

# Comparative analysis of cell populations involved in the proliferative and inflammatory processes in diffuse and localised pigmented villonodular synovitis

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**Summary.** The aim of the present study was a comparative quantitative evaluation of cell populations involved in the proliferative and inflammatory compartment in both localised and diffuse pigmented synovitis villonodularis (PVNS). 15 cases of each localised and diffuse PVNS were examined by flow cytometry, immunohistochemistry, double immunofluorescence and confocal microscopy with quantitative evaluation of CD3-, CD4-, CD8-, CD20-, CD57-, CD55-, CD68-, CD163- and h4Ph positive (+) cells. The proliferative compartment of localised and diffuse PVNS was mainly composed of double-positive CD68+/h4Ph+ (CD163+/CD55+) synoviocytes. The number of double-positive synoviocytes for macrophage and fibroblast markers was significantly higher in diffuse compared to localised PVNS. The accompanying inflammatory infiltrate showed a predominance of cytotoxic cells (CD8+, CD57+), whereby the number of CD3+ and CD20+ cells was significantly higher in localised PVNS. The number of CD57+ NK cells was significantly higher in diffuse PVNS. The proliferating macrophage-like synovial cells and the cytotoxic lymphocytes could contribute to the aggressive behaviour of localised and diffuse PVNS. Moreover, with regard to the quantitative differences in cell composition between diffuse and localised PVNS and their different clinical behaviour, further studies should continue to analyse localised and diffuse PVNS separately.

**Key words:** Cell populations, Localised PVNS, Diffuse PVNS

## Introduction

Pigmented villonodular synovitis (PVNS) represents a rare group of proliferative lesions in intraarticular or paratenosynovial tissue with progressive

destruction of the affected joints and articular bleeding (Durr et al., 2001). Most authors consider these lesions to be benign neoplasms derived from synovial cells with a high recurrence rate of 50-70% (Abdul-Karim, 1992; Darling et al., 1994; Flandry et al., 1994; Fassbender, 2002). Singular cases of PVNS with histological features of malignancy in recurrent lesions with pulmonary or nodal metastases possibly representing a distinct entity have been reported (Kobayashi et al., 1994; Layfield et al., 2000).

Microscopically, one can differentiate two basic forms of PVNS: a diffuse and a localised form. They both show similar morphological features with a diffuse or nodular (localised) isomorphic proliferation of synovial cells including giant cells with focal accumulations of lipid-loaded macrophages and deposits of intracellularly-stored iron pigment. The term "synovitis" is related to an accompanying inflammatory infiltrate, which is observed in both histological types of this lesion.

Clinical studies showed essential differences regarding the clinical behaviour, prognosis and recurrence rate of localised and diffuse PVNS reporting a more benign course of the localised form. Perka et al. (2000) analysed the clinical behaviour of PVNS of the knee joint and suggested that this lesion should be classified more strictly into a potentially neoplastic - diffuse form and a "reactive granuloma" - localised form.

There is little knowledge regarding the immunohistochemical characterisation of cell populations involved in PVNS. A previously published morphological study on localised PVNS showed a presence of macrophages and CD8+ T-cells in PVNS (Oehler et al., 2000). To our knowledge a detailed comparative quantitative analysis of the cell composition of the proliferative and inflammatory compartment in both forms of PVNS has not been conducted so far.

According to clinically well known differences in the outcome and recurrence of localised and diffuse PVNS, with a more aggressive behaviour of diffuse lesions, essential differences in the cell composition of

the inflammatory infiltrate and proliferating cells could be expected. The aim of the present study was a comparative quantitative evaluation of cell populations involved in the proliferative and inflammatory compartment in both localised and diffuse PVNS.

## Materials and methods

15 samples of synovial tissue of both localised PVNS and diffuse PVNS were investigated by immunohistochemistry, double immunofluorescence with confocal microscopy, and flow cytometry.

### Immunohistochemistry

All specimens have been investigated by immunohistochemistry.

The CD68 marker, which stains lysosomes and is therefore mainly found in phagocytosing cells, was used to identify macrophage-like synovial cells. For verification, we additionally used antibodies recognizing CD163, a marker for macrophage-like synovial cells, on a randomly taken subset of cases.

Prolyl 4-hydroxylase (h4Ph) plays a central role in the synthesis of all collagen types and is a putative marker for collagen producing cells (Nissi et al., 2001; Nokelainen et al., 2001). Therefore it has been used for detection of collagen synthesizing cells (fibroblasts or fibroblast-like synovial cells). The fibroblast-like synoviocytes express the decay-accelerating factor (CD55) in high level (Hamann et al., 1999). To identify specific fibroblast-like synoviocytes, we additionally used antibodies recognising CD55.

The following antibodies (all mouse anti-human) were used: CD68 (1:500 (clone KP1; Dako, Germany); CD20 (B-cell marker), 1:100 (clone L26, Dako); CD3 (T-cell marker), 1:1 (BioGenex, CA); CD8 (T8-cell marker), 1:10 (clone C8-144B; Dako); CD4 (T4-cell and monocytes-marker), 1:40 (clone 1F6; Loxo (Novocastra)); CD57 (native killer cell marker), 1:50 (clone NK-1; Zytomed); prolyl 4-hydroxylase (h4Ph-fibroblast marker), 1:400 (Medicorp, Montreal, Canada); CD163 (marker for synovial macrophages), 1:20 (Novo Castra, UK); and CD55 (marker for synovial fibroblasts), 1:50 (Bioscience, USA).

For screening of CD68, CD20, CD3, CD8, CD57, CD4 in the various samples immunostaining was processed automatically using an automated platform-Dako Autostainer.

The primary antibodies for these stainings were diluted in the antibody Diluent (Dako ChemMate™) and detection was made using a Dako ChemMate™ kit. Non-binding monoclonal mouse IgG1 was used as negative control. For screening of h4Ph, CD163 and CD55 expression the primary antibodies were diluted in 0.1M Na-phosphate buffer, pH 7.0, containing 0.5% protease free bovine serum albumin. H4Ph, CD55, CD163 single stainings were done using the conventional alkaline phosphatase anti-alkaline

phosphatase (APAAP) technique.

The sections were finally counterstained with hematoxylin and mounted.

### Double immunofluorescence and confocal microscopy

Double staining with CD163/h4Ph for proliferative synovial cells was performed on paraffin embedded sections, which were incubated with CD68 (clone PGM-1/IgG3; 1:100, Dako) in combination with anti-h4Ph (mouse IgG1/k; 1:400; Medicorp, Montreal, Canada). As secondary antibody, a combination of biotinylated goat anti-mouse IgG1 (1:100; Southern Biotech USA; distributed by Dunn, Asbach, Germany) and peroxidase-labelled sheep anti-mouse IgG3 (1:50; Serotec, UK9 was used, followed by streptavidin Cy3 (1:1000) and donkey anti-sheep Cy2 (1:50; both from Dianova, Hamburg, Germany).

Negative primary mouse mAb of IgG1 and IgG3 isotype (Dako) served as controls. Additionally, 3 specimens of both localised and diffuse PVNS were stained with double immunofluorescence for CD55/CD163. This staining was performed on frozen sections, which were incubated with 163 (IgG1; 1:20, Novo castra, UK) in combination with CD55 (IgG2a; 1:50, Bioscience, USA). As secondary antibody, a combination of biotinylated rabbit anti-mouse IgG1 (1:100; Zytomed, San Francisco, USA) and peroxidase-labelled sheep anti-mouse IgG2a (1:500; Binding Site, Birmingham, UK) was used, followed by streptavidin Cy3 (1:1000) and donkey Anti-sheep Cy2 (1:50; both from Dianova, Hamburg, Germany).

Negative primary mouse mAb of IgG1 and IgG2a isotype (Dako) served as controls. Confocal microscopy was performed using a Spectral Confocal Microscope (Leica TCS SL).

Positive cells were counted in 10 high power fields (HPF; 1 HPF = 0,159mm<sup>2</sup>) in neoplastic and normal synovial tissue. Confocal microscopy was performed on sections double stained for macrophage- and fibroblast marker (CD68/ h4Ph and CD163/CD55), using a spectral confocal Microscope (Leica TCS SL).

### Flow cytometry

Preparation of a single cell suspension (absolute cell number: 30,000-110,000) from paraffin-embedded material for DNA-flow cytometry was done according to the Hedley technique (Hedley et al., 1983) with modifications (Ehemann et al., 1999; Sergi et al., 1999). Multiparametric analysis was performed on a Galaxy pro cytometer (Partec, Münster, Germany) by stimulating the fluorochromes FITC, PE a 488nm argon laser (25mW, adjusted) and measuring the fluorescence intensities at 530/26nm, respectively. DNA stain was measured with a mercury lamp at 435nm. Green fluorescence macrophage, CD68/FITC Clone KP1 (DAKO, Denmark) was detected in the logarithmic mode, as was red fluorescence CD45 anti-Hie-1



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(Becton-Dickinson, Heidelberg, Germany). DNA-content was recorded in the linear mode.

For each measurement 10,000 cells were analysed.

### Statistic evaluation

Quantitative evaluation of positive stained cells in immunohistochemistry and double immunofluorescence was performed in 10 HPF. Quantitative evaluation of positive cells in flow cytometry was performed in percent.

Statistical evaluation was performed using the two-sided Wilcoxon rank test with a 0.95 confidence interval.

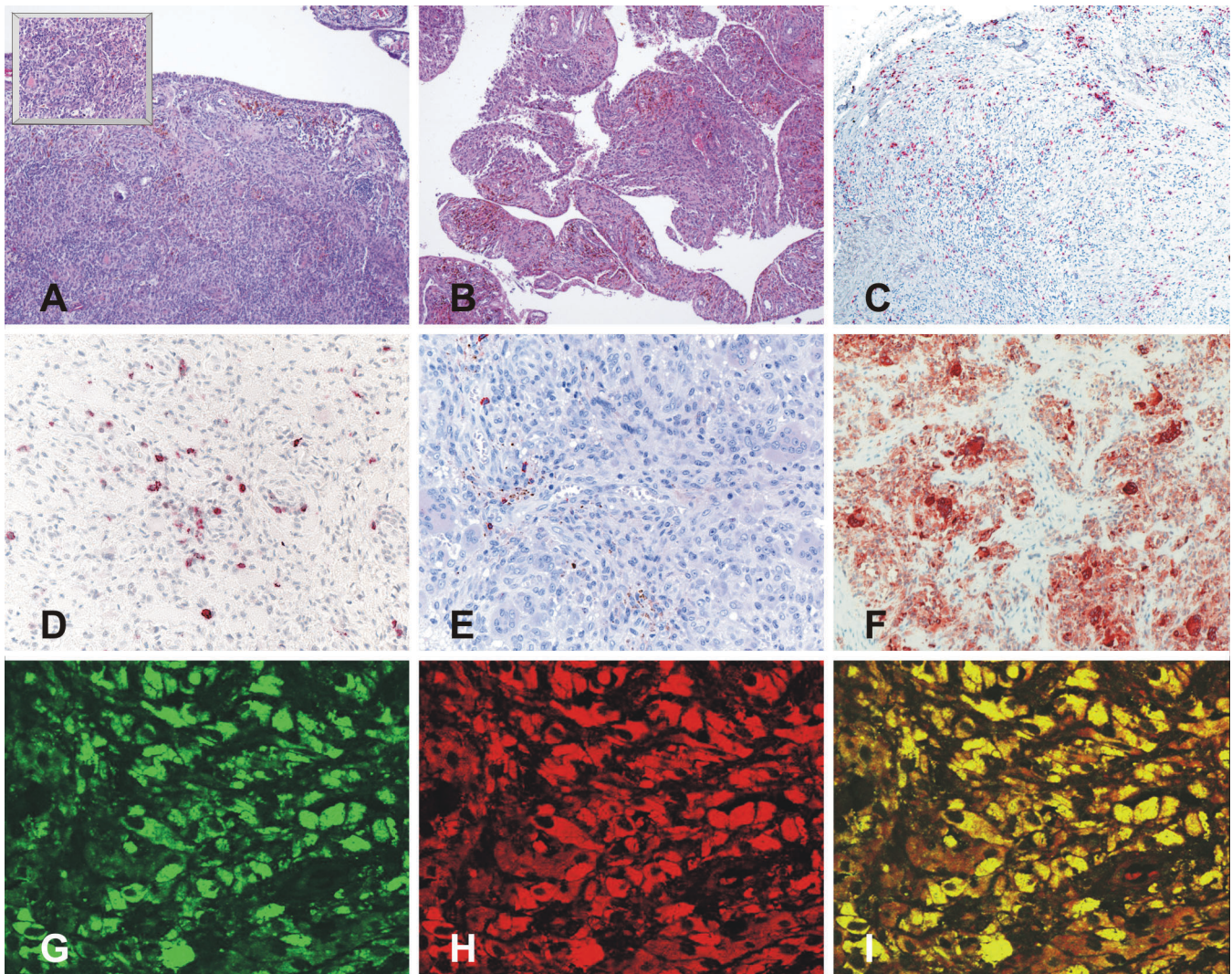
### Clinical data

Clinical data (duration of symptoms, recurrence, age and sex of patients) were analysed using a clinical questionnaire.

### Results

#### Diffuse PVNS

All specimens showed a similar distribution of the inflammatory cells with a clear predominance of CD8+ lymphocytes and CD57+ cells (native killer cells; NK)



**Fig. 1.** Representative areas of synovial tissue with localised and diffuse PVNS are shown for: **A.** HE-stain of localised PVNS. x 7,8. Insert: x 31,2 **B.** HE-stain of diffuse PVNS. x 7,8. **C.** numerous disseminated CD8+ lymphocytes were found in the proliferating synovial tissue (localised PVNS). x 7,8. **D.** Positive CD68-stain in the cytoplasm of most proliferating synovial cells and giant cells (localised PVNS). x 15,6. **E.** Distribution of CD57 in proliferating synovial tissue (diffuse PVNS). x15,6. **F.** only singular CD20+ B-cells were found in diffuse PVNS. x 15,6. **G.** Proliferating cells expressing CD68 in diffuse PVNS. x 260. **H.** Proliferating cells expressing h4Ph in the same area as in picture G in diffuse PVNS. x 260. **I.** Proliferating cells double positive for CD68 and h4Ph (yellow overlay) in diffuse PVNS. x 2604

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regardless of the duration of the symptoms (4 to 18 months, no recurrence), age (17 to 42 years) or sex of the patients. CD3+, CD8+ T-cells as well as NK-cells were distributed diffusely throughout the proliferating synovial tissue (Fig. 1e). The average number of CD20+ B-cells and CD4+ cells counted in ten high power fields was significantly lower than the number of cytotoxic lymphocytes (CD8+ T-cells and NK-cells) (Fig. 1f).

Analysis of CD68-versus CD45-positive cell populations using flow cytometry showed a clear predominance of CD68+ cells (63 %). Immunostaining using markers for macrophages (CD68, CD163) and fibroblasts (CD55, h4Ph) showed a positive cytoplasmic reaction in most of the proliferating synovial cells. Using double immunofluorescence, a predominance of synoviocytes double positive for macrophage and fibroblast markers (CD68+/h4Ph+ or CD163+/CD55+), in some areas up to 90% of the proliferating cells, was detected (Fig. 1g-i). In non-proliferating synovial tissue a few scattered CD68+ cells were found. No double positive cells were identified in these areas. The statistic data are summarised in Table 1.

### Localised PVNS

In localised as in diffuse PVNS, CD3+ and CD8+ lymphocytes were predominant in proliferating synovial tissue, regardless of the duration of the symptoms (4 to

13 months, one recurrence), age (21 to 45 years) or sex of the patients. The average number of CD4+ lymphocytes detected in ten high power fields was low. No statistically significant differences regarding the counts of CD8+ T-cells were found in localised versus diffuse PVNS (s. table 1).

The number of CD3+ and CD20+ lymphocytes was significantly higher in localised PVNS versus diffuse PVNS ( $p < 0.05$ ).

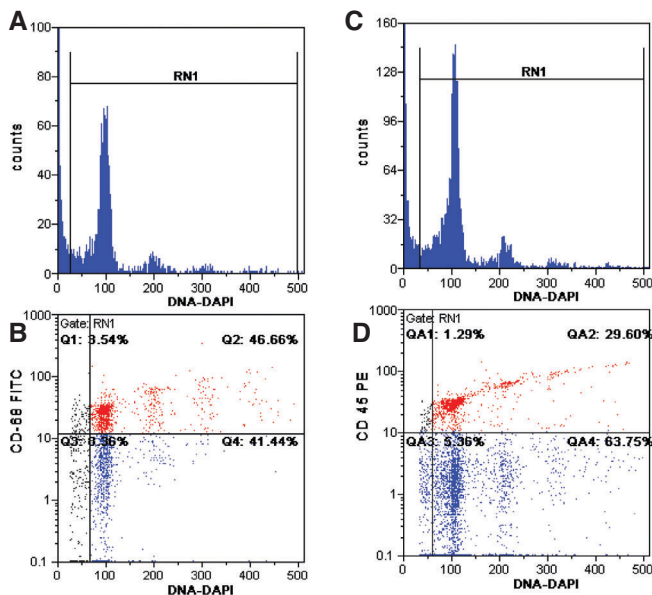
The number of CD57+ cells was significantly lower in localised compared to diffuse PVNS ( $p = 0.002$ ).

No statistically significant differences between localised and diffuse PVNS were found regarding the counts of CD45- and CD68-positive cells in flow cytometry.

The quantity and the distribution of CD68+ cells were similar in both localised and diffuse PVNS (Fig. 1d). Double-positive cells (CD68+/h4Ph+ and CD163+/CD55+) including some giant cells were distributed in small groups throughout the proliferative areas. The number of double positive cells was significantly lower in localised versus diffuse PVNS. The number of non-double-positive cells, staining either positive for macrophage (CD68, CD163) or for fibroblasts (h4Ph, CD66) markers was higher in localised compared to diffuse PVNS (Table 1).

### Discussion

Both localised and diffuse PVNS showed a similar cellular composition of the proliferative compartment and the accompanying inflammatory infiltrate, regardless of the duration of the symptoms, recurrence, age or sex of the patients. In spite of the homogenous pattern in conventional histological stains, immunohistochemical analysis revealed an intrinsic diversity of the proliferating synovial cell compartment.



**Fig. 2.** Histogram of cells isolated from synovial membrane with diffuse PVNS, stained for DNA content using DAPI (upper panels) (A, C) and two dimensional analysis of cells stained for CD68 (B) and CD45 (D) (lower panels). Fractions of cells positive for CD68 (46,66%) and CD45 (29,60%) are placed in quadrants Q1 (B) and QA1 (D). Negative cell populations for CD68 and CD45 are colored in blue. Per each analysis 10.000 cells are measured and calculated.

**Table 1.** Comparative quantitative evaluation and statistical analysis of examined cell populations by immunohistochemistry, double immunofluorescence and flow cytometry.

ANTIBODIES	DIFFUSE PVNS (Positive cells in 10 HPF)	LOCALISED PVNS (Positive cells in 10 HPF)	P VALUE
<i>Immunohistochemistry</i>			
CD4	19.83 ± 16.9	10.6 ± 5.20	0.31
CD8	267.5 ± 176.54	373.5 ± 104.27	0.31
CD3	98.83 ± 37.43	150.66 ± 57.46	0.041
CD57	75.33 ± 18.16	6.33 ± 4.08	0.002
CD20	19.66 ± 13.08	70.5 ± 38.077	0.026
<i>Double immunofluorescence</i>			
CD68	520.8 ± 277.15	849.31 ± 447.18	0.3871
H4Ph	810.19 ± 403.66	2106.06 ± 509.95	0.032
CD68/h4Ph	4102.98 ± 1320.98	2070.8 ± 831.27	0.015
<i>Flow cytometry</i>			
CD68	62.8 ± 9.54	71.21 ± 26.82	0.31
CD45	20.64 ± 18.13	26.21 ± 19.88	0.31



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The proliferating synovial cells included macrophage- as well as fibroblast-like cells by virtue of their immunohistochemical phenotype. In addition, there was a fraction of synoviocytes double-positive for both macrophage- and fibroblast- like markers. Synovial cells expressing macrophage markers (singular or in coexpression with fibroblast markers) constituted the predominant cell type in the proliferative compartment.

Although overall a similar cellular composition of proliferating synovial cells was seen, localised and diffuse PVNS differed considerably in the relative quantity of the analysed cells. Compared to the localised form, in diffuse tumours a significantly higher number of proliferating synoviocytes showing coexpression for macrophage/fibroblast markers was detected. A pathogenetic role of macrophage-like synovial cells seems to be important in both localised and diffuse PVNS. There is increasing evidence that the destruction of bone and cartilage can be due to the proliferation of synovial cells independent of an inflammatory reaction in diseases such as rheumatoid arthritis (Seemayer et al., 2001). The destructive potential of these cells by expression of cytokines and matrix metalloproteinases can mediate joint destruction and bleeding in PVNS (O'Keefe et al., 1998). It could be discussed as to whether these double positive cells contribute to the joint destruction and thereby contribute to the clinically observed more aggressive behaviour of diffuse PVNS.

The destructive effect could possibly be supplied by cytotoxic lymphocytes.

The inflammatory compartment showed a clear predominance of cytotoxic lymphocytes in both diffuse and localised PVNS. Notably, the average number of NK- cells was significantly higher in diffuse tumours. The predominance of the cytotoxic lymphocytes in diffuse and localised PVNS, and the relatively low number of B-lymphocytes and CD4+ cells, which play an important role in perpetuating the chronic inflammation in the synovial membrane (Lipsky and Davis, 1998), are not typical for a chronic unspecific inflammatory process.

The pathogenetic interaction between neoplastic and inflammatory cells in PVNS is not yet fully understood. Moreover, the neoplastic nature of the disease is still controversially discussed. Oehler et al. (2000) regard the perpetuated proliferation of synovial cells to be a result of the chronic inflammatory process. However, other authors consider these lesions to be benign neoplasms (Darling et al., 1994; Flandry et al., 1994; Fassbender, 2002).

The present study was not designed to investigate, whether PVNS represents a tumor-like or a true neoplastic lesion. However, the latter concept is supported by results of our own studies: we detected alterations in cell cycle, cell cycle related proteins and cell ploidy in many cases of PVNS (unpublished data).

The inflammatory infiltrate in diffuse PVNS included significantly more NK-cells compared to the localised form, and it could be speculated that NK-cells

intensify the joint destruction and bleeding seen in diffuse PVNS.

In summary, our analysis of cell populations involved in diffuse and localised PVNS showed an overall similar composition of cells in the proliferative and inflammatory compartment. These findings underline the concept of diffuse and localised PVNS being two forms of one disease (Fassbender, 2002). However, we found some significant quantitative differences regarding the immunohistochemical phenotype of proliferating synoviocytes as well as significant quantitative differences in the cell composition of the inflammatory compartment in diffuse compared to localised PVNS.

It could be discussed whether these findings play a role in the different reported clinical behaviour of localised and diffuse PVNS. The present study did not aim to investigate the functional activity or the interaction of the analysed cells. These aspects should be clarified in further studies. In view of the quantitative differences in cell composition between diffuse and localised PVNS and their different clinical behaviour, further studies should continue to analyse localised and diffuse PVNS separately.

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