SENTINEL LYMPH NODE IDENTIFICATION WITH MAGNETIC NANOPARTICLES

JOOST J. POUW

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This research is supported by the Dutch Technology Foundation STW, which is part of the Netherlands Organisation for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs (project number 10891).

Nederlandse titel:

Schildwachtklier identificatie met magnetische nanodeeltjes

Cover design: Joost Pouw

The basis for the cover image was created with The Biodigital Human (www.biodigital.com)

Printed by: Gildeprint – Enschede

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ISBN: 978-90-365-4021-6

DOI: 10.3990/1.9789036540216

<http://dx.doi.org/10.3990/1.9789036540216>

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DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
Prof. dr. H. Brinksma,
on account of the decision of the graduation committee,
to be publicly defended
on Friday the 29th of January 2016 at 14.45

by

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1

INTRODUCTION

Most solid malignancies have a tendency to spread through the lymphatic system, to locoregional lymph nodes [1, 2]. The presence of nodal metastasis is an important prognostic factor and is used to determine the choice of treatment of the individual patient in various malignancies, including breast cancer [3] and colorectal cancer [4]. Therefore, accurate nodal staging is of utmost importance for patients and clinicians. Currently, no non-invasive imaging modality is able to diagnose lymph node involvement with adequate accuracy [5, 6]. Surgical removal and histopathological examination is therefore the standard procedure for nodal staging.

The Sentinel Lymph Node (SLN) concept, first introduced by Cabanas in penile carcinoma [7], assumes the orderly progression of metastatic cells through the lymphatic system, and can be used to achieve accurate nodal staging. The SLNs are defined as the lymph nodes that receive direct lymphatic drainage from the tumour area (Fig. 1). Therefore the SLNs are most likely the first site of metastasis, if the tumour has spread. Consequently, the status of the SLNs reflects the status of the entire nodal basin, and nodal involvement of the higher echelon nodes can be ruled out if the SLNs are free of metastasis.

Figure 1 Schematic representation of a tumour and the draining lymphatic basin. The sentinel lymph nodes (SLN) receive direct lymphatic drainage from the tumour. Metastatic cells therefore first reach the SLNs before drainage progresses to the higher echelon nodes.

The SLN concept can be used with different purposes in different malignancies. In breast cancer, it is used to achieve nodal staging with minimal morbidity. In colorectal cancer, a SLN procedure can be used to improve staging accuracy rather than limit morbidity. Both procedures are technically very different, and pose different challenges. The use of magnetic nanoparticles for SLN identification to solve these challenges in both malignancies is described in this thesis.

1.1 THE SENTINEL LYMPH NODE IN BREAST CANCER

Sentinel Lymph Node Biopsy (SLNB), first described by Morton et al. in melanoma [8], is used as less invasive alternative to Axillary Lymph Node Dissection (ALND) for nodal staging in breast cancer patients [9]. In ALND all lymph nodes in the axilla are surgically removed, which is highly invasive and associated with morbidity [10-12]. With SLNB, accurate staging is achieved by removal and examination of a limited number of lymph nodes (the SLNs) instead of performing an ALND. The gold standard for SLNB is the combined technique, using an interstitially administered blue dye and radioisotope tracer (^{99m}Tc -nanocolloid) [12, 13]. The tracers are transported through the lymphatic system, to the draining SLNs. Lymphoscintigraphy is used for pre-operative localization of the SLNs. A handheld scintillation counter (gamma probe) and/or visual guidance are used for the intraoperative identification, and subsequent removal of the SLNs (Fig. 2). If histopathological examination of the SLNs reveals metastasis, often additional treatment of the axilla and/or adjuvant treatment is indicated [3]. If the SLNs are free of metastasis, no further axillary treatment is needed.

Figure 2 Overview of the sentinel lymph node biopsy procedure in breast cancer. (a) The blue dye and radioisotope tracer are injected in the breast. Subsequently the tracers are distributed to the SLNs through the lymphatic system. (b) Prior to surgery, the distribution of the radioisotope tracer is imaged using lymphoscintigraphy. This provides the surgeon with information on the number of SLNs and their location. The large arrow indicates the injection site, the small arrow indicates a SLN. (c) The SLNs are identified by the surgeon using visual guidance (blue dye) and a gamma probe (radiotracer). The SLNs and tumour are removed (inset) and the SLNs examined for the presence of metastasis. (Fig. 2a and c reproduced with permission. For the National Cancer Institute ©2010 Terese Winslow, U.S. Govt. has certain rights.)

Until recently a completion ALND was indicated in all breast cancer patients with SLN metastasis, however, three clinical trials drastically changed the role of nodal involvement in the choice of treatment. The IBCSG 23-01 trial [14] demonstrated that further axillary treatment is not needed in patients with micrometastatic (<2 mm) nodal involvement. The ACOSOG Z11 trial [15] showed that when there is limited macrometastatic (>2 mm) axillary

nodal involvement (1 or 2 positive SLNs) and a patient will receive whole breast irradiation (including part of the axilla) and adjuvant systemic therapy a completion ALND is no longer indicated [3]. The AMAROS trial [16] demonstrated that axillary radiotherapy achieves excellent and comparable axillary control as ALND in patients with positive SLNs. Although there is increasingly more evidence that patients with limited SLN involvement can be safely spared an ALND and the associated morbidity, in the absence of accurate non-invasive imaging modalities SLNB is still needed to achieve nodal staging.

The combined technique for SLNB is an effective procedure with high identification rate (>96%) and low false negative rate (<10%) [17]. However, the use of radioisotopes is associated with drawbacks. Use and disposal of radioisotopes is subject to stringent regulations, and many centres do not have access to radiotracers. Furthermore, the production of radiotracers is limited to a small number of reactors worldwide, potentially resulting in shortages. This combination of factors hampers the availability of the procedure worldwide. Therefore there is an important role for radioisotope-free alternative methods [18, 19].

1.2 THE SENTINEL LYMPH NODE IN COLORECTAL CANCER

The SLN procedure is not used routinely in colorectal cancer. Surgical treatment of colorectal cancer is fundamentally different from breast cancer. It consists of en-bloc resection of the affected colorectal segment and the complete adjacent lymphatic basin, regardless of the nodal status. All removed lymph nodes are subjected to conventional histopathological examination, after 24-48 hours of formalin fixation, to detect nodal involvement. Since the required extent of the resection and lymphadenectomy is dictated by the blood-supply [20], SLNB to limit the number of removed lymph nodes (and thus morbidity) has no purpose.

However, SLN Mapping (SLNM) can be used to improve staging accuracy by selecting a limited number of lymph nodes for more detailed histological analysis [21, 22]. Despite a good prognosis, up to 30% of the patients without nodal involvement develop recurrent disease within 5 years of surgery [23, 24]. This group could potentially benefit from adjuvant treatment. Various retrospective studies attribute the high recurrence rate to undetected (occult) nodal involvement [25-27]. These small (<2 mm) occult metastases can be detected with labour intensive and expensive histological examination. By only subjecting the SLNs to focussed histopathological examination nodal staging accuracy, and potentially treatment, of a large group of patients can be improved without considerably increasing labour and costs of the examination.

The en-bloc resection of the colorectal segment and adjacent nodal basin allows to perform an ex-vivo SLN procedure, shortly after the surgery. In the ex-vivo setting, a blue dye is generally used as tracer. Due to the fluidity of the dye, it distributes rapidly through the lymphatic system. Therefore, SLN identification must be performed shortly after tracer injection to prevent spread to higher echelon nodes. This does not comply with routine clinical practice, and hinders the widespread implementation of the procedure.

In summary, a large proportion of patients suffering from colorectal cancer is understaged of occult nodal involvement, and therefore potentially undertreated. SLNM can provide the means to accurately identify this group of patients. However, currently used tracers suffer from drawbacks and do not allow to perform ex vivo SLNM in a routine clinical workflow. This limits the widespread clinical implementation of the procedure. A tracer which allows for SLN identification during routine histopathological examination of the specimen (after formalin fixation), could provide the means to improve staging accuracy, and potentially treatment, of conventional node negative colorectal cancer patients, in routine clinical practice.

1.3 ALTERNATIVE TECHNIQUES FOR SENTINEL LYMPH NODE IDENTIFICATION

Different alternative SLN techniques have been developed and evaluated in the past years. In this paragraph, a short non-exhaustive overview of recently evaluated techniques will be given. In colorectal cancer Near Infrared (NIR) Fluorescence imaging was proposed as alternative to blue dye to improve ex vivo SLN Mapping. In breast cancer, NIR Fluorescence was evaluated as radioisotope-free alternative for in vivo SLNB. Additionally, Contrast Enhanced Ultrasound (CEUS) with a microbubble tracer was evaluated as minimal-invasive alternative technique. Finally, a magnetic technique using a magnetic nanoparticle tracer and handheld magnetometer was evaluated for SLNB in breast cancer.

1.3.1 Near Infrared fluorescence imaging

Indocyanine Green (ICG) is an FDA and EMA approved contrast agent for use in angiography, blood flow evaluation, and liver function assessment. It is used off-label in several clinical trials for SLN identification in different malignancies [28]. Under NIR excitation light, the tracer produces a fluorescent signal. The technique provides real time images of the lymphatic distribution of the tracer. The technique was evaluated both for ex vivo SLNM in colorectal cancer, and for in vivo SLNB in breast cancer.

Two studies performed ex vivo SLNM in colorectal cancer, with a fluorescent tracer not approved for in vivo use (HSA800 (IRDye 800CW conjugated to human serum albumin), and achieved excellent identification rates of 95% & 100% [29, 30]. The tracer visualizes the

distribution from injection site to sentinel nodes (Fig. 3). Although less prominent than with ICG, a disadvantage of the HSA800 tracer is the low molecular weight, enabling rapid lymphatic clearance and distribution to higher echelon nodes. This hampers the applicability of the technique for use after formalin fixation, in routine clinical practice.

Figure 3 Example of a SLNM procedure with a fluorescent tracer in the colon. (a) A colour image of the colon segment and part of the adjacent lymphatic basin is shown. (b) NIR-fluorescent image in which the injection site (arrow head), and a SLN (arrow) can be identified. A clear lymphatic vessel leading from the injection site to the SLN can be distinguished. (c) Merged image of (a) and (b). (Reproduced with permission from [29])

Multiple groups have used ICG as tracer for in vivo SLNB in breast cancer patients with identification rates ranging from 93.1% to 100% [18, 28]. The limited maximum imaging depth in tissue of approximately 1.5-2 cm [31, 32] does not allow to perform pre-operative imaging. The limited penetration also does not allow for transcutaneous localization of SLNs in most cases, however the technique can visualize afferent lymphatic vessel leading to the SLNs. A limitation of ICG is its low molecular weight, facilitating rapid lymphatic distribution and distribution to higher echelon nodes. This can result in the removal of more lymph nodes [18], and thus potentially increase morbidity. It also shortens the time-frame after injection in which the SLNB can be performed. A hybrid tracer in which ICG and ^{99m}Tc -nanocolloid are combined allows for pre-operative imaging, and overcomes the poor SLN retention of ICG [33]. However, due to the use of radioisotopes, these hybrid tracers are associated with the same drawbacks as radiotracers.

Experimental optimized fluorescent tracers have been developed to increase penetration depth, and facilitate lymph node retention [34, 35]. These optimized tracers could improve the technique, however currently ICG is the only fluorescent tracer approved for in vivo human use.

1.3.2 Contrast Enhanced Ultrasound

Contrast enhanced ultrasound (CEUS) was used for SLNB in breast cancer, and not in colorectal cancer. The technique uses a microbubble tracer for the visualization of afferent lymphatic vessels and SLNs. With an excellent penetration depth, CEUS can be used for pre-operative transcutaneous localization of the SLNs. A unique property of the technique is that it allows to perform ultrasound guided targeted biopsy of the SLNs, potentially

eliminating the need for axillary surgery. The first trials, however, report a success rate (successful identification on imaging and successful core biopsy) of approximately 90% and high false negative rates (33%-39%) [36, 37], therefore the technique cannot serve as replacement for SLNB surgery. The technique could play a role in addition to ultrasound guided biopsy of suspicious lymph nodes [38, 39], to improve pre-operative staging.

1.3.3 Magnetic nanoparticles for SLN identification

Superparamagnetic iron oxide (SPIO) nanoparticles are very suitable for use in biomedicine applications because of their biocompatibility and stability [40]. Several SPIO nanoparticle formulations have been approved for use in humans as intravenous (IV) administered MRI contrast agents [41, 42]. These magnetic tracers also have beneficial properties for SLN identification, and could overcome drawbacks of currently used tracers in both colorectal cancer and breast cancer.

1.3.3.1 Colorectal cancer

The stability and relatively large particles of magnetic nanoparticle tracers make them well suited for ex vivo use in colorectal cancer for SLN identification after formalin fixation. The retention in the sentinel lymph nodes of larger sized tracers [43, 44] and stability over time could overcome the need to immediately perform SLN identification after tracer injection, and thereby provide a method to improve staging accuracy in routine clinical practice.

We have demonstrated that the use of a magnetic nanoparticle tracer is technically feasible using a non-clinically applicable magnetometer [45]. Further clinical studies are needed to demonstrate the feasibility of the technique in a routine clinical workflow.

1.3.3.2 Breast cancer

After interstitial administration, the nanoparticles are distributed to the SLNs [46]. Their particle size of approximately 50-150 nm facilitates lymph node retention [47, 48]. The intrinsic penetrability of magnetic fields in human tissue could allow for in depth detection of the magnetic nanoparticles [49] and therefore a handheld magnetic probe (SentiMAG, Endomag Ltd, UK) was developed to localize the magnetic tracer during breast cancer surgery [50]. The commercially available magnetic tracer and handheld magnetometer are depicted in Figure 4.

Figure 4 Commercial magnetometer (SentiMAG®, Endomag Ltd, UK) consisting of a base unit with numerical display, and a handheld probe. The magnetic tracer (Sienna+®) consist of coated superparamagnetic iron oxide nanoparticles in sterile water for injection. (Photo courtesy Endomag Limited.)

The intraoperative use of a magnetic tracer and this handheld magnetometer was evaluated against the standard SLNB technique in four separate clinical trials [51-54]. Based on identification rates ranging from 94.4% to 98.3% all groups concluded that the magnetic technique was non-inferior to the standard technique for intraoperative SLN identification.

SPIO nanoparticles provide contrast on MRI, and can therefore be used as alternative to lymphoscintigraphy to obtain pre-operative imaging information on the number and location of SLNs [55]. In combination with the handheld magnetometer for intraoperative detection, it potentially provides an entirely radioisotope-free method for SLNB in breast cancer patients.

1.4 SCOPE AND OUTLINE OF THIS THESIS

As outlined in the previous paragraphs the currently used tracers for SLN identification in colorectal cancer and breast cancer suffer from limitations. The blue dye used in colorectal cancer does not allow to perform a SLN procedure during routine clinical practice, hampering widespread implementation of the procedure. In breast cancer, the use of radioisotopes limits the availability of the procedure worldwide. Magnetic nanoparticles have several advantageous properties for SLN identification, and could potentially resolve

these limitations in both malignancies. The aim of the research in this thesis is twofold. In colorectal cancer, the objective is to introduce a SLN procedure allowing to improve staging accuracy in a routine clinical workflow (Chapters 2 and 3). In breast cancer, we aim to introduce an entirely radioisotope-free method for SLNB (Chapters 4, 5 and 6).

In **Chapter 2** we describe the first step in developing a new technique to identify colorectal SLNs after ex vivo administration of a magnetic nanoparticle tracer. A non-clinical measuring protocol was developed to demonstrate and quantify accumulation of magnetic tracer in lymph nodes after ex vivo administration. Furthermore, quantitative requirements for a magnetometer to perform the procedure in routine clinical practice are formulated.

In **Chapter 3** a commercial magnetometer and magnetic tracer were used to perform ex vivo SLN identification in colorectal cancer, in a routine clinical workflow. The performance of the new technique was compared to the performance of a standard blue dye tracer. The identified SLNs (blue and magnetic) were subjected to serial sectioning and immunohistochemical staining to reveal occult metastases, to evaluate the ability of the technique to improve nodal staging accuracy.

In breast cancer, the intraoperative use of a handheld magnetometer and magnetic tracer achieved excellent identification rates, and was concluded to be non-inferior to the standard technique. Although different magnetic tracers are approved for use in humans, all groups used the same tracer. The technique can potentially be optimized by using a different tracer. In **Chapter 4** the performance of three magnetic nanoparticle tracers, approved for use in humans, is evaluated in an in vivo porcine model.

Unlike the gamma probe, the current generation magnetometers is not only sensitive to the magnetic tracer but also to the magnetic human body. This could limit the depth performance of the probes. In **Chapter 5** the depth performance of the current generation magnetometers is quantified in a tissue-mimicking phantom.

To be able to perform an entirely radioisotope-free SLNB procedure in breast cancer patients, an alternative for the pre-operative lymphoscintigraphy is needed. In **Chapter 6** the feasibility of using a magnetic tracer and MRI as alternative pre-operative imaging technique was evaluated. The performance of the new technique is compared to the current standard of lymphoscintigraphy. Furthermore the potential of the technique to non-invasively diagnose nodal involvement is explored.

Chapter 7 provides a general conclusion, and recommendations for improvement are given. The future perspective of magnetic nanoparticles in nodal staging is described.

1.5 REFERENCES

1. S. Li and Q. Li, *Cancer stem cells, lymphangiogenesis, and lymphatic metastasis*. Cancer Lett, 2015. **357**(2): p. 438-47.
2. S.A. Stacker, M.E. Baldwin, and M.G. Achen, *The role of tumor lymphangiogenesis in metastatic spread*. FASEB J, 2002. **16**(9): p. 922-34.
3. G.H. Lyman, S. Temin, S.B. Edge, L.A. Newman, et al., *Sentinel lymph node biopsy for patients with early-stage breast cancer: American Society of Clinical Oncology clinical practice guideline update*. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2014. **32**(13): p. 1365-83.
4. G. Quasar Collaborative, R. Gray, J. Barnwell, C. McConkey, et al., *Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study*. Lancet, 2007. **370**(9604): p. 2020-9.
5. K.L. Cooper, S. Harnan, Y. Meng, S.E. Ward, et al., *Positron emission tomography (PET) for assessment of axillary lymph node status in early breast cancer: A systematic review and meta-analysis*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2011. **37**(3): p. 187-98.
6. K.L. Cooper, Y. Meng, S. Harnan, S.E. Ward, et al., *Positron emission tomography (PET) and magnetic resonance imaging (MRI) for the assessment of axillary lymph node metastases in early breast cancer: systematic review and economic evaluation*. Health Technol Assess, 2011. **15**(4): p. iii-iv, 1-134.
7. R.M. Cabanas, *An approach for the treatment of penile carcinoma*. Cancer, 1977. **39**(2): p. 456-66.
8. D.L. Morton, D.R. Wen, J.H. Wong, J.S. Economou, et al., *Technical details of intraoperative lymphatic mapping for early stage melanoma*. Archives of surgery, 1992. **127**(4): p. 392-9.
9. A.E. Giuliano, D.M. Kirgan, J.M. Guenther, and D.L. Morton, *Lymphatic mapping and sentinel lymphadenectomy for breast cancer*. Annals of surgery, 1994. **220**(3): p. 391-8; discussion 398-401.
10. D.N. Krag, S.J. Anderson, T.B. Julian, A.M. Brown, et al., *Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial*. The lancet oncology, 2007. **8**(10): p. 881-8.
11. R.E. Mansel, L. Fallowfield, M. Kissin, A. Goyal, et al., *Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial*. Journal of the National Cancer Institute, 2006. **98**(9): p. 599-609.
12. U. Veronesi, G. Paganelli, G. Viale, A. Luini, et al., *A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer*. The New England journal of medicine, 2003. **349**(6): p. 546-53.
13. D.N. Krag, D.L. Weaver, J.C. Alex, and J.T. Fairbank, *Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe*. Surgical oncology, 1993. **2**(6): p. 335-9; discussion 340.

14. V. Galimberti, B.F. Cole, S. Zurrida, G. Viale, et al., *Axillary dissection versus no axillary dissection in patients with sentinel-node micrometastases (IBCSG 23-01): a phase 3 randomised controlled trial*. The lancet oncology, 2013. **14**(4): p. 297-305.
15. A.E. Giuliano, K.K. Hunt, K.V. Ballman, P.D. Beitsch, et al., *Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial*. JAMA, 2011. **305**(6): p. 569-75.
16. M. Donker, G. van Tienhoven, M.E. Straver, P. Meijnen, et al., *Radiotherapy or surgery of the axilla after a positive sentinel node in breast cancer (EORTC 10981-22023 AMAROS): a randomised, multicentre, open-label, phase 3 non-inferiority trial*. The lancet oncology, 2014. **15**(12): p. 1303-10.
17. T. Kim, A.E. Giuliano, and G.H. Lyman, *Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis*. Cancer, 2006. **106**(1): p. 4-16.
18. M. Ahmed, A.D. Purushotham, and M. Douek, *Novel techniques for sentinel lymph node biopsy in breast cancer: a systematic review*. The lancet oncology, 2014. **15**(8): p. e351-62.
19. A. Cousins, S.K. Thompson, A.B. Wedding, and B. Thierry, *Clinical relevance of novel imaging technologies for sentinel lymph node identification and staging*. Biotechnol Adv, 2014. **32**(2): p. 269-79.
20. G.H. Sakorafas, E. Zouros, and G. Peros, *Applied vascular anatomy of the colon and rectum: clinical implications for the surgical oncologist*. Surgical oncology, 2006. **15**(4): p. 243-55.
21. M.H. van der Pas, S. Meijer, O.S. Hoekstra, Riphagen, II, et al., *Sentinel-lymph-node procedure in colon and rectal cancer: a systematic review and meta-analysis*. The lancet oncology, 2011. **12**(6): p. 540-50.
22. E.S. van der Zaag, W.H. Bouma, P.J. Tanis, D.T. Ubbink, et al., *Systematic review of sentinel lymph node mapping procedure in colorectal cancer*. Annals of surgical oncology, 2012. **19**(11): p. 3449-59.
23. A.J. Bilchik, M. DiNome, S. Saha, R.R. Turner, et al., *Prospective multicenter trial of staging adequacy in colon cancer: preliminary results*. Archives of surgery, 2006. **141**(6): p. 527-33; discussion 533-4.
24. A. Figueredo, M.E. Coombes, and S. Mukherjee, *Adjuvant therapy for completely resected stage II colon cancer*. Cochrane Database Syst Rev, 2008(3): p. CD005390.
25. D. Iddings, A. Ahmad, D. Elashoff, and A. Bilchik, *The prognostic effect of micrometastases in previously staged lymph node negative (N0) colorectal carcinoma: a meta-analysis*. Annals of surgical oncology, 2006. **13**(11): p. 1386-92.
26. D.G. Nicastrì, J.T. Doucette, T.E. Godfrey, and S.J. Hughes, *Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature*. J Mol Diagn, 2007. **9**(5): p. 563-71.
27. E.S. van der Zaag, N. Kooij, M.J. van de Vijver, W.A. Bemelman, et al., *Diagnosing occult tumour cells and their predictive value in sentinel nodes of histologically negative patients with colorectal cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2010. **36**(4): p. 350-7.

28. L. Xiong, E. Gazyakan, W. Yang, H. Engel, et al., *Indocyanine green fluorescence-guided sentinel node biopsy: a meta-analysis on detection rate and diagnostic performance*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2014. **40**(7): p. 843-9.
29. M. Hutteman, H.S. Choi, J.S. Mieog, J.R. van der Vorst, et al., *Clinical translation of ex vivo sentinel lymph node mapping for colorectal cancer using invisible near-infrared fluorescence light*. Annals of surgical oncology, 2011. **18**(4): p. 1006-14.
30. B.E. Schaafsma, F.P. Verbeek, J.R. van der Vorst, M. Hutteman, et al., *Ex vivo sentinel node mapping in colon cancer combining blue dye staining and fluorescence imaging*. The Journal of surgical research, 2013. **183**(1): p. 253-7.
31. R.G. Pleijhuis, G.C. Langhout, W. Helfrich, G. Themelis, et al., *Near-infrared fluorescence (NIRF) imaging in breast-conserving surgery: assessing intraoperative techniques in tissue-simulating breast phantoms*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2011. **37**(1): p. 32-9.
32. I. Stoffels, J. Dissemond, T. Poppel, D. Schadendorf, and J. Klode, *Intraoperative Fluorescence Imaging for Sentinel Lymph Node Detection: Prospective Clinical Trial to Compare the Usefulness of Indocyanine Green vs Technetium Tc 99m for Identification of Sentinel Lymph Nodes*. JAMA Surg, 2015. **150**(7): p. 617-23.
33. B.E. Schaafsma, F.P. Verbeek, D.D. Rietbergen, B. van der Hiel, et al., *Clinical trial of combined radio- and fluorescence-guided sentinel lymph node biopsy in breast cancer*. The British journal of surgery, 2013. **100**(8): p. 1037-44.
34. J. Li, Z. Zhuang, B. Jiang, P. Zhao, and C. Lin, *Advances and perspectives in nanoprobe for noninvasive lymph node mapping*. Nanomedicine (Lond), 2015. **10**(6): p. 1019-36.
35. S.H. Kong, Y.W. Noh, Y.S. Suh, H.S. Park, et al., *Evaluation of the novel near-infrared fluorescence tracers pullulan polymer nanogel and indocyanine green/gamma-glutamic acid complex for sentinel lymph node navigation surgery in large animal models*. Gastric Cancer, 2015. **18**(1): p. 55-64.
36. S. Rautiainen, M. Sudah, S. Joukainen, R. Sironen, et al., *Contrast-enhanced ultrasound -guided axillary lymph node core biopsy: Diagnostic accuracy in preoperative staging of invasive breast cancer*. Eur J Radiol, 2015. **84**(11): p. 2130-6.
37. K. Cox, A. Sever, S. Jones, J. Weeks, et al., *Validation of a technique using microbubbles and contrast enhanced ultrasound (CEUS) to biopsy sentinel lymph nodes (SLN) in pre-operative breast cancer patients with a normal grey-scale axillary ultrasound*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2013. **39**(7): p. 760-5.
38. V. Altomare, G. Guerriero, R. Carino, C. Battista, et al., *Axillary lymph node echo-guided fine-needle aspiration cytology enables breast cancer patients to avoid a sentinel lymph node biopsy. Preliminary experience and a review of the literature*. Surgery today, 2007. **37**(9): p. 735-9.

39. S.C. Diepstraten, A.R. Sever, C.F. Buckens, W.B. Veldhuis, et al., *Value of preoperative ultrasound-guided axillary lymph node biopsy for preventing completion axillary lymph node dissection in breast cancer: a systematic review and meta-analysis*. Annals of surgical oncology, 2014. **21**(1): p. 51-9.
40. Q.A. Pankhurst, N.K.T. Thanh, S.K. Jones, and J. Dobson, *Progress in applications of magnetic nanoparticles in biomedicine*. Journal of Physics D: Applied Physics, 2009. **42**(22).
41. P. Reimer and B. Tombach, *Hepatic MRI with SPIO: detection and characterization of focal liver lesions*. European radiology, 1998. **8**(7): p. 1198-204.
42. P. Reimer, N. Jahnke, M. Fiebich, W. Schima, et al., *Hepatic lesion detection and characterization: value of nonenhanced MR imaging, superparamagnetic iron oxide-enhanced MR imaging, and spiral CT-ROC analysis*. Radiology, 2000. **217**(1): p. 152-8.
43. A.E. Merrie, A.M. van Rij, L.V. Phillips, J.I. Rossaak, et al., *Diagnostic use of the sentinel node in colon cancer*. Diseases of the colon and rectum, 2001. **44**(3): p. 410-7.
44. S. Saha, A.G. Dan, B. Berman, D. Wiese, et al., *Lymphazurin 1% versus 99mTc sulfur colloid for lymphatic mapping in colorectal tumors: a comparative analysis*. Annals of surgical oncology, 2004. **11**(1): p. 21-6.
45. M. Visscher, J.J. Pouw, J. van Baarlen, J.M. Klaase, and B. Ten Haken, *Quantitative analysis of superparamagnetic contrast agent in sentinel lymph nodes using ex vivo vibrating sample magnetometry*. IEEE transactions on bio-medical engineering, 2013. **60**(9): p. 2594-602.
46. L. Johnson, S.E. Pinder, and M. Douek, *Deposition of superparamagnetic iron-oxide nanoparticles in axillary sentinel lymph nodes following subcutaneous injection*. Histopathology, 2013. **62**(3): p. 481-6.
47. J.J. Pouw, M. Ahmed, B. Anninga, K. Schuurman, et al., *Comparison of three magnetic nanoparticle tracers for sentinel lymph node biopsy in an in vivo porcine model*. International journal of nanomedicine, 2015. **10**: p. 1235-43.
48. G. Mariani, L. Moresco, G. Viale, G. Villa, et al., *Radioguided sentinel lymph node biopsy in breast cancer surgery*. Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 2001. **42**(8): p. 1198-215.
49. Q.A. Pankhurst, J. Connolly, S.K. Jones, and J. Dobson, *Applications of magnetic nanoparticles in biomedicine*. J Phys D Appl Phys, 2003. **36**(13): p. R167-R181.
50. T. Joshi, Q.A. Pankhurst, S. Hattersley, A. Brazdeikis, et al., *Magnetic nanoparticles for detecting cancer spread*. 30th Annual San Antonio Breast Cancer Symposium – December 13–16, 2007 - Breast Cancer Research and Treatment, 2007. **106**(Suppl. 1): p. S129.
51. M. Douek, J. Klaase, I. Monypenny, A. Kothari, et al., *Sentinel node biopsy using a magnetic tracer versus standard technique: the SentiMAG Multicentre Trial*. Annals of surgical oncology, 2014. **21**(4): p. 1237-45.
52. M. Thill, A. Kurylcio, R. Welter, V. van Haasteren, et al., *The Central-European SentiMag study: sentinel lymph node biopsy with superparamagnetic iron oxide (SPIO) vs. radioisotope*. Breast, 2014. **23**(2): p. 175-9.

53. I.T. Rubio, S. Diaz-Botero, A. Esgueva, R. Rodriguez, et al., *The superparamagnetic iron oxide is equivalent to the Tc99 radiotracer method for identifying the sentinel lymph node in breast cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2014.
54. A. Pinero-Madrona, J.A. Torro-Richart, J.M. de Leon-Carrillo, G. de Castro-Parga, et al., *Superparamagnetic iron oxide as a tracer for sentinel node biopsy in breast cancer: A comparative non-inferiority study*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2015. **41**(8): p. 991-7.
55. J.J. Pouw, M.R. Grootendorst, R. Bezooijen, C.A. Klazen, et al., *Pre-operative sentinel lymph node localization in breast cancer with superparamagnetic iron oxide MRI: the SentiMAG Multicentre Trial imaging subprotocol*. The British journal of radiology, 2015: p. 20150634.

2

QUANTITATIVE ANALYSIS OF SUPERPARAMAGNETIC CONTRAST AGENT IN SENTINEL LYMPH NODES USING EX VIVO VIBRATING SAMPLE MAGNETOMETRY*

*This chapter is published as: Visscher M, **Pouw JJ**, van Baarlen J, Klaase JM, Ten Haken B. Quantitative analysis of superparamagnetic contrast agent in sentinel lymph nodes using ex vivo vibrating sample magnetometry. *IEEE Trans Biomed Eng.* 2013;60(9):2594-2602.

ABSTRACT

As the first step in developing a new clinical technique for the magnetic detection of colorectal sentinel lymph nodes (SLNs), a method is developed to measure the magnetic content in intact, formalin fixated lymph nodes using a Vibrating Sample Magnetometer (VSM). A suspension of superparamagnetic nanoparticles is injected ex vivo around the tumor in the resected colon segments. A selection of three lymph nodes is excised from the region around the tumor and is separately measured in the VSM. The iron content in lymph nodes is quantified from the magnetic moment curve using the Langevin model for superparamagnetism and a bimodal particle size distribution. Adverse, parasitic movements of the sample were successfully reduced by tight fixation of the soft tissue and using a small vibration amplitude. Iron content in the lymph nodes is detected with 0.5 μg accuracy and ranged from 1-51 μg . Histological staining confirmed iron presence. The current method of measuring intact biological tissue in a VSM is suitable to show the feasibility and merit of magnetic detection of SLNs in colorectal cancer. For clinical validation of magnetic SLN selection in colorectal cancer, a new magnetometer with high specificity for superparamagnetic nanoparticles is required.

2.1 INTRODUCTION

Magnetic nanoparticles have become increasingly important in both non-invasive and minimally invasive medical applications [1, 2]. Superparamagnetic nanoparticles have already been used as contrast agents in magnetic resonance imaging (MRI) for a long time [3, 4]. Furthermore, the use of magnetic nanoparticles for drug delivery [5-8] and hyperthermia treatment [9] remains under development. One of the new developments is the use of magnetic nanoparticles for sentinel lymph node (SLN) detection. In Japan and The United Kingdom, magnetic detection of sentinel lymph nodes using a handheld probe was developed for lung [10, 11] and breast cancer [12-14]. Similar experiments using a high- T_c SQUID gradiometer were demonstrated in a rat model [15]. A recent study shows the applicability of magnetic nanoparticles as contrast agent for photoacoustic imaging which can provide intra-operative lymph node staging [16].

The present clinical procedure of SLN detection includes selection of the lymph nodes that drain the tumor area by a technetium marker and blue dye to apply advanced microscopic analysis (ultrastaging) to detect metastasis [17, 18]. The presence of metastasis is important for disease staging and subsequent clinical decisions. SLN biopsy helps the pathologist to select nodes with the highest chance for (micro)metastasis. When no metastasis is found with normal hematoxylin and eosin (H&E) staining, ultrastaging - which is time consuming - can be exclusively restricted to the sentinel lymph nodes.

The introduction of magnetic nanoparticles in sentinel lymph node procedures can improve diagnosis and therapy for various tumors. In case of colorectal cancer, diagnosis can be improved by more specific selection of the SLNs. This can increase staging accuracy and subsequently, it can help to plan an adequate therapeutic path [19]. In breast cancer and melanoma magnetic SLN detection has to compete with the well performing, but logistically more complex, combined method using radioactive tracer and blue dye. Magnetic detection largely simplifies logistics and safety protocols and makes potentially as accurate SLN detection accessible for hospitals that do not have a department for nuclear medicine. In those hospitals significant therapeutic improvements can be achieved by introduction of a reliable SLN procedure.

In surgical procedures of colorectal cancer a complete colon segment is resected including all lymph nodes surrounding the tumor. Sentinel lymph node mapping (SLNM) for this type of cancer is still in development and is potentially highly beneficial [20-25]. The procedure is introduced to obtain a more precise diagnosis and is technically still developing regarding

tracers and surgical approach. The majority of studies use only a blue dye as contrast agent and are performed either in vivo or ex vivo [19]. A suspension of superparamagnetic iron oxide (SPIO) nanoparticles is an attractive alternative for both blue dye and technetium in colorectal cancer.

The added value of magnetic nanoparticles compared to the generally used technetium and blue dye tracers, is that they are stable and therefore detectable and quantifiable over time. The restricted lifetime of technetium-99m and the fluidity of blue dye limit the time frame of reliable detection of the SLN after surgery. The use of a physically more stable tracer allows ex vivo detection several hours after surgery. In such an ex vivo procedure, the SLN detection aims to make an accurate selection out of all harvested lymph nodes, rather than a search in a tissue mass for one specific tracer containing lymph node. All lymph nodes are individually selected as SLN based on the presence of magnetic tracer. This post-operative procedure reduces the burden on costly operating time.

Another advantage of a tracer with particles is to reduce the chance to select higher echelon nodes. The particles in a magnetic tracer are more easily trapped in the sentinel lymph node compared to the fluidic blue dye that may spread further to higher echelon nodes [26]. At present, it is still unknown whether these nanoparticles will end up in the SLNs (first echelon) after ex vivo injection. Physiological processes in the lymphatic system, like macrophage activity, are expected to stop soon after resection. Moreover, detection of ex vivo particle uptake can be limited because the lymph nodes in the mesenterium are rather small in size and ex vivo infiltration of particles might be low. The experiments in this first study have to show whether the nanoparticles can still accumulate in the SLNs in ex vivo circumstances.

The stability of a magnetic tracer provides the opportunity for a feasibility study to ex vivo magnetic sentinel lymph node detection in colorectal cancer in an extramural laboratory. Therefore, a clinically suitable instrument is not needed *a priori*. Detection of SPIO in a SLNM procedure serves to decide whether a particular lymph node is a candidate for additional microscopic analysis. The detection system has to give a decisive answer about the presence of tracer. Therefore a highly sensitive and specific detection system is required. Spatial imaging of tracer is inferior to a more reliable indicator of tracer presence. Therefore, magnetometry methods selectively sensitive for non-linear magnetic properties of SPIO are preferred over less specific laborious quantitative MRI techniques that are susceptible to assumptions about background signals from tissue, (geometry of) SPIO distribution and detection thresholds [27]. Different spectroscopic methods that have been developed to quantify SPIO content in cell samples, require sample digestion and are therefore not compatible with histopathologic analysis in a SLNM procedure [28].

In the present study, the SLNs were quantitatively analyzed using a standard vibrating sample magnetometer (VSM). Quantification of particle uptake serves to determine technical requirements for development of a clinically suitable magnetometer.

The magnetic analysis of fresh or formalin-fixated biological tissue using a VSM, is a challenging procedure. In several studies, magnetometry of biological tissue was achieved at rather low temperatures ($T < 273$ K) or after freeze-drying the sample to enable a firm fixation [29-34]. Such a procedure is problematic if the sample has to remain intact for clinical histological analysis. Therefore, in the present study a reliable, non-destructive VSM-method was developed to measure the magnetic content of SPIO particles in intact diamagnetic biological samples at room temperature. Despite the time-consuming and clinically impractical technique of VSM, the measurements provide important information for the development of a clinical magnetometer to replace the VSM in the methodology presented here.

The objective of the current study is first to show, with a limited number of experiments, the feasibility of magnetic nanoparticles as tracer for ex vivo SLNM in colorectal cancer. The second objective is to determine the quantitative requirements for a clinically suitable magnetometer, that can perform fast ex vivo analysis of colorectal lymph nodes. Since the focus in this study is on the technical feasibility of magnetic nanoparticles in ex vivo colorectal tissue, the patient-specific clinical results and their consequences are topic of future papers.

2.2 EXPERIMENTS

2.2.1 Superparamagnetic particles and clinical application

The Endorem MRI contrast agent (Guerbet Nederland B.V., Gorinchem, The Netherlands) is chosen as superparamagnetic tracer for identification of the SLNs. This tracer is a suspension of superparamagnetic iron oxide nanoparticles coated with dextran in a concentration of 11.2 mg iron per mL. The hydrodynamic particle size is reported in a range of 58-186 nm [35, 36]. Lymph nodes are harvested from resected tissue of patients with colorectal cancer who underwent a standard surgical procedure. Immediately after resection, the colon part containing the tumor is brought to a separate field and is injected submucosally around the tumor with 1.5-2.0 mL of Endorem and massaged for about 5 minutes to induce particle flow into the lymphatic system. Macrophage activity responsible for in vivo lymphatic processing of magnetic nanoparticles [37], is expected to stop immediately after resection. Therefore mechanical transport of particles through the interstitial space and the lymphatics should be maintained ex vivo to get the SLNs filled with

tracer. Since VSM analysis of all lymph nodes in each specimen would be very time consuming and magnetic detection of lymph nodes *in situ* was not possible, a parallel SLN selection procedure with blue dye is used. Patent Blue V (Guerbet Nederland B.V., Gorinchem, The Netherlands) is injected additionally after Endorem to enable the visual selection of SLNs by the pathologist. For each patient the blue lymph nodes nearest to the tumor, with a maximum of three, are considered as SLNs and are resected for analysis of iron content and placed in formalin for 24-72 hours. The local ethics committee of the hospital Medisch Spectrum Twente in Enschede was informed and agreed with the experimental procedure.

Figure 1 (a) Lymph node sample fixated with plastic system in glass tube. (b) VSM detection coil set with bore diameter 10.6 mm.

2.2.2 Sample placement

All samples are placed in a NMR glass tube (Wilmad-LabGlass, Vineland, New Jersey, USA) with an inner diameter of 8.16 mm and an outer diameter of 10 mm. To prevent uncontrolled movement during VSM-measurements, the samples are fixated between two plastic parts inside the tube (Fig. 1a). The upper part is adjustable in length to allow for different sample sizes; typically for lymph nodes between 2 and 10 mm. In addition, the soft lymphatic tissue with some surrounding fat can be compactly fixated. To reduce noise from

liquid movement, the level of remnant formalin in the tube is as low as possible. Automatic offset detection by the VSM-system itself is often not accurate because of low or absent magnetization in biological tissue. Therefore the axial distance from the bottom of the tube to the center of the sample is measured manually to determine the optimal VSM-offset position in the detection coil set.

2.2.3 VSM procedure

Measurements are performed using the VSM of a physical property measurement system (PPMS, Quantum Design Inc., San Diego, California, USA) with a maximum magnetic field capacity of $\mu_0 H = 9\text{T}$. The applied field range is lower ($\mu_0 H = 4\text{T}$) to prevent samples from large forces while approaching magnetic saturation for Endorem particles. The vibration frequency was 40 Hz, whereas the vibration amplitude was 0.5 mm. This low amplitude reduces the forces acting on the sample by a factor of 4, compared to the default amplitude of 2 mm. Consequently, noise caused by interfering, parasitic movements due to soft tissue is reduced. The lymph nodes are relatively large compared to most samples normally measured in a VSM. To fit the NMR tube containing the lymph node, a custom-made VSM detection coil was used with an inner diameter of 10.6 mm (see Fig. 1b)

To investigate sensitivity of magnetic detection and to calibrate the VSM for Endorem containing lymph nodes, a series of calibration samples was prepared. Small glass containers were filled with 15 μL diluted Endorem ranging from 1:1 to 1:150, which corresponds with 168 to 1.12 μg iron in a sample. In addition, some larger samples containing 500 and 1568 μg iron were used to increase accuracy of the calibration factor. Furthermore, a known Endorem sample is measured while immersed in formalin to investigate the noise contributions from free liquid formalin. Samples with Patent Blue V and formalin are measured to exclude the effect of superparamagnetic or ferromagnetic contributions when present in lymph node samples. To determine the correction for the demagnetization of the superconducting magnet [38], a paramagnetic palladium sample is measured in the same field range as applied to the lymph nodes.

2.2.4 Data analysis

VSM measurements of lymph nodes placed in the NMR tube with plastic fixation parts, are assumed to exhibit a superparamagnetic component originating from the nanoparticles, a diamagnetic component originating from the tissue and a paramagnetic component originating from the sample holder. Magnetic moment versus field curves of the sample were analyzed in MATLAB (The Mathworks Inc., Natick, Massachusetts, USA) by a parameter optimization of a model that includes the three different magnetic components.

Before the optimization, some pre-processing of the data was necessary to remove some additional effects from the data, which is explained hereafter.

In the first step, a correction is made to the measured field to compensate for demagnetization of the superconducting magnet in the PPMS [38]. The palladium measurement should theoretically show a strictly anhysteretic linear curve. Any hysteresis observed in this measurement can be attributed to the demagnetization of the magnet during the measurement. This causes an inaccurate field measurement that should be corrected to obtain coinciding ascending and descending branches in measurements of anhysteretic materials. To compensate for demagnetization of the superconducting magnet, a field correction of 1750 A/m is applied to each dataset. Then the assumption is made that no hysteresis is present in the Endorem sample at ambient temperatures [39] and the Langevin model for superparamagnetism can be applied.

The strength of the linear components in the measurements vary over different samples and are eliminated from the optimization by subtracting the linear approximation of the magnetic moment in the high field region. In most studies this component is determined by a 'background' measurement. There are three reasons why this cannot be done in the current study: (i) the magnetic contribution from tissue cannot be determined in a separate measurement before tracer administration and depends on the size of a lymph node and the amount of surrounding fat and thus differs for each lymph node, (ii) the amount of formalin surrounding the sample varies, (iii) since the variable size of the calibration samples and lymph nodes needs fine-tuning of the fixation system, the paramagnetic contribution of the sample holder in the detection coil differs from sample to sample. Therefore the sample dependent linear component is approximated by a linear fit of the data measured from 90% of the field maxima ($|H_{max}|$). The superparamagnetic component of the magnetic moment of the sample is assumed to be saturated in this region. Although this is not true for contributions of very small superparamagnetic particles, this approach can be used when the model describing the superparamagnetic component is subjected to the same procedure. Therefore, also the model is subjected to a linear subtraction, which is based on the slope of the modelled superparamagnetic component in the same high field range as the measured data. So, to obtain the most likely parameters describing the curvature of the magnetic moment curve, the model and the data are matched in the high field region by linear approximation, while the particle size distribution parameters in the fitting algorithm that describe the unsaturated non-linear superparamagnetic part, are optimized by minimization of the error between data and fit.

Asymmetry in the positive and negative branches of the measured curve were treated by an offset correction. Finally the magnetic moment curve is normalized in order to exclude

the saturation value from the parameters to be optimized. Then a normalized model for the superparamagnetic component can be compared with the normalized data.

The optimization procedure is now only dependent on the *shape* of the superparamagnetic components, which is determined by the particle characteristics in the sample. The superparamagnetic component is modelled by the Langevin model for superparamagnetism [40], described by

$$L(x_k H) = \coth(x_k H) - \frac{1}{x_k H} \quad (1)$$

with

$$x_k H = \frac{m_k \mu_0 H}{k_B T} \quad (2)$$

The constants μ_0 , k_B and parameter T represent vacuum permeability, the Boltzmann constant and absolute temperature (always 300 K in our case) respectively. The Langevin function is specific for a particle size with magnetic moment m_k [Am²] and depends on the applied magnetic field strength H [A/m]. Since the size of a magnetic nanoparticle determines its magnetic moment, a sample with a certain particle size distribution has also a certain magnetic moment distribution. Therefore the model describing the experimental data has to take into account a distribution of magnetic moments [41]. The magnetic moment of a superparamagnetic particle is related to its diameter D_k [m] by the bulk saturation magnetization M_s [A/m] of iron oxide Fe₃O₄ ($\mu_0 M_s = 0.60$ T, [41]) via

$$m_k = \frac{\pi D_k^3 M_s}{6} \quad (3)$$

For magnetic nanoparticles an unimodal log-normal particle size distribution is generally accepted [42], because it is physically very likely and can be explained by physical phenomena during the production process [43]. Furthermore, transmission electron microscopy results of Endorem indicated a log-normal core size distribution [39]. The numerical approach of the log-normal particle size distribution is defined as

$$f(D_k | D_1, \sigma_1) = \frac{1}{D_k \sigma_1 \sqrt{2\pi}} e^{\frac{-(\ln D_k - D_1)^2}{2\sigma_1^2}}, \quad k = (1, \dots, K) \quad (4)$$

where D_1 and σ_1 are the mean diameter and standard deviation of the associated normal distribution, respectively. The distribution is calculated for a broad range of K different particle diameters with diameter step size D_{step} . By substituting (3) for each D_k into (1) and (2) and multiplying each resulting Langevin function by its weight from the distribution $f(D_k|D_1, \sigma_1) \cdot D_{step}$ the contribution from each particle size is computed.

However, the model of the magnetic moment curve using a unimodal log-normal distribution for Endorem did not result in a suitable approach of the data. Especially in the region of the strongest curvature the model cannot match the data. Therefore the unimodal log-normal distribution cannot represent the core size distribution of Endorem and a core size distribution with other shape parameters has to be used. Since particle production processes often result in log-normal distributed populations, it is reasonable to add a second log-normal distribution in the fit, which gives more degrees of freedom to the modelling curve. The bimodality of the particle size distributions may originate from the production process of the nanoparticles. A chemical growth processes, such as precipitation used for Endorem production [39, 44], comprises initial nucleation and growth, after which some original (smaller) seeds may remain in the colloid, which gives rise to two lognormal distributed particle size populations [45]. In present analysis, the bimodal distribution is only a way to model the most probable experimental magnetic moment curve using the most relevant parameters of the size distribution. Implementation of a bimodal log-normal distribution requires three additional parameters to be optimized: a second mean and standard deviation for the distribution and the relative weight factors p and $(1 - p)$ for each distribution.

Finally, the sum of all modelled Langevin functions for the bimodal log-normal distribution describes the model to be optimized

$$m(H) = \sum_{k=1}^K n \frac{\pi D_k^3 M_s}{6} \cdot L(x_k H) \cdot f(D_k|D_1, D_2, \sigma_1, \sigma_2, p) \cdot D_{step}, k = (1, \dots, K) \quad (5)$$

where m represents the total field dependent magnetic moment of the sample and n the number of particles. This model as well as the data is normalized and the best parameters are determined by minimization of the root of the sum of squares of the logarithmic differences between the model $m(H)$ and measurement data $m_{sample}(H)$ [46]:

$$Error = \sqrt{\sum_{H=H_{min}}^{H_{max}} (\log |m(H)| - \log |m_{sample}(H)|)^2} \quad (6)$$

This minimization for five parameters is performed using the Nelder-Mead simplex algorithm, which is an unconstrained non-linear optimization algorithm implemented in the MATLAB software package [47]. The minimization gives optimal parameters for the particle size distribution.

After the optimum distribution has been determined, the original superparamagnetic component, which is lost in the normalization, can be reconstructed. The linear subtraction applied to the model is added again to both the normalized model and the normalized measured data. The total magnetic moment responsible for superparamagnetism in a sample is determined by the sum of magnetic moments of the individual particles. This can be derived from the factor that was used for normalization of the data. To finish the quantitative reconstruction of the superparamagnetic component, both model and data were multiplied by this factor, which is basically the saturated magnetic moment.

For relatively large linear contributions in lymph node measurements, the quantification of the superparamagnetic component is very sensitive for noise, since after linear correction the relatively small errors made at high fields have a large effect on the small amplitude of the superparamagnetic component. Therefore, reduction of movement noise is particularly important for the quantification of samples with low amounts of iron. Determination of all parameters of the bimodal particle size distribution is therefore not suitable for each individual lymph node measurement. For that reason, the parameters of the particle size distributions found for the calibration samples are averaged and used in the model to quantify the iron content in lymph nodes. This average bimodal distribution is based on all measurements of calibration samples with a fit error lower than 0.5 (see (6)). Thereby it is assumed that the particle size distribution of the superparamagnetic cores in the lymph nodes is the same as in the original tracer. The hydrodynamic size distribution of the particles that enter the lymph nodes might be different from the distribution in the original tracer, because the tissue and lymphatic system can be considered as a filter that may trap the larger particles. In the lymph node analysis presented here, the core size distribution in lymph nodes is assumed to be the same as in the original tracer, which supposes that hydrodynamic size is not directly related to magnetic core size. Finally, there remain three parameters to be estimated for the lymph node measurement. The first parameter is the saturated magnetic moment M_s , which corresponds to the amount of iron. The second parameter is the linear component χH , added to estimate the volumetric susceptibility χ of paramagnetic or diamagnetic material. The last estimate is an offset correction that is applied to correct for asymmetry.

2.2.5 Light microscopic analysis of lymph nodes

Following VSM-measurements, the lymph nodes are sliced (2-4 μm) for histological analysis by a pathologist. The presence of metastases is revealed by H&E and Cam 5.2 histological staining. Perls Prussian Blue staining is used to indicate iron content in the lymph nodes.

2.3 RESULTS AND DISCUSSION

2.3.1 Calibration and parameter modelling

Different samples with a known quantity of Endorem were used as reference measurement to calibrate the system, as well as to develop the parameter modelling of the total magnetic moment of a sample. The model achieved for the measured data and the accompanying bimodal particle size distribution is shown in Figure 2. For the average particle size distribution further used for lymph node quantification, the following parameters were found: $D_1 = 4.5 \text{ nm}$, $\sigma_1 = 0.47$, $D_2 = 8.3 \text{ nm}$, $\sigma_2 = 0.29$, $p = 0.52$. These values are in the same range as was found using a unimodal lognormal distribution for TEM analysis of Endorem nanoparticles [39, 48, 49]. The bimodal core size distribution has a more broadened peak compared to a unimodal lognormal distribution, but does not show two clear separate maximums. The use of the bimodal lognormal distribution does give more freedom to the shape of the distribution and does not implicate that there are two clearly distinguishable populations of particle sizes.

The deviation of the model from the measured data revealed a systematic measurement error (Fig. 2). The ascending and descending branches of the loop do not coincide, which causes dissimilar differences between the measurement data and the model. This may indicate hysteresis in the sample, but the asymmetric and inconsistent pattern of deviation argues for measurement errors.

Figure 2 The normalized magnetic moment versus field curve. The upper panel shows the normalized measurement and the curve of the optimized model on linear scale. The mid panel shows the difference between the model and the measured data. The negative and positive differences indicate that the model is well positioned in between the descending and ascending branch of the loop, showing some unphysical hysteresis due to measurement error. The lower panel on bilogarithmic scale gives more insight in the quality of measured data and the model in the low field region. Superparamagnetism is confirmed by the absence of significant hysteresis in the low field region. The bimodal lognormal particle size distribution that resulted in the best modelling curve is shown in the inset.

Since the saturated magnetization at a high field strength is used as calibration to estimate iron content in other samples, the model should be as precise as possible in this region. The calibration with the lowest amount of 1 μg iron could not accurately be quantified, but still shows a minor superparamagnetic component indicating the detection limit. The lowest amount of Endorem that could be quantified corresponds to 1.5 μg Fe with an error of ± 0.5 μg . This detection limit depends strongly on the quality of the measurement and the contribution of linear magnetic materials. The calibration constant used to quantify lymph node samples with a saturation field of $3.18 \cdot 10^6$ A/m was $7.76 \cdot 10^{-8}$ A·m² μg^{-1} .

Measurements of samples with Patent Blue V and formalin did not show any non-linear magnetic contribution that may interfere with the superparamagnetic contribution from particles accumulated in the tissue (results not shown). So, the presence of Patent Blue V and formalin in or around lymph node samples will not affect an accurate estimation of the superparamagnetic component from the tracer.

2.3.2 Lymph node analysis

The magnetic content in lymph nodes is determined based on the average particle size distribution found in the calibration samples. The Endorem mass in the lymph nodes is determined using the Langevin model with the bimodal distribution described in section 2.2.4. Although in most cases a significant linear contribution was present, a superparamagnetic non-linear component could be well estimated by the algorithm and therefore a background measurement became unnecessary. This is important, because a background measurement for the lymph nodes would even be impossible for this clinical application.

The magnetic moment curve of two lymph nodes is shown in Fig. 3. There is an obvious difference with the curve in Fig. 2 because of the linear contribution from sample holder and tissue. Both the superparamagnetic and the linear component are estimated by fitting the parameters m_s and χ respectively. The calibration constant derived from a series of known Endorem samples (see Section. 2.3.1) is used to determine the iron mass in the lymph node. Over all, from 13 patients and 33 lymph nodes included in the study, Endorem content was detected in 24 lymph nodes and was found in the range of 1.1 to 51.4 μg iron. The mean quantity of iron found in lymph nodes was 17.1 μg . Light microscopic analysis of the lymph nodes with Perls Prussian Blue staining confirmed iron presence in each lymph node that was detected by magnetometry (see Fig. 3). The iron presence was observed in the interstitial space in all but one lymph node. In that particular lymph node macrophages stained positive for iron.

a.

b.

c.

d.

Figure 3 Two examples of a VSM measurement of a lymph node containing Endorem and the corresponding microscopy images with Perls Prussian Blue staining. Endorem content corresponds with (a) 46.7 μg and (b) 11.9 μg iron. The green line indicates the model applied to the data points measured, including a linear (χ) and non-linear component with amplitude m_s . The corresponding histology images (c and d) with Perls Prussian Blue confirm the presence of SPIO and indicate interstitial spread of the particles throughout the sinuses of the lymph nodes.

Some measurements suffered from significant noise and possibly sample displacement. Lymph node samples with a substantial proportion of fat tissue are more susceptible to abusive, parasitic, lateral movements. This is overcome by a stronger fixation of the sample, resulting in lower noise and subsequent accurate quantification of the amount of iron. The remaining effect of motion-generated errors is represented in the error of the fit procedure (see (6)), which is on average 1.61 for lymph nodes compared to 0.24 for the calibration samples. However, for the present study this error is small enough to obtain a quantitative indication of Endorem filling of colorectal lymph nodes in an ex vivo sentinel node

procedure. Future systems for magnetic lymph node analysis need to be designed such that this kind of errors do not occur.

There are two possible reasons that some blue nodes that were selected as SLNs by definition, did not contain iron. First, the definition of the SLN may have failed by selecting lymph nodes that are not true SLNs. The probably more selective magnetic tracer has only reached the true SLNs in that case. This cannot be verified, since lymph node mapping is unable to reveal whether a lymph node is a first or higher echelon node. The second reason could be that the ex vivo circumstances reduced magnetic tracer migration towards lymph nodes. Therefore, some of the SLNs may be missed by the magnetic tracer. These aspects of the procedure should be investigated in a more elaborate clinical study that allows magnetic measurements on all the lymph nodes in a specimen.

Interestingly, the results show that ex vivo SLN mapping with magnetic nanoparticles is feasible. Lymphatic drainage of Endorem particles from the tumor in ex vivo colorectal tissue is possible by mechanical actuation, such as massage. Other physiological mechanisms of lymphatic transport, including macrophage uptake which is normally present in living tissue [37], are therefore not necessary for the selection of SLNs in colorectal cancer. After ex vivo injection, the particles flow via the interstitial space through the lymphatics to the SLNs, driven by mechanical pressure induced by massage. In in vivo cases SPIO accumulates normally in macrophages, but this activity is believed to cease soon after resection of the specimen. Other studies have shown the utility of ex vivo SLNM in colorectal cancer using a non-colloidal blue dye [20-25]. The present study has shown that despite the use of particles in ex vivo SLNM, the tracer ends up in lymph nodes. The use of particles might even be contributing to accurate sentinel node selection, since the chance of selection of second echelon nodes might be reduced. The specific clinical value of the use of magnetic nanoparticles in colorectal SLN mapping should be investigated in a more elaborate patient study.

The accumulated particles in SLNs are detectable by highly sensitive laboratory equipment. Although Endorem was a pragmatic choice for reasons of availability, it performed well as tracer for SLNs. However, further development of magnetic SLNM in colorectal cancer should consider the optimal magnetic and hydrodynamic particle size and composition. The success of technetium based SLN procedures has shown to be dependent on the particle size of the applied colloid [50, 51]. The development of magnetic nanoparticles with a higher (magnetic) yield, will lower the requirements for new clinical instruments to be developed or may increase the sensitivity of the procedure.

For several reasons, an experimental laboratory VSM-system is not suitable for clinical applications. The large magnetic fields and helium cooling, as well as sample mounting and long measuring time are significant drawbacks for clinical use of a VSM. Therefore further development of fast, high-sensitive magnetic detectors is desirable. Exploiting the non-linear behavior of the SPIO particles in AC-susceptometry or a frequency mixing technique [52], the detection can be very specific, which is mandatory for samples with unknown diamagnetic content. In colorectal cancer, the SLNs have to be selected out of a series of about 10-25 resected lymph nodes per patient. Therefore, a clinical magnetic detection instrument with high sensitivity and short processing time would enable pathologists to use their specific microscopy techniques for ultrastaging on magnetically selected SLNs, so as to find high-risk patients who may benefit from adjuvant therapy.

2.3.3 Other techniques of SPIO quantification

In literature several other techniques to quantify SPIO in biological samples are described. Besides magnetometry, optical and mass spectroscopy are used to analyse SPIO content in cell samples. Inductively coupled plasma spectroscopy is a highly sensitive but expensive method that is not suitable for routine sample analysis. These techniques are very sensitive for SPIO, but require sample digestion which is not compatible with histopathologic analysis in SLNM [28, 53, 54].

Since Endorem is developed as MRI contrast agent, the uptake of particles can be revealed by MRI. MRI techniques to quantify SPIO concentrations in samples are based on the field inhomogeneities produced by the particles. These field inhomogeneities can be quantified by measuring a reduction in relaxation time [53] or by model-based reconstruction based on a measured phase map [55, 56]. Boutry and colleagues could quantify magnetic nanoparticle content in cell samples by relaxometry [53], however their procedure is not applicable in SLNM because it requires sample digestion and thus impedes histopathologic analysis of intact samples. Problematic in SPIO quantification with MRI are other sources of field inhomogeneities in a sample, like gradient instabilities and tissue-tissue or air-tissue interfaces, that all may cause the contribution from SPIO nanoparticles to be indistinguishable [27, 56]. Therefore (background) measurements that allow identification of these other components are often required to determine the exact contribution from SPIO [55, 57, 58]. This makes MRI-procedures much more complex and time consuming, since in case of SLNM the SLNs also have to be measured before tracer administration. For ex vivo procedures this would postpone the time-critical tracer injection and therefore the identification rate of the procedure may become affected. Finally, MRI is an expensive technique which is less specific for non-linear magnetic properties and therefore less suitable for ex vivo SLNM with SPIO. Therefore magnetometry more selective for the

specific non-linear characteristics of SPIO can be much more accurate, less expensive and easier to implement in clinical practice.

2.4 CONCLUSION

Sentinel lymph node mapping using superparamagnetic nanoparticles is successfully applied in colorectal cancer patients. Although a dispersion of nanoparticles is used in the ex vivo tissue, the tracer ends up in lymph nodes. This study shows that non-destructive VSM-measurements on fresh or formalin-fixated lymph nodes, can reveal the magnetic properties inside, provided that the lymph nodes are firmly fastened. The non-linear superparamagnetic contribution arising from the magnetic nanoparticles in the tracer is distinguishable and quantifiable by modelling the magnetic moment curve with the Langevin model and a bimodal log-normal core size distribution. Furthermore, detection and selection of Endorem-filled SLNs in ex vivo colorectal tissue was proven to be possible by a detection limit of 1 μg iron. Selection of the SLN in colorectal cancer using a selective colloidal magnetic tracer can help to accurately intensify standard histopathological analysis by additional staining of those nodes that most probably contain metastases. To facilitate the clinical application of magnetic SLN detection in colorectal cancer, a clinical magnetometer has to be developed that allows quick and specific detection of the non-linear properties of superparamagnetic tracer in lymph nodes.

2.5 REFERENCES

1. Q.A. Pankhurst, N.K.T. Thanh, S.K. Jones, and J. Dobson, *Progress in applications of magnetic nanoparticles in biomedicine*. Journal of Physics D: Applied Physics, 2009. **42**(22).
2. S. Laurent, D. Forge, M. Port, A. Roch, et al., *Magnetic Iron Oxide Nanoparticles: Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications*. Chemical Reviews, 2008. **108**(6): p. 2064-2110.
3. C. Corot, P. Robert, J.M. IdÃ©e, and M. Port, *Recent advances in iron oxide nanocrystal technology for medical imaging*. Advanced drug delivery reviews, 2006. **58**(14): p. 1471-1504.
4. J.W.M. Bulte and D.L. Kraitchman, *Iron oxide MR contrast agents for molecular and cellular imaging*. NMR in Biomedicine, 2004. **17**(7): p. 484-499.
5. A. Ito, M. Shinkai, H. Honda, and T. Kobayashi, *Medical application of functionalized magnetic nanoparticles*. Journal of Bioscience and Bioengineering, 2005. **100**(1): p. 1-11.
6. T. Islam and L. Josephson, *Current state and future applications of active targeting in malignancies using superparamagnetic iron oxide nanoparticles*. Cancer Biomarkers, 2009. **5**(2): p. 99-107.
7. R. Ivkov, S.J. DeNardo, W. Daum, A.R. Foreman, et al., *Application of high amplitude alternating magnetic fields for heat induction of nanoparticles localized in cancer*. Clinical Cancer Research, 2005. **11**(19 II).
8. T.K. Jain, J. Richey, M. Strand, D.L. Leslie-Pelecky, et al., *Magnetic nanoparticles with dual functional properties: Drug delivery and magnetic resonance imaging*. Biomaterials, 2008. **29**(29): p. 4012-4021.
9. R. Hergt and S. Dutz, *Magnetic particle hyperthermia-biophysical limitations of a visionary tumour therapy*. Journal of Magnetism and Magnetic Materials, 2007. **311**(1 SPEC. ISS.): p. 187-192.
10. T. Nakagawa, Y. Minamiya, Y. Katayose, H. Saito, et al., *A novel method for sentinel lymph node mapping using magnetite in patients with non-small cell lung cancer*. Journal of Thoracic and Cardiovascular Surgery, 2003. **126**(2): p. 563-567.
11. Y. Minamiya, M. Ito, Y. Katayose, H. Saito, et al., *Intraoperative sentinel lymph node mapping using a new sterilizable magnetometer in patients with nonsmall cell lung cancer*. Annals of Thoracic Surgery, 2006. **81**(1): p. 327-330.
12. T. Joshi, Q.A. Pankhurst, S. Hattersley, A. Brazdeikis, et al., *Magnetic nanoparticles for detecting cancer spread*. 30th Annual San Antonio Breast Cancer Symposium – December 13–16, 2007 - Breast Cancer Research and Treatment, 2007. **106**(Suppl. 1): p. S129.
13. L. Johnson, Q. Pankhurst, A. Purushotham, A. Brazdeikis, and M. Douek, *Magnetic Sentinel Lymph Node Detection for Breast Cancer*. Cancer Research, 2011. **70**(24 Supplement): p. P1-01-23.
14. M. Shiozawa, A.T. Lefor, Y. Hozumi, K. Kurihara, et al., *Sentinel lymph node biopsy in patients with breast cancer using superparamagnetic iron oxide and a magnetometer*. Breast cancer, 2013. **20**(3): p. 223-9.

15. S. Tanaka, H. Ota, Y. Kondo, Y. Tamaki, et al., *Detection of magnetic nanoparticles in lymph nodes of rat by high T₂ SQUID*. IEEE Transactions on Applied Superconductivity, 2003. **13**(2 I): p. 377-380.
16. D.J. Grootendorst, J. Jose, R.M. Fratila, M. Visscher, et al., *Evaluation of superparamagnetic iron oxide nanoparticles (Endorem®) as a photoacoustic contrast agent for intra-operative nodal staging*. Contrast Media & Molecular Imaging, 2013. **8**(1): p. 83-91.
17. A.H. Strickland, N. Beechey-Newman, C.B. Steer, and P.G. Harper, *Sentinel node biopsy: an in depth appraisal*. Critical Reviews in Oncology/Hematology, 2002. **44**(1): p. 45-70.
18. P.J. Tanis, O.E. Nieweg, R.A. Valdés Olmos, and B.B.R. Kroon, *Anatomy and physiology of lymphatic drainage of the breast from the perspective of sentinel node biopsy*. Journal of the American College of Surgeons, 2001. **192**(3): p. 399-409.
19. E.S. van der Zaag, W.H. Bouma, P.J. Tanis, D.T. Ubbink, et al., *Systematic Review of Sentinel Lymph Node Mapping Procedure in Colorectal Cancer*. Annals of surgical oncology, 2012. **19**(11): p. 3449-3459.
20. A. Stojadinovic, P.J. Allen, M. Protic, J.F. Potter, et al., *Colon sentinel lymph node mapping: Practical surgical applications*. Journal of the American College of Surgeons, 2005. **201**(2): p. 297-313.
21. J.J. Tuech, P. Pessaux, F. Di Fiore, V. Nitu, et al., *Sentinel node mapping in colon carcinoma: In-vivo versus ex-vivo approach*. European Journal of Surgical Oncology, 2006. **32**(2): p. 158-161.
22. E.S. Van der Zaag, C.J. Buskens, N. Kooij, H. Akol, et al., *Improving staging accuracy in colon and rectal cancer by sentinel lymph node mapping: A comparative study*. European Journal of Surgical Oncology, 2009. **35**(10): p. 1065-1070.
23. P.M. van Schaik, J.C. van der Linden, M.F. Ernst, W.A.H. Gelderman, and K. Bosscha, *Ex vivo sentinel lymph node "mapping" in colorectal cancer*. European Journal of Surgical Oncology, 2007. **33**(10): p. 1177-1182.
24. C.T. Viehl, C.T. Hamel, W.R. Marti, U. Guller, et al., *Identification of Sentinel Lymph Nodes in Colon Cancer Depends on the Amount of Dye Injected Relative to Tumor Size*. World Journal of Surgery, 2003. **27**(12): p. 1285-1290.
25. J.H. Wong, S. Steineman, C. Calderia, J. Bowles, and T. Namiki, *Ex vivo sentinel node mapping in carcinoma of the colon and rectum*. Annals of surgery, 2001. **233**(4): p. 515-521.
26. V. Galimberti, S. Zurrada, M. Intra, S. Monti, et al., *Sentinel node biopsy interpretation: The Milan experience*. Breast Journal, 2000. **6**(5): p. 306-309.
27. W. Liu and J.A. Frank, *Detection and quantification of magnetically labeled cells by cellular MRI*. European Journal of Radiology, 2009. **70**(2): p. 258-264.
28. E.R. Dadashzadeh, M. Hobson, L. Henry Bryant, D.D. Dean, and J.A. Frank, *Rapid spectrophotometric technique for quantifying iron in cells labeled with superparamagnetic iron oxide nanoparticles: potential translation to the clinic*. Contrast Media & Molecular Imaging, 2013. **8**(1): p. 50-56.
29. W. Beyhum, D. Hautot, J. Dobson, and Q.A. Pankhurst, *Magnetic biomineralisation in Huntington's disease transgenic mice*. Journal of Physics: Conference Series, 2005. **17**(1): p. 50-53.

30. J. Dobson and P. Grassi, *Magnetic properties of human hippocampal tissue - Evaluation of artefact and contamination sources*. Brain Research Bulletin, 1996. **39**(4): p. 255-259.
31. D. Hautot, Q.A. Pankhurst, and J. Dobson, *Superconducting quantum interference device measurements of dilute magnetic materials in biological samples*. Review of Scientific Instruments, 2005. **76**(4).
32. D. Hautot, Q.A. Pankhurst, C.M. Morris, A. Curtis, et al., *Preliminary observation of elevated levels of nanocrystalline iron oxide in the basal ganglia of neuroferritinopathy patients*. Biochimica et Biophysica Acta - Molecular Basis of Disease, 2007. **1772**(1): p. 21-25.
33. J.L. Kirschvink, A. Kobayashi-Kirschvink, and B.J. Woodford, *Magnetite biomineralization in the human brain*. Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(16): p. 7683-7687.
34. Q. Pankhurst, D. Hautot, N. Khan, and J. Dobson, *Increased levels of magnetic iron compounds in Alzheimer's disease*. Journal of Alzheimer's Disease, 2008. **13**(1): p. 49-52.
35. I. Raynal, P. Prigent, S. Peyramaure, A. Najid, et al., *Macrophage Endocytosis of Superparamagnetic Iron Oxide Nanoparticles: Mechanisms and Comparison of Ferumoxides and Ferumoxtran-10*. Investigative Radiology, 2004. **39**(1): p. 56-63.
36. H.Y. Lee, S.H. Lee, C. Xu, J. Xie, et al., *Synthesis and characterization of PVP-coated large core iron oxide nanoparticles as an MRI contrast agent*. Nanotechnology, 2008. **19**(16).
37. M.A. Saksena, A. Saokar, and M.G. Harisinghani, *Lymphotropic nanoparticle enhanced MR imaging (LNMRI) technique for lymph node imaging*. European Journal of Radiology, 2006. **58**(3): p. 367-374.
38. D.X. Chen, O. Pascu, A. Roig, and A. Sanchez, *Size analysis and magnetic structure of nickel nanoparticles*. Journal of Magnetism and Magnetic Materials, 2010. **322**(24): p. 3834-3840.
39. C.W. Jung and P. Jacobs, *Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: Ferumoxides, ferumoxtran, ferumoxsil*. Magnetic Resonance Imaging, 1995. **13**(5): p. 661-674.
40. C.P. Bean and I.S. Jacobs, *Magnetic granulometry and super-paramagnetism*. Journal of Applied Physics, 1956. **27**(12): p. 1448-1452.
41. D.X. Chen, A. Sanchez, E. Taboada, A. Roig, et al., *Size determination of superparamagnetic nanoparticles from magnetization curve*. Journal of Applied Physics, 2009. **105**(8).
42. C.G. Granqvist and R.A. Buhrman, *Ultrafine metal particles*. Journal of Applied Physics, 1976. **47**(5): p. 2200-2219.
43. L.B. Kiss, J. Soderlund, G.A. Niklasson, and C.G. Granqvist, *New approach to the origin of lognormal size distributions of nanoparticles*. Nanotechnology, 1999. **10**(1): p. 25-28.
44. E.V. Groman, L. Josephson, and J.M. Lewis, *Biologically degradable superparamagnetic materials for use in clinical applications* U.S.P.a.T. Office, Editor. 1989, ADVANCED MAGNETICS, INCORPORATED

45. M. Blanco-Mantecón and K. O'Grady, *Grain size and blocking distributions in fine particle iron oxide nanoparticles*. Journal of Magnetism and Magnetic Materials, 1999. **203**(1-3): p. 50-53.
46. S. Biederer, T. Knopp, T.F. Sattel, K. Lüdtke-Buzug, et al., *Magnetization response spectroscopy of superparamagnetic nanoparticles for magnetic particle imaging*. Journal of Physics D: Applied Physics, 2009. **42**(20).
47. J. Lagarias, J. Reeds, M. Wright, and P. Wright, *Convergence Properties of the Nelder-Mead Simplex Method in Low Dimensions*. SIAM Journal on Optimization, 1998. **9**(1): p. 112-147.
48. J.E. Lima, A.L. Brandl, A.D. Arelaro, and G.F. Goya, *Spin disorder and magnetic anisotropy in Fe₃O₄ nanoparticles*. Journal of Applied Physics, 2006. **99**(8): p. 083908-10.
49. L.F. Gamarra, G.E.S. Brito, W.M. Pontuschka, J.B. Mamani, et al., *Study of the ferrofluid drying process for morphological and nanostructural characterization*. Brazilian Journal of Physics, 2007. **37**(4): p. 1288-1291.
50. M.R.S. Keshtgar and P.J. Ell, *Sentinel lymph node detection and imaging*. European Journal of Nuclear Medicine, 1999. **26**(1): p. 57-67.
51. M.H. Leidenius, E.A. Leppänen, L.A. Krogerus, and K.A.V. Smitten, *The impact of radiopharmaceutical particle size on the visualization and identification of sentinel nodes in breast cancer*. Nuclear medicine communications, 2004. **25**(3): p. 233-238.
52. H.J. Krause, N. Wolters, Y. Zhang, A. Offenhäusser, et al., *Magnetic particle detection by frequency mixing for immunoassay applications*. Journal of Magnetism and Magnetic Materials, 2007. **311**(1 SPEC. ISS.): p. 436-444.
53. S. Boutry, D. Forge, C. Burtea, I. Mahieu, et al., *How to quantify iron in an aqueous or biological matrix: a technical note*. Contrast Media & Molecular Imaging, 2009. **4**(6): p. 299-304.
54. P. Danhier, G. De Preter, S. Boutry, I. Mahieu, et al., *Electron paramagnetic resonance as a sensitive tool to assess the iron oxide content in cells for MRI cell labeling studies*. Contrast Media & Molecular Imaging, 2012. **7**(3): p. 302-307.
55. P. Cantillon-Murphy, L.L. Wald, M. Zahn, and E. Adalsteinsson, *Measuring SPIO and Gd contrast agent magnetization using 3T MRI*. NMR in Biomedicine, 2009. **22**(8): p. 891-897.
56. J. Langley, W. Liu, E.K. Jordan, J.A. Frank, and Q. Zhao, *Quantification of SPIO nanoparticles in vivo using the finite perturber method*. Magnetic Resonance in Medicine, 2011. **65**(5): p. 1461-1469.
57. R.M. Weisskoff and S. Kiihne, *MRI susceptometry: Image-based measurement of absolute susceptibility of MR contrast agents and human blood*. Magnetic Resonance in Medicine, 1992. **24**(2): p. 375-383.
58. P.H. Mills, T.K. Hitchens, L.M. Foley, T. Link, et al., *Automated detection and characterization of SPIO-labeled cells and capsules using magnetic field perturbations*. Magnetic Resonance in Medicine, 2012. **67**(1): p. 278-289.

3

EX VIVO SENTINEL LYMPH NODE MAPPING IN COLORECTAL CANCER USING A MAGNETIC NANOPARTICLE TRACER TO IMPROVE STAGING ACCURACY: A PILOT STUDY^{*}

^{*}This Chapter is submitted as: **Pouw JJ**, Grootendorst MR, Klaase JM, van Baarlen J, ten Haken B. *Ex vivo* sentinel lymph node mapping in colorectal cancer using a magnetic nanoparticle tracer to improve staging accuracy: A pilot study. to *Colorectal Disease*, and currently under review.

ABSTRACT

Aim: Nodal status is the most important prognostic factor in colorectal cancer (CRC). Small occult metastases remain undetected on conventional histopathological examination, potentially resulting in undertreatment of patients. *Ex vivo* sentinel lymph node mapping (SLNM) can be used to improve nodal staging accuracy. However currently used tracers suffer from drawbacks, which hampers implementation of the technique in routine clinical practice. Magnetic tracers have an optimal size for sentinel lymph node (SLN) retention and allow for objective quantitative selection of SLNs, and therefore have great potential for SLNM in CRC. This study evaluates the feasibility of *ex vivo* magnetic SLNM and compares the performance of this technique to blue dye SLNM.

Materials & Methods: Twenty-eight *ex vivo* SLNM procedures were performed in twenty-seven histological node-negative patients with CRC using a magnetic tracer and blue dye. A magnetometer was used to select magnetic SLNs after formalin fixation of the CRC specimen. Both magnetic and blue SLNs were subjected to serial sectioning and immunohistochemical staining to reveal occult metastases.

Results: At least one SLN was successfully identified in 27/28 (96%) and 25/28 (89%) of the cases with the magnetic technique and blue dye respectively. Isolated tumour cells were detected in ten patients. This was predicted with 100% sensitivity and accuracy using the magnetic technique, and with 91% sensitivity and 96% accuracy using the blue dye technique.

Conclusion: This study demonstrates that *ex vivo* magnetic SLNM is a feasible technique for use in routine clinical practice, which improves nodal staging accuracy of CRC patients.

3.1 INTRODUCTION

Over 1.3 million patients are annually diagnosed with colorectal cancer (CRC). In the more developed regions of the world, CRC is the second most common cause of cancer related death, accounting for over 300.000 deaths in 2012 [1].

Treatment with curative intent consists of en-bloc resection of the affected colorectal segment and adjacent lymphatic basin. Current guidelines demand that at least 12 lymph nodes (LNs) should be retrieved from the specimen and examined, with haematoxylin & eosin (H&E) staining of a single cut surface, for the presence of metastasis [2]. Presence of nodal metastasis is the most important prognostic factor, and determines if adjuvant chemotherapy is indicated. However, up to 30% of the node negative patients develop recurrent disease within 5 years of surgery with curative intent [3, 4]. Several retrospective studies attribute this high recurrence rate to the presence of occult nodal involvement, which remains undetected on conventional histopathological analysis [5-7]. Patients suffering from occult nodal involvement may therefore be understaged, potentially leading to undertreatment of the disease [6-8]. Although these small micrometastases (0.2-2 mm) and isolated tumour cells (ITCs, < 0.2 mm) can be detected by using so-called 'ultrastaging' (serial sectioning and immunohistochemical staining or molecular pathology techniques), ultrastaging is too expensive and time consuming to perform on the large number of LNs retrieved from the colorectal specimen in routine clinical practice. Since ultrastaging is not performed routinely, occult nodal involvement is missed. This results in inadequate staging, and potentially inadequate treatment of a large group of patients. The goal of this study is to provide a method to improve staging accuracy of conventional node negative CRC patients that can easily be implemented in routine clinical practice.

Sentinel lymph node mapping (SLNM) allows identification of LNs that receive direct lymphatic drainage from the tumour area; the so-called sentinel lymph nodes (SLNs). Since occult nodal involvement is predominantly found in these SLNs [5], the status of the SLNs is representative for the entire colorectal nodal basin. By subjecting only the SLNs to ultrastaging, the presence of occult metastasis can be accurately detected, without considerably increasing cost and labour, thus allowing for improved nodal staging.

At present, SLNM is performed by injecting a blue dye and/or radioisotope tracer peritumorally, followed by visual and/or gamma probe detection of discoloured and/or radioactive SLNs. The procedure can be performed *in vivo* as well as *ex vivo*, with a similar detection rate and accuracy [9, 10]. Aberrant lymphatic drainage is reported in only approximately 1-10% of the cases, and therefore a limited drawback when an *ex vivo* approach is used for SLNM. An important advantage of the *ex vivo* approach is that the patient is not exposed to the tracer, and therefore not at risk of developing an allergic

reaction [11, 12]. Furthermore, an *ex vivo* procedure does not add time to the surgery. Although the colloidal properties of radiotracers are beneficial for SLN detection [13, 14] they are generally not used in an *ex vivo* setting because the use of radioisotopes is strictly regulated and logistically challenging [15]. Blue dye is readily available and not limited by radiation safety legislation. However, due to the small size of the blue dye particles, they rapidly distribute through the lymphatic system, not only colouring the true SLNs but also higher echelon nodes. In routine clinical practice, all LNs are resected from the formalin fixed tissue by the pathologist, the day after surgery. Because of the distribution to higher echelon nodes and dilution of the blue dye in formalin, SLN identification must be performed shortly after injection of the tracer. Widespread clinical implementation of SLNM in CRC patients requires a tracer that allows for SLN detection after formalin fixation during routine pathological analysis, without regulatory and logistical barriers that hinder its day-to-day use.

Magnetic tracers have several advantageous properties for use in SLNM in CRC. We previously demonstrated that such magnetic tracers accumulate in the LNs after *ex vivo* administration in colorectal cancer, using non-clinical laboratory equipment [16]. Recently the use of a magnetic tracer was also evaluated for SLN biopsy in breast cancer patients [17]. Magnetic tracers consist of superparamagnetic iron oxide (SPIO) nanoparticles with a hydrodynamic size of 50-150 nm, which is favourable for SLN retention [18]. The colloidal properties of the tracer also prevent dilution from formalin fixation. Another advantage is that the presence of a magnetic tracer in LNs can be easily quantified with a handheld magnetometer [18], allowing objective discrimination between SLNs and higher echelon nodes. In addition, magnetic tracers do not suffer from regulatory and logistical barriers associated with the use of radioactivity, thus providing flexibility to perform SLNM. Because of these advantageous properties magnetic SLNM has great potential to improve nodal staging accuracy in routine clinical practice in CRC patients. In this study we evaluate the feasibility of using *ex vivo* magnetic SLNM in a routine clinical workflow, and compare this novel technique to *ex vivo* SLNM with blue dye.

3.2 MATERIALS AND METHODS

3.2.1 Patients

Patients with clinical stage I/II CRC scheduled for intended curative surgery were eligible for inclusion. The study was performed in accordance to the guidelines of the local ethics committee and all patients gave written informed consent. Exclusion criteria were presence of distant metastasis, intraoperative gross nodal involvement, and rectal cancer patients who underwent neoadjuvant (chemo)radiotherapy. Forty *ex vivo* SN procedures were performed in 39 patients; in one patient two tumours were resected separately. All patients

underwent a standard oncological resection and en-bloc lymphadenectomy dictated by the location of the tumour. Presence of nodal metastasis was detected by conventional histological analysis in 12 of these 39 patients. Since the goal of this study is to improve staging accuracy of the patients without nodal metastasis on conventional histological analysis these 12 patients were excluded for further analysis. The remaining 27 node negative patients (28 procedures) comprise the cohort of this study.

3.2.2 Sentinel lymph node mapping procedure

Immediately after resection, the fresh surgical specimens were sent to the pathology department. Colonic specimens were opened antimesenterically by the pathologist or technician, and rectal specimens were opened on the anterior border opposite of the mesorectum, leaving the mesorectum intact. The tumour was located, and the diameter determined.

First, a total of 2 mL of magnetic tracer was injected submucosally in 4 sites circumferentially around the tumour. In the first 10 procedures an MRI contrast agent, Endorem® (Guerbet B.V., Gorinchem, The Netherlands), was used as magnetic tracer. In the remaining 18 procedures, a magnetic tracer optimized for SLN localization [18], Sienna+® (Endomagnetics Ltd, Cambridge, UK), was used. Endorem® consist of SPIO nanoparticles with a mean hydrodynamic diameter of 111 nm and contains 11.2 mg iron (Fe)/mL. Sienna+® has a concentration of 28 mg Fe/mL and the nanoparticles have a mean hydrodynamic diameter of 59 nm. A gentle massage of the injection sites was performed for 3 minutes to promote *ex vivo* tracer distribution. Subsequently, an optimized dose of 0.5 - 2 mL Patent Blue V (Guerbet B.V., Gorinchem, The Netherlands) was injected at the same sites (0.5 mL/cm of tumour diameter [19]), followed by a massage of 5 minutes. The magnetic tracer was injected first, because we expected more difficult distribution of the viscous magnetic tracer compared to the fluidly blue dye.

After administration of the tracers the specimen was formalin fixated for 24-72 hours. Following fixation, all LNs were harvested from the specimens according to the standard clinical protocol. All harvested LNs were individually placed on the probe of a handheld magnetometer, SentiMAG® (Endomagnetics Ltd., Cambridge, UK), to select the LNs with uptake of magnetic tracer (Fig. 1). The probe was balanced in the air before each measurement to compensate for any drift of the signal. The signal on the display of the system, which is proportional to the amount of magnetic tracer [18, 20], was used for quantification of tracer uptake. SLNs were defined as LNs with uptake of magnetic tracer. If more than three nodes with tracer uptake were identified, the three nodes with the highest SPIO uptake were designated as SLNs. Blue nodes were also defined as SLNs, if more than three blue nodes were detected, the first three blue nodes closest to the tumour were selected as SLNs. This definition is similar to the definition used by Faerden et al. [21].

Figure 1 Selection of lymph nodes with uptake of magnetic tracer. A blue formalin fixated lymph node is placed on the handheld probe (covered with a surgical glove) of the magnetometer with plastic tweezers. The numerical display of the system is used to quantify the uptake of magnetic tracer.

A procedure was considered successful if at least one SLN was identified. If ITCs and/or micrometastasis was found in a LN, but not in one of the SLNs, this was considered to be a false negative result.

3.2.3 Histopathological analysis

All LNs were embedded in paraffin and stained with H&E to evaluate for tumour involvement. In addition to this conventional staging, SLNs of the node negative patients were subjected to ultrastaging; the SLNs were additionally sectioned at 250 μ m intervals, and coloured with Cam5.2, an anti-body against keratin, and H&E. Positive cells were categorised as isolated tumour cells (<0.2 mm) or micrometastasis (0.2 - 2 mm). Rare single positive staining cells with immunohistochemical staining lacking cytological characteristics of malignancy were considered negative. In the first ten procedures ultrastaging was performed on all resected LNs, to rule out false negative results. If no false negative results were detected in this series, ultrastaging of the non-SLNs would be omitted in the remaining procedures.

3.3 RESULTS

Twenty-eight *ex vivo* SLNM procedures were performed in 27 patients who underwent intended curative surgery for colorectal cancer and were lymph node negative on routine histological staining. Patient and tumour characteristics are provided in Table 1.

Table 1 Patient and tumour characteristics of the included patients

Characteristic	N
Gender	
Male	13
Female	14
Age in years (mean $\pm$ SD, range)	73 $\pm$ 6 (63-84)
Tumour size in cm (mean $\pm$ SD, range)	4.3 $\pm$ 2.7 (1-11.5)
<i>Tumour location</i>	
Cecum	5
Ascending colon	7
Transverse colon	1
Descending colon	1
Sigmoid colon	11
Rectum	3
<i>Depth of tumour invasion</i>	
T1	4
T2	6
T3	15
T4	3

At least one SLN was successfully identified in 27/28 procedures with the magnetic tracer, and in 25/28 procedures with the blue dye, resulting in an identification rate of 96% for the magnetic technique and 89% for the blue dye technique. The outcomes of the SLNM procedure are listed in Table 2. A total of 607 LNs were dissected, of which 199 (mean 7.4, range 1-16) were magnetic, and 205 (mean 8.2, range 3-36) were blue. More than three LNs with tracer uptake (blue and/or magnetic) were found in all but three procedures. The uptake of magnetic tracer in the LNs could be easily quantified with the handheld

magnetometer, and allowed for an objective selection of three SLNs with the highest uptake in all procedures. A total of 120 SLNs were selected from all nodes with tracer uptake (blue and/or magnetic).

Table 2 Sentinel lymph node mapping procedure and ultrastaging results

Characteristic	N
Identification rate	
Magnetic	28/28 (100%)
Blue	25/28 (89%)
Number of dissected nodes (mean $\pm$ SD, range)	21.7 $\pm$ 11.6 (7 - 54)
Magnetic	7.4 $\pm$ 4.1 (1-16)
Blue	8.2 $\pm$ 6.6 (3-36)
Total number of SLNs	120
Magnetic & Blue	33
Magnetic	46
Blue	41
Histology after ultrastaging	
Negative	18
Isolated tumour cells	10
Micrometastasis	0
False negatives after ultrastaging	
Magnetic	0
Blue	1

Of the 120 SLNs, 33 were selected as both magnetic and blue SLNs, 46 were selected as magnetic SLN only and 41 were selected as blue SLN only (Fig. 2). However, only 11 magnetic SLNs were not coloured blue, and only 11 blue SLNs were not magnetic.

Ultrastaging of the SLNs by serial sectioning and immunohistochemical staining revealed the presence of ITCs in 10/27 conventional node negative patients. No micrometastases were detected. The magnetic technique accurately predicted the presence of occult tumour cells in all patients. However, in one patient ITCs were found solely in a magnetic SLN that was not blue, resulting in a false negative result with the blue dye. No false-negative results were identified when ultrastaging all the non-SLNs of the first ten patients, therefore ultrastaging of the non-SLNs was omitted in the remaining procedures of this pilot study.

Figure 2 Number of magnetic and/or blue sentinel lymph nodes as identified with the different techniques.

3.4 DISCUSSION

Presence of nodal metastasis is the most important prognostic factor in CRC, and is used to determine if a patient should receive adjuvant treatment. Hence, accurate nodal staging is of great importance. SLNM can be used to improve nodal staging accuracy by identifying the nodes at risk of harbouring occult metastasis, and subjecting these nodes to ultrastaging. This study demonstrates the feasibility of a magnetic technique for *ex vivo* SLNM to improve nodal staging accuracy in routine clinical practice. An identification rate of 96% was achieved with the magnetic tracer, which is in line with the previously reported average identification rate of 94% with blue dye and/or radioisotopes [15, 22].

The identification rate with the magnetic technique was higher than with the blue dye (96% vs. 89% respectively). There are two possible explanations for this difference. Firstly, the magnetometer is able to detect very low amounts of tracer, whereas small amounts of blue dye are difficult to distinguish visually. Secondly, formalin fixation of the tissue can dilute the blue dye and thus lower the concentration so it is no longer visible with the eye resulting in a failed procedure. On the contrary, the colloidal properties of the magnetic tracer prevent dilution, once it is taken up by the LNs. Therefore, the magnetic technique does not suffer from dilution of the tracer, allowing for identification of SLN with small amounts of magnetic tracer, even after formalin fixation.

Although the magnetic tracer consists of larger particles, there was no significant difference in the mean number of blue nodes (8.2) and mean number of nodes with magnetic tracer uptake (7.4). This is also most likely because of the high sensitivity of the magnetometer for small amounts of magnetic tracer, as it could result in detection of higher echelon nodes with very limited tracer uptake. The majority of non-SLNs with uptake of magnetic tracer (77/120 non-SLNs) displayed a signal <10% of the signal of the most magnetic SLN of that procedure, supporting the assumption that also higher echelon nodes with small amounts of magnetic tracer uptake were detected. This results in a high number of magnetic LNs. A unique property of the magnetic technique is that the quantitative signal allows for objective selection of the SLNs. Furthermore, unlike with the blue dye, a standardized dose of 2 mL of SPIO was used independent of the tumour diameter. Dose optimization of the magnetic tracer is likely to reduce the number of magnetic nodes as well, as it will restrict tracer distribution to higher echelon nodes.

A large discordance between the nodes selected as SLN was observed between the magnetic and blue technique (Fig. 2). Of the 120 SLNs, 33 were selected as both magnetic and blue SLNs, 46 were selected as magnetic SLN only and 41 were selected as blue SLN only. However, only a small proportion of magnetic SLNs (11/46) were not coloured blue and a similar small proportion of blue SLNs were not magnetic (11/41). Therefore the observed discordance is likely to arise from the differences in the definitions used for the selection of the magnetic and blue SLNs; the magnetic SLNs were selected based on an objective functional definition (amount of tracer uptake) and the blue SLNs were selected based on an anatomical definition (closest to the tumour). In some procedures it was difficult to determine which lymph nodes were located closest to the tumour, emphasizing the subjective nature of this definition.

Ultrastaging of the SLNs revealed ITCs in 10/27 patients (37%), which is concordant with previous studies [5]. Presence of occult metastasis was accurately predicted with the magnetic technique in all 10 patients (100%), and in 9 out of 10 patients (90%) with the blue dye technique. Ultrastaging did not reveal any micrometastasis. To date no prospective

trials have been performed to determine whether patients with ITCs/micrometastasis would benefit from adjuvant treatment. However a recent meta-analysis concluded that patients with micrometastasis have worst prognosis compared to node negative patients and patients with ITCs [23]. Although no micrometastases were detected, the feasibility of the technique to accurately select the lymph nodes harbouring occult metastasis was demonstrated. Based on these results we advise to perform a study with a higher number of patients to determine the clinical relevance of the introduction of the magnetic technique.

An alternative technique for SLNM, using a near-infrared (NIR) fluorescent tracer and imaging system, has been described previously in literature. This fluorescent imaging technique allows to visualize tracer distribution from the injection sites to the sentinel nodes, and has been used both *in vivo* and *ex vivo* in colorectal cancer [24-26]. Both Schaafsma et al. and Hutteman et al. used an *ex vivo* approach, and achieved a sensitivity of 80% and 92 % for macrometastasis respectively. However they did not perform ultrastaging on the sentinel nodes to detect presence of occult tumour cells and therefore their outcomes cannot be compared to our results. A limitation of this technique is that fluorescent tracers do not allow for SLN detection after formalin fixation due to migration to higher echelon nodes [25]. Furthermore, the required imaging systems are complex. These drawbacks make the method less suitable for colorectal SLNM to improve staging accuracy in routine clinical use.

This study demonstrated the use of a magnetic tracer for *ex vivo* sentinel node selection. There is also growing interest for *in vivo* SLNM in colon cancer. Rather than improving staging accuracy, the goal of an *in vivo* procedure is to allow for a limited resection of the increasing number of early stage tumours [27] or patient tailored determination of the extent of the lymphadenectomy [28]. Since the magnetic tracer Sienna+® is approved for *in vivo* use, and the magnetometer has a favourable detection depth of 2-3 cm's, it could be used for this purpose in the future. However, currently no laparoscopic magnetometer is available yet.

A limitation of the current study is the small number of patients, in particular the small number of patients with occult metastasis. Larger studies are needed to validate the advantages of the magnetic technique over the blue dye technique in terms of staging accuracy.

In conclusion, this study demonstrates that *ex vivo* magnetic SLNM is a feasible technique for use in routine clinical practice. The magnetic tracer can be detected in the LNs after formalin fixation, and the quantitative signal of the magnetometer can be used to objectively distinguish SLNs from higher echelon nodes. The magnetic technique achieved

an excellent identification rate, sensitivity and accuracy, and was superior to the blue dye technique. The technique can be used to improve staging accuracy of CRC patients in routine clinical practice.

3.5 REFERENCES

1. J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. Int J Cancer, 2015. **136**(5): p. E359-86.
2. S.B. Edge and C.C. Compton, *The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM*. Annals of surgical oncology, 2010. **17**(6): p. 1471-4.
3. A.J. Bilchik, M. DiNome, S. Saha, R.R. Turner, et al., *Prospective multicenter trial of staging adequacy in colon cancer: preliminary results*. Archives of surgery, 2006. **141**(6): p. 527-33; discussion 533-4.
4. A. Figueredo, M.E. Coombes, and S. Mukherjee, *Adjuvant therapy for completely resected stage II colon cancer*. Cochrane Database Syst Rev, 2008(3): p. CD005390.
5. E.S. van der Zaag, N. Kooij, M.J. van de Vijver, W.A. Bemelman, et al., *Diagnosing occult tumour cells and their predictive value in sentinel nodes of histologically negative patients with colorectal cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2010. **36**(4): p. 350-7.
6. D. Iddings, A. Ahmad, D. Elashoff, and A. Bilchik, *The prognostic effect of micrometastases in previously staged lymph node negative (NO) colorectal carcinoma: a meta-analysis*. Annals of surgical oncology, 2006. **13**(11): p. 1386-92.
7. D.G. Nicastrì, J.T. Doucette, T.E. Godfrey, and S.J. Hughes, *Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature*. J Mol Diagn, 2007. **9**(5): p. 563-71.
8. G. Quasar Collaborative, R. Gray, J. Barnwell, C. McConkey, et al., *Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study*. Lancet, 2007. **370**(9604): p. 2020-9.
9. J.J. Tuech, P. Pessaux, F. Di Fiore, V. Nitu, et al., *Sentinel node mapping in colon carcinoma: in-vivo versus ex-vivo approach*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2006. **32**(2): p. 158-61.
10. T.F. Wood, S. Saha, D.L. Morton, G.J. Tsioulis, et al., *Validation of lymphatic mapping in colorectal cancer: in vivo, ex vivo, and laparoscopic techniques*. Annals of surgical oncology, 2001. **8**(2): p. 150-7.
11. S. Thevarajah, T.L. Huston, and R.M. Simmons, *A comparison of the adverse reactions associated with isosulfan blue versus methylene blue dye in sentinel lymph node biopsy for breast cancer*. Am J Surg, 2005. **189**(2): p. 236-9.
12. C. Bezu, C. Coutant, A. Salengro, E. Darai, et al., *Anaphylactic response to blue dye during sentinel lymph node biopsy*. Surgical oncology, 2011. **20**(1): p. e55-9.
13. S. Saha, A.G. Dan, B. Berman, D. Wiese, et al., *Lymphazurin 1% versus 99mTc sulfur colloid for lymphatic mapping in colorectal tumors: a comparative analysis*. Annals of surgical oncology, 2004. **11**(1): p. 21-6.
14. A.E. Merrie, A.M. van Rij, L.V. Phillips, J.I. Rossaak, et al., *Diagnostic use of the sentinel node in colon cancer*. Diseases of the colon and rectum, 2001. **44**(3): p. 410-7.

15. M.H. van der Pas, S. Meijer, O.S. Hoekstra, Riphagen, Il, et al., *Sentinel-lymph-node procedure in colon and rectal cancer: a systematic review and meta-analysis*. The lancet oncology, 2011. **12**(6): p. 540-50.
16. M. Visscher, J.J. Pouw, J. van Baarlen, J.M. Klaase, and B. Ten Haken, *Quantitative analysis of superparamagnetic contrast agent in sentinel lymph nodes using ex vivo vibrating sample magnetometry*. IEEE transactions on bio-medical engineering, 2013. **60**(9): p. 2594-602.
17. M. Douek, J. Klaase, I. Monypenny, A. Kothari, et al., *Sentinel node biopsy using a magnetic tracer versus standard technique: the SentiMAG Multicentre Trial*. Annals of surgical oncology, 2014. **21**(4): p. 1237-45.
18. J.J. Pouw, M. Ahmed, B. Anninga, K. Schuurman, et al., *Comparison of three magnetic nanoparticle tracers for sentinel lymph node biopsy in an in vivo porcine model*. International journal of nanomedicine, 2015. **10**: p. 1235-43.
19. C.T. Viehl, C.T. Hamel, W.R. Marti, U. Guller, et al., *Identification of sentinel lymph nodes in colon cancer depends on the amount of dye injected relative to tumor size*. World J Surg, 2003. **27**(12): p. 1285-90.
20. B. Anninga, M. Ahmed, M. Van Hemelrijck, J. Pouw, et al., *Magnetic sentinel lymph node biopsy and localization properties of a magnetic tracer in an in vivo porcine model*. Breast cancer research and treatment, 2013. **141**(1): p. 33-42.
21. A.E. Faerden, O.H. Sjo, S.N. Andersen, B. Hauglann, et al., *Sentinel node mapping does not improve staging of lymph node metastasis in colonic cancer*. Diseases of the colon and rectum, 2008. **51**(6): p. 891-6.
22. E.S. van der Zaag, W.H. Bouma, P.J. Tanis, D.T. Ubbink, et al., *Systematic review of sentinel lymph node mapping procedure in colorectal cancer*. Annals of surgical oncology, 2012. **19**(11): p. 3449-59.
23. D.A. Sloothak, S. Sahami, H.J. van der Zaag-Loonen, E.S. van der Zaag, et al., *The prognostic value of micrometastases and isolated tumour cells in histologically negative lymph nodes of patients with colorectal cancer: a systematic review and meta-analysis*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2014. **40**(3): p. 263-9.
24. M. Hutteman, H.S. Choi, J.S. Mieog, J.R. van der Vorst, et al., *Clinical translation of ex vivo sentinel lymph node mapping for colorectal cancer using invisible near-infrared fluorescence light*. Annals of surgical oncology, 2011. **18**(4): p. 1006-14.
25. B.E. Schaafsma, F.P. Verbeek, J.R. van der Vorst, M. Hutteman, et al., *Ex vivo sentinel node mapping in colon cancer combining blue dye staining and fluorescence imaging*. The Journal of surgical research, 2013. **183**(1): p. 253-7.
26. C. Hirche, Z. Mohr, S. Kneif, S. Doniga, et al., *Ultrastaging of colon cancer by sentinel node biopsy using fluorescence navigation with indocyanine green*. Int J Colorectal Dis, 2012. **27**(3): p. 319-24.
27. R.A. Cahill, S. Perretta, J. Leroy, B. Dallemagne, and J. Marescaux, *Lymphatic mapping and sentinel node biopsy in the colonic mesentery by Natural Orifice Transluminal Endoscopic Surgery (NOTES)*. Annals of surgical oncology, 2008. **15**(10): p. 2677-83.

28. S. Saha, G. Johnston, A. Korant, M. Shaik, et al., *Aberrant drainage of sentinel lymph nodes in colon cancer and its impact on staging and extent of operation*. Am J Surg, 2013. **205**(3): p. 302-5; discussion 305-6.

4

COMPARISON OF THREE MAGNETIC NANOPARTICLE TRACERS FOR SENTINEL LYMPH NODE BIOPSY IN AN *IN VIVO* PORCINE MODEL *

*This Chapter is published as: **Pouw JJ**, Ahmed M, Anninga B, Schuurman K, Pinder SE, Van Hemelrijck M, Pankhurst QA, Douek M, ten Haken B. Comparison of three magnetic nanoparticle tracers for sentinel lymph node biopsy in an *in vivo* porcine model. *Int J Nanomedicine* 10:1235-1243 (2015)

ABSTRACT

Introduction: Breast cancer staging with sentinel lymph node biopsy (SLNB) relies on the use of radioisotopes, which limits the availability of the procedure worldwide. The use of a magnetic nanoparticle tracer and a handheld magnetometer provides a radiation-free alternative, which was recently evaluated in two clinical trials. The hydrodynamic particle size of the used magnetic tracer differs substantially from the radioisotope tracer, and could therefore benefit from optimization. The aim of this study was to assess the performance of three different sized magnetic nanoparticle tracers for SLNB within an *in vivo* porcine model.

Materials & Methods: Sentinel lymph node biopsy was performed within a validated porcine model using three magnetic nanoparticle tracers, approved for use in humans, (ferumoxytol, with hydrodynamic diameter $d_H = 32$ nm; Sienna+, $d_H = 59$ nm; and ferumoxide, $d_H = 111$ nm) and a handheld magnetometer. Magnetometer counts (transcutaneous and *ex vivo*), iron quantification (vibrating sample magnetometry) and histopathological assessment were performed on all *ex vivo* nodes.

Results: Transcutaneous ‘hotspots’ were present in 12/12 cases within 30 minutes of injection for the 59 nm tracer, compared to 7/12 for the 32 nm tracer and 8/12 for the 111 nm tracer, at the same timepoint. *Ex vivo* magnetometer counts were significantly greater for the 59 nm tracer than for the other tracers. Significantly more nodes per basin were excised for the 32 nm tracer compared to other tracers, indicating poor retention of the 32 nm tracer. Using the 59 nm tracer resulted in a significant higher iron accumulation compared to the 32 nm tracer.

Conclusion: The 59 nm tracer demonstrated rapid lymphatic uptake, retention in the first nodes reached and accumulation in high concentration, making it the most suitable tracer for intra-operative sentinel lymph node localization.

4.1 INTRODUCTION

Breast cancer is the most common cancer among women, with approximately 1.67 million new patients diagnosed annually worldwide and accounting for 25% of all cancer cases [1]. Breast cancer predominantly spreads via the lymphatic system to locoregional lymph nodes, meaning that the status of these lymph nodes is important for staging of the disease, and determining prognosis [2-4].

The sentinel lymph nodes (SLN) are defined as the first draining nodes from a primary tumor, and therefore are most likely to be the first site of lymphatic metastasis. Sentinel lymph node biopsy (SLNB) with the 'combined technique' of radioisotope and blue dye is the standard method for determining axillary staging in early-stage breast cancer patients with a clinically and radiologically negative axilla [3, 5-9].

The combined technique is performed by injecting a radioactive tracer (^{99m}Tc nanocolloid) and blue dye interstitially, either peritumorally or periareolarly. The tracers are distributed through the lymphatic system, to the draining SLNs. The tracers are then detected within the SLNs by the surgeon using a handheld scintillation counter (gamma probe) and/or visually. The gamma probe is first used to transcutaneously localize the SLNs and determine the optimal incision site. Post-incision both the blue dye and gamma probe guide the surgeon to the SLNs. After identification, the SLNs are removed and sent for histopathological examination. With an identification rate of 96% and a false negative rate of 5-10% it is an effective procedure with low morbidity [3].

Although the currently used technique performs well, it has drawbacks. The use of radioisotopes is subject to stringent regulations on training of staff and handling and disposal of radioactive material. Furthermore the six-hour half-life of ^{99m}Tc limits theatre scheduling. Most importantly, many hospitals in the world do not have access to radioisotopes. Consequently only approximately 60% of the patients in the western world and negligible numbers in the rest of the world have access to SLNB [10]. This has led to the search for other techniques, not reliant on the use of radioisotopes [11].

Recently the use of a magnetic tracer and a handheld magnetometer was evaluated against the 'combined technique' as a radiation free alternative in two separate clinical trials [12, 13]. A superparamagnetic iron oxide (SPIO) tracer was administered interstitially in the breast, followed by identification of the SLNs with a handheld magnetometer. The magnetic technique was found to be non-inferior to the 'combined technique' with identification rates of 94.4% - 98.0% [12, 13]. However, there was a discordance in SLNs identified between the magnetic and combined techniques that ranged between 2.0% [13], and 6.9% [12], – potentially leading to false-negative staging with the magnetic technique. A smaller study by Shiozawa et al. [14] used a similar SPIO tracer and different magnetometer,

comparing SPIO and blue dye only – no radioisotope was used. They found an SLN identification rate of 90% for the SPIO in combination with blue dye, with a discordance rate of 16.7% and demonstrated the viability of the technique for performing SLNB in the absence of radioisotopes.

Physical and chemical properties including shape, coating material and particle size influence the distribution of nanoparticles *in vivo* [15, 16]. ^{99m}Tc nanocolloid has a mean particle size of approximately 8 nm whilst the particle size of the magnetic tracer used for the magnetic technique (Sienna+[®], Endomagnetics UK), is much larger with a mean size of approximately 60 nm [17, 18]. Since particle size is the most important property influencing lymphatic uptake and lymph node retention [19], the identification of different SLNs by the two techniques could possibly arise due to differences in particle size of the radioisotope and magnetic tracer. In order to optimize the clinical application of the magnetic technique for SLNB, we evaluated the performance of three different sized SPIO-based formulations - licensed for human use- within an *in vivo* porcine model.

4.2 MATERIALS AND METHODS

4.2.1 Tracers/dynamic light scattering

Three SPIO-based licensed formulations with a broad range in particle size were used as magnetic tracers in this study. Feraheme[®]/Rienso[®] (Takeda, Japan) consists of ferumoxytol, 30 mg Fe/mL and is an FDA approved drug used for the intravenous treatment of iron deficiency anaemia in adult patients with chronic kidney disease. The particles have a magnetic core of maghemite ($\gamma\text{-Fe}_2\text{O}_3$) surrounded by a polyglucose sorbitol carboxymethylether coating. Sienna+[®] (Endomagnetics, UK) contains 27 mg Fe/mL and is a CE-marked magnetic tracer intended to mark and locate SLNs in cancer patients. The magnetic core consists of magnetite and maghemite ($\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$), with a carboxydextran coating. Finally, Endorem[®]/Feridex[®] (Guerbet, France) consisting of ferumoxide, 11.2 mg Fe/mL is licensed as an MRI contrast agent for liver imaging, with a magnetite core coated with dextran.

All magnetic tracers were diluted to the same iron concentration of 11.2 mg Fe/mL to facilitate comparison of performance. Sterile water for injection and a pipette were used to prepare the dilutions. The hydrodynamic particle size of all tracers was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, UK).

4.2.2 Animals and Surgery

Ethical permission for animal experimentation was granted by the IRCAD Ethics Review Board (Strasbourg, France) under reference number 38.2012.01.047. A previously

developed and validated porcine model which closely resembles human lymphatic drainage was used to evaluate the performance of the different magnetic tracers for SLNB [20]. At the IRCAD Institute (Strasbourg, France), after induction of anaesthesia, mini-pigs were injected with one of the three different magnetic tracers subcutaneously bilaterally into the areolae of the third inguinal mammary glands. A total of 0.5 mL (5.6 mg Fe) of each diluted tracer was injected on each side, in six mini-pigs, for each tracer (18 mini-pigs in total; 36 injections).

Transcutaneous magnetometer measurements using a handheld magnetometer (SentiMAG[®], Endomagnetics Ltd, UK) were undertaken in the inguinal region, where the draining lymph nodes are located. Measurements were performed at 5, 10, 15, 30, 45, 60 and 240 minutes after administration of the tracer. The sites at which uptake of SPIO was successfully detected ('hotspots') were marked using a permanent marker.

Bilateral SLNB of the inguinal region was undertaken 4 hours after injection. The incision was made at the site of the 'magnetic hotspot'. If no 'hotspot' was located, a blind incision was made in the inguinal region. Post-incision, the handheld magnetometer was used to identify the SLNs *in vivo*. After identification, lymph nodes demonstrating SPIO uptake were removed, and the *ex vivo* magnetometer count recorded. Lymph nodes with a magnetometer count higher than 10% of the 'hottest node' were defined as SLNs, as was used in the SentiMAG Multicentre Trial [12], and in accordance with standard clinical practice [21].

After completion of the SLNB procedure, a groin node clearance (GNC) was performed. *Ex vivo* magnetometer measurements were performed on the GNCs to determine if there were any further SLNs, missed initially. All resected lymph nodes were fixed in formalin and sent to the University of Twente (Enschede, The Netherlands) for iron quantification and subsequently to King's College London (London, UK) for histopathological evaluation.

4.2.3 Quantification of iron content (VSM)

The quantification of magnetic tracer in the excised and formalin-fixed sentinel lymph nodes was performed using vibrating sample magnetometry (VSM) on a Physical Properties Measuring system (PPMS, Quantum Design Inc., San Diego, CA, USA). An applied magnetic field of 4.0T was used to bring the magnetic iron oxide nanoparticles to saturation. The amount of magnetic tracer in the lymph nodes was determined by comparing the obtained amplitude of the saturation magnetization to known calibration samples of each of the tracers. The iron content was reported as the mass of iron (Fe) in the node, present in the form of maghemite or magnetite. VSM allows quantification with 0.5 µg Fe accuracy and is non-destructive, which allows subsequent histopathological analysis of the same samples [22].

4.2.4 Histopathology

The nodes were then transferred intact to King's College London (London, UK) where they were embedded in wax, thinly sliced and stained with haematoxylin & eosin (H&E) and with Perl's Prussian blue for iron. Iron staining within each node was graded, using a previously validated 5-point grading scale (*0=none, 1=minimal, 2=mild, 3=moderate, 4=marked*) [20], by an experienced pathologist and a second observer (SP; BA or MA).

4.2.5 Statistical analysis

We conducted a two-sided test ($\alpha=0.05$) expecting a difference of 50 μg (SD: 30) in iron content readings between each magnetic tracer. When performing a total of 12 procedures (six mini-pigs) for each magnetic tracer, these 36 procedures provided us with a power of 82% to detect this difference. The relationship between continuous variables was calculated using Pearson's correlation coefficient, associations between categorical data using the Chi-squared test and associations between categorical and continuous variables using analyses of variance (ANOVA). All statistical analyses were performed using Statistical Analysis Systems (SAS) release 9.3 (SAS Institute, Cary, NC).

4.3 RESULTS

4.3.1 Dynamic light scattering

The Z-averaged hydrodynamic diameter of the magnetic tracers ferumoxytol, Sienna+ and ferumoxide was determined to be 32 nm, 59 nm and 111 nm respectively. When the size distributions were evaluated graphically (Fig. 1), significant intra tracer heterogeneity in particle size was observed. This is reflected by a polydispersity index of 0.179, 0.181 and 0.266 for the 32 nm tracer, 59 nm tracer and 111 nm tracer respectively.

Figure 1 Particle size distribution of the three magnetic tracers by dynamic light scattering. The particle size (logarithmic scale is shown against the intensity.

4.3.2 Surgery

A total of 36 SLNB procedures were performed in 18 mini-pigs, and SLN identification was successful in all cases. *In vivo* magnetic ‘hotspots’ from the draining inguinal lymph nodes were successfully identified transcutaneously prior to surgical excision using the handheld magnetometer in all cases (12/12) for the 32 nm tracer and the 59 nm tracer, and in all but one case (11/12) for the 111 nm tracer (240 minutes after injection). The number of procedures in which a transcutaneous hotspot was successfully identified increased with time after injection for all three tracers (Fig. 2). There were 7/12 and 8/12 successfully identified magnetic ‘hotspots’ after 30 minutes using the 32 nm tracer and 111 nm tracer respectively, compared to the 59 nm tracer with magnetic ‘hotspots’ in all cases (12/12).

Figure 2 Relationship between the time after injection of the magnetic tracer and successful identification of transcutaneous hotspot.

A total of 77 SLNs were identified with the handheld magnetometer during surgery and subsequently excised. Thirty-five SLNs were identified using the 32 nm tracer (mean 2.9, SD 1.2), 20 SLNs with the 111 nm tracer (mean 1.7, SD 0.9) and 22 SLNs (mean 1.8, SD 0.8) with the 59 nm tracer (Fig. 3 (a)). A statistically significant difference between the tracer used, and the number of SLNs identified was observed overall ($P=0.0099$) (Fig. 3 (a)). More nodes per basin were excised with the 32 nm tracer compared to the 111 nm tracer ($P<0.001$) and the 59 nm tracer ($P=0.03$).

The distribution of magnetometer counts demonstrated a statistically significant difference between the 3 tracers overall ($P<0.0001$) (Fig. 3 (b)). Multi comparisons demonstrated that *ex vivo* magnetometer counts were significantly higher with the 59 nm tracer compared to the other two tracers ($P<0.05$).

Figure 3 Boxplots of the relationship between the magnetic tracer and: **(a)** the number of excised sentinel lymph nodes during surgery; **(b)** the ex vivo magnetometer counts of the excised sentinel lymph nodes; **(c)** the iron content measured of the excised sentinel lymph nodes by vibrating sample magnetometry. The circle symbols represent outliers; the diamond symbols represent the mean value.

4.3.3 Quantification of iron content (VSM)

The iron content of the *ex vivo* SLNs was recorded using VSM (Fig. 3 (c)). Multiple comparisons between the tracers demonstrated a significant higher iron content for the 59 nm tracer compared to the 32 nm tracer ($P<0.05$) only. The mean iron content of the SLNs was determined to be 106 (SD 70) μg , 179 (SD 159) μg and 265 (SD 206) μg for the 32 nm tracer, 111 nm tracer and 59 nm tracer respectively. A linear relationship between the iron content of the *ex vivo* SLNs and the handheld magnetometer counts was observed for all tracers (Fig. 4) – 32 nm tracer ($r = 0.93$; $P<0.001$), 111 nm tracer ($r = 0.95$; $P<0.001$), 59 nm tracer ($r = 0.93$; $P<0.001$). Figure 4 demonstrates a difference in sensitivity of the magnetometer for the different tracers. The magnetometer count is 2.8 times higher for the 59 nm tracer compared to the same amount of iron for the 111 nm tracer and 1.3 times higher for the 32 nm tracer compared to the 111 nm tracer.

Figure 4 Correlation between the iron content as determined by vibrating sample magnetometry and the *ex vivo* magnetometer counts for the different magnetic tracers.

4.3.4 Histopathology

The iron was distributed predominantly in the subcapsular space peripherally within the cortex, subcapsular space and sinuses (Fig. 5). The iron deposition from the 111 nm tracer was mostly confined to macrophages within the nodes (Fig. 5 (a)), whereas the 59 nm tracer demonstrated more free iron granules (particularly peripherally) (Fig. 5 (c)). Only very sparse, small islands of iron within macrophages were visible for the 32 nm tracer (Fig. 5 (b)). There was a significant difference in the grade of the iron content of excised SLNs between the three different tracers overall ($P<0.0001$) (Fig. 6). The amount of iron deposition was significantly greater with the 111 nm tracer and 59 nm tracer ($P<0.05$) compared to the 32 nm tracer.

Figure 5 Iron distribution within nodes on histopathology using Perl's Prussian blue staining for iron and haematoxyline & eosine. Magnification 2x, with inserts at 20x magnification. (a) Node containing the 111 nm tracer; (b) node containing the 32 nm tracer; (c) Node containing the 59 nm tracer.

Figure 6 Number of excised sentinel lymph nodes (SLNs) per grade of iron distribution, for the three different magnetic tracers. Grade 0 = none, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, and Grade 4 = marked.

4.4 DISCUSSION

Rapid uptake of a magnetic tracer after interstitial injection is important to maximise transcutaneous hotspot detection and enable injection in the theatre suite, facilitating theatre scheduling. Transcutaneous identification of ‘hotspots’ was possible in most procedures with the 59 nm tracer by 10 minutes from injection, and in all except one procedure (with the 111 nm tracer), by 240 minutes. By 30 minutes from injection all ‘hotspots’ were already identifiable using the 59 nm tracer. The greater sensitivity of the magnetometer for the 59 nm tracer for a given quantity of iron compared to the other two tracers (Fig. 4), facilitates hotspot detection.

Discoloration of the skin was present at the injection site using all tracers, but this was not quantified during this study. Our animal model did not allow to evaluate whether the skin discoloration would fade away over time or persist as a tattoo. Although superficial administration of a SPIO tracer can result in skin tattooing, the current clinical studies using SPIO have also not reported adequate follow-up data to fully quantify this [12-14]. However, any tattooing is anticipated to be temporary rather than permanent but may persist for several months - similar to that expected for blue dye [23].

Any tracer used for SLNB must be retained by the SLNs and not pass on to higher echelon nodes in order to avoid excessive excision of normal nodes. Within this study, a statistically significantly greater number of nodes was excised using the 32 nm tracer compared to the

other two tracers. This is a distinct disadvantage of the smaller particle sized magnetic tracer. It suggests that rather than being retained within the SLN after entering through the afferent duct, it passes through the efferent system and onto higher echelon nodes. This is reflected by the small islands of sparsely-distributed iron within the nodes after administration of the 32 nm tracer on histopathology (Fig. 5 (b)). By applying the “10% rule” and excising those nodes with only 10% of the *ex vivo* count of the hottest excised node using radioisotope, Martin *et al.* [24] demonstrated that a mean SLN harvest of 1.96 nodes was associated with a false negative rate (FNR) of 5.8%. In the SentiMAG Multicentre Trial using the 59 nm tracer, 2.02 nodes were excised per procedure using the magnetic technique. Excising more than two SLNs during sentinel node biopsy is not proven to be beneficial except after primary systemic therapy [25]. Our data suggests that the small sized 32 nm tracer would result in removal of an excessive number of normal nodes.

In addition to retention, the accumulation of magnetic tracer within the SLNs is essential for intra-operative identification. The 59 nm tracer demonstrated statistically significant higher *ex vivo* magnetometer counts compared to the other two tracers, but only a statistically significant greater iron content over the 32 nm tracer. This is explained by the higher sensitivity of the used magnetometer for the 59 nm tracer compared to the other two tracers. The higher sensitivity for the 59 nm tracer is due to differences in magnetic properties of the tracers, arising from differences in size and material of the magnetic cores [26].

By injecting the magnetic tracer 24 hours before surgery one may be able to improve the iron uptake by the SLNs and thereby improve the performance of a tracer. However, extending the period between injection and surgery could allow adverse SPIO migration to higher echelon nodes beyond the SLN. Therefore this is likely only suitable for the larger 111 nm tracer - within this context. However, to optimize the time between injection and surgery for each tracer, formal assessment of individual tracers for SLNB when injected at different times before surgery is needed.

The variation in the accumulation of iron within nodes, between different tracers was demonstrated on histopathology. There was significantly less iron deposition in nodes from the 32 nm tracer compared to the other tracers, with only sparse, small islands of iron derived from the 32 nm tracer being observed in the nodes. There was no difference in the grading of nodes between the 59 nm tracer and the 111 nm tracer on histopathology. In both cases the iron was distributed in the subcapsular space and sinuses of nodes, consistent with previous histopathological studies [20, 27]. However, the 111 nm tracer was found to be deposited mainly within macrophages – demonstrating its ability to activate the mononuclear phagocytic system (MPS) – compared to the 59 nm tracer, which although demonstrating phagocytosis within the node also displayed extensive free iron granules

peripherally. These two processes of activating the MPS and free iron granule deposition facilitate iron accumulation and hence SLN identification using a handheld magnetometer.

Different studies have recently evaluated the influence of magnetic nanoparticle size on lymphatic uptake in rodent models [28-30]. Tracers with particle sizes ranging from 4-1000 nm were used in these studies. The purpose of these studies was to optimize tracer uptake in the SLNs to facilitate pre-operative localization with MRI, rather than intraoperative detection. Mori et al. [30] used 50, 100, 200 and 1000 nm sized particles and concluded that particles of 200 nm and larger are not suitable in view of lack of uptake within 24 hours. Iida et al. [28] compared the performance of 4, 8 and 20 nm citrate coated nanoparticles, and concluded that the 20 nm particles were best retained in the SLNs and accumulated in the highest concentration. However, the 20 nm tracer was the largest used, and therefore it is possible that uptake could be further optimized by increasing the particle size. Kjellman et al. [29] used monodisperse magnetic nanoparticles with a Polyethylene glycol (PEG) coating with sizes of 15, 27 and 57 nm. The 15 nm particles were observed to accumulate in the SLNs the fastest, and in the highest concentrations. A 15 nm PEG coated tracer approved for use in humans is currently not available, and was not used in our study. Therefore, the efficiency of the 15 nm tracer cannot be compared to our results.

During the course of the animal experimentation there were no signs of adverse events developing within the animals as a consequence of the magnetic tracer administration. When magnetic tracers are used as MRI contrast agents, doses of 25-100 mg are typically administered. Animal studies have demonstrated no acute or sub-acute toxicity when 150 times this dose has been administered [31]. Consequently, iron overload would not be possible within this model due to the small amounts of iron injected (5.6 mg).

In this study we evaluated the performance in SLNB of three magnetic tracers approved for human use. However, only the 59 nm tracer is currently CE-marked for the purpose of SLNB in Europe. The 111 nm and 32 nm tracers are licensed as an MRI contrast agent and an intravenous treatment of iron-deficiency anaemia respectively. Apart from dilution of the tracers to standardise iron concentrations, no changes were made to the constituents to facilitate the translation of the results to clinical practice. The formulation of the tracers is very similar; all are aqueous suspensions of magnetic nanoparticles in water, with a pH level ranging from 5-9. The most remarkable difference between the tracers is the particle size, however differences in coating and other constituents are also evident. The coating of the 111 nm tracer and 59 nm tracer are both dextran-based (dextran and carboxydextran respectively), however a polyglucose sorbitol carboxymethylether coating is used in the 32 nm tracer. Although the coating is known to be a factor influencing *in vivo* nanoparticle distribution, particle size was demonstrated to be the most significant factor for interstitially administered tracers [19, 29, 32-34]. The poor performance of the PEG-coated 32 nm tracer

compared to the other tracers is therefore likely explained by a combination of the particle size distribution and the coating. The observed differences between the dextran-coated tracers are most likely explained by the different particle size distributions of these tracers.

4.5 CONCLUSION

Currently, the 59 nm tracer is the best performing magnetic nanoparticle tracer, approved for human use, in sentinel lymph node biopsy with a handheld magnetometer. The particles of the 59 nm tracer distribute rapidly from the injection site to the SLNs, allowing transcutaneous localization within 30 minutes in contrast to the 240 minutes for the 32 nm tracer and 111 nm tracer. The particles of the 59 nm tracer are retained in the first nodes reached in contrast to the particles of the 32 nm tracer. Finally the particles accumulate in high concentrations, facilitating intraoperative localization. The rapid distribution, retention in the first nodes reached, and accumulation in high concentration make the 59 nm tracer the most suitable magnetic tracer for sentinel lymph node biopsy.

4.6 CONFLICTS OF INTEREST STATEMENT

Although one of the authors (QAP) fulfils a part-time paid advisory role as Chief Scientist (Physics) for Endomagnetics Ltd., his role in this work has been purely academic. The remaining authors have no disclosures to make and have no financial or personal relationships with other people or organizations that could inappropriately influence their work.

4.7 REFERENCES

1. S.I. Ferlay J, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11* [Internet]. 2013 01/10/2014]; Available from: <http://globocan.iarc.fr>.
2. A.E. Giuliano, D.M. Kirgan, J.M. Guenther, and D.L. Morton, *Lymphatic mapping and sentinel lymphadenectomy for breast cancer*. Annals of surgery, 1994. **220**(3): p. 391-8; discussion 398-401.
3. T. Kim, A.E. Giuliano, and G.H. Lyman, *Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis*. Cancer, 2006. **106**(1): p. 4-16.
4. D.N. Krag, D.L. Weaver, J.C. Alex, and J.T. Fairbank, *Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe*. Surgical oncology, 1993. **2**(6): p. 335-9; discussion 340.
5. G. Gill, S.T.G.o.t.R.A.C.o. Surgeons, and N.C.T. Centre, *Sentinel-lymph-node-based management or routine axillary clearance? One-year outcomes of sentinel node biopsy versus axillary clearance (SNAC): a randomized controlled surgical trial*. Annals of surgical oncology, 2009. **16**(2): p. 266-75.
6. D.N. Krag, S.J. Anderson, T.B. Julian, A.M. Brown, et al., *Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial*. The lancet oncology, 2007. **8**(10): p. 881-8.
7. R.E. Mansel, L. Fallowfield, M. Kissin, A. Goyal, et al., *Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial*. Journal of the National Cancer Institute, 2006. **98**(9): p. 599-609.
8. U. Veronesi, G. Paganelli, G. Viale, A. Luini, et al., *A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer*. The New England journal of medicine, 2003. **349**(6): p. 546-53.
9. G. Zavagno, G.L. De Salvo, G. Scalco, F. Bozza, et al., *A Randomized clinical trial on sentinel lymph node biopsy versus axillary lymph node dissection in breast cancer: results of the Sentinella/GIVOM trial*. Annals of surgery, 2008. **247**(2): p. 207-13.
10. J. Rescigno, J.C. Zampell, and D. Axelrod, *Patterns of axillary surgical care for breast cancer in the era of sentinel lymph node biopsy*. Annals of surgical oncology, 2009. **16**(3): p. 687-96.
11. M. Ahmed, A.D. Purushotham, and M. Douek, *Novel techniques for sentinel lymph node biopsy in breast cancer: a systematic review*. The lancet oncology, 2014. **15**(8): p. e351-62.
12. M. Douek, J. Klaase, I. Monypenny, A. Kothari, et al., *Sentinel node biopsy using a magnetic tracer versus standard technique: the SentiMAG Multicentre Trial*. Annals of surgical oncology, 2014. **21**(4): p. 1237-45.
13. M. Thill, A. Kurylcio, R. Welter, V. van Haasteren, et al., *The Central-European SentiMag study: sentinel lymph node biopsy with superparamagnetic iron oxide (SPIO) vs. radioisotope*. Breast, 2014. **23**(2): p. 175-9.

14. M. Shiozawa, A.T. Lefor, Y. Hozumi, K. Kurihara, et al., *Sentinel lymph node biopsy in patients with breast cancer using superparamagnetic iron oxide and a magnetometer*. Breast cancer, 2013. **20**(3): p. 223-9.
15. C. Fang and M. Zhang, *Multifunctional Magnetic Nanoparticles for Medical Imaging Applications*. Journal of materials chemistry, 2009. **19**: p. 6258-6266.
16. J. Wang, J.D. Byrne, M.E. Napier, and J.M. DeSimone, *More effective nanomedicines through particle design*. Small, 2011. **7**(14): p. 1919-31.
17. I.R. Jimenez, M. Roca, E. Vega, M.L. Garcia, et al., *Particle sizes of colloids to be used in sentinel lymph node radiolocalization*. Nuclear medicine communications, 2008. **29**(2): p. 166-72.
18. L.M. O'Brien, R. Duffin, and A.M. Millar, *Preparation of ^{99m}Tc-Nanocoll for use in sentinel node localization: Validation of a protocol for supplying in unit-dose syringes*. Nuclear medicine communications, 2006. **27**(12): p. 999-1003.
19. M.A. Swartz, *The physiology of the lymphatic system*. Advanced drug delivery reviews, 2001. **50**(1-2): p. 3-20.
20. B. Anninga, M. Ahmed, M. Van Hemelrijck, J. Pouw, et al., *Magnetic sentinel lymph node biopsy and localization properties of a magnetic tracer in an in vivo porcine model*. Breast cancer research and treatment, 2013. **141**(1): p. 33-42.
21. K.M. McMasters, T.M. Tuttle, D.J. Carlson, C.M. Brown, et al., *Sentinel lymph node biopsy for breast cancer: a suitable alternative to routine axillary dissection in multi-institutional practice when optimal technique is used*. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2000. **18**(13): p. 2560-6.
22. M. Visscher, J.J. Pouw, J. van Baarlen, J.M. Klaase, and B. Ten Haken, *Quantitative analysis of superparamagnetic contrast agent in sentinel lymph nodes using ex vivo vibrating sample magnetometry*. IEEE transactions on bio-medical engineering, 2013. **60**(9): p. 2594-602.
23. Y. Masannat, H. Shenoy, V. Speirs, A. Hanby, and K. Horgan, *Properties and characteristics of the dyes injected to assist axillary sentinel node localization in breast surgery*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2006. **32**(4): p. 381-4.
24. R.C. Martin, 2nd, M.J. Edwards, S.L. Wong, T.M. Tuttle, et al., *Practical guidelines for optimal gamma probe detection of sentinel lymph nodes in breast cancer: results of a multi-institutional study. For the University of Louisville Breast Cancer Study Group*. Surgery, 2000. **128**(2): p. 139-44.
25. T. Kuehn, I. Bauerfeind, T. Fehm, B. Fleige, et al., *Sentinel-lymph-node biopsy in patients with breast cancer before and after neoadjuvant chemotherapy (SENTINA): a prospective, multicentre cohort study*. The lancet oncology, 2013. **14**(7): p. 609-18.
26. M. Visscher, S. Waanders, H.J.G. Krooshoop, and B. ten Haken, *Selective detection of magnetic nanoparticles in biomedical applications using differential magnetometry*. Journal of Magnetism and Magnetic Materials, 2014. **365**: p. 31-39.
27. L. Johnson, S.E. Pinder, and M. Douek, *Deposition of superparamagnetic iron-oxide nanoparticles in axillary sentinel lymph nodes following subcutaneous injection*. Histopathology, 2013. **62**(3): p. 481-6.

28. S. Iida, K. Imai, S. Matsuda, O. Itano, et al., *In vivo identification of sentinel lymph nodes using MRI and size-controlled and monodispersed magnetite nanoparticles*. Journal of magnetic resonance imaging : JMRI, 2013. **38**(6): p. 1346-55.
29. P. Kjellman, R. in 't Zandt, S. Fredriksson, and S.E. Strand, *Optimizing retention of multimodal imaging nanostructures in sentinel lymph nodes by nanoscale size tailoring*. Nanomedicine : nanotechnology, biology, and medicine, 2014. **10**(5): p. 1089-95.
30. Y. Mori, M. Umeda, M. Fukunaga, K. Ogasawara, and Y. Yoshioka, *MR contrast in mouse lymph nodes with subcutaneous administration of iron oxide particles: size dependency*. Magnetic resonance in medical sciences : MRMS : an official journal of Japan Society of Magnetic Resonance in Medicine, 2011. **10**(4): p. 219-27.
31. M. Swartz, *The physiology of the lymphatic system*. Advanced Drug Delivery Reviews, 2001. **50**: p. 3-20.
32. A.E. Hawley, S.S. Davis, and L. Illum, *Targeting of Colloids to Lymph-Nodes - Influence of Lymphatic Physiology and Colloidal Characteristics*. Advanced drug delivery reviews, 1995. **17**(1): p. 129-148.
33. C. Oussoren, J. Zuidema, D.J.A. Crommelin, and G. Storm, *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection .2. Influence of liposomal size, lipid composition and lipid dose*. Bba-Biomembranes, 1997. **1328**(2): p. 261-272.
34. A. Maiseyeu and V. Bagalkot, *In vitro uptake of apoptotic body mimicking phosphatidylserine-quantum dot micelles by monocytic cell line*. Nanoscale Res Lett, 2014. **9**(1): p. 176.

5

A PHANTOM STUDY QUANTIFYING THE DEPTH PERFORMANCE OF A HANDHELD MAGNETOMETER FOR SENTINEL LYMPH NODE DETECTION^{*}

^{*}This chapter is submitted as: Pouw JJ, Bastiaan DMC, Klaase JM, ten Haken B. Depth performance of a handheld magnetometer for sentinel node biopsy. to *Journal of Surgical Research*, and currently under review.

ABSTRACT

Background: The use of a magnetic nanoparticle tracer and handheld magnetometer for sentinel lymph node biopsy (SLNB) was recently introduced to overcome drawbacks associated with the use of radioisotope tracers. Unlike the gamma probe, the used magnetometers are not only sensitive to the tracer, but also the diamagnetic human body. This potentially limits the performance of the magnetometer when used clinically.

Materials and methods: A phantom, mimicking the magnetic and mechanical properties of the human axilla, was constructed. The depth performance of two current generation magnetometers was evaluated in this phantom. LN-phantoms with tracer uptake ranging from 5-500 μg iron were placed at clinically relevant depths of 2.5, 4 and 5.5 cm. Distance-response curves were obtained to quantify the depth performance of the probes.

Results: The depth performance of both probes was limited. In the absence of diamagnetic material and forces on the probe (ideal conditions) a LN-phantom with high uptake (500 μg iron) could first be detected at 3.75 cm distance. In the phantom, only superficially placed LNs (2.5 cm) with high uptake (500 μg iron) could be detected from the surface. The penetration depth was insufficient to detect LNs with lower uptake, or which were located deeper.

Conclusion: The detection distance of the current generation magnetometers is limited, and does not meet the demands formulated by the European Association for Nuclear Medicine for successful transcutaneous SLN localization. Future clinical trials should evaluate whether the limited depth sensitivity is of influence to the clinical outcome of the SLNB procedure.

5.1 INTRODUCTION

Sentinel lymph node biopsy (SLNB) is the standard of care for staging the axilla of early-stage breast cancer patients. It achieves equal overall and disease free survival compared to axillary lymph node dissection, while it reduces morbidity [1-3]. The current standard for SLNB is the combined technique, which uses a radioisotope tracer (^{99m}Tc -nanocolloid) and blue dye. Both tracers are injected interstitially in the breast, and subsequently distributed to the sentinel lymph nodes (SLNs). In the operating theatre, the surgeon detects the tracers within the SLNs using a gamma probe and/or visually by blue colorization of the node(s). First, the SLNs are transcutaneously localized with the gamma probe to determine the optimal incision site. Post incision, the SLNs are identified and removed. The combined technique performs very well with an identification rate of 96% and a false-negative rate of approximately 5% to 10% [4].

This is explained by multiple factors; the high sensitivity of the probe to low amounts of tracer, penetration depth of several centimetres, and specificity to the tracer. Despite these advantages, the use of radioactivity is also associated with drawbacks. Firstly, the handling and disposal of radioactive material is subject to the stringent regulations. Secondly, the 6h half-life of the ^{99m}Tc tracer complicates theatre scheduling. Finally, the radiotracer is only produced in a limited number of reactors, hampering availability of the procedure worldwide. These drawbacks have stimulated the search for alternative radioisotope-free techniques for SLNB [5]. The use of a magnetic nanoparticle tracer and handheld magnetometer is one of these alternative techniques. The magnetic tracer consists of superparamagnetic iron oxide (SPIO) nanoparticles, which have a long shelf life. The use is not restricted by radiation related regulations, and therefore overcomes the drawbacks associated with the use of radioisotopes.

The use of a magnetic tracer and handheld magnetometer for SLNB was recently evaluated in several clinical trials [6-9]. The used magnetometer (SentiMAG[®], Endomag Ltd., UK) is a magnetic susceptometer, meaning that the device is not only sensitive to the magnetic tracer, but to all magnetic materials present near the probe; e.g. human tissue. The magnetic tracer produces a positive signal in vicinity of the probe whilst the diamagnetic tissue produces a negative signal. The resulting displayed signal is the sum of both the positive and negative components. Therefore the positive signal from the tracer can be 'obscured' by the negative signal from the tissue, potentially resulting in failed sentinel lymph node detection. Since the response of this magnetometer is highly distance dependent, this poses a fundamental limitation for the detection of deeply located sentinel nodes with low tracer uptake [10].

To compensate for the undesired diamagnetic signal, the probe is balanced on the skin, away from the injection site. The measured negative signal from the tissue in this position is added to the displayed signal for compensation. However, during SLN localization the probe is not used in a static position. By pressing the probe against the skin or placing it in the incision the amount and geometry of the tissue in vicinity of the probe changes, and therefore the magnetic tissue contribution is not constant during the SLNB procedure. This can result in a false positive signal when no tracer is present, or obscure the signal when tracer is present.

The goal of this study is to quantify this balancing effect, and to evaluate whether it limits axillary sentinel lymph node detection. We developed a phantom with magnetic and mechanical properties similar to human tissue, in which lymph node phantoms filled with magnetic tracer can be placed at clinically relevant depths. The performance of the magnetometer was evaluated with different quantities of magnetic tracer placed at different depths in this phantom.

5.2 MATERIALS AND METHODS

The performance of the magnetometer used in the previous clinical trials in breast cancer patients and the currently sold version were evaluated in this study. The SentiMAG[®] system consist of a handheld probe (previously used version with a diameter of 24 mm; Probe 1) (currently sold version with a diameter of 18 mm; Probe 2) (Fig. 1a), and a base unit which displays the measured signal.

5.2.1 Phantom

To evaluate the performance of the magnetometers a phantom resembling the magnetic and mechanical properties of the human axilla was constructed. A Perspex container (0.25x0.25x0.25 m) was filled with a 0.9% saline solution. The susceptibility of water (-9.05×10^{-6}) matches the magnetic susceptibility of tissue (-11×10^{-6} to -7×10^{-6}) [11], therefore saline was used as diamagnetic medium. To simulate mechanical tissue properties, a double layer of thin latex sheet (0.38 mm) was spanned across a Perspex cover with a circular aperture (diameter 60 mm) in the centre. When the probe is pressed in the phantom, forces are exerted on the probe as during clinical transcutaneous hotspot detection. Furthermore, the sheet deforms in a similar geometry as tissue, mimicking the tissue deformation during an SLNB procedure. A scale bar on the side of the container was used to determine the depth of the probe in the phantom. Five height adjustable nylon wires were used to place a lymph node phantom at the desired depth. The phantom with a lymph node, and the probe pressed in the phantom is displayed in Figure 1b.

Figure 1 **(a)** A picture of both magnetometer probes used in this study. Probe 1 (Bottom) with a diameter of 24 mm was used in the reported clinical trials [6-9], Probe 2 (Top) with an 18 mm diameter is the currently sold version. **(b)** The phantom filled with saline, in which a lymph node phantom is placed. The probe is pressed through the circular aperture, covered by latex sheets. The deformation around the probe, mimicking tissue deformation, is visible. The yellow scale bar is used to measure the depth of the probe in the phantom. The magnetometer base unit (left) displays a positive signal due to presence of magnetic tracer.

Hollow PVC beads with an internal volume of 35 μL (Pharmabotics Ltd., UK) developed for use in a training simulator for SLNB with radioisotopes were used as lymph node phantoms [12]. Lymph node phantoms containing different amounts of iron were made by filling the beads with a dilution of magnetic tracer (Sienna+[®], Endomag Ltd., UK, 27.9 mg iron/mL) and demineralised water.

5.2.2 Lymph node phantoms iron content and depth-placement

Before each measurement, a lymph node phantom was placed at a clinically relevant depth, determined based on the study of Mathelin et al. [13]. In their study, a ruler was used during surgery to measure the depth of all SLNs before excision in 11 breast cancer patients. The reported depth of the SLNs ranged from 1.5 to 8.5 cm. When only the depth of the most superficial SLN per patient is analysed, this results in a mean depth of 4.0 cm (SD 1.8, range 1.5-7.5 cm). In our study the LN phantoms were therefore placed at a depth of 2.5 cm (superficial SLNs), 4.0 cm (intermediate SLNs) or 5.5 cm (deep-seated SLNs) to evaluate the performance of the magnetometer in different clinical scenarios.

Besides the depth, the iron content of the LN phantoms was also varied to simulate SLNs with low, intermediate or high tracer uptake. Currently, there is no quantitative data available on the uptake of magnetic tracer by SLNs in humans. Waddington et al. [14] report a radiotracer uptake ranging from 0.0038 – 5.14% of the injected dose (corrected for decay). However, the vast majority (> 75%) of the SLNs in their study demonstrated an uptake <0.67% of the injected dose. When an equal uptake for magnetic tracers is assumed, this results in an iron quantity of 372 μg . Therefore, LN phantoms with high, intermediate and low uptake were filled with 500 μg , 50 μg and 5 μg iron respectively.

5.2.3 Phantom measurements

First, the performance of the probes was assessed in optimal conditions, e.g in the absence of diamagnetic medium and forces on the probe. LN phantoms were placed in the empty container without latex cover at a depth of 5.5 cm. The magnetometer was balanced in the air. The distance response curve of the system for the different quantities of iron was recorded for different Probe – Lymph Node distances with 0.25 cm steps.

Secondly, the phantom was filled with saline and covered with the latex sheets to mimic tissue. The probe was balanced while it was placed on the surface of the phantom to compensate for the diamagnetic signal. A distance response curve was obtained for the different LN phantoms, with the LN phantoms placed at 2.5, 4.0 and 5.5 cm (Fig. 2, top panels). Measurements were performed by pressing the probe in the phantom, with 0.25 cm steps, starting at the surface of the phantom until the LN phantom was reached (Probe – LN distance = 0 cm, Fig. 2, bottom panels). Each measurement was repeated five times.

The distance at which the magnetometers were able to detect the LN phantom was determined in air, and in the tissue mimicking phantom. The Probe – Lymph Node distance at which the magnetometer signal was > 0 was defined as the maximum detection depth. The forces on the probe were measured with a scale underneath the phantom, and used to calculate the elastic modulus of the phantom.

Figure 2 Schematic representation of the phantom measurements, with lymph node phantoms placed at 2.5, 4.0 and 5.5 cm depth. The top panels display the start of the measurement, with the probes placed at the surface. The middle panels illustrate the difference in tissue geometry for the same Probe – Lymph node distance, for the different lymph node depths. The bottom panels represent the situation for a Probe – Lymph node distance of 0 cm, for the different depths.

5.3 RESULTS

The distance response curve in air (Fig. 3a) confirms that the magnetometer signal is highly distance dependent. Furthermore, it shows that Probe 2 has a higher mass sensitivity (SentiMAG signal/ μg iron) than Probe 1. To be able to better compare the performance of both probes, the amplitude of the signal of Probe 2 was scaled to match Probe 1. The obtained signal with 500 μg iron at distances of 1.75 – 3.0 cm was used to determine the scaling factor. Scaled Figure 3b shows that both probes perform very similar in terms of detection depth. In air, both probes achieved a detection distance of 3.75 cm for the 500 μg LN, 2.25 cm (Probe 1) and 2.5 cm (Probe 2) for the 50 μg LN, and 1.25 cm with both probes for the 5 μg LN. The signal from Probe 2 was less stable compared to Probe 1, reflected by the higher standard deviations (Fig. 3b).

Figure 3 **(a)** Distance response curve of the two probes for 5, 50 and 500 μg of iron, under ideal conditions (e.g. in absence of diamagnetic medium and forces on the probe). **(b)** Magnified distance response curve in which the amplitude of the signal of Probe 2 is scaled to match Probe 1.

When the LN phantoms were placed in a diamagnetic environment, the depth performance of the probes decreased compared to the measurements in air. The insertion of the probe in the diamagnetic material results in a negative signal. The probe must be brought closer to the LN to overcome this negative signal, resulting in decreased depth performance. Probe 1 displayed better depth-sensitivity compared to Probe 2 in the tissue mimicking phantom. Only the superficially placed LN phantom (2.5 cm depth), with high tracer uptake (500 μg iron) could be detected from the surface (Fig. 4a) with both probes.

The detection distance of the 500 μg iron phantom placed at intermediate depth (4.0 cm) decreased to 3.4 and 3.0 cm for Probe 1 and 2 respectively, and decreased even further to 2.9 and 2.4 cm when a deep-seated LN was mimicked (5.5 cm depth). The difference in performance for the different LN depths arises because the probe must be pressed deeper in the phantom to achieve the same Probe – LN distance as with the more superficial placed LNs (Fig. 2, middle panels). This results in a different tissue geometry and forces on the probe, and thus a difference in (negative) signal.

The LN phantom with intermediate uptake (50 μg iron) was detectable rather close from the surface (2.2 and 1.9 cm) when it was placed 2.5 cm deep. When it was placed deeper (4.0 and 5.5 cm (Fig. 4b and c), the detection distance decreased below 1.6 cm. The LNs with low tracer uptake were difficult to detect in all situations, with detection distances <1 cm for all depths.

Measurements with Probe 1 resulted in more stable signals compared to Probe 2. With Probe 2, signals smaller than ~50 were very difficult to record as they fluctuated rapidly. The measurements were performed by bringing the probe to the desired depth, and immediately recording the result. The variability of the signals is reflected in the very large standard deviations of the obtained signal. The variability of the signal from Probe 2 is much larger in the tissue mimicking phantom, than in air.

The forces on the probe were measured with a scale underneath the phantom, and used to calculate a Stress-strain curve (Fig. 5). The elastic modulus was determined to be 212 kPa at 18% strain.

Figure 4 Distance response curve of the two probes for 5, 50 and 500 μg of iron, in the tissue mimicking phantom for different lymph node depths (a) 2.5 cm, (b) 4.0 cm and (c) 5.5 cm

Figure 5 Stress-strain curve of the tissue mimicking phantom. The dotted line is a fit to the data which serves as a guide to the eye.

5.4 DISCUSSION

SLNB with a magnetic nanoparticle tracer and handheld magnetometer was recently introduced as alternative to the combined technique for axillary staging in early stage breast cancer. Unlike the gamma probe, the used magnetometers are not only sensitive to the tracer, but also to the diamagnetic human body. The tissue contribution limits the sensitivity of the devices [10]. This study is the first to quantify the depth performance of the current generation magnetometers, using a tissue mimicking phantom.

The depth performance of both probes was limited. In ideal conditions the maximum Probe – Lymph Node distance at which the LNs could be detected, for both probes, was 3.75 cm for LNs with high iron uptake (500 μg iron). In the phantom, mimicking the transcutaneous hotspot detection, only superficially located LNs (2.5 cm depth) with high iron uptake could be detected from the surface. The penetration depth was insufficient to detect LNs with lower amounts of iron, or which were located deeper. Pressing the probe into the phantom to reduce the distance to the LNs did not resolve this problem, and reduced the detection distance compared to the detection distance in air. The detection distance of the 50 μg LN, placed at 4.0 or 5.5 cm was below 1.6 cm indicating that it is very difficult, if not impossible, to detect these LNs transcutaneously.

The EANM and SNMMI practice guideline for lymphoscintigraphy and sentinel node localization in breast cancer [15] requires that the sensitivity of the detector is sufficient to

identify a weakly active SLN when attenuated by up to 5 cm of soft tissue. Furthermore, SLNs can be located up to 8.5 cm deep [13]. Therefore the depth performance of the current generation magnetometers does not meet the requirements to successfully locate SLNs transcutaneously. A magnetic detection technique insensitive to the human body was developed [16], and could potentially overcome this problem. However, a handheld system which uses this technology is not yet commercially available. Alternatively, pre-operative SPIO-MRI is able to localize the SLNs after injection of the tracer [17], and can be used to determine the optimal incision site as replacement of the transcutaneous measurement.

Different assumptions were made in the construction of the phantom, measurement protocols and interpretation of the data. In the phantom, we used a homogeneous diamagnetic medium, allowing for accurate balancing of the system. In patients, the magnetic susceptibility of tissue varies [11], introducing inaccuracies. We used a signal threshold of zero as detection limit. Since the obtained signals were not stable in the tissue phantom, especially for Probe 2, this is likely to be a very optimistic value. The highly variable signal of Probe 2 in the tissue environment could potentially hinder interpretation of the signal in clinical use. Furthermore, in our experiments the location of the SLNs was known, allowing for precise alignment of the probe with the LNs. This combination of factors will consequently result in a lower detection distance in clinical practice. Manual positioning of the probe could introduce variations in the results, however this reflects the clinical use of the system. A latex sheet with an elastic modulus of 212 kPa was used in the phantom, this is well within the range of reported elastic moduli of 20 – 558 kPa of different components of breast tissue at 20% strain [18]. To our knowledge, there is no data available on the elastic modulus of the axilla of breast cancer patients, and therefore the forces on the probe in our phantom could deviate from the clinical situation.

Although we found that the depth performance of the current generation of magnetometers is in theory insufficient for adequate transcutaneous hot spot detection, Pinero-Madrone et al. [9] report a 95.5% magnetometer hot spot detection rate in their trial which compared the use of a magnetometer to gamma probe. A small study in 22 patients reports a hot spot detection rate of 64% [19]. Other comparative trials do not report quantitative data [6-8], however Douek et al. mention that axillary hot spot detection is technically difficult. The high transcutaneous hot spot detection rate reported by Pinero-Madrone with the magnetometer, despite the limited depth performance, could have different explanations. In our phantom, balancing was performed under ideal, reproducible circumstances. One of possible scenarios is that the probe is pressed slightly into the tissue during balancing, and is not pressed during the actual measurement, resulting in a false positive signal. However, this is speculation, and more clinical data on the transcutaneous

detection rate is needed to evaluate the in vivo transcutaneous performance. Despite the limited detection depth of the current generation magnetometers in our phantom, the different comparative phase 2 clinical trials report excellent, non-inferior intraoperative detection rates for the magnetic technique compared to the combined technique. This poses the questions whether, in contrast to the EANM guideline a detection depth <5 cm is sufficient, and whether pre-operative/transcutaneous localization is necessary to achieve successful intraoperative SLN identification. Future randomized controlled clinical trials are needed to answer these questions.

In conclusion, we demonstrated that the detection distance of the current generation magnetometers does not meet the demands formulated by the EANM for successful transcutaneous hotspot detection. Use in a diamagnetic tissue environment is of influence to the signal, and decreases the detection distance of the system. Surgeons using the system should be aware of the influence of tissue, and the factors potentially leading to false positive or false negative signals. As an alternative to the transcutaneous hot spot detection, SPIO-MRI can be used for the pre-operative localization of the SLNs. Data from randomized controlled clinical trials is needed to evaluate the clinical consequences of the depth limitations of the current generation magnetometers in clinical practice.

5.5 REFERENCES

1. D.N. Krag, S.J. Anderson, T.B. Julian, A.M. Brown, et al., *Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial*. The lancet oncology, 2007. **8**(10): p. 881-8.
2. R.E. Mansel, L. Fallowfield, M. Kissin, A. Goyal, et al., *Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial*. Journal of the National Cancer Institute, 2006. **98**(9): p. 599-609.
3. U. Veronesi, G. Paganelli, G. Viale, A. Luini, et al., *A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer*. The New England journal of medicine, 2003. **349**(6): p. 546-53.
4. T. Kim, A.E. Giuliano, and G.H. Lyman, *Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis*. Cancer, 2006. **106**(1): p. 4-16.
5. M. Ahmed, A.D. Purushotham, and M. Douek, *Novel techniques for sentinel lymph node biopsy in breast cancer: a systematic review*. The lancet oncology, 2014. **15**(8): p. e351-62.
6. M. Douek, J. Klaase, I. Monypenny, A. Kothari, et al., *Sentinel node biopsy using a magnetic tracer versus standard technique: the SentiMAG Multicentre Trial*. Annals of surgical oncology, 2014. **21**(4): p. 1237-45.
7. I.T. Rubio, S. Diaz-Botero, A. Esgueva, R. Rodriguez, et al., *The superparamagnetic iron oxide is equivalent to the Tc99 radiotracer method for identifying the sentinel lymph node in breast cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2014.
8. M. Thill, A. Kurylcio, R. Welter, V. van Haasteren, et al., *The Central-European SentiMag study: sentinel lymph node biopsy with superparamagnetic iron oxide (SPIO) vs. radioisotope*. Breast, 2014. **23**(2): p. 175-9.
9. A. Pinero-Madrone, J.A. Torro-Richart, J.M. de Leon-Carrillo, G. de Castro-Parga, et al., *Superparamagnetic iron oxide as a tracer for sentinel node biopsy in breast cancer: A comparative non-inferiority study*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2015. **41**(8): p. 991-7.
10. M. Visscher, S. Waanders, J. Pouw, and B. ten Haken, *Depth limitations for in vivo magnetic nanoparticle detection with a compact handheld device*. Journal of Magnetism and Magnetic Materials, 2015. **380**: p. 246-250.
11. J.F. Schenck, *The role of magnetic susceptibility in magnetic resonance imaging: MRI magnetic compatibility of the first and second kinds*. Med Phys, 1996. **23**(6): p. 815-50.
12. M.R. Keshtgar, D.W. Chicken, W.A. Waddington, W. Raven, and P.J. Ell, *A training simulator for sentinel node biopsy in breast cancer: a new standard*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2005. **31**(2): p. 134-40.

13. C. Mathelin, S. Salvador, D. Huss, and J.L. Guyonnet, *Precise localization of sentinel lymph nodes and estimation of their depth using a prototype intraoperative mini gamma-camera in patients with breast cancer*. Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 2007. **48**(4): p. 623-9.
14. W.A. Waddington, M.R. Keshtgar, I. Taylor, S.R. Lakhani, et al., *Radiation safety of the sentinel lymph node technique in breast cancer*. Eur J Nucl Med, 2000. **27**(4): p. 377-91.
15. F. Giammarile, N. Alazraki, J.N. Aarsvold, R.A. Audisio, et al., *The EANM and SNMMI practice guideline for lymphoscintigraphy and sentinel node localization in breast cancer*. Eur J Nucl Med Mol Imaging, 2013. **40**(12): p. 1932-47.
16. M. Visscher, S. Waanders, H.J.G. Krooshoo, and B. ten Haken, *Selective detection of magnetic nanoparticles in biomedical applications using differential magnetometry*. Journal of Magnetism and Magnetic Materials, 2014. **365**: p. 31-39.
17. J.J. Pouw, M.R. Grootendorst, R. Bezooijen, C.A. Klazen, et al., *Pre-operative sentinel lymph node localization in breast cancer with superparamagnetic iron oxide MRI: the SentiMAG Multicentre Trial imaging subprotocol*. The British journal of radiology, 2015: p. 20150634.
18. T.A. Krouskop, T.M. Wheeler, F. Kallel, B.S. Garra, and T. Hall, *Elastic moduli of breast and prostate tissues under compression*. Ultrason Imaging, 1998. **20**(4): p. 260-74.
19. H. Tafazal, M. Dakka, R. Attwal, D. Clarke, et al., *P103. The use of SentiMag in identifying the sentinel lymph node: Warwick experience*. European Journal of Surgical Oncology (EJSO), 2015. **41**(6): p. S55-S56.

6

PRE-OPERATIVE SENTINEL LYMPH NODE LOCALIZATION IN BREAST CANCER WITH SUPERPARAMAGNETIC IRON OXIDE-MRI: THE SENTIMAG MULTICENTRE TRIAL IMAGING SUB PROTOCOL*

*This Chapter is published as: Pouw JJ, Grootendorst MR, Bezooijen R, Klazen CAH, de Bruin WI, Klaase JM, Hall-Craggs MA, Douek M, ten Haken B. *Pre-operative sentinel lymph node localization in breast cancer with superparamagnetic iron oxide-MRI: The SentiMAG Multicentre Trial imaging sub protocol*. The British journal of radiology, 2015. **88**(1056): p. 20150634

ABSTRACT

Objective: Sentinel Lymph Node Biopsy (SLNB) with a superparamagnetic iron oxide (SPIO) tracer was shown to be non-inferior to the standard combined technique in the SentiMAG Multicentre Trial. The MRI sub protocol of this trial aimed to develop a magnetic alternative for pre-operative lymphoscintigraphy (LS). We evaluated the feasibility of using Magnetic Resonance Imaging (MRI) following administration of magnetic tracer for pre-operative localization of sentinel lymph nodes (SLNs) and its potential for non-invasive identification of lymph node (LN) metastases.

Methods: Patients with breast cancer scheduled to undergo SLNB were recruited for pre-operative LS, SPECT-CT and SPIO-MRI. T1-weighted-TSE and T2-weighted-GRE sequences were used before and after interstitial injection of magnetic tracer into the breast. SLNs on MRI were defined as LNs with signal drop and direct lymphatic drainage from the injection site. LNs showing inhomogeneous SPIO-uptake were classified as metastatic. During surgery a handheld magnetometer was used for SLNB. Blue or radioactive nodes were also excised. The number of SLNs and MR assessment of metastatic-involvement were compared to surgical and histological outcomes.

Results: 11 patients were recruited. SPIO-MRI successfully identified SLNs in 10/11 patients vs. 11/11 patients with LS/SPECT-CT. One patient had metastatic involvement of four LNs and this was identified in one node on pre-operative MRI.

Conclusion: SPIO-MRI is a feasible technique for pre-operative localization of SLNs, and in combination with intraoperative use of a handheld magnetometer provides an entirely radioisotope-free technique for SLNB. Further research is needed for evaluation of MRI characterisation of LN-involvement using subcutaneous injection of magnetic tracer.

6.1 INTRODUCTION

Sentinel lymph node biopsy (SLNB) is standard care for surgical staging of the axilla in clinically and radiologically node-negative patients with breast cancer [1-3]. The gold standard for SLNB is the combined technique, using a radioisotope and blue dye to identify the sentinel lymph nodes (SLNs) intraoperatively using a gamma probe and/or by visual identification of blue dye. Planar lymphoscintigraphy (LS), or optionally SPECT-CT, provides the surgeon with pre-operative information on the location and number of SLNs. Although the value of pre-operative LS has been questioned in breast cancer [4], it is required for SLNB in melanoma in order to decide which lymphatic basin(s) require surgical exploration. Furthermore, LS is still the standard of care in breast cancer in many countries, and the European Association of Nuclear Medicine recommends the procedure because of the potential added value over the use of the gamma probe alone [5]. However, the use of radioisotopes has important drawbacks: it exposes patients and medical staff to radiation and is governed by stringent legislation. Furthermore, LS provides low spatial resolution, unlike MRI.

Three separate trials have evaluated a radioisotope-free surgical technique for SLNB, using an interstitially administered superparamagnetic iron oxide (SPIO) tracer in combination with a handheld magnetometer, and demonstrated an identification rate non-inferior to the combined technique [6-8]. To be able to perform an entirely radioisotope-free SLNB procedure, an alternative for LS is needed. SPIO contrast agents are known to provide 'negative contrast' on T2*-weighted MRI since the uptake of the tracer results in a drop of signal intensity. Intravenously injected SPIOs were first used as MRI contrast agent for the identification of metastases in the liver and spleen [9, 10]. Several animal and patient studies have demonstrated that if SPIOs are injected interstitially, they are taken up by SLNs, and therefore SPIO-MRI can be used as an alternative to SPECT-CT for pre-operative localization of SLNs. However, all these studies still rely on the use of radioisotopes for intraoperative detection of the sentinel nodes [11-15]. To date, only one study has involved both pre-operative SPIO-MRI and intraoperative SLN detection with a handheld magnetometer in 9 patients [16]. However, this study did not compare the MRI technique with the gold standard of the combined technique.

The application of magnetic nanoparticles is not necessarily limited to localization of SLNs as it has the potential to identify metastatic involvement of lymph nodes (LNs). Intravenously administered USPIOs are taken up in the healthy LN tissue and not in metastatic areas, and consequently areas affected by tumour have relatively high signal intensity on T2-weighted images compared to normal tissue [17-19]. If localization of SLNs could be coupled with accurate detection of metastasis, it could potentially provide an alternative to current routine surgical axillary staging, i.e. SLNB [20]. Although SLNB is a

minimally-invasive procedure, it is associated with morbidity, e.g. lymphedema, loss of arm mobility, numbness and pain [21, 22]. Ultrasmall superparamagnetic iron oxide (USPIO)-MRI has been successfully used as a non-invasive method for evaluating metastatic LN involvement in various types of cancer including breast cancer [17-19]. A non-invasive method that accurately diagnoses SLN metastases would prevent patients undergoing unnecessary SLNB, thereby decreasing physical and psychological morbidity, and treatment costs [21-23].

In this imaging sub protocol of the SentiMAG Multicentre trial (NTR3238, <http://www.trialregister.nl>) both SPIO-MRI and intraoperative SLN detection using subcutaneous injection of magnetic tracer and a handheld magnetometer were performed, and quantitatively compared to the current gold standard of LS/SPECT-CT and SLNB with the combined technique. This is believed to be the first time such a comparison has been made, and that the magnetic tracer has been used for imaging. The purpose of this study is to demonstrate the feasibility of SPIO-MRI for pre-operative localization of SLNs in patients with breast cancer as an alternative to LS and/or SPECT-CT, and to evaluate its potential to identify LN metastasis.

6.2 MATERIALS AND METHODS

6.2.1 Patients

Between July 2012 and March 2013, patients with histologically confirmed breast cancer who were clinically and radiologically node-negative and scheduled to undergo SLNB were eligible for inclusion. Patients were recruited after Ethics Committee approval had been obtained (ref NL39018.044.11) and after informed consent. Exclusion criteria were known intolerance to iron or dextran compounds, iron overload disorder and the standard MRI exclusion criteria. Furthermore, patients scheduled for a one-day protocol were excluded for logistical reasons.

6.2.2 Planar lymphoscintigraphy and SPECT-CT

The day before surgery, patients received two peritumoural, 0.5 mL injections of ^{99m}Tc -albumin-colloid (Nanocoll®; GE Healthcare, Eindhoven, The Netherlands) with a total activity of approximately 140 MBq, as per standard practice. LS was performed 2 hours after injection using an E-cam dual-head gamma camera (Siemens, Erlangen, Germany). Anterior and anterior-oblique images were acquired. In addition to LS, which is the standard of care, SPECT-CT (Symbia T6, Siemens, Erlangen, Germany) was performed. Imaging was performed in the same position as MRI: patients were placed in the prone position on an MR breast-imaging mattress, with the arms adducted and parallel to the body. This positioning facilitates comparison of SPECT-CT images and MR images post-acquisition.

6.2.3 SPIO-MRI

The day before surgery, MRI was performed with a 1.5T system (Intera, Philips, Best, The Netherlands). Patient positioning was similar to SPECT-CT (see above). The SENSE Breast-7 coil was used for 8 patients and the SENSE Body coil for 3 patients, the latter being used for larger patients.

The magnetic tracer Sienna+® (Endomag Ltd, Cambridge, UK), is a CE-marked injectable medical device, consisting of a sterile aqueous suspension of SPIO-particles in injectable water, containing circa 27 mg iron/mL. The particles have a carboxydextran coating and a mean hydrodynamic diameter of 59 nm (Z-averaged diameter) [24].

Before tracer injection, transverse images were obtained using T1-weighted-TSE sequence (TR/TE 734/16 ms; FA 90°; slice thickness 3.0 mm; FOV 30 cm; matrix 320x320; imaging time approx. 4 min). In addition, a T1-weighted-TSE sequence in the coronal plane (TR/TE 727/16 ms; FA 90°; slice thickness 3.0 mm; imaging time 3 min) was obtained to distinguish LNs located closely to each other.

While the patient was still positioned in the scanner, a periareolar subcutaneous injection of 2 ml magnetic tracer (diluted with 3 ml normal saline) was administered followed by 5 min massage. In 9/11 patients, the magnetic tracer was administered after injection of the radioisotope, and in 2/11 patients the magnetic tracer was injected first. In the first 2 patients the injection site was not massaged, in the remaining 9 patients, the injection site was massaged for 3-5 minutes, to promote lymphatic drainage. Post-contrast imaging of the breast and axillary region was started approximately 5 minutes after injection.

Pre- and post-contrast T2*-weighted-GRE images (TR/TE 500/4.6 ms; FA 18°; FOV 30 cm; matrix 400x400; slice thickness 3.0 mm; imaging time 5 min) were acquired in the transverse plane to localize the LNs with SPIO-uptake and assess the SPIO-distribution within the LNs. This sequence was repeated post-injection until SPIO-uptake in LNs was observed.

The total imaging time (including time required for tracer injection) of approximately 30 minutes for the SPIO-MRI was comparable to the imaging time (approximately 25 minutes without time for injection) for SPECT-CT. LS was less time consuming with an imaging time of approximately 10-15 minutes.

6.2.4 Image analysis

LS and SPECT-CT images were evaluated on a Syngo Multimodality Workplace (Siemens, Erlangen, Germany) by a nuclear medicine physician (WdB). LNs demonstrating uptake of the radioactive tracer and presenting with an afferent lymphatic vessel leading from the injection site to the LN are defined to be SLNs. If no afferent vessel can be identified, the

first LN appearing from the injection site is defined to be SLN. The number of SLNs and their location was registered both for LS and SPECT-CT.

The MRI data was analysed independently by two radiologists experienced in breast imaging (RB, CK) using a DynaCAD workstation (Philips, Best, The Netherlands). Any disagreements were resolved by consensus. All axillary LNs were identified on the T1-TSE-weighted images, and their number and anatomical level (in relation to pectoralis minor muscle) noted. The pre- and post-contrast T2*-GRE-weighted images were compared to identify LNs with decreased signal intensity due to SPIO-uptake. The number and anatomical level of LNs demonstrating SPIO-uptake were recorded. LNs showing SPIO-uptake from direct lymphatic drainage of the injection site were considered to be SLNs on SPIO-MRI, according to the definition of radioactive SLNs. The number of SLNs and their locations were registered.

6.2.5 MR criteria for detection of metastases

SLNs showing heterogeneous SPIO-uptake were classified as metastatic; nodes with homogeneous uptake were classified as non-metastatic. The classification was compared to the histopathology results of the resected SLNs.

6.2.6 Surgery

SLNB was performed as previously described [6]. Patent Blue V (Guerbet, France) was administered intraoperatively after induction of anaesthesia. The blue dye was administered with a periareolar injection. The surgeon initially used a handheld magnetometer (SentiMAG®, Endomagnetics, Cambridge, UK) for SLNB. A gamma probe (Europrobe 3, Euromedical Instruments, Le Chesnay, France) was used for subsequent confirmation of the magnetometer results. The magnetometer consists of a base unit and a handheld probe with a diameter of 24 mm. The device generates an alternating magnetic field, which magnetizes the SPIO particles in vicinity of the probe. The resulting change in magnetization can be used as a measure for the amount of tracer present. The obtained signal is shown on the numerical display and produces an audible signal. Any iron-containing and/or radioactive and/or blue SLNs were removed. A correlation was made between the number of SLNs identified on LS/SPECT-CT and SPIO-MR, and the number of radioactive SLNs and magnetic SLNs removed during surgery. Routine histological assessment was performed on all resected SLNs to determine the presence of metastasis.

6.3 RESULTS

The study recruited 11 patients; eight with invasive ductal carcinoma and three with ductal carcinoma in situ. The mean age of the patients was 58.5 years (range 45-71 years). In 10/11 patients (91%), SLNs were successfully identified by SPIO-MRI. The injection site, lymphatic tracts and SLNs could be identified on post-contrast T2*-weighted images (Fig. 1, Fig. 2).

Figure 1 The different MR sequences and SPECT-CT of a single patient. (a) A T1-weighted image showing the anatomy and morphology, one axillary LN is visible (arrow). (b) The same axillary LN, visible (arrow), on a pre-contrast T2-weighted scan. (c) A large decrease in signal is observed at the periareolar injection site. The axillary LN also shows a signal decrease due to uptake of SPIO and is therefore considered SLN (large arrow). A SPIO-filled lymphatic vessel draining from the injection site to the SLN is indicated with the small arrow. (d) The radioactive SLN (large arrow) identified by SPECT-CT corresponds to the SLN identified by MRI.

An artefact at the injection site was seen in all patients; however, this did not affect axillary imaging in any patient. The same LNs could be identified both before and after massage of the injection site in all patients. A total of 21 SLNs (mean 1.9, range 0-4) were identified on MRI. LS/SPECT-CT identified a total of 13 SLNs (mean 1.2, range 1-2). In all patients, the number of SLNs on LS and SPECT-CT was concordant.

SLNs were successfully identified during surgery using the handheld magnetometer and the combined technique in 11/11 patients. A total of 22 SLNs were excised in 11 patients (mean 2.0, range 1-4). Of these, 11 nodes were magnetic, radioactive and blue, 8 were magnetic and radioactive, and 3 were solely magnetic. A correlation between the number of SLNs identified by pre-operative imaging and the number of SLNs removed during surgery is shown in Table 1.

The pre-operative MRI showed the same number of magnetic SLNs as found during surgery in 6/11 patients (55%). Of the remaining five patients, 3 patients showed one additional SLN on MRI that was not identified during surgery and one patient showed one additional SLN removed during surgery that was not identified on MRI. In one patient (patient 2, Table 1), no SLNs were identified on MRI; however, 3 magnetic SLNs were removed during surgery.

LS/SPECT-CT accurately predicted the number of radioactive SLNs identified during surgery in 6/11 patients (55%). Four patients had one additional SLN removed during surgery that was not identified on nuclear imaging. In one patient (patient 2, Table 1), two additional radioactive SLNs were removed during surgery.

Histopathological analysis of the resected SLNs revealed nodal involvement in 2 patients (patient 1 & 2, Table 1). Patient 2 (Table 1) was excluded in the analysis of nodal metastases because no SPIO-uptake in the LNs was observed during MRI examination. All 4 magnetic SLNs of patient 1 harboured macrometastasis; 1 of these 4 SLNs showed heterogeneous SPIO-uptake (Fig. 3) and was correctly classified as metastatic. Two histopathologically node-negative patients were falsely classified as metastatic.

Figure 2 Post contrast T2-GRE-weighted images of three patients. **(a)** One SLN with magnetic tracer uptake (arrow). **(b)** Two SLNs with magnetic tracer uptake (large arrow) with a lymphatic vessel (small arrow) draining from the periareolar injection site. **(c)** Two closely located SLNs (arrow) result in one large area with decreased signal.

Figure 3 A SLN showing inhomogeneous SPIO-uptake. The arrow indicates the SLN on **(a)** T1-TSE-weighted image, **(b)** pre contrast T2-GRE-weighted image and **(c)** post contrast T2-GRE weighted image. The position of the LN is different in the post contrast scan due to movement of the patient during the procedure.

Table 1: Correlation between the number of SLNs identified by pre-operative MRI and LS/SPECT-CT, and the number of magnetic and radioactive SLNs removed during surgery.

Patient	SLNs on SPIO-MRI (n)	SLNs on LS/SPECT-CT (n)	Magnetic SLNs resected (n)	Radioactive SLNs resected (n)
1	4	1	4	1
2	0	1	3	3
3	1	1	1	1
4	3	1	2	2
5	2	1	2	2
6	3	1	2	2
7	2 ^a	1 ^b	1	1 ^b
8	1	1	2	2
9	2	2	2	2
10	1 ^a	1	1	1
11	2	2	2	2

^aIn these patients one additional LN with iron uptake was identified, but it was not clear whether these LNs received lymphatic drainage directly from the injection site or from one of the SLNs. These LNs were therefore not classified as SLNs

^bOne additional parasternal SLN was identified by LS/SPECT-CT. The intercostal space was not explored during surgery and the parasternal node was therefore not removed, and not shown in this table.

6.4 DISCUSSION

This study demonstrates the feasibility of SPIO-MRI for pre-operative localization of SLNs. SPIO-MRI successfully identified SLNs in 10/11 patients compared to 11/11 patients with the standard combined technique. The concordance between the number of nodes identified by imaging and during surgery was 55% for both the magnetic and the combined technique.

Poor spatial resolution of LS is a recognised drawback of SLNB with radioisotopes. Several animal and patient studies with SPIO-MRI have been performed to improve pre-operative localization of SLNs to provide a surgical roadmap [11-15]. However, these studies still rely on the use of radioisotopes for intraoperative detection of the sentinel nodes. Shiozawa et al. were the first to present an entirely radioisotope-free procedure, using SPIO-MRI for pre-operative localization and a handheld magnetometer for intraoperative detection of SLNs [16]. They successfully identified SLNs with MRI in 9/9 patients, however MRI only accurately predicted the number of SLNs in 3/9 (33%) patients. We achieved a concordance between the number of nodes identified by imaging and during surgery of 55%, equal to the combined technique. Shiozawa et al. used a different, possibly less sensitive, magnetometer which could explain this difference.

In the first two patients in our study, highly time-variable distribution of SPIO was observed. In the first patient, SPIO-uptake was seen immediately after injection. However, in the second patient, no SPIO-uptake was observed in LNs on MRI 35 minutes after injection. Nevertheless, a low-signal lymphatic tract extending towards the axilla was seen 22 minutes after injection of SPIO. Three magnetic SLNs were identified during surgery the next day, so it is apparent that lymphatic drainage was slow, and it is most likely that SLNs would have been detected by MRI if scanning had continued for a longer period. Other studies have shown that many factors (e.g. BMI, age, tumour size) affect the identification rate of SLNs, and it is hypothesized that these factors also alter lymphatic drainage [25-27]. To improve drainage of the tracer, i.e. to prevent unsuccessful SLN uptake of SPIO, a massage of the injection site was performed in the remaining 9 patients. This is in line with the practice guideline of the EANM/SNMMI [5]. Immediate tracer uptake in the SLNs was observed in the 9 remaining patients, indicating that massaging improves lymphatic drainage and should therefore be performed for this application.

Concordance rates between the number of nodes identified by imaging and during surgery of the combined technique reported in the literature range from 39-73% [28-31], which is in line with our findings. In all the (5/5) discordant procedures LS/SPECT-CT underestimated the number of SLNs while SPIO-MRI overestimated the number of SLNs in 3/5 discordant procedures. A possible explanation for this observation is the limited resolution of LS;

closely located SLNs may appear as one hotspot. The high resolution of MRI allows to distinguish these closely located SLNs. The fact that MRI is more sensitive to very small concentrations of SPIO than the handheld magnetometer may result in a higher number of SLNs on imaging compared to surgery. The differences in the number of SLNs identified by LS/SPECT-CT and MRI could also be explained by difference in lymphatic drainage from the peritumoural and periareolar injection sites. There remains considerable debate about the different drainage patterns. Large differences between the drainage patterns from the different sites were recently reported in a small study [32], while Caruso et al. [33] concluded that the same SLNs were reached from different injection sites. In our study, the SLNs identified during surgery were both magnetic and hot in all but one patient, indicating similar drainage patterns, and thus supporting the conclusion drawn by Caruso et al.

The clinical significance of a discordance between the number of SLNs found on imaging and during surgery remains unclear. In a recent retrospective study, no higher axillary recurrence rate was found when fewer SLNs were identified during surgery than were seen on LS [31].

The magnetic tracer generated an artefact at the injection site in the breasts of all patients. This was not noted by Shiozawa et al. [16] and Motomura et al. [34], using a similar magnetic tracer (Resovist®). Motomura et al. administered approximately 150x less magnetic tracer compared to our study, which could explain this finding. However, Shiozawa et al. used a similar dosage to that in our study. Although the artefact at the injection site did not affect axillary imaging, future work should evaluate whether this artefact persists, and focus on limiting the artefact by optimising dosage, injection site and scan parameters. The technique should not be used in patients who will subsequently require breast MRI surveillance.

The secondary goal of this study was to evaluate the potential of SPIO-MRI to identify LN metastasis pre-operatively. An accurate method could alter the current treatment pathway of patients with early breast cancer by sparing patients without nodal involvement an unnecessary SLNB procedure, while patients with nodal involvement can directly undergo axillary lymph node dissection without having an SLNB procedure. In this study only 1/10 patients presented with (macro)metastases. One of the 4 involved SLNs of this patient was accurately classified as metastatic on MRI but there were false negative results in the other 3 nodes. Moreover, two patients were falsely classified as node-positive. Since the primary goal of this study was the localization of SLNs rather than their characterization, choices in the protocol were made which differed from previously used methods for the characterization of SLNs. The trials involving intravenous injected USPIOs [17-19] all performed post-contrast scans after a minimum waiting time of 24h to ensure that the magnetic nanoparticles could transport to and distribute through the LNs. Motomura et al.

used a similar interval of 18-24 hours before post-contrast imaging was performed in their study with interstitial administered SPIO [34]. To be able to perform our procedure in the current clinical schedule for SLNB, we performed post-contrast scanning immediately after injection. As described earlier, this was suitable for localization of SLNs but may not be ideal for LN characterization. Johnson et al. observed a significant correlation between the time of SPIO-injection and LN resection, and the SPIO-distribution in the LN [35]. A longer time interval between injection and resection resulted in distribution throughout the whole LN. A possible explanation for the false positive results in this study is that distribution through the whole LN was not yet complete at the time of scanning.

Since the handheld magnetometer is not as sensitive for low SPIO-concentrations as MRI, a high dose of SPIO was used to facilitate intraoperative detection with the handheld magnetometer. High SPIO-concentrations do not only shorten T2-relaxation in their vicinity, but also influence relaxation in the surrounding voxels. As a result of this 'blooming effect', LNs with inhomogeneous SPIO-uptake can appear to show complete homogeneous uptake, resulting in false negative findings. Shiozawa et al. used a similar dosage and waiting time (45 mg iron, 20 minutes) as we did and obtained 100% sensitivity, specificity and accuracy [16]. They included 9 patients of whom only one had metastasis in two SLNs. Further evaluation is essential to find the optimal dosage and timing for characterization of involved LNs.

The use of a SPIO-MRI for SLNB has several advantages. It provides the surgeon with detailed anatomical information on the location of the SLNs, which serves as a detailed surgical roadmap. When combined with the intraoperative use of a handheld magnetometer, the need for radioisotopes can be completely eliminated. The magnetometer does not achieve equal transcutaneous penetration depth as the gamma probe, however the pre-operative SPIO-MRI can be used to determine the optimal incision site. Accurate localization may also facilitate the introduction of targeted removal of SLNs under local anaesthetic, further reducing the morbidity associated with a surgical intervention. In addition, the technique has the potential to non-invasively characterize the involvement of the sentinel nodes, although further optimization is needed. In the future the ability to non-invasively characterize the SLNs could eliminate the need for axillary surgery in part of the patients.

Since this study was a feasibility study, the number of included patients is small and insufficient to perform meaningful statistical analysis. A limited number of patients was recruited, because patients could participate in the surgical trial without participating in this imaging sub protocol. Furthermore a lack of access to MRI scanning slots limited the number of recruited patients. Larger prospective studies to validate and optimize both pre-operative SPIO-MRI and the surgical magnetic SLNB are needed. Secondly, the number of patients

with nodal metastases is too small to draw valid conclusions about the efficacy of SPIO-MRI to characterise SLN involvement.

6.5 CONCLUSIONS

This study demonstrated that SPIO-MRI for pre-operative localization of SLNs is a feasible alternative to LS/SPECT-CT, and in combination with the intraoperative use of a handheld magnetometer provides an entirely radioisotope-free technique for SLNB in breast cancer patients. Further research is needed for evaluation of MRI characterisation of LN-involvement using subcutaneous injection of magnetic tracer.

6.6 REFERENCES

1. U. Veronesi, G. Paganelli, G. Viale, A. Luini, et al., *A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer*. The New England journal of medicine, 2003. **349**(6): p. 546-53.
2. R.E. Mansel, L. Fallowfield, M. Kissin, A. Goyal, et al., *Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial*. Journal of the National Cancer Institute, 2006. **98**(9): p. 599-609.
3. D.N. Krag, S.J. Anderson, T.B. Julian, A.M. Brown, et al., *Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial*. The lancet oncology, 2007. **8**(10): p. 881-8.
4. A. Goyal and R.E. Mansel, *Does imaging in sentinel node scintigraphic localization add value to the procedure in patients with breast cancer?* Nuclear medicine communications, 2005. **26**(10): p. 845-7.
5. F. Giammarile, N. Alazraki, J.N. Aarsvold, R.A. Audisio, et al., *The EANM and SNMMI practice guideline for lymphoscintigraphy and sentinel node localization in breast cancer*. Eur J Nucl Med Mol Imaging, 2013. **40**(12): p. 1932-47.
6. M. Douek, J. Klaase, I. Monypenny, A. Kothari, et al., *Sentinel node biopsy using a magnetic tracer versus standard technique: the SentiMAG Multicentre Trial*. Annals of surgical oncology, 2014. **21**(4): p. 1237-45.
7. M. Thill, A. Kurylcio, R. Welter, V. van Haasteren, et al., *The Central-European SentiMag study: sentinel lymph node biopsy with superparamagnetic iron oxide (SPIO) vs. radioisotope*. Breast, 2014. **23**(2): p. 175-9.
8. I.T. Rubio, S. Diaz-Botero, A. Esgueva, R. Rodriguez, et al., *The superparamagnetic iron oxide is equivalent to the Tc99 radiotracer method for identifying the sentinel lymph node in breast cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2014.
9. D.D. Stark, R. Weissleder, G. Elizondo, P.F. Hahn, et al., *Superparamagnetic iron oxide: clinical application as a contrast agent for MR imaging of the liver*. Radiology, 1988. **168**(2): p. 297-301.
10. R. Weissleder, P.F. Hahn, D.D. Stark, G. Elizondo, et al., *Superparamagnetic iron oxide: enhanced detection of focal splenic tumors with MR imaging*. Radiology, 1988. **169**(2): p. 399-403.
11. N. Kitamura, S. Kosuda, K. Araki, M. Tomifuji, et al., *Comparison of animal studies between interstitial magnetic resonance lymphography and radiocolloid SPECT/CT lymphoscintigraphy in the head and neck region*. Annals of nuclear medicine, 2012. **26**(3): p. 281-5.
12. R. Madru, P. Kjellman, F. Olsson, K. Wingardh, et al., *99mTc-labeled superparamagnetic iron oxide nanoparticles for multimodality SPECT/MRI of sentinel lymph nodes*. Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 2012. **53**(3): p. 459-63.

13. D. Mizokami, S. Kosuda, M. Tomifuji, K. Araki, et al., *Superparamagnetic iron oxide-enhanced interstitial magnetic resonance lymphography to detect a sentinel lymph node in tongue cancer patients*. Acta oto-laryngologica, 2013. **133**(4): p. 418-23.
14. T.R. McCauley, M.D. Rifkin, and C.A. Ledet, *Pelvic lymph node visualization with MR imaging using local administration of ultra-small superparamagnetic iron oxide contrast*. Journal of magnetic resonance imaging : JMRI, 2002. **15**(4): p. 492-7.
15. S. Iida, K. Imai, S. Matsuda, O. Itano, et al., *In vivo identification of sentinel lymph nodes using MRI and size-controlled and monodispersed magnetite nanoparticles*. Journal of magnetic resonance imaging : JMRI, 2013. **38**(6): p. 1346-55.
16. M. Shiozawa, S. Kobayashi, Y. Sato, H. Maeshima, et al., *Magnetic resonance lymphography of sentinel lymph nodes in patients with breast cancer using superparamagnetic iron oxide: a feasibility study*. Breast cancer, 2014. **21**(4): p. 394-401.
17. M.G. Harisinghani, J. Barentsz, P.F. Hahn, W.M. Deserno, et al., *Noninvasive detection of clinically occult lymph-node metastases in prostate cancer*. The New England journal of medicine, 2003. **348**(25): p. 2491-9.
18. M. Memarsadeghi, C.C. Riedl, A. Kaneider, A. Galid, et al., *Axillary lymph node metastases in patients with breast carcinomas: assessment with nonenhanced versus uspio-enhanced MR imaging*. Radiology, 2006. **241**(2): p. 367-77.
19. S.C. Michel, T.M. Keller, J.M. Frohlich, D. Fink, et al., *Preoperative breast cancer staging: MR imaging of the axilla with ultrasmall superparamagnetic iron oxide enhancement*. Radiology, 2002. **225**(2): p. 527-36.
20. M. Ahmed, S.I. Usiskin, M.A. Hall-Craggs, and M. Douek, *Is imaging the future of axillary staging in breast cancer?* European radiology, 2014. **24**(2): p. 288-93.
21. H. Verbelen, N. Gebruers, F.M. Eeckhout, K. Verlinden, and W. Tjalma, *Shoulder and arm morbidity in sentinel node-negative breast cancer patients: a systematic review*. Breast cancer research and treatment, 2014. **144**(1): p. 21-31.
22. M.B. Teshome, K.V.; McCall, L.M.; Cormier, J.N.; Giuliano, A.E.; Hunt, K. *Long-term Incidence of Lymphedema after Sentinel Lymph Node Dissection for Early Stage Breast Cancer: ACOSOG Z0010 (Alliance)*. in Society of Surgical Oncology 67th Annual Cancer Symposium. 2014.
23. Y. Meng, S. Ward, K. Cooper, S. Harnan, and L. Wyld, *Cost-effectiveness of MRI and PET imaging for the evaluation of axillary lymph node metastases in early stage breast cancer*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2011. **37**(1): p. 40-6.
24. J.J. Pouw, M. Ahmed, B. Anninga, K. Schuurman, et al., *Comparison of three magnetic nanoparticle tracers for sentinel lymph node biopsy in an in vivo porcine model*. International journal of nanomedicine, 2015. **10**: p. 1235-43.
25. A.B. Chagpar, R.C. Martin, C.R. Scoggins, D.J. Carlson, et al., *Factors predicting failure to identify a sentinel lymph node in breast cancer*. Surgery, 2005. **138**(1): p. 56-63.
26. D. Gschwantler-Kaulich, M. Riegler-Keil, E. Ruecklinger, C.F. Singer, et al., *Factors influencing the identification rate of the sentinel node in breast cancer*. European journal of cancer care, 2011. **20**(5): p. 627-31.

27. C.E. Cox, E. Dupont, G.F. Whitehead, M.D. Ebert, et al., *Age and body mass index may increase the chance of failure in sentinel lymph node biopsy for women with breast cancer*. The breast journal, 2002. **8**(2): p. 88-91.
28. M. Carmon, D. Hain, J. Shapira, and E. Golomb, *Preoperative lymphatic mapping does not predict the number of axillary sentinel lymph nodes identified during surgery in breast cancer patients*. The breast journal, 2006. **12**(5): p. 424-7.
29. M.A. Mathew, A.K. Saha, T. Saleem, N. Saddozai, et al., *Pre-operative lymphoscintigraphy before sentinel lymph node biopsy for breast cancer*. Breast, 2010. **19**(1): p. 28-32.
30. C.B. Teal, J.P. Slocum, E.A. Akin, and T.A. Kelly, *Correlation of lymphoscintigraphy with the number of sentinel lymph nodes identified intraoperatively in patients with breast cancer*. American journal of surgery, 2005. **190**(4): p. 567-9.
31. J.H. Volders, R.F. van la Parra, C.D. Bavelaar-Croon, P.C. Barneveld, et al., *Discordance between number of scintigraphic and perioperatively identified sentinel lymph nodes and axillary tumour recurrence*. Breast, 2014.
32. F. Noushi, A.J. Spillane, R.F. Uren, R. Cooper, et al., *High discordance rates between sub-areolar and peri-tumoural breast lymphoscintigraphy*. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology, 2013. **39**(10): p. 1053-60.
33. G. Caruso, C. Cipolla, R. Costa, A. Morabito, et al., *Lymphoscintigraphy with peritumoral injection versus lymphoscintigraphy with subdermal periareolar injection of technetium-labeled human albumin to identify sentinel lymph nodes in breast cancer patients*. Acta radiologica, 2014. **55**(1): p. 39-44.
34. K. Motomura, M. Ishitobi, Y. Komoike, H. Koyama, et al., *SPIO-enhanced magnetic resonance imaging for the detection of metastases in sentinel nodes localized by computed tomography lymphography in patients with breast cancer*. Annals of surgical oncology, 2011. **18**(12): p. 3422-9.
35. L. Johnson, S.E. Pinder, and M. Douek, *Deposition of superparamagnetic iron-oxide nanoparticles in axillary sentinel lymph nodes following subcutaneous injection*. Histopathology, 2013. **62**(3): p. 481-6.

7

GENERAL CONCLUSION & FUTURE PERSPECTIVE

This thesis describes the use of magnetic nanoparticles for sentinel lymph node identification. The aim of the research was twofold. In colorectal cancer, the objective was to introduce a procedure allowing to improve staging accuracy in a routine clinical workflow. In breast cancer, we aimed to introduce an entirely radioisotope-free method for SLNB. In this chapter, first the main conclusions are recapitulated and discussed. Finally, technical recommendations and the future perspective of the use of magnetic nanoparticles in lymph node staging are given.

7.1 COLORECTAL CANCER

As discussed in Chapters 2 and 3, ex vivo sentinel lymph node mapping (SLNM) in colorectal cancer can potentially be used to cost-efficiently improve staging accuracy, and thereby potentially improve treatment of a large group of patients. Currently used tracers suffer from significant drawbacks when used in a standard clinical workflow, hampering widespread implementation of the technique in routine clinical practice. With advantageous properties as an optimal size for lymph node retention and excellent stability, magnetic nanoparticles are a promising alternative to resolve these drawbacks. In Chapter 2 we demonstrated that magnetic nanoparticles are taken up by lymph nodes after ex vivo administration. It was proven possible to quantitatively detect magnetic nanoparticles in lymph nodes after formalin fixation, confirming the stability. Although a non-clinically applicable time-consuming measurement technique (VSM) was used in a limited number of patients, the results supported the potential of magnetic nanoparticles for ex vivo SLNM in colorectal cancer and warranted further clinical studies with a magnetometer suitable for clinical use.

The use of such a commercial magnetometer, developed for clinical use, was evaluated in Chapter 3. The study demonstrated that a magnetic nanoparticle tracer can be used for SLNM in a routine clinical workflow with an excellent identification rate, superior to a standard blue dye tracer. The magnetic technique accurately selected SLNs with occult nodal involvement in all patients, indicating that the technique can improve nodal staging accuracy in a routine clinical workflow. The clinical impact, and cost-effectiveness of the introduction of the technique must be investigated in future studies, involving a larger number of patients. With the work in this thesis we have established the feasibility of using ex vivo magnetic SLNM to improve staging accuracy of colorectal cancer patients, in routine clinical practice.

7.2 BREAST CANCER

The availability of the current standard technique for SLNB in breast cancer worldwide is limited due to the use of radioisotopes. We aimed to introduce an entirely radioisotope-free alternative for SLNB in breast cancer patients, using a magnetic nanoparticle tracer. The combined technique is a very effective method for SLNB, therefore alternative techniques must meet high demands to be considered as feasible alternative. Chapters 4, 5 and 6 were dedicated to the optimization of the magnetic technique for SLNB to meet these high demands, in a broad sense. This was achieved by optimization of the choice of tracer, by analysing and resolving shortcomings and pitfalls of the intraoperative technique, and development and evaluation of a pre-operative MR imaging method.

A number of magnetic nanoparticle tracers are approved for human use. The particle size of the previously used magnetic nanoparticle tracer differs substantially from the radioisotope tracer, and could possibly benefit from optimization. We used an in vivo porcine model to evaluate the performance of three tracers on the clinically important aspects of distribution speed, SLN retention and accumulation in high concentration. We demonstrated that a 59 nm sized tracer is the best performing tracer.

The similar mode of operation of the handheld magnetometer and gamma probe requires little adjustment from the surgeon when using the technique. However, an important difference is that magnetometers are not only sensitive to the tracer but also the magnetic human body, potentially resulting in ambiguous signals. In a phantom study (Chapter 5), we demonstrated that a diamagnetic tissue environment limits the depth performance of the system. It was shown that the detection distance of the current generation magnetometers does not meet the demands formulated for gamma probes for successful transcutaneous hotspot identification. The pre-operative MRI protocol described in Chapter 6 can resolve this limitation by providing pre-incision information on the location of the SLNs.

Although the necessity of pre-operative imaging has been questioned in breast cancer, it is the standard of care in many countries. Therefore, an alternative for lymphoscintigraphy is needed to provide an entirely radioisotope-free SLNB technique. In Chapter 6 we demonstrated that SPIO-MRI can serve as a feasible alternative to lymphoscintigraphy in breast cancer patients. As a secondary goal, the potential of SPIO-MRI to non-invasively diagnose lymph nodes metastasis to eliminate the need for surgical axillary staging, was explored in this chapter. The number of patients with nodal metastases was too small to draw conclusions about the efficacy of the technique for this purpose.

In summary, by analysing and resolving shortcomings and pitfalls of the existing intraoperative magnetic technique for SLNB our research made important contributions to make the introduction of an entirely radioisotope-free technique for SLNB in breast cancer patients possible.

7.3 RECOMMENDATIONS AND FUTURE PERSPECTIVE

A handheld magnetometer developed for intraoperative SLN localization was used to quantify magnetic tracer uptake in individual colorectal lymph nodes. Although the handheld magnetometer allowed to perform a quick quantification, the device was not developed for this purpose. The lymph nodes must be precisely placed on the probe to obtain reproducible and comparable results. The technique would benefit from a dedicated device, in/on which lymph nodes can be easily and reproducibly placed.

In this thesis, ex vivo colorectal SLNM was used to improve nodal staging accuracy given that the complete nodal basin is removed, irrespective of the nodal status. With a growing interest for localized excision of early rectal cancer, and early colon cancer there is a role for in vivo SLNM. An in vivo SLNM procedure would allow to perform a localized resection of the tumour, and achieve accurate nodal staging by removing only the SLNs. However, the current magnetometer is not suitable for laparoscopic use. Development of a laparoscopic magnetometer would provide the means to perform localized resections in colorectal cancer, without compromising the ability to determine the nodal status. A laparoscopic magnetometer would also enable the possibility to perform a SLN procedure in various other pelvic and abdominal malignancies, without the logistical constraints of radioisotopes.

While the current generation magnetometers achieve an excellent intraoperative identification rate, they could benefit from technical improvements. Firstly, the sensitivity to diamagnetic tissue limits the depth performance of the system, and can result in false-positive or false-negative signals. Secondly, the magnetometer cannot be used together with regular surgical steel instruments. The Diffmag measurement technology is insensitive to diamagnetic tissue, and can be used near standard surgical equipment. A handheld magnetometer using this technique could therefore resolve two important drawbacks of the current magnetometer. It would provide a more user friendly and potentially better performing technique. A magnetometer using this technique was developed, however has not yet been used clinically.

The use of magnetic nanoparticles for in vivo SLNB in breast cancer was explored in this thesis. In centres performing blue dye only- SLNB due to lack of access to radioisotopes, the magnetic technique (with optional pre-operative SPIO-MRI) provides a valuable complementation. Randomized controlled clinical trials must be conducted to confirm the non-inferiority of the technique to the combined technique before it can serve as alternative. In these trials, pre-operative imaging with SPIO-MRI and intraoperative identification using the handheld magnetometer should be compared to lymphoscintigraphy and intraoperative gamma probe detection.

The application of magnetic nanoparticles for in vivo SLNB is not necessarily limited to breast cancer, but could also be used in other malignancies. In addition to the advantages of a radioisotope-free procedure, the possibility to perform high resolution pre-operative imaging could be especially beneficial in malignancies with a more complex lymphatic drainage pattern such as melanoma and head and neck cancer. It could provide detailed anatomical information on the location of the SLNs and thereby aid intraoperative identification. With radioisotopes, identification of SLNs close to the injection site is especially challenging because the activity of the SLN is obscured, the so-called shine through phenomenon. Future work should explore the possibilities and value of SLN localization with magnetic nanoparticles in these cases. More research is needed to limit the injection site artefact by optimising dosage and scan parameters to aid identification of SLNs in close proximity to the injection site.

As described in the introduction there is increasingly more evidence that removal of lymph nodes with limited involvement does not improve locoregional control or survival in patients treated with whole breast radiation and adjuvant systemic therapy. Since MRI is fundamentally limited by its resolution, this establishes the conditions for the technique to potentially completely replace surgical SLNB in the future. Since most patients receive whole breast radiation after breast conserving surgery and/or adjuvant therapy based on tumour characteristics one can debate the necessity to detect micrometastatic disease. We have evaluated the potential of SPIO-MRI to non-invasively detect SLN metastases as a secondary goal in Chapter 6. A dedicated study to optimize tracer dosage, injection site and timing of MR scanning should be performed to assess the potential of the technique to serve as replacement for surgical lymph node staging in breast cancer patients.

SUMMARY

Most solid malignancies have a tendency to spread through the lymphatic system to locoregional lymph nodes. Presence of metastasis is an important prognostic factor, and is used to determine the optimal treatment of the patient. The sentinel lymph nodes (SLNs) receive direct lymphatic drainage from the tumour area, and are therefore most likely the first site of metastasis, if present. The SLNs therefore play a crucial role in the staging and treatment of cancer. This thesis describes the use of magnetic nanoparticles for SLN identification in colorectal- and breast cancer. **Chapter 1** describes the disadvantages of currently used tracers for SLN identification. A short overview of alternative tracers and techniques, and their beneficial properties and disadvantages, is given. The aim of the research in this thesis is twofold. In colorectal cancer, the objective is to introduce a SLN procedure allowing to improve nodal staging accuracy in a routine clinical workflow after ex vivo administration of magnetic nanoparticles (Chapters 2 and 3). In breast cancer, we aim to introduce an entirely radioisotope-free method for in vivo SLN Biopsy (SLNB) (Chapters 4, 5 and 6).

COLORECTAL CANCER

Ex vivo SLN Mapping (SLNM) in colorectal cancer can potentially be used to cost-efficiently improve nodal staging accuracy, and thereby potentially improve treatment of a large group of patients. However, currently used experimental techniques suffer from limitations, hampering widespread clinical implementation. A new technique using magnetic nanoparticles has the potential to overcome these limitations. The development of this technique for ex vivo SLN identification in colorectal cancer is described in Chapters 2 and 3. As a first step the feasibility of using magnetic nanoparticles as tracer for ex vivo SLN identification was evaluated in **Chapter 2**. An MRI contrast agent (Endorem®) was injected around the tumour, after resection of the colorectal segment in thirteen patients. Vibrating sample magnetometry (VSM) was performed on a selection of the resected LNs to quantitatively evaluate the uptake of magnetic tracer. It was proven possible to quantitatively detect magnetic nanoparticles in the LNs. Although a time consuming non-clinically applicable measurement technique (VSM) was used, the results supported the potential of magnetic nanoparticles for ex vivo SLNM in colorectal cancer, and warranted further clinical studies with a magnetometer suitable for clinical use.

In **Chapter 3** the results of a study in which such a magnetometer was used are described. Twenty-eight ex vivo SLNM procedures with a magnetic tracer were performed in twenty-

seven patients (in one patients two procedures were performed). SLNM was also performed with blue dye as a control. A commercially available handheld magnetometer developed for clinical use (SentiMAG®) was used to select magnetic SLNs after formalin fixation of the colorectal segment. Selection of SLNs after formalin fixation allows to easily implement the technique in routine clinical practice. At least one SLN was successfully identified with the magnetic technique in 96% of the cases vs. 89% with the blue dye. Isolated tumour cells were found in the SLNs of ten patients. These tumour cells would not have been detected in routine clinical practice. The magnetic technique achieved 100% sensitivity and accuracy, while the blue dye technique achieved 91% sensitivity and 96% accuracy. These results indicate that the magnetic technique can improve nodal staging accuracy in a routine clinical workflow. The clinical impact, and cost-effectiveness of the introduction of the technique must be investigated in future studies, involving a larger number of patients.

BREAST CANCER

In breast cancer, currently a radioisotope tracer is used for pre- and intraoperative identification of SLNs. Lymphoscintigraphy is used for pre-operative imaging, and a handheld scintillation-counter (gamma probe) is used for the intraoperative identification. However, the worldwide availability of radioisotopes, and therefore this technique for SLNB, is limited. Therefore there is a clinical need for radioisotope-free alternative techniques. We aimed to introduce such an alternative for SLNB in breast cancer patients using a magnetic nanoparticle tracer. Because the radioisotope tracer performs very well in terms of identification rate and false negative rate, alternative techniques must meet high demands to be considered as feasible alternative. Chapters 4, 5 and 6 describe different aspects of the optimization of the magnetic technique for SLNB to meet these demands.

A number of magnetic nanoparticle tracers are approved for human use. However, the performance of only one of these tracers has been evaluated clinically. The particle size of this tracer differs substantially from the radioisotope tracers currently used. Therefore the magnetic tracer could benefit from optimization. In **Chapter 4**, the performance of three magnetic nanoparticle tracers approved for human use (ferumoxylol, with hydrodynamic diameter $d_H = 32$ nm; Sienna+, $d_H = 59$ nm; and ferumoxide, $d_H = 111$ nm) was evaluated in an in vivo porcine model. A total of 36 SLNB procedures using a handheld magnetometer were performed in 18 mini-pigs. Tracers were evaluated on the clinically important aspects of distribution speed, SLN retention and accumulation in high concentration. We demonstrated that the currently used 59 nm sized tracers performs the best on all these aspects.

An important difference between the currently used gamma probe and the magnetometer is that the latter is not only sensitive to the tracer but also to the magnetic human body. This can potentially result in ambiguous signals and limit the depth performance. This is especially important to achieve transcutaneous localization of the SLN to determine the optimal incision site. The depth performance of the SentiMAG magnetometer was evaluated in the phantom study described in **Chapter 5**. A phantom, mimicking the magnetic and mechanical properties of the human axilla, was constructed. Lymph node phantoms with tracer uptake ranging from 5-500 μg iron were placed at clinically relevant depths, and the depth performance of the magnetometers was evaluated. In the phantom, only superficially placed LNs (2.5 cm) with high uptake (500 μg iron) could be detected from the surface. The penetration depth was insufficient to detect LNs with lower uptake, or which were located deeper. The detection distance of the current generation magnetometers is limited, and does not meet the demands formulated by the European Association for Nuclear Medicine for successful transcutaneous SLN localization. Future clinical trials should evaluate whether the limited depth sensitivity is of influence to the clinical outcome of the SLNB procedure.

Although the necessity of pre-operative imaging has been questioned in breast cancer, it is the standard of care in many countries. Therefore, an alternative for lymphoscintigraphy is needed to provide an entirely radioisotope-free SLNB technique. Furthermore, a technique that provides pre-incision information on the number of SLNs and their location can be used to resolve the limited transcutaneous depth sensitivity of the current generation magnetometers. The development and evaluation of a pre-operative imaging protocol, using the MRI contrast properties of the magnetic tracer, is described in **Chapter 6**. Eleven patients with breast cancer scheduled to undergo SLNB were recruited for pre-operative lymphoscintigraphy, SPECT-CT and MRI with magnetic tracer (SPIO-MRI). T1-weighted-TSE and T2-weighted-GRE sequences were used before and after interstitial injection of magnetic tracer into the breast. SLNs on MRI were defined as LNs with signal drop and direct lymphatic drainage from the injection site. LNs showing inhomogeneous tracer-uptake were classified as metastatic. During surgery a handheld magnetometer was used for SLNB. SPIO-MRI successfully identified SLNs in 10 out of 11 patients vs. 11 out of 11 patients with lymphoscintigraphy/SPECT-CT. One patient had metastatic involvement of four LNs and this was identified in one node on pre-operative MRI. We concluded that SPIO-MRI is a feasible technique for pre-operative localization of SLNs, and in combination with intraoperative use of a handheld magnetometer provides an entirely radioisotope-free technique for SLNB.

In **Chapter 7**, the main conclusions are recapitulated and discussed. In summary, with the work in this thesis we have established the feasibility of using ex vivo magnetic SLNM to

improve staging accuracy of colorectal cancer patients, in routine clinical practice. Furthermore, by analysing and resolving shortcomings and pitfalls of the existing intraoperative magnetic technique for SLNB our research made important contributions to make the introduction of an entirely radioisotope-free technique for SLNB in breast cancer patients possible. Finally, technical recommendations to improve the magnetic technique in colorectal- and breast cancer are given. The possible application in other malignancies is described and the future perspective of the use of magnetic nanoparticles in non-invasive lymph node staging are given.

SAMENVATTING

De meeste kwaadaardige tumoren hebben de neiging zich via het lymfatisch systeem te verspreiden naar locoregionale lymfeklieren. De aanwezigheid van uitzaaiingen is een belangrijke prognostische factor, die wordt gebruikt om de optimale behandeling van de patiënt te bepalen. De schildwachtklieren (SWK) ontvangen directe lymfatische drainage vanuit het gebied van de tumor. Als er sprake is van uitzaaiingen dan zijn de SWK het meest waarschijnlijk de eerste plaats waar ze optreden. De SWK spelen daarom een cruciale rol bij het bepalen van het stadium en optimale behandeling van de ziekte. Dit proefschrift beschrijft het gebruik van magnetische nanodeeltjes (MND) voor het identificeren van de SWK bij dikkedarmkanker en bij borstkanker. **Hoofdstuk 1** beschrijft de nadelen van de huidige tracers die gebruikt worden voor SWK identificatie. Een kort overzicht van alternatieve tracers en technieken, en hun voor- en nadelen worden gegeven.

Het doel van het onderzoek in dit proefschrift is tweeledig. Het eerste doel is bij darmkanker een SWK procedure te ontwikkelen die het mogelijk maakt om in de dagelijkse klinische praktijk de lymfeklierstadiëring te verbeteren na ex vivo toediening van MND (Hoofdstuk 2 en 3). Het tweede doel is om voor borstkanker een volledig stralingsvrije (zonder radio-isotopen) methode voor in vivo SWK identificatie te introduceren (Hoofdstuk 4, 5 en 6).

DIKKEDARMKANKER

De ex vivo SWK procedure kan bij darmkanker in potentie gebruikt worden om kosteneffectief zeer kleine lymfeklier-uitzaaiingen te detecteren die met regulier onderzoek verborgen blijven (verbeteren van de zogenoemde nauwkeurigheid van lymfeklierstadiëring), en daarmee mogelijk de behandeling van een grote groep patiënten verbeteren. De huidige gebruikte experimentele technieken hebben beperkingen die wijdverspreide klinische implementatie belemmeren. Een nieuwe techniek, gebruik makend van MND, heeft het potentieel deze beperkingen te overwinnen. De ontwikkeling van deze techniek voor ex vivo SWK identificatie in darmkanker wordt beschreven in Hoofdstuk 2 en 3. Als eerste stap werd de haalbaarheid van het gebruik van MND als tracer voor ex vivo SWK identificatie geëvalueerd in **Hoofdstuk 2**. Na resectie van het colorectale segment werd bij 13 patiënten een MRI contrastmateriaal (Endorem®) geïnjecteerd rondom de tumor. Om kwantitatief de opname van de magnetische tracer te evalueren werd zogenoemde 'vibrating sample magnetometry' (VSM) toegepast op een deel van de verwijderde lymfeklieren. Er werd aangetoond dat het mogelijk is MND kwantitatief te detecteren in de lymfeklieren. Hoewel een tijdrovende niet-klinisch toepasbare

meettechniek (VSM) werd gebruikt, ondersteunen de resultaten de potentie van MND voor ex vivo SWK identificatie in darmkanker, en rechtvaardigen ze verdere klinische studies met een magnetometer geschikt voor klinisch gebruik.

De resultaten van een studie waarin een dergelijke magnetometer werd gebruikt worden beschreven in **Hoofdstuk 3**. Er werden 28 ex vivo SWK procedures met een magnetische tracer uitgevoerd bij 27 patiënten (bij één patiënt werden twee procedures uitgevoerd). Ter vergelijking werd ook een SWK procedure uitgevoerd met blauwe kleurstof. Een commercieel beschikbare ‘handheld’ magnetometer, ontwikkeld voor klinisch gebruik (SentiMAG®), werd gebruikt om magnetische SWK te selecteren, na formaline-fixatie van het colorectale segment. Een dergelijke selectie van SWK na formaline-fixatie laat eenvoudige implementatie van de techniek in de dagelijkse klinische praktijk toe. In 96% van de gevallen werd tenminste één SWK met succes geïdentificeerd met de magnetische techniek tegen 89% met de blauwe kleurstof. Geïsoleerde tumorcellen werden in de SWK van tien patiënten gevonden. Deze tumorcellen zouden in de reguliere klinische praktijk niet gevonden zijn. De magnetische techniek behaalde een sensitiviteit en nauwkeurigheid van 100%, terwijl de blauwe-kleurstof techniek een sensitiviteit van 91% en een nauwkeurigheid van 96% behaalde. Deze resultaten geven aan dat de magnetische techniek in de dagelijkse klinische praktijk zeer kleine lymfeklier-uitzaaiingen kan helpen detecteren die met regulier onderzoek verborgen blijven. De klinische invloed en de kosteneffectiviteit van de introductie van de techniek moeten in latere studies met een grotere groep patiënten, worden onderzocht.

BORSTKANKER

Momenteel wordt bij borstkanker een radio-isotoop tracer gebruikt voor de pre- en intra-operatieve identificatie van de SWK. Lymfoscintigrafie wordt gebruikt voor de preoperatieve afbeelding en een handheld scintillatieteller (gamma-probe) wordt gebruikt voor de intra-operatieve detectie van de SWK. Maar de wereldwijde beschikbaarheid van radio-isotopen, en daarmee deze techniek voor SWK identificatie, is beperkt. Daarom is er een klinische behoefte aan alternatieve radio-isotoop vrije technieken. We streefden de introductie van een dergelijke alternatieve techniek voor SWK identificatie bij borstkanker na met het gebruik van een MND tracer. Omdat de radio-isotoop tracer erg goed presteert op het gebied van identificatiepercentage en vals-negatief percentage, moeten alternatieve technieken aan zeer hoge eisen voldoen om als haalbaar alternatief beschouwd te worden. Hoofdstuk 4, 5 en 6 beschrijven verschillende aspecten van de optimalisatie van de magnetische techniek om aan deze eisen te voldoen.

Een aantal MND tracers zijn goedgekeurd voor gebruik in mensen. Echter is de prestatie van maar één van deze tracers klinisch beoordeeld. De deeltjesgrootte van deze tracer verschilt substantieel van de goed presterende radio-isotoop tracers die nu gebruikt worden. Een magnetische tracer met dezelfde deeltjesgrootte als de radio-isotoop tracers zou daardoor mogelijk de techniek kunnen verbeteren. De prestaties van drie MND tracers die goedgekeurd zijn voor gebruik in mensen (ferumoxitol, met hydrodynamische diameter $d_H = 32$ nm; Sienna+, $d_H = 59$ nm; en ferumoxide, $d_H = 111$ nm) werden geëvalueerd in een in vivo varkens model (**Hoofdstuk 4**). Een handheld magnetometer werd gebruikt om een totaal van 36 SWK procedures uit te voeren in 18 mini-varkens. De tracers werden geëvalueerd op de klinisch belangrijke aspecten van distributiesnelheid, SWK retentie en de accumulatie in hoge concentraties. We hebben laten zien dat de huidige gebruikte 59 nm tracer het best presteert op al deze aspecten.

Een belangrijk verschil tussen de huidig gebruikte gamma-probe en magnetometer is dat de laatste niet alleen gevoelig is voor de tracer maar ook voor het diamagnetische menselijk lichaam. Dit kan mogelijk resulteren in meerduidige signalen en bovendien de dieptegevoeligheid beperken. Dit is vooral van belang om transcutane lokalisatie van de SWK te bewerkstelligen en zo de optimale plek van incisie te bepalen. **Hoofdstuk 5** beschrijft een fantoomstudie waarin de dieptegevoeligheid van de SentiMAG magnetometer werd geëvalueerd. Een fantoom met magnetische en mechanische eigenschappen van de menselijke oksel werd geconstrueerd. Lymfeklier-fantomen met traceropname variërend van 5 tot 500 μg ijzer werden op klinisch relevante dieptes geplaatst, en de dieptegevoeligheid van de magnetometers werd geëvalueerd. In het fantoom konden alleen oppervlakkig geplaatste lymfeklier-fantomen (2.5 cm) met hoge opname (500 μg ijzer) worden gedetecteerd vanaf het oppervlak. De indringdiepte bleek ontoereikend om lymfeklier-fantomen met minder opname, of op grotere diepte te detecteren. De dieptegevoeligheid van de huidige generatie magnetometers is beperkt en voldoet niet aan de eisen die gesteld zijn door de Europese Associatie voor Nucleaire Geneeskunde om succesvolle transcutane SWK lokalisatie te bewerkstelligen. Toekomstige klinische studies zouden moeten evalueren of de beperkte dieptegevoeligheid van invloed is op de klinische uitkomst van de SWK procedure.

Hoewel de noodzaak van preoperatieve afbeeldingstechnieken wordt betwist, is het onderdeel van de standaard behandeling in veel landen. Daarom is er een alternatief voor de lymfoscintigrafie nodig om een compleet radio-isotopen vrije SWK techniek te kunnen bieden. Bovendien kan een techniek die pre-incisie informatie verschaft over het aantal SWK en hun locatie gebruikt worden om de beperkte dieptegevoeligheid van de huidige generatie magnetometers te compenseren. De ontwikkeling en evaluatie van een

preoperatief afbeeldingsprotocol, waarin de MRI contrasteigenschappen van de magnetische tracer worden benut, wordt beschreven in **Hoofdstuk 6**. Bij elf borstkankerpatiënten die ingeroosterd waren om een SWK procedure te ondergaan werden preoperatieve lymfoscintigrafie, SPECT-CT en MRI met magnetische tracer (SPIO-MRI) toegepast. T1-gewogen-TSE en T2-gewogen-GRE sequenties werden voor en na de interstitiële toediening van de magnetische tracer in de borst gebruikt. SWK op MRI werden gedefinieerd als klieren met afname van signaal en directe lymfatische drainage vanuit de injectieplek. Lymfeklieren met inhomogene traceropname werden als uitgezaaid beschouwd. Een handheld magnetometer werd tijdens de operatie gebruikt voor SWK identificatie. SPIO-MRI identificeerde succesvol de SWK in tien van de elf patiënten tegen elf patiënten met lymfoscintigrafie en de SPECT-CT. Eén patiënt had uitzaaiingen in vier lymfeklieren, en dit werd geïdentificeerd in één klier op de preoperatieve MRI. We concludeerden dat SPIO-MRI een haalbare techniek is voor de preoperatieve lokalisatie van SWK, en dat de techniek in combinatie met het intra-operatieve gebruik van een handheld magnetometer een compleet radio-isotoop vrije techniek voor SWK identificatie biedt.

In **Hoofdstuk 7** worden de hoofd conclusies geresumeerd en bediscussieerd. Samenvattend hebben we met het in dit proefschrift beschreven onderzoek de haalbaarheid van het gebruik van magnetische SWK identificatie om de nauwkeurigheid van lymfeklierstadiëring bij dikkedarmkanker patiënten in de dagelijkse klinische praktijk te verbeteren aangetoond. Daarnaast heeft ons onderzoek, door tekortkomingen en valkuilen van de bestaande intra-operatieve techniek voor magnetische SWK identificatie in kaart te brengen en op te lossen, een belangrijke bijdrage geleverd om de introductie van een compleet radio-isotoop vrije SWK identificatie techniek bij borstkanker mogelijk te maken. Tot slot worden technische aanbevelingen gegeven die de magnetische techniek in borstkanker en in dikkedarmkanker kunnen verbeteren. De mogelijkheden voor de toepassing van de techniek in andere kankersoorten wordt beschreven en het toekomst perspectief van het gebruik van MND voor non-invasieve lymfeklierstadiëring wordt beschreven.

DANKWOORD

Tot slot wil ik graag een aantal mensen bedanken die een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift.

Als eerste wil ik mijn co-promotor dr. Bennie ten Haken bedanken voor de mogelijkheid om na mijn afstuderen als promovendus binnen zijn groep het werk voort te zetten. Ik heb onze samenwerking, en de vrijheid die je me daarin gaf als zeer prettig en leerzaam ervaren. Je onbevangen technische blik op het klinische onderzoek waren in onze discussies vaak zeer verfrissend. Dank daarvoor! Daarnaast gaat mijn dank ook uit naar mijn promotor prof. Theo Ruers. Je werd pas in een later stadium bij mijn promotie betrokken. Ik waardeer je bereidheid om als mijn promotor op te treden en de wijze waarop je mij de ruimte hebt gegeven dit proefschrift af te ronden.

Ik wil de leden van de promotiecommissie bedanken voor het bestuderen en beoordelen van mijn proefschrift, en voor hun aanwezigheid bij mijn verdediging. Especially, I would like to thank prof. Quentin Pankhurst for the evaluation of my thesis and coming over to the Netherlands for the defence. Additionally, our discussions and your insightful suggestions in the STW users committee meetings and throughout the whole project were very valuable and much appreciated.

De overige leden van de STW gebruikerscommissie (en hun bedrijven) en de program officer ben ik ook dankbaar voor hun inbreng.

In het bijzonder wil ik dr. Joost Klaase bedanken voor de klinische begeleiding tijdens mijn promotie, en voor de mogelijkheid om klinisch onderzoek te doen op de afdeling Chirurgie van het Medisch Spectrum Twente. Met je klinische ervaring en kennis heb je me veel geleerd en in staat gesteld om klinisch relevant onderzoek te doen. Ik ben de overige chirurgen van de afdeling Chirurgie ook zeer erkentelijk voor hun enthousiasme om mee te werken aan verschillende onderzoeken, en de gastvrijheid waarmee ik altijd op de OK ontvangen ben. Specifiek dank aan dr. Eino van Duyn voor de hulp bij de tracer injecties in de MRI. Dank gaat ook uit naar Anja Stam van het Researchbureau heelkunde, je hulp bij veel administratieve en organisatorische aspecten heeft ervoor gezorgd dat alle studies soepel verlopen zijn.

Voor de totstandkoming van hoofdstuk 6 van dit proefschrift hebben de afdeling Radiologie en Nucleaire Geneeskunde van het MST een belangrijke rol gespeeld. Ik wil dr. Roland Bezooijen bedanken voor het enthousiasme om in de avonden samen een MRI protocol te ontwikkelen, en dr. Klazen en dr. Bezooijen voor de beoordeling van de scans. Daarnaast wil ik de radiodiagnostisch laboranten, en vooral Aletta Goolkate-Geerligts, bedanken voor het uitvoeren van de scans. Ik ben nucleair geneeskundige dr. Wieger de Bruin dankbaar

voor de flexibiliteit en het meedenken, en om het onderzoek zo efficiënt mogelijk te plannen tussen het reguliere programma van de nucleaire geneeskunde.

I would like to thank dr. Michael Douek and his group for the pleasant collaboration in the SentiMAG Multicentre trial and the animal study. Working on the first trial that evaluated the magnetic technique in breast cancer patients was really inspiring. Michael, I would also like to thank you for critically reviewing the papers we collaborated on. Your insightful suggestions improved the quality of the work.

Patholoog dr. Joop van Baarlen van Laboratorium Pathologie Oost-Nederland heeft vele extra coupes beoordeeld, en altijd de ruimte gegeven om metingen en onderzoek in zijn lab te doen. Daar ben ik je zeer dankbaar voor. Daarnaast wil ik de analisten van dit laboratorium bedanken voor hun flexibiliteit die soms nodig was om mijn onderzoek mogelijk te maken.

Mijn collega's van de NIM-groep wil ik bedanken voor de plezierige werksfeer door de jaren heen. Ik heb, vooral op technisch gebied, veel van jullie geleerd. Daarnaast heb ik de pauzes en lunches die we samen doorbrachten altijd als erg gezellig ervaren. Ook dank ik de verschillende secretaresses die door de jaren heen van alles voor me geregeld hebben. Ik ben vooral Tanja Gerrits dankbaar voor de hulp met allerlei zaken rondom mijn promotie in het laatste jaar. Ook wil ik alle studenten die ik tijdens mijn promotie heb mogen begeleiden bedanken, ik hoop dat jullie minstens zo veel van mij geleerd hebben als ik van jullie!

Bijzondere aandacht gaat uit naar de paranimfen die mij op de dag van de verdediging bijstaan. Ik vind het heel leuk dat ik tijdens mijn verdediging niet alleen vanuit de zaal gesteund zal worden door familie, maar ook vanaf het podium door mijn broer. Michiel, dank hiervoor! Martijn, dr. Visscher, ik heb de eer genoten om jou als paranimf bij te staan, en vind het erg leuk dat we de rollen nu kunnen omdraaien. Tijdens mijn afstuderen was je al nauw betrokken bij mijn werk, ik kan me nog goed de eerste experimenten op de OK en de eindeloze VSM-metingen in de Hogekamp herinneren. Ongelofelijk hoe veel verder we nu zijn. Ik heb veel van je geleerd, en onze samenwerking altijd als zeer prettig ervaren.

Ik wil mijn ouders bedanken voor hun interesse en bovenal steun door de jaren heen. Daarnaast wil ik mijn vader bedanken voor het kritisch doornemen van een aantal stukken van dit proefschrift. Pap en mam, dank jullie wel!

Lieve Jorien, als laatste maar belangrijkste. Je bent altijd oprecht geïnteresseerd in mijn werk, maar weet ondertussen misschien wel meer van sentinel nodes en nanodeeltjes dan je lief is. Dank je wel voor je begrip in drukke periodes, en je onvoorwaardelijke steun. Ik ben heel benieuwd wat de toekomst ons samen allemaal gaat brengen!

Joost, December 2015, Enschede

ABOUT THE AUTHOR

Joost Jacob Pouw was born on January 9th, 1985 in Doetinchem, the Netherlands. He obtained his Bachelor and Master degree (November 2010) in Technical Medicine (Track Robotics & Imaging) at the University of Twente, Enschede, the Netherlands. His graduation project, entitled 'Ex vivo magnetic sentinel lymph node detection in colorectal cancer' was performed at the Medisch Spectrum Twente, Enschede, the Netherlands, in collaboration with the NIM-Magnetic detection group from the University of Twente.

After obtaining his Master degree, he started as a PhD candidate at the MIRA Institute for Biomedical Engineering and Technical Medicine. His project focused on radiation free localization and diagnosis of sentinel lymph nodes with magnetic nanoparticles. The project involved clinical studies conducted at the Medisch Spectrum Twente in breast- and colorectal patients. The research was performed in collaboration with the Department of Surgery of Medisch Spectrum Twente and the Division of Cancer studies, King's College London.

LIST OF PUBLICATIONS

JOURNAL ARTICLES

J.J. Pouw, M.R. Grootendorst, R. Bezooijen, C.A. Klazen, W.I. De Bruin, J.M. Klaase, M.A. Hall-Craggs, M. Douek, and B. Ten Haken, *Pre-operative sentinel lymph node localization in breast cancer with superparamagnetic iron oxide MRI: the SentiMAG Multicentre Trial imaging subprotocol*. The British journal of radiology, 2015. **88**(1056): p. 20150634.

J.J. Pouw, M. Ahmed, B. Anninga, K. Schuurman, S.E. Pinder, M. Van Hemelrijck, Q.A. Pankhurst, M. Douek, and B. Ten Haken, *Comparison of three magnetic nanoparticle tracers for sentinel lymph node biopsy in an in vivo porcine model*. International journal of nanomedicine, 2015. **10**: p. 1235-43.

M. Ahmed, B. Anninga, **J.J. Pouw**, S. Vreemann, M. Peek, M. Van Hemelrijck, S. Pinder, B. Ten Haken, Q. Pankhurst, and M. Douek, *Optimising magnetic sentinel lymph node biopsy in an in vivo porcine model*. Nanomedicine: nanotechnology, biology, and medicine, 2015. **11**(4): p. 993-1002.

M. Visscher, S. Waanders, **J. Pouw**, and B. ten Haken, *Depth limitations for in vivo magnetic nanoparticle detection with a compact handheld device*. Journal of Magnetism and Magnetic Materials, 2015. **380**: p. 246-250.

B. Anninga, M. Ahmed, M. Van Hemelrijck, **J. Pouw**, D. Westbroek, S. Pinder, B. Ten Haken, Q. Pankhurst, and M. Douek, *Magnetic sentinel lymph node biopsy and localization properties of a magnetic tracer in an in vivo porcine model*. Breast cancer research and treatment, 2013. **141**(1): p. 33-42.

M. Visscher, **J.J. Pouw**, J. van Baarlen, J.M. Klaase, and B. Ten Haken, *Quantitative analysis of superparamagnetic contrast agent in sentinel lymph nodes using ex vivo vibrating sample magnetometry*. IEEE transactions on bio-medical engineering, 2013. **60**(9): p. 2594-602.

D.J. Grootendorst, R.M. Fratila, **J.J. Pouw**, G.C. Langhout, B. ten Haken, R.J.A. van Wezel, S. Rottenberg, W. Steenbergen, S. Manohar, T.J.M. Ruers, *Photoacoustic staging of nodal metastases using SPIOs: comparison between in vivo, in toto and ex vivo imaging in a rat model*. Accepted for publication in Biomedical Spectroscopy and Imaging

Submitted manuscripts:

J.J. Pouw, M.R. Grootendorst, J.M. Klaase, J. van Baarlen, B. ten Haken, *Ex vivo sentinel lymph node mapping in colorectal cancer using a magnetic nanoparticle tracer to improve staging accuracy: A pilot study*. Submitted to Colorectal Disease (Under review).

J.J. Pouw, D.M.C. Bastiaan, J.M. Klaase, B. ten Haken, *Depth performance of a handheld magnetometer for sentinel node biopsy*. Submitted to Journal of Surgical Research (Under review).

CONFERENCE CONTRIBUTIONS

Proceedings article:

J. Pouw, R. Fratila, A. Velders, B. ten Haken, Q. Pankhurst, and J. Klaase, *Ex Vivo Magnetic Sentinel Lymph Node Detection in Colorectal Cancer with a SPIO Tracer*, in *Magnetic Particle Imaging*, 2012. p. 181-185

Oral contributions and posters (first author contributions selected):

J.J. Pouw, M. Ahmed, B. Anninga, K. Schuurman, M. Douek, B. ten Haken, *Performance of three different clinical magnetic nanoparticle tracers for sentinel lymph node detection*, 10th international Conference on the Scientific and Clinical Applications of Magnetic Carriers, Dresden, Germany, June 2014 (oral)

J.J. Pouw, M.R. Grootendorst, B. ten Haken, R. Bezooijen, W.I. de Bruin WI, J.M. Klaase, M. Douek, *Pre-operative MRI localisation of the sentinel lymph node in patients with breast cancer after interstitial injection of a magnetic tracer*, 5th International Symposium on Cancer Metastasis and the Lymphovascular System and the 8th International Sentinel Node Society (ISNS) Meeting, San Francisco, USA, May 2013 (poster)

J.J. Pouw, B. ten Haken, J. van Baarlen, J.M. Klaase, *Ex vivo sentinel lymph node selection in colorectal cancer using a superparamagnetic iron oxide (SPIO) tracer*, 5th International Symposium on Cancer Metastasis and the Lymphovascular System and the 8th International Sentinel Node Society (ISNS) Meeting, San Francisco, USA, May 2013 (poster)

J. Pouw, R. Fratila, A. Velders, B. ten Haken, Q. Pankhurst, and J. Klaase, *Ex Vivo Magnetic Sentinel Lymph Node Detection in Colorectal Cancer with a SPIO Tracer*, 2nd International Workshop on Magnetic Particle Imaging, Lubeck, Germany, March 2012 (oral)

OTHER CONTRIBUTIONS

J.J. Pouw, M. Heijblom, J.M. Klaase, R. Bezooijen, B. ten Haken, *Radiation free localization and diagnosis of lymph nodes in cancer patients*, STW Annual congress, Nieuwegein, October 2014 (oral and poster) *Second place 'Simon Stevin Leering' prize*

J.J. Pouw, B. ten Haken, *Stralingsvrije Lokalisatie van Sentinel Nodes m.b.v. Magnetische Nanodeeltjes*, Albert Schweitzer Hospital, Wetenschapsdag, Dordrecht, October 2011 (invited oral)

ISBN: 978-90-365-4021-6