Splenic studies: prevention of pneumococcal disease on organisational, clinical and experimental level

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Chapter 7

How to diagnose hyposplenia:
comparison of different functional tests

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submitted
Abstract

Background.
Asplenic patients at risk for pneumococcal sepsis. Patients with hyposplenic function, such as associated with sickle cell disease (SCD), are also at risk. However, tests to assess splenic function are lacking. Aim of this study was to compare diagnostic methods for determining splenic function.

Methods.
Eighteen patients with (heterozygous and homozygous) SCD, and eight splenectomized patients were compared to ten controls. All subjects underwent spleen scintigraphy, after which functional splenic volumes (FSV) were calculated. FSV was compared to immunological function, presence of Howell Jolly bodies (HJB), percentages of pitted red cells (PIT) and B cell-subsets.

Results.
Heterozygous SCD patients had increased splenic volumes, but diminished FSV, homozygous SCD patients were asplenic. Splenectomized and homozygous SCD patients had a strongly reduced phagocytic and immunological function. Heterozygous SCD patients had reduced anti-polysaccharide responses without an increase in PIT. FSV correlated significantly with phagocytic and immunological function. HJB were indicative of splenic dysfunction, HJB absence was not indicative of normal functioning splenic tissue.

Conclusions.
Heterozygous SCD patients have diminished FSV, whereas homozygous patients are asplenic. Although visualizing HJB is methodologically advantageous to PIT, both are valid biomarkers of splenic dysfunction. The amount of nonswitched memory B cells is strongly correlated to FSV.
Introduction

The spleen plays an important role in the defense system against invading pathogens, especially encapsulated bacteria. Upon splenectomy, patients are historically known to be at risk for overwhelming infection, the so-called “Overwhelming post-splenectomy infection” (OPSI) or “Post-splenectomy sepsis” (PSS), which is associated with considerable mortality, described to be as high as 80%.

Whereas splenectomized individuals are evidently at risk for infection, there is a much larger, heterogeneous group of patients that has diminished splenic function or functional asplenia, associated with systemic disorders such as sickle cell disease (SCD), inflammatory bowel disease (IBD) and celiac disease. In patients with homozygous sickle cell disease (HbSS), the function of the spleen is clearly diminished and a spectacular improvement in life expectancy has been observed following the introduction of a vaccination program and the administration of prophylactic antibiotics in children with HbSS. In milder forms of compound SCD, such as HbSC and HbSβ+ thalassemia, as well as in celiac disease, Crohn’s disease and ulcerative colitis, splenic function is less clear and a standard test to assess spleen function is lacking.

Based on the different functions of the spleen, there are several methods to determine the level of dysfunction of the organ. With respect to the phagocytic function of the spleen, radionuclide tests such as scintigraphy and clearance of labeled erythrocytes have been used, as well as morphological analysis of peripheral blood smears on the presence of Howell Jolly bodies and the number of pitted red cells. The immunological function of the spleen can be analyzed by determining the immune response upon vaccination or by evaluation of specific B cell subsets.

To our knowledge, no studies have been performed directly comparing all these modalities of splenic function in hyposplenic as well as asplenic patients.

In the present study, we aimed to compare the diagnostic methods described above.

Methods

Study subjects

This clinical study was designed as an exploratory observational study. The study was approved by the Medical Ethical Committee of the Academic Medical Center (AMC, Amsterdam) and written informed consent was obtained from all participants. A total of 36 subjects (aged 18-65 years) were included in the study: 18 patients with sickle cell disease were recruited from the AMC outpatient clinic (i.e. 10 compound heterozygous (HbSC) and 8 homozygous (HbSS) -SCD patients), 8 patients that underwent total surgical removal (without auto-transplantation) of the spleen after (non-iatrogenic) trauma were identified in the AMC and Onze Lieve Vrouwe Gasthuis (OLVG, Amsterdam), and 10 healthy controls. Subjects were excluded if they had: any disease known to possibly affect immunity or splenic function, pneumococcal vaccination within the last 2 years, a
SCD crisis within the last 3 months or an episode of overwhelming sepsis in their medical history. After inclusion and medical screening, patients underwent all tests described below at day 1 and a second blood sample was collected at day 14.

**Scintigraphy and blood clearance**

Fifteen minutes after injection of 4 mg/2 mL Sn-pyrophosphate (Technescan, Coviden, Petten, the Netherlands), 20 ml blood was drawn and mixed with acid citrate dextrose. Red blood cells were incubated with 250 MBq of technetium-99m ($^{99mTc}$) pertechnetate for 30 minutes and heated to 49.5°C for 25 minutes. After incubation and denaturation, the supernatant was removed and 0.9% saline was added up to a volume of 20 ml. After centrifugation and removal of supernatant with excess pertechnetate, 80 MBq in 3 ml of these autologous, heat-altered Tc-labeled erythrocytes was reinjected. Cell plasticity was tested using Schleier&Schuell filtration paper (Whatman, Sigma-Aldrich, Zwijndrecht, the Netherlands), where heat-altered red blood cells lose the ability to pass.

Patients were imaged on a dual head gamma camera (GE Infinia 2, GE Healthcare, Den Bosch, the Netherlands) equipped with low energy all purpose collimators. Dynamic imaging started upon reinjection and continued for 30 min (60 frames of 10 sec/frame followed by 20 frames of 60 sec/frame, 128 matrix). Single photon emission computed tomography (SPECT) combined with low-dose CT was performed 30 min after reinjection, to obtain high resolution images of liver and spleen. Finally, a 1-minute planar scintigraphy was performed 60 minutes after reinjection of cells. Meanwhile, blood samples were drawn after reinjection (i.e. at t = 0, 1, 2, 3, 4, 5, 10, 20, 30 and 60 minutes), to determine the plasma clearance of heat-altered cells, and half life values were calculated. The activity remaining in the syringe after reinjection as well as the blood samples were measured in a gamma scintillation counter (Cobra II, Perkin Elmer, Waltham, USA). The results were corrected for physical decay.

Splanic *functional* volume was computed by dividing the percentage of splenic uptake of the injected dose of labeled erythrocytes, as measured by SPECT, by the total splenic volume (measured by CT), and is therefore given in % uptake/cm$^3$.

**Howell Jolly bodies**

HJ bodies (HJB) were visualized in the peripheral blood smear of fresh EDTA blood by a Pappenheim (basophilic) staining (combination of May-Grünwald and Giemsa). Presence of HJB was manually determined by technicians of the laboratory of Hematology of the AMC (Amsterdam).

**Pitted red cell counts**

Red cells bearing vacuoles, so-called pits, can be visualized by interference contrast microscopy (DIC) as previously described. Two drops of fresh heparin blood were fixed in 2 ml 0.1% glutaraldehyde, pH 7.4. Images were collected with a Leica TCS SP2 AOBS confocal microscope
(Mannheim, Germany) with 40X oil immersion optics. One thousand red cells were examined on a standard dry preparation. Counting pitted red cells (PIT) was done by the same two investigators independently blinded for groups (AP and BC), and mean PIT percentages were calculated.

**Vaccinations**

Concomitant administration of three vaccines was performed in all subjects: PPV-23 (Pneumo23®, Sanofi Pasteur MSD), tetanus toxoid (Tetanusvaccin®, Nederlands Vaccin Instituut) and immunocyanin (Immucothel®, Biosyn Arzneimittel GmbH, Fellach, Germany). Vaccination with immunocyanin induces a primary T cell dependent immune response, without memory; tetanus toxoid (TT) induces a secondary T cell dependent memory response; pneumococcal polysaccharide (PS) induces a secondary T cell independent memory B cell response. 1 mg of immunocyanin was administered subcutaneously, 1 ml of PS and 0.5 ml of TT were administered in the deltoid muscle of separate arms. One hour before as well as 14 days after immunization, blood was drawn to analyze specific antibody production.

**Determination of antigen-specific antibodies**

Serum was stored at −20°C until use. Pre- and postvaccination serum samples were analysed simultaneously by an ELISA determining IgG antibody levels specific for pneumococcal polysaccharides (anti-PS, against the complete pool of 23 polysaccharide antigens present in the vaccine), tetanus toxoid (anti-TT) and immunocyanin (anti-KLH, keyhole limpet haemocyanin). Anti-PS titers were reported as a percentage relative to a serum pool of healthy donors which was set at 100. Anti-TT antibody titers (purified TT was obtained from RIVM, Bilthoven, the Netherlands) were quantified and reported as kU/L. Anti-KLH (Keyhole Limpet Haemocyanin antigen, Sigma-Aldrich, St. Louis, MO, USA) antibody titers were quantified and reported in U/L. For all antigen-specific antibodies, the ratio was calculated by dividing individual post- and pre-immunization titers which reflects the response to vaccination. A ratio of less than twofold was considered to be non-protective, whereas individuals with a ratio of fourfold or greater were considered as good responders.

**Immunofluorescence and flowcytometry**

For determination of absolute numbers of T, B, and NK-lymphocytes, a 6-color immunofluorescence assay was used, and whole blood samples were measured on a FACS (Fluorescence-Activated Cell Sorting, FacsCanto II)- machine, with FacsCanto software (all from BD Biosciences, Erembodegem, Belgium). For flowcytometric immuno-phenotyping of B cells, isolated PBMCs were resuspended in PBS containing 0.5% (w/v) BSA, and 200,000 PBMCs were incubated with fluorescent label–conjugated mAbs using saturating concentrations. APC-conjugated CD20, PerCP Cy 5.5–conjugated CD19, and PE-conjugated IgD were from BD Biosciences and FITC-conjugated CD27 was from Sanquin (Amsterdam, the Netherlands). Within CD19⁺CD20⁺ B cells,
naïve cells are defined as IgD⁻CD27⁻, nonswitched memory B cells are IgD⁻CD27⁺ and switched memory B cells are IgD⁻CD27⁺.

Statistics
Statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were used to calculate patient characteristics. To determine differences between two non-parametric variables, p-values were calculated by Mann-Whitney U test. When comparing lymphocyte counts (using flowcytometry), differences between mean cell counts were tested for significance by independent T-test, following Levene’s test for equality of variances. Differences between PIT percentages between the 4 patient groups were calculated by a one-way ANOVA (Kruskal-Wallis). Pearson’s correlation coefficients (r) were calculated to describe the degree of association between two variables. If the variables had a non-parametric distribution, a Z-transformation was performed to satisfy test requirements. A two-tailed p-value of less than 0.05 indicated significance.

Results
Patient characteristics are shown in Table 1. In all participants the full set of diagnostic procedures was performed. Mean splenic volume after splenectomy was 33 ml due to the presence of splenosis (ectopic splenic tissue) in all splenectomized patients. In patients with HbSC, despite an increase in total splenic volume, the mean functional splenic volume was decreased as compared with controls.

Spleen scintigraphy
Uptake curves of labeled erythrocytes were compared in spleen and liver of all participants, at 15, 30 and 60 minutes post-injection. In healthy controls (HC), after 60 minutes 70% of the injected dose had been taken up by the spleen (see Table 1), and 20% by the liver, implicating that 10% remained within the circulation, or within tissue macrophages that are part of the reticulo-endothelial system (RES). The splenic uptake curves of HbSC patients were comparable to HC. HbSS patients and patients after splenectomy, had significantly reduced uptake at all time points. Splenectomized patients had more uptake in the liver as compared to HbSS patients at 60 minutes post injection (31 versus 16%) (data not shown).
Since we found that the total splenic volume in HbSC patients was increased as compared to HC (see Table 1) whereas the uptake curves were comparable, we calculated the functional splenic volumes by dividing the percentage of the injected dose in the spleen 60 minutes post-injection by the total splenic volume on CT.
<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>HbSC</th>
<th>HbSS</th>
<th>Splx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients [N]</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age [mean (range)]</td>
<td>30  (20-65)</td>
<td>29  (19-45)</td>
<td>27  (20-56)</td>
<td>45  (34-56)</td>
</tr>
<tr>
<td>Gender [% male]</td>
<td>60</td>
<td>50</td>
<td>37,5</td>
<td>75</td>
</tr>
<tr>
<td>Time since splx [mean years (range)]</td>
<td>-</td>
<td>-</td>
<td>11 (4-15)</td>
<td></td>
</tr>
<tr>
<td>Splenic volume (ml) [median (range)]</td>
<td>209</td>
<td>471</td>
<td>7,6</td>
<td>33</td>
</tr>
<tr>
<td>Total splenic uptake 60 minutes p.i. (%) [mean (range)]</td>
<td>70</td>
<td>67</td>
<td>2 ***</td>
<td>6 ***</td>
</tr>
<tr>
<td>Mean half life clearance values (minutes)</td>
<td>13</td>
<td>17</td>
<td>403 ***</td>
<td>44 ***</td>
</tr>
<tr>
<td>Functional splenic volume (% uptake/cm³) [median (range)]</td>
<td>0.32</td>
<td>0.13 ***</td>
<td>0 ***</td>
<td>0.15 **</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics.

HC= healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy. p.i. = post-injection. Splenic volumes were measured by CT. Half-time values of labeled erythrocytes were calculated using plasma clearance curves of each patient (see Methods section). Functional splenic volume is shown in % of Tc-uptake per cm³, computed by division of the total Tc-uptake by the total splenic volume (as described in the Methods section). Significance was tested by Mann-Whitney U test, as compared to healthy controls: ** p< 0.01, and *** p< 0.005.

Howell Jolly bodies

HJB were visible on peripheral blood smears of one HC (10%), and one HbSC patient (10%). 6 HbSS patients (75%), and 4 Splx patients (50%) had HJB present. The relation between functional splenic volume and the presence of HJB regardless of patient group is shown in Figure 1.

HJB were present only when the splenic functional volume dropped below 0.30. However, even at low volumes of functional splenic tissue, patients did not uniformly showed HJB in their blood smear.

Pitted red cell counts

Mean percentages of PIT are shown in Table 2. Compared to healthy controls, HbSS and splenectomy (Splx) patients had significantly increased percentages of PIT (p< 0.005). There was a significant inverse correlation between functional spleen volume and the percentage of PIT in the peripheral blood smear (r = -0.53, p= 0.001). (Figure 2).
Figure 1. Relation between functional splenic volume and presence of Howell-Jolly bodies.
HC= healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy. Dashed line represents a functional splenic volume of 0.30, above this level no HJb are observed.

<table>
<thead>
<tr>
<th>Percentage of PIT</th>
<th>HC</th>
<th>HbSC</th>
<th>HbSS</th>
<th>Splx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>5,9 ± 2,9</td>
<td>8,1 ± 4,9</td>
<td>29,5 ± 13,5</td>
<td>16,2 ± 9,3</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>5,0</td>
<td>6,4</td>
<td>27,0</td>
<td>14,2</td>
</tr>
<tr>
<td>Range</td>
<td>1,0 – 9,5</td>
<td>1,6 – 15,4</td>
<td>16,2 – 54,9</td>
<td>7,5 – 31,2</td>
</tr>
<tr>
<td>p-value, compared to HC</td>
<td>-</td>
<td>0,28</td>
<td>&lt; 0,0001</td>
<td>0,002</td>
</tr>
<tr>
<td>p-value, all groups</td>
<td>&lt; 0,0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Percentages of pitted red cells (PIT).
Values are given in mean percentage ± SD and geometric means.
P-values of mean PIT percentages as compared to HC were computed by Mann Whitney U test, differences between all four groups by one-way ANOVA (Kruskal-Wallis) on Z-transformed data. HC= healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy.
Figure 2. Correlation between the percentage of pitted red cells (PIT) and functional splenic volume. Correlation coefficient ($r$) is -0.53 ($p=0.001$). Reference line indicates PIT of 16%. HC = healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy.

**Immune response**

Responses to immunizations with immunocyanin, tetanus toxoid and PPV-23 are shown in Table 3. Although the HbSC and HbSS patients had increased pre-vaccination levels of anti-immunocyanin, no differences in post-vaccination responses were detected. Unexpectedly, antibody responses after vaccination with TT were significantly increased in the HbSC and HbSS patients, as compared to both HC and Splx patients. Regarding the T cell independent response to pneumococcal polysaccharide (PS), Splx patients still showed a sufficient response, but responses of both HbSC and HbSS patients were significantly diminished as compared to those of HC.
<table>
<thead>
<tr>
<th>Titer response</th>
<th>HC</th>
<th>HbSC</th>
<th>HbSS</th>
<th>Splx</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-IC</td>
<td>GMT</td>
<td>GMT</td>
<td>GMT</td>
<td>GMT</td>
</tr>
<tr>
<td>pre</td>
<td>475,8</td>
<td>773,3 *</td>
<td>1179,2 *</td>
<td>824,6</td>
</tr>
<tr>
<td>post</td>
<td>1320,2</td>
<td>1768,1</td>
<td>3139,0</td>
<td>2455,6</td>
</tr>
<tr>
<td>fold</td>
<td>2,69</td>
<td>2,29</td>
<td>2,66</td>
<td>2,98</td>
</tr>
<tr>
<td>increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-TT</td>
<td>1,8</td>
<td>1,6</td>
<td>1,0</td>
<td>1,3</td>
</tr>
<tr>
<td>pre</td>
<td>12,0</td>
<td>30,9 **</td>
<td>31,8 *</td>
<td>13,0</td>
</tr>
<tr>
<td>post</td>
<td>6,52</td>
<td>19,36</td>
<td>32,29 *</td>
<td>9,85</td>
</tr>
<tr>
<td>fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-PS</td>
<td>86,3</td>
<td>187,9</td>
<td>134,8</td>
<td>252,6 *</td>
</tr>
<tr>
<td>pre</td>
<td>347,0</td>
<td>291,2</td>
<td>214,3</td>
<td>592,4</td>
</tr>
<tr>
<td>post</td>
<td>4,02</td>
<td>1,55 *</td>
<td>1,59 ***</td>
<td>2,22</td>
</tr>
<tr>
<td>fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Responses to immunizations. Values are given as geometric mean titers. Fold increase ratios were calculated by dividing post with pre values. P-values were determined by Mann-Whitney U test for each group as compared to HC. * indicates p< 0.05, ** indicate p< 0.01 and *** indicate p< 0.005. IC = immunocyanine vaccine, TT = tetanus toxoid, PS = polysaccharide. Pre = before vaccination, post = 2 weeks after vaccination. HC= healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy.

**Flowcytometric analysis**

Flowcytometric analysis of lymphocytes is shown in Table 4. Several T-lymphocyte subsets, NK cells and B cells were increased in HbSS and Splx patients as compared to HC and HbSC. There were no differences in counts pre-or post-immunization (data not shown). Furthermore, there were no significant differences in T and B cell subset ratios before and after immunization between the patient groups (neither in absolute cell counts, nor in percentages, data not shown). Individual percentages of nonswitched memory B cells for each patient group are shown in Figure 3A. Remarkably, HbSS and Splx-patients had significantly reduced mean IgD⁺CD27⁺ cell counts (nonswitched memory B cells). There was a significant positive correlation between functional splenic volume and percentage of IgD⁺CD27⁺ cells, both before immunization (r = 0.625, p< 0.01, see Figure 3B) and after immunization (r =0.768, p< 0.01, data not shown). The ratios of post and pre-immunization values of IgD⁺CD27⁺ cells were not different between groups (p= 0.911, data not shown).
<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>HbSC</th>
<th>HbSS</th>
<th>Splx</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, 10⁹/L</td>
<td>7.1 ± 1.8</td>
<td>6.4 ± 3.0</td>
<td>7.4 ± 3.4</td>
<td>8.7 ± 1.5</td>
</tr>
<tr>
<td>Absolute cell counts (10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>1.36 ± 0.45</td>
<td>1.06 ± 0.34</td>
<td>1.70 ± 0.55</td>
<td>2.21 ± 0.65**</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>0.52 ± 0.33</td>
<td>0.33 ± 0.12</td>
<td>0.50 ± 0.26</td>
<td>0.69 ± 0.35</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>0.74 ± 0.30</td>
<td>0.68 ± 0.28</td>
<td>1.09 ± 0.31*</td>
<td>1.50 ± 0.35***</td>
</tr>
<tr>
<td>NK-cells</td>
<td>0.25 ± 0.09</td>
<td>0.24 ± 0.23</td>
<td>0.43 ± 0.27</td>
<td>0.58 ± 0.24***</td>
</tr>
<tr>
<td>B cells (CD19+)</td>
<td>0.24 ± 0.08</td>
<td>0.31 ± 0.20</td>
<td>0.51 ± 0.20***</td>
<td>0.59 ± 0.28**</td>
</tr>
<tr>
<td>B-cell subsets (% of CD19+/CD20+ B cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgD+CD27-</td>
<td>65.9 ± 13.5</td>
<td>61.3 ± 15.5</td>
<td>80.2 ± 9.6*</td>
<td>77.6 ± 7.5*</td>
</tr>
<tr>
<td>IgD+CD27+</td>
<td>15.5 ± 10.1</td>
<td>13.4 ± 7.2</td>
<td>4.0 ± 1.9**</td>
<td>7.2 ± 3.5*</td>
</tr>
<tr>
<td>IgD-CD27+</td>
<td>15.8 ± 5.6</td>
<td>22.0 ± 9.0</td>
<td>13.1 ± 6.9</td>
<td>12.8 ± 6.1</td>
</tr>
</tbody>
</table>

Table 4. Flowcylometric analysis of lymphocytes in whole blood of healthy controls (HC), heterozygous sickle cell patients (HbSC), homozygous sickle cell patients (HbSS) and splenectomized patients (Splx).

Values are given in mean ± SD. P-values were calculated as compared to HC by independent T-test, following Levene’s test for equality of variances, * indicates p<0.05, ** indicate p<0.01 and *** indicate p<0.005. Only pre-immunization samples were used. WBC = white blood count. IgD⁺CD27⁻ = naive nonswitched B cells, IgD⁺CD27⁺ = nonswitched memory B cells, IgD⁻CD27⁻ = switched memory B cells.
Figure 3. Relation between nonswitched memory B cells and splenic function.

(A). Individual nonswitched memory B cell percentages of total CD19⁺/CD20⁺ B cells, for each group.
(B). Relation between the percentage of nonswitched memory B cells and functional splenic volume (FSV). Correlation between Z-transformed values of functional splenic volume and IgD⁻CD27⁻ B cells was calculated by Spearman’s rho (r), ** indicates p < 0.01.

HC= healthy controls, HbSC = patients with heterozygous SCD, HbSS = patients with homozygous SCD, Splx = patients after splenectomy.
Discussion

We found that all patients after total splenectomy had remaining functional splenic tissue using scintigraphy. This might be related to splenosis due to the trauma that caused the splenic damage, and the subsequent splenectomy. In HbSC patients, splenic volume appeared to be increased. This was not associated with an increased functional splenic volume (FSV), resulting in a total splenic uptake after 60 minutes that was comparable to healthy controls, despite the enlarged size. Although half life values of labeled erythrocytes yielded differences between groups, these values are not sensitive for indicating splenic function, since the liver and the RES partially contribute to blood clearance.

The presence of HJB was significantly associated with diminished FSV, although the absence of HJB was not indicative of normal functioning splenic tissue as assessed by scintigraphy. This is in line with previous observations by Gotthardt et al, failing to demonstrate a relation between the presence HJB and splenic function or size as measured by scintigraphy or ultrasonography. 15 We found a highly significant inverse correlation between pitted red cell percentages and FSV. This confirms previous observations in which counting PIT has been suggested to be a reliable method to determine hyposplenic function, although most data are derived from a single research group (Corazza). In a recent study by Rogers et al. similar findings are described in children with sickle cell anemia 16, however a percentage of >4.5% PIT was suggested to be indicative of absent splenic function. Our study rather indicates a percentage of 16% PIT to be indicative of hyposplenic function, which appears to be in accordance with previous studies 17,18. A major drawback of this elegant method however, is the DIC microscope. Its use is complex, and interpreting the results is difficult, thus it will probably be difficult to implement counting PIT as a reliable method for diagnosing hyposplenia.

Antibody titers of anti-immunocyanin (primary response, i.e. no memory), anti-TT (T cell dependent), and anti-PS (T cell independent memory B cell response) were determined. Surprisingly, there was an increased antibody response after vaccination with TT in the HbSC and HbSS patients, as compared to both HC and Splx patients. Future studies are needed to elucidate whether this is due to altered T-memory cell function or, for instance, to a change in T-regulatory function. Also, sickle cell patients showed a reduced response to pneumococcal vaccination as compared to HC and splenectomized patients. This could indicate either a primary hyporesponsiveness to repeated pneumococcal vaccination, as described by O’Brien 19, but may also be related to the increased baseline anti-PS titers due to previous vaccinations. Indeed, although recent vaccination (<2 years) was an exclusion criteria, all HbSC and HbSS patients had been immunized against pneumococci previously. Remarkably, normal primary and secondary antibody responses were found in the splenectomized patients.

Naive B cells (IgD+CD27+) can differentiate into memory B cells (CD27+), which comprise 30-60% of the B-cell pool. Differentiation results in either development of switched memory IgD-CD27+ B cells, in the germinal center under influence of T helper cells, or development of nonswitched
memory IgD+IgM+CD27+ B cells which takes place in the splenic stromal cells and is T cell independent. Here, we confirm that switched memory B cells, which are derived from the germinal centers, are present at normal frequency in asplenic individuals and sickle cell patients. However, nonswitched memory B cells were significantly reduced in HbSS and splenectomized patients, confirming that these cells require the spleen for their generation and survival. This could be related to the increased susceptibility for infections in patients with reduced splenic function, since nonswitched memory IgD+IgM+CD27+ B cells have shown to be protective against mortality of *S. pneumoniae* after splenectomy.

Since decreased levels of nonswitched B cells are also described in patients suffering from other immune deficiencies, such as Common Variable Immunodeficiency Syndrome, this test is not specific for diagnosing hyposplenia. However, the highly significant correlation between the percentage of IgD+CD27+ cells and functional splenic volume might be helpful in screening for splenic dysfunction. The validation of a cut-off value would need evaluation in larger patient cohorts. Although all Splx patients had splenosis on scintigraphy, in some even associated with some rest-functional capacity, this was not accompanied by adequate IgD+CD27+ B cell counts. It is therefore uncertain to what level patients with splenosis are protected from invasive pneumococcal disease, which may be related to anatomical/vascular differences.

All measured lymphocyte subsets (CD3+, CD8+, and CD4+ T cells, NK and B cells) were increased in HbSS and splenectomized patients as compared to healthy controls. A possible explanation for this increase is that the spleen is missing as a storage site (sequestration) for these cells.

Several limitations of this study have to be considered. The present study was designed as an exploratory observational cohort study, with relatively small patient numbers. Therefore, our results need to be confirmed in a larger trial. Furthermore, it would be interesting to complete these data with parameters of patients after angio-embolization of the spleen, which is now a common surgical procedure. Although we have considered using these results to suggest diagnostic recommendations in clinical practice, however, group sizes were too small for proposing cut-off values, which need to be validated first.

In conclusion, we demonstrate that a diminished functional splenic volume correlated with both a reduced phagocytic function as represented by PIT percentages, a reduced anti-PS response upon pneumococcal vaccination, and reduced numbers of nonswitched B cells. T cell dependent immune responses to vaccinations were not related to FSV. The absence of HJB was not indicative of normal functioning splenic tissue. Whether these functional tests can also be correlated to the risk of overwhelming infection with encapsulated bacteria remains to be demonstrated.

Patients with HbSS must be considered to have functional asplenia. In patients with HbSC, phagocytic function is unimpaired, and a normal percentage of nonswitched B cells is accompanied by a reduced antibody response upon pneumococcal vaccination, suggesting a reduced immunological function in these patients.
References


