2.0 Materials and Methods

2.1 Study sample and sampling method

This prospective study was performed with approval from the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg, South Africa (Appendix A - B). Ethics approval number M090101. The study was conducted at Chris Hani Baragwanth and Charlotte Maxeke Johannesburg Academic hospitals, Johannesburg, South Africa, both large, tertiary, urban, public-sector hospitals.

Fifty FNAs of primary or metastatic adenocarcinoma of the lung and metastatic adenocarcinoma to liver or lymph nodes were performed from February 2009 to December 2009. Palpable masses e.g. superficial lymph nodes are aspirated by cytology staff trained in FNA technique. Deep-seated masses are aspirated by radiology personnel, mainly radiology registrars, who receive exceedingly variable instruction on FNA technique. Material from lymph node, lung and liver masses was aspirated for conventional smears and cell block preparation (refer to Appendix C) simultaneously. Four slides were made from two needle passes. As per this cytology unit's routine procedure, two were fixed immediately in alcohol for Papanicolaou (Pap) staining (refer to Appendix D), and two were air dried for Diff-Quik staining (refer to Appendix E). Samples collected for the cell block were either from an additional dedicated needle aspiration and / or needle rinse of the existing needle passes. These were stored in Shandon's Formal –Fixx® solution for at least 12 hours (maximum of 48 hours) before processing using the Shandon Cytoblock Kit®. Even if a dedicated extra aspiration was

performed, the other needle passes were rinsed in all cases to obtain the highest possible cellular yield.

A comparison between the grading (on an ordinal scale) of cellularity, morphological preservation, architectural preservation, immunocytochemical staining intensity and presence of background staining was performed on paired FNA smears and cell block samples (refer to Table 2.1). Each arm of the paired analysis was performed blindly (separately and at different times) by experienced cytologists without knowledge of the grading outcome of the other.[50, 51] All the samples were assessed by the candidate (Shehnaz Khan) under a double-headed microscope together with one of two experienced cytologists (Drs Tanvier Omar and Pam Michelow) who alternated. This was in an effort to reduce the workload of these experienced cytologists who work in an extremely understaffed cytology unit.

The cellularity, morphology and architecture of each cell block sample was evaluated under the microscope using the Haematoxylin and Eosin (H/E) stain (refer to Appendix F) and that of the FNA smear using the Pap stain and/or Diff-Quik stain especially where the Pap stain was not available, since the best Pap slide containing the most representative material was usually used for split slide conventional immunocytochemistry (ICC) assay (refer to Appendix G). This technique involves de-staining Pap stained FNA smears (refer to Appendix H) followed by splitting the slide and re-staining for the respective ICC tests.

The grading system was modified, as detailed in table 2.1, from the work of Bhatia & Dey et al 2008,[51] Goldstein & Bosler et al 2007 [52] and Liberman & Weidner 2000 [53] respectively. The only modification being the assessment of nuclear and cellular detail which was not graded under a separate category but included in the grading of morphological preservation. The cellularity, morphological preservation, architectural preservation and immunocytochemistry on FNA and cell block samples were graded as detailed in table 2.1.

Table 2.1: The grading system [51,52,53]

SCORE	DESCRIPTION
Cellularity [51]	Cellularity was qualitatively graded as follows:
0	No cells
1+	low (tumour cells represent <10% of cells present)
2+	moderate (tumour cells represent 10-50% of cells present)
3+	high (tumour cells represent >50% of cells present).
Morphological Preservation [51]	Examination of presence or absence of crisp, well-preserved clear nuclear chromatin, nuclear margin, cytoplasm contents and cytoplasmic membrane.
0	Poorly preserved
1+	Well preserved
Architectural Preservation [51]	Examination of presence or absence of tissue architecture as evidenced by cellular relationship with each other e.g. a honeycomb arrangement in adenocarcinoma, or molding in neuro-endocrine carcinomas.
0	absent
1+	present
ICC [51, 52, 53]	Focal staining refers to positive staining of cells concentrated to specific area/s of the sample; diffuse staining refers to positive staining of cells spread throughout the sample.
0	negative / absent staining
1+	focal weak intensity < 10% of tumour cells showing positivity
2+	focal moderate intensity 10-50% of tumour cells showing positivity
3+	focal strong intensity > 50% of tumour cells showing positivity
4+	diffuse weak intensity < 10% of tumour cells showing positivity
5+	diffuse moderate intensity 10-50% of tumour cells showing positivity
6+	diffuse strong intensity > 50% of tumour cells showing positivity
Background Staining [51, 52, 53]	Examination of presence or absence of background staining in relation to the smear / section.
0	no background staining
1	mild background staining (< 10% of smear / section)
2	moderate background staining (10-50% of smear / section)
3	severe background staining (> 50% of smear / section)

All ICC assays included the use of appropriately optimized primary antibodies in the presence of positive and negative controls. The known positive controls were respective formalin fixed paraffin embedded tissue for the relevant antibodies used as recommended by the manufacturer. The negative control comprised an additional slide of the test case in which the primary antibody step was omitted and Dako Antibody Diluent (S2022) was applied to the section instead. The Dako Real Envision Detection System (K5007) was used in all assays (Table 2.2).

Table 2.2: Primary antibodies: source and specifications

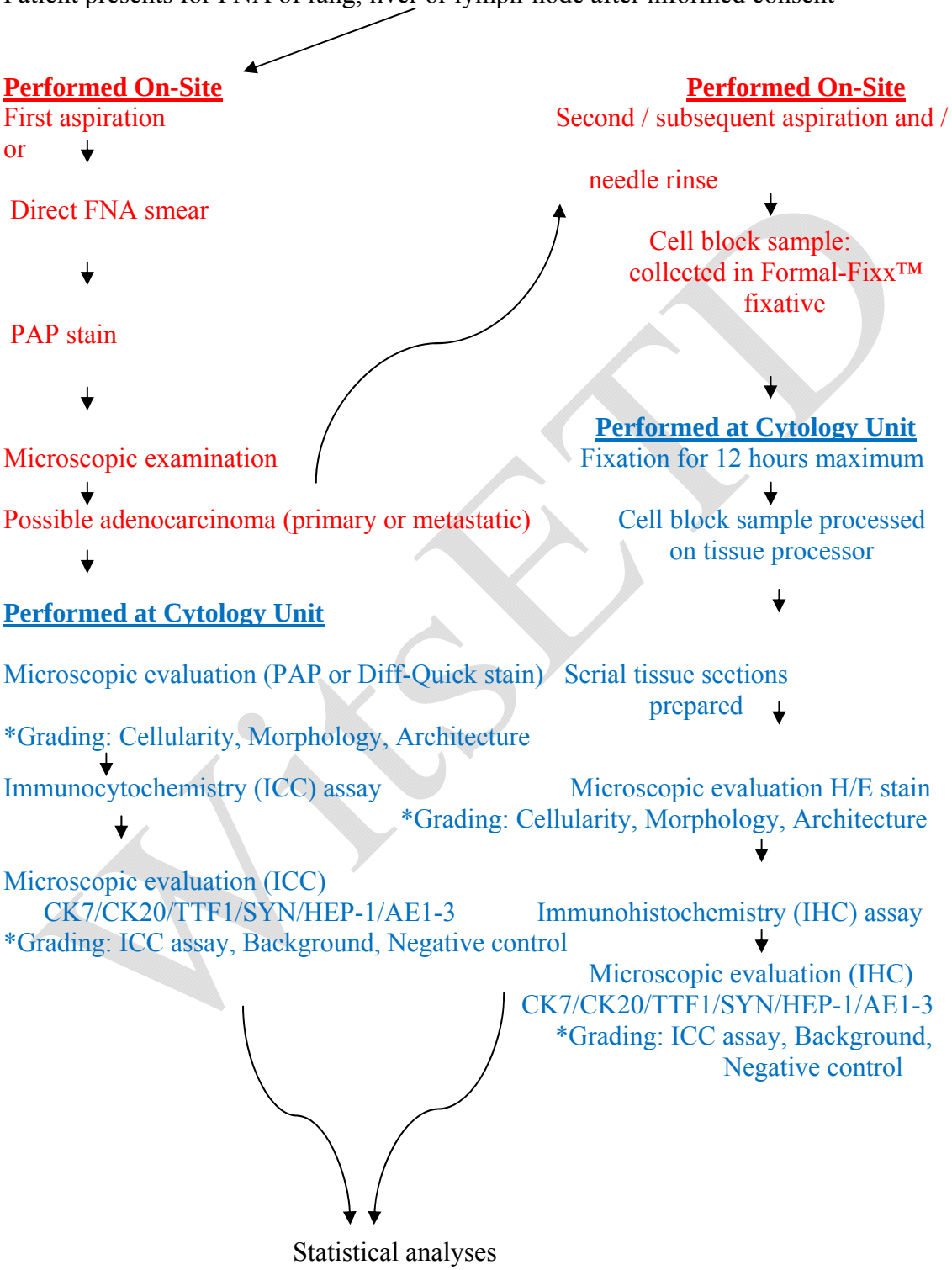
Primary Antibody	Source / Catalogue no.	Dilution	Epitope Retrieval Method	Enhancement
CK7	Dako / M7018	1:100	Tris-Edta Buffer, pH9.00	Pressure cooker
CK20	Invitrogen / 08-1200	Prediluted	Edta Buffer, pH8.00	Microwave
TTF1	Novocastra / NCL-L-TTF1	1:200	Edta Buffer, pH8.00	Microwave
Hep-1	Dako / M7158	1:50	Tris-Edta Buffer, pH9.00	Pressure cooker
AE1/3	Dako / M3515	1:50	Citrate buffer, pH6.1	Pressure cooker
Synaptophysin	Invitrogen / 18-0130	1:200	Nil	Nil

Invitrogen Corporation, 542 Flynn Road, Camarillo, CA 93012 USA

Novocastra Laboratories Ltd, Balliol Business Park West, Benton Lane, Newcastle upon Tyne, NE12 8EW, UK.

Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark

2.2 The flow of processes in this study



2.3 Statistical method used

The data were analysed using Stata Statistical Software 2007: Release 10, College Station, Tx: StataCorp. The agreement between the two methods of sample preparation was assessed with respect to the quality of definition of cytomorphological characteristics and ICC. The Kappa statistic (K) was used to measure inter-rater agreement. Kappa values > 0.75 represented excellent agreement beyond chance; values < 0.4 represented poor agreement beyond chance and values between 0.4 and 0.75 represented moderate agreement.[54] P-Values ≤ 0.05 were considered to be statistically significant.[54] (Appendix I refers). The statistical evaluation and interpretation was assisted by Professor Petrus Johannes Becker MSc (Pret), PhD (Unisa) Biostatistics Unit, Medical Research Council , South Africa and Division of Clinical Epidemiology, University of Pretoria, South Africa.

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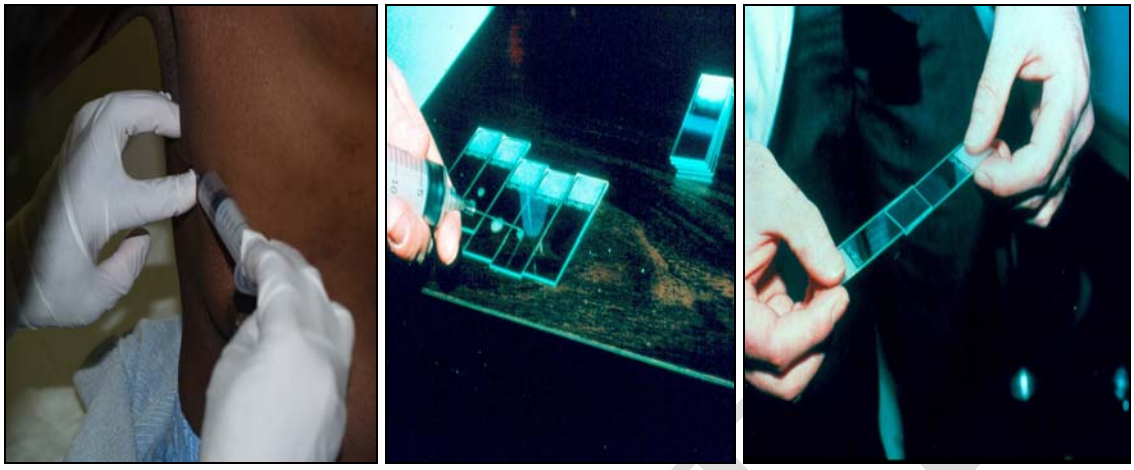

Figure 2.1 FNA performed on site (I). Direct smears prepared (II; III).

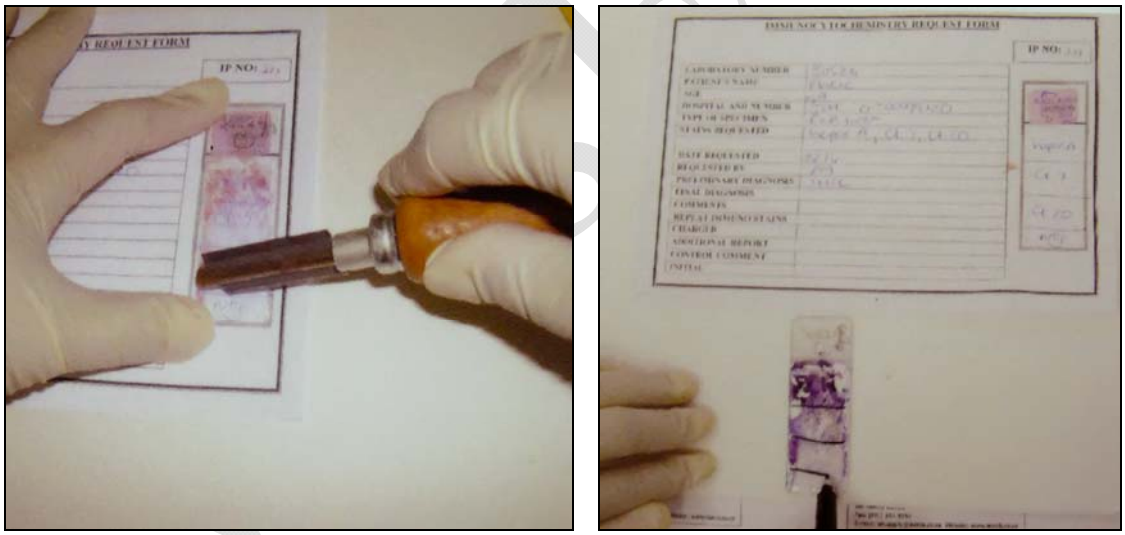

Figure 2.2 Split slide immunocytochemistry: Glass slides containing representative material stained with Papanicolaou stain are cut into respective segments with a tungsten carbide knife (I). These glass segments are stained using the split-slide immunocytochemistry technique and thereafter re-assembled accordingly (II).

Figure 2.3 Fixative for cell block samples (Shandon Formal-Fixx), consumables, reagents from Shandon Cytoblock kit and complete cell block.

Figure 2.4 Shandon Cytoblock® kit and consumables.