

Materials and Methods

Patients

Skin tissue from 30 patients with hidradenitis suppurativa (HS), in whom surgical excision was indicated, were included in this study. In all HS patients, samples were taken from representative inflammatory HS lesions and from adjacent healthy-appearing skin (1 cm from the lesional skin border), which presented the intraindividual control tissue. We included skin tissue from 30 healthy subjects to serve as interindividual controls.

Of the 30 patients with HS, 13 (43.3%) were female, and 17 (56.7%) were male, with a mean age of 40.1 (± 10.8) years. Disease severity was assessed by both the Hurley classification system and the more precise modified Hidradenitis Suppurativa Score (mHSS, as previously described in detail) [19]. Seventeen (56.7%) of the 30 patients were in Hurley stage II, and 13 (43.3%) were in Hurley stage III, with an overall mean mHSS of 58 ± 72.6 . In total, 22/30 (73.3%) patients were current smokers. The mean body mass index was $30 (\pm 6.2) \text{ kg/m}^2$. A positive family history in a first- or second-degree relative was reported by 5/30 (16.7%) patients. None of the patients had systemic HS therapy with antibiotic or immunosuppressive agents within 4 weeks before inclusion. In the healthy control group, 12/30 (40%) were female, and 18/30 (60%) were male, with a mean age of 75.2 (± 11.4) years. Overall, the mean body mass index in the healthy control group was $27.3 (\pm 5) \text{ kg/m}^2$, and 6/30 (20%) were current smokers. The Ethical Review Board of the Ruhr-University Bochum, Germany, approved this study (registration No. 5076-14), which was conducted according to the Declaration of Helsinki. Informed consent was obtained from all study subjects.

Immunohistochemical Analysis and Scoring

After drying overnight at 37°C, sections (4 μm thick) of formalin-fixed, paraffin-embedded tissues were deparaffinized in Rotihistol (10 min, room temperature [RT], 2 times), and subsequently hydrated through graded alcohol series. For antigen retrieval, sections were heated in Target Retrieval Solution pH6 (S2369; Dako, Glostrup, Denmark) in a steamer for 20 min. After cooling the samples to RT and a brief washing in Wash Buffer (S330630; Dako), sections were blocked for unspecific protein binding with 1.5% Casein-PBS solution for 15 min at RT. We used the following commercially available primary antibodies from Active Motif (La Hulpe, Belgium): mouse monoclonal 5-methylcytosine (5-mC) antibody (1:500) and rabbit polyclonal 5-hydroxymethylcytosine (5-hmC) antibody (1:500). As negative control, sections were incubated without a primary antibody. After incubation of the primary antibody for 30 min at RT in a moist chamber, the antigen was stained red by the use of the Dako REALTM Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (K5005; Dako) according to the manufacturer's protocol. For a blue nuclear counterstaining, specimens were

incubated in hematoxylin (S202084; Dako) for 1 min followed by 5 min of incubation in tap water. Finally, samples went through a series of ascending alcohol concentrations and were mounted with Entellan (Merck, Darmstadt, Germany). During the immunohistochemistry staining procedure several staining results were recorded and compared to obtain the optimized protocol for 5-hmC. Although we made every effort to optimize the used protocol, we observed weak positive cytoplasmic staining in all 3 groups. We used the protocol modification with the lowest cytoplasmic staining.

Samples were excluded from the analysis if the epidermis was nonexistent (1 skin sample in the healthy control group and 3 samples in the perilesional HS group).

For analysis, the stained slides were scanned at 20× magnification using the Nanozoomer whole slide scanner from Hamamatsu (Herrsching am Ammersee, Germany); the images were evaluated by using the viewer software NDP.view2 (Hamamatsu). For scoring, the entire epidermis was evaluated by 2 investigators (S.H. and L.S.) using the H-score system (ranging from 0 to 300) [20]. The average scores of the 2 investigators were used for statistical analysis. Five fields were chosen at random at 40× magnification. The staining intensity in the cell nuclei was scored (0, negative; 1, weak staining; 2, intermediate staining; 3, strong staining). The total number of cells in each field and the number of cells stained at each intensity were counted. The intensity of the stain was multiplied by the percentage (0--100) of keratinocytes, which indicated staining intensity: ($1 \times [\% \text{ cells with weak staining}] + 2 \times [\% \text{ cells with intermediate staining}] + 3 \times [\% \text{ cells with strong staining}]$).

Statistical Analysis

Continuous data are presented as means \pm standard deviations or medians (interquartile ranges) as appropriate. Analysis of data distribution was assessed by the D'Agostino-Pearson test. In cases of nonnormally distributed variables, the Mann-Whitney U test for independent samples and the Wilcoxon test for dependent samples were used. In cases of normal distributions, data were analyzed by the 2-sided paired or unpaired *t* test, as appropriate. For correlation analysis, the Pearson correlation coefficient (*r*) test or Spearman's coefficient of rank correlation (ρ) were used as appropriate. The level of significance for all statistical tests was $p < 0.05$. MedCalc software version 15.2 (MedCalc, Mariakerke, Belgium) was used for statistical analysis.