

Optimising Magnetic Sentinel Lymph Node Biopsy in an *in vivo* Porcine Model

(Short Title: Magnetic Sentinel Node Biopsy in a Porcine Model)

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ABSTRACT

The magnetic technique for sentinel lymph node biopsy (SLNB) has been evaluated in several clinical trials, albeit under proscribed conditions. An *in vivo* porcine model was therefore developed to optimise the magnetic technique by evaluating the effect of differing volume of magnetic tracer and time of injection. A total of 48 sentinel node procedures were undertaken and 423 nodes (109 sentinel nodes) retrieved. There was a significant correlation between magnetometer counts and the tracer content of excised sentinel lymph nodes (SLNs) ($r=0.82$; $p<0.001$). Total numbers of tracer-containing SLNs increased with increasing volumes of tracer ($p<0.001$). Transcutaneous magnetometer counts increased with increasing time from injection of tracer ($p<0.0001$) and reached a plateau within 60 minutes. A non-statistically significant trend was observed between volume of tracer injected and tracer content of SLNs ($p=0.07$). We infer that increasing tracer volume and injecting prior to surgery improves transcutaneous 'hotspot' identification, but that very high injection volumes lead to an increase in the number of nodes excised.

INTRODUCTION

Sentinel lymph node biopsy (SLNB) is the standard of care for axillary staging of breast cancer patients with a clinically and radiologically normal axilla [1-6]. The gold-standard technique is the 'combined technique' with interstitial injection of technetium-labelled nanocolloid and blue dye into the breast. SLNB offers the benefits of minimally invasive surgery (less morbidity) and a low false negative rate. [4, 7] However, the reliance upon radioisotopes has drawbacks in terms of radiation exposure, the short (six hour) half-life of technetium 99m (^{99m}Tc), handling and disposal of radioisotopes, the training of medical staff and legislative requirements. Perhaps for these reasons, and despite the incidence of cancer rising, over the last decade the performance of the SLNB procedure has reached a plateau with around 60 per cent of an estimated 500,000 patients in the Western world having access to the procedure. [8] This figure drops to 5 per cent in China and is minimal in the rest of the world. [9] This has led to interest in the development of novel techniques not reliant upon radioisotopes (our Lancet reference in press – will be published by the time this is accepted) **which are currently restricted uptake of dye by higher echelon nodes [10-12] and high false negative rates. [13] (This seems a non-sequitur – what does it mean?)** The magnetic technique, developed by Douek et al (ref), is one of the most promising alternatives. A sterile, aqueous suspension of superparamagnetic carboxydextran-coated iron oxide (SPIO) is injected interstitially into the breast and travels to the axillary lymph nodes. It is distributed within the sinuses, subcapsular space and parenchyma of the nodes. [14]. High power microscopic examination has revealed iron predominantly sequestered within macrophages. Once taken up by macrophages in the mononuclear phagocyte system of the lymphatics, the magnetic tracer is believed to be broken down and distributed across iron stores in the body. [15] The magnetic tracer can be detected intra-operatively using a handheld magnetometer [16-18].

Magnetic SLNB for breast cancer has been demonstrated as a feasible technique in three published clinical trials. [16-18] The largest trial, the SentiMAG Multicentre Trial, [17] recruited 161 patients (170 SLNB procedures) and found that the magnetic technique was non-inferior to the standard dual technique. This was confirmed by a later study, the Central-European SentiMag Study, [18] of 150 patients. However, the false negative rate has been unacceptably high in studies of magnetic SLNB. **Shiozawa *et al* [16] (would be good here to list how many patients, and maybe give this trial a name to - to be consistent with the other two)** reported a false negative rate of 17 per cent using the magnetic technique and even the SentiMAG Multicentre Trial, [17] which was found to be non-inferior to the dual technique for SLN identification demonstrated a false negative rate of 8 per cent and 4 per cent for the magnetic and dual techniques respectively. The Central-European SentiMag Study [18] identified a lower false negative rate for the magnetic technique of 3 per cent *versus* 9 per cent for the standard radioisotope technique, inconsistent with the previous studies. The higher false negative rate in the standard technique in this trial may be explained by the omission of blue dye, which is known to improve the SLN identification rate and lower the false negative rate of the dual technique. [7, 19] All studies injected the magnetic tracer periareolarly after induction of general anaesthesia, with Shiozawa *et al* [16] injecting 1.6 mL Resovist (Bayer Health Care Osaka, Japan; 27.9 mg iron/mL), and the other two trials both using 2 mL Sienna+ (27mg iron/mL) diluted in 3 mL normal saline. [17, 18]

In order to make the magnetic technique a creditable alternative to the current dual technique, it is essential that we understand the behaviour of magnetic tracers from the injection site to distribution within the lymphatic basin. Outstanding issues include the optimal volume of magnetic tracer to administer and timing of injection prior to surgery. By identifying these factors it would be possible to optimise the SLN identification rate of the magnetic technique,

reduce the false negative rate and prevent excision of higher echelon SLNs. Our group previously developed a porcine model which closely replicates human size as well as vasculature and lymphatic drainage. This model was used to successfully demonstrate the feasibility of magnetic SLNB using 16 mini-pigs and 32 SLNB procedures. [20] Anninga and Ahmed *et al* [20] identified a significant correlation between magnetometer counts and the magnetic tracer content of *ex vivo* SLNs and the significant association between the grading of *ex vivo* SLNs for their tracer content and magnetometer counts. [20] This model had therefore been validated for the purpose of magnetic SLNB. Based upon our previous experience, it was possible to determine an adequate sample size via power analysis to identify the optimal volume of magnetic tracer required to ensure maximal SLN tracer uptake and therefore optimise clinical SLN identification and potentially reduce false negative rates using the magnetic technique.

MATERIALS AND METHODS

This study was conducted at the IRCAD institute, Strasbourg (France), King's College London (United Kingdom) and the Universiteit Twente, Enschede (The Netherlands). Ethical permission was granted for animal experimentation, by the IRCAD Ethics Review Board, Strasbourg, France (Reference number: 38.2013.01.056). Mini-pigs used for the IRCAD laparoscopic general surgical skills course were surgically prepped and anaesthetized for the purpose of the course. Prior to commencement of the laparoscopic skills course a magnetic tracer (Sienna+, Endomagnetics UK; 27mg iron/mL) was injected subcutaneously into the areolar of the left and right 3rd inguinal mammary glands in 24 mini-pigs.

The performance of SLNB in porcine model:

Magnetic tracer was injected in escalating volumes between 0.06 mL and 2 mL neat. A handheld magnetometer (SentiMag, Endomagetics UK) was then used to localize any *in vivo* signal from draining inguinal lymph nodes up to 60 minutes after injection using 15-minute intervals and these repeated again 4 hours later on completion of the laparoscopic skills course. Bilateral groin SLNB was undertaken at the site of magnetic 'hot spots' (*Fig.1*). All lymph nodes with a magnetometer count higher than 10 per cent of the hottest node were considered to be SLNs and were excised, with *ex-vivo* counts also recorded. Once the SLNB was completed, a groin node clearance was performed to remove all lymph nodes from each groin basin. The harvested SLNs were fixed in formalin and sent to Universiteit Twente, Enschede (The Netherlands), where the quantification of magnetic tracer in each excised node was performed using vibrating sample magnetometry (VSM) on a Physical Properties Measuring system (PPMS, Quantum Design Inc., San Diego, CA, USA). The measurements were performed using a magnetic field of 4.0 T, which is required to bring the magnetic iron oxide (maghemite, $\gamma\text{-Fe}_2\text{O}_3$) nanoparticles to saturation. The amount of magnetic tracer in the lymph nodes was determined by comparing the obtained amplitude of the magnetization to known calibration samples, and was reported as an 'iron content', i.e. the mass of Fe in the node, present in the form $\gamma\text{-Fe}_2\text{O}_3$. The nodes from the groin clearances were also fixed in formalin and sent to King's College London (United Kingdom) where they underwent careful dissection to accurately determine the total number of nodes in each clearance specimen and random sampling magnetometer counts to ensure no SLNs were present in the specimens.

Statistical analysis:

Based upon data accrued from our previous feasibility study [20] a power calculation was performed to determine sample size. We conducted a two-sided test ($\alpha=0.05$) expecting a difference of 50 μg (SD: 30) in iron content readings between different volumes of tracer. When performing a total of 6 procedures (3 mini-pigs) for each volume of magnetic tracer (0.06-2.0

mL), these 48 procedures provided us with a power of 82% to detect this difference. The correlation between continuous variables was calculated using the Pearson's correlation coefficient (r) and associations between categorical and continuous variables using analyses of variance (ANOVA).

RESULTS

A total of 48 SLNB procedures followed by 48 groin node clearances were performed on 24 mini-pigs. All 48 were successful and at least 1 'hot' node was identified in each procedure. *In vivo* magnetic 'hot spots' from the draining inguinal lymph nodes were identified transcutaneously prior to surgical incision using the handheld magnetometer, with all volumes of administered magnetic tracer. A total of 423 nodes were harvested (mean 8.8 nodes (SD 2.7) per groin, range 4-14), of which 109 were SLNs (mean 2.2 nodes (SD 1.4) per groin, range 1-7). A significant linear relationship was demonstrated between the handheld magnetometer counts and the iron content of excised SLNs recorded on VSM ($r=0.82$; $p<0.001$) (*Fig.2*).

The impact of the volume of magnetic tracer injected:

Increasing the volume of magnetic tracer injected did not result in a significant difference in the magnetometer counts of the excised SLNs ($P=0.37$) (*Fig.3a*). There was a trend (not statistically significant; $P=0.07$) observed between the volume of the magnetic tracer injected and the iron content of the excised SLNs assessed with VSM ($P=0.07$) (*Fig.3b*). However, there was a significant correlation between the percentage iron-uptake (relative to injected dose) of the excised SLNs and the volume of magnetic tracer injected ($P<0.001$) (*Fig.3c*). This is demonstrated by a reduction in the percentage iron-uptake (amount of iron taken up by each

node, relative to the amount injected) from a mean of 25 per cent for 0.06 mL volume of magnetic tracer to less than 2 per cent for 2 mL of magnetic tracer.

Increasing the volume of magnetic tracer from 0.06 mL to 2 mL resulted in a significant increase in the mean number of SLNs excised from 1 to 4 ($P < 0.001$) (*Fig. 3d*).

The relationship between the volume of magnetic tracer and time elapsed since injection:

A significant correlation was observed between transcutaneous magnetometer counts over the sentinel node and the time elapsed since injection of magnetic tracer. The longer the time since injection, the stronger was the transcutaneous magnetic hot spot ($P < 0.0001$) (*Figs. 4a*). The mean magnetometer count at 240 minutes post-injection was double that of the count at 5 minutes. There was no significant difference between the transcutaneous magnetometer counts and volumes of magnetic tracer injected between 5 and 60 minutes post-injection (*Figs. 4b(i)-(v)*). However, at 240 minutes from injection, there was a significant correlation between increasing volume of magnetic tracer and higher magnetometer counts ($P < 0.009$) (*Fig. 4b(vi)*).

Iron content of excised SLNs measured by VSM (μg):

The iron content of *ex vivo* SLNs was in the range of 41 to 1431 μg (mean 463.9 μg (SD 401)). The peak distribution of iron content per excised SLN was in the range of 101 and 200 μg , representing 23 per cent of the iron content of all nodes (*Fig. 5*). A total of 71 per cent of excised SLNs possessed iron contents below 600 μg .

DISCUSSION

The magnetic technique for SLNB successfully identified at least 1 SLN in all 48 porcine groins in which it was performed. This study confirmed a significant correlation between the handheld magnetometer counts and the iron content of *ex vivo* SLNs as recorded on VSM measurements. The Pearson's Correlation (r) of 0.82 (*Fig.2*) was in keeping with our previous study in which $r=0.86$, [20] reaffirming the ability of handheld magnetometers to quantify iron content in-vivo. A future application of this would be in the axillary staging of breast cancer using SPIO-enhanced magnetic resonance imaging (MRI). By using the handheld magnetometer for clinical quantification of iron content of excised SLNs and subsequent performance of *ex vivo* MRI of these nodes, it would be possible to optimise the volumes of magnetic tracer required to allow satisfactory characterisation of SLNs, minimizing the artefact observed with higher concentrations of SPIO. By allowing characterisation of SLNs to be performed on MRI, it would be possible to allow patients with metastatically involved axillary nodes to be directed immediately to surgery and those without involvement to undergo observation (without the problem of post-operative scarring), eradicating the need for an unnecessary invasive SLNB. [21]

We did not identify any significant correlation between handheld magnetometer counts or iron content of *ex vivo* SLNs and the volume of magnetic tracer injected (*Figs 3a and b*). This suggests that current practice of using 2 mL of magnetic tracer as in the SentiMAG Multicentre Trial is excessive, [17] with SLNB being possible with much lower volumes. Our results demonstrate that the lowest volume of magnetic tracer injected of 0.06 mL resulted in a mean iron uptake of the excised SLNs of 25 per cent compared to less than 2 per cent for the maximal volume of 2 mL ($P<0.001$) (*Fig.3c*). This decrease in percentage iron uptake was mirrored by an increase in the number of excised SLNs in the porcine model (*Fig.3d*). It has been shown that the iron content within SLNs after interstitial magnetic tracer injection is predominately distributed

within the subcapsular space and sinuses of the lymph nodes. [14, 20] Our results suggest that the porcine SLNs have a saturation limit for the iron content distributed within them. Once this saturation point is exceeded the iron is passed on to the next echelon SLN, which once its saturation point is exceeded will similarly result in passage to the next nodes. The distribution of the iron content of the excised SLNs demonstrated that 71 per cent of nodes have iron content below 600 μg , with the peak distribution of iron content being between 101-200 μg (*Fig.5*). This would suggest that the majority of porcine SLNs have their saturation limit within this range. The volume of 0.06 mL resulted in a median of 1 SLN excised, compared to 4 (range 2-7) for 2 mL ($P<0.001$). This suggests that by varying the volume of magnetic tracer it is possible to determine the echelon level of SLNs excised and the number retrieved. Our results would suggest that by using a volume of 0.5 mL it is possible to excise a median of 2 SLNs (range 1-3), which would be ideal in most clinical scenarios. Should other clinical situations require greater numbers of SLNs to be excised – such as in the performance of SLNB after primary systemic therapy in breast cancer (where elevated false negative rates are known to be an issue) [22] – an increased volume of 2 mL could be administered to provide a median of 4 nodes. This ability to control the level of echelon nodes excised may prove an advantage over other developing novel techniques for SLNB, such as indocyanine green (ICG) fluorescence, in which studies have demonstrated the mean number of excised SLNs ranging from 1.75-5.4 for the same volume of 1 mL ICG administered. [12, 23, 24]

Clinically, more than 2 mL of magnetic tracer has not been used. Furthermore, the nodal saturation point in humans is likely to be higher than that in mini-pigs. Our results would suggest that if we continued to increase the volume injected beyond a saturation point, we would increase the number of sentinel nodes retrieved. Likewise if we injected lower volumes of magnetic tracer we may ultimately reach a volume where almost all iron injected would be taken up by a draining inguinal lymph node. If we base this upon our results where the lowest

uptake by a node to allow it to be identified by the magnetometer was 41 µg of iron, this would correlate with an injection of 0.002 mL of magnetic tracer. The important finding is that the lower volumes studied from 0.06 mL allow feasible SLN identification comparable to volumes up to 2 mL and by manipulating these volumes it is possible to determine the level of draining echelon SLNs that are identified.

The benefit of magnetic SLNB is that it eradicates the need for nuclear medicine input for the administration of radioisotopes. This means that the control of the performance of SLNB is directly within the hands of the operating surgeon who administers the magnetic tracer. All 3 published clinical studies [16-18] for the use of the magnetic technique injected the magnetic tracers after induction of general anaesthetic, periareolarly into the breast. Although the optimal timing of the magnetic tracer is not yet known, our results suggest that a pre-operative injection (4 hours or more prior to surgery) is likely to significantly improve the percutaneous magnetometer count and by doing so, improve the identification rate. We performed transcutaneous magnetometer counts at 15-minute intervals, commencing 5 minutes after injection up to 60 minutes and then again at 240 minutes, just before performing surgery. The timing of the transcutaneous magnetometer counts from the draining inguinal lymph nodes after injection of the magnetic tracer was significantly associated with the overall value of the magnetometer counts themselves. The transcutaneous magnetometer counts were significantly greater for all volumes injected (*Fig.4a*). However, a significant increase in the transcutaneous magnetometer count was only observed after 240 minutes from injection of magnetic tracer (*Figs 4b(i)-(v)*). (*Fig. 4b(vi)*). This also has important clinical implications for the magnetic technique in order to allow a transcutaneous magnetic 'hotspot' to be identified and therefore the optimal positioning of the skin incision for technical and aesthetic outcomes in SLNB. The handheld magnetometer counts demonstrate a reducing logarithmic relationship ($r=-0.97$; $P<0.001$) for increasing depth of injection [15]. Therefore, to assist in transcutaneous

hotspot detection in the axilla, which can vary greatly upon the depth of location of the SLN, it is essential that maximal iron uptake is achieved to attain the greatest possible transcutaneous magnetometer counts. Our study demonstrates that a gap of 240 minutes from injection to transcutaneous magnetometer count readings will result in the highest transcutaneous magnetometer counts to be recorded for all volumes of magnetic tracer injected (*Fig. 4a*). However, by 60 minutes post-injection the mean transcutaneous magnetometer count was 5000 counts compared to just 6000 at 240 minutes when all volumes of tracer were compared (*Fig. 4a*). This suggests a plateau in the transcutaneous magnetometer counts has been reached and that any prolonged delay between injection and surgery beyond 60 minutes may not be clinically relevant. However, it is important to mention that caution must be taken in attempting to optimise the transcutaneous 'hotspot' count as it can compromise other outcomes for magnetic SLNB. By increasing both the delay between injection and surgery and volume of magnetic tracer injected, there is a risk of increasing average node retrieval, by the unnecessary removal of higher echelon nodes.

There are practical issues to implementation of this delay between injection and performance of SLNB. Firstly, significantly increasing the time gap between surgery and injection would mean injecting pre-operatively, which, in the case of a periareolar injection, could cause discomfort. Increasing the volume of magnetic tracer injected (periareolar) could cause increase discolouration and discomfort. A practical alternative approach is to evaluate intratumoral injection of the magnetic tracer under ultrasound-guidance using local anaesthetic, for synchronous lesion localisation and sentinel node biopsy – as is currently being evaluated within the UKCRN MagSNOLL Multicentre Trial [25].

CONCLUSION

The magnetic technique for SLNB requires further optimisation prior to evaluation within a randomised controlled trial. In a porcine model, magnetic SLNB is feasible even at very low volumes of injected magnetic tracer (0.06-2 mL). Injection of higher volumes of magnetic tracer, beyond a saturation point, results in an increase in node retrieval rate. An injected tracer volume of 0.5 mL, providing a median number of 2 SLNs (range 1-3), is ideal for most clinical situations. Pre-operative injection of magnetic tracer (4 hours prior to surgery), improves identification of transcutaneous 'hot spot', facilitating sentinel node identification. Further trials should evaluate pre-operative injection and lower volumes of injected magnetic tracer.

Acknowledgements

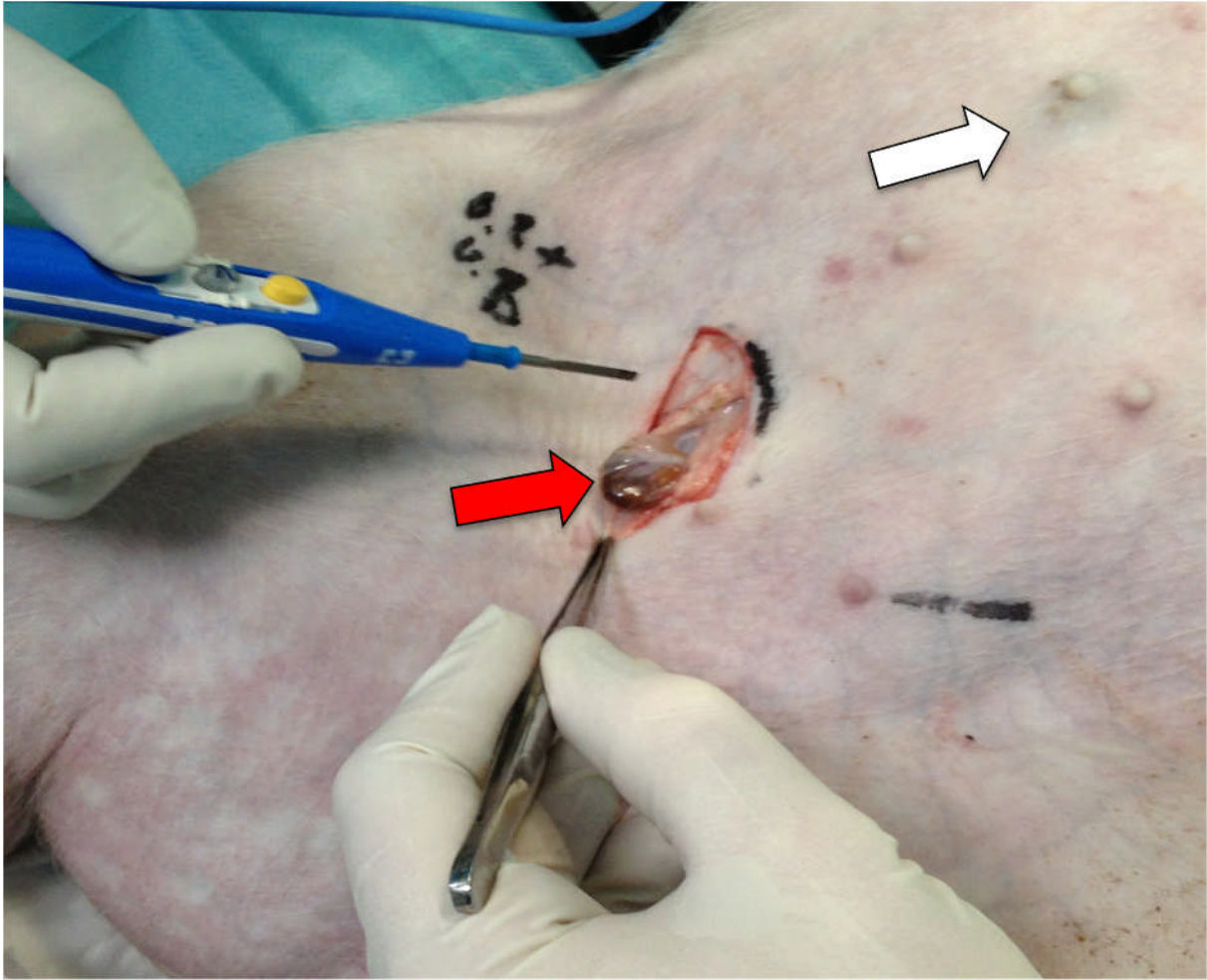
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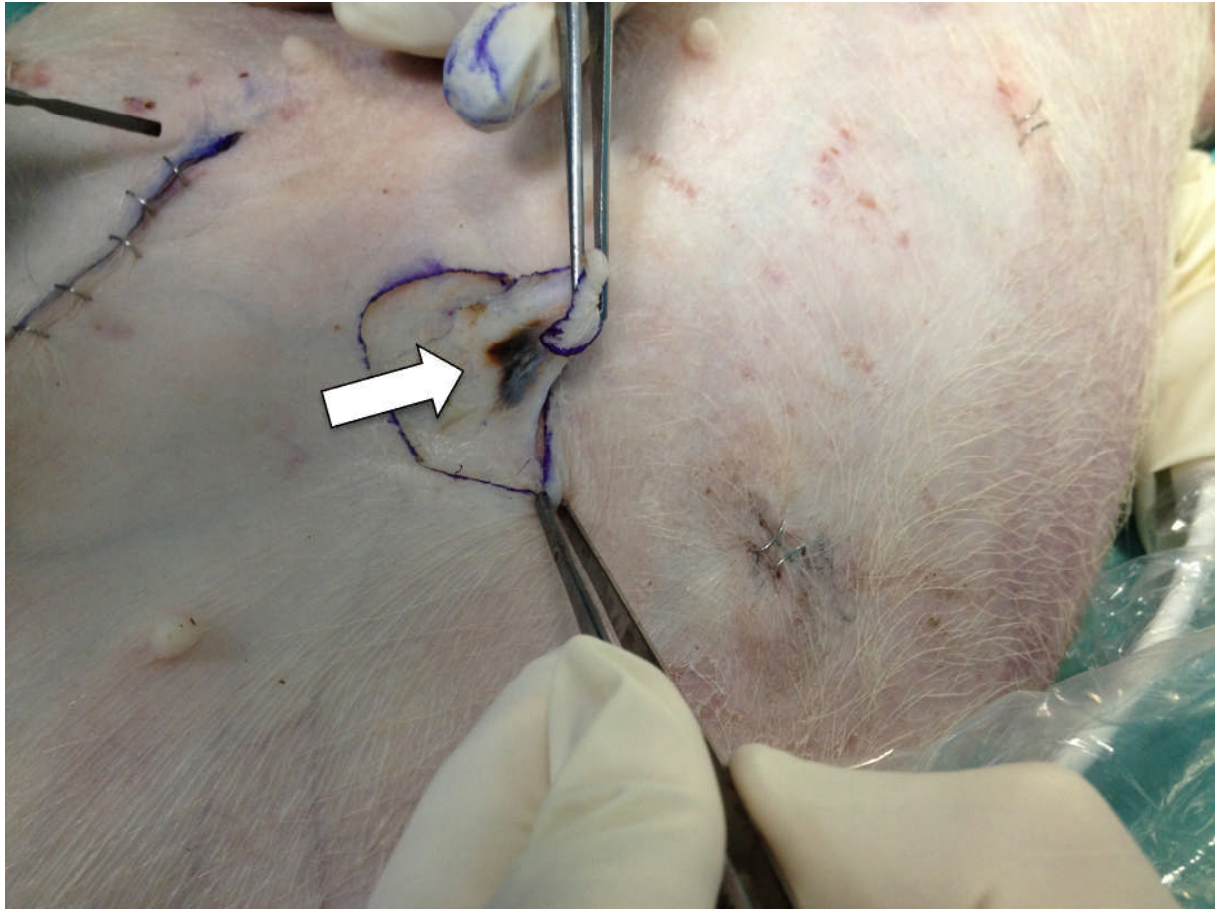
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Figure 1. The operative procedures performed:



a) *In vivo* SLNB with the injection site indicated with the white arrow and the brown discolored node *in-vivo* indicated with the red arrow



b) *In vivo* magnetic-guided excision of the mammary gland injection site (white arrow indicates brown-staining from injection of magnetic tracer).

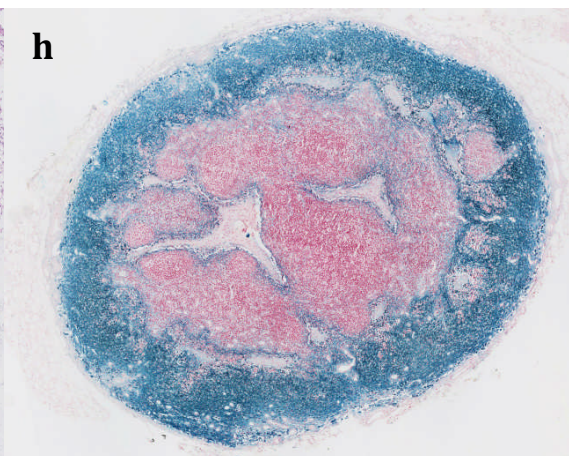
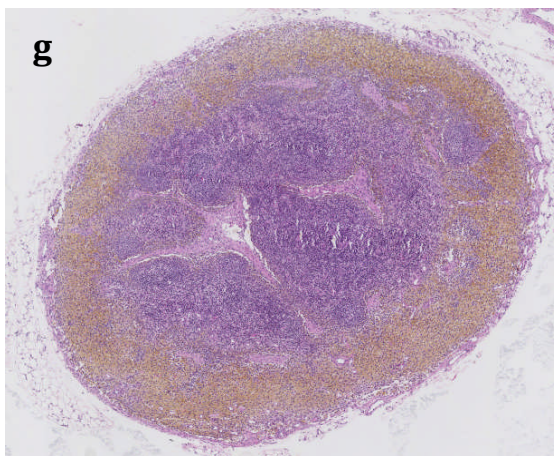
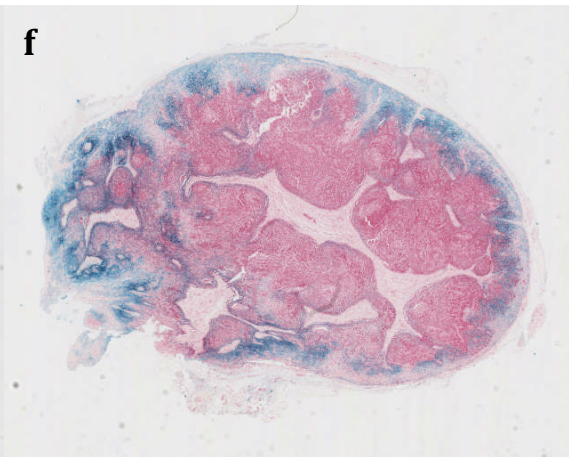
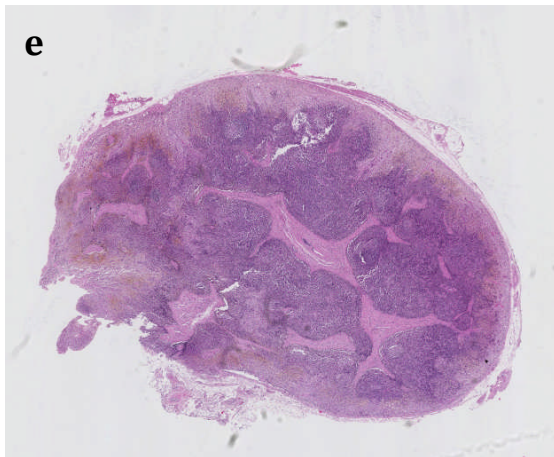
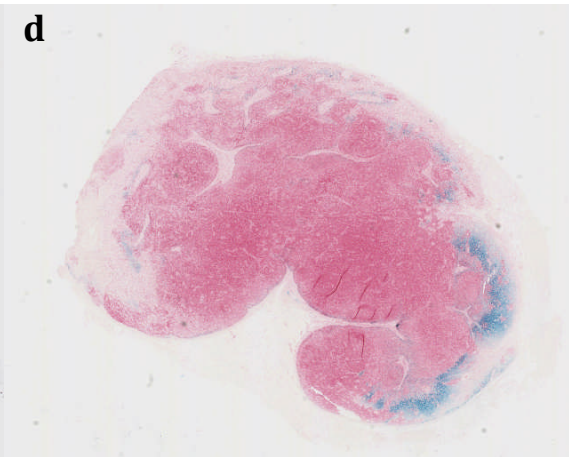
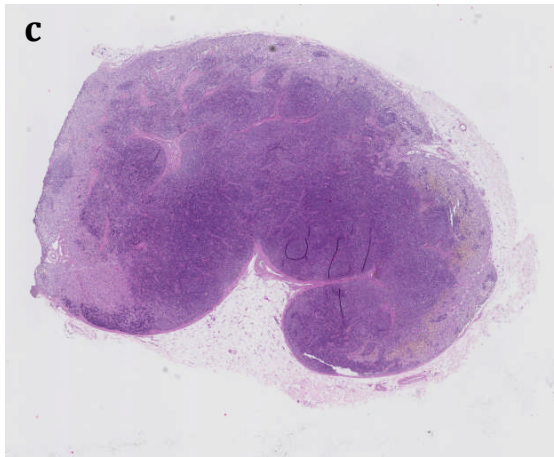
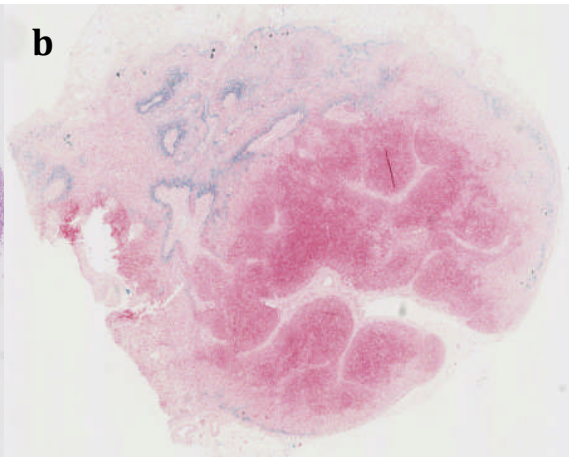
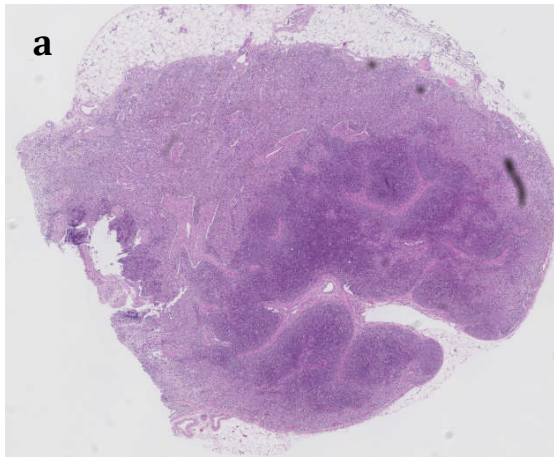
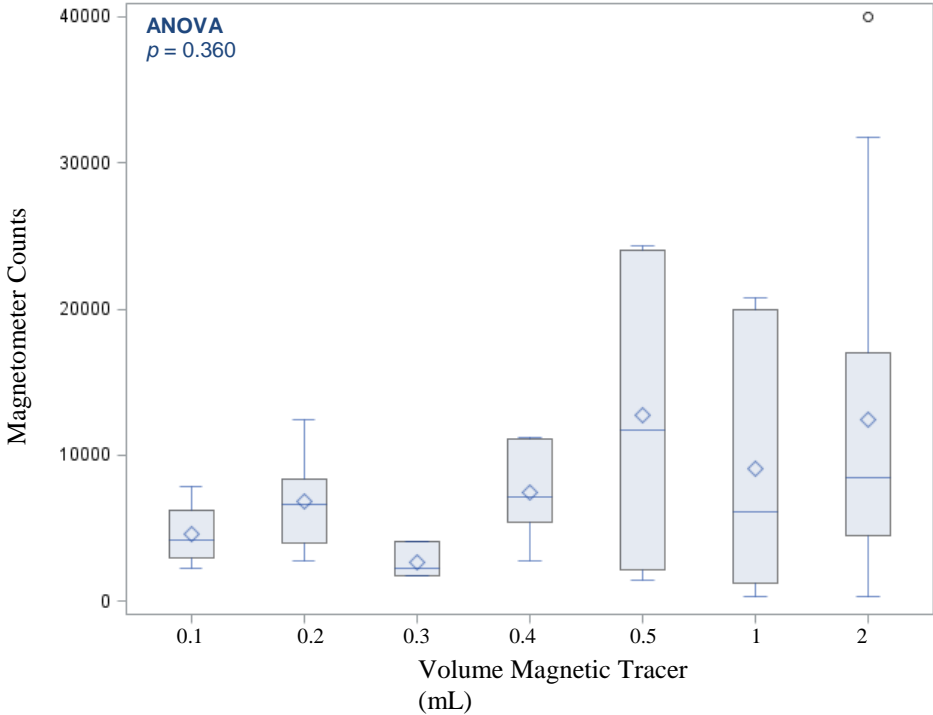
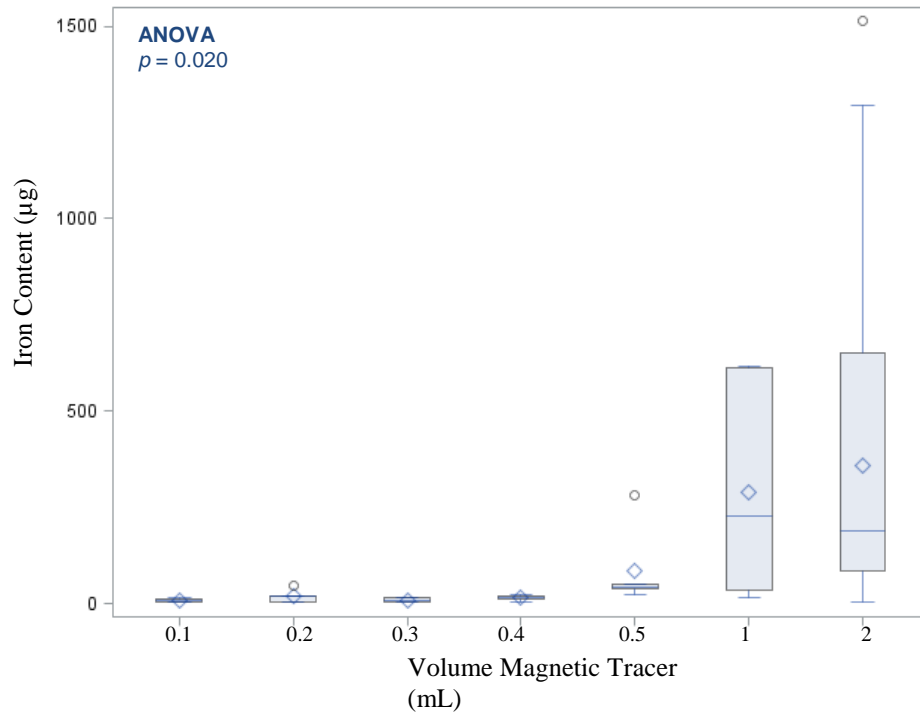


Figure 2: Examples of sentinel lymph nodes that were scored 1 to 4 after H&E (a,c,e and g) and Perl's (b,d,f and h) staining clearly showing the brown discoloration in the cortex of the nodes

Figure 3. The relationships for variations in the volume of magnetic tracer:



a) Boxplot of the volume of magnetic tracer *versus* magnetometer counts from excised sentinel lymph nodes



b) Boxplot of the volume of magnetic tracer *versus* iron content of sentinel lymph nodes

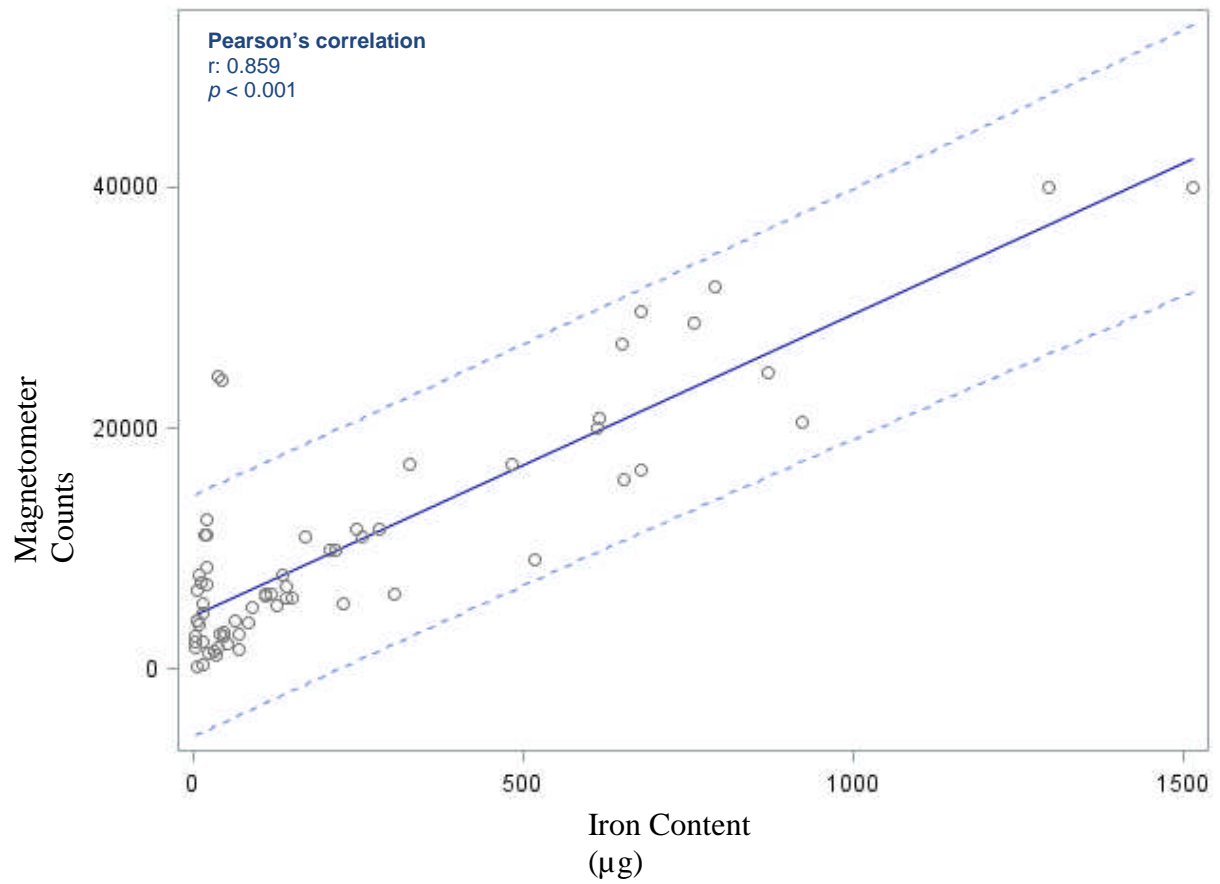
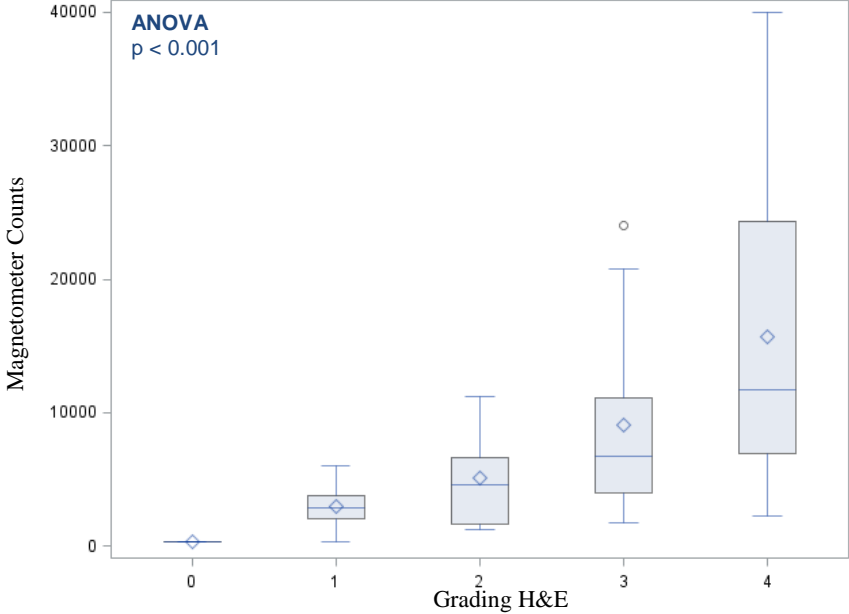
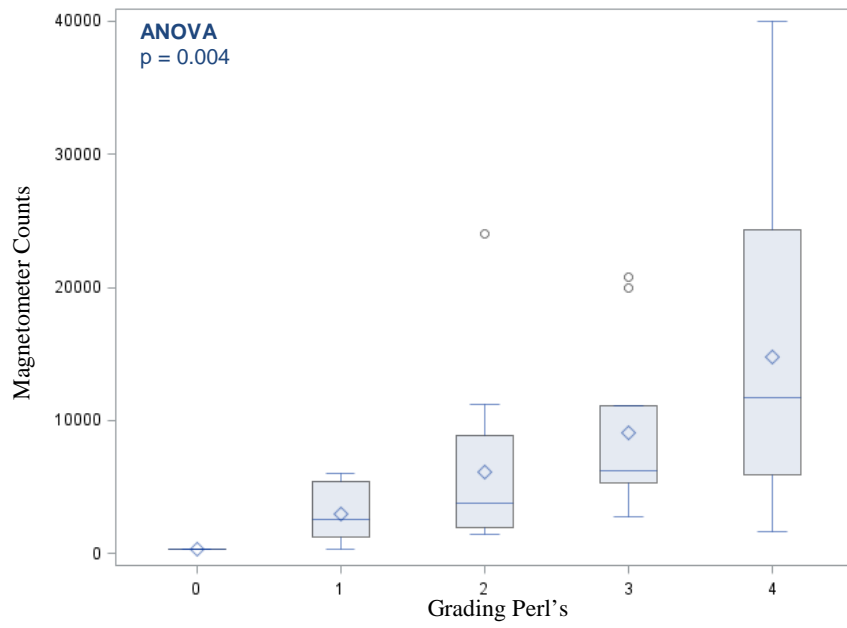


Figure 4. Graph demonstrating the relationship between the iron content and magnetometer counts of excised sentinel lymph nodes

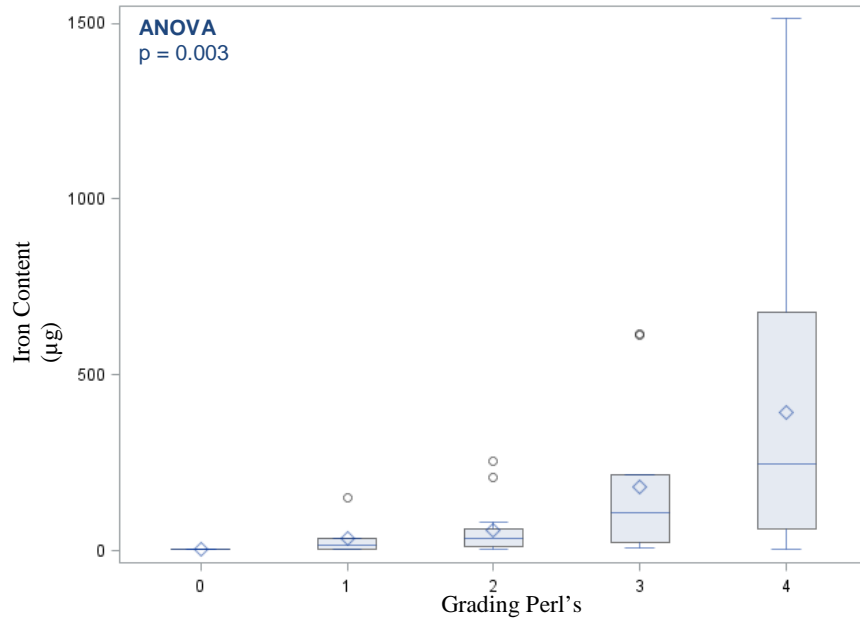
Figure 5. The relationships for the histopathological grading of excised sentinel lymph nodes:



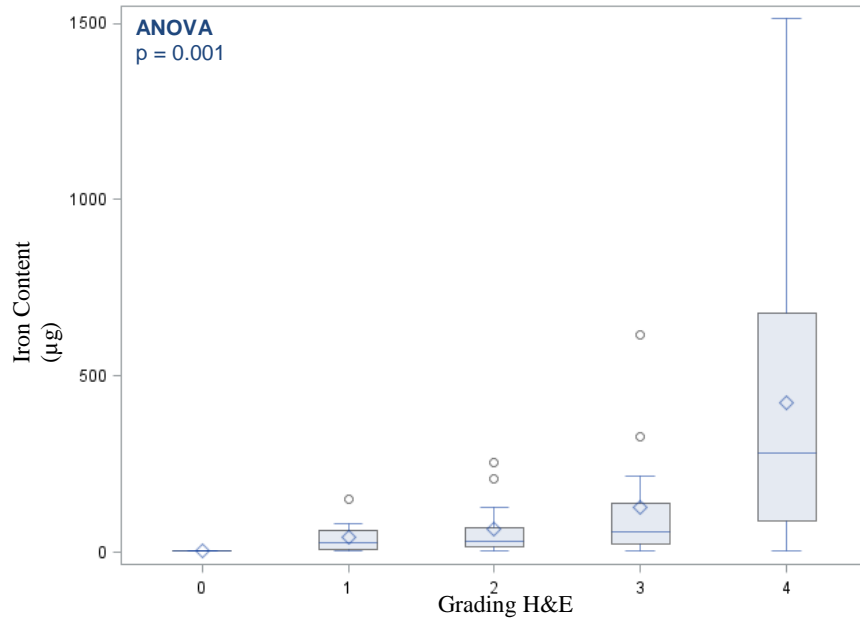
a) Histopathological grading (H&E) *versus* magnetometer counts of excised sentinel lymph nodes



b) Histopathological grading (Perl's) *versus* magnetometer counts of excised sentinel lymph nodes

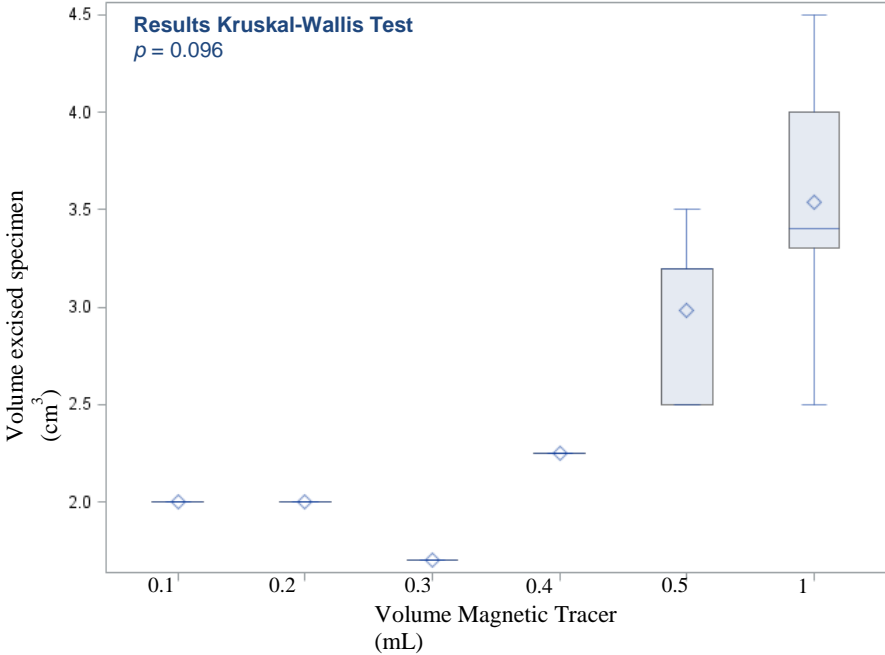


c) Histopathological grading (Perle's) *versus* iron content of excised sentinel lymph nodes

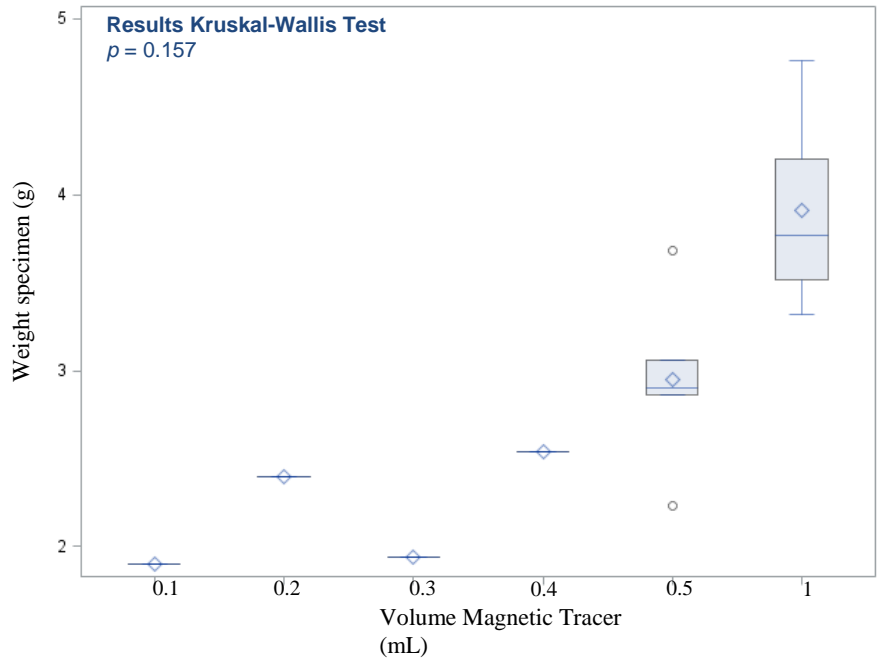


d) Histopathological grading (H&E) *versus* iron content of excised sentinel lymph nodes

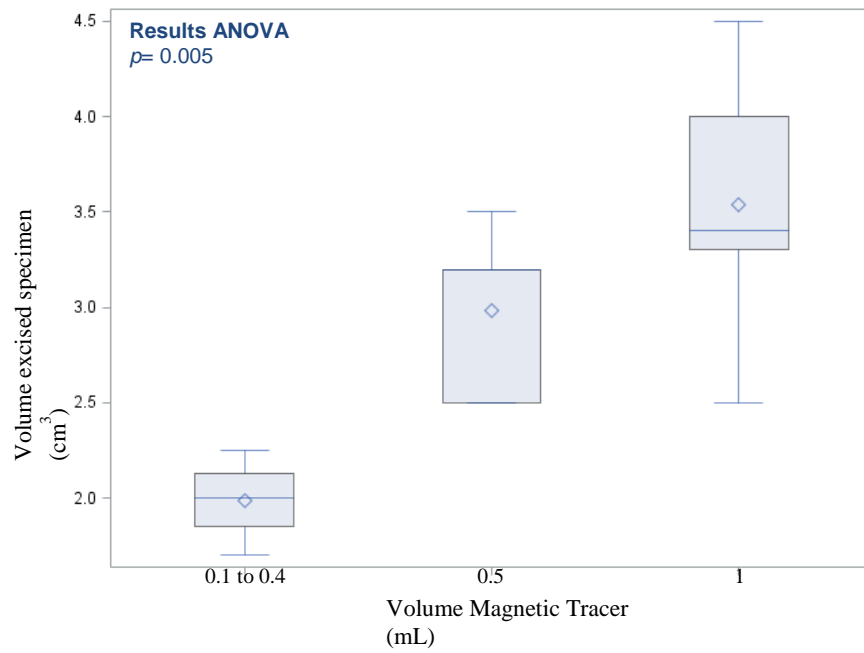
Figure 6. The relationships of the volume of magnetic tracer injected and excised injection site specimens:



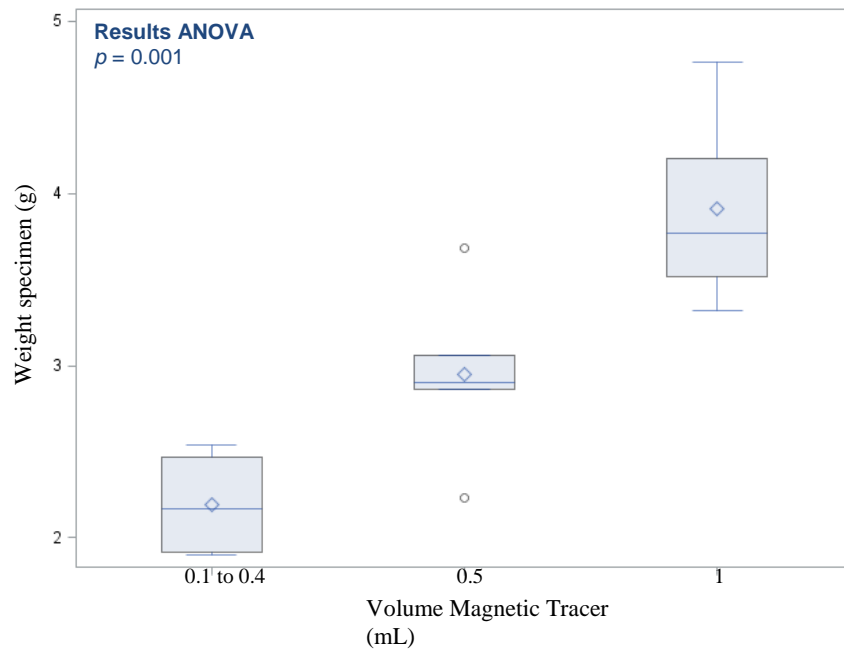
a) Volume of magnetic tracer injected versus the volume of excised specimens



b) Volume of magnetic tracer injected versus the weight of excised specimens

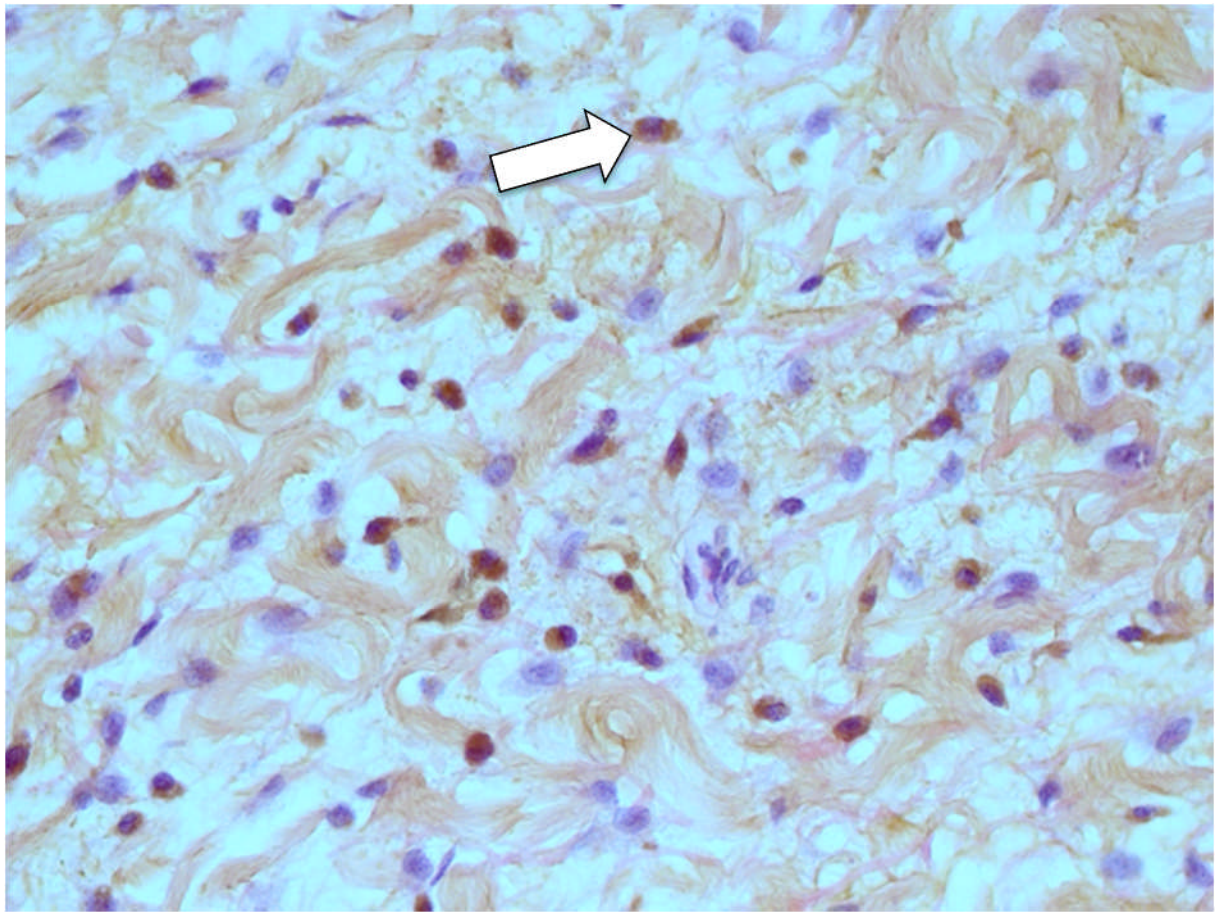


c) Volume of magnetic tracer injected versus the volume of excised specimens with 0.1 to 0.4 mL considered as a single group for analysis

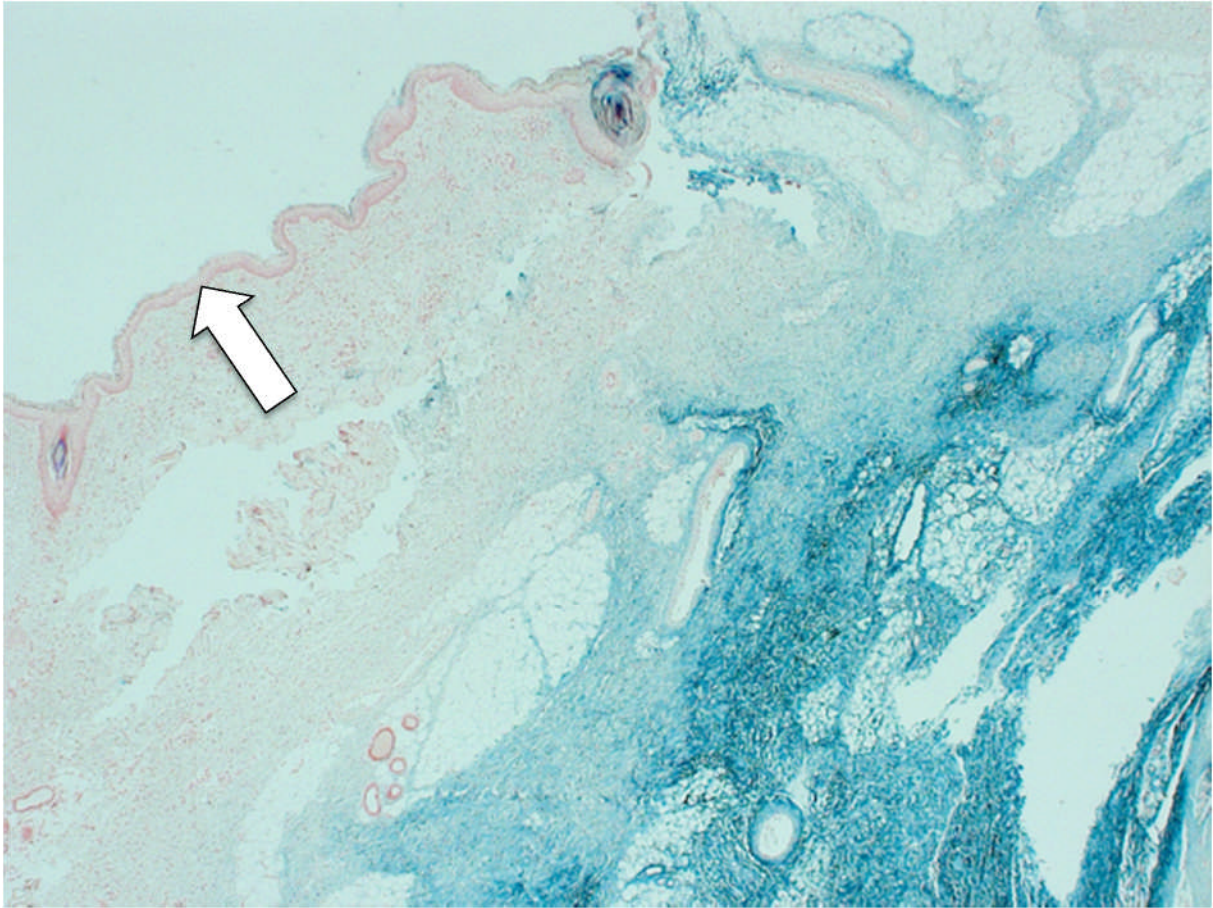


d) Volume of magnetic tracer injected and weight of excised specimen with 0.1 to 0.4 mL considered as a single group for analysis

Figure 7. Histopathological assessment of the magnetic tracer injection sites:



a) The presence of intermittently dispersed brown stained macrophages (white arrow) at the site of injection (magnification x40).



b) Perl's staining of the excised injection site demonstrating the blue discolouration of the stained iron deep to the skin surface (white arrow) surrounding the lactiferous ducts (low power).