CHAPTER 7

Performance of $^{89}$Zr-labeled-rituximab PET as an imaging biomarker to assess CD20 targeting:

a pilot study in patients with relapsed/refractory diffuse large B cell lymphoma

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ABSTRACT

Purpose: Treatment of patients with diffuse large B cell lymphoma (DLBCL) includes rituximab, an anti-CD20 monoclonal antibody (mAb). Insufficient tumor targeting might cause therapy failure. Tumor uptake of $^{89}$Zirconium ($^{89}$Zr)-mAb is a potential imaging biomarker for tumor targeting, since it depends on target antigen expression and accessibility. The aim of this pilot study was to describe the performance of $^{89}$Zr-labeled-rituximab PET to assess CD20 targeting in patients with relapsed/refractory DLBCL.

Methods: Six patients with biopsy-proven DLBCL were included. CD20 expression was assessed using immunohistochemistry (IHC). 74 MBq $^{89}$Zr-rituximab (10 mg) was administered after the therapeutic dose of rituximab. Immuno-PET scans on day 0, 3 and 6 post injection (D0, D3 and D6 respectively) were visually assessed and quantified for tumor uptake. Spearman's rank correlation coefficient was used to assess the correlation between tumor uptake of $^{89}$Zr-rituximab and ranking of CD20 expression in biopsies.

Results: Tumor uptake of $^{89}$Zr-rituximab and CD20 expression were concordant in 5 patients: for one patient, both were negative, for the other four patients visible tumor uptake was concordant with CD20-positive biopsies. Intense tumor uptake of $^{89}$Zr-rituximab on PET ($SUV_{peak}=12.8$) corresponded with uniformly positive CD20 expression on IHC with in one patient. Moderate tumor uptake of $^{89}$Zr-rituximab (range $SUV_{peak}=3.2-5.4$) corresponded with positive CD20 expression on IHC in three patients. In one patient tumor uptake of $^{89}$Zr-rituximab was observed ($SUV_{peak}=3.8$), while the biopsy was CD20-negative. Overall, a positive correlation was observed between tumor uptake of $^{89}$Zr-rituximab and CD20 expression in the biopsied tumor lesions ($r_s=0.83$, $p=0.04$, $n=6$).

Conclusions: This study provides evidence for the use of $^{89}$Zr-rituximab-PET as an imaging biomarker to assess CD20 targeting, given the observed correlation between tumor uptake of $^{89}$Zr-rituximab and CD20 expression in biopsies. Therefore, $^{89}$Zr-rituximab-PET allows for further studies relating tumor targeting to clinical benefit of rituximab treatment in individual patients.
INTRODUCTION

DLBCL is an aggressive, potentially fatal, but curable form of lymphoma. It is the most common lymphoma subtype, representing 30% of all lymphoma. This malignancy develops from the B-cells in the lymphatic system and is characterized by expression of CD20, a transmembrane protein. The function of CD20 is still unknown, but as it is only expressed on B cells and not on other tissues, it is a useful target for treatment. Rituximab, an anti-CD20 mAb, is currently incorporated in all first line and subsequent treatment regimens. The introduction of rituximab in first line treatment has improved the prognosis of a three-year event-free survival (EFS) from 59% to 79% for patients of 18 to 60 years old. However, patients with relapsed/refractory DLBCL have a three-year overall survival (OS) of only 49% (1). Early relapse (<12 months) and prior rituximab treatment are associated with a worse outcome, with a three-year EFS of 21% versus 47%, suggesting rituximab resistance. Although it is standard practice to include rituximab in second line treatment, it is unclear whether individual patients benefit from repeated rituximab treatment. To obtain clinical benefit from mAb treatment tumor targeting is required. It comprises target antigen expression, as well as a drug that reaches and binds to the target.

Target expression of CD20 is assessed by IHC on a single tumor biopsy as part of the routine work up to confirm the diagnosis of DLBCL, or to confirm relapsed/refractory disease (2). [Fluorine-18]-2-fluoro-2-deoxy-D-glucose (18F-FDG)-PET is incorporated in staging and response evaluation of DLBCL (3), but provides no information on expression of CD20.

Molecular imaging with 89Zirconium (89Zr)-labeled mAbs, also known as immuno-PET, allows for visualization and quantification of tumor uptake and whole body biodistribution of 89Zr-mAbs. Since tumor uptake depends on target expression and accessibility, it is a potential imaging biomarker for tumor targeting.

Two clinical trials on immuno-PET with 89Zr-labeled mAbs have reported whether tumor uptake on immuno-PET and target expression in biopsies are correlated. These studies were on 89Zr-bevacizumab, an anti-endothelial growth factor (VEGF)-A mAb, in patients with breast cancer (5) and 89Zr-labeled anti-membrane-bound surface glycoprotein mesothelin (MSLN) mAb in patients with pancreatic and ovarian cancer (6). Correlations between a measure of tumor uptake and a measure of target status were reported, to provide evidence that immuno-PET may be used as an imaging biomarker to assess tumor targeting.
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The aim of this pilot study was to describe the performance of $^{89}\text{Zr}$-labeled-rituximab PET as an imaging biomarker to assess CD20 targeting in patients with relapsed/refractory diffuse large B cell lymphoma, by correlating tumor uptake of $^{89}\text{Zr}$-rituximab with CD20 expression in biopsied tumor lesions.

MATERIALS AND METHODS

Data used in this pilot study was obtained as part of an ongoing main trial (registered in the Dutch Trial Register http://www.trialregister.nl, NTR 3392) with formal ethical approval from the Medical Ethics Committee of the VU University Medical Center, Amsterdam, The Netherlands (approval date July 2012, reference 2012/165). The study was performed in compliance with the principles of the Declaration of Helsinki. All patients signed an informed consent form.

Patients with a primary diagnosis of CD20-positive DLBCL, relapsed after or refractory to first line treatment with rituximab combined with anthracycline-based chemotherapy (R-CHOP), were eligible for inclusion. A $^{18}\text{F}$-FDG-PET scan was performed as routine clinical staging before start of second line treatment (7).

Patients were included before start of second line treatment with rituximab combined with cisplatin-based chemotherapy. Inclusion criteria consisted of age $\geq$ 18 years, WHO performance score 0-2 and eligibility for high dose chemotherapy and autologous stem cell transplant. Patients with known central nervous system (CNS) involvement were not eligible.

Tumor biopsies were obtained to confirm refractory/relapsed disease before start of second line treatment. IHC was performed, including at least staining for CD20, CD79a, CD3 and MIB1 to support the diagnosis. As part of the routine work-up CD20 expression was reported as either present (+) or absent (-). In addition, CD20 expression was assessed semi-quantitatively as:

A. Uniformly positive in all tumor cells.
B. Heterogeneously positive, ranging from positive in the majority of cells to positivity limited to sparse groups of cells.
C. Absent, ranging from extremely sparse groups of CD20-positive cells to completely absent in all tumor cells, with a positive internal control sample and CD79a-positive.

A qualitative assessment was made for membranous or granular staining patterns. Tumor biopsies were assessed by a pathologist blinded for immuno-PET results.
\(^{89}\text{Zr}\)-rituximab-PET in lymphoma

\(^{89}\text{Zr}\) (T½ = 78.4 hrs) (BV Cyclotron VU, Amsterdam, the Netherlands) was coupled to rituximab via the bifunctional chelator N-succinyl-desferal. Methods used for radiolabeling have been described previously (8-10). The radiochemical purity was assessed by instant thin layer chromatography (iTLC) and high-performance liquid chromatography (HPLC). The immunoreactive fraction was assessed by Lindmo binding assay using either Ramos (patient 1, 2, 3) or Su-DHL4 (patient 4, 5, 6) cells. The endotoxin content was determined according to pharmacopeia using an endosafe PTS reader. Sterility of each \(^{89}\text{Zr}\)-rituximab batch was assured by performing a media fill immediately after final filter sterilization of each batch. The radiochemical purity, immunoreactivity and endotoxin content of every batch were assessed prior to administration to a patient. Manufacturing and radiolabeling were performed according to Good Manufacturing Practice (GMP) standards.

Patients received a therapeutic dose of rituximab (range 700-1000 mg) on day 1 of cycle 1 of second line treatment, within 2 hours followed by \(^{89}\text{Zr}\)-rituximab (10 mg, 74 MB +/- 10%) as an intravenous bolus injection. Venous blood samples were scheduled at t=120 min (D0), 72 hrs (D3) and 144 hrs (D6) post injection (p.i.). Whole blood and plasma radioactivity concentrations were measured with a gamma well counter (Wallac 1480 Wizard, Turku, Finland). Radioactivity measurements obtained with venous blood samples were correlated with imagederived blood pool measurements. Percentage injected dose (%ID) in blood pool on D6 was calculated using image derived radioactivity concentrations and total blood volume (11). Tracer availability is commonly defined as the concentration of the tracer in the plasma fraction, therefore the total activity in plasma needs to be calculated and compared to the total activity in whole blood.

Total activity in plasma on D6 was calculated from the venous blood samples, using standard hematocrit values (0.45 for males and 0.4 for females) and total blood volume. Total activity in whole blood was calculated from the venous blood samples and total blood volume. A two-tailed paired t-test was used to test for a difference between both measures of total activity.

Immuno-PET scans (Gemini TF-64/Ingenuity TF-128; Philips Healthcare, Best, the Netherlands), were acquired at D0, D3 and D6 p.i., and reconstructed as described previously (12). Each whole body immuno-PET scan was preceded by a 35 mAs low-dose computed tomography (ldCT) scan for attenuation and scatter correction. Immuno-PET scans were evaluated by a nuclear medicine physician, blinded for the \(^{18}\text{F}\)-FDG-PET and biopsy results. Lesions were considered positive
in case of focal uptake exceeding local background. Immuno-PET scans were classified as positive (moderate or intense, at the D6 scan) or negative for tumor uptake of $^{89}$Zr-rituximab in the biopsied tumor lesion. Thereafter, the immuno-PET scans were compared with the $^{18}$F-FDG-PET scans to confirm tumor localization. Tumor volumes of interest (VOIs) were manually delineated on immuno-PET scans at D3 and D6, using a semi-automatic in-house software tool. Peak (i.e. average value of a 12mm sphere positioned within the VOI so as to obtain the highest value) and mean activity concentrations ($AC_{\text{peak}}$ and $AC_{\text{mean}}$, respectively) were derived per tumor VOI. For $AC_{\text{mean}}$ standard deviations (SD) were derived per VOI. Blood pool VOIs were delineated using a fixed size VOI of the aortic arch (volume of 1.6 mL), on the IdCT and imported to the PET images. $AC_{\text{mean}}$ was derived per blood pool VOI. Standardized uptake values ($SUV_{\text{peak}}$ and $SUV_{\text{mean}} \pm \text{SD}$, respectively) were calculated for tumor lesions, and decay corrected to the time of injection. Tumor to blood ratio's (T/B) for tumor lesions were calculated as tumor AC on D6 divided by image derived blood pool AC on D6. To assess tumor uptake over time, $SUV_{\text{peak}}$ D6/D3 ratios were calculated.

Patients were ranked based on the level of CD20 expression in order to correlate biopsy results to tumor uptake of $^{89}$Zr-rituximab, defined on PET images. Spearman's rank correlation coefficient ($r_s$) as well as the two-tailed p-value were calculated to explore the relation between tumor uptake of $^{89}$Zr-rituximab, measured as $SUV_{\text{peak}}$, and the level of CD20 expression in the biopsied lesions. Statistical tests were performed using SPSS Statistics Version 22 (IBM software).

**RESULTS**

Six patients with a primary diagnosis of CD20-positive DLBCL, with refractory or relapsed disease after first line treatment with R-CHOP, were included. Patient characteristics are summarized in Table 1.

Patient 2 and 6 had primary refractory disease, with a partial remission (PR) after R-CHOP. The other patients had relapsed disease, of whom two patients (1 and 3) had an early relapse within 1 year after R-CHOP. In all patients $^{18}$F-FDG-PET scans were obtained for staging, before start of second line treatment. All patients were subsequently treated in second line with rituximab combined with high dose cytarabine, cisplatin and dexamethasone (R-DHAP).
Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Response to first line</th>
<th>Ann Arbor stage at relapse</th>
<th>Disease localization at relapse on $^{89}$T-FDG-PET</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>23</td>
<td>complete remission</td>
<td>IV A</td>
<td>liver</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>55</td>
<td>partial remission</td>
<td>III B</td>
<td>cervical, para-iliac, spleen</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>46</td>
<td>complete remission</td>
<td>III B</td>
<td>supraclavicular, mediastinal, hilar, mesenteric, retro-peritoneal, para-iliac, inguinal</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>46</td>
<td>complete remission</td>
<td>II A</td>
<td>retro-peritoneal</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>47</td>
<td>complete remission</td>
<td>I E</td>
<td>nasopharynx</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>69</td>
<td>partial remission</td>
<td>III A</td>
<td>submandibular, retro-clavicular, axillary, inguinal</td>
</tr>
</tbody>
</table>

Administration of $^{89}$Zr-rituximab was well tolerated by all patients. Four patients underwent all scans according to protocol. Due to chemotherapy induced nausea, only a scan of the head and neck area could be obtained in patient 2, and blood sampling at D3 and 6 was not feasible. Patient 6 cancelled the D3 scan due to diarrhea and nausea, most likely caused by an infectious entero-colitis. For patient 3 no venous blood sample was obtained at D3.

$^{89}$Zr-rituximab in blood pool, liver, spleen and kidneys was evident at D0, decreasing over time in all patients (Figure 1).

Figure 1 Example of whole body distribution of $^{89}$Zr-rituximab over time
Maximum intensity projections at D0, D3 and D6 p.i. for patient 3.
All sites of positive tumor uptake identified at D6 were also observed at D3, whereas no tumor uptake was identified on D0. At D6 on average 27.6% ± 5.7% ID of $^{89}$Zr-rituximab was still circulating in blood pool. The total activity at D6 as derived from plasma samples was not significantly different from the whole blood samples (n=5). Image derived and venous sampled whole blood activity concentrations were correlated with an $R^2$ of 0.98 and slope of 0.85.

All biopsied tumor sites showed uptake of $^{18}$F-FDG and DLBCL localization was confirmed by pathology. IHC was negative for CD20 expression in patient 1 and 6 (Figure 2), and positive in the other patients (Figure 3). Patients were ranked based on the intensity level and pattern of CD20 expression.

Tumor uptake of $^{89}$Zr-rituximab and CD20 expression on IHC were concordant in five patients: for patient 1, both were negative (Figure 4 and 2A), for the other four patients visible tumor uptake was concordant with CD20-positive biopsies. Intense visual tumor uptake of $^{89}$Zr-rituximab on PET was observed in patient 3, corresponding with uniformly positive CD20 expression on IHC (Figure 5 and 3D). $S_{\text{peak}}$ for this tumor lesion on D6 was 12.8. CD20 expression on IHC was also observed in patient 2 (Figure 3A), 4 (Figure 3B) and 5 (Figure 3c), concordant with tumor uptake of $^{89}$Zr-rituximab. $S_{\text{peak}}$ for these tumor lesions on D6 was 3.2, 5.4, 3.4, respectively. In patient 6 tumor uptake of $^{89}$Zr-rituximab was observed ($S_{\text{peak}}$ on D6 = 3.8) (Figure 6) while a core needle biopsy was CD20 negative (Figure 2B). Tumor uptake over time ($S_{\text{peak}}/D6/D3$ ratio) was calculated and ranged between 0.6 and 1.4. See Table 2 for additional PET uptake measures.

Overall, a positive correlation was observed between tumor uptake of $^{89}$Zr-rituximab, measured as $S_{\text{peak}}$, and the CD20 expression ranking ($r_s = 0.83$, $p=0.04$, $n = 6$).

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Figure 2 Biopsies: absence of CD20 expression
A) Core-needle biopsy of DLBCL (liver) in patient 1 shows completely absent CD20 expression (rank 1).
B) Core-needle biopsy of DLBCL (axillary lymph node) in patient 6 showing almost completely absent CD20 expression: extremely sparse groups of CD20-positive tumor cells with granular staining pattern (rank2).
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Figure 3: Biopsies: presence of CD20 expression

A) Excision biopsy of DLBCL (cervical lymph node) in patient 2 shows heterogeneously positive CD20 expression in sparse groups of cells, granular staining pattern (rank 3).
B) Excision biopsy of DLBCL (nasopharynx) in patient 5 showing heterogeneously positive CD20 expression in the majority of cells, membranous staining pattern (rank 4).
C) Excision biopsy of DLBCL (retroperitoneal lymph node) in patient 4 showing uniformly positive CD20 expression, membranous staining pattern (rank 5).
D) Excision biopsy of DLBCL (inguinal lymph node) in patient 3 showing uniformly positive CD20 expression, membranous staining pattern (rank 6).

Table 2: Additional quantitative tumor uptake measures of 89Zr-rituximab in the biopsied tumor lesions

<table>
<thead>
<tr>
<th>Patient</th>
<th>SUV&lt;sub&gt;mean&lt;/sub&gt; ±SD</th>
<th>Tumor to blood ratio</th>
<th>SUV&lt;sub&gt;peak&lt;/sub&gt; ratio D6/D3</th>
<th>VOI (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2.3 ± 0.4</td>
<td>NA*</td>
<td>0.6</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>9.1 ± 2.6</td>
<td>4.7</td>
<td>1.4</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>4.3 ± 0.9</td>
<td>1.6</td>
<td>1.1</td>
<td>7.6</td>
</tr>
<tr>
<td>5</td>
<td>2.5 ± 0.4</td>
<td>1.1</td>
<td>1.0</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>3.5 ± 0.5</td>
<td>0.8</td>
<td>NA*</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*NA = not available

Figure 4: No tumor uptake on 89Zr-rituximab-PET, concordant with CD20 expression in biopsy. Axial images: from left to right: attenuation corrected PET, low dose CT and fused PET/CT image of patient 1.
A) 89Zr-rituximab-PET shows no tumor uptake concordant with a CD20-negative liver biopsy.
B) Corresponding tumor location on 18F-FDG-PET.
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Figure 5 Example of tumor uptake on $^{89}$Zr-rituximab-PET, concordant with CD20 expression in biopsy. Axial images: from left to right attenuation corrected PET, low dose CT and fused PET/CT image of patient 3.
A) $^{89}$Zr-rituximab-PET shows intense tumor uptake concordant with a CD20-positive biopsy (inguinal lymph node).
B) Corresponding tumor location on $^{18}$F-FDG-PET.

Figure 6 Tumor uptake on $^{89}$Zr-rituximab-PET, discordant with CD20 expression in biopsy. Axial images: from left to right attenuation corrected PET, low dose CT and fused PET/CT image of patient 6.
A) $^{89}$Zr-rituximab-PET shows tumor uptake discordant with a CD20-negative biopsy (axillary lymph node).
B) Corresponding tumor location on $^{18}$F-FDG-PET.
Therapy failure in patients with relapsed/refractory DLBCL may be caused by insufficient CD20-mediated tumor targeting of rituximab. To elucidate the role of tumor targeting, development of an imaging biomarker to assess tumor targeting of rituximab is needed.

To our knowledge, this is the first study to report the performance of $^{89}$Zr-rituximab-PET as an imaging biomarker for CD20 targeting by correlating tumor uptake of $^{89}$Zr-rituximab as defined with PET and CD20 expression in biopsied tumor lesions. Tumor biopsies were obtained as routine work-up and tumor uptake on immuno-PET was evaluated at the biopsy sites. Overall, a positive correlation between tumor uptake of $^{89}$Zr-rituximab and CD20 expression in biopsies was observed.

In one patient, tumor uptake of $^{89}$Zr-rituximab was discordant with a CD20-negative biopsy. A possible explanation for the discrepancy is that the tumor site was biopsied in a $^{18}$F-FDG-PET positive, $^{89}$Zr-rituximab-PET negative part. IHC is the current gold standard for determination of CD20 expression, however heterogeneity in target expression within and between tumor lesions may not be detected by a single biopsy. Practical limitations of tumor biopsies are the invasiveness of the procedure and the fact that the tumor is not always safely accessible.

Tumor uptake was quantified in regions with focal uptake exceeding local background. SUV$_{peak}$ is commonly used as a measure of tumor uptake, but reflects only the highest uptake in a small part of the tumor. Manual delineation aims to capture total tumor uptake of $^{89}$Zr-rituximab, and allows for the derivation of SUV$_{mean}$, its standard deviation and VOI volume. In this study the ranking of PET uptake was identical for SUV$_{peak}$ and SUV$_{mean}$. For $^{18}$F-FDG-PET in lymphoma, the Deauville criteria are used to define tumor uptake using liver and mediastinal blood pool as reference region (15). The observed tumor to blood ratios in this study (range 0.8-4.7) indicate a difference in an uptake criterion based on local contrast versus bloodpool as reference region. To develop a clinically relevant criterion for positive tumor uptake of $^{89}$Zr-rituximab further studies are required, linking tumor uptake to clinical outcome to rituximab treatment.

A limitation of our study is that the amount of circulating CD20+ B cells, which could influence tracer availability for tumor targeting, was not measured. However, tracer availability in the blood pool could be derived accurately from the
image data and was found to be more than 25% ID at D6. By using the blood samples this activity was found to be present in the plasma fraction. Therefore, the presence of a significant CD20 antigen sink hampering tracer availability for tumor targeting can be ruled out in this study.

So far, two other clinical studies have been published on the use of $^{89}$Zr-labeled anti-CD20 with focus on prediction of toxicity for radio-immunotherapy treatment planning in patients with B cell lymphoma (13, 14).

The current study provides evidence for the use of $^{89}$Zr-rituximab-PET as an imaging biomarker to assess CD20 targeting. These results allow for further studies to assess whether $^{89}$Zr-rituximab-PET is able to predict which patients will or will not respond to repeated rituximab treatment, and select which patients will benefit from a change of treatment (dose optimization, switch to a different targeted therapy or ADC). Novel treatment options emerge, including new anti-CD20 mAbs as obinutuzumab and ofatumumab with enhanced capacity for cytotoxicity as well as mAbs for other targets, for instance the anti-CD38 mAb daratumumab, and antibody-drug conjugates (ADC’s) as brentuximab vedotin, an anti-CD30 mAb linked to the antimitotic agent monomethyl auristatin E (MMAE). Molecular imaging with immuno-PET is a promising strategy to guide individualized treatment to improve efficacy, reduce toxicity and costs of mAb treatment.

CONCLUSION

This study provides evidence for the use of $^{89}$Zr-rituximab-PET as an imaging biomarker to assess CD20 targeting, given the observed correlation between tumor uptake of $^{89}$Zr-rituximab and CD20 expression in biopsies. Therefore, $^{89}$Zr-rituximab-PET allows for further studies relating tumor targeting to clinical benefit of rituximab treatment in individual patients.
REFERENCES


SUPPLEMENTARY DATA

Supplemental Figure 1 Total activity (in MBq) in whole blood and plasma on D6.