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CYTOLOGY AND ASSOCIATED HISTOLOGY OF BILIARY AND PANCREATIC DUCT STRICTURES: HOW GOOD IS OUR CORRELATION?

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of

Master of Medicine in the branch of Anatomical Pathology

Johannesburg, 2015

DECLARATION

I, Kirstin Janine Fearnhead (neé Coetzee), declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Anatomical Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



20th May 2015.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

None.

ABSTRACT

Endoscopic Retrograde Cholangiopancreatography (ERCP) with bile and pancreatic duct brushings is a first-line diagnostic investigation in patients with pancreatic carcinoma and cholangiocarcinoma presenting with obstructive jaundice. Biliary brushing cytology has a high specificity approaching 100%, but sensitivity is only modest, with reported ranges in the literature of 42-88%.

This cross-sectional retrospective descriptive study determined the correlation between pancreatobiliary duct brushing cytology and the gold standard of histological diagnosis on specimens received in the Department of Anatomical pathology from Chris Hani Baragwanath and Charlotte Maxeke Johannesburg Academic Hospitals over a seven-year period from April 2006 until October 2014.

A total of 208 patients were eligible, having a median age of 55 years and a male:female ratio of 1,39. We demonstrated a disease prevalence of 48% in our cohort. Our sensitivity = 63%, specificity = 96%, positive predictive value (PPV) = 94%, negative predictive value (NPV) = 74%, and overall accuracy = 80%. These findings are in line with, and compare favourably to, those reported in previous studies on this subject.

We can conclude that biliary brushing cytology in our context is a highly specific and moderately sensitive diagnostic test, which is valuable in confirming malignancy in patients with pancreatobiliary strictures. Its continued use as a first-line diagnostic procedure in this clinical context is justified, and our Cytology Department is providing a useful and valid service to clinicians and patients alike.

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DEFINITIONS AND ABBREVIATIONS

Definitions

Validity: The ability of a test to measure that which it is supposed to measure.[1] The validity of a test is measured by the sensitivity and specificity.

Sensitivity: ("Positive in disease") The ability of a test to correctly classify an individual as 'diseased'.[1]

Specificity: ("Negative in health") The ability of a test to correctly classify an individual as 'disease-free'.[1]

Positive predictive value (PPV): This is the percentage of patients with a positive test who actually have the disease.[1] It gives an indication of how many of the test positives are true positives.

Negative predictive value (NPV): This is the percentage of patients with a negative test who do not have the disease.[1] It gives an indication of how many of the test negatives are true negatives.

Accuracy: This is a statistical measure applied to how well a binary test correctly identifies or excludes a condition.[2] The accuracy is the proportion of true results (true positives and true negatives) among the total number of cases examined.

Abbreviations

CHBH:	Chris Hani Baragwanath Hospital.
CI:	Confidence interval.
CMJAH:	Charlotte Maxeke Johannesburg Academic Hospital.
CT:	Computerised tomography.
DIA:	Digital image analysis.
DNA:	Deoxyribonucleic acid.
ERBC:	Endoscopic retrograde brush cytology.
ERCP:	Endoscopic retrograde cholangiopancreatography.

EUS: Endoscopic ultrasound.

FISH: Fluorescent in-situ hybridisation.

FNA: Fine-needle aspiration.

FNAC: Fine needle aspiration cytology.

H&E: Haematoxylin and eosin.

IMP3: Insulin-like growth factor (IGF) mRNA-binding protein-3.

IPMN: Intraductal papillary mucinous neoplasm.

IPN-B: Intraductal papillary neoplasm of the bile ducts.

ISH: In-situ hybridisation.

K-RAS: Kirsten rat sarcoma virus.

LOH: Loss of heterozygosity.

MANEC: Mixed adenocarcinoma/neuroendocrine carcinoma.

MCN: Mucinous cystic neoplasm.

N/C ratio: Nuclear to cytoplasmic ratio.

NEC: Neuroendocrine carcinoma.

NET: Neuroendocrine tumour.

NHLS: National Health Laboratory Services.

NOS: Not otherwise specified.

PanNET: Pancreatic neuroendocrine tumour.

Pap: Papanicolaou.

PCR: Polymerase chain reaction.

PDAC: Pancreatic ductal adenocarcinoma.

PSC: Primary sclerosing cholangitis.

PTC: Percutaneous transhepatic cholangiography.

pVHL: von Hippel Lindau gene product.

RC: Routine cytology.

ROSE: Rapid onsite evaluation.

SCC: Squamous cell carcinoma.

SPN: Solid pseudopapillary neoplasm.

Tri-7: Trisomy 7.

US: Ultrasound.

CHAPTER 1

1.0 INTRODUCTION

Pancreatic cancer is a primary malignancy of the pancreatic ductal system. It is a disease that is often fatal, accounting for almost a third of a million deaths in 2012, worldwide.[3] This is largely attributable to its propensity for delayed presentation, with irresectable disease and metastases.

Cholangiocarcinoma is a primary malignancy of the biliary tree (bile ducts), and is commonly subdivided into those tumours arising in the intrahepatic biliary tree, and those arising in the extrahepatic biliary tree. Both worldwide and in a South African context, cholangio-carcinoma is relatively less common than pancreatic cancer. However, its clinical presentation, particularly the extrahepatic variety, is similar to pancreatic cancer, namely obstructive jaundice.

Gallbladder cancer and ampullary adenocarcinomas, although less common than both of the aforementioned tumours, also present in a similar fashion.

After imaging such as ultrasound (US) and Computerised Tomography (CT) scans, Endoscopic Retrograde Cholangiopancreato-graphy (ERCP) is the next most common investigation undertaken in patients presenting with obstructive jaundice.[4] The ERCP findings in these patients are often those of a stricture of one of the hepatobiliary or pancreatic ducts.

Strictures of the extrahepatic biliary tree and pancreatic duct may be attributable to underlying benign (inflammatory, calculous) or malignant pathologies.[5] Whilst many biliary strictures discovered at ERCP are malignant, distinguishing these from the benign cases is crucial, as the appropriate treatment will usually differ drastically.

Biliary brushing cytology can be performed at the time of ERCP by the passage of a specialized cytology collection brush through the endoscope. This enables cytological assessment of the mucosa lining the stricture at the time of ERCP, and considerably enhances the diagnostic information obtained, without the addition of a separate invasive procedure to obtain a cytological sample. As such, brush cytology has become the diagnostic modality of choice for strictures of the pancreaticobiliary ducts. Biliary tract brushing cytology has been proven to have superior sensitivity in disease detection than biliary duct washings or bile fluid cytology.[6] A number of studies have documented the high specificity, modest sensitivity and reasonable accuracy of the technique.[7-11]

The specificity of biliary tract brushing cytology is relatively high, and varies between 92% and 100%,[12] although it is quoted in most studies and reviews as being nearly 100%.[6,7,12] The sensitivity of the procedure, on this other hand, is somewhat disappointing, and has ranged from a dismal 8.3% in one series,[8] to 89% in more recent studies,[5] with most sensitivity values falling within the range 42% to 88%.[7]

There has been a single previous study of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of brush cytology in the South African context.[12] This was performed by Smith, Balabyeki, Leiman and Wright in the ERCP unit of the Helen Joseph hospital in Johannesburg during the late 1990s. Their findings, published in 2002, indicated an overall sensitivity of 70%, specificity of 100%, PPV of 100%, NPV of 57% and accuracy of 78%, when only malignant diagnoses were regarded as being positive.[12] When patients with cytology diagnoses classified as suspicious were included as having positive cytology, the sensitivity increased to 82,8%, NPV to 70,6% and accuracy to 87,8%.[12]

Although biliary brushing cytology has been enthusiastically taken up by the pancreaticobiliary units at both Charlotte Maxeke Johannesburg and Chris Hani Baragwanath Hospitals, no further studies on ERCP brush cytology in the South African setting have since been performed.

The aim of this investigation was therefore to obtain more contemporary data regarding the performance of bile and pancreatic brushing cytology, when compared with diagnoses rendered on histology, in a similar local setting, with an aim to improving the service if necessary.

In this research report, I shall detail sequentially the aims, objectives, materials and methods of my investigation into the correlation between the cytology of bile duct brushings performed at two Johannesburg hospitals over the past seven years, and the associated histology. This will be followed by my results, illustrated with tables and graphs where possible, a discussion of the meaning of the results and the research as a whole, and a final conclusion.

CHAPTER 2

2.0 AIMS AND OBJECTIVES

2.1 Aims

The use of bile and pancreatic duct brushing cytology has become the procedure of choice in the investigation of a patient with biliary obstruction. The Cytology Unit of the Department of Anatomical Pathology, University of the Witwatersrand and NHLS receives many bile and pancreatic duct brushings annually. Although the abovementioned data from Smith *et al* published in 2002 indicate fair sensitivity, NPV and accuracy of this diagnostic procedure in our clinical setting, we have no indication of the quality of more current results. We wish to establish, based on more recent data, whether this cytology unit is rendering a good service to patients and clinicians alike.

This study aims to determine our correlation between bile and pancreatic duct brushing cytology and the "gold standard" of histological diagnosis in patients with strictures of the pancreatobiliary ducts.

2.2 Objectives

1. To determine the validity, as measured by sensitivity and specificity, of results obtained by this cytology unit in assessing endoscopic brushings of the biliary and pancreatic ducts in patients with strictures.
2. To ascertain the positive and negative predictive values (PPV and NPV) and accuracy of this diagnostic test in our current setting.
3. To further assess the predictive value of an "atypical" cytological diagnosis by assessing how this cytological category correlates with the final histopathological diagnosis. This may direct adjustments in cytologic screening criteria.

CHAPTER 3

3.0 LITERATURE REVIEW

3.1 Epidemiology and demographics of pancreatic adenocarcinoma and cholangiocarcinoma

The latest data from Globocan, published by the International Agency for Research on Cancer (IARC) indicate that the estimated worldwide incidence of pancreatic cancer in 2012 was 337 872 new cases, with 8067 of these occurring in Sub-Saharan Africa.[3] Testimony to the lethality of this disease, pancreatic cancer death rates are almost equivalent to its incidence, and were placed at 330 372 worldwide and 7789 in Sub-Saharan Africa in 2012.[3] The age-standardised rate (ASR) for pancreatic cancer is quoted as 4.2 per 100 000 population globally, whilst that in Sub-Saharan Africa is 1.8.[3]

According to the latest available South African National Cancer Registry (NCR) data for 2008, there were 121 new cases of pancreatic cancer in males reported in this year, with 103 new cases reported in females.[13] The ASR is quoted as 0.74 for males and 0.48 for females, with lifetime risks of 1 in 1022 for males and 1 in 1619 for females.[13]

The highest rates of pancreatic ductal carcinoma have been recorded among African Americans and the indigenous people of Oceania.[14] The incidence is reported to be approximately 50% higher in men than in women, and most patients fall between the ages of 60 and 80 years.[14]

Similar data for cholangiocarcinoma is not available on Globocan or in the NCR. Data collection and analysis is somewhat complicated by the subdivision into the topographically distinct intrahepatic and extrahepatic subtypes. Intrahepatic subtypes may be classified together with other liver tumours while extrahepatic bile duct tumours are often combined

with pancreatic tumours. Thus the incidence may be somewhat under-reported. Like pancreatic cancer, however, incidence and mortality rates are similar, due to its poor prognosis.[15] The highest incidence of cholangiocarcinoma has been recorded in northeast Thailand, where it is 96 per 100 000 men; rates in the Western world are approximately 100 times less.[15]

Given that cancers of the pancreas, gallbladder, extrahepatic bile ducts and ampulla of Vater demonstrate shared embryologic origin, differentiation pathways, epidemiological factors and microscopic features, a field effect in carcinogenesis in this anatomical region has been suggested.[16] Cigarette smoking is the best known risk factor for pancreatic cancer, with 20-30% of cases in men and 10% of cases in women said to be attributable to tobacco smoking.[14] It has also been suggested that obesity, low physical activity, high saturated fat intake, low intake of fresh fruit and vegetables and heavy drinking may place individuals at greater risk for pancreatic cancer.[14] Chronic pancreatitis, most particularly the hereditary forms, increases the risk of pancreatic cancer more than tenfold.[14] A relative risk of 1.5 to 3 has been observed in diabetics, and there is a reported threefold relative risk for patients who have had a gastrectomy.[14]

Risk factors for carcinoma of the extrahepatic bile ducts (extrahepatic cholangiocarcinoma) include primary sclerosing cholangitis (PSC), ulcerative cholangitis (UC), and abnormal choledochopancreatic junction.[16] The purported reason for the high disease incidence in south-east Asia is the high rate of infestation with the liver flukes *Opisthorcis viverrini* and *Clonorchis sinesis* in this population.[16] Gallbladder cancer has been linked to cholelithiasis, diffusely calcified gallbladder wall ("porcelain gallbladder"), pancreatobiliary maljunction, familial adenomatous polyposis (FAP), UC and PSC.[17]

3.2 ERCP brushing cytology

3.2.1 Description of the technique

ERCP is a procedure which combines upper gastro-intestinal endoscopy with contrast-enhanced X-ray for use in the diagnosis and treatment of diseases of the pancreatic and biliary ducts.[18] Together with percutaneous transhepatic cholangiography (PTC), it came into clinical use during the 1960's.[19]

During ERCP, a flexible endoscope with an attached light source is inserted via the mouth through the oesophagus and stomach, and into the duodenum.[18] Air insufflation of the stomach and duodenum assists in visualisation of the Ampulla of Vater (duodenal papilla),[18] which is the opening of the confluence of the main pancreatic duct and common bile duct into the second part of the duodenum. The ampulla is then cannulated by the passage of a blunt-ended catheter through the endoscope, and contrast medium is hereby injected into the ductal system, enabling its visualisation by X-ray video (fluoroscopy) and detection of strictures and biliary obstructions.[18] (**Figure 1.**)



Figure 1: ERCP
Hepatobiliary surgeon performing ERCP in the endoscopy suite at CHBH.

Diagnostic procedures which can be performed at ERCP include bile aspiration cytology, bile duct brushing cytology, and endoscopic forceps biopsy. Possible therapeutic procedures include removal of gallstones, and insertion of expandable plastic biliary drainage stents to overcome blockages and relieve obstructive jaundice.[18]

The simplest method of obtaining a cytology specimen at ERCP is aspiration of bile duct juice via a catheter in the bile duct.[20] This technique enables cytological examination of only the naturally exfoliated biliary mucosal cells already present within the bile.[20] It follows that the sensitivity of this procedure is disappointingly low, falling within the range of 6-32%.[20] Although one study by Mohandas et al reported that dilatation of the malignant biliary strictures to 10F gauge increased in the sensitivity of bile fluid cytology to from 27% to 63%,[21] these findings were not reproduced by similar studies; some have hence called into question the validity of these results, citing the possibility of case selection and tumour bias in the Mohandas study.[22] One advantage of bile fluid cytology, however, is that this technique can be performed outside of the ERCP setting if applied to specimens collected via a chronic percutaneous biliary drainage catheter.[20]

The technique of bile duct brushing cytology by passage of a cytology brush through the endoscope was first described by Osnes *et al* in 1975.[12] Various advances in ERCP technology and adaptations of the endoscope since this first description allowed for brush cytology to take centre stage for a long period as the diagnostic modality of choice for strictures of the pancreatobiliary ducts. More recently, endoscopic ultrasound (EUS) guided fine-needle aspiration (FNA) has emerged as a supplementary technique for the acquisition of cytology material from the pancreas and bile duct system, and this is now the procedure of choice for the diagnosis of pancreatic malignancy.[20]

Biliary brushing cytology uses a standard cytology brush measuring 1.5cm long with bristles orientated at 90° on a 6F sheath; a newly-designed brush which is 5cm long and boasts stiffer bristles orientated at 45°on a 7F sheath may also be used.[20] The brush is wire-guided through a stricture and deployed through a second lumen across the stricture so that the

bristles scrape against the surface mucosa and remove cells. Following sampling, the brush is retracted into a sheath and removed from the endoscope so that the material can be smeared onto a glass slide and fixed, (**Figure 2**) or rinsed into a fixative material for liquid-based cytology.[20]



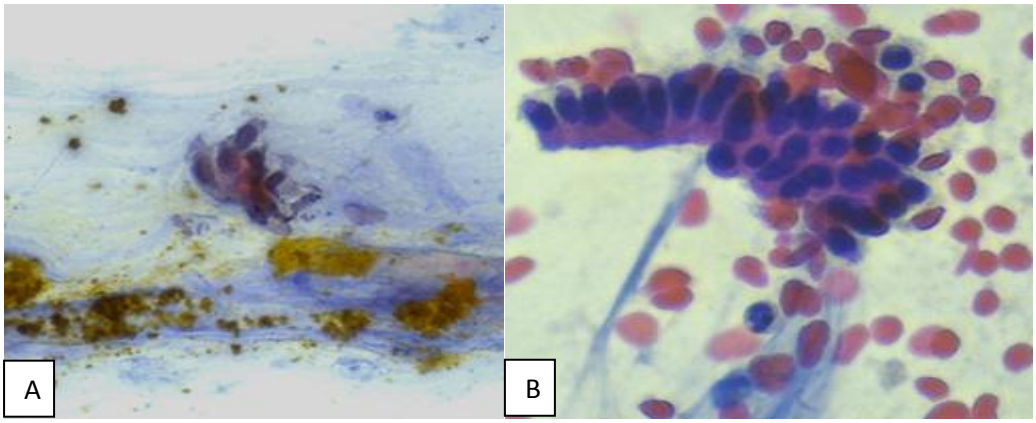
Figure 2: Slide creation
Smearing of cytology specimen obtained at ERCP.

Brushing cytology as described above can be applied to strictures both of the bile and the pancreatic ducts. Pancreatic duct brushing carries a significant risk of pancreatitis, which can be reduced by stent placement following the brushing procedure.[20]

In this research report, the term *bile duct brushing* will be used interchangeably with *pancreatic duct brushing*, and will be assumed to encompass the brush cytological sampling of any part of the pancreatobiliary ductal system.

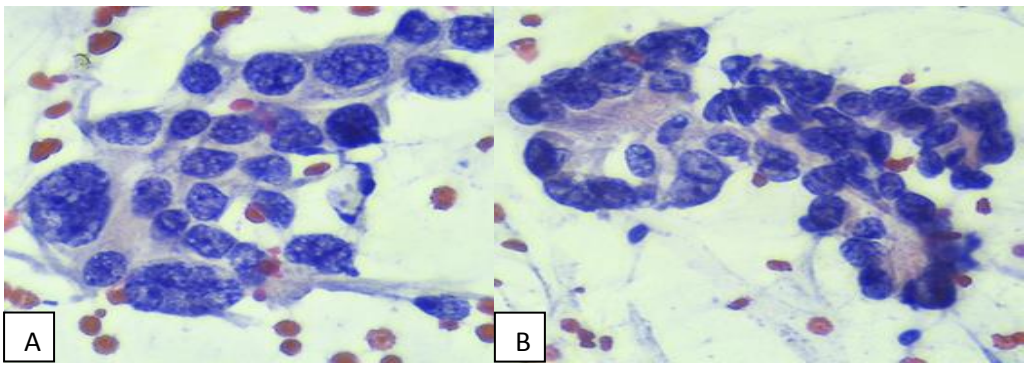
3.2.2 Cytological diagnostic criteria in bile duct brushing cytology

Benign ductal epithelium in brushing specimens appears as flat, well-cohesed, monolayer sheets with maintained polarity. The composite epithelial cells have well-defined cell borders, dense eosinophilic cytoplasm and a central nucleus with even contours and finely granular, pale chromatin; nucleoli are inconspicuous. Background bile appears as amorphous greenish yellow material.[19] (**Figures 3A and 3B.**)



Figures 3A and 3B: Benign bile duct epithelium
Benign ductal epithelium arranged in well-cohesed strips and honeycomb sheets in which polarity has been maintained, in a background of bile. (Papanicolaou stain, 200X and 400X magnification, respectively)

The most common malignancy, by far, of the pancreatobiliary system is adenocarcinoma. However, on occasion, other tumours such as non-Hodgkin lymphomas and neuroendocrine tumours (NETs) can be encountered. Adenocarcinoma smears show free lying solitary, malignant cells and/or three-dimensional aggregates.[19] Within the clusters, the tumour cell nuclei are piled up, overlapped and crowded. There is nuclear enlargement, the nuclei have smooth or irregular contours, the chromatin is coarse and hyperchromatic and the nucleoli are conspicuous.[19] The nuclear to cytoplasmic (N/C) ratio is usually high, but can vary. Cytological criteria reported to most accurately and reproducibly predict malignancy in bile duct brushings are: chromatin clumping, increased N/C ratio, and either nuclear moulding or loss of honeycombing.[19] Even when sparse, 3-D clusters of epithelioid cells with marked atypia warrant a diagnosis of malignancy when seen in bile duct brushing cytology.[19] (**Figure 4.**)



Figures 4A and 4B: Malignant cytology in an adenocarcinoma
Groups of malignant cells showing cellular crowding, overlapping, irregular nuclear borders and chromatin distribution, and prominent nucleoli. (Papanicolaou stain, both 400X magnification)

Malignancy must be distinguished from benign reparative and reactive atypia. In reactive atypia, cells are arranged in cohesive monolayer sheets with retained honeycombing. Although there may be slight overlapping of cells in places, the polarity of cells is usually maintained, and cells show streaming cytoplasm with intact cell borders.[19] The nuclei are mildly enlarged and round to oval in shape and show smooth nuclear contours with fine, even chromatin. The prominence of nucleoli varies, and although nucleoli are usually inconspicuous, macro-nucleoli can occur. The smear background may be inflamed or contain bile pigment, cholesterol or crystals.[19] (**Figure 5.**)

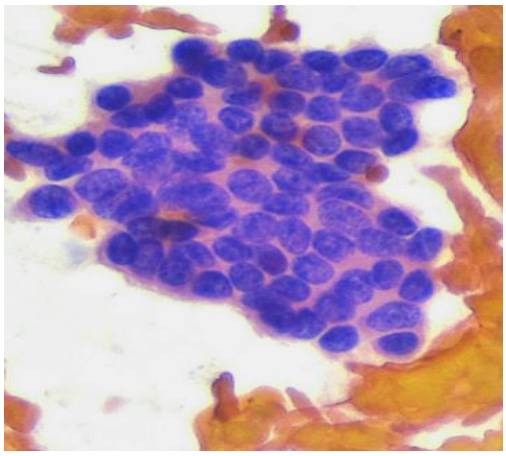


Figure 5: Reactive bile duct epithelium
Slightly enlarged nuclei, smooth nuclear borders, finely granular chromatin and occasional discernible nucleoli. (Papanicolaou stain, 400X magnification)

Dysplastic cell groups usually show more marked architectural disturbances (cellular crowding and overlapping), and higher degrees of nuclear atypia with higher N/C ratios and chromatin abnormalities than reactive atypia.[19]

Inadequate smears are defined as those specimens that provide no diagnostic information about the lesion which has been sampled.[23] The main reasons for designation of a brushing specimen as inadequate are scanty cellularity or no cellular material present, and obscurement of the material by blood, air-drying or other artefact. (**Figures 6A and 6B.**)

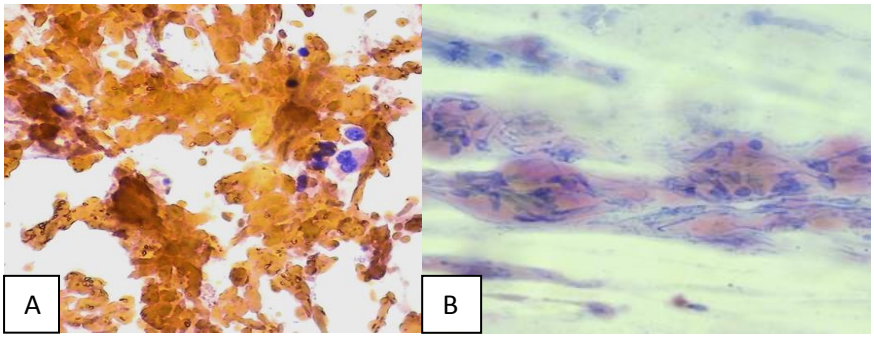


Figure 6A: Inadequate smear, obscured by blood
(Papanicolaou stain, 100X)

Figure 6B: Air-drying artefact
(Papanicolaou stain, 400X)

3.2.3 Terminology for reporting pancreatobiliary cytology

In 2014, the Papanicolaou Society of Cytopathology published guidelines on standardized terminology and nomenclature for pancreatobiliary cytology.[23] Their proposed terminology scheme outlines six diagnostic categories: non-diagnostic, negative (for malignancy), atypical, neoplastic: benign or other, suspicious (for malignancy) and positive/malignant. The proposed nomenclature system is intended for use with brushing cytology of pancreatobiliary strictures and with EUS-FNA of pancreatic masses and cysts. (**Table 1**, adapted from reference 23.) As this is a very new classification system, its performance has yet to be assessed.

Table 1: Proposed pancreatobiliary terminology classification scheme

I	Non-diagnostic: Provides no diagnostic or useful information about the solid or cystic lesion sampled. Any cellular atypia precludes use of this diagnostic category.
II	Negative (for malignancy): Sample contains adequate cellular and/or extracellular tissue to evaluate or define a lesion that is identified on imaging. When using this category, one should give a specific diagnosis when practical. Specific diagnoses include: benign pancreatobiliary tissue in the setting of vague fullness and no discrete mass, acute or chronic pancreatitis, autoimmune pancreatitis, pseudocyst, lymphoepithelial cyst, and accessory spleen.
III	Atypical: This category is heterogeneous and includes cases with reactive changes, low cellularity, premalignant changes (dysplasia) and cases assigned to this category due to observer caution in diagnosis. This category should only be applied when there are cells present with cytoplasmic, nuclear

	or architectural features that are not consistent with normal or reactive cellular changes of the pancreas or bile ducts, and are insufficient to classify them as a neoplasm or suspicious for a high-grade malignancy. The findings are insufficient to establish an abnormality explaining the lesion seen on imaging. Follow-up evaluation is warranted.
IV	IVA – Neoplastic: Benign: The cytological specimen is sufficiently cellular and representative, with or without the context of clinical, imaging and ancillary studies, to be diagnostic of a benign neoplasm (e.g. a serous cystadenoma).
	IVB – Neoplastic: Other: This category defines a neoplasm that is premalignant, such as intraductal papillary neoplasm of the bile ducts (IPN-B), intraductal papillary mucinous neoplasm (IPMN), or mucinous cystic neoplasm (MCN) with low, intermediate or high-grade dysplasia by cytological criteria. It also denotes a low-grade malignant neoplasm such as well-differentiated pancreatic neuroendocrine tumour (PanNET) or solid pseudopapillary neoplasm (SPN). While mucinous epithelium in biliary brushing specimens may indeed represent a neoplastic change, given the lack of evidence-based literature on the cytological interpretation, histology and management of these lesions, low-grade mucinous change of the biliary should remain in the "atypical" rather than the "neoplastic" category.
V	Suspicious (for malignancy) "SFM": A specimen is SFM when some, but an insufficient number, of the typical features of a specific high-grade, aggressive malignant neoplasm are present. It refers mainly, but not exclusively, to pancreatic ductal adenocarcinoma (PDAC). The cytological features raise a strong suspicion for malignancy, but the findings are qualitatively and/or quantitatively insufficient for a conclusive diagnosis, or tissue is not present for ancillary studies to define a specific neoplasm. The morphologic features must be sufficiently atypical that malignancy is considered more probable than not.
VI	Positive or malignant: A group of neoplasms that unequivocally display malignant cytologic characteristics and include PDAC and its variants, cholangiocarcinoma, acinar cell carcinoma, high-grade neuroendocrine carcinoma (small cell and large cell), pancreatoblastoma, lymphomas, sarcomas and metastases to the pancreas.

Category IV: neoplastic, benign or other, is a diagnostic category which is rarely applicable in bile duct brushing specimens and will be generated most often by EUS-FNA of pancreatic lesions. With the exception of this category, the diagnostic categories suggested by the Pap society and listed in Table 1, conform to those used by cytopathologists reporting pancreatobiliary cytology in the Cytology Unit of the Dept of Anatomical Pathology, Faculty of Health Sciences, University of the Witwatersrand and National Health Laboratory Services (NHLS), Johannesburg.

3.2.4 Usefulness of brushing cytology in diagnosing biliary and pancreatic malignancy: published data

Although biliary tract brushing cytology has superior sensitivity in detection of malignancy than biliary duct washings or bile fluid cytology,[6] its overall sensitivity remains suboptimal, and falls within the range of 42-88%.[7]

In the first study to describe the technique, Osnes *et al* reported on 17 patients having ductal abnormalities at ERCP who underwent what they termed Endoscopic Retrograde Brush Cytology (ERBC). Eight samples were diagnosed as malignant and a further 2 as suspicious. Six of 7 patients who underwent surgical excision had a malignant diagnosis, with another patient having a pancreatic malignancy proven at autopsy. They concluded that bile duct brushings at ERCP could provide useful information in the management of patients with biliary tract lesions.[6]

However, the procedure as described by Osnes *et al* was technically challenging and was not popularized until 1989, when Foutch *et al* described placement of a guidewire across the stricture to assist cytology sample collection with a standard cytology brush.[25] The introduction of a Geenen cytology brush mounted on a flexible-tipped wire in 1990 by Venu *et al*[26] added further impetus to the modality's uptake by hepatobiliary surgeons worldwide.

Many studies on the operating characteristics (sensitivity, specificity, positive and negative predictive values, technical success/adequacy rates and accuracy) of pancreatobiliary brush cytology have since been published. The results of some of these are summarized in **Table 2**.

Table 2: Summarised data on efficacy of biliary brush cytology in detecting pancreatobiliary malignancy

Author	Patients (N)	Technical success / adequacy %	Sensitivity			Specificity %	Accuracy %	PPV	NPV
			Bile duct cancer %	Pancreatic cancer %	Overall %				
Sawada* (1989)[22]	72	100	N/A	85	85	100	-	100	-
Venu (1990)[26]	53	94	80	60	69	100	-	100	-
Scudera (1990)[27]	25	100	100	50	60	100	-	100	38
Rabinowitz ** (1990)[22]	65	100	62	N/A	62	100	-	100	-
Foutch (1990)[25]	34	100	100	60	60	100	-	100	-
Ryan (1991)[28]	69	90	44	30	44	100	-	-	-
Howell (1992)[29]	29	-			8.3	100	-	100	-
Kurzawinski (1993)[22]	39	100	60	65	69	100	-	100	-
Yap (1994)[22]	52	100	75	42	54	92	-	-	-
Ferrari (1994)[30]	74	95	20	66	56	100	70	100	51
Ryan* (1994)**[31]	48	100	30	45	42	92	94	35	55
Enayanti (1996)[32]	50	99		-	33	100	73	100	68
Mansfield (1997)[10]	43	95	75	50	54	100	-	100	-
Trent (1999)[9]	31	100	-	-	71	100	84	100	81
Jaiwala (2000)[33]	133	-	47	42	48	90	-	94	33
Stewart (2001)[34]	406	96	-	-	60	98	75	98	61
Smith (2002)[12]	41	98	20	79	70	100	78	100	57

Author	Patients (N)	Technical success / adequacy %	Sensitivity			Specificity %	Accuracy %	PPV	NPV
			Bile duct cancer %	Pancreatic cancer %	Overall %				
Ylagan § (2003)[8]	142	-	-	-	31/ 42	100	-	100	-
Volmar (2005)[5]	1118	99	53	51	66	89	76	89	67
Elek (2005)[11]	103	-	27	14	20	100	74	100	33
Sheehan § (2007)[7]	113	92/100			39/53	100/100	68/72	100/ 100	61/ 60
Mahmoudi (2008)[35]	199	-	-	-	61	100	-	100	-
Hart# (2010)[36]	64	-	-	-	33	100	81	100	49
Chadwick (2013)[37]	104	-			46	93	70	86	65

(Table adapted from reference 22, updated.)

PPV = positive predictive value.

NPV = negative predictive value.

* Specimens collected from the pancreatic duct; all patients had pancreatic cancer.

** Specimens were obtained by the percutaneous route; all patients had cholangiocarcinoma.

*** Compared flow cytometry to routine cytometry. Values stated are for routine cytology.

§ Compared Thinprep® to conventional cytology. Values are stated as conventional/ThinPrep®.

Compared conventional cytology alone to cytology with IMP3 staining. Values are for cytology alone.

As can be inferred from the table, the specificity of biliary tract brushing cytology is high (mostly 100%[12] with a lowest reported value of 92%[6,7,12]), but its sensitivity is disappointing (between 8.3%[8] and 89%,[5] mostly in the range 42% to 88%[7]). Differential sensitivities for the detection of pancreatic cancer and cholangiocarcinoma are 38% to 56% and 63% to 75%, respectively,[12] depending on whether single or multiple sampling methods are employed.[10]

Reported reasons for the only moderate sensitivity of bile duct brushing cytology include both errors of sampling (limitations due to anatomical location of lesions, lesion size, extensive fibrosis or surface ulceration, and extrinsic tumours surrounding the bile ducts but not infiltrating them, with overlying benign epithelium) and errors of interpretation (subtle

changes in well-differentiated lesions, poor cellular preservation, failure to pay adequate attention to the smear background and unfamiliarity with diagnostic criteria).[6, 19] Another contributing factor is the rendering of negative diagnoses on suboptimal or inadequate specimens.[19]

In a study comparing various methods of cytological sampling of biliary strictures, Mansfield *et al*[10] performed a prospective evaluation in which they included material gathered by means of a cytology brush, the screw-like mechanism of the Sohendra stent remover, and the retrieved stent itself, with submission of both slides and washings from each of these; fluid cytology was also performed on 20ml of aspirated bile in each case. Adequacy rates of brush cytology and material from blocked stents were 96% and 84%, respectively, compared to only 44% adequacy of the bile fluid samples. They reported the sensitivity of brush cytology alone to be 44%, with an increase to 54% with all cytology methods combined, when cytological diagnoses of *malignant* or *suspicious for malignancy* were included as positives. Differential analysis of pancreatic carcinoma and cholangiocarcinoma diagnoses revealed 50% sensitivity for the diagnosis of the former, and 75% sensitivity for the diagnosis of the latter.[10]

Several investigators have focused on the added benefit of using the ThinPrep® method of specimen preparation in comparison to conventional cytology smears.[7,8] This method of liquid-based cytology requires that the cytology brush and its sample are deposited into a liquid fixative, instead of smeared directly on to a slide. The liquid and its cellular contents are then centrifuged, pipetted and filtered by a machine and transferred to a slide in a 20mm diameter circle.[37] The result is better cellular preservation and elimination of background debris and drying artefact, with a higher rate of sample adequacy.[37] Although one study by Sheehan et al found that Thinprep® smear preparation increased the sensitivity of the modality from 39% to 53%,[7] a similar study by Ylagan et al found comparable sensitivities between the two techniques.[11] However, the latter group documented an increase in overall sensitivity when specific and well-defined cytologic criteria were employed in the diagnosis.[11]

3.2.5 Ancillary techniques applied to brushing cytology material

Several ancillary diagnostic modalities have been investigated insofar as they may improve the sensitivity of biliary and pancreatic duct brushings. These include digital image analysis (DIA), various immunocytochemical antibodies, and molecular analysis including Fluorescent in-Situ Hybridisation (FISH) and K-RAS genetic mutational analysis.[24]

Digital Image Analysis (DIA)

Digital Image Analysis (DIA) uses spectrophotometry with generation of a histogram by a quantitative DNA analysis program to identify abnormalities of DNA content (ploidy) in brush cytology samples from bile duct strictures. Whilst diploid results are in keeping with benign and reactive conditions, aneuploidy and tetraploidy are supportive of malignancy. The reported specificity of DIA in detection of malignancy is excellent, but the sensitivity is only moderate, and its diagnostic accuracy is therefore comparable to that of routine cytology. The diagnostic sensitivity of DIA was comparable to that of routine cytology (RC) for patients with Primary sclerosing cholangitis (PSC), and greater than RC for patients without PSC.[39]

Immunocytochemistry

The immunocytochemical markers which have been shown to be most helpful in the distinction of benign from malignant biliary epithelium are S100P, von Hippel Lindau gene product (pVHL), CD10 and insulin-like growth factor (IGF) mRNA-binding protein-3 (IMP3).[24] Tretiakova *et al* [40] showed complete loss of CD10 immunostaining in 66% and 97% of high-grade dysplasia and adenocarcinomas, respectively, compared to to 5.7% and 4.6% loss in reactive biliary epithelium and normal epithelium, respectively. A potential pitfall, however, was that up to 94.3% of reactive epithelium exhibited discontinuous CD10 expression, with positive cells varying from 20% to 80%, such that careful attention to the pattern of staining, as well as morphological correlation, are crucial in interpretation of this marker.[40]

Levy *et al*[41] demonstrated the utility of an immunohistochemical panel of S100P, pVHL and IMP3, with 70% of adenocarcinomas showing a S100P+/IMP3+ /pVHL− staining pattern, 15% showing with a S100P+/IMP3−/pVHL− pattern and 5% showing a S100P−/IMP3+/pVHL− pattern.[41] Individually, S100P showed nuclear and cytoplasmic staining in 90% of adenocarcinomas, IMP3 was positive in 77.5% of adenocarcinomas, and pVHL was completely negative in 92.5% of adenocarcinomas.[41] Hart *et al*[36] found that the sensitivity of immunocytochemical staining for IMP3 in the detection of malignancy in biliary brushings was superior to routine cytologic evaluation of a Pap-stained specimen. Moreover, the combined use of biliary brushing cytology and IMP3 immunocytochemistry provided sensitivity results which were superior to the use of either method alone, with maintenance of the 100% specificity.[36]

Fluorescent In-Situ Hybridisation (FISH)

A commercially available set of DNA probes which was developed for the detection of urothelial carcinoma in urine samples (UroVysion; Abbott Molecular, USA) has been applied to biliary brushing samples by several investigators, with considerable success.[24] The UroVysion test consists of DNA probes to the pericentromeric regions of chromosomes 3, 7, and 17 and to the 9p21 band location of the p16 tumor suppressor gene, each fluorescently labelled in a different colour, and detects changes in copy number of these three chromosomes, as well as p16 gene deletion, all of which are known to be present in bladder cancer.[42] These same genetic aberrations may also be seen in other carcinomas. Boldorini *et al*,[43] used the following criteria for malignancy when applying the UroVysion test to bile duct brushings: ≥5 cells with polysomy (a gain of two or more of the four probes) or ≥10 cells with trisomy of chromosome 7 or 3 (three copies of chromosome 7 or 3, and ≤2 of the other three probes). They documented FISH to be more sensitive than routine cytology (90% vs 77%), although the difference was only statistically significant if those cases placed in the "suspicious" cytology category were considered as negatives.[43]

Using a cohort of 86 patients with indeterminate biliary strictures who were stratified according to whether or not they had PSC, Levy *et al*[39] found that FISH provided the greatest diagnostic sensitivity of any of the tissue sampling techniques, particularly when considering

trisomy of chromosome 7 (Tri-7) as indicative of malignancy. However Tri-7 was the cause of all the FISH false positives in the PSC group, decreasing the test's sensitivity in this cohort, indicating that in this group it may be preferable to regard Tri-7 as benign. They found that, depending on how suspicious cytology results were interpreted, composite DIA/FISH (when considering Tri-7 as benign) increased the sensitivity one- to fivefold in PSC patients versus RC, and two- to fivefold in patients without PSC.[39]

Kipp *et al*[44] found an overall sensitivity for detection of cancer in cytology specimens of 50% for FISH, with sensitivities of 63% and 41% for pancreatic adenocarcinoma and cholangiocarcinoma, respectively. Their FISH results were categorized as disomic (negative for malignancy), trisomy (equivocal for malignancy), or polysomy (positive for malignancy).[44]

Fluorescent In-Situ Hybridisation (FISH) is currently advocated by the Papanicolaou Society of Cytopathology as the ancillary technique which affords the greatest increase in sensitivity without compromising specificity over that obtainable by conventional cytology alone.[24]

<u>K-RAS mutational analysis</u>

An activating point mutation in codon 12 of the K-RAS (Kirsten rat sarcoma) oncogene is one of the earliest steps in what is now a well-defined and accepted stepwise molecular progression model of pancreatic ductal adenocarcinoma, and has been documented in more than 90% of pancreatic cancers.[45] Although K-RAS mutational analysis on bile duct brushings has shown promise in the quest to increase overall sensitivity, its exact diagnostic utility remains uncertain at this point.[24]

Sturm *et al*[46] in 1998 used semi-nested PCR with mutant enrichment to detect K-RAS codon 12 mutations in histology sections from pancreaticoduodenectomy specimens and *postoperative* ampullary brushings, and found mutations to be present in 71% of the carcinomas, which included pancreatic, ampullary and cholangioacrcinomas. There was 86% concordance between the mutational analysis on the whole tissue samples and the

cytological samples, and the discrepancies were attributed by the authors to intratumoural heterogeneity and sampling error.[46]

In a different study in 1999, Sturm *et al*[47] studied 312 consecutive patients with extrahepatic biliary stenosis and found that the sensitivities of cytology and K-RAS mutational analysis to detect malignancy were 36 and 42%, respectively, and 62% combined.[47] Mutational analysis had a higher sensitivity for pancreatic carcinoma (67%) than for gallbladder, bile duct and ampullary carcinomas (27%). The specificity of cytology was 98%, and the specificity of the mutational analysis and of both tests combined was 89%.[47]

In a 2010 study already quoted above, Kipp *et al* demonstrated a combined sensitivity of 86% for K-RAS mutation and fluorescence in situ hybridization (FISH) analysis in the diagnosis of pancreatic adenocarcinoma on brush cytology samples.[44]

Khalid *et al*[48] investigated genetic material acquired from brush cytology specimens and microdissected surgical malignant and normal tissue from 26 patients with biliary strictures who underwent ERCP with brush cytology. They developed a panel of 12 polymorphic microsatellite markers linked to six tumour suppressor genes, and performed polymerase chain amplification (PCR) on the material. They compared the PCR products for LOH and K-ras codon 12 mutations, and found that they were able to discriminate reactive from malignant cells with 100% sensitivity, specificity, and accuracy.[48]

The drawback of K-RAS testing is that, as K-RAS mutations are one of the earliest steps in pancreatic carcinogenesis, mutations can be detected in preinvasive lesions and dysplastic epithelium, including in low-grade dysplasia/pancreatic intraepithelial neoplasia 1A, and 1B.[44] K-RAS mutations have also been detected in chronic pancreatitis.[24] As such, it is the current view point of the Papanicolaou Society of Cytopathology that:

> *"additional studies will be necessary to determine the value of KRAS mutational analysis in both bile duct stricture cytology samples and FNA of solid pancreatic*

masses, but current data do not support KRAS testing of solid pancreatic masses and bile duct strictures as a useful ancillary test for diagnosis". [24]

3.2.6 Correlation of atypical cytology with outcome

Some studies have investigated the significance of a diagnosis of atypia on biliary brushing cytology in terms of its correlation with final outcome.[5,32-34,37] The case groupings into benign, atypical, suspicious and malignant categories have varied amongst studies. Some investigators have chosen to include all equivocal diagnoses, including "atypical", "dysplastic" and "suspicious/suggestive of malignancy" in the atypical category,[34] whilst others have split the atypical category into "atypical, favour reactive", atypical, not otherwise specified (NOS)" and "atypical, suspicious"[37] or "low-grade atypia" and "high-grade atypia".[33] Jaiwala *et al*[33] generated separate performance characteristics for scenarios when atypia was considered positive cytology, when only high-grade atypia was considered positive, and when atypia was excluded altogether.[33] Stewart *et al* considered a cytological diagnosis of atypical to be negative,[34] whilst Volmar *et al* regarded atypicals as inconclusive, and excluded these cases from their calculation of performance characteristics.[5]

In the above studies, cases classified as atypical on cytology had malignancy rates of between 44 and 81%. The large range of malignancy here is almost certainly related to the varying definition of "atypical", including cases with findings as disparate as "reactive atypia" and "suspicious for malignancy". Those investigating differential performance characteristics with inclusion and exclusion of atypicals found that inclusion of this category increased cytology's sensitivity at the expense of specificity: the latter dropped as low as 66% when all atypicals were considered positive.[33,37]

3.3 Diagnostic gold standards in pancreatobiliary malignancy

Histology results are often used as the diagnostic gold standard when the operating characteristics of a cytology test are being investigated. In the case of pancreatobiliary cytology, it is important to note that the curative surgery of choice is a pancreatico-duodenectomy (Whipple's procedure). This is a major surgical procedure with significant associated morbidity and mortality.[49] Only about 20% of pancreatic cancer patients are candidates for this procedure, which can be performed only if the disease is potentially resectable (i.e. confined to the head of the pancreas and with no vascular involvement or spread to liver, lungs or abdominal cavity), and which cannot be undertaken in very ill patients with a poor performance status.[50]

As the majority of patients with cancers of the pancreas, biliary tree or ampullary region will not be candidates for surgical resection, histological analysis of resection specimens is often not an ideal gold standard in the case of pancreatobiliary cytology. For this reason, many investigators have supplemented the final histological diagnosis with other information in determining which patients were, in fact suffering from malignant disease. For instance, in the study by Smith et al, a combination of intraoperative fine needle aspiration cytology (FNAC) of a mass lesion, histology on resection specimens, clinical or radiological evidence of metastatic disease, or malignant clinical course at six month follow-up were all used as the gold standard against which the cytology results were measured.[12] Many other studies have used a similar combination of radiology, intraoperative tissue diagnosis, resection histology, ERCP-guided and laparotomy biopsy results, autopsy findings and final clinical course to obtain a composite gold standard.[5,7-12,21,26,28-39,41,43,46-48]

Endobiliary forceps biopsy can be performed to obtain tissue for histology at the time of ERCP to improve the sensitivity of tissue sampling.[11, 20, 33] One study suggested that the combination of mini forceps biopsy (where a small forceps is placed through a patent ampulla and guided by fluoroscopy to the area of concern) with brushing cytology increased the overall sensitivity by 15-25% compared with either method alone.[20] Endobiliary forceps biopsy has been reported to have a sensitivity of between 37%[11] and 81%[22], with 88%

sensitivity for cholangiocarcinomas and 71 % for pancreatic neoplasms.[22] The sensitivity, accuracy and negative predictive values of standard and mini forceps biopsy have been reported as 29,4%, 53.8% and 42.8% and 76.5%, 84.6% and 69.2%, respectively,[20] which is not much different to the performance of cytology. Of note, then, is that this method of tissue sampling does not have a 100% sensitivity for detection of malignancy, and caution should be exercised when using histology *biopsy* results as a gold standard.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Study design

This cross-sectional retrospective descriptive study was performed on cytology and histology records retrieved from the National Health Laboratory Services (NHLS) Disalab database for Chris Hani Baragwanath Hospital (CHBH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) for the time period April 2006 to April 2013.

4.2 Place of study

The laboratory setting was the cytology and histopathology units of the division of Anatomical Pathology at the University of the Witwatersrand. The clinical setting was two tertiary referral centres, namely CHBH in Soweto, and CMJAH in Parktown.

4.3 Study population

The study population comprised all adult patients over the age of 18 of either sex who underwent biliary and pancreatic duct brushing cytology during the stated 7-year (84-month) period at either of the two hospitals under study.

4.4 Inclusion and exclusion criteria

The inclusion criteria were as follows:

- All adult patients of age 18 years or older were included.
- Patients with both pancreatobiliary brushing cytology and corresponding histology records retrieved by a computer-based record search for same were included.

The exclusion criteria were as follows:

- Patients less than 18 years of age were excluded.
- Patients with biliary/pancreatic duct brushing cytology only, without corresponding histopathology, were excluded.
- Patients who underwent pancreatobiliary brushing having histology results from anatomical sites other than the pancreas, liver, bile ducts, Ampulla of Vater or associated lymph nodes (i.e. histology pertaining to other and unrelated pathologies) were excluded.
- Patients with biliary and pancreatic brushings who were not seen at the two hospitals under study were excluded.

4.5 Methods

4.5.1 Data retrieval and entry

The bile duct brushing cases reported by the Cytology department during the study time frame were identified by a computer record search. The cases were identified by an administrative code unique to bile duct and pancreatic duct brushings, entered into the NHLS database at the time of receipt of the specimens. This search was performed by the NHLS Information Technology (IT) services after completion of the relevant form providing details of the research protocol and ethics approval.

All cases so identified were then subjected to a second computer-based search for appropriate histology. This search involved a cross-check of the patient's name and date of birth in the NHLS Disalab histology database. Those cases with cytology and no corresponding histology were excluded.

Data points were captured from each case that had both cytology and histology records, and entered into an anonymized Excel 2010 spreadsheet, as follows:

1. Each case was allocated a **study number**, starting at 1 and proceeding chronologically.
2. Patient **age**.

3. Patient **sex**.
4. **Clinical presentation** (e.g. obstructive jaundice).
5. **Cytology laboratory reference number**.
6. **Cytology diagnosis** (in words).
7. **Cytology adequacy** (numbered 0,1 or 2).
8. **Cytology diagnostic category** (numbered from 3 to 6).
9. **Histology reference number**.
10. **Histology specimen type and site**.
11. **Histology diagnosis** (in words).
12. **Histology adequacy** (numbered 10, 11 or 12).
13. **Histology diagnostic category** (numbered from 13 to 16).
14. Further **comments** as necessary.

(The codes for the cytology and histology categories and blank data capture sheet are attached in **Appendix 1.**)

4.5.1.1 Use of histology in data retrieval

- Where more than one histology result appeared for any patient, and there was a discrepancy in those results, the excision or autopsy result was used in preference over any biopsy results.
- Where several biopsy results were present for any patient without a final excision or autopsy, and there was a discrepancy between those results, any malignant diagnosis was used as the final histological "gold standard" diagnosis.
- In some cases there was positive cytology with negative biopsy histology only, and mention was made in the histology report to the effect that "given the clinical/radiological suspicion of a mass lesion, the biopsy may not be representative of the pathology present". These cases were excluded from the final analysis.

4.5.1.1 Use of cytology in data retrieval

Where there was more than one cytology result for any patient, and there was a discrepancy in the diagnoses rendered, the cytology with the date that was closest to that the histology report (i.e. contemporaneous procedures) was used.

4.5.2 Data analysis

The gold standard against which the cytology was evaluated was the final histological diagnosis, preferably that rendered on an excision specimen (although all relevant histology results were recorded for each case).

Positive results were those of malignancy. Included in the positive category were all cytology cases classified as suspicious for malignancy (category 5). Those categorized as atypical (category 4) were placed in a separate category. Benign diagnoses (category 3) were categorized as negative.

For each case, a decision was recorded as to whether the cytology result tested against the histology gold standard yielded a true positive, false positive, true negative or false negative result, as follows:

1. Brushings positive, histology positive = true positive, value “a”.
2. Brushings positive, histology negative = false positive, value “b”.
3. Brushings negative, histology positive = false negative, value “c”.
4. Brushings negative, histology negative = true negative, value “d”.
5. Inadequate cytology = value “e”
6. Atypical cytology = value “f”

After calculation of the total numbers of each of the above four categories, a “two-by-two” contingency table, was created, as illustrated below in **Table 3**:

Table 3: Example of two-by-two contingency table

	Histology positive	**Histology Negative**
Cytology positive	a	b
Cytology negative	c	d

A separate calculation was performed on the final histological diagnosis of those cases accorded atypical values on cytology ("f"), insofar as what percentage of these cases had a final diagnosis of malignancy on histopathology, and the percentage that was ultimately accorded a benign diagnosis.

4.5.3 Reproducibility of the data

The bile duct brushing cytology diagnoses are rendered by experienced and trained specialist cytopathologists with more than five years' experience in the field.
The histological diagnoses are rendered by two separate individuals: first an Anatomical Pathology registrar (trainee), with variable experience, and thereafter by a registered consultant.

As the histological material on excision specimens is, for the most part, the entire pancreas with attached bile ducts, duodenum and distal stomach (Whipple's procedure), the error inherent in rendering diagnoses on small tissue samples is largely eliminated.

ERCP-directed biopsy diagnoses are subject to inherent difficulties of sampling error, and variable adequacy. Often the material submitted for histology from ERCP biopsy procedures is crushed and scanty, rendering accurate histological diagnosis impossible. The result is that ERCP forceps biopsy has reported sensitivities ranging from 37-81%,[11,22] showing a significant proportion of false negative diagnoses. For this reason, we excluded from the final analysis those cases where there was a clinical suspicion of malignancy and the cytology was positive, but the only available histology was a biopsy that did not confirm malignancy.

The majority of the cases have a final histological diagnosis of pancreatic ductal adenocarcinoma, which is a fairly straightforward and reproducible diagnostic category.

4.5.4 Statistical analysis

Descriptive statistics in the form of mean, median and standard deviation were used to describe the patient ages, and frequency analysis was used to describe the gender of the patients.

The cytological and histological diagnoses were correlated using simple statistical calculations for sensitivity, specificity, positive predictive value, negative predictive value, accuracy, and prevalence as follows:

a. $Sensitivity = \frac{a}{a+c}$
b. $Specificity = \frac{d}{b+d}$
c. $Positive\ predictive\ value = \frac{a}{a+b}$
d. $Negative\ predictive\ value = \frac{d}{c+d}$
e. $Accuracy = \frac{a+d}{a+b+c+d}$
f. $Prevalence = \frac{a+c}{a+b+c+d}$

For statistical purposes, numbers of cases categorized as inadequate for cytological assessment were delineated and placed in a separate category. The total number of inadequate cases was calculated as a percentage of the total number of cases.

The percentage of cases categorized as atypical was also calculated with reference to the total case number. The proportions of cases in this category which were ultimately accorded malignant and benign histological diagnoses, respectively, were then calculated.

4.6 Ethical considerations

Ethics clearance has been granted by the Human Research Ethics Committee (Medical) and Faculty of Health Sciences of the University of Witwatersrand, Postgraduate Office: certificate number M110720 (**Appendix 2**).

As this is a retrospective study, which was performed on archived departmental records and reports, no additional interventions were undertaken insofar as individual patients are

concerned. No new tests were performed on the specimens, and no additional costs were incurred by the patients, hospitals or laboratory.

The data entry spreadsheet was kept anonymous and cases were entered according to the NHLS cytology or histology "case number", as well as allocated a separate number for the purposes of this study. Only patient age, sex, clinical presentation, cytological and histological diagnoses and categories were recorded. Clinical details such as HIV-status were not recorded on the data capture spreadsheet. Patient confidentiality was strictly maintained at all times.

There was no interaction between the principle investigator and the patients for inclusion in the study, and hence no opportunity for obtaining verbal or written consent. However, strict anonymity was maintained and patient names were not divulged during or after the study. This information was kept in a locked cupboard in Dr. K. Fearnhead's private office at the Medical School campus.

CHAPTER 5

5.0 RESULTS

5.1 Sample size

A total of 227 cases with cytology and corresponding histology results from the hospitals under study during the stated time period were identified and the reports retrieved. Of these, 19 were excluded as they had positive cytology and negative biopsy histology, with biopsies that were suspected to be false negatives (i.e. not fully representative of the underlying pathology). The final cohort, therefore, comprised 208 cases (N = 208).

5.2 Demographics

The patients' ages ranged from 21 to 84 years, with both the mean and the median patient age falling at 55 years. The mean age is 54,8 ± 12,7 years. The age distribution is shown in **Figure 7.**

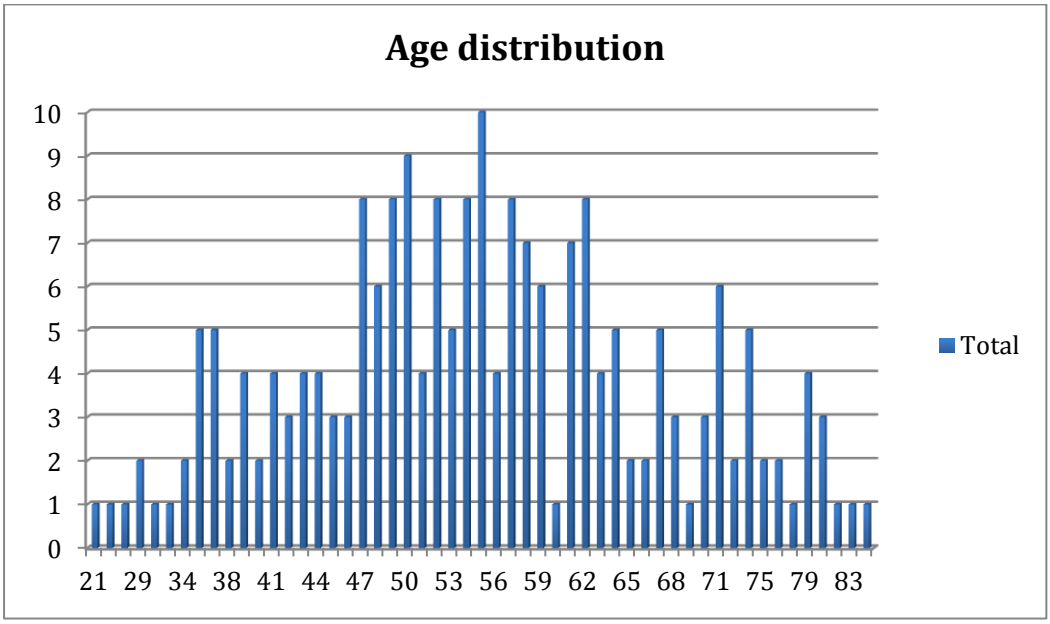


Figure 7: Age distribution

There were a total of 121 males and 87 females, with a male: female ratio of 1,39. (**Figure 8.**)

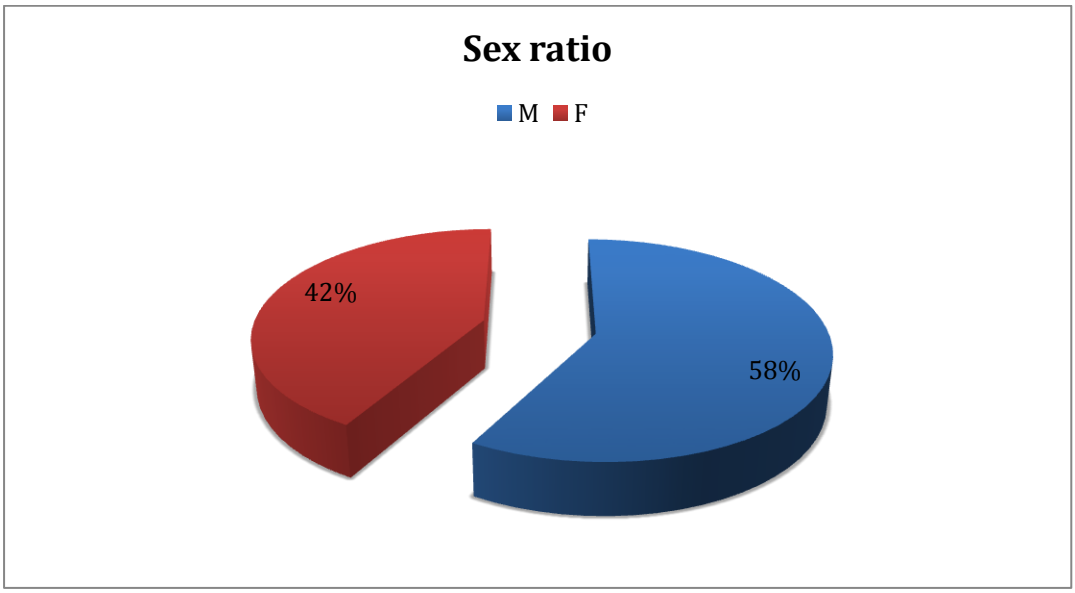


Figure 8: Sex ratios

5.3 Presenting symptoms

17 patients (8,17%) presented with abdominal pain, 140 (67,3%) with jaundice, of whom 2 (0,96%) had Mirizzi syndrome, 6 (2,88%) with vomiting or gastric outlet syndrome, 6 (2,88%) with loss of weight, and 4 (1,92%) with fever. In 27 patients (12,98%), the presenting complaint was not stated.

5.4 Adequacy rates

5.4.1 Cytology adequacy

A total of 10 of the bile duct brushings (4,81%) were classified as inadequate and could not be assessed cytologically, 23 (11,06%) as suboptimal, and 175 (84,13%) as adequate. (**Figure 9.**)

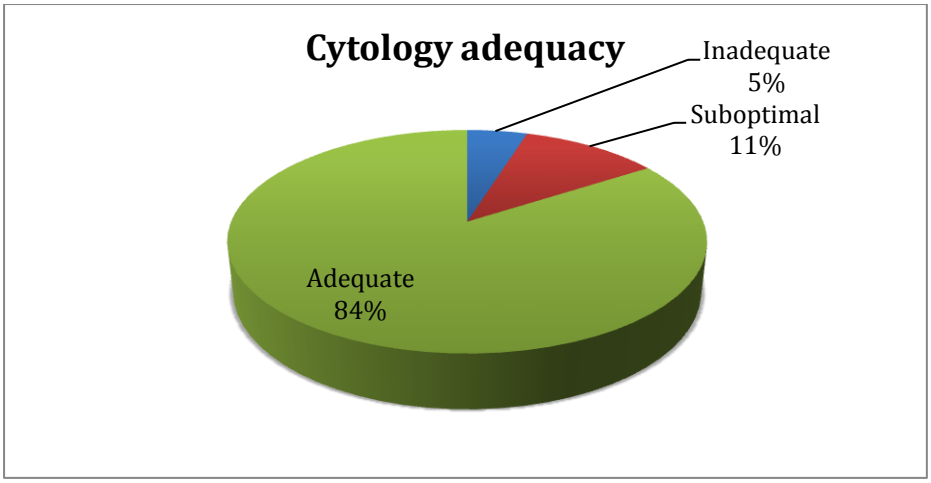


Figure 9: Cytology adequacy

Of the 23 suboptimal cytologies, 8 (34,78%) were diagnosed as benign, 14 (60,87%) as atypical, 1 (4,35%) as suspicious for malignancy, and none (0%) were diagnosed as outright malignant.

5.4.2 Histology Adequacy

A single histology case (0,48%) was classified as inadequate for assessment, and this was due to its not having been submitted in formalin. Six cases (2,88%) were classified as suboptimal, for reasons such as the presence of crush artefact, or the scanty/superficial nature of the biopsy material; all of these were biopsy specimens. The remaining 201 cases (96,63%) were classified as adequate for diagnostic purposes. (**Figure 10.**)

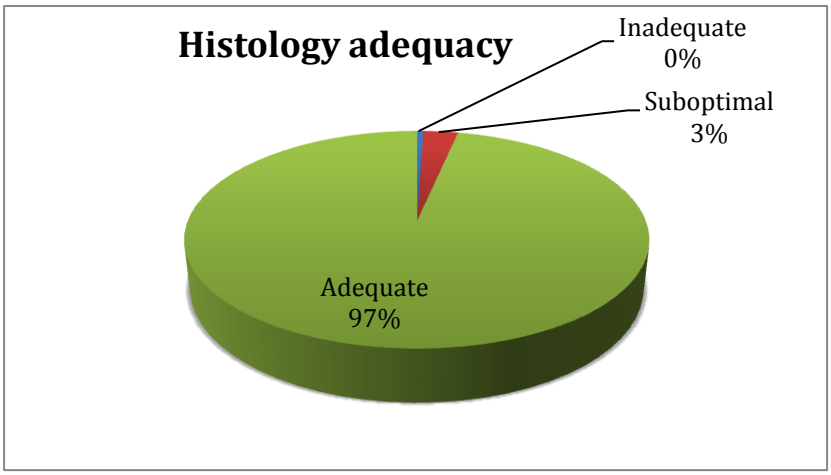


Figure 10: Histology adequacy

5.5 Validity of bile duct brushing cytology: sensitivity, specificity, accuracy and predictive values

Cases classified as malignant or suspicious for malignancy on cytology were included as positive cytological diagnoses. The numbers and percentages of true and false positives and negatives, respectively, were as follows:

1. True positives: 44 cases = 21,15%
2. False positives: 3 cases = 1,44%
3. True negatives: 74 cases = 35,58%
4. False negatives: 26 cases = 12,50% (**Figure 11.**)

The numbers of cases with inadequate and atypical cytology (which were classified separately and not placed in the four categories above), were as follows:

1. Inadequate cytology: 10 cases = 4,81%
2. Atypical cytology: 51 cases = 24,52% (**Figure 11.**)

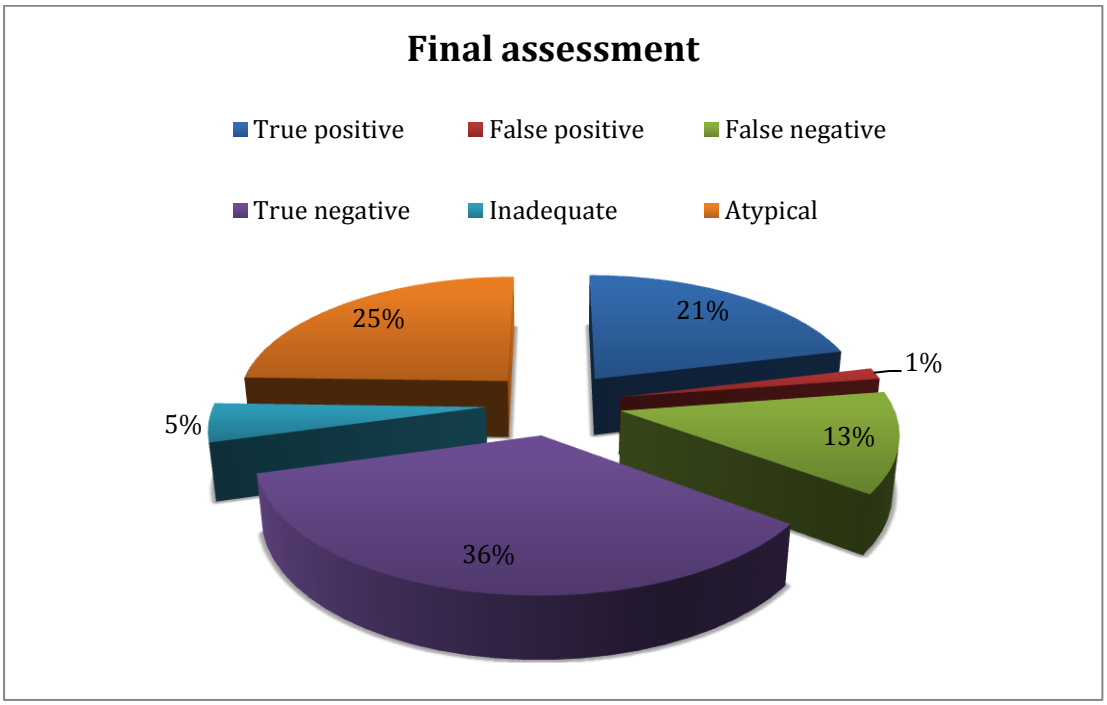


Figure 11: Final assessment

5.5.1 Sensitivity

Bile duct brushing cytology's sensitivity is the extent to which it can correctly diagnose malignancy in patients with malignant bile and pancreatic duct strictures. It is complementary to the false negative rate. Given a moderate number (n=26, 13%) of false negatives in our cohort, it follows that the sensitivity will be only moderate to good, but not excellent. Our calculated sensitivity was 62,86%, with a 95% confidence interval (CI) of 50,43% to 73,86%.

Differential sensitivities for pancreatic adenocarcinoma, cholangiocarcinoma and ampullary carcinoma are as follows:

1. Pancreatic carcinoma: 57,89%
2. Cholangiocarcinoma: 60,00%
3. Ampullary carcinoma: 85,71%

5.5.2 Specificity

The specificity of bile duct brushing cytology informs about how many of the patients with positive cytology will ultimately turn out to have a malignancy, and it is inversely correlated to the false positive rate. Given an only 1% false positive rate (n=3), it follows that the specificity of bile duct brushing cytology is high. Our calculated specificity was 96,10% (95% CI = 88,27%-98,99%).

5.5.3 Positive predictive value (PPV)

For any positive bile duct brushing cytology result, the probability that it is a true positive equates to the positive predictive value (PPV). Our calculated PPV was 93,62% (95% CI = 81,44%-98,34%).

5.5.4 Negative predictive value (NPV)

For any negative bile duct brushing cytology result, the probability that it is a true negative equates to the test's negative predictive value (NPV). Our NPV was 74% (95% CI = 64,10%-82,03%).

5.5.5 Accuracy

The accuracy of brushing cytology equates to the number of "true" (correct) results, either positive or negative, in the total number of results. Cytology's calculated accuracy in our study is 80,27%, meaning that 80,27% of all results were true reflections of the patient's disease status. The 95% confidence interval is 66,90% to 93,64%.

The final results of the validity calculations are summarised in **Table 4**.

Table 4: Summarised validity measures

Sensitivity	62,86% (95% CI = 50,43-73,86%)
Specificity	96,10% (95% CI = 88,27-98,99%)
Positive predictive value (PPV)	93,62% (95% CI = 81,44-98,34%)
Negative predictive value (NPV)	74,00% (95% CI = 63,10-82,03%)
Accuracy	80,27% (95% CI = 75,68-84,86%)

These values were verified and confidence intervals calculated using the following resource: http://www.vassarstats.net/clin1.html, accessed on 2nd February 2015 at 10:58am.

5.6 Prevalence of malignant disease

The prevalence rate of malignancy in our patient cohort was 47,62% (95% CI = 39,38-55,98%).

5.7 Histological diagnosis

5.7.1 Histology sample types

The different histological specimens received and their numbers are listed below. As many patients underwent open surgery, many had several histology specimens received from the same procedure.

Biopsy samples

The biopsy samples consisted of:

1. Pancreatic biopsies (i.e. core or wedge biopsies of a specific mass in the head, body or tail of the pancreas): n = 4
2. Ampullary/periampullary/duodenal (forceps) biopsies: n= 87
3. Liver biopsies (core or wedge): n= 24
4. Biopsies of distant sites in suspected metastatic disease (including omental biopsies, umbilical and lymph node biopsies): n=7
5. Biopsies of gallbladder masses: n=2

Excision samples:

The various excision procedures performed included the following:

1. Partial pancreatectomy (Frey's procedure or other): n=14
2. Whipple's pancreaticoduodenectomy: n=34
3. Gallbladder (cholecystectomy +/- excision of bile ducts): n=41
4. Partial hepatectomy or hepatic segmentectomy: n=3
5. Distal gastrectomy: n=1

Autopsies:

Three patients underwent postmortem examinations.

5.7.2 Final histological diagnosis

Malignant diagnoses

A total of 98 patients had a diagnosis of malignancy on histology. Of these, the final histological diagnoses were as follows (given in absolute numbers and as percentages of the total number of malignant diagnoses):

1. Pancreatic adenocarcinoma: n=29 (29,59%)
2. Cholangiocarcinoma: n=13 (13,27%)
3. Ampullary carcinoma: n=17 (17,35%)
4. Mucinous carcinoma: n=3 (3,06%)
5. Gallbladder carcinoma: n=4 (4,08%)
6. Neuroendocrine carcinoma: n=2 (2,04%)
7. Mixed adenocarcinoma/neuroendocrine carcinoma (MANEC): n=1 (1,02%)
8. Lymphoma: n=3 (3,06%)
    a. Burkitt lymphoma: n=1 (1,02%)
    b. Plasmablastic lymphoma: n=1 (1,02%)
    c. Diffuse Large B-Cell Lymphoma (DLBCL): n=1 (1,02%)
9. Metastatic carcinoma: n=1 (1,02%)
10. Squamous cell carcinoma (SCC): n=1 (1,02%)
11. Dysplasia/adenocarcinoma in-situ/adenoma/other preinvasive lesion: n=6 (6,12%)
12. Signet ring adenocarcinoma: n=2 (2,04%)
13. Adenocarcinoma, not otherwise specified (NOS): n=16 (16,33%)

The single case of metastatic carcinoma was in a 43-year-old female patient who was known with stage IV breast cancer, with metastases to the chest wall, orbital soft tissues, liver, bone, bone marrow and porta hepatis, the latter causing obstructive jaundice.

The single case of SCC was in a 48-year-old female patient, and was diagnosed on a biopsy of the ampulla of Vater. A comment was made in the histology report that this may represent either a primary or a metastatic SCC, or could be part of an adenosquamous carcinoma.
Of the 6 preinvasive/dysplastic lesions, two were tubulovillous adenomas of the ampulla, one with low-grade and one with high-grade dysplasia.

Benign diagnoses

There were 107 patients with benign diagnoses on histology, two of whom were diagnosed with a non-malignant tumour.

The spectrum of benign, non-neoplastic diagnoses was as follows (given in absolute numbers and as percentages of the total number of benign diagnoses):

1. Duodenitis/inflammation of the ampulla and benign peptic ulcers: n=29 (27,62%)
2. Chronic pancreatitis: n=17 (16,19%)
3. Cholecystitis and cholangitis: n=33(31,43%)
4. Granulomatous inflammation NOS: n=2 (1,90%)
5. Inflammation NOS: n=3 (2,86%)
6. Specific infections:
    a. Microsporidiosis: n=2 (1,90%)
    b. Schistosomiasis: n=2 (1,90%)
7. Hepatic abscess: n=1 (0.95%)
8. Normal tissue: n=23 (21,90%)

The two non-malignant tumours were both intraductal papillary mucinous neoplasms (IPMN's).

There were three equivocal histological diagnoses that were classified as atypical (0,48%). All were on biopsy specimens, and two of the three were classified as suboptimal specimens. One of these was a superficial biopsy showing features of an atypical papillary lesion, and the other two showed glandular atypia worrisome for malignancy.

5.8 Atypical cytology: correlation with final outcome

A total of 51 cases, constituting 24,52% of the total cohort, were classified as atypical. Of these, 26 were ultimately classified as benign disease on histology and 25 were ultimately diagnosed as malignant. Therefore, of cases classified as atypical on cytology, roughly half (50,98%) represented benign disease and the other half (49,02%) represented malignant disease.

CHAPTER 6

6.0 DISCUSSION

6.1 The major findings of the study

We set out to determine the validity measures of brushing cytology when compared to histology in specimens received from two Johannesburg hospitals over a seven-year period. Our study shows a very good correlation between biliary brushing cytology and histology in this setting, with values that are in line with those reported in previous studies. We have demonstrated excellent specificity and positive predictive values (PPV) of 96% and 94%, respectively, with a false positive rate of only 1%. Our sensitivity and negative predictive values (NPV), on the other hand, are more modest: 63% and 74%, respectively, with a 13% false negative rate. We have shown our overall accuracy to be 80%. Twenty five percent of cases were classified as atypical, of which 51% were ultimately accorded benign and 49% malignant diagnoses.

Eighty four percent of specimens were deemed adequate for cytological assessment, with 11% suboptimal and the remaining 5% inadequate or unsuitable for assessment.

6.2 The meaning of the findings

The high specificity and corresponding high PPV indicate that a positive brushing cytology is strongly predictive of the presence of malignancy, and there is a low rate of false positive diagnoses. The lower sensitivity and NPV indicate that a negative brushing cytology by no means rules out the possibility of a pancreatobiliary malignancy in an individual patient. This confirms biliary brushing cytology's previously reported operating characteristics as a strong "rule-in" test, but only a moderate to weak "rule-out" test.

We set out to establish whether a cytology diagnosis of "atypical" was more predictive of benignity or malignancy, and found it to be neither. A quarter (25%) of all cytology specimens were classified as atypical. This accounts for a significant proportion of cases being rendered an indeterminate diagnosis on cytology. An example of one of our atypical cases, together with examples of cases with benign and malignant cytology for comparison, is shown in **Figure 6.**

Of the atypical cases, 51% were ultimately proven to have benign disease, with 49% having malignant disease – an almost 50:50 split. Thus the cytological diagnostic category of "atypical" appears to carry no predictive value or correlation with final outcome in our setting, and comprises an even mix of reactive/inflammatory atypia and neoplastic change.

Given that over half (61%) of cytology specimens deemed suboptimal for interpretation were classified as atypical, it appears that suboptimal quality specimens select for an indeterminate diagnosis.

6.3 Relation of the findings to previous studies

6.3.1 Cohort size and demographics

This is one of the largest cohorts of patients which has to date been studied for validity measures of biliary brushing cytology. The studies by Volmar *et al*[5] and Stewart *et al,*[33] featuring 971 and 406 patients, respectively, comprised bigger patient numbers, but most other studies[7-12, 21-22, 35-32, 35, 37] featured smaller cohorts. Mahmoudi *et al* featured a similar cohort of 199 patients.[34]

Our patient demographics are comparable to those reported in many of the previous studies, with slight differences. Our reported mean patient age of 55 years falls below that reported in most studies,[5,7,12,25,30, 33,35,37-38] whose mean age ranges from 62[37] to 75[25] years; it is slightly higher than the mean age of 52 years reported by Trent *et al.*[9] The reported male:female ratios are quite variable, with some studies having a greater number of female patients and a ratio below zero,[30,35] whilst the majority, like us, reported a slight excess of male patients and ratios between 1.0 and 1.4.[5,7,9,21,31,33,39]

6.3.2 Operating characteristics

We showed a cytology adequacy rate of 84% and histology adequacy of 97%. The former falls below the reported "technical success" rates of 92% to 100% of all the studies summarised in **Table 2**. However, if we combined our adequates with suboptimal specimens, we achieve a technical success rate of 95%.

Our sensitivity, at 62,86%, compares favourably with that reported in previous studies, and is higher than that reported in 18 of the 21 papers summarized in **Table 2**. The average of the overall sensitivities of all the studies in **Table 2** is 51,78%. Similar can be said for our NPV of 74%.

Our specificity of 96,1% is below the 100% reported in 15 of the 21 studies in Table 2, but above that reported by Yap *et al,*[22] Volmar *et al,*[5] Ryan *et al,*[31] Jaiwala *et al*[33] and Chadwick *et al,*[37] and falls at the high end of the reported range of 89-100%.[22] This analysis also holds true for our PPV of 93,62%.

The accuracy of cytology in our context is 80,27%. This is lower than the 94%, 84% and 81% accuracy reported by Ryan *et al*,[31] Trent *et al*,[9] and Hart *et al*,[36] respectively, but higher than the accuracies of the eight other studies listed in **Table 2** in which an accuracy figure was reported.

Our results are slightly inferior to those reported in the only previous South African study on this subject.[12] Their sensitivities of 70% when only malignant diagnoses were considered positive, and 83% when both suspicious and malignant diagnoses were considered positive are superior to ours, but still fall in the moderate range. Their perfect specificity of 100% is also superior to our value of 96%. Overall, however, our validity measures are roughly comparable to theirs.

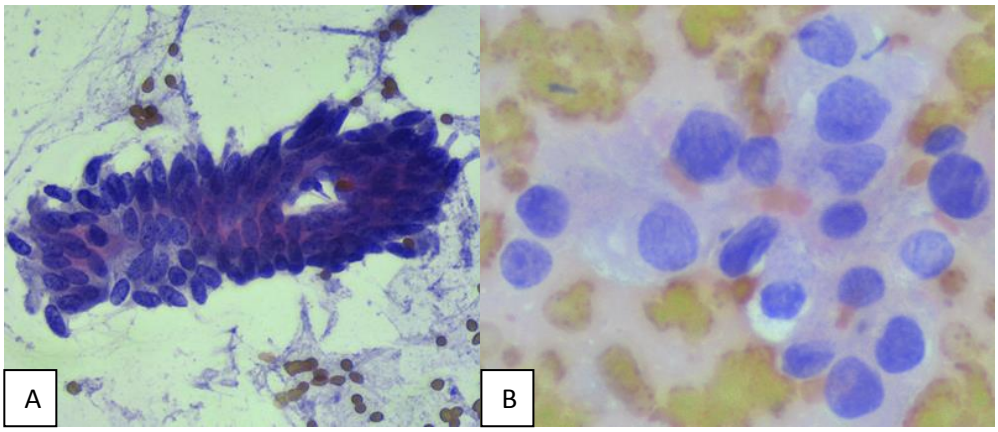
6.3.3 Atypical cytology: analysis and comment

Our atypical cytologies comprised 25% of all specimens, and these cases were analysed separately in terms of their final outcomes. Given the differences in both the classification of atypicals and in their use in the calculations in the other studies cited in this report, meaningful comparison is difficult. Well over half (60%) of suboptimal specimens were accorded an atypical cytological diagnosis, in keeping with the fact that a definitive diagnosis is often precluded in specimens of borderline and suboptimal adequacy, due to difficulties in accurate interpretation. In part, then, our high rate of aytpicals on cytology may be attributable to our roughly one in ten rate of suboptimal specimens.

Uncertainties regarding diagnostic criteria would contribute to a high proportion of atypicals. However, it is the practice of the cytologists in our Cytology Department to consign a case to the atypical category only when the features are not consistent with either benign/reactive or suspicious/malignant epithelium, as mandated by the new Papanicolaou Society terminology guidelines. (**Table 1.**) It is hoped that, following publication of these guidelines last year, new studies will emerge documenting proportions of cases consigned to each category in cytology departments around the world, such that we may gauge our results by this yardstick.

We have shown that, in our practice, there is no correlation between a cytological diagnosis of atypical and the final outcome: there is a roughly 50/50 benign/malignant split in the final

analysis; this is comparable to the results of a random “coin toss”. This speaks to the fact that the category of atypical is something of a waste-basket for cases showing reactive/ inflammatory atypia, degenerative atypia in air-dried and scanty samples, and neoplastic change (often in samples of suboptimal adequacy) which falls short of that required for a designation of “suspicious for malignancy” or “malignant. (**Figure 12.**)



Figures 12A and 12B: Atypical epithelium

Atypical epithelium, showing slight crowding and overlap with moderate nuclear: cytoplasmic ratio (A), and smooth nuclear outlines with fine to moderately coarse chromatin (B). (Papanicolaou stain, 200X and 400X, respectively)

For one quarter of specimens to be given an equivocal cytological diagnosis is a detractor from the clinical usefulness of the test, and ways to reduce cytological atypicals need to be considered in order to improve our service. Possible changes would be increased training for the ERCP operators in specimen collection and submission, to reduce the rate of suboptimal specimens. However, given the inherent long time delay in the brushing of the lesion and the retraction of the brush into the sheath and retrieval through the upper gastrointestinal tract out through the mouth, biliary brushing specimens are subject to a greater amount of air-drying artefact than other cytology specimens. Increased training would be of some benefit in less experienced endoscopists, but would perhaps not decrease the rate of suboptimal specimens substantially overall.

Rapid on-site evaluation (ROSE) is an intervention that has been shown to improve the quality of specimens obtained at cytology and thus improve cytological accuracy overall.[51]

ROSE has for several years been performed by members of the Cytology Department in image-guided fine needle aspirates (FNAs), to good effect. Since 2014, ROSE has also been extended to intraoperative FNAs of pancreatic masses and ERCP-guided biliary brushing cytology. Preliminary assessment suggests that this is improving the service and decreasing inadequate/suboptimal specimens. As our study time frame ends before this change, the effect would not be evident in our cohort.

6.4 Alternative explanation of the findings

Our sensitivity and NPV would have been lower had we excluded those cases classified cytologically as "suspicious for malignancy", and our already-high specificity and PPV would have increased slightly. Likewise, including the "atypicals" as either positive or negative cytology would have resulted in drastically different operating characteristics. In this regard, however, it is common practice to regard a diagnosis of "suspicious for malignancy" as positive, whilst a diagnosis of "atypical" is widely accepted to be indeterminate.

We must bear in mind that issues of clinical variability can lead to the sensitivity and specificity of a diagnostic test changing in relation to the prevalence of a disease.[52] For instance, cohorts with a higher disease prevalence may be composed of patients with more severe clinical manifestations, such that the performance characteristics of a diagnostic test may be better in these populations than in those with lower prevalences. Our sensitivities and overall accuracy being higher than those reported in previous studies may, in part, be attributable to a higher prevalence of malignancy in our study population.

6.5 Study Limitations

Exclusive correlation with histology as a gold standard in this study introduces a potential bias toward missing cases with an ultimate malignant clinical course, albeit with benign histology. As cases with malignant cytology and benign *biopsy* histology would have been the main contributors to this bias, it was decided to exclude those cases where there was

discordance between cytology and biopsy histology. Those cases excluded on this basis included all but one of the "suboptimal" histologies. However, a percentage of our cases with benign brushing cytology and benign biopsy histology could, potentially, still have represented false negatives by both analyses.

Independent review of cytology, as performed in some prior studies on this topic,[5,11,34] was not performed in this study. This could potentially have allowed for reclassification of a proportion of the false positive or false negative cytologies, and may have improved our operating characteristics marginally.

6.6 Clinical relevance of the findings

In our context, a negative or atypical biliary brushing cytology in a patient with a stricture or mass lesion of the pancreatobiliary system must be regarded as equivocal for malignancy, as we have shown that there remains an appreciable incidence of malignancy in these patients. In particular, we have shown that atypical cytology is not predictive of malignancy or benignity in any way. Further investigation, with repeat cytology, biopsy histology, or definitive management based on the constellation of clinical and radiological findings is appropriate in this patient subset.

On the other hand, brushing cytology that is suspicious for malignancy or malignant can be regarded as diagnostic of malignancy, given that our PPV and specificity values approach 100%. This can obviate the need for further invasive diagnostic procedures in these patients, and suggests that definitive management decisions can be based on a positive cytology diagnosis.

Inadequate cytologies provide no diagnostic information, and remain a challenge with which we must contend. Suboptimal cytologies predict for inconclusive diagnoses, and should also be eliminated as far as possible.

6.7 Suggestions for further research

Implementation of more rigorously defined diagnostic terminology as espoused by the Papanicolaou society's 2014 standardised nomenclature for pancreatobiliary cytology will undoubtedly impact on reporting practices, and may affect the validity of brushing cytology results. Studies prospectively classifying or retrospectively reclassifying our cases according to this scheme will be of value in this regard.

A formal analysis of the recently instituted policy of ROSE for biliary brushing cytology at Chris Hani Baragwanath Hospital will be instructive in assessing whether this improves validity measures. Of particular interest will be whether this practice, in reducing inadequate and suboptimal specimens, has any benefit in reducing numbers of cases consigned to the diagnostically unhelpful atypical category.

CHAPTER 7

7.0 CONCLUSION

This study has investigated the validity measures of biliary brushing cytology performed at two Johannesburg hospitals over a seven-year period. We have shown a moderate sensitivity and a moderate NPV of 63% and 74%, respectively. Our specificity was an impressive 96%, and we demonstrated an equally satisfactory PPV of 94%. All validity measures reported by us are in line with figures reported in several international studies and one local study into these parameters. Biliary brushing cytology as performed in our local context, therefore, is a highly specific and moderately sensitive diagnostic tool. A positive diagnosis in the correct clinical context is confirmatory of malignancy, whilst an atypical or negative diagnosis mandates further investigation.

Continued use of biliary brushing cytology as a first-line diagnostic procedure in this clinical context is justified, and our Cytology Department is providing a useful and valid service to clinicians and patients alike.

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APPENDIX 1: DATA COLLECTION CODES AND SHEET

<u>Appendix 1a:</u> Numerical codes to be used in data capture for cytology and histology category

	Cytology	**Histology**
Inadequate	0	10
Suboptimal (Diagnosis made but sample may not be fully representative of underlying lesion)	1	11
Adequate	2	12
Benign	3	13
Atypical	4	14
Suspicious	5	15
Malignant	6	16

Appendix 1b: Blank data collection sheet

Study number	Age	Sex	Clinical presentation	Cytology ref #	Cytology adequacy[1]	Cytology diagnosis	Cytology Category[1]	Histology Ref #	Histology specimen type and site	Histology Adequacy[1]	Histology diagnosis	Histology category	Comments
			e.g. obstructive jaundice		0 or 1		2, 3, 4, 5, 6		e.g. peripancreatic lymph node excision	10 or 11		12, 13, 14, 15, 16	

[1] See Appendix 1a.

APPENDIX 2: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Kristin Coetzee

CLEARANCE CERTIFICATE	M110720
PROJECT	Cytology and Associated Histology of Biliary and Pancreatic Duct Strictures: How Good is Our Correlation?
	(Previously Dr Nicola Scholfield)
INVESTIGATORS	Dr Kristin Coetzee
DEPARTMENT	Department of Anatomical Pathology
DATE CONSIDERED	29/07/2011
DECISION OF THE COMMITTEE*	Appoved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 11/08/2014 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr Pamela Michelow

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3: TURNITIN SIMILARITY REPORT

