female
72 cancer with more than 1.300.000 cases diagnosed each year worldwide. [1] It is also the
73 leading cause of cancer-related deaths in women to date.[1] It has been demonstrated that
74 both the extensive genetic alterations that are observed in epithelial cancer cells [2] and the
75 composition of the stromal compartment can influence the progression of BC in a clinically
76 relevant manner. [3] These results highlight the complexity of this heterogeneous disease
77 and also represent a major challenge in the development of targeted therapeutics.
78 Accumulating evidence indicates that tumour growth and progression are dependent on
79 the malignant potential of epithelial cancer cells and on the multidirectional interactions of
80 factors produced by cell types that form a local tumour milieu. These include adipocytes,
81 tumour-associated fibroblasts, endothelial cells, and immune cells. All of these cell types
82 produce networks of cytokines and growth factors that are present in the local
83 microenvironment. [4-7] The importance of the tumour microenvironment in cancer growth
84 and progression is widely accepted, yet the origin and significance of signaling cross-talk
85 between cancer cells and the cells that constitute the supporting tumour interstitium,
86 including immune cells, remains poorly understood. An important component of immune
87 cells is the population of tumour-infiltrating lymphocytes (TILs). The presence of TILs is
88 generally accepted as a prognostic factor for achieving a pathological complete response
89 in BC patients following neoadjuvant chemotherapy (For a review see ref. 8). Moreover,
90 access of the peritumoural space and tumour islet by TILs has been shown to correlate
91 with good prognosis in various cancers, including ovarian carcinoma, [8] colon cancer, [9] and
92 BC. [10, 11]

93 The complex composition of cell types in a tumour microenvironment enables a
94 network of cytokines and growth factors to modulate the progression of malignant cells. [12]

Cytokine-mediated, multidirectional signaling events between cancer cells and leukocytes in the tumour-stroma milieu are generally implemented through the tumour interstitial fluid (TIF). Interstitial fluid forms at the interface between circulating bodily fluid and intracellular fluid, and provides an environment that facilitates the exchange of ions, proteins, cytokines, and growth factors between various cellular components within the interstitial space. Biomolecules that derive from cancer cells and stromal cells can also accumulate in TIF via secretion, exosome-mediated secretion, and membrane shedding. Thus, interstitial fluid represents a valuable resource for the discovery of novel biomarkers and therapeutic targets. [12-14]

Interstitial fluid may also provide insight into the regulatory mechanisms and functions of secretion-related processes during tumour development. The local tumour space accumulates secretome components at much higher concentrations compared with serum, and proximal lesion sampling and -*omic* profiling of tumour-associated fluid are two promising approaches for identifying novel candidate biomarkers. [12] We previously developed a procedure for recovering TIF from fresh BC tissue specimens and performed a comprehensive, gel-based proteome characterization of BC interstitial fluids for a systematic search of potential biomarkers. As a result, a 9-protein signature profile with a higher abundance in TIF compared to normal counterparts was identified. [12, 15, 16] Furthermore, in a preliminary study, a number of these cytokines were detected and measured in breast TIF using a cytokine-specific antibody array. [16] A similar approach has been used by others to dissect the pathological role of interstitial molecules in cell migration, extracellular matrix reorganization, tumour microenvironment formation, morphogenesis, and immunity. [12-14, 17, 18]

Over the last decade, accumulating evidence has demonstrated a role for infiltrating leukocyte populations in BC progression. [10, 19] In contrast, very little is known

120 about the *in vivo* origin of the cytokines associated with TILs, tumour subtypes, and clinical
121 outcome. Here, we present the results of a comprehensive array-based analysis of 27
122 cytokines and growth factors in a large cohort of breast TIF, matched NIF, and serum
123 samples. To our knowledge, this is the first large-scale study to profile various cytokines
124 and growth factors secreted into the local interstitium of breast tumours in order to
125 characterise a local cytokine response in a tumour microenvironment. This approach
126 provides the basis for discriminating a systemic cytokine response that is induced by a
127 primary cytokine reaction in a tumour niche and can be directly associated with
128 malignancy. The main objectives of the present study were to: (i) identify and compare the
129 abundance of cytokines and growth factors present in malignant versus normal interstitial
130 fluids; (ii) characterise the cytokine profiles of various tumour subtypes, (iii) identify a
131 possible correlation between cytokines present in TIF with subpopulations of tumour-
132 associated TILs, (iv) identify cytokines exhibiting a significant association with TIF and
133 matched serum, and (v) identify a possible correlation between the cytokine profile of
134 breast TIF and clinical outcome.

135 **2. Results**

136 **2.1. Analysis of tumour-secreted cytokines and the tumour microenvironment:**
137 **Comparative cytokine profiling of TIF and normal interstitial fluid (NIF)**

138 A quantitative comparison of the most prominent cytokines in breast TIF compared to
139 NIF was performed. A total of 27 cytokines (Supplementary Table 1) were measured
140 across both sets of samples using a multiplex bead-based immune assay (Luminex). The
141 amount of each sample that was loaded was normalised based on total protein
142 concentration. The resulting cytokine concentrations detected in the proximal fluids were
143 then log-transformed to achieve a similar data distribution across all of the samples. A
144 paired analysis using matched samples identified 11 cytokines that were significantly

145 elevated in the TIF samples compared with the NIF samples: interleukin (IL)-7, IL-10,
146 fibroblast growth factor (FGF)2, IL-13, interferon (IFN)-γ-inducible protein (IP-10), IL-1
147 receptor antagonist (IL-1RA), platelet-derived growth factor (PDGF)β, IL-1β, chemokine
148 ligand 5 (RANTES), vascular endothelial growth factor (VEGF), and IL-12 (Figure 1A). IL-6
149 was the only cytokine with a slight, yet significantly lower expression level in the TIF
150 samples compared with the NIF samples (Figure 1A).

151 To further characterise the origin and intra-tissue localization of differentially expressed
152 cytokines, an immunohistochemical (IHC) analysis of selected tissue sections prepared
153 from matched tumour and normal samples was performed. The tissue samples were
154 selected based on the criterion of having high or low levels of the cytokines of interest
155 detected in TIF samples compared to NIF samples, as well as the availability of
156 corresponding tissue samples and specific antibodies. Thus, IHC staining was performed
157 for IL-10, IP-10, IL-1RA, PDGFβ, RANTES, and VEGF (Figure 1B). In Supplementary
158 Figure 1, representative IHC results for several matched tumour/normal tissue samples
159 are presented. Between 8 and 17 matched samples were stained for each of the six
160 cytokines in order to confirm the similarity of the IHC patterns observed in the normal and
161 tumour samples. A brief summary of the data is presented in a table at the bottom of
162 Supplementary Figure 1. In the non-malignant breast tissue sections, very few infiltrating
163 immune cells were observed (data not shown). Moreover, expression of IL-10, IP-10, IL-
164 1RA, PDGFβ, RANTES, and VEGF were mainly restricted to the ductal epithelial cells
165 (Figure 1B), while their intracellular localization was primarily observed in the cytoplasm in
166 both the luminal and basal cell layers. An exception was RANTES whose expression was
167 substantially associated with the myoepithelial cells. In the stained tumour lesions, IP-10,
168 IL-1RA, PDGFβ, RANTES, and VEGF exhibited moderate to strong staining intensities in a
169 vast majority of the lesions analyzed (Figure 1B, lower panel and Supplementary Figure

170 1). Furthermore, these cytokines often exhibited higher expression levels in the tumour
171 cells than in the TILs (as shown for IP-10 and RANTES, Figure 2). In contrast, expression
172 of IL-10 was detected in ducts of the normal tissues, while the cancer cells exhibited a
173 lower staining intensity compared to the other five cytokines that were assessed (Figure
174 1B and Supplementary Figure 1).

175 A complete list of all the samples analyzed in this study, including the
176 histopathological, biochemical, and clinical parameters evaluated, are presented in
177 Supplementary Table 2.

178 **2.2 Cytokines in the tumour interstitium were associated with breast tumour**
179 **subtype and TILs**

180 The role of TILs in BC subtypes has been found to be heterogeneous. [20] Therefore,
181 haematoxylin/eosin staining and IHC staining were performed to estimate the extent and
182 type of lymphocyte infiltration present in the four major breast tumour subgroups identified
183 among the lesions examined. First, the total number of TILs present in the tissue sections
184 were scored with haematoxylin/eosin staining. Next, TIL subpopulations were
185 characterised by performing IHC staining with antibodies specific for particular classes of
186 lymphocytes: T-lymphocytes (anti-CD3 antibodies), T-helper lymphocytes (anti-CD4
187 antibodies), cytotoxic T-lymphocytes (anti-$CD8^+$ antibodies), and tumour-associated
188 macrophages (TAMs) (anti-CD68 antibodies). The data listed in Table 1 show that Luminal
189 A lesions had lower frequencies of TILs and $CD3^+$ cells compared to the Luminal B and
190 triple-negative breast cancer (TNBC) lesions. Similar results have been reported in other
191 studies. [10] In contrast, the levels of cytotoxic T-lymphocytes ($CD8^+$) detected were not
192 statistically significant, while levels of tumor-associated macrophages (TAMs) ($CD68^+$)
193 significantly differed between the Luminal A and TNBC lesions (Table 1).

194 We further analyzed the 27 cytokines across HER2, Luminal A, Luminal B and TNBC
195 subtypes. Significantly higher levels of IP-10 and RANTES were detected in the TNBC
196 tissues than in the Luminal A tissues (Figure 2A). As described above, the TNBC tissues
197 analyzed in this study were characterised by a substantially higher rate of TILs compared
198 to the Luminal A tissues (Table 1). The IP-10 and RANTES expression data were then
199 categorised according to high versus low levels of TILs and $CD3^+$, $CD4^+$, and $CD8^+$ TIL
200 subsets across all four breast tumour subtypes. The tumours characterised by a high
201 proportion of $CD3^+$ TILs exhibited significant higher levels of IP-10 and RANTES than TIF
202 samples with low $CD3^+$ TILs (Figure 2B). IHC staining of corresponding tissue sections
203 further showed that expression of both IP-10 and RANTES were generally detected in
204 tumour cells and TILs with relatively similar or slightly higher intensity (Figure 2, C and D),
205 irrespective of tumour subtype. These findings imply that TILs may also contribute to the
206 levels of soluble cytokines detected in breast TIF. When IP-10 and RANTES levels were
207 corrected according to the TIL scoring that was performed with the ComBat function of the
208 SVA package, greater similarity was observed among the patterns of IL-10 and RANTES
209 expression for the various tumour subtypes (Figure 2E). Thus, despite the data that show
210 IP-10 and RANTES are expressed by tumour cells, TILs also appear to contribute to the
211 total pool of secreted IL-10 and RANTES detected, particularly for the TNBC subtype.

212 **2.3. Association of TIF cytokines with morphological and clinicopathological**
213 **parameters**

214 To identify potential associations between groups of cytokines with similar profiles
215 and tumour subtype, immune cell infiltration and patient survival were subjected to
216 unsupervised hierarchical clustering for all 27 cytokines of interest. TIF-associated
217 cytokines were correlated by using K-means clustering. The corresponding heatmap is
218 presented in Figure 3A and three major cytokine clusters are shown. Most of the cytokines

219 that were present at low levels in the TIF samples (IL-17-IL15, eotaxin, IL-2, granulocyte-
220 macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1
221 (MCP-1), macrophage inflammatory protein (MIP)-1A, IL-4, and IFN-γ) were clustered
222 (cluster a). Similarly, the cytokines that exhibited medium levels of expression were
223 clustered (cluster b). A greater degree of correlation was observed among the cytokines
224 that were highly abundant, namely IP-10, IL-1β, IL-12, IL-9, PDGFβ, IL-10, IL-13, and IL-7
225 (cluster c). All of the cytokines in cluster c were also identified as being highly abundant in
226 the TIF samples compared with the NIF samples in the experiments described above
227 (Figure 1A). In particular, clusters 1 and 3 included TIF samples with high levels of
228 cytokines (cytokine cluster c). In contrast, cluster 2 included samples with a lower
229 abundance of cytokines. Cluster 3 was characterised by a higher infiltration of $CD8^+$ cells
230 and no particular association with tumour subtype was observed (Figure 3B). The
231 clinicopathological parameters, tumour grade, patient age, tumour stage, and tumour size,
232 did not significantly differ among the clusters. Furthermore, no association between
233 disease-free survival (DFS) and the patient clusters were identified according to the log-
234 rank test (Figure 3C).

235 **2.4. Secretion of IL-5 in the tumour interstitium was associated with poor prognosis**
236 **in the BC patients examined**

237 As emphasised above, it is well-known that immune cells, particularly TILs,
238 influence BC patient survival and therapy response. [10] Considering that TILs also
239 contribute to the secretion of cytokines into the tumour interstitium, we hypothesised that
240 cytokines released by a tumour may influence immune signaling to affect tumour
241 progression and disease course. To evaluate whether TIF cytokines are related to patient
242 prognosis, a survival analysis was performed for the entire dataset of 27 cytokines. For
243 this, the cytokines that were detected in the TIF samples were split into two groups

according to their expression level (e.g., high versus low as described in Materials and Methods) and then were compared with DFS. A log-rank test analysis only identified a significant association for IL-5 ($P < 0.001$; Figure 4A). The patients with high levels of IL-5 ($n = 12$) had a survival rate of 12%, with a median survival of 68.2 months and a hazard ratio of 4.17. The patients with low levels of IL-5 ($n = 66$) had a 5-year survival rate of 92%, with a median survival of 115.8 months. No association with survival have been found for each tumor subtype separately (data not shown). There was a modest trend for a positive correlation between higher levels of IL-5 in TIF and serum (Figure 4B), thereby implying that tumour-derived IL-5 could have a prognostic value in a serum analysis. However, no survival association was identified for serum levels of IL-5 (data not shown).

2.5. Correlation of cytokine levels in TIF and serum: the potential contribution of tumour-derived cytokines to the serum cytokine pool

Previous studies have shown that certain cytokines are abundant in the serum of BC patients compared with the serum of healthy individuals. [21] Studies in a mouse model of BC have also demonstrated that in the early stages of tumour progression, components of the tumour microenvironment gain access to the bloodstream. [39] Both sets of results suggest that blood-based tests have the potential to detect a host's response against a malignant tumour in its early stages. To investigate whether cytokines secreted into TIF contribute to cytokine levels in serum, Pearson's correlation coefficient was applied to the cytokine data obtained from the TIF and NIF samples. A positive, yet modest, correlation was identified for IL-7, IL-1β, IL-10, and PDGFβ in the TIF samples compared with the NIF samples (Figure 5). These results are consistent with the concept that serum levels of these cytokines are affected by the secretion of cytokines from the microenvironment of a tumour into the TIF and then into the blood.

3. Discussion

To the best of our knowledge, this study is the first to comprehensively profile a spectrum of various cytokines and growth factors in the local tumour interstitium of BC patients. A multiplex-array platform was used to comparatively assess a total of 27 cytokines and growth factors in interstitial fluid samples recovered from cancerous tissues (n = 78) and from corresponding normal tissues sampled from the vicinity of the cancer tissues (n = 43). This integrated approach allowed us to profile the cytokine landscape directly from the local tumour-environment space as a primary response to tumour metabolism, including inflammatory immune responses. These data also provide a basis for discriminating a local tumour response from systemic cytokine reactions that may be caused by stimuli not directly related to malignancy.

Eleven cytokines and growth factors were found to be consistently elevated in the breast TIF samples examined compared with the matched NIF samples. These included: IL-7, IL-10, FGF2, IL-13, IP-10, IL-1RA, PDGFβ, IL-1β, RANTES, VEGF, and IL-12. Increased levels of these cytokine/growth factors in the tumour interstitium reflect the patients' response to a growing tumour. When six of these cytokines were further examined in IHC analyses of available tissues, the contribution of these cytokines by immune cells proximal to the cancer cells appeared to be potentially greater than the contributions of the other stromal components to the total pool of cytokines. In the correlation analysis that was performed for all 27 cytokines across the four main breast subtypes characterised (i.e., HER2, Luminal A, Luminal B, and TNBC), levels of IP-10 and RANTES appeared to differentiate the TNBC subgroup from the Luminal A group. Furthermore, it should be noted that even though epithelial cancer cells in the TNBC lesions displayed high levels of both cytokines, the contribution of cytokines by the TILs to the total pool of secreted factors potentially accounts for the observed differences.

294 However, the high levels of IL-1β, IL-1RA, IL-7, IL-10, IL-12, IL-13, FGF2, PDGFβ, and
295 VEGF that were measured in the TIF samples suggest that these cytokines and growth
296 factors were generated in a local tumour niche as a general response to tumour
297 progression, independent of a specific association with immune subpopulations or tumour
298 subtype.

299 The available literature regarding a role for RANTES (CCL5) in BC is rather
300 controversial. Tumour-derived RANTES has been associated with many clinical
301 specimens of breast and cervical cancers and higher plasma levels of RANTES have been
302 identified in patients with progressive and more advanced diseases than in patients in
303 remission. [22-24] Moreover, an analysis of core needle biopsies from 113 invasive BCs
304 revealed that the mean concentration of RANTES was significantly higher in the group of
305 patients with axillary lymph node metastasis compared with those without. [25] In contrast,
306 the results from two murine mammary tumour models did not show a correlation between
307 tumour-derived RANTES expression and tumour growth rate or metastatic capacity. [26, 27]
308 In the present study, elevated levels of RANTES in the tumour interstitium of the TNBC
309 lesions were partly consistent with the results of a recent publication where TNBC cell
310 invasiveness was found to be promoted by RANTES produced by breast peritumoural
311 adipose tissue. [28] Thus, additional large-scale studies are needed to determine the
312 diagnostic and/or prognostic value of RANTES expression in BC patients.

313 Previous studies have shown that serum levels of IP-10 (CXCL10) are elevated in
314 BC patients compared to controls, [29] and also in patients with other malignancies. [30, 31]
315 Here, we provide evidence that breast tumour tissues secrete more IP-10 than non-
316 tumoural tissues in the same patient. Higher IP-10 secretion also correlated with T-cell
317 infiltration, particularly in the TNBC subtype. Previously, positive IHC staining of IP-10 in
318 BC sections correlated with a higher infiltration of T-cell lymphocytes (CD4 and CD8), [32]

319 thereby suggesting a role for IP-10 in lymphocyte recruitment. Interestingly, experimental
320 evidence has also demonstrated that IP-10 secretion by BC cells is a strong
321 chemoattractant for regulatory T-cells (γδTreg). Correspondingly, *in vivo* neutralization of
322 IP-10 has been found to inhibit the migration and trafficking of γδTreg into breast tumour
323 sites. [33] Importantly, Cxcl10 expression has been found to be of pivotal relevance for the
324 efficacy of anthracycline treatments that induce the production of type I IFNs by malignant
325 cells. For example, when the function of Cxcl10 was compromised via inactivation of
326 mediators of its signaling pathway or via neutralization of its receptor, Cxcr3, anthracycline
327 treatments did not achieve optimal therapeutic responses. [34] The present data and those
328 of others suggest that this may be due to the role of CXCL10 in lymphocyte recruitment.
329 However, additional studies are needed to elucidate the details of this possible
330 mechanism.

331 A subset of the cytokines analyzed in the present study have been shown to be
332 related to the progression of BC and other cancer types. For example, PDGF signaling is
333 recognised as being relevant for the cancer biology axis due to its experimentally
334 documented effects on malignant cells and on other cells of the tumour microenvironment.
335 [35] In the present study, PDGFβ was found to be expressed in normal mammary gland
336 tissues, particularly in the myoepithelial cell layer, and its expression was exacerbated in
337 cancer cells and in other components of the tumour stroma, including immune cells. These
338 results are consistent those of another study, [36] and also highlight the role of expression
339 levels of PDGFβ in relation to clinical outcome. For example, for tumours that express high
340 levels of PDGFβ, both *in vitro* and *in vivo* inhibition of PDGFβ has been found to prevent
341 pericyte loss and vascular permeability, thereby leading to a decrease in metastasis
342 formation. [37]

343 It was recently demonstrated that IL-1RA that is synthesised by Gr-1+ myeloid cells
344 is able to prevent the onset of senescence in a PTEN-null prostate tumour model. [38] In the
345 same study, patients with high levels of IL-1RA did not respond to chemotherapy and
346 experienced a shorter DFS period compared with patients with lower levels of IL-1RA. [38] In
347 the present study, IL-1RA was abundant in the TIF samples, with both cancer cells and
348 TILs contributing to the high levels observed. However, we did not identify any association
349 between IL-1RA levels and patient survival. The latter observation is most likely due to the
350 relatively low number of samples available and the reduced number of events.

351 IL-7 is required for the normal development of T cells in mice and humans and is
352 also needed for the maintenance of $CD4^+$ and $CD8^+$ T cells, thereby promoting expansion
353 of both naïve and memory T cells.[39] Early evidence showed that IL-7 was able to stimulate
354 the proliferation of $CD4^+$ TILs that were extracted from colorectal cancer biopsies. [40] In
355 normal breast tissues, low levels of *IL-7* transcripts have been found, while *IL-7* transcripts
356 are generally absent in BC cell lines. In contrast, IL-7 receptor (*IL7R*) transcripts have
357 been found in both BC cell lines and in normal breast tissue. [41] Consistent with these
358 previous observations, BC tissues were found to express higher levels of IL-7 than the
359 normal breast tissues that were examined in the present study.

360 IL-10 is a molecule with immunosuppressive and immunostimulatory properties. In
361 diffuse large B-cell lymphoma [42] and gastric cancer [43], elevated plasma levels of IL-10
362 have correlated with poor prognosis. A strong correlation between BC progression and IL-
363 1β levels has also been observed. [44] In a study by Kurtzman et al. [45] elevated levels of IL-
364 1β were observed in 90% of invasive BCs, with cellular localization of IL-1β observed in
365 both cancer cells and stromal cells. In general, expression of IL-1β has been associated
366 with more aggressive phenotypes in breast tumours. [46, 47]

367 The use of inflammatory mediators as biomarkers is not straightforward since they
368 are often present at higher levels in both cancers and non-neoplastic
369 pathologies/conditions. However, certain inflammatory mediators may be generated as
370 part of a general response to cancer. In mouse models of BC, cancer progression evokes
371 a rapid physiological response from the tumour microenvironment, including immune
372 response signaling. These changes induce a release of proteins into the plasma, including
373 cytokines, angiogenic factors, and extracellular matrix components. [39] Moreover, this
374 release has been found to occur before the onset of a clinically detectable cancer. [48] In the
375 present study, PDGFβ, IL-7, IL-1β, and IL-10 exhibited an association between their levels
376 in TIF samples and their levels in matched sera. These results support the hypothesis that,
377 for a subset of BC patients, an increase in serum levels of cytokines is due to the
378 production of these cytokines within a tumour. Thus, providing a readout of biological
379 processes that are directly associated with cancer development/progression. Additional
380 studies of large series of samples are needed to confirm these results and to determine
381 their potential usefulness for achieving a reliable diagnosis of BC.

382 It has been well-characterised that the activation of $CD8^+$ cells is mediated by the
383 T_h1-response, and this process plays an important role in the treatment of BC either by
384 conventional, or targeted, therapy in combination with radiotherapy. [10, 49] In contrast, a low
385 density of T cells has been associated with poor prognosis for both colorectal cancer [9, 50]
386 and BC. [51] Here, high levels of IL-5 expression in TIF samples were identified as a factor in
387 poor prognosis. IL-5 is a hallmark cytokine of the T_h2 response that is associated with
388 allergies and parasitic infections. IL-5 also has a prominent role in the promotion of B cell
389 and eosinophil differentiation and proliferation. [52] Correspondingly, cumulative evidence
390 supports a critical role for IL-5 in cancer prognosis. In lung cancer models, depletion of IL-
391 5 reduced metastasis, while the administration of recombinant IL-5 to IL-5 knockout mice

392 significantly increased pulmonary metastasis. [53] Similarly, exogenous administration of IL-5
393 to mice was found enhance malignant pleural effusions, a pathological consequence of
394 cancer that is predominantly observed in lung and breast adenocarcinomas. [54] In bladder
395 cancer, IL-5 expression is associated with a muscle-invasive phenotype, [55] while *in vitro*,
396 IL-5 treatment increased the migration and invasion capacities of bladder cancer cells via
397 the MMP-9/NF-κB/AP-1 pathway. [56] A previous study also demonstrated that BCs with a
398 higher metastatic capacity express significantly higher levels of *IL-5* mRNA, and these
399 results are consistent with the present results. [57] In addition, it was recently shown that BC
400 patients with high serum levels of IL-5 had a higher frequency of positive lymph nodes. [58]
401 The latter results are consistent with the present findings as well, and also suggest a role
402 for IL-5 in BC metastasis. There was no association identified between serum levels of IL-
403 5 and patient survival in the present study. However, it is possible that the relatively low
404 number of samples available and the reduced number of events may have contributed to
405 this result. In an independent BC cohort (MicMa), [59, 60] IL-5 levels were assessed using the
406 same technology used in the present study and a non-significant tendency towards a bad
407 prognosis was observed in patients with high serum levels of IL-5 expression (unpublished
408 data, Jabeen *et al.*, personal communication). Therefore, further studies are needed to
409 confirm the role of IL-5 and patient prognosis.

410 In the present study, IL-4 was not identified as a prognosis factor, yet it is
411 considered another hallmark modulator of the T_h2 response. [61] Moreover, similar to IL-5, a
412 role for IL-4 in the promotion of invasive and metastatic behavior of BC cells has been
413 proposed, [62, 63] thereby supporting a role for T_h2 signaling and its detrimental response.
414 Enabling of a T_h1 response appears to be related to a higher frequency of mutation rates
415 in mismatch repair-deficient tumours, where it has been shown that mismatch-repair status
416 predicts the clinical benefit of blocking immune checkpoints with pembrolizumab. [64] This

417 observation also strongly supports the hypothesis that a high number of mutation-
418 associated neo-antigens are more likely to stimulate an immune response against a
419 tumour. However, it remains unclear whether a low rate of mutations is sufficient to
420 establish a T_h2 response in tumours, or if this process depends on other mechanisms that
421 have yet to be identified. Based on the evidence presented here that IL-5 is associated
422 with a poor prognosis in BC cases, and the observations published by other authors that
423 T_h2 cells and other T_h2-associated cytokines promote the invasion and metastasis, [65]
424 support for therapeutic strategies that inhibit or reverse the T_h2 response in tumours to
425 improve patient survival is provided. [65]

426 **4. Conclusion**

427 The exacerbated production and secretion of cytokines and growth factors by
428 cancer cells and tumour-infiltrating immune cells is a consistent feature of BC tissues.
429 Here, we provide evidence that tumour-infiltrating lymphocytes are contributors to the total
430 pool of secreted cytokines, and in some cases, the extent of these secretions are BC
431 subtype-dependent. Furthermore, the leakage of tumour-produced cytokines into the
432 bloodstream may account for the higher levels of certain cytokines in the serum of BC
433 patients. Of particular interest is the finding that the intratumour levels of IL-5, a T_h2-
434 cytokine, were associated with poor prognosis in the group of BC patients that was
435 examined. Consequently, further studies are needed to confirm and address the biological
436 and clinical relevance of IL-5 in human BC.

437 **5. Materials and methods**

438 **2.1 Clinical samples: tumour tissues, matched non-malignant tissues, and serum**

439 Fresh samples of tumour tissue and non-malignant tissue distant (about 5 cm) to
440 the tumour margin were collected from patients defined as high-risk according to the

441 Danish Breast Cooperative Group (www.dbcg.dk accessed 22.10.2009 [16]) that underwent
442 a mastectomy between 2003 and 2012 as part of the Danish Center for Translational
443 Breast Cancer Research program. All of the patients presented a unifocal tumour with an
444 estimated size of more than 20 mm in diameter and none of the patients had a history of
445 breast surgery or had received preoperative treatment. The age range of the selected
446 cohort was 32–84 years (median age= 68.5 years). Patients were followed after surgery
447 and cancer-specific survival was measured from the date of primary surgery until the date
448 of death from BC. The date and cause of death were assigned in accordance with the
449 Danish Cancer Registration System and the Danish Register of Cause of Death. Death
450 records were complete up to 2014-10-08 and served as the censor date. Registered
451 clinicopathological data for the patients were available from the Department of Pathology,
452 Rigshospitalet, Copenhagen University Hospital, Denmark. This study was conducted in
453 compliance with the Helsinki II Declaration and written informed consent was obtained
454 from all participants. This project was approved by the Copenhagen and Frederiksberg
455 regional division of the Danish National Committee on Biomedical Research Ethics (KF 01-
456 069/03).

457 At the time of collection, each tumour biopsy and matched non-malignant tumour
458 biopsy were divided into two pieces. One piece was stored at −80 °C and was
459 subsequently prepared as a FFPE sample that was sectioned, mounted on glass slides,
460 and stained for histological characterization, tumour subtyping, TIL scoring, and IHC
461 studies. The second biopsy piece was placed in PBS at 4 °C within 30–45 min of surgical
462 excision and then was subjected to interstitial fluid recovery (see below).

463 Matched sera were obtained from women that were enrolled in the Danish Center
464 for Translational Breast Cancer Research program and underwent surgery between 2001

465 and 2006. Blood samples were collected preoperatively following a standardised protocol.
466 [66] The samples had only undergone one freeze/thaw cycle before they were analyzed.

467 **2.2 Histological assessment of tissue biopsies: IHC and breast tumour subtyping**

468 IHC analysis was performed as described previously to conduct histological
469 characterizations of the tissue samples collected. [16] First, small FFPE blocks were
470 prepared from 2-3 various parts of the tissue piece and the sections were stained with a
471 CK19 (*KRT19*) antibody. Tissue morphology and estimates of tumour cell content were
472 made. [15] A visual assessment of tumour-stroma percentages were evaluated as previously
473 described. [67] All of the slides were blindly reviewed (IIG, PSG).

474 Subtype scoring of the tumour tissues as Luminal A, Luminal B, HER2, or TNBC
475 was performed based on the estrogen receptor (ER), progesterone receptor (PgR), HER2,
476 and Ki67 status of each tissue in accordance with the St. Gallen International Breast
477 Cancer Guidelines. [68] For tumour stratification, the ER- and PgR-positive cases were
478 considered negative when the percentage of nuclear immunoreactivity within the invasive
479 cancer cells was $< 1\%$. The cases with $\geq 1\%$ of the invasive cancer cells positively stained
480 were classified as positive. Cases were considered HER2-positive if their membrane
481 positivity was 3+ and/or the fluorescence *in situ* hybridization (FISH) ratio of HER2 to
482 CEP17 was ≥ 2.0. For a HER2 IHC score of 2+, this was also evaluated by FISH and a
483 value < 2.0 was considered negative and a value ≥ 2.0 was considered positive. Mean
484 Ki67 expression was used for subtype estimation and the cutoff for Ki67 positivity was
485 assigned in accordance with currently accepted criteria. [69] Ki67 index values were
486 measured using the open access web application, ImmunoRatio, to perform automated
487 image analysis. [70] The list of patients analyzed in this study, including sample type
488 collected and tumour subtype identified, are presented in Supplementary Table 2. In
489 Supplementary Table 4, the antibodies used in this study are listed. For tumour subtyping,

490 antibodies recognizing ER, PgR, HER2, and Ki67 were used. For TIL subpopulation
491 scoring, antibodies recognizing CD3, CD4, CD8, CD45 and CD68 were used. For cytokine
492 detection, antibodies recognizing RANTES, PDGFβ, IP-10, IL-1RA, IL10, and VEGF were
493 used. Standardization of the dilution, incubation, and development times appropriate for
494 each antibody allowed an accurate comparison of expression levels in all cases. In all of
495 the antibody staining studies conducted, positive and negative control slides were
496 analyzed in parallel, with the latter incubated with PBS instead of primary antibodies.

497 **2.3 Estimation of TILs and their subpopulations**

498 The proportion of TILs in tissue sections was evaluated in accordance with the
499 recommendations of the International TILs Working Group 2014. [71] An assessment of
500 overall inflammatory reactions and the number of lymphoid cells present within biopsies
501 were determined for haematoxylin and eosin-stained sections according to a previously
502 described protocol [72] that included three categories for scoring of the stainings: (1+):
503 absence of a lymphocyte infiltrate, (2+): partial infiltration by lymphocytes, and (3+):
504 lymphocyte-predominant BC depending on the observed distribution of lymphocyte
505 localization (see Supplementary Figure 2 and Supplementary Table 2). IHC analyses were
506 also performed to examine the most prominent components of the immune
507 microenvironment in the breast tumours examined. The distribution of TILs was evaluated
508 with IHC according to the detection of $CD3^+$ cells, $CD4^+$ cells, and $CD8^+$ cells to identify T
509 cells, helper T cells, and cytotoxic T cells, respectively. Scoring of these stainings was
510 performed as previously reported [73-76], with the same cut-off criteria used for the positively
511 stained cells as described above: 1+ (> 10%), 2+ (10–50%), 3+ (> 50%). These scores
512 were independently and blindly assigned (IIG, PSG) and any discrepancies were resolved
513 by consensus. The macrophage marker, CD68, was also evaluated with the same criteria.

For each immune cell population that was analyzed, the expression results were dichotomized as low (< 10%) and high (> 10%).

2.4 Recovery of TIF

TIF and NIF samples were extracted from small surgically resected breast tumour pieces and from normal breast epithelial tissues that were collected proximal to the tumour cells, respectively, as previously described. [77] Briefly, for each sample, approximately 0.1–0.3 g of clean tissue was cut into small pieces (~1 mm^3 each), washed twice in cold PBS to remove blood and cell debris, and then incubated in PBS for 1 h at 37 °C in a humidified CO_2 incubator. The samples then were centrifuged consecutively at 1000 rpm and 5000 rpm for 2 min and 20 min, respectively, each at 4 ^{o}C. After the supernatants were carefully aspirated, total protein concentration for each sample was determined with the Bradford assay. [78]

2.5 Luminex xMAP assay

A total of 27 cytokines, including ILs, chemokines, growth factors, IFN, and tumour necrosis factor (TNF), were analyzed in a 27-plex commercially available cytokine panel from Bio-Rad (Lot #: 5029511) (Supplementary Table 1). Interstitial fluids obtained from 78 breast tumour tissues and 43 normal breast tissues, as well as 25 serum samples (see above), were analyzed. Total protein concentrations were determined for each sample in a series of control standard dilutions as instructed by the manufacturer. The same amount of each sample was than analyzed with the Luminex xMAP 200 platform. The results obtained were then collected and processed with Bio-Plex Manager 6.0 (Bio-Rad).

2.6 Data normalization and statistics

Statistical analysis was performed using the R statistical programming environment. For data normalization, the observed concentrations were log transformed using a pseudo count of 0.5. Next, the significant abundance of each cytokine in tumour samples versus normal samples was calculated using a paired *t*-test. *P*-values were adjusted for multiple hypothesis testing using Bonferroni correction. Associations between immune subpopulations (e.g., TILs and CD markers) and tumour subtypes were assessed using Fisher's exact test and an X^2 test. Immune subpopulations with scores ≥ 2 and < 2 were labeled as high and low, respectively. Correlation of cytokine levels between TIF and serum samples were computed using Pearson's correlation coefficient. To address TILs as a source of variation for selected cytokines, TIF correction according to TIL status was performed using the ComBat function of the SVA package. [79] The levels of cytokines were analyzed using ANOVA to test the difference of the mean between the tumour subtypes. Clustering of TIF samples according to cytokine levels was performed using K-means clustering with $k = 3$.

2.7 Survival analysis

To divide the samples assessed into groups according to high versus low cytokine secretion, the R-package MaxStat was used. [80] A 10-fold cross-validation was then performed by dividing the data set into ten parts and the cutoff value from 9 of the parts was used to assign a group label to the tumours of the 10th part. Survival analysis in R was also performed. [81] Statistical significance of the curves obtained was determined by using the log-rank test. DFS was measured from the time of surgery until the date of first recurrence or the date of death from BC. The patients that survived or died due to other causes were censored.

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798

799

Figure legends

Figure 1. Differential abundance of cytokines in TIF and NIF samples. A) Cytokines differentially presented in NIF and TIF samples. Pairs of samples with at least one missing value were excluded from this analysis. Paired t-test, adjusted *P*-values: $*P < 0.05$; $***P < 0.001$. **B)** IHC images showing expression of IL-10, IP-10, IL-1RA, PDGFβ, RANTES and VEGF in representative pairs of tissue sections corresponding to the same NIF and TIF pair. Red arrows show positive staining in ductal epithelial cells within normal and malignant lesions. Scale bar = 100 µm.

Figure 2. Expression of IP-10 and RANTES among breast cancer subtypes. **A)** Expression levels of IP-10 and RANTES according to tumour subtype; ANOVA test, $*P < 0.05$. **B)** Expression of IP-10 and RANTES according to immune cell infiltration status;unpaired t test $**P < 0.01$; $***P < 0.001$. **C)** Representative IHC images of serial sections showing the expression of IP-10 and RANTES in cancer cells (blue arrows) and in areas with lymphocyte infiltration (red arrows; CD45, CD3 and CD8 markers) in a Luminal B tumour. Scale bar = 100 µm. **D)** Representative IHC images showing cancer cells (blue arrows) and TILs (red arrows) expressing IP-10 and RANTES in a HER2 and Luminal B tumour section, respectively. Scale bar = 20 µm. **E)** Expression levels of IP-10 and RANTES among tumour subtypes adjusted according to TILs infiltration using the ComBat function.

Figure 3. Hierarchical clustering. A) Heatmap of clustered cytokines (columns) and TIF samples (rows). Minimum and maximum normalised levels are shown in yellow and grey, respectively. K-means was used as the clustering method. **B)** Association between TIF clusters, tumour subtypes and immune cell subpopulations. **C)** Kaplan-Meier plot illustrating DFS survival in patients with breast cancer according TIF clusters (N = 78), analyzed using a log-rank test.

Figure 4. IL-5 and breast cancer survival. **A)** Kaplan-Meier DFS survival curves illustrating survival in patients with breast cancer according to IL-5 log-expression in TIF samples (N = 78). **B)** Correlation analysis for IL-5 between TIF and serum levels (N = 13).

831 **Figure 5. Correlation between cytokine levels in TIF and serum.** Correlations were
832 calculated using Pearson's correlation coefficient (R).

833

834

835

836 **Table 1**. Association between breast cancer subtypes, total level of TILs, lymphocytes
837 subpopulations, and macrophages

Immune cell subpopulation	Frequencies per tumour subtype N (%)*				
	HER2	Lum A	Lum B	TNBC	Total
TILs Low	2 (29)	28 (72)	7 (37)	1 (8)	38 (49)
TILs High	5 (71)	11 (28)	12 (63)	12 (92)	40 (51)
CD3 Low	4 (80)	36 (92)	12 (66)	7 (58)	59 (80)
CD3 High	1 (20)	3 (8)	6 (34)	5 (42)	15 (20)
CD4 Low	3 (50)	33 (84)	13 (72)	5 (39)	54 (71)
CD4 High	3 (50)	6 (16)	5 (28)	8 (61)	22 (29)
CD8 Low	5 (83)	35 (90)	14 (77)	9 (75)	63 (84)
CD8 High	1 (17)	4 (10)	4 (23)	3 (25)	12 (16)
CD68 Low	5 (83)	31 (80)	11 (61)	5 (42)	52 (69)
CD68 High	1 (17)	8 (20)	7 (39)	7 (58)	23 (31)

Subtypes	*P* values**				
	TILs	CD3	CD4	CD8	CD68
Lum A - LumB	0.01	0.021	ns	ns	ns
Lum A - HER2	0.027	ns	ns	ns	ns
Lum A - TNBC	<0.001	0.012	0.002	ns	0.025
Lum B - HER2	ns	ns	ns	ns	ns
Lum B - TNBC	ns	ns	ns	ns	ns
TNBC - HER2	ns	ns	ns	ns	ns

838 *Number of samples and % for each subtype are presented
839 ** Fisher's exact test; ns = not significant
840

A

IL−7 *** N=41
IL−10 *** N=34
FGF2 *** N=39
IL−13 *** N=42
IP−10 *** N=29
IL−1RA *** N=26

PDGFβ *** N=25
IL−1β *** N=42
IL−6 * N=42
RANTES * N=28
VEGF * N=23
IL−12 * N=42

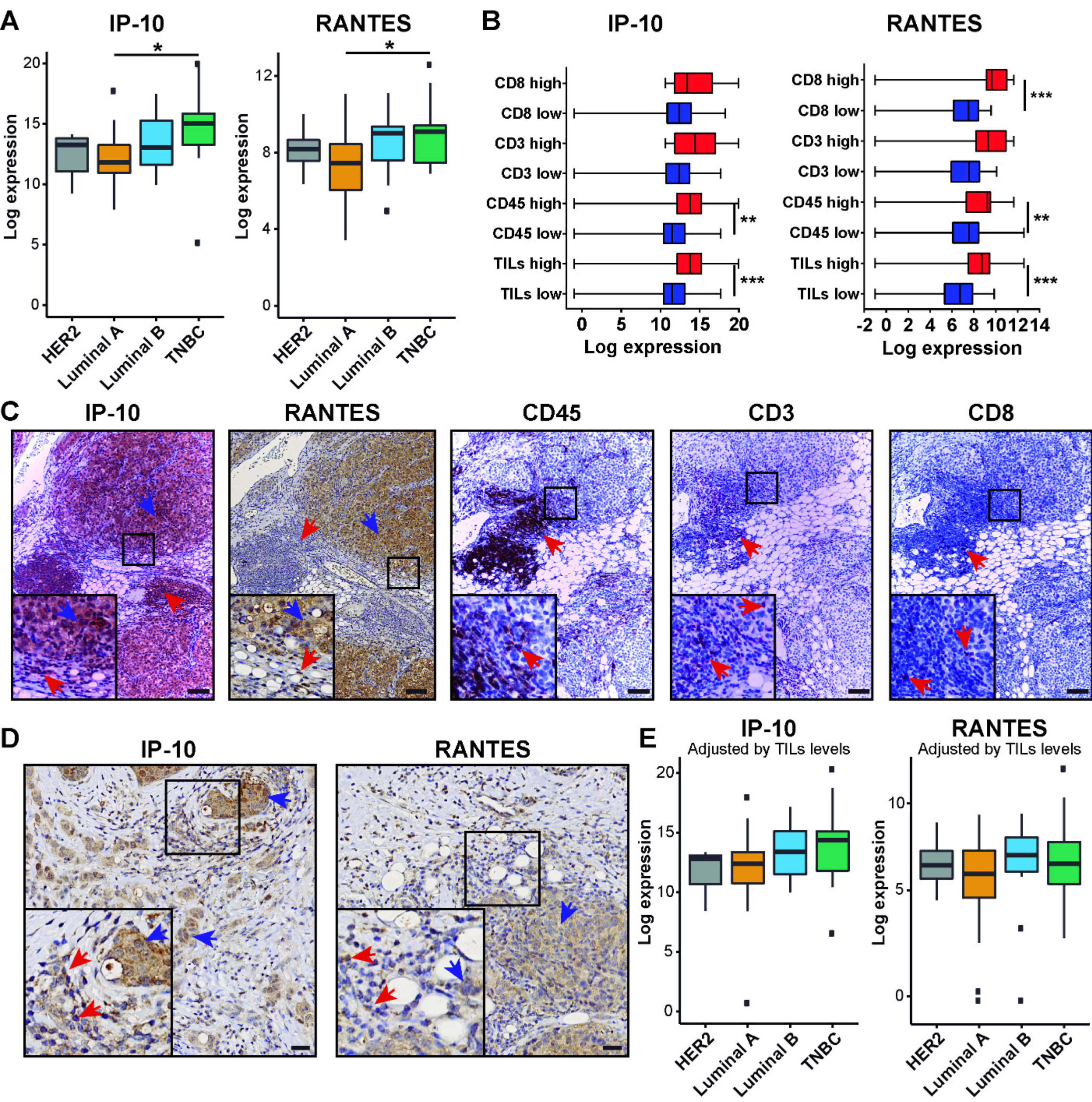
Log expression

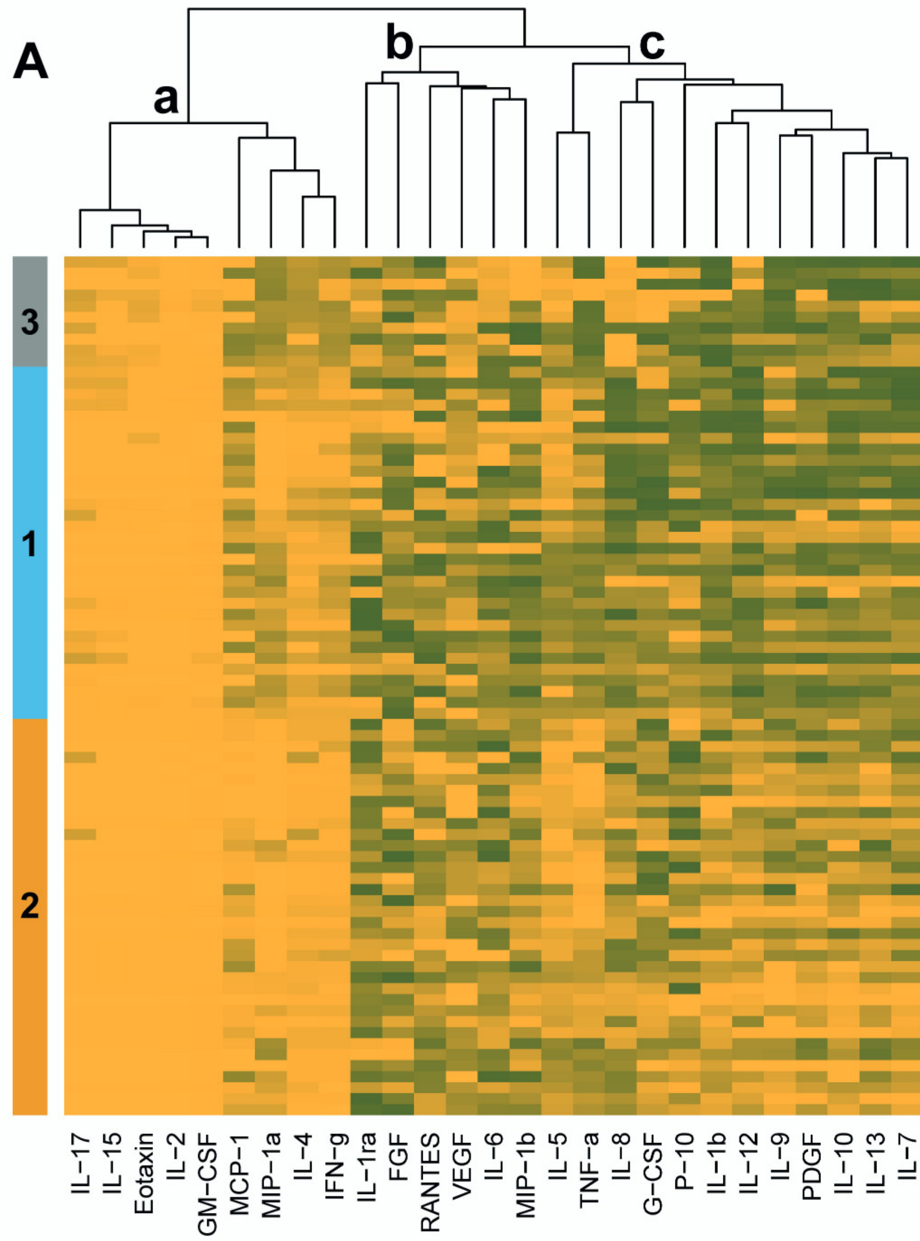
NIF TIF

B

Normal Tumour Normal Tumour

IL-10
IP-10
IL-1RA
PDGFβ
RANTES
VEGF


A
IP-10
RANTES
Log expression
HER2
Luminal A
Luminal B
TNBC
B
CD8 high
CD8 low
CD3 high
CD3 low
CD45 high
CD45 low
TILs high
TILs low
Log expression
C
IP-10
RANTES
CD45
CD3
CD8
D
IP-10
RANTES
E
IP-10
Adjusted by TILs levels
RANTES
Adjusted by TILs levels

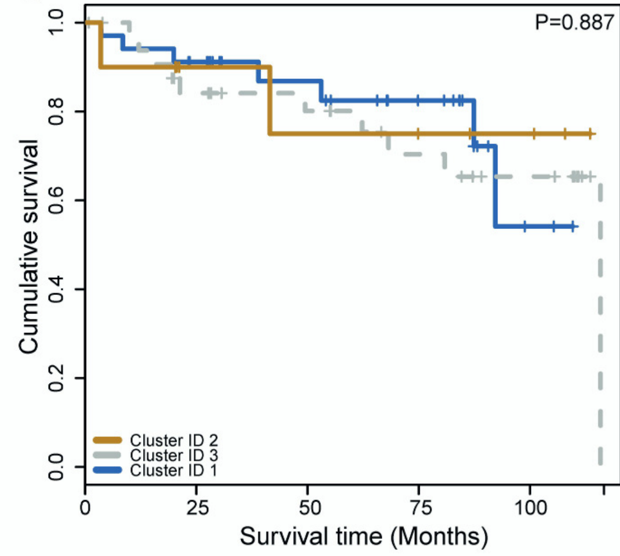


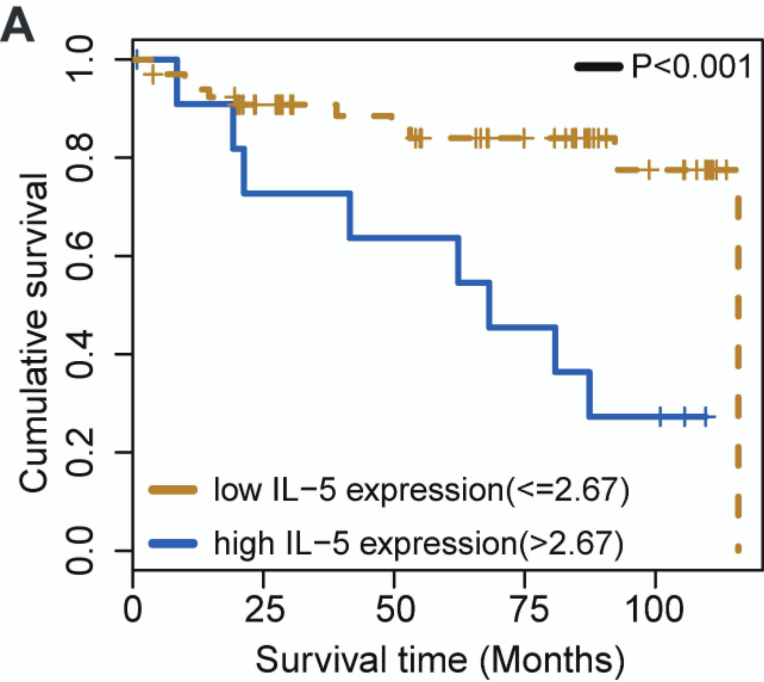
B

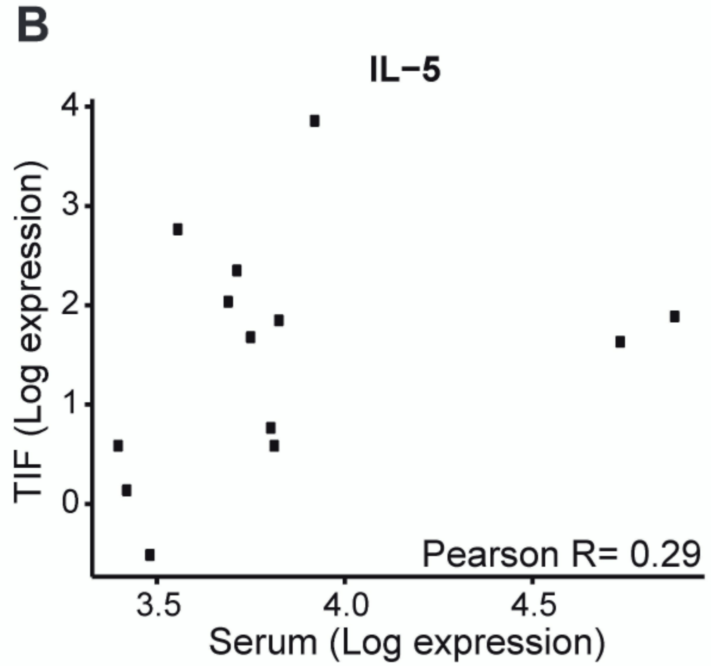
Associations between subtypes, immune infiltrates and clusters

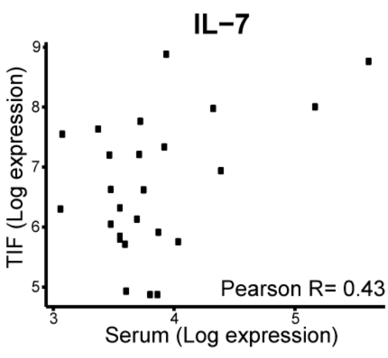
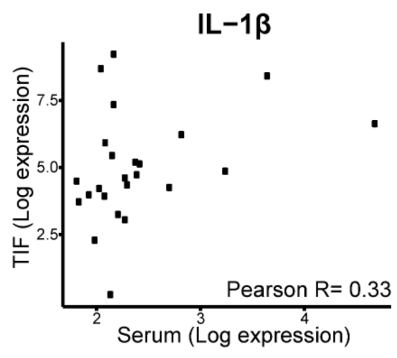
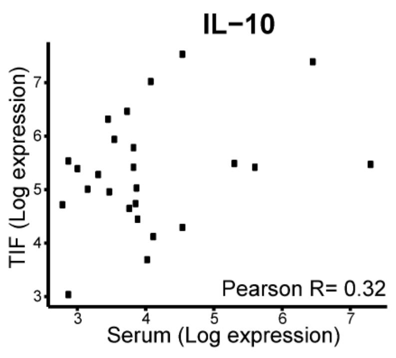
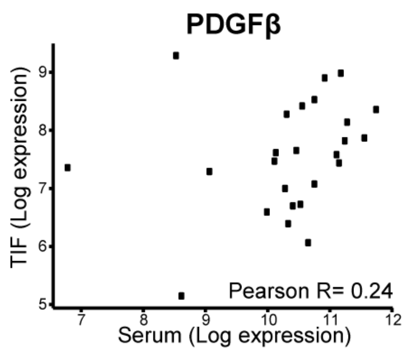
		Cluster			
		1	2	3	*P value
Subtype	LumA	22	15	2	ns
	LumB	7	8	4	
	HER2	3	3	1	
	TNBC	4	6	3	
TILs	Low	16	17	5	ns
	High	20	15	5	
CD3	Low	27	24	8	ns
	High	9	5	1	
CD4	Low	24	22	8	ns
	High	12	8	2	
CD8	Low	30	28	5	0.02
	High	6	2	4	
CD68	Low	26	19	7	ns
	High	10	11	2	

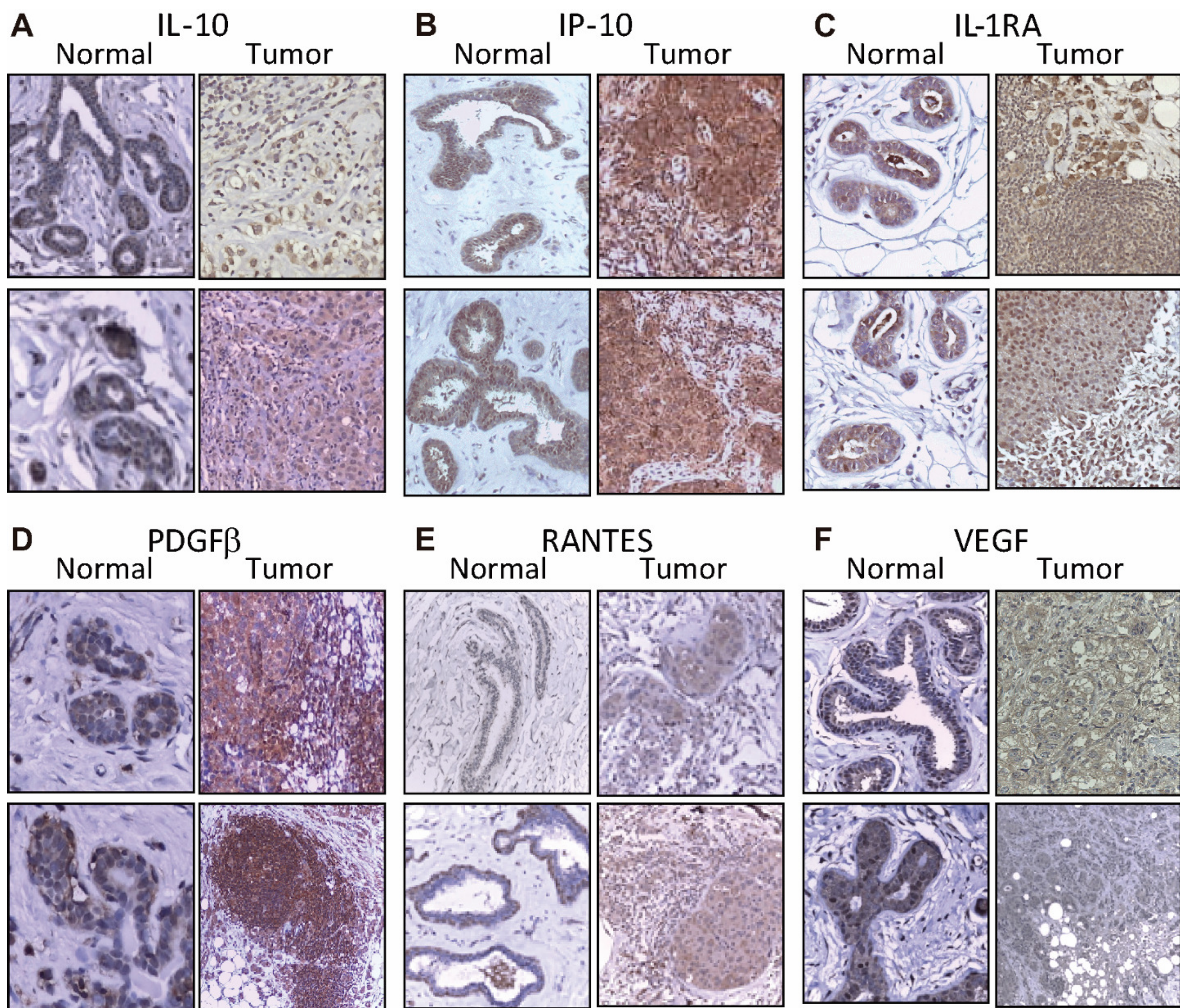
*X^2 square test; ns=not significant




A
P<0.001
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
low IL−5 expression(<=2.67)
high IL−5 expression(>2.67)
0
25
50
75
100
Survival time (Months)

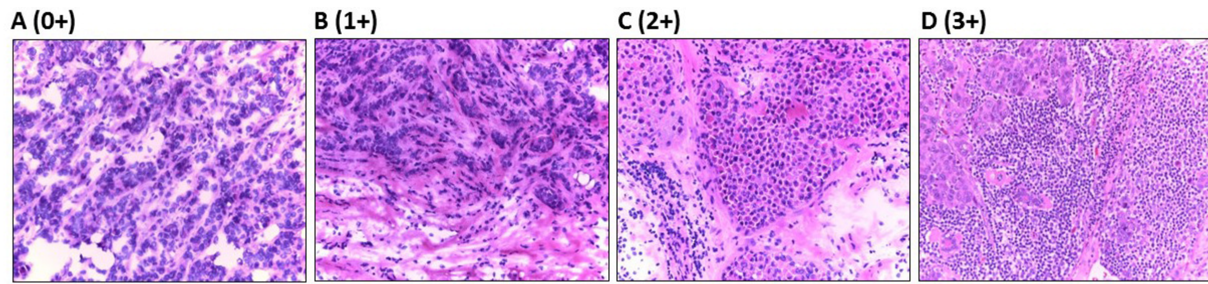

B
IL−5
TIF (Log expression)
4
3
2
1
0
Pearson R= 0.29
3.5
4.0
4.5
Serum (Log expression)


IL−7
TIF (Log expression)
Serum (Log expression)
Pearson R= 0.43

IL−1β
TIF (Log expression)
Serum (Log expression)
Pearson R= 0.33

IL−10
TIF (Log expression)
Serum (Log expression)
Pearson R= 0.32

PDGFβ
TIF (Log expression)
Serum (Log expression)
Pearson R= 0.24



Summary of immunohistochemistry validation pairs of sections NIF / TIF			
	Normal	**Tumor**	
Cytokine	**Normal ducts**	**Tumoral cells**	**Tumoral leukcocytes**
IL-10	Low (+) to moderate (++) staining in >90% of cells in 8 of 8 samples. Cytoplasmic staining in myoepithelial and luminal cells	Low (+) cytoplasmic staining in >90% of cells in 16 of 16 samples.	Low (+) cytoplasmic staining in 16 of 16 samples.
IP-10	Low (+) to moderate (++) staining in 11 of 13 samples. Cytoplasmic staining in myoepithelial and luminal cells.	Moderate (++) to strong (+++) cytoplasmic staining in >90% of 13 of 13 samples	Low (+) to moderate (++) cytoplasmic staining in 8 of 13 samples
IL-1RA	Low (+) to moderate (++) staining in >50% of cells in 10 of 11 samples. Cytoplasmic staining in myoepithelial and luminal cells, with occasional nuclear staining.	Moderate (++) to strong (+++) cytoplasmic staining in >90% of 11 of 11 samples	Low (+) cytoplasmic staining in 5 of 11 samples
PDGF	Low (+) to moderate (++) staining in 15 of 17 samples. Cytoplasmic staining in myoepithelial and luminal cells.	Moderate (++) to strong (+++) cytoplasmic staining in >90% of 16 of 17 samples	Moderate (++) cytoplasmic staining in 11 of 17 samples
RANTES	Moderate (++) staining intensity in >90% of cells in 11 of 17 samples. Staining more intense in myoepithelial cells than in luminal cells.	Moderate (++) cytoplasmic staining in >90% of cells in 15 of 17 samples.	Low (+) staining cytoplasmic staining in 11 of 17 samples.
VEGF	Low (+) to moderate (++) staining in >90% of cells in 15 of 15 samples. Cytoplasmic staining in myoepithelial and luminal cells, with occasional nuclear staining	Low (+) to moderate (++) cytoplasmic staining in >90% of cells in 17 of 17 samples.	Low (+) to moderate (++) cytoplasmic staining in 16 of 16 samples. TILs equally intense than tumoral cells

Supplementary figure 1. Immunohistochemical validation of selected cytokines. A) IL-10 expression in 2 pairs of Normal/Tumour tissue sections. B) IP-10 expression in 2 pairs of Normal/Tumour tissue sections. C) IL-1RA expression in 2 pairs of Normal/Tumour tissue sections. D) PDGFβ expression in 2 pairs of Normal/Tumour tissue sections. E) RANTES expression in 2 pairs of Normal/Tumour tissue sections. F) VEGF expression in 2 pairs of Normal/Tumour tissue sections. Table depicts the staining summary for each cytokine among the Normal/Tumour pairs analyzed.



Supplementary figure 2. Examples of TILs distribution within tumour biopsies based on Haematoxylin & Eosin staining. The scoring was done based on the analysis of the entire biopsy section. The representative areas for each scoring is shown. (A) Luminal A tumour lacking of TILs infiltration (0+), (B) Luminal B tumour having low TILs infiltration (1+), (C) Luminal B/HER2 enriched tumour with intermediate TILs infiltration (2+) and (D) TNBC tumour with high proportion of TILs (3+).

Supplementary Table 1. Cytokines and growth factors evaluated in this study

Cytokine	Gene symbol	Gene name
MCP-1	CCL2	Chemokine (C-C motif) ligand 2
MIP-1a	CCL3	Chemokine (C-C motif) ligand 3
MIP-1b	CCL4	Chemokine (C-C motif) ligand 4
RANTES	CCL5	Chemokine (C-C motif) ligand 5
Eotaxin	CCL11	Chemokine (C-C motif) ligand 11
IL-8	CXCL8	Chemokine (C-X-C motif) ligand 8
IP-10	CXCL10	Chemokine (C-X-C motif) ligand 10
FGF	FGF2	Fibroblast growth factor 2 (basic)
VEGF	VEGFA	Vascular endothelial growth factor A
PDGFβ	PDGFB	Platelet-derived growth factor beta polypeptide
GM-CSF	CSF2	Colony-stimulating factor 2
G-CSF	CSF3	Colony-stimulating factor 3
TNF-a	TNF	Tumour necrosis factor
IFN-γ	IFNG	Interferon, gamma
IL-1RA	IL1RN	Interleukin 1 receptor antagonist
IL-1b	IL1B	Interleukin 1, beta
IL-2	IL2	Interleukin 2
IL-4	IL4	Interleukin 4
IL-5	IL5	Interleukin 5
IL-6	IL6	Interleukin 6
IL-7	IL7	Interleukin 7
IL-9	IL9	Interleukin 9
IL-10	IL10	Interleukin 10
IL-12	IL12A	Interleukin 12
IL-13	IL13	Interleukin 13
IL-15	IL15	Interleukin 15
IL-17	IL17A	Interleukin 17

DCTB number	TIF (78)	CODE FOR TREATMENT
DCTB 052	YES	BWHC1
DCTB 054	YES	No data
DCTB 057	YES	BWHA1
DCTB 058	YES	BWHA1
DCTB 060	YES	BWHC
DCTB 061	YES	KHAC25
DCTB 062	YES	No data
DCTB 063	YES	KHAC25
DCTB 064	YES	BWHA1
DCTB 065	YES	KHAC25
DCTB 066	YES	BWHC; BWGC1
DCTB 069	YES	BWHC; BWHA1
DCTB 070	YES	KHAC25
DCTB 072	YES	BWHC; BWHC1
DCTB 073	YES	BWHC; BWGC1
DCTB 074	YES	BWHA1
DCTB 076	YES	KHAC25
DCTB 077	YES	BWHA1;BWHA2; BWGC1
DCTB 078	YES	BWHA1
DCTB 079	YES	BWHA1
DCTB 080	YES	BWHA1; BWHC1
DCTB 081	YES	BWHA1
DCTB 083	YES	BWHA2; BWHC1; BWHC2
DCTB 084	YES	BWHA1; BWGC1
DCTB 085	YES	KHAC25
DCTB 086	YES	BWHC1
DCTB 088	YES	BWHC1
DCTB 091	YES	BWHC1; BWGC7
DCTB 094	YES	BWHA1; BWGC1
DCTB 102	YES	BWHC1
DCTB 104	YES	BWHC1; BWHB40
DCTB 105	YES	BWHC1
DCTB 106	YES	KHAC25
DCTB 109	YES	KHAC25
DCTB 110	YES	BWHC1
DCTB 111	YES	BWHA1
DCTB 112	YES	BWHA1
DCTB 115	YES	BWHC2; BWHC20
DCTB 116	YES	KHAC25
DCTB 117	YES	KHAC25
DCTB 118	YES	BWHA1; BWHC1
DCTB 119	YES	BWHC1
DCTB 120	YES	BWHA1; BWHA2; BWGC5
DCTB 122	YES	BWHC1
DCTB 124	YES	BWHA1; BWHC1
DCTB 125	YES	BWHC2
DCTB 127	YES	BWHA1; BWGC7A
DCTB 128	YES	BWHA1
DCTB 129	YES	BWGC5
DCTB 131	YES	BWHC1
DCTB 133	YES	BWGC5
DCTB 155	YES	BWHA1; BWHA2; BWGC5

DCTB 156	YES	BWHA1; BWHA2
DCTB 157	YES	KHAC25
DCTB 158	YES	BWGC7A
DCTB 161	YES	BWHA1; BWHA2; BWGC5
DCTB 199	YES	BWGC7; BWGC7A
DCTB 200	YES	KHAC25
DCTB 201	YES	BWHA1; BWHA2; BWHC1; BWGC7A
DCTB 202	YES	KHAC25
DCTB 203	YES	BOHE20A; BWHA139; BWHA202
DCTB 223	YES	BOHJ13; BWHA1; BWHA2; BWGC5; BOHE20A
DCTB 229	YES	BWGC7; BWGC7A; BWHA247; BOHE20A
DCTB 231	YES	BOHE20A; BWGC5 BWHA139; BWHA208
DCTB 232	YES	BOHE20A; BWHC; BWHA139; BWHA208
DCTB 234	YES	BWHA1; BWHA2
DCTB 235	YES	BWHC1
DCTB 237	YES	BOHE20A; BWHC; BWHA139; BWHA208; BWHA
DCTB 258	YES	BWGC5A
DCTB 264	YES	BWHC1
DCTB 279	YES	BWHA1; BWHA2; BWHC1; BWGC5
DCTB 289	YES	KHAC25
DCTB 290	YES	BWGC5A
DCTB 293	YES	BWGC5A
DCTB 295	YES	BWGC1; BWGC5; BWGC5A
DCTB 302	YES	KHAC25
DCTB 374	YES	KHAC25
DCTB 383	YES	BOHE20A; BWHA105; BWHA208

TYPE OF TREATMENT
Treatment with antiestrogen
No data
Basic cytostatic treatment
Basic cytostatic treatment
Hormonal cancer therapies
Radical mastectomy. No postmastectomy treatment
No data
Radical mastectomy. No postmastectomy treatment
Basic cytostatic treatment
Radical mastectomy. No postmastectomy treatment
Hormonal cancer therapies. Conventional external beam radiation
Hormonal cancer therapies. Basic cytostatic treatment
Radical mastectomy. No postmastectomy treatment
Hormonal cancer therapies. Treatment with antiestrogen
Hormonal cancer therapies. Conventional external beam radiation
Basic cytostatic treatment
Radical mastectomy. No postmastectomy treatment
Basic cytostatic treatment. Complex cytostatic treatment. Conventional external beam radiation
Basic cytostatic treatment
Basic cytostatic treatment
Basic cytostatic treatment. Treatment with antiestrogen
Basic cytostatic treatment
Complex cytostatic treatmnet. Treatment with antiestrogen. Treatment with enzyme inhibitor
Basic cytostatic treatment. Conventional external beam radiation
Radical mastectomy. No postmastectomy treatment
Treatment with antiestrogen
Treatment with antiestrogen
Treatment with antiestrogen. Radiation therapy with gating
Basic cytostatic treatment . Conventional external beam radiation
Treatment with antiestrogen
Treatment with antiestrogen.Treatment with bisfosfonat
Treatment with antiestrogen
Radical mastectomy. No postmastectomy treatment
Radical mastectomy. No postmastectomy treatment
Treatment with antiestrogen
Basic cytostatic treatment
Basic cytostatic treatment
Treatment with enzyme inhibitor. Treatment with Exemestane
Radical mastectomy. No postmastectomy treatment
Radical mastectomy. No postmastectomy treatment
Basic cytostatic treatment. Treatment with antiestrogen
Treatment with antiestrogen
Basic cytostatic treatment. Complex cytostatic treatment.Individual conformal radiation therapy
Treatment with antiestrogen
Basic cytostatic treatment; Treatment with antiestrogen
Treatment with enzyme inhibitor
Basic cytostatic treatment. Radiation therapy with gating (IGRT)
Basic cytostatic treatment
Individual conformal radiotherapy
Treatment with antiestrogen
Individual conformal radiotherapy
Basic cytostatic treatment. Complex cytostatic treatment. Individual conformal radiotherapy

Basic cytostatic treatment. Complex cytostatic treatment
Radical mastectomy. No postmastectomy treatment
Radiation therapy with gating (IGRT)
Basic cytostatic treatment. Complex cytostatic treatment. Individual conformal radiation therapy
Radiation therapy with gating, Radiation therapy with gating (IGRT)
Radical mastectomy. No postmastectomy treatment
Basic cytostatic treatment. Complex cytostatic treatment. Conventional external beam radiation. Radiation th
Radical mastectomy. No postmastectomy treatment
Treatment with pegfilgrastim. Treatment with (CE). Treatment with paclitaxel
Treatment with trastuzumab. Basic cytostatic treatment. Complex cytostatic treatment. Individual conformal ra
Radiation therapy with gating. Radiation therapy with gating (IGRT). Treatment with (TC). Teatment with pegfil
Treatment with pegfilgrastim. Individual conformal radiation therapy Treatment with (CE). Treatment with doce
Treatment with pegfilgrastim.Hormonal cancer therapies. Treatment with (CE). Treatment with docetaxel
Basic cytostatic treatment. Complex cytostatic treatment
Treatment with antiestrogen
Treatment with pegfilgrastim. Hormonal cancer therapies. Treatment with (CE). Treatment with docetaxel. Tr
Individual conformal radiation therapy, image guided radiotherapy (IMRT)
Antineoplastic treatment with antiestrogen
Basic cytostatic treatment. Complex cytostatic treatment. Treatment with antiestrogen. Individual conformal ra
Radical mastectomy. No postmastectomy teratment.
Individual conformal radiation therapy, image guided radiotherapy (IGRT)
Individual conformal radiation therapy, image guided radiotherapy (IGRT)
Individual conformal radiotherapy. Individual conformal radiation therapy, image guided radiotherapy (IGRT)
Radical mastectomy. No postmastectomy teratment.
Radical mastectomy. No postmastectomy teratment.
Treatment with pegfilgrastim. Treatment with cyclophosphamid. Treatment with docetaxel

Type	Size	Gr	Her2-IHC	Her2-FISH	ALN	ER	PgR	AR	Nuclear Ki67 (%)
L	40	2	3+		N- 0/11	ER-	PgR-	AR+	No slide
D	40	1	1+		N+ 1/7	ER+	PgR-	AR+	4.1
D	30	3	3+		N+ 27/31	ER-	PgR-	AR-	38.3
D	20	3	3+		N+ 9/20	ER-	PgR-	AR-	21.4
D	20	3	3+		N+ 9/20	ER-	PgR-	AR-	7.5
D	30	2	2+	1,2	N+ 3/14	ER+	PgR-	AR+	4.5
D	40	2	2+	1,5	N+ 7/11	ER+	PgR+	AR+	4.9
L	25	2	2+	1,31	N+ 1/13	ER+	PgR+	AR-	2.6
D	16	2	2+	1,46	N- 0/13	ER+	PgR+	AR-	5.8
Tu/Kr	23	1	1+		N+ 23/25	ER+	PgR+	AR+	0.7
L	70	2	1+		N- 0/10	ER+	PgR+	AR+	7
D	25	2	0		N+ 3/15	ER+	PgR+	AR+	3.8
D	33	3	3+		N+ 3/11	ER-	PgR+	AR+	21
D	25	3	2+	2,75	N+ 1/15	ER+	PgR+	AR+	15.3
L	50	1	1+		N+ 14/15	ER+	PgR-	AR+	3.8
D	21	3	1+		N+ 3/22	ER-	PgR-	AR-	No slide
D	30	2	2+	1,21	N- 0/20	ER+	PgR-	AR+	5.1
L	30	2	2+	1,39	N+ 11/17	ER+	PgR-	AR+	5.6
D	32	3	0		N+ 14/17	ER-	PgR-	AR-	8.8
Apocrine	35	1	1+		N- 0/17	ER-	PgR-	AR+	56
D	40	3	3+		N+ 1/15	ER+	PgR+	AR+	18.5
L	50	2	1+		N+ 8/15	ER+	PgR+	AR+	2.8
D	45	2	2+	1.19	N+ 10/16	ER+	PgR+	AR+	12.1
D	18	2	0		N+ 3/11	ER+	PgR+	AR-	15.7
D	30	2	2+	1.69	N+ 3/16	ER+	PgR-	AR+	23
D	110	2	1+		N+ 20/20	ER+	PgR+	AR+	13
D	35	2	2+	1.48	N+ 8/15	ER+	PgR+	AR-	5.2
D	30	2	3+		N+ 13/16	ER+	PgR-	AR+	14.8
D	21	3	3+		N+ 3/13	ER-	PgR-	AR+	13.3
D	60	2	0		N+ 1/11	ER+	PgR-	AR+	6.1
D	55	1	1+		N+ 12/12	ER+	PgR+	AR-	2
D	60	2	0		N+ 13/18	ER+	PgR+	AR+	11
D	22	2	0		N- 0/7	ER+	PGR+	AR+	10
D	20	1	0		N+ 4/10	ER+	PgR+	AR+	3.6
D	40	2	2+	0.14	N+ 18/18	ER+	PGR-	AR-	4.4
D	23	2	3+		N+ 3/9	ER-	PgR-	AR+	5.3
D	15	2	0		N- 0/15	ER+	PgR+	AR+	7.3
L	22	2	1+		N+ 4/10	ER+	PgR+	AR+	8.8
D	60	3	2+	1.33	N+ 19/22	ER+	PgR-	AR-	26
D	30	3	1+		N+ 8/15	ER+	PgR+	AR+	6.5
L	33	2	1+		N+ 10/12	ER+	PgR+	AR+	10.4
D	35	3	1+		N+ 10/10	ER+	PgR+	AR+	11.8
D	17	3	0		N+ 3/9	ER-	PgR-	AR+	16
D	18	1	1+		N+ 5/10	ER+	PgR+	AR+	1.5
L	25	3	3+		N+ 14/24	ER+	PgR+	AR+	8.3
L	40	2	1+		N- 0/14	ER+	PgR+	AR+	3.2
D	38	2	2+	2.27	N+ 1/13	ER+	PGR-	AR+	13.2
D	21	2	0		N- 0/19	ER+	PgR+	AR-	10.4
D	100	2	0		N+ 3/5	ER+	PgR+	AR+	8.9
D	25	3	2+	6.1	N+ 3/5	ER+	PgR+	AR+	18.7
D	28	3	1+		N+ 4/10	ER+	PgR+	AR+	22.7
D	50	2	1+		N+ 4/10	ER+ ER+	PgR+	AR+	7.2

L	50	2	0		N+ 3/9	ER+	PgR+	AR+	10.2
L	19	2	1+		N+ 1/10	ER+	PgR+	AR+	1.6
D	32	3	0		N- 0/1	ER-	PgR-	AR+	22
D	40	3	0		N+ 4/10	ER-	PgR-	AR-	67.6
L	16	2	0		N+ 1/35	ER+	PgR-	AR+	5.5
D	25	3	1+		N- 0/1	ER-	PgR-	AR+	12.2
D	10	3	2+	1.00	N+ 2/22	ER+	PgR+	ND	2.2
D	22	2	0		N- 0/1	ER+	PgR+	AR+	20.5
D	35	3	0		N+ 5/10	ER+	PgR+	AR+	26.8
D	35	3	3+		N- 0/3	ER-	PgR-	AR+	17.1
D	45	3	1+		N+ 1/13	ER-	PgR-	AR-	78
D	25	3	2+	1.03	N+ 1/13	ER+	PgR+	AR+	34.2
D	35	3	0		N+ 1/3	ER+	PgR-	AR+	79.9
D	28	3	2+	0.96	N- 0/2	ER-	PgR-	AR-	50.6
L	30	2	2+	1.08	N- 0/11	ER+	PgR-	AR+	3.6
D	24	3	1+		N+ 7/13	ER-	PgR-	AR-	66.2
D	22	3	1+		N+ 11/14	ER+	PgR-	AR+	13.4
D	40	3	3+		N+ 25/25	ER+	PgR-	AR+	11.3
L	70	2	1+		N+ 13/16	ER+	PgR+	AR+	18.6
Metaplasia adeno squamose	75		2+	0.98	N+ 9/10	ER-	PgR-	AR-	88
D	30	2	2+	1.28	N+ 7/14	ER+	PgR+	AR+	100
D	26	3	2+	1.44	N+ 1/11	ND	PgR+	AR+	54
D	34	3	2+	1.26	N+ 22/23	ER+	PgR+	AR+	73.6
D	29	3	2+	1.36	N+ 1/4	ER+	PgR+	AR+	13.9
Metapl. Carc.	50		2+	Norm.	N- 0/1	ER-	PgR-	AR+	13.3
D	45	3	1+		N+ 5/16	ER-	PgR-	AR-	65.4

Tumor subtype	Reccurency Date (2014-10-08)
HER2	2011 (24/10): IDC (righ breast) and metastases
Lum A	
HER2	
HER2	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
HER2	2006 (5/5): IDC (righ breast and metastases)
Lum A	2005 (31/8): IDC and metastases in lung + malignant tumor cells from neck
Lum A	
TNBC	
Lum A	
Lum A	2005 (5/12): Bronchi mucosa and malignant tumor cells (cytologi)
TNBC	
TNBC Apo	
Lum B/HER2 enriched	2012 (9/5) + 2013 (5/8): malignant tumor cells in LN from neck (cytologi)
Lum A	2013 (13/5): ILC (left breast)
Lum A	2010 (23/3): Metastases and pleura adenocarcinoma
Lum A	
Lum A	
Lum A	
Lum A	
Lum B/HER2 enriched	
HER2	
Lum A	
Lum A	2008 (14/7): IDC and metastases in liver + lung
Lum A	
Lum A	
Lum A	
Lum A	
HER2	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	2007 (27/1): metastases - IDC (rigth breast)
TNBC	
Lum A	
Lum B/HER2 enriched	2009 (26/3): Metastases in skin (chest); 2009 (12/8): ILC skin (thorax) 2012 (24/8): LN from axil-malignant tumor cells
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	

Lum A	2012 (24/5): ILC (left breast) and metastases 2014 (16/4): Metastases in femur
Lum A	
TNBC (ER conflict)	
TNBC	
Lum A	
TNBC (ER conflict)	2014 (31/1): Adenocarcinoma - pancreas (primary tumor)
Lum A	
Lum A	
Lum A	
HER2	
TNBC	
Lum A	2013 (20/9): Metastases in liver
Lum A	
TNBC	
Lum A	
TNBC (ER conflict)	2012 (3/9): Metastases in lung 2013 (15/2): Malignant tumor cells in ploural fluid (cytologi)
Lum A	
Lum B/HER2 enriched	2013 (4/4): IDC (left breast) and metastases 2014 (9/5): Malignant tumor cells in LN from axil (cytologi)
Lum B	
TNBC (ER conflict)	
Lum A	
Lum A	
Lum A	
Lum A	
Lum A	
TNBC	2014 (12/8): Metastases and malignant tumor cells and IDC from skin on chest

Outcome (Date from Patient Registry:2014-10-08)	Primary cause of death due to the breast cancer	Tumor cells (%)
Dead 2013 (6/10)		≥70%
Dead 2005 (3/12)	Malignant neoplasm of breast	≥70%
		≥50%
		≥70%
		≈40%
Dead 2006 (5/6)	Malignant neoplasm of breast	≥50%
Dead 2005 (9/8)		≥50%
Dead 2012 (27/7)		≥70%
		≥70%
Dead 2012 (30/3)	Malignant neoplasm of breast	<10%
		≈40%
Dead 2013 (16/10)		≥70%
Dear 2009 (21/02)	Malignant neoplasm of breast	≥50%
Dead 2009 (21/12)	Malignant neoplasm of breast	≥70%
Dead 2008 (25/1)	Malignant neoplasm of breast	≈40%
		≈40%
Dead 2011 (31/7)	Malignant neoplasm of breast	≥70%
Dead 2006 (31/3)	Malignant neoplasm of breast	≥50%
Dead 2008 (25/6)	Malignant neoplasm of breast	≥50%
		≥70%
		≥70%
		≥50%
Dead 2012 (31/5)	Malignant neoplasm of breast	≥50%
Dead 2010 (29/12)	Malignant neoplasm of breast	>70%
Dead 2007 (8/3)	Malignant neoplasm of breast	≥50%
Dead 2006 (1/3)	Malignant neoplasm of breast	≥50%
Dead 2007 (10/2)		≥70%
Dead 2008 (29/10)	Malignant neoplasm of breast	≥50%
		≥50%
		≈40%
Dead 2008 (10/7)	Malignant neoplasm of breast	≈40%
Dead 2009 (08/08)		≥70%
Dead 2008 (05/07)		≥50%
		≥70%
		≥50%
		>70%
		≥50%
Dead 2008 (28/2)		≥70%
Dead 2007 (4/3)		≥50%
		≥70%
Dead 2008 (04/1)		≥70%
Dead 2010 (29/12)	Malignant neoplasm of breast	≥70%
		≥70%
		≥70%
		≥50%
		≥70%
		≥70%
Dead 2014 (13/2)		≥70%
		≥70%
Dead 2011 (5/3)		≥70%
Dead 2009 (25/8)	Malignant neoplasm of breast	≥70%
		≥70%

		≥70%
		≥70%
		≥70%
		≥70%
		≥70%
		≈40%
		≥70%
Dead 2012 (18/1)		>70%
		≥70%
		≥70%
		≥70%
		≥70%
		≥70%
		≥70%
		≥70%
Dead 2013 (7/4)		≈40%
		≥70%
		≥70%
		≈40%
Dead 2012 (14/5)	Malignant neoplasm of breast	≥70%
		≥70%
		≥70%
Dead 2013 (20/3)		≥70%
		≥70%
		≥70%
		≥70%

Total TILs (1+ to 3+: bases on HE performed at the time of tumor collection and TIF preparation)	CD45 (1+-3+ scoring as specified in Mat and Methods)	CD3 (1+-3+ scoring as specified in Mat and Methods)
0	0	0
0	0	0
2+ (indside tumor)	No tissue left	No tissue left
2+ (indside tumor)	2+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
0	0	0
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indsinde tumor)	0	0
1+ (indside tumor)	2+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	3+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	No tissue left	No tissue left
0	0	0
2+ (indside tumor)	0	0
3+ (indside tumor)	3+ (indside tumor)	0
3+ (indside tumor)	0	1+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	0	0
1+ (indside tumor)	No tissue left	No tissue left
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	0	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	0	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	0
1+ (indside tumor)	0	0
1+ (indside tumor)	0	0
2+ (indside tumor)	2+ (indside tumor)	0
0	0	0
0	0	0
0	1+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	0
0	1+ (indside tumor)	0
2+ (indside tumor)	2+ (indside tumor)	0
0	0	0
2+ (indside tumor)	2+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	0
2+ (indside tumor)	1+ (indside tumor)	0
0	0	0
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
3+ (indside tumor)	3+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	0	0

2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
3+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	0
3+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
3+ (indside tumor)	1+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	3+ (indside tumor)
1+ (inside tumor)	1+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
0	0	0
3+ (indside tumor)	0	2+ (indside tumor)
1+ (inside tumor)	1+ (indside tumor)	0
1+ (inside tumor)	1+ (indside tumor)	0
2+ (indside tumor)	0	0
0	0	0
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	3+ (indside tumor)	1+ (indside tumor)

CD4 (1+-3+ scoring as specified in Mat and Methods)	CD8 (1+-3+ scoring as specified in Mat and Methods)	CD68 (1+-3+ scoring as specified in Mat and Methods)
0	0	0
0	0	2+ (indside tumor)
No tissue left	No tissue left	No tissue left
Neg	2+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	0	1+ (indside tumor)
0	0	0
0	1+ (indside tumor)	0
0	0	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
0	0	0
0	0	0
1+ (indside tumor)	1+ (indside tumor)	0
2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
0	No tissue left	No tissue left
0	0	1+ (indside tumor)
0	1+ (indside tumor)	2+ (indside tumor)
3+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	3+ (indside tumor)
1+ (indside tumor)	0	2+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
0	0	0
No tissue left	No tissue left	No tissue left
1+ (indside tumor)	2+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	2+ (indside tumor)	0
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	0	2+ (indside tumor)
1+ (indside tumor)	0	0
0	0	0
0	0	0
1+ (indside tumor)	1+ (indside tumor)	0
0	0	0
0	0	0
0	0	0
2+ (indside tumor)	0	0
0	0	0
2+ (indside tumor)	0	1+ (indside tumor)
0	0	0
0	0	2+ (indside tumor)
0	1+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	0	1+ (indside tumor)
0	0	1+ (indside tumor)
0	0	1+ (indside tumor)
3+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
3+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	0	2+ (indside tumor)
3+ (indside tumor)	2+ (indside tumor)	1+ (indside tumor)
0	1+ (indside tumor)	2+ (indside tumor)
0	1+ (indside tumor)	2+ (indside tumor)

1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
0	0	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	3+ (indside tumor)	2+ (indside tumor)
3+ (indside tumor)	3+ (indside tumor)	1+ (indside tumor)
0	1+ (indside tumor)	0
	1+ (indside tumor)	0
0	0	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	
3+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)
3+ (indside tumor)	2+ (indside tumor)	1+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	0
3+ (indside tumor)	2+ (indside tumor)	0
1+ (indside tumor)	0	1+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	3+ (indside tumor)
2+ (indside tumor)	2+ (indside tumor)	2+ (indside tumor)
1+ (indside tumor)	1+ (indside tumor)	0
1+ (indside tumor)	2+ (indside tumor)	3+ (indside tumor)
0	0	1+ (indside tumor)
0	1+ (indside tumor)	1+ (indside tumor)
0	0	2+ (indside tumor)
0	0	0
2+ (indside tumor)	1+ (indside tumor)	2+ (indside tumor)
2+ (indside tumor)	1+ (indside tumor)	1+ (indside tumor)

Supplementary Table 3. Number of cases analyzed listed according to the type of sample and pathological features of the tumor samples.

		NIF(43)	TIF(78)	Serum (25)
Subtype	Luminal A	19	39	9
	Luminal B	14	19	7
	HER2	2	7	4
	TNBC	8	13	5
ER status	ER positive	32	56	16
	ER negative	11	22	9
PgR status	PR positive	26	41	12
	PR negative	17	37	13
HER2 status	HER2 negative	6	13	6
	HER2 positive	37	65	19
Grade	I	3	7	0
	II	18	36	10
	III	22	35	15

Supplementary table 4: Antibodies used in this study

Marker	Antibody	Dilution	Vendor
CK19	Monoclonal mouse (clone 4E8)	1:1000	ThermoFischer Scientific
Ki67	Monoclonal mouse (clone MIB-1)	1:200	DAKO
ER	Monoclonal mouse (clone 1D5)	1:200	DAKO
PGR	Monoclonal mouse (synthetic peptide directed towards the N-terminal end)	1:200	DAKO
HER2	Polyclonal rabbit (HercepTest)	1:300	DAKO
CD3	Polyclonal rabbit (synthetic peptide from the intracellular part of the ε-chain of human CD3)	1:200	DAKO
CD4	Monoclonal mouse (clone IS 649)	1:25	DAKO
CD8	Monoclonal mouse (clone C8/144B)	1:100	DAKO
CD45	Monoclonal mouse (clone 2B11+PD7/26)	1:400	DAKO
CD68	Monoclonal mouse (clone PG-M1)	1:100	DAKO
RANTES	Monoclonal mouse (clone 50013-5; LS-B6426)	1:400	LSBio
PDGFb	Rabbit polyclonal (ab23914)	1:100	Abcam
IP-10	Rabbit polyclonal (ab9807)	1:100	Abcam
IL-1RA	Rabbit polyclonal (HPA001482)	1:30	Sigma
IL10	Monoclonal mouse (SC-8438)	1:100	Santa Cruz
VEGF	Monoclonal mouse (ab68334)	1:100	Abcam