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# Cadherin-11 mRNA transcripts are frequently found in rheumatoid arthritis peripheral blood and correlate with established polyarthritis

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**Abstract** Rheumatoid arthritis (RA) synovial fibroblasts hyperexpress the mesenchymal cadherin-11, which is involved also in tumor invasion/metastasis, whereas anti-cadherin-11 therapeutics prevent and reduce experimental arthritis. To test the hypothesis that cadherin-11 is aberrantly expressed in RA peripheral blood, 100 patients (15 studied serially) and 70 healthy controls were analyzed by real-time reverse transcription-PCR. Cadherin-11 mRNA transcripts were detected in 69.2% of moderately/severely active RA, versus 31.8% of remaining patients ($p = 0.001$), versus 17.1% of controls ($p < 0.0001$). Notably, cadherin-11 positivity correlated significantly and independently only with established (>1 year) polyarthritis (>4 swollen tender joints), by multivariate logistic regression analysis including various possible clinical/laboratory factors. Rare cells of undefined nature, detected by flow cytometry following CD45(−) enrichment, strongly expressed surface cadherin-11 (estimated 10–50 cells/ml of blood) in 5/6 patients with polyarticular established disease versus 1/6 patients with early RA. Studies on the potential pathogenic role of circulating cells expressing cadherin-11 in RA are warranted.
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## 1. Introduction

Rheumatoid arthritis (RA) is a progressive autoimmune systemic disease characterized by chronic synovial inflammation in multiple joints which leads to cartilage damage and bone erosions. Numerous different cell types, such as T and B lymphocytes, macrophages, chondrocytes, osteoclasts and synovial fibroblasts (SFs), also known as

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type-B or fibroblast-like synoviocytes, are involved in the destructive process. Recent advances in the fields of synovial immunologic processes, cytokines, intracellular signaling pathways and mesenchymal tissue responses, have led to a better understanding of RA complex pathophysiology [1].

A recent important advance has been the elucidation of the potential role of activated SFs in mediating RA progression and particularly their capacity to migrate to non-affected joints through the bloodstream and to invade distant cartilage. Although so far there is no evidence for SF migrating from one joint to another in human RA, experimental studies have shown that transfer to the knee joint of immortalized SFs from an immune-independent animal model of RA induced an arthritis-like disease in healthy mice, whereas these SFs migrated to most tissues throughout the body, including peripheral joints [2]. Moreover, the subcutaneous implantation of RA patient-derived SFs (RASFs) in immunodeficient mice revealed their active movement to the naive cartilage via the vasculature, independently of the site of application of RASFs into the mouse, leading to a marked destruction of the target cartilage [3]. Taken together, these experimental results support the hypothesis that RASFs, based on their unique potential to migrate via the vasculature toward hitherto unaffected cartilage, may be one of the key pathophysiological factors that facilitate and drive the progression from oligo- to polyarticular disease in humans.

Another crucial advance in understanding RA pathogenesis has been the identification of the cadherin-11, an adhesion molecule also involved in tumor invasion and metastasis [4,5], as essential in the formation and organization of the synovial membrane and for subsequent inflammation. The mesenchymal cadherin-11, known also as osteoblast cadherin [6], provides cellular adhesion between SFs and was initially thought to be selectively expressed on these cells [7]. Cadherin-11-null mice demonstrated an average 50% reduction of clinical arthritis caused by the transfer of arthritogenic K/BxN serum, as well an 80% reduction in cartilage erosion, indicating that SFs have a critical role in the course of inflammatory arthritis. Moreover, anti-cadherin-11 therapeutics not only prevented acute arthritis, but also ameliorated established arthritis in this experimental model [8]. These experimental results revealed a central role of cadherin-11 in determining the behavior of SFs and provided evidence on why RA involves the joints [9] by showing that, a) the synovium is not acting as an innocent victim of infiltrating inflammatory cells but rather as an innkeeper that regulates their entry and their potentially harmful inflammatory behavior, and that, b) cartilage damage can result from the synovial tissue's own capacity to attach, migrate over, and invade into local environment. Notably, cadherin-11 expression is also increased in lung fibroblasts from patients with RA-associated interstitial lung disease [10], whereas a recent study has shown aberrant cadherin-11 expression in hematopoietic cells, namely in alveolar macrophages derived from patients with pulmonary fibrosis [11].

Against the background described above we tested the hypothesis that aberrant expression of cadherin-11, possibly deriving from pathogenic cells such as circulating RASFs and/or hematopoietic cells with synovium/cartilage invading potential, can be found in the peripheral blood of patients with active polyarticular RA.

## 2. Patients and methods

### 2.1. Dermal fibroblast cultures and spiking experiments

The primary Human Dermal Fibroblast cell line isolated from adult skin (HDFa) (Invitrogen, Life Technologies Corporation, USA) was cultured and served as positive control for cadherin-11 expression at mRNA and protein level, as well as for determination of the RT-PCR sensitivity in cell spiking experiments after serial dilution at $10^4$, $10^3$, $10^2$, 10, 1 and 0 cells per ml of blood.

### 2.2. Peripheral blood and synovial fluid samples

Peripheral blood was obtained from consecutive consenting RA patients attending our Rheumatology outpatient Clinics, who met the 1987 American College of Rheumatology classification criteria, and had clinically active synovitis ($n$ = 85) or quiescent disease ($n$ = 15) (Table 1). Active synovitis was defined by the presence of at least one simultaneously swollen and tender joint. Detailed clinical examination, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) measurements were performed and the disease activity index-28 (DAS28-ESR) was calculated. Fifteen patients with active RA and DAS28-ESR $>$ 3.1 at baseline (moderate or severe disease activity) were randomly chosen and re-examined after 2–4 months. Peripheral blood samples obtained from 70 blood donors, gender and age (+/−5 years) matched to patients younger than 66 years, served as healthy control samples. In addition, synovial fluid samples were obtained from 5 RA patients with clinical knee joint effusion. The study was approved by the Laikon Hospital ethics committee and all subjects provided informed consent according to the Declaration of Helsinki.

### 2.3. RNA extraction and cDNA synthesis

Freshly drawn synovial fluid or peripheral blood samples (3 ml) were subjected to erythrocyte lysis (10 mM KHCO3, 155 mM NH4Cl, 0.1 mM EDTA, pH 7.4) and RNA was extracted using 1 ml of TRI Reagent TR-118 (RT-118; MRC Inc. Cincinnati, OH, USA) according to the manufacturer's instructions. One microgram of each RNA was reverse transcripted to cDNA using 200 U of M-MuLV Reverse Transcriptase (Finnzymes), 0.5 μg oligo DT Primers (Fermentas, GmbH, Germany), 0.5 mM dNTPs (HT Biotechnology, Cambridge, UK) and 4 U ribonuclease inhibitor (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

### 2.4. Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR

The obtained cDNA was amplified by RT-PCR using 1x Kapa 2G Robust HotStart Ready Mix (KAPA Biosystems) according to the manufacturer's instructions or quantified by QRT-PCR using 1x Kapa SYBR FAST qPCR kit (KAPA Biosystems) in selected cycling conditions (95 °C for 30 s, 95 °C for 3 s, 63.3 °C for 30 s, for 38 cycles). B-actin served as an internal control (F: CCTCGTCTTTGCCGA R: TGGTGCCTGGGGCG). In

**Table 1** Demographics, clinical features, laboratory parameters and treatment modalities at peripheral blood sampling from 100 patients with RA enrolled in the study and stratified by the level of disease activity (DA)[a].

| | All $n = 100$ | Remission $n = 15$ | Low DA $n = 7$ | Moderate DA $n = 38$ | Severe DA $n = 40$ |
|---|---|---|---|---|---|
| Women (%) | 75 | 73 | 71 | 76 | 75 |
| Age (mean ± SD) | 56 (±15) | 55 (±13) | 57 (±19) | 57 (±16) | 55 (±15) |
| Disease duration (mean ± SD) | 8,6 (±10,3) | 7,6 (±8,1) | 4,9 (±3,3) | 9,2 (±12) | 8,9 (±10,1) |
| Polyarthritis (%) | 53 | 0 | 0 | 47 | 88 |
| ESR 1st h (mean ± SD) | 38 (±27) | 23 (±13) | 34 (±35) | 35 (±25) | 55 (±23) |
| RF positivity (%) | 61 | 80 | 29 | 55 | 65 |
| CRP mg/ml (mean ± SD) | 21 (±34) | 10 (±14) | 20 (±43) | 22 (±40) | 33 (±32) |
| DMARDs (%) | 55 | 67 | 43 | 55 | 53 |
| Biologics (%) | 37 | 53 | 43 | 42 | 25 |
| Untreated/low dose corticosteroids (%) | 33 | 7 | 29 | 32 | 45 |
| Cadherin-11 positivity in RT-PCR (%) | 61 | 27 | 43 | 68 | 70 |

[a] Remission = DAS28 < 2.6; low DA = DAS28 2.6–3.2; moderate DA = DAS28 3.2–5.1; severe = DAS28 > 5.1; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CRP, C-reactive protein; DMARDs, disease modifying antirheumatic drugs.

all cases primers sets were designed de novo and checked for specificity using National Cancer for Biotechnology Information (NCBI) BLAST search tool (Cadh11F: CATGGGCACCA TGAGAAGGGC, Cadh-11R: GCCTGAGCCATCAACGTGTACTG) and used at a concentration of 0.2 μM each. Relative differences in cadherin-11 mRNA expression levels between peripheral blood and synovial fluid from the same patient, as well as between serial peripheral blood samples, were estimated as fold changes using ddCT method and b-actin as reference gene. Difference of cadherin-11 mRNA expression levels between subgroups of patients was performed by comparing mean ± SD of individual difference between threshold cycle values (dCT).

### 2.5. Immunohistochemistry of synovial tissue

Cadherin-11 immunostaining was performed on 4 μm sections of paraffin-embedded RA synovial tissues obtained from our tissue archive using the 2-step peroxide conjugated polymer technique (Envision kit, DAKO Corp., Carpinteria, CA). Following antigen retrieval (30 mm in citrate buffer PH 6.0 at 750 W) the anti-cadherin-11 mAb (clone 1A5, Abnova, Taiwan) diluted 1:100 was applied overnight at 4 °C. Staining was visualized using DAB as chromogen. Sections from the same specimen in which the primary antibody was substituted with non-immune serum served as negative control.

### 2.6. Flow cytometry analysis

Synovial fluid samples (0.2 ml) were incubated with 1 μg of the anti-cadherin-11 mAb described above (clone 1A5, Abnova) or purified mouse IgG1 (k Isotype Ctrl, Biolegend, San Diego) for 30 min at room temperature and then stained with 0.4 μg secondary antibody (PE Goat anti-mouse IgG, BioLegend, San Diego, CA) and 20 μl anti-CD45 (FITC anti-human CD45, Biolegend) for 20 min at RT, according to the manufacturer's instructions and analyzed in Cyflow ML Flow Cytometer (Partec Gmbh, Germany).

Peripheral blood samples (7 ml) were subjected to erythrocyte lysis and incubated with 5 μg of human anti-CD45 (purified anti-human CD45, Biolegend) for 30 min at 4 °C and then incubated with 35 mg of un-conjugated magnetic beads (Biomag, goat anti-mouse IgG, Polysciences, GmbH Eppelheim,) for another 30 min at 4 °C. Magnetic bead/CD45 positive cell complexes were removed using a magnetic separator (Biomag, multistep magnetic separator, GmbH) for 10 min. The remaining cell suspensions were analyzed by flow cytometry as described above.

### 2.7. Statistical analysis

Chi square test with Yate's correction, unpaired *t*-test, as well as univariate and multivariate logistic regression analysis, including demographic, clinical and laboratory factors and treatment modalities that could affect cadherin-11 positivity in real-time quantitative RT-PCR, were used as appropriately; $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Cadherin-11 gene is frequently expressed in RA-derived peripheral blood

As revealed by cell spiking experiments using the HDFa cell line our RT-PCR method was sensitive enough to detect mRNA for cadherin-11 from 10 HDFa cells per ml of blood (Fig. 1A). Using this method, cadherin-11 mRNA was amplified by RT-PCR in all 5 samples of total RNA extracted from freshly drawn synovial fluid derived from knee joints of patients with active RA (not shown). Subsequently, we searched for cadherin-11 mRNA transcripts by this method in peripheral blood derived from healthy donors (Fig. 1B) and RA patients with clinical synovitis (Fig. 1C), in the majority of whom the presence of cadherin-11 mRNA transcripts was evident. The corresponding bands' density of cadherin-11 RT-PCR products lied between the densities which denoted 10 and 100 cadherin-11 expressing HDFa cells/ml of blood in spiking experiments shown in Fig. 1A.

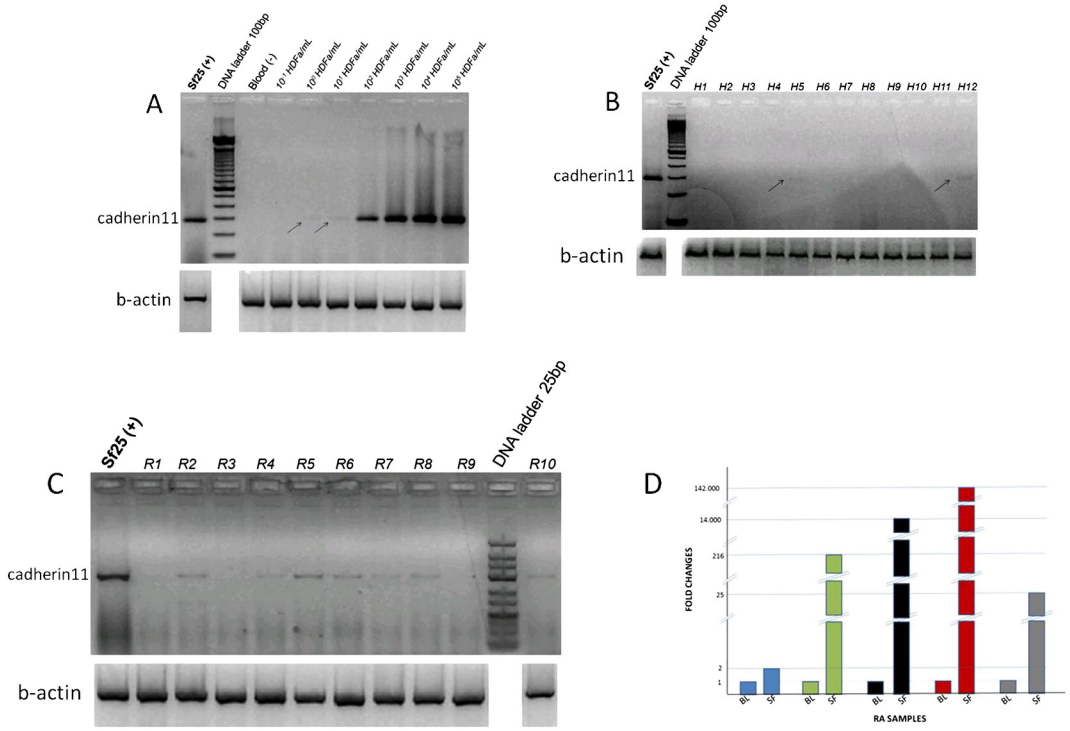


**Figure 1** A. Cadherin-11 expressing human dermal fibroblasts (HDFa) cell-spiking experiment reveals that the sensitivity of RT-PCR to detect mRNA transcripts lies between 1 and 10 cells/ml (black arrows). Total RNA extracted from RA knee joint-derived synovial fluid (Sf25) served as positive control (first lane). B. Only 2 of 12 peripheral blood samples derived from healthy blood donors (H1–H12) expressed by RT-PCR cadherin-11 mRNA, appearing as faint bands (black arrows). C. In contrast, cadherin-11 mRNA transcripts appear in 7 of 10 peripheral blood samples derived from patients with RA (R1–R10) using RT-PCR. D. Cadherin-11 mRNA expression level in knee joint-derived synovial fluid is always stronger than the corresponding level in peripheral blood from patients with RA ($n$ = 5), estimated by real-time quantitative RT-PCR as fold changes using ddCT method and b-actin as reference gene.

### 3.2. Cadherin-11 mRNA transcripts in RA-derived peripheral blood are more frequently found in active disease.

Based on the findings described above, we next employed the real-time quantitative RT-PCR in paired peripheral blood/synovial fluid samples from 5 patients. As shown in Fig. 1D, cadherin-11 expression level in blood was always weaker by several folds than in the corresponding synovial fluid sample.

The rates of cadherin-11 mRNA detection by real-time quantitative RT-PCR in 100 peripheral blood samples derived from patients in remission (DAS28 < 2.6), severely active (DAS28 > 5.1), moderately active (DAS28 ranging between 3.2 and 5.1) disease, and patients with low disease activity (DAS28 ranging between 2.6 and 3.2) are shown in Table 1. Collectively, cadherin-11 mRNA was detected in 69.2% of moderately or severely active disease ($n$ = 78), versus 31.8% of patients with low disease activity or in remission ($n$ = 22) ($p$ = 0.001), versus 17.1% of healthy controls ($p$ < 0.0001) (Fig. 2).

However, within positive samples, individual values of DAS28 did not correlate with the corresponding cadherin-11 mRNA blood levels, estimated by the dCT method, by regression analysis. Moreover, mean dCT values were comparable between positive samples either from patients with severely active disease ($n$ = 28, 17.69 + 1.64), the remaining patients ($n$ = 33, 17.94 + 1.78) or healthy controls ($n$ = 8, 17.46 + 0.98).

On the other hand, in the majority of those 15 patients with moderately or severely active RA at baseline who were examined serially, the changes in disease activity between baseline and follow-up paralleled the changes of cadherin-11 level of detection by real-time quantitative RT-PCR. More specifically, cadherin-11 mRNA transcripts became undetectable in 6 patients, whereas mRNA levels decreased in 3 additional patients (#1–8, 14, Table 2) as disease became inactive (patients #1–4) or DAS28 decreased (patient # 5–8, 14) after 2–4 months from baseline. Accordingly, cadherin-11 mRNA transcripts were undetectable at baseline in one patient with moderate disease activity but were detected at follow-up, as disease activity increased to severe levels (patient # 9, Table 2).

### 3.3. Established polyarthritis correlates independently with cadherin-11 mRNA transcripts found in RA-derived peripheral blood

ESR levels were comparable between those 61 RA patients with detectable cadherin-11 (39 + 27 at the 1st hour) and the

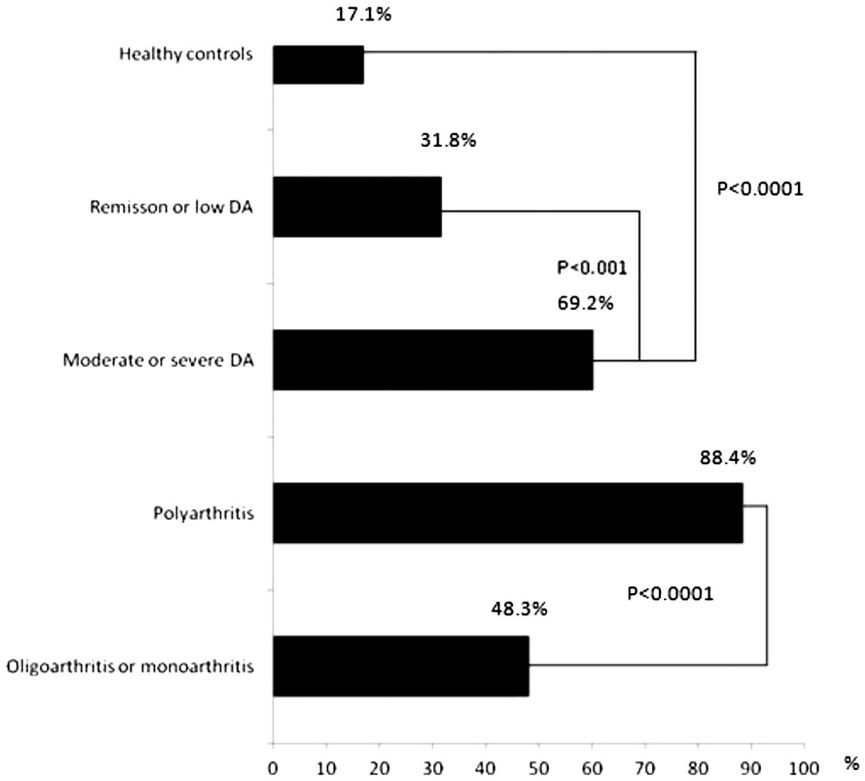


**Figure 2** Rates of cadherin-11 detection by real-time RT-PCR in peripheral blood derived from healthy blood donors (controls), patients with RA in remission or with low disease activity, and patients with moderately or severely active disease. The comparison between those active patients with polyarthritis and those with mono/oligoarthritis at the time of sampling is also shown.

remaining 39 patients (41 + 27) studied at baseline; CRP levels were also comparable (22.8 + 28.4 versus 16.5 + 17.7 mg/ml, respectively). Those patients with RF-positive disease were almost equally distributed between cadherin-11 positive and negative samples (56% versus 44%, respectively). Regarding treatment modalities, no significant differences were found between cadherin-11 positive and negative patients in terms of non-biologic DMARDs administration (56% versus 44%), biologic agents in general (49% versus 51%) or anti-TNF agents (50% versus 50%).

Interestingly, cadherin-11 positivity was associated with longer disease duration; patients who had been tested positive had almost double RA duration than the remaining patients (10.2 + 11.9 versus 6.0 + 6.6 years, respectively, $p = 0.027$). Moreover, we observed that among those 13 active patients with disease of less than 1 year duration (early RA) only 5 were positive for cadherin-11 (38.4%) versus 52 among the remaining 72 patients (72.2%) with active disease (established RA) ($p = 0.017$). By stratifying the baseline samples obtained from patients with established RA and clinically active synovitis by the presence of mono/oligoarthritis ($n = 29$) versus polyarthritis (>4 swollen tender joints, $n = 43$) we found a highly significant difference in cadherin-11 positivity (48.3% versus 88.4%, respectively ($p < 0.0001$) (Fig. 2).

Finally, these results were confirmed by multivariate logistic regression analysis of the baseline samples after controlling for treatments. In the model various factors that could influence the presence of cadherin-11 mRNA transcripts in peripheral blood, such as gender, age, DAS-28 score, ESR and CRP (after logarithmic transformation in order to be normalized), rheumatoid factor positivity, anti-CCP antibody positivity, early versus established disease, mono/oligoarthritis versus polyarthritis, and treatment modalities such as anti-TNF treatment, other biologic treatment and any treatment versus no treatment, were entered. It was indeed found that only two factors could independently correlate with cadherin-11 positivity in real-time quantitative RT-PCR, namely, the presence of RA of more than one year duration (established disease) ($p = 0.018$, odds ratio 6.39, 95% confidence intervals 1.37 to 29.84) and the presence of polyarthritis ($p = 0.010$, odds ratio 6.63, 95% confidence intervals 1.57 to 28.02) at the time of sampling.

### 3.4. Identification of rare cells expressing surface cadherin-11 in RA-derived synovial fluid and peripheral blood

Detailed immunohistochemical studies in human synovitis had shown that cadherin-11 is abundantly expressed in the intimal lining layer and the synovial sublining in inflamed tissues from patients with RA [10]. Cadherin-11 immunoreactivity was confirmed in both the RA hyperplastic synovial lining cells as well as in sublining fibroblasts and around or within the blood vessels (Fig. 3A). Using the same anti-cadherin-11 antibody in flow cytometry analysis of control HDFa cells cadherin-11 expression on their surface was clearly shown (Fig. 3B).

As representatively shown in Fig. 3C, double stain flow cytometry analysis of RA-derived synovial fluid samples

**Table 2** Changes in individual disease activity from baseline (moderate or severe) assessed by DAS28 (ESR) and after 2–4 months, and corresponding changes of cadherin-11 mRNA blood levels by real-time quantitative RT-PCR in 15 patients with RA who were examined serially.

| Patient #, gender/age (y) | DAS28 baseline/ follow-up | RT-PCR result baseline/ follow-up | mRNA fold-change from baseline |
|---|---|---|---|
| #1, W/26 | 5.3/2.3 | +/− | n/a |
| #2, W/60 | 6.1/2.5 | +/− | n/a |
| #3, W/63 | 4.9/1.9 | +/− | n/a |
| #4, W/45 | 4.8/2.2 | +/− | n/a |
| #5, M/37 | 4.7/2.8 | +/− | n/a |
| #6, M/80 | 6.3/4.4 | +/+ | 0.86 |
| #7, M/56 | 5.3/2.9 | +/+ | 0.75 |
| #8, M/58 | 6.1/3.3 | +/+ | 0.22 |
| #9, M/67 | 3.8/5.9 | −/+ | n/a |
| #10, W/67 | 5.3/4.8 | +/+ | 2.9 |
| #11, W/67 | 3.4/3.1 | +/+ | 9.65 |
| #12, W/28 | 5.5/5.2 | −/+ | n/a |
| #13, W/31 | 3.5/3.0 | −/− | n/a |
| #14,W/61 | 4.8/4.3 | +/− | n/a |
| #15, W/60 | 4.7/4.9 | −/− | n/a |

W, woman; M, man; n/a, not applicable.

revealed that rare cells strongly expressing surface cadherin-11 were consistently present. The majority of these cells co-expressed the pan-haemopoietic marker CD45 (72.7% + 9.6%, range 62% to 85%, $n = 5$). Finally, by double stain flow cytometry analysis following CD45(−) enrichment, cadherin-11 expressing cells were also identified in peripheral blood from 5/6 patients with established RA (as representatively shown in Fig. 3D) versus in 1/6 patients with early polyarticular RA, and in none of 6 healthy blood donors. Interestingly, about 60% of cadherin-11 positive cells found in the peripheral blood of these RA patients did not co-express CD45 (59.2% + 15.0%, range 32% to 73%, $n = 6$).

## 4. Discussion

Studies in RA patients have shown that synovial inflammation is associated with high levels of cadherin-11 expression which can be reversed by prednisone [10]. Other studies have demonstrated that cadherin-11 may operate in RASFs to increase cell invasiveness and migration [12], as may also happen in various types of tumor cells [4,5]. Therefore, to test our hypothesis and assuming that cadherin-11 expressing circulating cells would be rare, we first asked whether cadherin-11 is aberrantly expressed in the peripheral blood from RA patients at the mRNA level. PCR-based methods have been widely used for the detection, for example, of rare circulating tumor cells in peripheral blood [13,14]. To avoid false-positive results a highly overexpressed marker in tumor cells compared with normal cells should be chosen [13]. Once detected by RT-PCR, mRNA markers are indicative of the presence of viable cells in the sample examined. By using such a method we identified the presence of cadherin-11 mRNA transcripts in 7 of 10 peripheral blood samples derived from RA patients (Fig. 1C), indicating that viable cadherin-11 expressing cells circulate in their vasculature.

We next analyzed a large number of peripheral blood samples by real-time quantitative RT-PCR and found that cadherin-11 mRNA was 4-fold more frequently found in patients with moderately/severely active RA than healthy donors (Fig. 2). False-positive results could have been arisen as a consequence of introduction in the circulation of dermal fibroblasts during blood sampling [13], but the highly significant difference clearly suggests an association between the presence of cadherin-11 expressing cells in the blood and clinically active synovitis in patients with RA. The difference in cadherin-11 mRNA quantities between patients and controls, which could have been expected, did not reach significance probably due to the high sensitivity of the real-time quantitative RT-PCR method employed and/or the small number of positive control samples.

As confirmed by multivariate logistic regression analysis the detection of cadherin-11 mRNA transcripts in RA peripheral blood was independent of age, gender, ESR or CRP levels, autoantibody status or treatment modalities but correlated significantly and independently with the presence of poly-arthritis in patients with established RA (Fig. 2). Given the potential role of cadherin-11 in erosive disease, which tends to be RF positive, the lack of correlation between the presence of cadherin-11 mRNA transcripts and RF positivity could be attributed to the small number of patients with the low disease activity being all but one RF negative. Significant upregulation of cadherin-11 expression by cytokines that are crucially involved in RA has been shown in experimental studies. For example, cadherin-11 expression on RASFs is highly increased *in vitro* ""by IL-17 [15], and TNF, albeit at the protein and not the mRNA level [10]. Upon engagement of cadherin-11 RASFs secrete IL-6 [16] and synthesize several matrix metalloproteinases that are key to cartilage erosion in RA [17]. Moreover, there are dramatic synergistic effects with TNF and IL-1 on IL-6 induction upon cadherin-11 engagement [16].

The presence of cadherin-11 expressing cells in RA-derived synovial fluid (Fig. 3C) and peripheral blood (Fig. 3D) was verified by double stain flow cytometry using CD45, a lineage marker of bone-marrow derived cells. According to spiking experiments using dermal fibroblasts (Fig. 1B), we indirectly estimate that in synovial fluids the number of cadherin-11 expressing cells varied between 100 cells/ml and over 1000 cells/ml. The estimated number of cadherin-11 expressing cells was several-fold lower in peripheral blood and varied between 10 and 50 cells/ml of blood. Similar very low cell numbers were also estimated by extrapolating our flow-cytometry findings in synovial fluids and peripheral blood samples. We believe that the rarity of these cells did not allow us to demonstrate cadherin-11 expression at the protein level in a large number of additional experiments by Western analysis of synovial fluid and peripheral blood samples from RA patients (data not shown). Accordingly, whether cadherin- 11 is functional in these cells, which can be examined by engagement of cadherin-11 and measurement of pro-inflammatory molecules and matrix metalloproteinases *in vitro*, as shown by Noss et al. (17) remains unknown.

Co-expression of cadherin-11 and CD45 by flow cytometry was observed on RA synovial fluid-derived cells, as has also been reported on disaggregated fresh *ex vivo* RA synovial

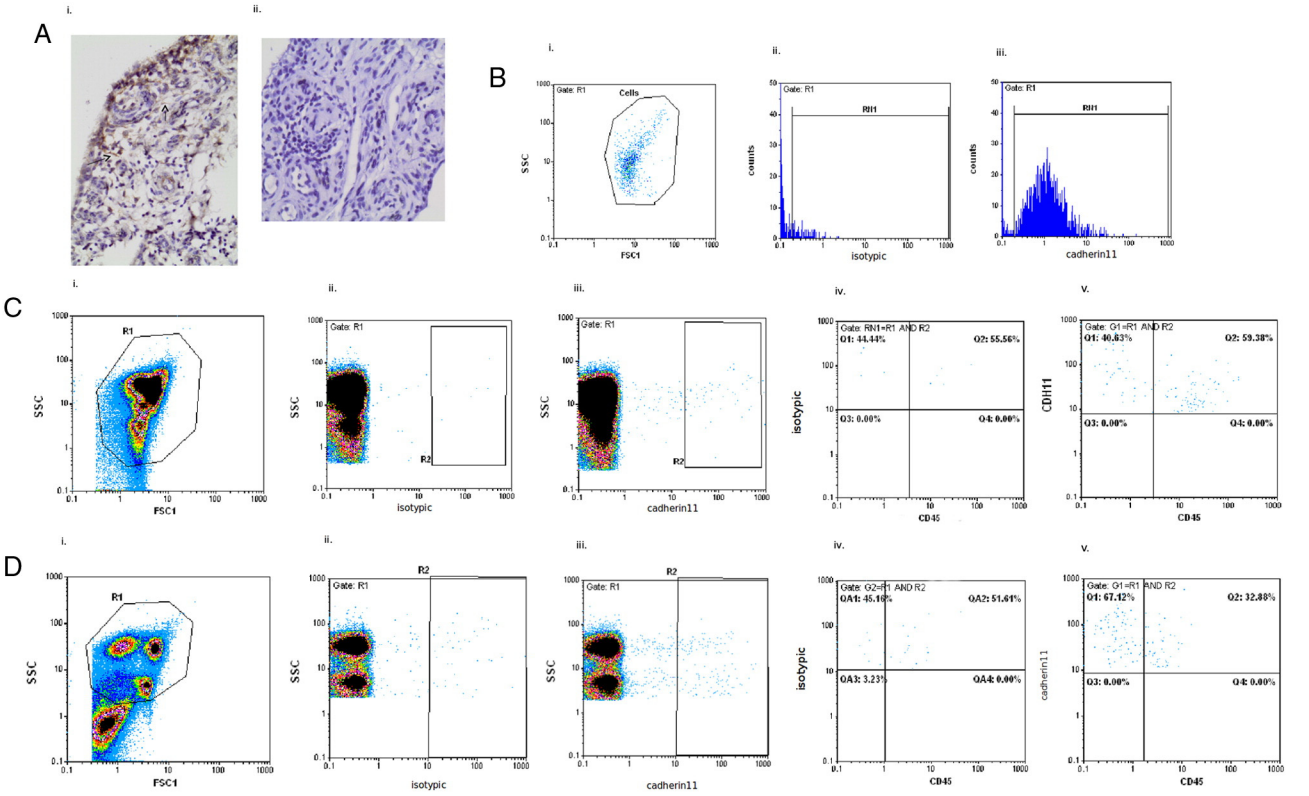


**Figure 3** A. Cadherin-11 immunolocalization in the hyperplastic intimal synovial lining, sublining fibroblasts (black arrows), as well as around and within blood vessels in a representative patient with RA; note that plasma cells are not stained with the anti-cadherin-11 mAb (i); negative control (ii). B. Cadherin-11 expression on HDFa cells confirmed by flow cytometry. Gated cells according to forward and side scatter (i), purified mouse IgG1 staining (isotypic control) (ii) and anti-cadherin-11 mAb staining (iii) reveal surface expression in approximately 80% of HDFa cells. C. Representative flow cytometry analysis for cadherin-11 and pan-hematopoetic marker CD45 expression of synovial fluid derived from RA knee joint. Cells were gated according to forward and side scatter (i), and after staining with purified mouse IgG1 (isotypic control) (ii), or anti-cadherin-11 mAb (iii), and both anti-45 mAb and the respective isotypic control (iv), a portion (40.6%, upper left) of cadherin-11 positive (+) cells do not co-express CD45 (v). D. Representative flow cytometry analysis for cadherin-11 expression in peripheral blood derived from a patient with established RA after CD45 negative enrichment. Blood cells (lympho, mono, granulo-cytes) were gated according to forward and side scatter (i), and after staining with purified mouse IgG1 (isotypic control) (ii), or anti-cadherin-11 mAb (iii), and both anti-45 mAb and the respective isotypic control (iv), a portion (67.1% upper left) of cadherin-11 positive (+) cells do not co-express CD45 (v).

tissue [7]. About 70% of these cells co-expressed CD45, indicating their hematopoietic origin. On the other hand, an average of 60% cadherin-11 positive cells found in peripheral blood of 6 of 12 RA patients lacked CD45 expression. In additional triple stain flow cytometry experiments employing also mAbs which are used to characterize synovial cells, such as mAbs against CD90 and CD106 (not shown), as well as in additional cytospin immunofluorescence experiments using anti-cadherin-11, anti-CD90 and antiCD106 mAbs (not shown), we were unable to obtain reproducible, convincing results allowing us to conclude on whether some of the rare peripheral blood cells that express cadherin-11 but lack CD45 expression [18] truly represent circulating synovial cells. Such cells could hypothetically enter the peripheral circulation as the synovium is being transformed into a hyperplastic, invasive tissue with new vessel formation. However, the experiments reported herein cannot define the exact nature of circulating cadherin-11 expressing cells. In our opinion, possibly circulating RASFs can be detected and accurately characterized only by fluid phase biopsies from RA patients. Using this novel method the different morphology of synovial cells compared to haematopoietic cells can be demonstrated. Recently, this method has been successfully employed for demonstration of circulating tumor cells in peripheral blood from patients with metastatic cancers [19], as well as of circulating endothelial cells after myocardial infarction [20].

On the other hand, cadherin-11 can be expressed on bone marrow-derived cells, such as alveolar macrophages under certain conditions [11]. Very recently, macrophages expressing cadherin-11 were also found in the skin of patients with systemic sclerosis [21]. Interestingly, prolonged *in vitro* cultures (2–4 weeks) of bone marrow plasmacytoid dendritic cells from patients with RA and osteoarthritis resulted in cadherin-11 neo-expression [22]. Also, in our hands cadherin-11 neo-expression by flow cytometry was observed, albeit in rare cells, in healthy-derived peripheral blood mononuclear cells after activation *in vitro* with phytohemaglutinin (data not shown). Finally, circulating fibrocytes could also explain cadherin-11 and CD45 co-expression, despite the fact that expression of cadherin-11 on them has not been studied. Fibrocytes are CD45(+) monocyte-derived cells that express features of both a macrophage and a connective tissue cell [23]

and are able to migrate to joints and influence the onset of disease processes in collagen-antibody induced arthritis [24]. Therefore, circulating hematopoietic cells with synovium/cartilage invading potential may also partly explain the independent correlation of cadherin-11 mRNA transcripts in RA peripheral blood with established polyarthritis.

To conclude, the results presented herein indicate that detection of cadherin-11 mRNA transcripts is frequent in the peripheral blood of RA patients with clinical synovitis, correlates only with the presence of polyarthritis in established disease and is not related with levels of autoantibodies, inflammation markers or treatment modalities. Since specific blood markers of synovial inflammation are essentially lacking [25], cadherin-11 mRNA may serve as the first putative biologic marker of polyarthritis. These results further support the notion that cadherin-11 is a legitimate therapeutic target in RA [26,27]. Along this line, the anti-cadherin-11 mAb SDP051 is anticipated to enter clinical trials in RA in the near future. Finally, further study on the potential pathogenic role of circulating cells that express cadherin-11 in human RA, as already has been shown for RASFs in mice, is warranted.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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