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Kimbung, Siker; Kovács, Anikó; Bendahl, Pär-Ola; Malmström, Per; Fernö, Mårten; Hatschek, Thomas; Hedenfalk, Ingrid

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LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

## **Claudin-2 is an independent negative prognostic factor in breast cancer and specifically predicts early liver recurrences**

Siker Kimbung<sup>1,2</sup>, Anikó Kovács<sup>3</sup>, Pär-Ola Bendahl<sup>1</sup>, Per Malmström<sup>1,4</sup>, Märten Fernö<sup>1</sup>,  
Thomas Hatschek<sup>5</sup>, and Ingrid Hedenfalk<sup>1,2</sup>.

<sup>1</sup>Division of Oncology, Department of Clinical Sciences, Lund, Lund University, Sweden.

<sup>2</sup>CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden.

<sup>3</sup>Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden.

<sup>4</sup>Skåne Department of Oncology, Skåne University Hospital, Lund, Sweden.

<sup>5</sup>Department of Oncology and Pathology, Karolinska Institutet and Karolinska University Hospital, Sweden.

**Corresponding author:** Associate Professor Ingrid Hedenfalk, Division of Oncology, Department of Clinical Sciences, Lund, Lund University, Medicon Village, SE-22381 Lund, Sweden. Phone: +46-46-2220652. Fax: +46-46-147327. E-mail: [Ingrid.Hedenfalk@med.lu.se](mailto:Ingrid.Hedenfalk@med.lu.se)

**Running Title:** Claudin-2 expression predicts breast cancer hepatic recurrences

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**Abbreviations:** BCSS, breast cancer specific survival; CI, confidence interval; *CLDN2*, Claudin-2 mRNA; ER, estrogen receptor; HR, hazard ratio; IHC, immunohistochemistry; LiMFS, liver metastasis-free survival; LNM, lymph node metastasis; OR, odds ratio; PR, progesterone receptor; RFS, relapse-free survival; and TMA, tissue microarray.

## **Abstract**

**Background:** Predicting any future metastatic site of early-stage breast cancer is important as it significantly influences the prognosis of advanced disease. This study aimed at investigating the potential of claudin-2, over-expressed in breast cancer liver metastases, as a biomarker for predicting liver metastatic propensity in primary breast cancer.

**Methods:** Claudin-2 expression was analyzed in two independent cohorts. Cohort 1 included 304 women with metastatic breast cancer diagnosed between 2002-2007, while cohort 2 included 237 premenopausal women with early-stage node-negative breast cancer diagnosed between 1991-1994. Global transcriptional profiling of fine-needle aspirates from metastases was performed, followed by immunohistochemical analyses in archival primary tumor tissue. Associations between claudin-2 expression and relapse site were assessed by univariable and multivariable Cox regression models including conventional prognostic factors. Two-sided statistical tests were used.

**Results:** *CLDN2* was significantly up-regulated ( $P < 0.001$ ) in liver metastases compared to other metastatic sites. Claudin-2 protein was more frequently expressed in primary tumors from patients who subsequently developed liver metastases ( $P = 0.02$ ) and high expression was associated with a shorter metastasis-free interval (cohort 1, HR=1.4, 95% CI=1.0–1.9; cohort 2, HR=2.2, 95% CI=1.3-3.5). Specifically, a significantly shorter interval between primary tumor diagnosis and liver-specific recurrence was observed among patients with high levels of claudin-2 expression in the primary tumor (cohort 1, HR=2.3, 95% CI=1.3–3.9).

**Conclusion:** These results suggest a novel role for claudin-2 as a prognostic biomarker with the ability to predict not only the likelihood of a breast cancer recurrence, but more interestingly, the liver metastatic potential of the primary tumor.

**Keywords:** Breast cancer, liver metastasis, claudin-2, prognostic biomarker

## 1. Introduction

Despite advances in management and the favorable prognosis of patients with early breast cancer, metastases are frequently diagnosed and the anatomical location of the metastases is correlated to the length of survival after recurrence (Imkampe et al., 2007; Largillier et al., 2008; Yardley, 2010). With the exception of the brain, recurrence in the liver is prognostic of the worst outcome relative to loco-regional, bone or lung relapses (Goldhirsch et al., 1988; Imkampe et al., 2007; Pentheroudakis et al., 2006; Yardley, 2010). Approximately 50% of all patients diagnosed with metastatic breast cancer develop hepatic metastases (Mano et al., 2005; Singletary et al., 2003; Solomayer et al., 2000) and there is evidence purporting an increasing trend in breast cancer liver metastases (Kennecke et al., 2010). However, the molecular determinants of site-specific metastatic preferences and factors accounting for heterogeneity in response to treatment and outcome are yet to be comprehensively established. A better understanding of these factors will likely influence decisions about surveillance and adjuvant therapy, as well as treatment of advanced disease.

Conventional clinico-pathological markers are used to assess the risk of recurrence. In addition, gene expression signatures stratifying patients according to recurrence risk (reviewed in (Sotiriou and Pusztai, 2009)) and more specifically, predicting the propensity of relapsing in bone (Kang et al., 2003), lung (Minn et al., 2005) and brain (Bos et al., 2009) have been published. However, because experimental models incompletely capture the relevant genetic complexity and the contribution of the host tumor microenvironment, studies using biopsies from metastases may be more suitable for identifying site-specific predictive biomarkers. Recently, we performed comparative genome-wide transcriptional profiling of a consecutive series of breast cancer metastases with one of the specific objectives being to identify potential liver metastasis genes (Kimbung *et al.*, Manuscript in preparation).

Remarkably, we observed that contrary to the down-regulation of many genes involved in cell

adhesion and matrix re-modeling in liver metastases, *CLDN2*, a member of the same gene family, was significantly over-expressed. Over-expression of *CLDN2* was also recently observed in an experimental mouse model of breast cancer liver metastases (Tabaries et al., 2011), as well as in a limited series of clinical samples of breast cancer liver metastases (Tabaries et al., 2012), with accompanying data supporting the involvement of claudin 2 in the establishment and out-growth of breast cancer cells in the liver microenvironment. These data motivated the design of the present study, which was aimed at investigating if the high expression of *CLDN2* observed in liver metastases, is also a trait of primary breast cancers that recur in the liver. Furthermore, we sought to explore associations with conventional prognostic factors for breast cancer and patient outcome, with particular focus on the potential of claudin-2 as a biomarker for predicting liver metastatic propensity in primary breast cancer.

## **2. Materials and Methods**

### **2.1 Patients and tumors**

This study was approved by the regional ethics committees at all participating sites.

#### **2.1.1 Cohort 1**

The test cohort consisted of 304 women with metastatic breast cancer who were enrolled in a randomized phase III trial conducted between 2002 and 2007 in Sweden, comparing two different first-line chemotherapy regimens (Hatschek et al., 2012). Patients with brain metastases, HER2 amplified tumors, or other malignancies diagnosed within five years of enrolment were excluded from the trial. Complete information on the study design, patient characteristics and trial outcome has been reported (Hatschek et al., 2012). The median follow-up for the endpoints relapse free survival (RFS) and breast cancer specific survival (BCSS) was 6.0 and 9.7 years respectively, for patients alive at last update.

### **2.1.2 Cohort 2**

The prognostic value of claudin-2 was further evaluated in an independent cohort of 237 premenopausal women with early-stage lymph-node negative breast cancer included in a prospective study evaluating the prognostic value of the S-phase fraction (Malmstrom et al., 2001). Adjuvant treatment was administered to only 29 (12%) patients. Detailed information on treatment and evaluation of tumor pathological markers has been previously reported (Klintman et al., 2010; Malmstrom et al., 2001). Median follow-up was 10.6 and 18.3 years for RFS and BCSS, respectively.

## **2.2 Transcriptional analyses**

Fine-needle aspirates from metastatic lesions from different anatomical sites were collected prior to treatment of metastatic disease whenever possible (cohort 1) and subjected to whole-genome transcriptional profiling. Tumor cellularity was assessed by a pathologist on Giemsa stained, ethanol-fixed, cytopsin preparations and only samples with high (>50%) tumor cell content were included in the final analyses. Total RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Valencia, CA), integrity analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and hybridized onto custom made Affymetrix HuRSTA-2a520709 gene chips. Raw intensity gene expression levels were processed and normalized using the robust multichip average (RMA) algorithm. After normalization, a probe presence filter was applied to select only probes present in  $\geq 90\%$  of assays. Gene-specific expression intensities were summarized by merging probes based on gene symbols, and genes with expression below the median expression threshold for Y-chromosome genes were filtered out from the dataset, leaving a total of 16,112 genes for inclusion in subsequent analyses. Finally, data were log<sub>2</sub> transformed and mean-centered across the entire dataset. All data processing and normalization steps were performed in the R environment ([www.r-project.org](http://www.r-project.org)). Ninety-one

out of 122 samples passed all quality assessments and were included in subsequent analyses. Differentially expressed genes and biological processes between the liver metastases and other metastatic sites were identified using the Significance Analysis of Microarrays (SAM) and DAVID tools (Huang da et al., 2009a, b), respectively. The gene expression data are available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under the accession number GSE46141.

### **2.3 Tissue microarrays (TMA) and immunohistochemistry (IHC)**

Archival formalin-fixed paraffin-embedded (FFPE) primary tumor blocks were collected. Two representative 0.6 (cohort 1) or 1.0 (cohort 2) mm cores were extracted from the donor blocks and assembled in separate TMA blocks. Regional lymph node metastases (LNMs) from patients in cohort 1 were similarly assembled in a TMA. Whenever pathological markers were examined, both core biopsies were evaluated, and results from the core with the highest/strongest positivity were recorded. Investigators were always blinded to outcome.

### **2.4 Evaluation of standard pathological markers**

Estrogen (ER) and progesterone (PR) receptor status were analyzed by IHC and cytosol based biochemical assays for cohort 1 and 2 respectively, as previously described (Chebil et al., 2003; Malmstrom et al., 2001). Antibodies were purchased from Ventana (ER, clone SP1; PR, clone 1E2) and staining was performed with the Ventana Benchmark ULTRA (Ventana Medical Systems, Tucson, AZ). Re-evaluation of histological grade was performed following the Elston and Ellis criteria as described (Malmstrom et al., 2001). Proliferation was assessed by the Ki67 index, using the MIB-1 antibody (K5001, Dako, Copenhagen, Denmark). A cut-

off of  $\geq 20\%$  was used to indicate high Ki67 (Klintman et al., 2010). All scorings were performed independently by board certified breast pathologists.

## **2.5 Claudin-2 immunohistochemistry**

A mouse monoclonal antibody specific for claudin-2 (12H12, Invitrogen, Sweden) was used at a 1:400 dilution. This antibody has previously been used for the evaluation of claudin-2 expression by IHC in several studies (Dhawan et al., 2011; Kim et al., 2008; Soini, 2004, 2005; Szasz et al., 2010). Immunohistochemical reactions were performed following the manufacturer's protocol and the Envision horseradish peroxidase rabbit/mouse kit and the Dakocytomation Autostainer (DAKO) system was used. Staining was detected as a membranous and cytoplasmic granular reaction. Non-neoplastic human kidney tissue was included as positive control. Each sample was given a semi-quantitative score from 0-2 for the proportion of tumor cells staining positive [0 (<10%), 1 (11-50%), and 2 (>50%)] and 0-3 for the intensity of tumor cell staining [0 (absent), 1 (weak), 2 (moderate), and 3 (strong)]. The proportion and intensity scores were combined by addition to obtain a final score ranging from 0-5. No consensus for choice of cut-off for claudin-2 scoring was found in the literature. Therefore, in this study, a total score of  $\geq 3$  was considered as high expression and scores  $< 3$  as low expression, representative of the majority of these studies (Dhawan et al., 2011; Soini, 2005; Szasz et al., 2010; Tabaries et al., 2011; Tabaries et al., 2012).

## **2.6 Statistical analyses**

Patients and tumor characteristics were compared across the claudin-2 expression groups using the  $\chi^2$  and Mann-Whitney U or one-way analysis of variance tests for categorical and continuous variables, respectively. Odds ratios (OR) were computed by logistic regression



modeling and the McNemar test was used to assess differences between paired primary tumors and regional LNMs. RFS, liver metastasis-free survival (LiMFS) and BCSS were the primary, secondary and tertiary end-points, respectively. RFS included recurrence to any site, LiMFS included only liver recurrences, and BCSS included breast cancer specific death as an event. The differences between the claudin-2 groups for each end-point were summarized using hazard ratios estimated in both univariable and multivariable Cox-proportional hazards models (see Appendix Methods A.1 for further details). Proportional hazards assumptions were checked by graphical methods. All P-values correspond to two-sided statistical tests and values  $<0.05$  were considered significant. The statistical software package IBM SPSS Statistics 19 (IBM Corporation, NY) was used.

### **3. Results**

#### **3.1 Patient and tumor characteristics**

Flow charts of the cohorts and a summary of primary tumor characteristics for patients in cohort 1 are presented in Appendix Fig A.1 and Appendix Table A.1. Figure 1A illustrates an inferior post-recurrence survival in patients with liver compared to non-liver recurrences in cohort 1 (Log-rank;  $P=0.006$ ). The poor outcome for patients with liver metastases remained significant (Figure 1B; Log-rank  $P=0.02$ ) after stratifying the patients with non-liver metastases into three groups based on the most advanced metastatic site recorded (loco-regional, bone and lung, respectively). Liver recurrences were rare (18 cases) in cohort 2, thus the distributions of patient and tumor characteristics by claudin-2 expression but not by site of relapse were explored in this cohort.

### 3.2 Claudin-2 expression and associations with clinico-pathological characteristics

A total of 91 breast cancer metastases from 6 specific anatomical sites [liver (n=16), bone (n=5), lung (n=2), lymph node (n=39), local [breast (n=11) and skin (n=17)], and ascite (n=1)] were included in the search for differentially expressed genes associated with hepatic recurrence. SAM analyses revealed 733 (423 up-regulated and 307 down-regulated) significantly differentially expressed genes between liver metastases and other sites. There was an enrichment of genes associated with cell adhesion and matrix re-modeling among the significantly down-regulated genes in the liver metastases (Figure 2). In contrast, *CLDN2* expression was found to be significantly up-regulated in liver metastases compared to other sites (Figure 3A; Mann-Whitney;  $P < 0.001$ , and Figure 3B; Kruskal Wallis;  $P = 0.007$ ). Following the notion that transcriptional profiles of primary tumors and metastases from a patient are very similar (Harrell et al., 2012; Weigelt et al., 2003), we investigated if *CLDN2* was up-regulated in metastases derived from patients diagnosed with liver metastases compared to non-liver involvement irrespective of the anatomical location of the metastatic lesion that was profiled. *CLDN2* was thus found to be significantly over-expressed in metastases from patients with liver involvement compared to those without (Figure 3C; Mann-Whitney  $P = 0.001$ , and Figure 3D; Kruskal Wallis  $P = 0.06$ ).

Next, we investigated (in cohort 1) if the high *CLDN2* expression observed in the hepatic metastases could be a trait acquired from the primary tumors, potentially priming them for selective colonization of the liver. Of the 191 evaluable cases, 134 (70%) were classified as high claudin-2 expressing (Table 1 and Figure 4). Notably, a significant association between high claudin-2 expression in the primary tumor and liver relapse was found (OR=2.1, 95% CI=1.1-4.0).

Other associations between claudin-2 and conventional breast cancer prognostic factors were then explored. High expression of claudin-2 was found to be significantly associated with

positive nodal status (OR=2.1, 95% CI=1.1-3.9) in cohort 1, while significant positive associations between claudin-2 expression and high histological grade (grade 3; OR=3.0, 95% CI=1.6–5.7), high proliferation (high Ki67; OR=4.4, 95% CI=2.3-9.0), and younger age (<50 years; OR=2.0, 95% CI=1.1-3.7) were observed in cohort 2 (Table 1).

### **3.3 Claudin-2 expression and tumor progression: correlation between primary and lymph node metastasis**

Paired data from primary tumors and LNMs were available from 107 cases in cohort 1. Discordant claudin-2 expression was observed in 32 pairs [30% (McNemar; P=0.02)], the majority of which changed from low expression in the primary tumor to high expression in the LNM [23/32 (72%)]. Subgroup analyses revealed that significant discordant expression was only demonstrated among ductal carcinomas (n=83, McNemar; P=0.02). In contrast, no difference in the expression pattern was observed in lobular carcinomas (McNemar; P=0.5), as 15/17 evaluable cases displayed concordant high expression.

### **3.4 Claudin-2 expression in relation to recurrence and breast cancer death**

Uni- and multivariable Cox proportional hazards ratio estimates of the difference between the claudin-2 groups for RFS, LiMFS and BCSS, respectively are shown in Tables 2-4. Twenty-year survival estimates are reported.

The median RFS was significantly shorter (3.6 years vs. 5.7 years) for the high claudin-2 group in both univariable (HR=1.4, 95% CI=1.0-1.9) and multivariable analyses (Tables 2-3) in cohort 1. Histological grade, ER status, tumor size, axillary lymph node status and age at primary diagnosis were other independent factors significantly correlated with a shorter RFS in multivariable models. In cohort 2, high claudin-2 expression was prognostic for shorter

RFS (HR=2.2, 95% CI=1.3-3.5) in univariable analyses. Age, HER2 status and histological grade were also significant in univariable analyses, with age and HER2 status remaining significant independent factors in multivariable models (Table 4).

Next, we investigated if claudin-2 expression in the primary tumor was prognostic for the diagnosis of liver metastases in cohort 1. Univariable analyses revealed a substantial decrease in the median time to liver metastasis diagnoses from 12.1 years in the low expressing group to 5.9 years in high expressing groups (Tables 2-3, HR=2.3, 95% CI=1.3–3.9). Claudin-2 remained the strongest independent liver metastasis risk factor in multivariable analyses (HR=2.0, 95% CI=1.1–3.8).

In addition, there was a trend towards higher risk of death from breast cancer among patients with high claudin-2 expression in univariable analyses (cohort 1: Appendix Table A.2; HR=1.4, 95% CI=0.98–2.1 and cohort 2: Table 4; HR=1.3, 95% CI=0.76–2.3).

#### **4. Discussion**

Our study reveals that *CLDN2* is frequently over-expressed in breast cancer liver metastases, and in addition conclusively demonstrates that primary tumors from patients who are diagnosed with hepatic recurrences also frequently express high levels of claudin-2 protein. Most importantly, for the first time, we provide evidence that claudin-2 is a potential prognostic factor for predicting the likelihood of a breast tumor to relapse specifically in the liver, and is furthermore a general predictor of early breast cancer recurrences.

While it is known that cancer cells preferentially metastasize to specific organs, the molecular mechanisms driving this organ-specific tropism are not well understood. Gene expression signatures that predict bone (Kang et al., 2003), lung (Minn et al., 2005) and brain (Bos et al., 2009) metastases from breast cancer have been published, but no signature for liver metastasis

is currently available despite the adverse clinical outcome of patients with hepatic metastases as demonstrated by us herein, and others (Imkampe et al., 2007; Largillier et al., 2008; Yardley, 2010). Although these gene signatures have contributed greatly to the understanding of metastasis organotropism, there is a need to identify the most informative and robust candidate genes among these signatures, which may be used as surrogate biomarkers in more convenient assays such as IHC. In concordance with previous experimental mouse model studies of breast cancer (Erin et al., 2009; Tabaries et al., 2011) we observed that decreased expression of cell adhesion and tight junction genes (including *DSG2*, *CLDN4*, *CLDN8*, *POSTN*, *THBS2*) may be a trait of breast cancer liver metastases. Interestingly however, like Tabaries and colleagues, we show that claudin-2 is over-expressed in breast cancer liver metastases, highlighting a potentially important role of claudin-2 in the development of liver metastases in these patients. Importantly, our study further demonstrates that this is an attribute of primary tumors, as a significantly higher proportion of patients with liver metastases also displayed high claudin-2 levels in their primary tumors. Additionally, Tabaries *et al.*, (Tabaries et al., 2011; Tabaries et al., 2012) provided the functional evidence characterizing *CLDN2* as a breast cancer liver metastasis virulence gene that endows circulating breast cancer cells with enhanced capacity to adhere, survive, and proliferate in the hepatic microenvironment. Taken together, these studies compel us to propose that claudin-2 is a novel and functionally relevant biomarker for predicting liver metastases.

In order for circulating tumor cells to seed metastases, interactions between tumor cells and the microenvironment are critical. Claudin-2 is a unique member of the claudin family of transmembrane cell adhesion proteins and is selectively expressed in leaky epithelia (Escaffit et al., 2005; Reyes et al., 2002). Available data indicate that it is highly expressed and plays a role in the onset and progression of colorectal cancer (Dhawan et al., 2011), lung cancer (Peter et al., 2009), and inflammatory bowel disease (Ridyard et al., 2007; Weber et al., 2008). There are limited but controversial data on the expression of claudin-2 in breast

cancer, and its role in disease progression and prognosis has not been extensively studied. While it is reported to be expressed in about 50% of primary breast carcinomas (Soini, 2004, 2005; Thakur et al., 2007), one study reported down-regulation of claudin-2 in up to 93% of primary breast cancers compared to adjacent normal breast tissue (Kim et al., 2008). The recently described poor prognosis claudin-low subtype of breast cancer is characterized by down-regulation of claudins 3, 4 and 7, and is enriched with triple-negative tumors (Prat et al., 2010). We found claudin-2 to be expressed in 70% of tumors in cohort 1 and 51% of tumors in cohort 2. The distribution of claudin-2 in cohort 2 in our study is in line with previous studies (Soini, 2004, 2005; Thakur et al., 2007) and in addition, we found a significant positive association between high claudin-2 expression and poor prognostic factors including high histological grade, younger age and high proliferation, confirming the negative prognostic effect of its expression in breast cancer. The higher proportion of claudin-2 positive tumors seen in cohort 1 reflects the conservative selection bias of the clinical trial, resulting in an enrichment of patients with an inferior prognosis within this cohort. On the one hand, this provided sufficient statistical power to study the liver metastatic potential of the biomarker, while on the other hand, because the exclusion criteria of the trial are linked to prognosis, this may have confounded the statistical estimates towards the null hypothesis, partly explaining the absence of a significant statistical association between claudin-2 expression and other poor prognostic factors in cohort 1.

Claudin-2 expression in matched primary tumors and lymph node metastases in relation to clinico-pathological features and outcome has been previously studied (Szasz et al., 2010), showing loss of expression in the LNMs among lobular cancers only. Similarly and consistent with another previous study (Soini, 2004), we did not observe any significant differences in expression in ductal vs. lobular, amongst primary tumors. In contrast to the previous study however, increased expression of claudin-2 in LNMs compared to primary tumors was observed among ductal tumors. This could suggest that claudin-2 may facilitate ductal breast

cancer dissemination, a hypothesis supported by results from studies in colorectal (Dhawan et al., 2011) and lung cancer (Peter et al., 2009). Claudin-2 facilitates the conversion of tight junctions from a compact to a leaky strand phenotype (Furuse et al., 2001; Singh et al., 2007), suggesting that over-expression may increase the permeability of epithelial structures, thereby enabling access to factors in the microenvironment necessary for tumor growth, invasion and metastasis. It remains to be investigated if claudin-2 can be targeted therapeutically to prevent dissemination and outgrowth of liver metastases. Of interest, preclinical studies have shown that claudin-2 expression can be down-regulated by inhibition of EGFR and PI3K using specific antibodies and inhibitors (Bos et al., 1997; Dhawan et al., 2011), providing additional support for the use of these compounds, many of which are currently being evaluated in clinical trials. However, because of the limited number of cases with matched primary tumor and LNM data in our study (n=107) and that of Szasz, *et al.*, (n=97) larger studies are required to better understand the significance of these findings.

Notably, we observed a positive association between high claudin-2 expression in the primary tumor and a significantly shorter recurrence-free interval, and a trend towards higher risk of death was noted. Importantly, claudin-2 remained a significant independent prognostic factor for RFS in multivariable analyses. The prognostic value of claudin-2 expression in primary breast tumors has been previously studied (Szasz et al., 2010), but no significant association with survival was observed. Cohort 1 in the present study included only patients with advanced disease, biasing the effect estimates towards the null hypothesis. Notwithstanding, the negative prognostic power of claudin-2 was confirmed in the independent cohort of premenopausal women with early-stage node-negative disease.

Most importantly, for the first time, we present data showing that high expression of claudin-2 in primary tumors predicts shorter time to develop liver metastases. Associations between site of relapse and molecular subtype have been reported (Kennecke et al., 2010; Smid et al.,

2008), but the significant overlap between relapse sites across subtypes compromises their predictive power and warrants the identification of supplementary site-specific biomarkers. In multivariable analyses (cohort 1) including ER status, histological grade, nodal status, age at primary diagnosis and tumor size, only claudin-2 and tumor size remained independently significant for liver metastases. While we observed a marginal increase in the liver metastatic risk among patients with larger tumors, Kannecke and colleagues (Kannecke et al., 2010), reported a significant association between large tumor size and lower risk of liver and brain seeding. Although our findings are consistent with the metastatic model purporting that an aggressive potential can be reflected by a large volume (Norton and Massague, 2006), it does not explain the propensity for liver-specific colonization. Importantly, claudin-2 was the strongest predictor for time to liver recurrence. It remains to be verified if it is also functionally important in mediating the early stages of tumor invasion or whether it serves as a passenger biomarker for the liver metastatic potential of a tumor at the primary site. We found claudin-2 expression to have limited value in predicting liver metastatic potential in colorectal cancer, most likely due to high overall levels of expression in colorectal carcinomas (data not shown).

Despite improvements in breast cancer survival, distant recurrences are not uncommon and remain incurable. Our data provide evidence projecting claudin-2 as a novel breast cancer prognostic biomarker with application for predicting not only the likelihood of a tumor to recur, but more interestingly its liver metastatic potential. We have uncovered novel correlations, corroborated previous data and observed important discrepancies. The inconsistencies between our results and some of the previous studies may be partly attributed to differences in the patient cohorts with respect to clinico-pathological characteristics and follow up time, sample size, as well as the choice of analytical and statistical methods. Nevertheless, the analogous negative prognostic effect of claudin-2 observed in the two cohorts despite their clinical differences, and the significance of our results for improving



personalized management of breast cancer warrants further investigation in larger population based cohorts which better capture the heterogeneity in biology and outcome of breast cancer.

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## Figure Legends

**Figure 1.** Kaplan-Meier representation of post-recurrence survival according to site of relapse in cohort 1. A) Patients were stratified by presence (liver) or absence (other) of liver metastases. B) Patients with non-liver metastases (breast, lymph-node, skin, bone, lung and ascite) were further stratified into three groups according to the most distant metastatic site. P values are from two-sided Log-rank tests.

**Figure 2.** Supervised analysis comparing transcriptional profiles of liver metastases to non-liver metastases (breast, lymph-node, skin, bone, lung and ascite). A summary of significantly differentially altered cell adhesion and matrix-remodeling genes is presented. Red corresponds to up-regulated genes and green corresponds to down-regulated genes within the heatmap. The color scale represents the mean centered Log<sub>2</sub> expression of the genes. Black in the top bar represents liver metastases and gray represents other metastases.

**Figure 3.** Claudin-2 mRNA expression. A-B) Box plots comparing *CLDN2* expression between liver and non-liver (breast, lymph-node, skin, bone, lung and ascite) metastatic lesions in cohort 1. The specific anatomical location of the profiled metastases was taken into consideration. C-D) Box plots comparing *CLDN2* expression between patients presenting with liver metastases vs. non-liver metastases. Patients were categorized in to four groups associated with prognosis and this stratification considered only the most advanced metastatic site recorded and not the specific anatomical location of the metastatic lesion profiled [local; locally advanced or regional metastases in the lymphnodes or skin, bone; skeletal metastases with or without loco-regional metastases, lung; plural metastases with or without skeletal and loco-regional metastases, liver; hepatic metastases with or without plural, skeletal or loco-regional metastases. The open circles and asterisks in the figures represent mild and extreme outliers respectively for each group in each comparison. All statistical tests are two-sided.

**Figure 4.** Claudin-2 protein expression. Representative images of immunohistochemical staining of primary breast cancers showing A) deficient (<10% positive tumor cells) and B) high (>50% positive tumor cells) claudin-2 expression, respectively.

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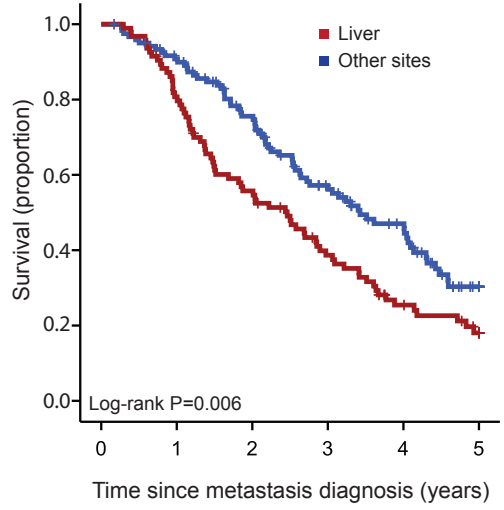
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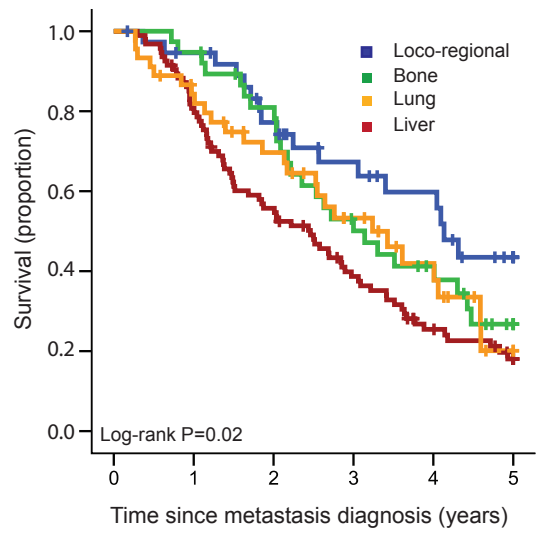
Figure 1

A



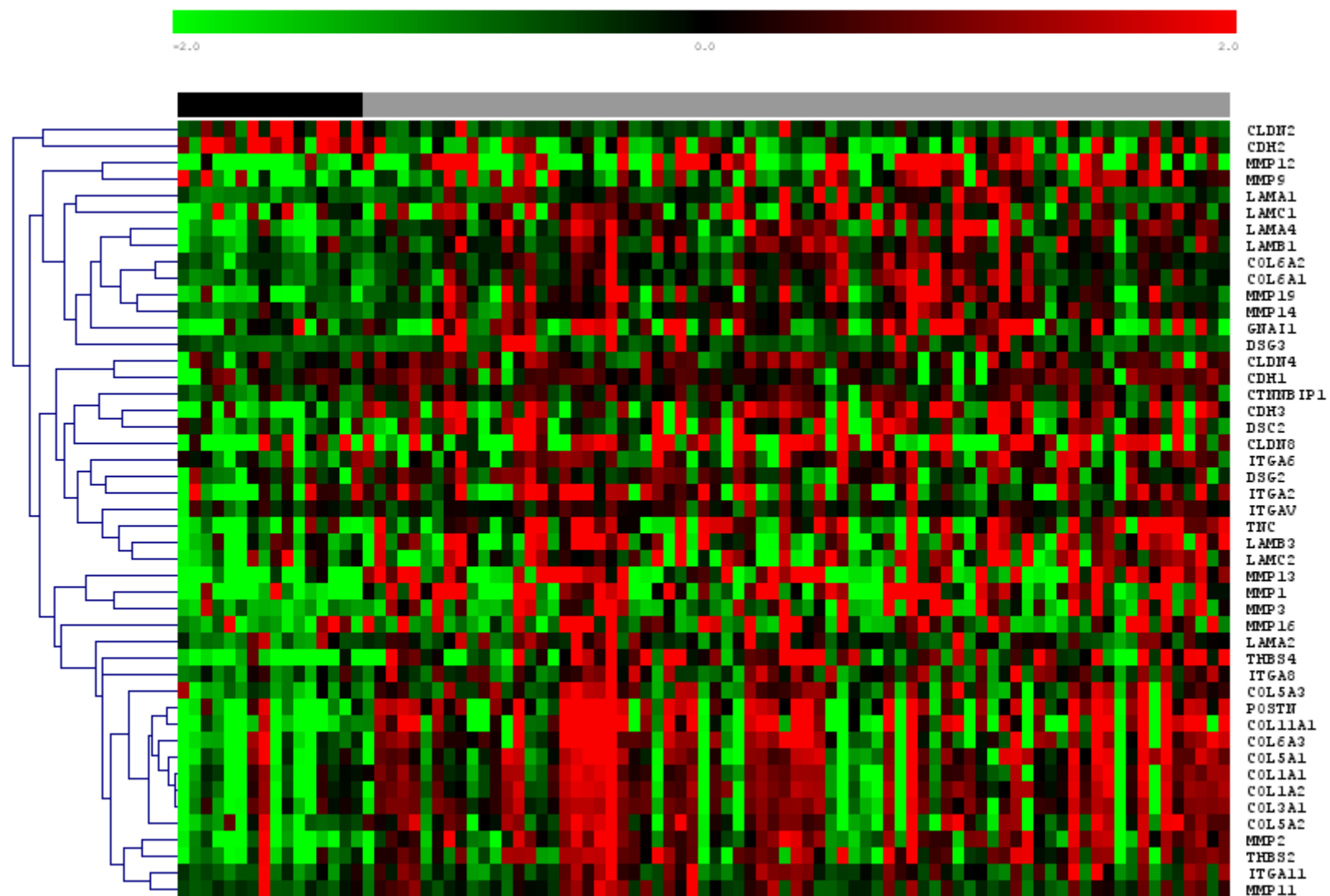
No. at risk	0	1	2	3	4	5
Liver	94	75	51	33	19	11
Other sites	121	106	82	54	36	12

B

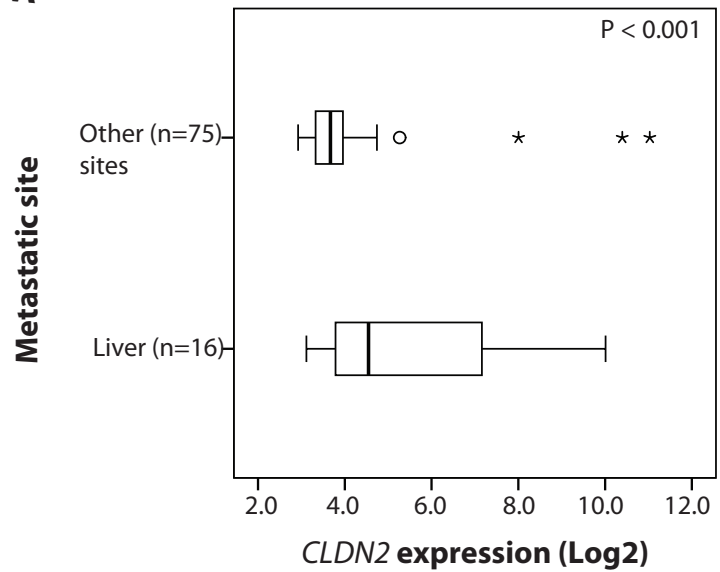


No. at risk	0	1	2	3	4	5
Liver	94	75	51	33	19	11
Lung	45	36	27	17	10	2
Bone	38	36	29	18	12	4
Loco-regional	38	34	36	19	14	6

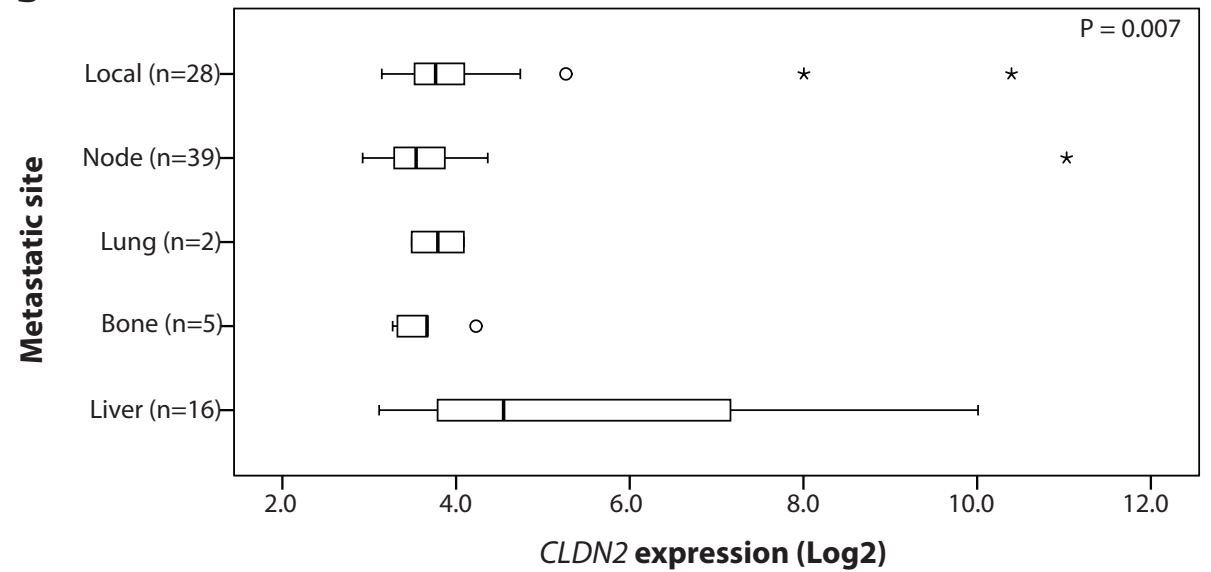
Figure 2



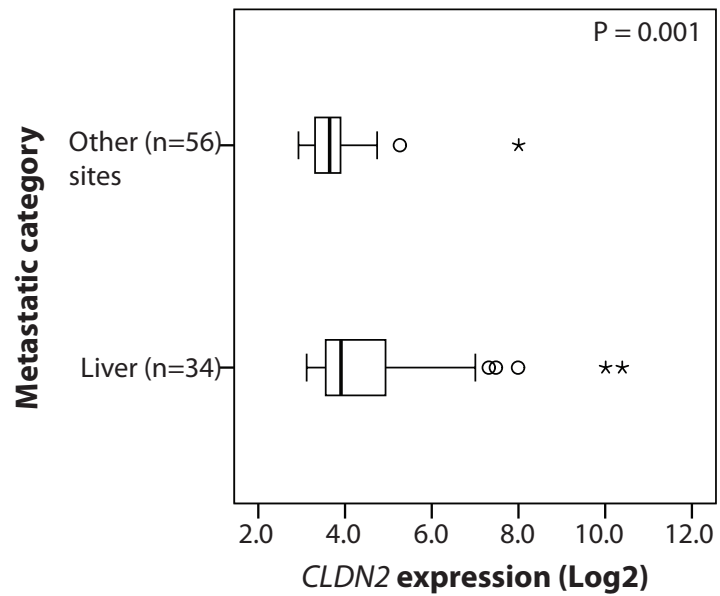
**Figure 3**  
**A**



**B**



**C**



**D**

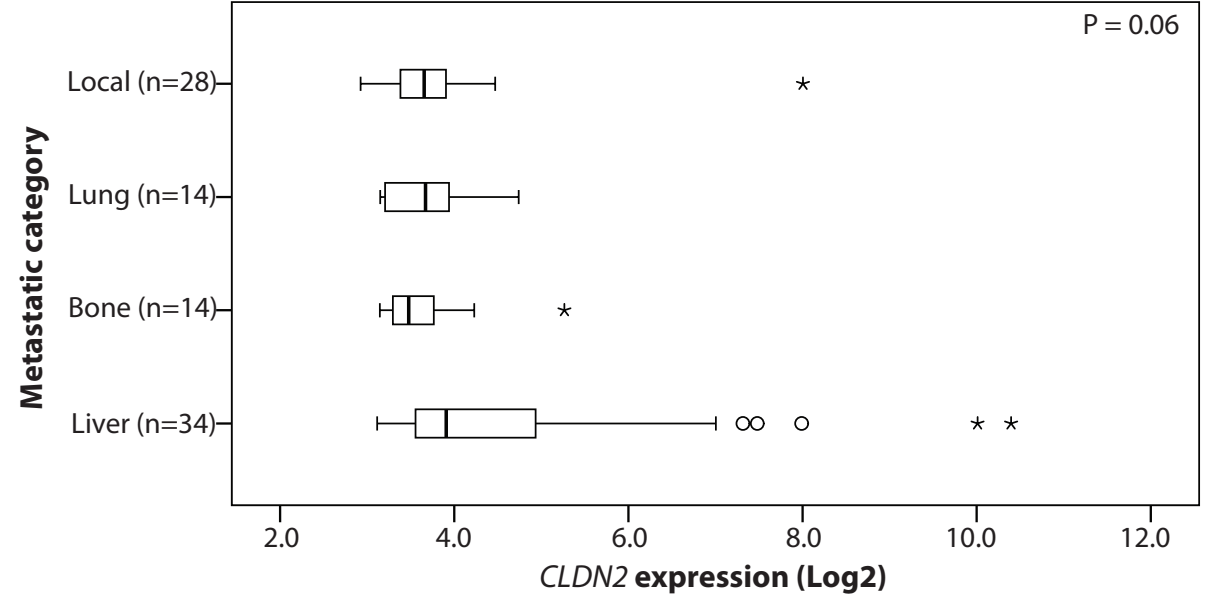
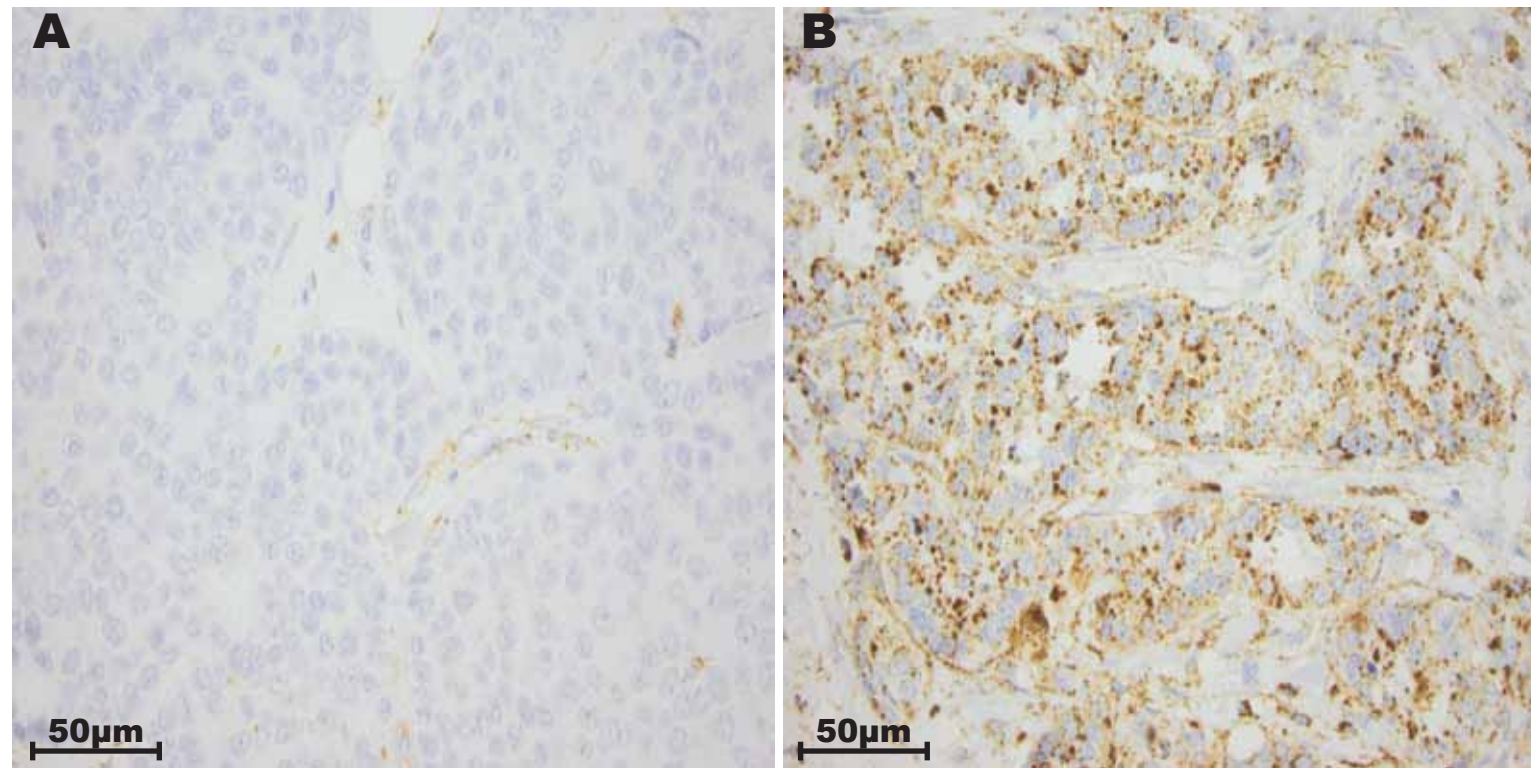


Figure 4



**Table 1. Associations between claudin-2 protein expression and other conventional breast cancer prognostic factors in cohorts 1 and 2.**

Prognostic Factor	Cohort 1		Cohort 2	
	% in high CLDN2 (N high/N total)		% in high CLDN2 (N high/N total)	
	CLDN2+ 70% (134/191)	P	CLDN2+ 51% (107/208)	P
<b>Age</b>				
< 50 years	73% (62/85)	0.45	56% (86/154)	<b>0.03</b>
≥ 50 years	68% (72/106)		39% (21/54)	
<b>ER</b>				
Positive	71% (107/150)	0.93	49% (67/136)	0.39
Negative	71% (24/34)		56% (40/72)	
<b>PR</b>				
Positive	64% (67/104)	0.06	51% (74/145)	0.86
Negative	78% (59/76)		52% (33/63)	
<b>Tumour size</b>				
≤ 2.0 cm	68% (54/79)	0.61	49% (77/156)	0.30
> 2.0 cm	72% (79/110)		58% (30/52)	
<b>Nodal status</b>				
N0	60% (37/62)	<b>0.03</b>	51% (107/208)	-
N+	75% (94/125)		0	
<b>Histological grade</b>				
1/ 2	78% (56/72)	0.16	43% (61/143)	<b>&lt; 0.001</b>
3	68% (66/97)		69% (43/62)	
<b>Ki67</b>				
High	65% (41/63)	0.29	77% (44/57)	<b>&lt; 0.001</b>
Low	73% (85/117)		43% (56/129)	
<b>Site of relapse</b>				
Liver	79% (66/84)	<b>0.02</b>	50% (9/18)	0.90
Non-Liver	64% (68/107)		52% (98/190)	

Abbreviations: CLDN2, claudin-2; ER, Estrogen Receptor; PR, Progesterone Receptor. P = P-value from  $\chi^2$  test for association in 2x2 tables. Cases with missing data were not included in the analyses.

**Table 2. Median survival in relation to the expression of claudin-2 in cohort 1.**

	<b>n</b>	<b>Events</b>	<b>Median (yrs)</b>	<b>95% CI</b>	<b>P</b>
<b>RFS</b>					<b>0.03</b>
Low CLDN2	55	55	5.7	4.5 - 6.9	
High CLDN2	126	126	3.6	2.9 - 4.2	
<b>LiMFS</b>					<b>0.002</b>
Low CLDN2	55	11	12.1	8.3 - 15.8	
High CLDN2	126	63	5.9	3.8 - 7.9	
<b>BCSS</b>					0.06
Low CLDN2	57	41	10.6	7.6 - 13.5	
High CLDN2	134	97	6.6	5.4 - 7.8	

Abbreviations: CLDN2, Claudin-2; RFS, Relapse-Free Survival; LiMFS, Liver Metastasis -Free Survival; BCSS, Breast Cancer Specific Survival; CI, Confidence Interval; yrs, years.

Table 3. Recurrence-free survival (RFS) and liver metastasis free survival (LiMFS) in cohort 1.

	RFS						LiMFS					
	Univariable			Multivariable			Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
<b>CLDN2</b> (High vs Low)	1.4	1.0 – 2.0	<b>0.03</b>	1.5	1.0 – 2.2	<b>0.03</b>	2.3	1.3 – 3.9	<b>0.003</b>	2.0	1.1 – 3.8	<b>0.03</b>
<b>Age</b> (>50 yrs vs ≤50 yrs)	2.3	1.7 – 3.2	<b>&lt;0.001</b>	2.4	1.7 – 3.5	<b>&lt;0.001</b>	1.6	1.0 – 2.5	<b>0.04</b>	1.4	0.81 – 2.3	0.23
<b>ER</b> (Neg vs Pos)	2.0	1.4 – 3.0	<b>&lt;0.001</b>	2.0	1.3 – 3.3	<b>0.004</b>	1.2	0.58 – 2.4	0.68	1.3	0.58 – 3.1	0.49
<b>Histological grade</b> (3 vs 1/2)	1.6	1.2 – 2.2	<b>0.002</b>	1.6	1.1 – 2.3	<b>0.01</b>	1.1	0.70 – 1.7	0.66	1.3	0.79 – 2.2	0.29
<b>Nodal status</b> (N+ vs N0)	1.7	1.2 – 2.2	<b>0.001</b>	1.4	1.0 – 2.1	0.05	1.5	0.94 – 2.3	0.09	1.2	0.69 – 2.0	0.54
<b>Tumor size</b> (> 2.0 cm vs ≤ 2.0 cm)	1.6	1.2 – 2.2	<b>0.001</b>	1.4	1.0 – 2.0	<b>0.04</b>	1.4	0.92 – 2.2	0.12	1.7	1.0 – 2.9	<b>0.04</b>

Abbreviations: HR, Hazards Ratio; CI, Confidence Interval; CLDN2, Claudin-2; ER, Estrogen Receptor.

**Table 4. Recurrence-free survival (RFS) and breast cancer specific survival (BCSS) in cohort 2.**

	RFS						BCSS					
	Univariable			Multivariable			Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
<b>CLDN2</b> (High vs Low)	2.2	1.3 – 3.5	<b>0.002</b>	1.4	0.83 – 2.4	0.20	1.3	0.76 – 2.3	0.32	0.78	0.42 – 1.4	0.42
<b>Age</b> (years)	0.92	0.88 – 0.96	<b>&lt;0.001</b>	0.93	0.89 – 0.98	<b>0.004</b>	0.9	0.87 – 0.95	<b>&lt;0.001</b>	0.92	0.87 – 0.97	<b>0.002</b>
<b>ER</b> (Neg vs Pos)	1.5	0.98 – 2.4	0.06	1.4	0.86 – 2.4	0.17	1.3	0.78 – 2.3	0.3	1.35	0.73 – 2.5	0.34
<b>Histological grade</b> (3 vs 1/2)	1.9	1.2 – 3.0	<b>0.004</b>	1.3	0.77 – 2.2	0.32	2.3	1.4 – 3.9	<b>0.002</b>	1.9	0.98 – 3.5	0.06
<b>HER2</b> (Pos vs Neg)	2.8	1.6 – 5.1	<b>0.001</b>	2.1	1.1 – 4.0	<b>0.02</b>	3.9	2.0 – 7.5	<b>&lt;0.001</b>	2.9	1.5 – 5.8	<b>0.003</b>
<b>Tumor size</b> (>2.0 cm vs ≤2.0 cm)	1.2	0.7 – 1.9	0.56	1	0.56 – 1.8	0.99	1.3	0.75 – 2.4	0.33	1.2	0.6 – 2.4	0.59

Abbreviations: HR, Hazards Ratio; CI, Confidence Interval; CLDN2, Claudin-2; ER, Estrogen Receptor.



**Appendix Table 1**

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**Appendix Table 2**

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**Appendix Figure 1**

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## Appendix Methods

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