# PROTOCOL TITLE:

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| **Haematuria Biomarker Study (HaBio)**  **Study ID No:** |

### ORECNI no: 11/NI/0164

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ABBREVIATIONS

8-OHdG 8-hydroxydeoxyguanosine

A-FABP adipocyte-faty acid binding protein

AUC Area under the curve

BCH Belfast City Hospital

BPE Benign prostate enlargement

BTA Bladder Tumour Antigen

CAH Craigavon Area Hospital

CCRCB Centre for Cancer Research and Cell biology

CEA Carcino-Embryonic Antigen

CK Cytokeratin

CRF Case Report File

CRP C-reactive protein

CTA Clinical Trial Administrator

CV Coefficient of variation

CXCL1 Chemokine (C-X-C motif) Ligand 1

DPU Day Procedure Unit

ECMC Experimental Cancer Medicine Centre

EGF Epidermal Growth Factor

FDA Food and Drug Administration

FDP Fibrinogen Degradation Product

FISH Fluorescence *in situ* hybridisation

FPSA Free Prostate Specific Antigen

GCP Good Clinical Practice

HA Hyaluronidase

HTA Human Tissue Act

IHC immunohistochemistry

IL Interleukin

IQR Inter-quartile range

MCM 5 Mini chromosome maintenance 5

MCP-1 monocyte chemoattractant protein-1

MMP matrix metalloproteinase 9

NIB Northern Ireland Biobank

NICaN Northern Ireland Cancer Network

NICTC Northern Ireland Cancer Trials Centre

NGAL neutrophil-associated gelatinase lipocalin

NMP22 Nuclear Matrix Protein 22

NSE Neuron Specific Enolase

PAI-1 Plasminogen activator inhibitor type 1

pERK phospho extracellular signal regulated kinase

PKR Protein kinase R

PPI Patient public involvement

PPP Proven Predicted Probability

PSA Prostate Specific Antigen

REC Research Ethical Committee

RFC Random Forest Classifier

ROC Receiver Operating Characteristic

SOP Standard Operating procedure

STARD Standards for the Reporting of Diagnostic Accuracy Studies

TGF-β Transforming Growth Factor-β

TIMP Tissue Inhibitor of MetalloProteinases

TM Thrombomodulin

TMA Tissue MicroArray

TNF-α Tumour Necrosing Factor- α

TNFR1 Tumour Necrosing Factor Receptor 1

TPA Tissue-type plaminogen activator

TPS Tryptase

TPSA Total Prostate Specific Antigen

TURB Transurethral resection of the bladder

UBC Urinary Bladder Cancer

VEGF Vascular Endothelial Growth Factor

vWF Von Willebrand Factor

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### 1. Background & Rationale

### 1. 1 Background – Clinical need for diagnostic biomarkers for haematuria

Haematuria is a very common presenting symptom to medical practitioners. It is termed frank or macroscopic haematuria if it is visible to the patient. However, when medical or nurse practitioners dipstick an otherwise normal sample, haematuria can also be detected. This is termed dipstick or invisible haematuria.

There is an urgent clinical need for additional evidence-based risk stratification modalities to inform triage and/or management decisions for patients who present with haematuria (Mostafid, 2010).

Haematuria in its visible and invisible forms can represent a disease process within the urinary tract. These disease processes can range from benign causes such as urinary infection or stone disease to malignant causes such as bladder cancer. Indeed, it is common for no obvious cause to be identified after detailed investigations. These investigations include cystoscopy and radiological imaging of the upper urinary tract.

Cystoscopy allows direct observation of the bladder, but it is both invasive and uncomfortable for the patient. If a suspicious region is seen a biopsy is needed. Cystoscopy does not allow for upper track visualisation, does not always detect small areas of carcinoma *in situ,* can give false positive results, is embarrassing for the patient and can be biased by the risk category of the patient (van der Aa, 2010).

Cytology testing on voided urine samples examines the appearance of cells from urine. Cytology has a low sensitivity and therefore cannot be used to stratify high or low risk haematuria.

There is a strong clinical need for urine based tests which can at least risk stratify and if possible, be diagnostic. The drive therefore is to use biomarker profile patterns in urine as a way to diagnose the cause of the haematuria. Biomarker profiles may fail to differentiate between all possible causes of haematuria, but it would be a huge advancement if benign and malignant causes were differentiated. Therefore a significant focus of the recruitment drive in this study will be bladder cancer patients.

Currently only three biomarkers are FDA approved. Nuclear Matrix Protein 22 (NMP22) (Lotan, 2009), Bladder Tumour Antigen (BTA) (Kinders, 1998) and Fibrinogen Degradation Product (FDP) (Johnston, 1997) are FDA approved protein-based biomarker assays, but none to date, has sufficient diagnostic accuracy, on their own, to replace cystoscopy or cytology. Similarly to NMP22, Aurora kinase is a key regulator of mitosis. There is a fluorescence *in situ* hybridisation (FISH) test for gene amplification of Aurora kinase A, which was reported to achieve 97% specificity and 87% sensitivity (Park, 2008). However, this assay involves laborious and complex steps which preclude its translation to a high throughput format, appropriate for screening large numbers of patients. Similarly, other diagnostic tests for bladder cancer, telomerase and FISH, are complex assays which cannot be used without expert personnel and equipment.

Randox Laboratories is an international clinical diagnostics company offering innovative solutions to laboratories worldwide. The company has developed four FDA approved analysers, (Evidence ™), all are CE marked. The analysers have varying throughput capacity for the use of their patented biochip array technology. Testing is performed on 0.1ml plasma, serum or urine which is applied to a biochip which simultaneously measures multiple biomarkers, providing results in 1-2 hours (Figure 1).



**Figure 1: General assay procedure using Evidence Investigator**

Procedures are manual and very similar to performing an ELISA. First, assay reagents and sample are added to the ready to use biochips within the carriers. The carriers are then incubated at 37°C with orbital shaking. Following incubation, the carriers are washed to remove any unbound material from the biochips. Signal reagent is then added to each biochip before the carrier is imaged using the evidence Investigator imaging module. The light emitted from each test region is quantified and results are automatically calculated by the custom designed software.

To date, no single biomarker has the diagnostic capability to replace cystoscopy, the gold standard for detection of bladder cancer. Thus, there is a need for a device that can measure multiple biomarkers simultaneously, handle high throughput screening of patient samples, is non-invasive, deliver results within hours, is robust and has high sensitivity and specificity for bladder cancer. The Randox biochip arrayed with the correct combination of biomarkers would address this need (Figure 1).

### We completed a pilot study between 2006 and 2008 which recruited 181 patients with haematuria. Using data from 157 of these patients, we demonstrated the feasibility of creating diagnostic algorithms for bladder cancer in patients presenting with haematuria (Abogunrin *et al* Cancer 2012).

### 1.2 Feasibility Study Summary

BACKGROUND

In the feasibility study, we appraised 23 biomarkers previously associated with bladder cancer in 157 patients (Figure 2). These biomarkers represented diverse carcinogenic pathways (Table 1).

Slide1.TIF

**Figure 2: Feasibility Study Flow Chart**

|  |  |
| --- | --- |
| **Pathways** | **Biomarkers** |
| Angiogenesis | HA, IL-1α, IL-1β, IL-8, VEGF, vWF |
| Apoptosis | FAS, NMP22 |
| Differentiation | CEA, CK18 |
| Glycolysis | NSE |
| Growth | EGF, HA, IL-1α, IL-1β, IL-8, NMP22 |
| Immune | BTA, IL-2, IL-4, IL-8 |
| Inflammation | CRP, HA, IL-6, IL-8, NGAL, MCP-1, TNFα |
| Metastases | CEA, HA, IL-8, MMP-9, MMP-9 NGAL complex, NGAL |

**Table 1: Study Pathways investigated in the Feasibility Study**

BTA Bladder Tumour Antigen, CEA Carcino-Embryonic Antigen, CK18 CytoKeratin 18, IL InterLeukins, CRP C-Reactive Protein, EGF Epidermal Growth Factor, HA Hyaluronidase, MCP-1 Monocyte Chemoattractant Protein-1, MMP-9 Matrix MetalloProteinase-9, NGAL Neutrophil-Associated Gelatinase Lipocalin, NMP22 Nuclear Matrix Protein 22, NSE Neuron Specific Enolase, TNF-α Tumor Necrosis Factor alpha, TM ThromboModulin, VEGF Vascular Endothelial Growth Factor, vWF von Willebrand Factor.

Our aim in the feasibility study was to determine whether single biomarkers and/or multivariate algorithms significantly improved on the predictive power of an algorithm based on demographics for prediction of bladder cancer in patients presenting with haematuria.

METHODS

Twenty-two biomarkers in urine and Carcino-embryonic antigen (CEA) in serum were evaluated using ELISAs and biochip array technology in two patient cohorts; 80 patients with bladder cancer and 77 controls with confounding pathologies. We used Forward Wald binary logistic regression analyses to create algorithms based on demographic variables designated Prior Predicted Probability (PPP) and multivariate algorithms which included PPP as a single variable. Areas under the Curve (AUC) were determined from Receiver Operating Characteristic (ROC) curves for single biomarkers and algorithms.

RESULTS

Following univariate analysis, nine biomarkers were differentially expressed (T-test; p < 0.05). CEA AUC = 0.74; Bladder Tumor Antigen BTA AUC = 0.74; and Nuclear matrix protein (NMP22) = 0.79. PPP included age and smoking years; AUC = 0.76. An algorithm including PPP, NMP22 and Epidermal Growth Factor (EGF) significantly improved AUC to 0.90 when compared to PPP. The algorithm including PPP, BTA, CEA and thrombomodulin (TM) increased AUC to 0.86. Sensitivities = 91%, 91% and specificities = 80%, 71%, respectively, for the algorithms.

CONCLUSION

Addition of biomarkers representing diverse carcinogenic pathways can significantly impact on the ROC statistic based on demographics. Benign Prostate Hyperplasia was a significant confounding pathology and identification of non-muscle invasive bladder cancer remains a challenge.

PUBLICATIONS

Frank Emmert-Streib, Funso Abogunrin, Ricardo de Matos Simoes, Brian Duggan, Mark W Ruddock,Cherith N Reid, Owen Roddy, Lisa White, Hugh F O’Kane, Declan O’Rourke, Neil H Anderson, Thiagarajan Nambirajan, Kate E Williamson. Collectives of diagnostic biomarkers identify high risk subpopulations of haematuria patients: exploiting heterogeneity in large-scale biomarker data. *BMC Medicine* 2013, **11**:12 doi:10.1186/1741-7015-11-12

Cherith N. Reid, Michael Stevenson, Funso Abogunrin, Mark W. Ruddock, Frank Emmert-Streib, John V. Lamont, Kate E. Williamson (2012)[Standardization of Diagnostic Biomarker Concentrations in Urine: The Hematuria Caveat](http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0053354) Research Article PLOS ONE doi: 10.1371/journal.pone.0053354

Funso Abogunrin, Hugh F O’Kane, Mark W Ruddock, Michael Stevenson, Cherith N Reid, Joe M O’Sullivan, Neil H Anderson, Declan O’Rourke, Brian Duggan, John V Lamont, Ruth E Boyd, Peter Hamilton, Thiagarajan Nambirajan, Kate E Williamson. The impact of biomarkers in multivariate algorithms for bladder cancer diagnosis in patients with haematuria. [Cancer](http://www.ncbi.nlm.nih.gov/pubmed/21918968) (2012) 118(10):2641-50. DOI: 10.1002/cncr.26544

**1.3 Haematuria Biomarker Study (HaBio)**

BACKGROUND

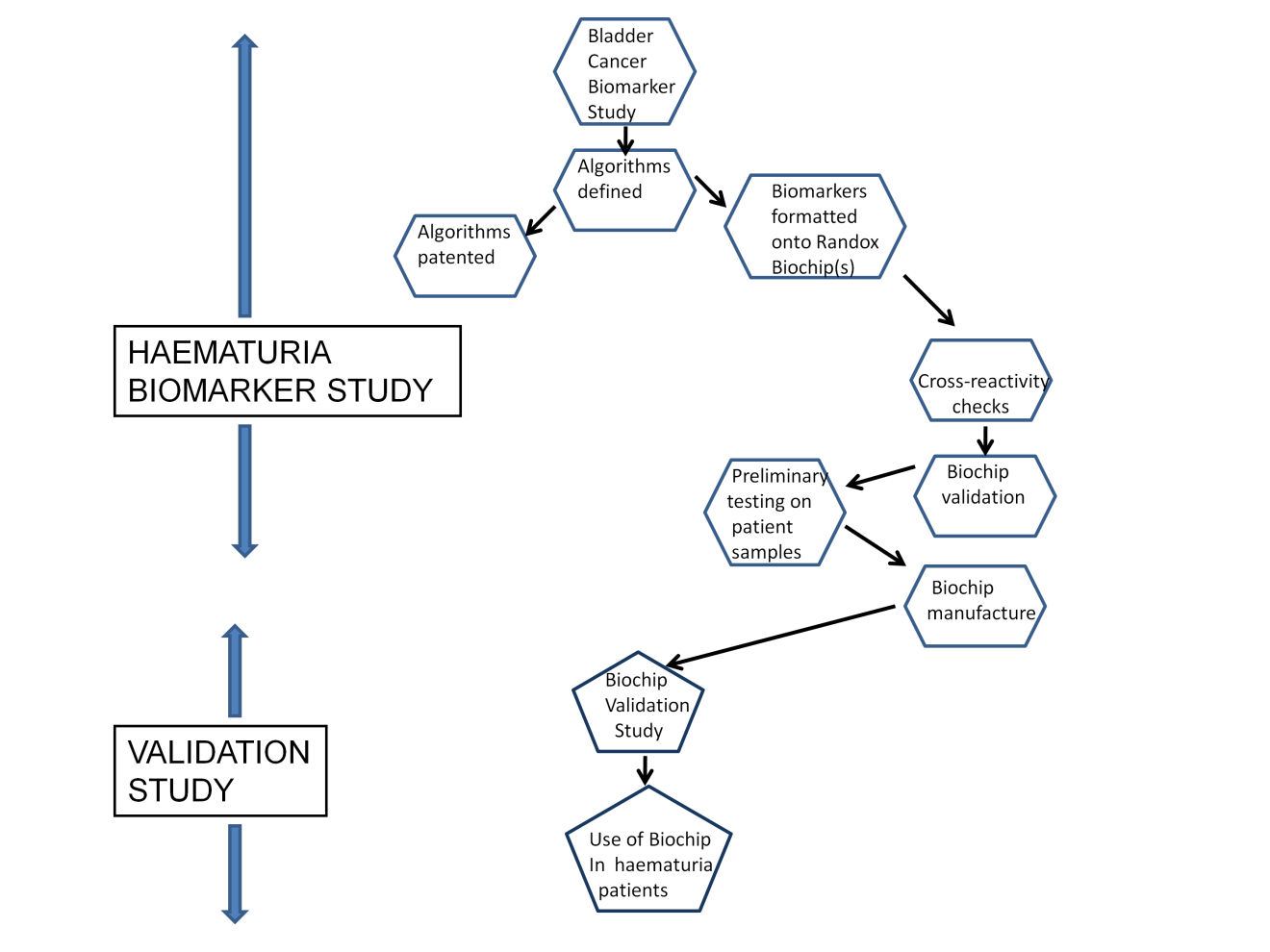
Having established the feasibility of diagnostic algorithms for bladder cancer in haematuria patients, we have designed a large Haematuria Biomarker Study (HaBio) which will recruit 999 patients in hospitals throughout Northern Ireland.

The aim of HaBio is to define algorithms in a larger group of patients using an expanded panel of biomarkers. These algorithms will require validation in a separate Validation Study (outside the scope of this proposal) (Figures 3) which will be undertaken after optimisation of the biochip (Figure 4).

**Figure 3 Sequence of studies to establish a bladder cancer biochip.tif**

**Figure 3: Sequence of studies to establish a Bladder Cancer Biochip**

Patients were recruited to a feasibility study between 2006 and 2008. This study reported that addition of biomarkers to a diagnostic algorithm for bladder cancer based on demographics, significantly enhanced the Receiver Operating Characteristics statistic (Abogunrin et al, 2012). We designed HaBio to define two or more algorithms for the diagnosis of bladder cancer in 999 haematuria patients. The best algorithm(s) will be formatted onto the Randox biochip(s) for validation in a subsequent Validation Study.



**Figure 4: Optimisation of biochip and its future validation**

The aim of HaBio is to create two or more algorithms which detect bladder cancer in haematuria patients. Algorithms will be defined following both classical statistical analyses and bioinformatics approaches . The biomarkers which contribute to algorithms chosen for development will be formatted onto biochips for subsequent checks and optimisation. Subsequent steps such as biochip manufacture and testing in a larger independent study will be outside the remit of HaBio.

We will recruit 999 patients with haematuria to a case control study. One third of the patients (n = 333) will have bladder cancer. The remaining 666 patients will have negative results from all investigations for bladder cancer, and will act as controls in the study. Recruitment will always prioritise bladder cancer patients. Two controls will be matched to each cancer patient. Matching with respect to age, gender and where possible, smoking vs non-smoking will be informed by a bespoke algorithm.

The study has been designed as a case control study to ensure that large numbers of bladder cancer are sampled. It is important to include large numbers of bladder cancer to demonstrate that the biomarker in question can rule the disease out; this approach reduces false positives. The “costs” of failing to diagnose against the “costs” of a false positive have to be balanced. Our study will recruit bladder cancers and controls (patients with confounding pathologies) as independent groups. Our design embraces the prevalence concept in that all recruited patients will have a history of haematuria and therefore a 1:20 risk of bladder cancer. The HaBio study will bridge the gap between Proof of Concept and a future Validation Study (Figure 5).

Figure 5 - Proof of concept.tif

**Figure 5: Proof of Concept to Biochip**

We have demonstrated proof of concept in the feasibility study. We screened ~60 biomarkers as possible contributors to a diagnostic algorithm. We have undertaken preliminary analyses on these biomarkers and have now identified 58 biomarkers that will be measured on all patients’ samples. If there is adequate blood/urine remaining we may add additional biomarkers.

RECRUITMENT STRATEGIES

Recruitment predictions are based on figures from the Northern Ireland Cancer Registry (Table 2).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Year of**  **diagnosis** | | **Number of cases** | **% all cancers**  **ex. NMSC** | **European age standardised incidence rate per 100,000 persons (95% CI)** | **World age standardised incidence rate per 100,000 persons (95% CI)** | |
| **1993** | 207 | | 3.3 | 11.6 (10.0,13.3) | 7.7 (6.6,8.9) |
| **1994** | 196 | | 3.2 | 10.8 (9.2,12.4) | 7.0 (6.0,8.1) |
| **1995** | 209 | | 3.4 | 11.6 (9.9,13.2) | 7.7 (6.6,8.8) |
| **1996** | 262 | | 4.1 | 14.2 (12.4,15.9) | 9.3 (8.1,10.5) |
| **1997** | 163 | | 2.6 | 8.8 (7.4,10.2) | 5.9 (4.9,6.9) |
| **1998** | 234 | | 3.6 | 12.5 (10.8,14.1) | 8.2 (7.0,9.3) |
| **1999** | 215 | | 3.4 | 11.5 (10.0,13.1) | 7.7 (6.6,8.8) |
| **2000** | 211 | | 3.2 | 11.1 (9.5,12.6) | 7.3 (6.3,8.4) |
| **2001** | 182 | | 2.8 | 9.2 (7.8,10.6) | 6.0 (5.1,7.0) |
| **2002** | 174 | | 2.6 | 8.7 (7.3,10.0) | 5.6 (4.7,6.5) |
| **2003** | 227 | | 3.2 | 11.3 (9.8,12.8) | 7.3 (6.3,8.4) |
| **2004** | 215 | | 3.0 | 10.5 (9.1,12.0) | 6.9 (5.9,7.8) |
| **2005** | 193 | | 2.7 | 9.2 (7.9,10.6) | 6.0 (5.1,6.9) |
| **2006** | 225 | | 3.0 | 10.5 (9.1,11.9) | 6.7 (5.8,7.7) |
| **2007** | 193 | | 2.4 | 8.9 (7.7,10.2) | 5.8 (4.9,6.7) |
| **2008** | 208 | | 2.6 | 9.3 (8.0,10.6) | 6.0 (5.1,6.9) |

### Table 2 Bladder cancer figures from the Northern Ireland Cancer Registry 1993 - 2008

The number of new bladder cancer cases recorded by the Northern Ireland Cancer Registry ranged from 163 in 1997 to 262 in 1996. Our recruitment strategy is calculated on the basis of ~180 TCCB cases/year which equates to 450 TCCB over 30 months.

http://www.qub.ac.uk/research-centres/nicr/Data/OnlineStatistics/Bladder/

All urology consultants in Northern Ireland have been briefed on the HaBio study and are very supportive. They are happy for patients under their care to be recruited. Recruitment will begin in Belfast City Hospital and then expand into the other hospitals in Northern Ireland. A highly coordinated approach and good communication will be developed by the Clinical Committee. The objective is to establish protocols that will ensure that recruitment is effective and efficient. For example, in BCH newly diagnosed bladder cancer patients attend a pre-assessment clinic and this will be a good point of contact for the Research Nurses.

Recruitment rates will be monitored through the Clinical Committee who submit quarterly reports to the Steering Committee. Recruitment will go through troughs and peaks and this will be considered when gauging progress. Recruitment of bladder cancer patients will be the priority. Three months into the study recruitment will be reviewed. We predict 95% recruitment success because the trial is non-interventional and patients will only be required to provide urine and serum. If there is a shortfall in recruitment we will either extend the duration of project or add additional recruitment centres.

PROTEIN ANALYSES

All protein analyses will be undertaken “in triplicate” in urine or serum by Randox personnel at their laboratories in Crumlin. At all times throughout the study, Randox personnel will be blinded to all patient data. Randox Laboratories is an international clinical diagnostics company offering innovative solutions to laboratories worldwide. The company has extensive expertise and experience in the field of protein analyses having developed four FDA approved analysers, (Evidence ™), all are of which are CE marked. The analysers have varying throughput capacity for the use of their patented biochip array technology (Fitzgerald, 2005). Analyser testing is performed on 0.1ml plasma, serum or urine which is applied to a biochip which simultaneously measures multiple biomarkers, providing results in 1-2 hours (Figure 1). More than 30 of the biomarkers selected for testing (Table 3) will be measured using commercial ELISAs as these proteins are not yet validated in biochip format.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Biomarker** | **Supplier /assay format** | **Analysis Matrix** | **Urine**  **Quantity** | **Serum quantity** | **Pathway(s)** | **References** |
| 8-hydroxydeoxyguanosine (8-OHdG) | USCN/  96 well | Urine | 100 ul x 3 | - | Oxidative  DNA damage | Ksiazek, 2008  Spriggs, 2010 |
| γ synuclein | USCN/ 96 well | Urine |  | 100 ul x 3 | Growth, Invasion, Metastases | Ahmad, 2007 |
| Adipocyte-fatty acid binding protein (A-FABP) | Biovendor / 96 wells | Serum | - | 100 ul x 3 | Intracellular lipid transport, Metabolism, Signal transduction | Boiteux, 2009 |
| + # Bladder Tumour Antigen (BTA)  TRAK  (quantitiative test)  human complement factor H-related protein (hCFHrp) | Polymedco  / 96 wells | Urine | 25 ul x 3 | - | Immune | Kinders , 1998 |
| Calreticulin | USCN/ 96 well | Urine | - | 100 ul x 3 | Stress  Auto immune  Ca 2+signalling | Kageyama, 2004  Spriggs, 2010 |
| CD44 | Abcam/ 96 well | Serum | - | 100ul x 3  (40 x dilution needed) | Metastases | Golshani, 2008 |
| #Carcino-embryonic antigen (CEA) | Randox Biochip | Serum | Tumour PSA panel | - | Differentiation | Margel, 2006 |
| Cytokeratin (CK) 20 | Cusabio / 96 well | Urine | - | 100 ul x 3 | Differentiation | Jiang, 2001 |
| #Cleaved CK 18  M30 Apoptosense | Enzo/ 96 well | Serum | - | 25 ul x 3 | Apoptosis | Leers, 1999 |
| Clusterin | R&D systems/ 96 well | Urine | 50 ul x 3  (4 x dilution needed) | - | Apoptosis  Adhesion | Poon, 2000 |
| #C-reactive protein (CRP) | Randox Biochip | Urine | Cerebral II panel | 100 ul x 3 | Inflammation | Trichopoulos, 2006 |
| Chemokine (C-X-C motif) Ligand 1  (CXCL1)(GROα) | R&D systems/ 96 well | Serum | - | 200 ul x 3 | Angiogenesis, Inflammation | Salcedo, 2003 |
| Cystatin C | R&D systems/ 96 well | Urine | - | 100 ul x 3 | Protease inhibitor | Janoskwi, 2001 |
| Cystatin B | USCN / 96 Well | Urine | - | 100 ul x 3  (0-10 x dilution needed) | Protease inhibitor | Feldman, 2009 |
| #D-dimer | Randox Biochip | Urine | Cerebral II panel | - | Coagulation | Tefferi, 1989 |
| EN2 | ELISA | Urine | - | 100 ul x 3 | HOX gene family | Morgan, 2011 |
| #Epidermal Growth Factor (EGF) | Randox Biochip | Urine | Cytokine 1 panel | - | Growth | Saika, 2000 |
| #FAS | Raybio / 96 Well | Urine | 100 ul x 2 | - | Apoptosis | Svatek, 2006 |
| #Hyaluronidase (HA) | Echelon /96 well | Urine | 100 ul x 2 | - | Angiogenesis | Golshani, 2008 |
| IFNγ | Randox biochip | Urine |  |  | Immune |  |
| IL-10 |  | Urine |  |  |  |  |
| #IL-1α | Randox Biochip | Urine | Cytokine 1 panel | - | Inflammation  Angiogenesis  Growth | Seddighzadeh 2003 |
| #IL-1β | Randox Biochip | Urine | Cytokine 1 panel | - | inflammation  angiogenesis  growth | Ahirwar, 2009 |
| #IL-2 | Randox Biochip | Urine | Cytokine 1 panel | - | Immune | Marits, 2006 |
| #IL-4 | Randox Biochip | Urine | Cytokine 1 panel | - | Immune | Satyam, 2009 |
| #IL-6 | Randox Biochip | Urine | Cytokine 1 panel | - | Inflammation | Leibovici, 2005 |
| #IL-8 | Randox Biochip | Urine | Cytokine 1 panel | - | Immune response | Leibovici, 2005 |
| IL-13 | Randox Biochip | Urine | Cytokine 5  panel | - | Inflammation | Cohen, 2011 |
| IL-3 | Randox Biochip | Urine | Cytokine 5  panel | - | Immune | Yang, 1986 |
| IL-7 | Randox Biochip | Urine | Cytokine 5  panel | - | Growth Factor | Heufler, 1993 |
| IL-12 p70 | Randox Biochip | Urine | Cytokine 5  panel | - | Immune | Gillessen, 1995 |
| IL-23 | Randox Biochip | Urine | Cytokine 5  panel | - | Inflammation | Lankford, 2003 |
| LASP-1 | Cusabio | Serum | - | 3 x 100 ul | Ion transport | Payton, 2012 |
| Mini chromosome maintenance 5 (MCM 5) | USCN /  96 well | Urine | - | 100 ul x 2 | DNA replication | Stoeber, 2002 |
| Midkine | EIAAB /  96 well | Serum/  urine | - | 100 ul x 2 | Embryonic marker,  Inflammation,  Renin angiotensin system | Hobo, 2009 |
| #Monocyte chemoattractant protein -1(MCP-1) | Randox Biochip | Urine | Cytokine 1 panel | - | Inflammation | Vazquez-Lavista, 2009 |
| #Matrix metalloproteinase 9 (MMP9) | Randox Biochip | Urine | Cerebral II panel | - | Metastases | Eissa, 2007 |
| #MMP9-NGAL complex | R&D systems/  96 well | Urine | 50 ul x 2 | - | Metastases | Roy, 2008 |
| #NGAL | Randox Biochip | Urine | Cerebral II panel | - | Inflammation,  Metastases | Monier, 2002 |
| #Nuclear mitotic protein 22 (NMP22) | Matritech Dip Test ( 1 test POC) | Urine | 250 ul | - | Apoptosis, Growth | Lotan, 2009 |
| #Neurone Specific Enolase (NSE) or γ enolase | Randox Biochip | Urine | Cerebral II panel | - | Glycolysis | Hobarth, 1992 |
| phospho extracellular signal regulated kinase  pERK | Biotang/  96 well | Urine | **-** | 10 ul x 2 | Translation inhibition | Spriggs, 2010 |
| Protein Kinase R (PKR) | USCN /  96 well | Urine |  | 100 ul x 2 | Translation inhibition | Spriggs, 2010 |
| Plasminogen activator inhibitor type 1 (PAI-1)  SERPINE 1 | Symansis/ 96 well | Serum | - | 100 ul x 2 | Angiogenesis | Becker, 2010 |
| Proepithelin (Progranulin) | Stratech | Urine |  | 50 ul x 2 | Growth, Migration | Monami, 2009 |
| #prostate specific antigen (PSA) (free) | Randox Biochip | Serum | - | Tumour PSA panel | Prostate cancer | Control |
| #prostate specific antigen (PSA) (total) | Randox Biochip | Serum | - | Tumour PSA panel | Prostate cancer | Control |
| S100A4 | MBL International / 96 well | Serum | - | 100 ul x 2 | Metastases | Levett, 2002 |
| Tissue Inhibitor of Metalloproteinases MMP9 (TIMP-MMP9) | Randox ELISA | Serum/  Urine | To be established (100 ul x 2) | - | Invasion, Metastases | Eissa, 2007 |
| #Transforming Growth Factor-β 1(TGF-β1) | R&D systems / 96 well | Urine | - | 100ul x 2 | Immune, Differentiation, Growth, Metastases | Eder, 1996 |
| #Thrombomodulin (TM ) | Randox Biochip | Urine | Cerebral II panel | - | Metastasis | Obama, 1999; Meyers-Irvin, 2005 |
| #Tumour necrosis factor alpha ( TNFα) | Randox Biochip | Urine | Cytokine 1 panel |  | Immune, inflammation | Leibovici, 2005 |
| sTNFR1 | Randox Biochip | Urine | Cytokine IV panel | - | Apoptosis,  Inflammation | Gregorc,2007 |
| sTNFR2 | Randox Biochip | Urine | Cytokine IV panel | - | Apoptosis,  Inflammation | Gregorc,2007 |
| Tissue type plasminogen activator (TPA) | Assay Pro  /96 well | Urine | 50ul x 2 | - | Differentiation, Proliferation | Senatore, 1990 |
| Tryptase (TPS) | USCN / 96 well | Urine | - | 100 ul x 2 | Apoptosis | Boman, 2001  Payne, 2004 |
| Urinary Bladder Cancer (UBC) (CK8 and CK18) | IDL | Urine | **-** | 100 ul x 2 | Apoptosis | Mungan, 2000 |
| #Vascular endothelial growth factor (VEGF) | Randox Biochip | Urine | Cytokine I panel | - | Angiogenesis | Crew, 1999 |
|  |  |  |  |  |  |  |

**Table 3: Biomarker panel**

For this study we have selected biomarkers on the basis of their performance in the pilot study, current scientific evidence of their involvement in pathways associated with bladder carcinogenesisor of their discovery in recent proteomic screening. Further biomarkers will be added, as appropriate. In addition, osmolarity, protein, e creatinine, and micro-albumuria will be measured in urine fromall patients. Micro-albumuria is a marker of renal disease. PSA is included in the panel to identify patients with benign prostate enlargement or prostate cancer and CEA is a useful biomarker of both bladder cancer and smoking. If patients have abnormal levels of microalbuminuria, PSA or CEA a letter will be send to their urology consultant advising him/her of the levels measured by Randox Laboratories and that these biomarkers should be re-assessed. This may improve the accuracy of the final diagnosis for these patients. The accuracy of final diagnosis is very critical to our interpretation of the accuracies of diagnostic classifiers. Following analyses of the Quality Control experiments, some biomarkers were not informative and these biomarkers have therefore been excluded..

We will assess biomarkers in urine or serum as indicated. Volumes of urine and serum that will be required.

# 26 biomarkers were previously assessed in the pilot study (Abogunrin F et al, Cancer 2012). +Either BTA TRAK or BTA STAT will be assessed.

The biomarkers listed in Table 3 represent diverse pathways known to be involved bladder carcinogenesis (Table 4) (Hanahan & Weinberg, 2011). In addition, we have included biomarkers associated with thrombosis and hypertension due to their particular relevance to haematuria patients.

|  |  |
| --- | --- |
| **Pathways** | **Biomarkers** |
| Apoptosis | Cleaved CK18, FAS, NMP22, clusterin, sTNFR1, TPS |
| Angiogenesis | CXCL1, HA, IL-1α, IL-1β, IL-8, PAI-1, VEGF |
| Coagulation | d-Dimer, IL-1β, TM, TNFα, VEGF |
| Differentiation | CEA, CK20 TGF-β1, TPA |
| DNA/oxidative damage or stress | 8-OHdG, calreticulin |
| Growth and sustained proliferation | γ synuclein, EGF, HA, IL-1α, IL-1β, IL-7, IL-8, MCM5, NMP22, Proepithelin , TGF-β1, TPA |
| Glycolysis | NSE |
| Hox gene | EN2 |
| Hypertension | Midkine |
| Immune | BTA, IL-2, IL-3, IL-4, IL-6, IL-8, IL-12p70, TGF-β1 |
| Inflammation | CRP, CXCL1, HA, IL-6, IL-8, IL-13, IL-23, Midkine, NGAL, MCP-1, TNFα, sTNFR1 |
| Invasion/metastases | γ synuclein, CD44, CEA, cystatin B, HA, IL-8, MMP-9, MMP-9 NGAL complex, NGAL, Proepithelin, S100A4, TIMP-MMP9 TGF-β1 |
| Ion Transport | LASP-1 |
| Nutrition/metabolism | A-FABP, PERK, PKR, Cystatin C |

**Table 4: HaBio Biomarker Pathways**

We have selected 58 biomarkers for assessment in the HaBio Study (Table 3). These biomarkers characterise the diverse pathways involved in carcinogenesis (Hanahan and Weinberg 2011).

8-OHdG 8-hydroxydeoxyguanosine, A-FABP Adipocyte-faty acid binding protein, BTA Bladder Tumour Antigen, CEA Carcino-Embryonic Antigen, CK18 CytoKeratin 18, IL InterLeukins, CRP C-Reactive Protein, CXCL1 Chemokine (C-X-C motif) Ligand 1, EGF Epidermal Growth Factor, HA Hyaluronidase, MCM5 Mini chromosome maintenance 5, MCP-1 Monocyte Chemoattractant Protein-1, MMP-9 Matrix MetalloProteinase-9, NGAL Neutrophil-Associated Gelatinase Lipocalin, NMP22 Nuclear Matrix Protein 22, NSE Neuron Specific Enolase, PERK phospho extracellular signal regulated kinase, PKR Protein Kinase R, PAI-1 Plasminogen activator inhibitor type 1, TGF-β1 Transforming Growth Factor-β 1, TIMP-MMP9 Tissue Inhibitor of Metalloproteinases MMP9, TNF-α Tumour Necrosis Factor alpha, sTNFR1 soluble TNF-α, TM ThromboModulin, TPA Tissue type plasminogen activator, TPS Tryptase, UBC Urinary Bladder Cancer, VEGF Vascular Endothelial Growth Factor. Micro albuminuria will be measured to improve the accuracy of the final diagnosis.

PATHOLOGY

To confirm bladder cancer, a Consultant Pathologist will undertake a review of diagnostic pathology for all patients recruited. The pathologist will record pathological grade and stage and note the presence/absence of inflammatory infiltrate together with any other relevant information. Similarly, a Consultant Cytopathologist will undertake a review of diagnostic cytology and record diagnosis and note the presence/absence of inflammatory cells. Immunohistochemistry on serial sections will be used to identify protein and other microscopic constituents within representative tumour and histologically “normal” urothelial samples.

Northern Ireland Biobank (NIB)

Northern Ireland Biobank (NIB) have ethical approval (ORECNI 11/NI/0013) to retrieve archived pathological samples and to create tissue microarrays using tissue surplus to diagnostic need. Tissue samples will include diagnostic samples taken at the time of recruitment and any additional biopsies that are assessed pathologically during the time-frame of the study; that is recurrent tumours. Haematoxylin and eosin stained sections can be annotated by a pathologist to indicate the areas for TMA core, DNA and RNA extraction. Once TMAs have been created researchers can apply for sections from the TMA and aliquots of DNA and RNA through application to the NIB. Haematoxylin and eosin, and immunohistochemically stained sections can be scanned so that virtual images can be assessed remotely by the researcher or their collaborators. For the purposes of this study the Chief Investigator will submit a NIB application requesting access to sections from TMAs created from tumour and normal tissue samples and to DNA and RNA extracted from the tumour and normal tissue sections, from the patients recruited to HaBio. Sections will be immunostained to detect biomarker expression and assessed using light or confocal microscopy. DNA will be extracted from tumour samples from all the cancer patients according to NIB protocols and from biopsies taken from patients recruited to HaBio as controls, when available. mRNA will be extracted from a subset of tumours (5-10) and from 5 biopsies from patients recruited as controls for microarray analyses. DNA and mRNA extracted from histologically normal tissue from HaBio controls, when available, will act as controls. This work will be undertaken in the Northern Ireland Molecular Pathology Laboratory. The NIB Information Management System (IMS) will track the blocks and slides at all times. None of the HaBio investigators will be storing HTA relevant material at any time. Virtual images will be used for the assessment of the immunostained and haematoxylin and eosin sections.

**1.4 Definition of key biomarkers**Key biomarkers will be defined as biomarkers which fulfil one of the following:

* contribution to algorithms/classifiers created in the feasibility or HaBio studies
* AUC > 0.65 as individual biomarkers of bladder cancer in the feasibility or HaBio studies

### 2 Aim, Objectives and Trial Design

### 2.1. Aim

To define an algorithm(s) for the diagnosis of bladder cancer in haematuria patients

### 2.2. Objectives

PRELIMINARY BIOMARKER ANALYSES OBJECTIVES

1. To validate that the control biomarkers FPSA and TPSA are gender selective

2. To determine stability of each biomarker when maintained for up to 6h on ice prior to freezing

3. To identify “potentially informative” biomarkers with satisfactory ranges i.e., < 75% of the measurements are thresholds or limits of detection (LOD)

4. To identify biomarkers with satisfactory CVs

5. To determine the inter-site variability of biomarker measurements

6. To determine stability of biomarkers in blood when centrifugation is undertaken at room temperature

7. To determine stability of biomarkers in blood when centrifugation is delayed for up to three hours

RECRUITMENT OBJECTIVES

1. To obtain consent in accordance with NICTC SOPs
2. To recruit 999 patients; 333 patients with pathologically proven bladder cancer; and 666 controls.
3. To collect urine and serum from all recruited patients
4. To complete a Recruitment Form for all patients

PRIMARY OBJECTIVES

1. To define individual sensitivities and specificities for all key biomarkers based on data from ~999 patients presenting with haematuria
2. To create a diagnostic algorithm based on demographics, lifestyle, medications and smoking to act as a bench mark for the diagnostic algorithms
3. To create diagnostic algorithms combining individual biomarker data
4. To define sensitivities and specificities for diagnostic algorithm(s)
5. To create a biochip(s) formatted with the protein biomarkers which contribute to the algorithm(s)
6. To optimise the biochip(s)
7. To gain understanding of the underlying pathobiology of bladder carcinogenesis using both classical statistics and novel statistical approaches to analyse the extensive database that will be generated
8. To obtain follow-up data on all of the patients three years after their recruitment

SECONDARY OBJECTIVES

1. To create algorithms which differentiate between benign causes of haematuria patients such as BPE, stones, kidney disease and inflammatory disease (these algorithms may require validation is a separate future study)
2. To create algorithms which differentiate “high” and “low” risk sub-populations.
3. To determine levels, patterns of expression and cell type location of key biomarkers using appropriate immunohistochemistry (IHC) on sections from

Tissue Microarrays obtained under the auspices of the Northern Ireland BioBank.

1. Determine the association between grade, stage and inflammation and the presence/absence of key biomarker immunoreactivity
2. To determine the association between measured biomarker levels in urine or serum and intensity of immunoreactivity in tumour samples.
3. To determine the association of immunohistochemical profiles and progression.
4. To determine molecular profiles of the tumour and urothelium for the 333 bladder cancer patients and for bladder tissue removed from controls, when available.
5. To determine the association between IHC profiles and gene / mRNA expression and clinical data.
6. To identify patients with a high risk of kidney disease using biomarker profiles.

PATIENT PUBLIC INVOLVEMENT OBJECTIVES

1. To include patient representatives on the Trial Steering Committee
2. To hold a meeting at the end of the trial to disseminate the results and hear comments from the patients who participated.

OBJECTIVES FOR FUTURE STUDIES

1. To validate the optimised diagnostic biochip as a means to stratify “low risk” patients presenting with haematuria
2. To design a clinical study in haematuria patients to validate biomarker(s) and/or algorithms as predictors of pathologies other than bladder cancer in haematuria patients.

### 2.3 Study Design

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| --- |
| The trial is a case control study (Figure 6). Nine hundred and ninety-nine patients aged between 40 and <86 years, with a history of haematuria and cystoscopy in the last six months and who satisfy the inclusion criteria will be recruited at haematuria clinics, dedicated recruitment sessions, at pre-assessment clinics, as inpatients prior to transurethral resection of the bladder (TURB) or at planned cystoscopy. Patients will be recruited from Northern Ireland hospitals. Controls will be –ve cystocopy/ –ve Uro Ca pathology/ -ve other investigations for bladder cancer. Two control patients will be matched to each bladder cancer patient with respect to age, gender and where possible, smoking status. |

### 

### HaBio design protocol Fig 6.tif Figure 6 HaBio Study design

### The duties of the CRNs/ HaBio clinicianare boxed in red; the duties of the CTA are highlighted in orange; the duties of the technician are boxed in brown and this person will also play a key role in the boxes highlighted in blue. The analyses of proteins will all be undertaken at Randox (green box). Components associated with pathology and / or NIB are boxed in blue and those activities associated with statistics in purple. Activities undertaken by clinicians are boxed in grey. The activities in the yellow box will take place take place after statistical analyses have been completed. Randox will validate the biochip using samples collected during HaBIo.

HaBio will be a three-way collaborative study between QUB, Randox Laboratories and Northern Ireland hospitals (Figure 7). The outlined project has been designed to address a clinical need i.e., to develop a combination of protein biomarkers which can detect bladder cancer in haematuria patients. A wide and extensive range of biomarkers will be evaluated providing a great opportunity for scientists at QUB to gain greater understanding of the pathobiology underlying bladder carcinogenesis. Randox Laboratories have the technology to create a biochip arrayed with the identified biomarkers and thus create the biochip required to translate the study’s findings to the clinic.

Figure 7 - HaBio WorkFlow chart.tif

**Figure 7: HaBio Work Flow**

HaBio has been funded by Invest NI and will be an official Experimental Cancer Medicine centre (ECMC) study approved by Northern Ireland Cancer Trial Centre. Patients will be recruited from Northern Ireland hospitals.

This trial will be conducted in compliance with the principles of the Declaration of Helsinki, the principles of good clinical practice (GCP) and Standards for the Reporting of Diagnostic Accuracy Studies (STARD) recommendations (Bossuyt et al, 2003a & b).

### 3 Selection and Withdrawal of Patients

### 3.1 Inclusion Criteria

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| --- |
| Inclusion criteria for 333 BLADDER CANCER patients  Patients with newly diagnosed or recurrent bladder cancer or a suspicion of bladder cancer, will be recruited prior to TURB.   * Written informed consent to participate in the study * Aged between 40 and <86 years * Current haematuria or a history of haematuria * With an active bladder * Cystoscopy within the last 6 months or planned cystoscopy * No cystoscopy within 48h prior to recruitment * No treatment for bladder cancer in the three weeks prior to recruitment * No history of cancers other than bladder cancer, that are currently active * Suspicion of bladder cancer or proven bladder cancer   Inclusion criteria for 666 control patients  These patients will be recruited following negative cystoscopy and negative findings for other bladder cancer investigations.   * Written informed consent to participate in the study * No history of bladder cancer * No history of cancer, that is active * Current haematuria or a history of haematuria * With an active bladder * Negative cystoscopy within the last 3 months, or planned cystoscopy * No cystoscopy within 48h prior to recruitment * Ability to participate in study procedures |

### 3.2 Selection of Participants

|  |
| --- |
| All eligible patients attending the participating hospitals will be informed of the study and invited to participate. |

### 3.3 Withdrawal of Subjects

### Patients can withdraw from the study at any time by informing the Chief Investigator or one of the Research Nurses / Clinician. Each patient’s withdrawal will be recorded and followed up with a letter informing the patient that their samples have been destroyed and that their data have been deleted from file as appropriate. If a patient withdraws they will be replaced where possible.

### 3.4 Expected Duration of Trial.

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| --- |
| Recruitment to HaBio will commence in early autumn 2012 and will last 30 months. Protein and data analyses will be undertaken over three years from the commencement of recruitment i.e., until the summer 2015. All patient notes will be reviewed 3 years after recruitment. Therefore we expect the study will continue until the summer of 2018. |

### 4. Habio Action Plan

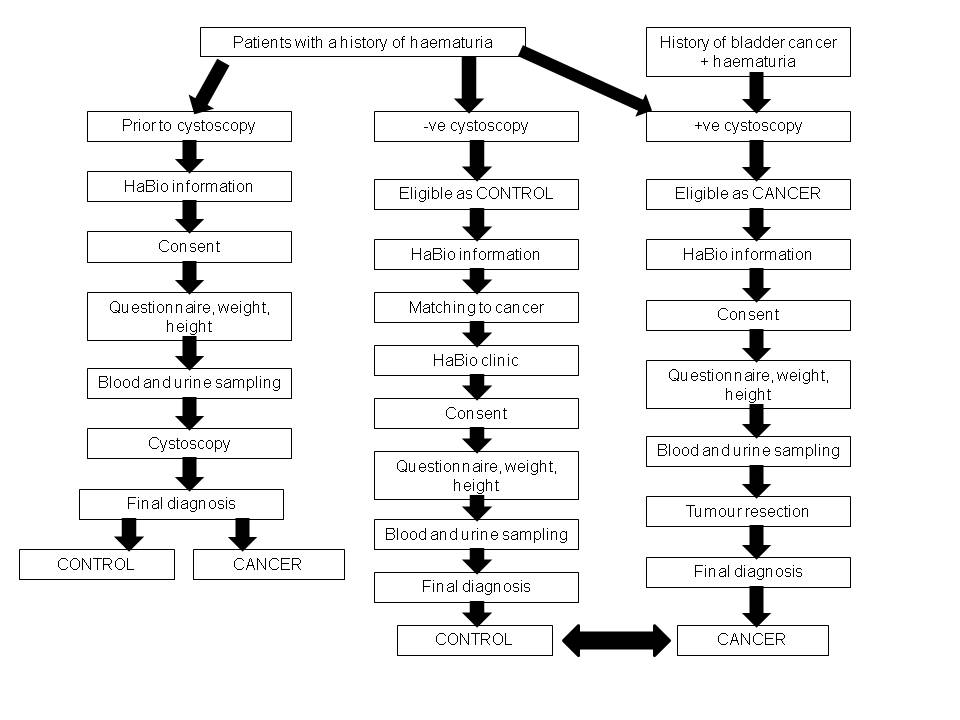
**4.1 Routine clinical procedures on haematuria patients**

Cystoscopy and ultrasound. High risk patients will also routinely have IVP and CT. Tissue removed during cystoscopy is routinely sent for pathological assessment. If tumour is identified, patients undergo transurethral resection of the bladder (TURB). The pathologist assesses the TURB resection samples to make a final diagnosis.

**4.2 HaBio Research procedures**

The following procedures will be undertaken for research purposes: Urine and blood collection; height, weight and blood pressure measurements; collection of data as per recruitment form; collation of diagnostic pathology, cytology reviews and investigation results for final diagnosis; immunohistochemistry on Tissue Microarrays, and DNA and mRNA extraction from samples surplus to diagnostic need; and follow up review three years after recruitment. Construction of Tissue Microarrays and DNA and mRNA extraction will be undertaken in the Northern Ireland Molecular Pathology Laboratory according to established protocols.

Patients with a history of haematuria will be recruited to HaBio. The main action points are illustrated in Figure 8.

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**Figure 8 HaBio Action Points**

Patients with haematuria are referred to the day procedure unit (DPU) for assessment. All patients undergo cystoscopy. When cystoscopy is negative (middle column ), patients will be eligible to be recruited as controls to HaBio. When cystoscopy is positive the patients will be eligible to be recruited as cancers However, for some of these patients subsequent biopsy will confirm benign disease and when this occurs these patients will be re-designated as one of the 666 controls and an additional cancer patient may be recruited unless 333 patients with bladder cancer have already been recruited. Some patients may be recruited as cancers prior to cystoscopy or cystoscopy/biopsy; these patients will be confirmed as controls or cancers when their final diagnosis is confirmed. Recruitment will be closely monitored to ensure that we maintain matching of controls to cancers. Patients with pathologically confirmed bladder cancer will be designated as cancers and all other patients will be designated as controls. Many of the bladder cancer patients will attend a pre-assessment clinic where they can be identified for recruitment. Some patients with newly diagnosed cancer are admitted to hospital without attending the pre-assessment clinic. Patients with a history of bladder cancer are regularly reviewed and at review these patients undergo cystoscopy. If their cystoscopy is positive this indicates the presence of a recurrent tumour. These patients will then be eligible to be recruited to HaBio as cancer patients. All patients may be posted HaBio information prior to recruitment.

**5 Study Procedures**

At all times the principles of GCP will be adhered to. The storage and handling of urine and blood samples will be undertaken in accordance with the ethical approval obtained for this study. Tissue samples will not be stored by HaBio investigators who will only access virtual images of stained sections. Samples relevant to HTA will be tracked using the Information Management System of NIB.

**5.1 Recruitment**

COLLECTION OF RECRUITMENT AND REVIEW DATA

The Research Nurses will collate demographic, clinico-pathological data and information about all treatments onto the Recruitment Form. The patient will be asked questions about their lifestyle, hobbies and pastimes, the number of times they pass urine and whether they have any pain, their occupation(s), medication(s) and whether they have ever been exposed to hazardous chemicals. The nurse will record the weight and height of each patient. Blood pressure will be measured up to three times. A copy of the completed Consent Form will be inserted into the patient notes. The Research Nurses/clinicians will also collect and record investigation results for the Review Form. Clinicians will review the clinical notes to define the final diagnosis for each patient. After discussions between Mr Brian Duggan and Consultant Nephrologist, Professor Peter Maxwell, we have included assessment of microalbuminuria. Microalbuminuria is a marker of renal disease (BAUS haematuria guidelines 2008; Goldstein 2012) and if abnormal levels are detected by the Randox analysis the patient’s consultant will be sent a letter reporting the abnormal levels detected during the analyses in the HaBio study. The consultant will be advised to check the abnormal levels. Similarly, if abnormal PSA or CEA levels are detected, the patient’s consultant will be sent a letter advising them of the raised levels. This letter will be placed into the patient’s notes. . This may benefit the patient because renal disease, prostate disease and other cancers may be detected earlier. It may also increase the accuracy of final diagnoses for patients recruited to HaBio which is an important element within HaBio because defining accurate classifiers is dependent on accurate diagnoses for each patient. The final diagnosis will be recorded onto the last page of the Review Form by a clinician. This last page will be placed into the patient’s notes. Three years after recruitment, or as soon as possible thereafter, the patient’s notes will be reviewed and follow-up information recorded.

SAMPLE PROCESSING

For each patient, a sealed bag labelled with study’s ethical number will contain all the necessary tubes pre-labelled (bar coded) for each patient. The Research nurse will collect approximately 25ml of urine and approximately 35ml blood from 979 patients. The 20 patients recruited for the quality control assessment, undertaken at the beginning of the study, will be consented for approximately 25ml urine and approximately 45ml blood. One of the nurses or the technician will process and aliquot samples which will be held on ice until freezing in alarmed – 80oC freezers. Frozen samples will be collected and transported on dry ice to Randox Laboratories by Randox personnel approximately once a month. Urine and serum in aliquots, when available, will be retained in freezers in CCRCB by NIB. These samples will be used for optimisation of biochips and may be accessed by Randox if there is a need to repeat any analyses. From certain hospitals, samples may be directly transported to Randox for aliquoting and storage. Samples will be stored in -80oC freezers at Randox until analysis. Samples will have been aliquoted into samples sizes that prevent freeze/ thawing. Appropriate records detailing the number of urine and serum samples at each location will be created and maintained by the Research Nurses and HaBio Technician as per standard NICTC SOPs. A letter will be sent to each patient’s GP informing them that their patient has been recruited to the study. A copy of this letter will be added to the patient’s notes.

**5.2 Monitoring**

Monitoring of this trial to ensure compliance with Good Clinical Practice and scientific integrity will be managed and oversight retained by the Research Governance Framework, Belfast Health and Social Care Trust and the Policy Office QUB. Yearly reports will be compiled by the Clinical Trials Administrator (CTA) and sent to the Office for Research Ethics Committees Northern Ireland, QUB Research Policy Office and the Belfast Health and Social Care Trust. The study is non-interventional and will be monitored in line with Trust and University requirements.

**6 Biochip optimisation**

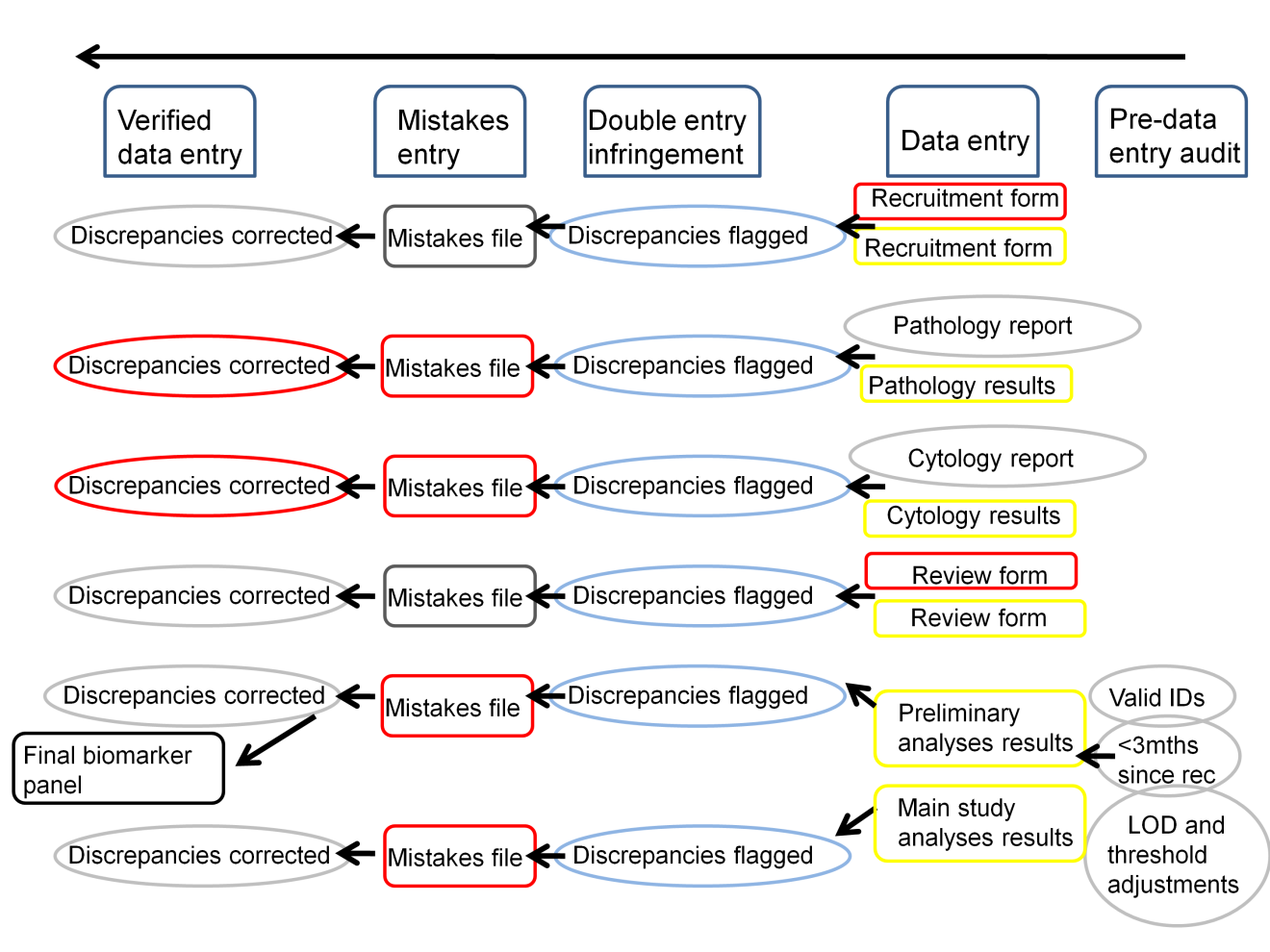
The process of biochip validation is summarised by a validation plan, which describes the entire process in detail. In brief, this is to enable performance data and stability to be established for the final list of chosen biomarkers, to be incorporated onto the biochip.

The performance data includes extensive investigations, including recovery, precision, sensitivity, specificity, shipping and concentration considerations. The stability data includes details such as accelerated and reconstituted investigations, calibrations, frozen calibrators and controls.

At each stage the data will be subjected to a series of technical reviews and following approval at each stage, the biochip will be ready for release. The relevant documentation will be completed for the transfer of the device from design to manufacturing.

**7 Database**

**7.1 Main Study database**

Delegated study personnel will enter the data onto the the database in Centre for Cancer Research and Cell Biology. The database is on a dedicated computer which is password protected and data entry, access and download will be traceable back to the individual user. Any database entry will use the unique study number allocated to each patient for anonymity. Double entry will automatically highlight discrepancies in the data entry. This will facilitate the corrections of mistakes. 

**Figure 9: Database Work Flow Chart**

A culture of meticulous attention to detail/ no blame will be nurtured. Rigorous audit and attention to mistakes which will be verified by source documentation will be essential. Data from the Recruitment and Review forms, Preliminary Biomarker Analyses experiments, pathology and cytology reviews and all protein analyses will be double entered; one entry will be completed by the CTA (orange) and the second entry by one of the Research Nurses (red) ,the Chief Investigator (gray), the HaBio clinician or HaBio technician. Follow up information will be entered into the anonymised database by the Chief Investigator, Research Nurses or HaBio Clinician, as appropriate. Results from biomarker and tissue analyses will be uploaded as appropriate according to SOPs. LOD limit of detection.

Inconsistencies and errors in data entry will be reviewed once a week by QUB personnel. Separate logs of discrepancies, errors or other highlighted entries will be maintained in room G10 in CCRCB by the CTA. All inconsistencies will be logged, dated, checked against source data or documentation and outcomes recorded. Randox Laboratories will monitor biomarker analyses according to their internal procedures.

Categorical variables have defined entry categories and entries falling outside these require addition by the CTA. Measurements for each biomarker have defined limits of detection (LOD) and thresholds.

At the end of the study, the Chief Investigator will give Randox an anonymised database in Excel format.

**8 Statistics**

### 8.1 Sample Size

Under certain assumptions i.e. a moderate 0.5 correlation between biomarkers of interest, a sample of 333 from the positive group and 666 from the negative group achieve 80% power to detect a difference of 0.0422 between the area under the ROC curve (AUC) under the null hypothesis of 0.8500 and an AUC under the alternative hypothesis of 0.8922 using a two-sided z-test at a significance level of 0.05000. The data are discrete (rating scale) responses. The AUC is computed between false positive rates of 0.000 and 1.000. The ratio of the standard deviation of the responses in the negative group to the standard deviation of the responses in the positive group is 1.000.

**8.2 Data downloads and preparation**

Data will be downloaded weekly from the computer in CCRCB onto an iron key and transferred to a computer in NICTC.g.

Statistical analyses will be supervised by the two HaBio statisticians, Mr Michael Stevenson and Dr Frank Emmert-Streib.

Following audit and correction of errors flagged within the database, we will run cross tabulations to check for any anomalies/discrepancies in the data. Possible errors will be checked and corrected using source data. All changes will be made to the original databases as stored in CCRCB.

All scale variables will be assessed for normal distribution. Data that is not normally distributed will be transformed before classical statistical analyses using SPSS.

**8.3 Analyses of demographic data**Data from the Recruitment Form will be explored using univariate analyses prior to multivariate modelling. Data will be grouped as follows:

Smoking years will be grouped into 0, 1-10yrs, 11-20yrs, 21-30yrs and >30yrs and similarly smoking quantity into 0, 0-10, 11-20, 21-30 and > 30 cigarettes/day.

Alcohol consumption will be grouped into none, < 5 units, ≥5 – 9 units, 10-15 units, > 15 units.

Current and past occupations will be grouped into low, medium and high risk of bladder cancer. For individual patients with more than one occupation, the occupation with the highest risk will be used to define the risk category. The moderate risk group will include firemen, engineers, meat processing workers, carpet cleaners, refuse collectors, mechanical engineers, tobacco factory workers, road service workers, bar managers, mechanics, labourers, construction workers, lorry drivers and those working in photographic processing. The high risk group will include painters, wood lathe operators, dye mixers, coal miners, coal delivery men and joiners and those occupations associated with a sedentary lifestyle (Ji 2005). Exposure to chemicals, weedkillers, dyes, paints, leather, metal, rubber or car mechanics will be grouped accordingly.

Hobbies and pastimes will be probed if there is a possible link to hazardous chemical exposure. They will be classified as active or sedentary.

Frequency of passing urine at night will be grouped into 0, 1, 2 and > 2 times and during the day as ≤ 3, 4-6 times, > 6.

Medications taken at the time of recruitment, will be grouped into 14 categories: anti hypertensives (AH), anti-cholesterol, anti-platelets, anti-ulcer, BPH therapy, i.e. alpha blocker and 5 alpha reductase inhibitor, anti-asthma, analgesics, anti-depressants, anti-inflammatory, anti-diabetes, anxiolytics, anti-coagulants and vitamins.

Using the above groupings cell sizes will be explored. Where cells contain < 30 items logical combination of groupings and re-coding will be considered. We will then investigate mean biomarker levels across groupings and explore homogeneity of variances. These preliminary analyses will inform subsequent modelling to define which factors effect individual biomarker levels and ultimately the diagnostic algorithm.

Forward Wald binary logistic regression analyses will be used to create a diagnostic algorithm for bladder cancer in haematuria patients based on demographics, lifestyle and clinicopathological variables.

**8.4 Preliminary analyses of biomarker data**

We will undertake a preliminary analysis using data from 999 patients to reduce the dimensionality using T-test and stepwise Forward Wald binary logistic regression analyses. This approach will identify smaller effect sizes because the maximum number of cases will be employed in the analyses to identify the biomarkers for inclusion in the subsequent analyses to create the algorithms.

We will use univariate analyses to determine significant differences between controls and bladder cancer (p < 0.10). Receiver operating characteristic (ROC) curves will be generated to determine sensitivities and specificities for individual biomarkers which are shown to be significantly different between the control and TCCB groups (p = < 0.05). The cutoff limit for each biomarker will be the measured level which yielded the highest efficacy (ie, sensitivity – (1 – specificity)). We will enter biomarkers (~40) into a Forward Wald binary logistic regression analyses (cut-off probability for case classification = 0.5) to create a diagnostic algorithm. Biomarkers which do not achieve 0.1 significance following univariate analyses and do not contribute to the 999 patient equation will not be entered into subsequent regression analyses.

**8.5 Diagnostic algorithms**

All the data will be divided into ten groups of 99 patients with 1:2 cases to controls plus one case in each group. We will run 10 models. Algorithms will be created (as described in 7.4) using cohorts of 900 patients and tested on the remaining 99 patients. We will average the AUCs and obtain a combined standard error = 0.1\*(SE12 +SE22 + SE32 +SE42 +SE52 +SE62 +SE72 +SE82 +SE98 +SE102) 0.1. For the ten algorithms, ROC curves will be generated by inserting the predicted probabilities, generated from the Forward Wald analyses, as the test variable. We will use the coordinate points of the ROC curves to determine the cut-off point. ROC curves plot “sensitivity” against “1 – specificity” for each value. We will paste the coordinate points into an Excel file and then use Pythagoras’ theorem to determine the distance of each point from the top of the y-axis of the ROC curve, i.e., Distance = (1-sensitivity)2 + (1-specificity)2. The minimum distance maximises the specificity for each biomarker/algorithm and will be used as the cut-off point to delineate positive from negative test results. As it is expected that the key biomarkers identified from these groups may differ slightly, we will undertake a final Forward Wald binary logistic regression analysis to identify the components of the final diagnostic algorithm(s) for testing in the biochip format.

**8.6 False positives in Confounding Pathologies**

It will be important to establish which confounding pathologies have the highest proportion of false positives when the algorithm is applied. Following investigations control patients will be sub-classified into the following confounding pathologies - “no diagnosis”, “benign pathologies”, “stones and inflammation”, “renal disease”, “BPE”, “muscle invasive bladder cancer” or “non-muscle invasive bladder cancer”. The final diagnosis will be based primarily, but not exclusively on the results from cystoscopy plus pathology, PSA, renal disease biomarkers and radiological imaging of the upper urinary tract. USS will be used to identify BPE. Cross-tabulations of confounding pathologies against expected TCCB (predicted probability > 0.50) and controls (predicted probability ≤ 0.50) will be explored. If < 30 cases occur in one or more categories data will be recoded or combined as appropriate Predicted probabilities against final disease classifications will be plotted as scatter charts.

**8.7 Algorithms for Confounding Pathologies**

When a patient presents with frank painless haematuria the priority is to identify the 1:20 with bladder cancer (Mostafid 2010). However, it would be very useful for stratification and management strategies if those with specific confounding pathologies could also be identified. We will use binary logistic regression analyses methods as described for the generation of the diagnostic algorithm, to identify equations for patients who have the most prevalent confounding pathologies; “no diagnosis”, “stones and inflammation” and BPH. Diagnosis of renal disease will be on the basis of interpretation of micro-albuminuria measured in urine and the final diagnosis following nephrology referral, if appropriate. Other groupings are likely to have insufficient numbers. From the pilot study data we estimate that we will recruit 52 patients with cancers other than bladder cancer, 104 patients with BPE, 147 patients with stones or inflammation, 52 with other benign pathologies and 311 patients who will either be diagnosed with renal disease or will have “no diagnosis”. Individual biomarkers or combinations of biomarkers with potential to identify any of the confounding pathologies will be further investigated in larger studies.

**8.8 Computational biology and machine learning**

Alternative statistical and computational approaches to analyses of the comprehensive dataset will be developed, under the supervision of Dr Emmert-Streib (CCRCB). Analyses using these novel approaches will increase our understanding of the underlying pathobiology of bladder carcinogenesis, complement classical statistical analyses and generate hypotheses for exploration in laboratory experiments.

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**9.0 Development and Marketing of the Haematuria Diagnostic Biochip**

As the Haematuria Diagnostic Biochip will be a multianalyte test, to date there is no direct competition in the market. As confirmed by consultant urologists, internet and literature searches, tests under development are generally single analyte tests. On the basis of delivering the sensitivity and specificity to that of cystoscopy, there currently is no available test. There is freedom in the market to deliver a test to substitute cystoscopy in low risk patients.

Entry of the Haematuria Diagnostic test array into the UK/ European market would require the relevant In Vitro Diagnostics approval, which is CE Marking. Randox Laboratories has obtained CE Marking for the Evidence ™ and Evidence Investigator ™ biochip arrays. In addition, FDA approval has been obtained for use of the analysers in the US, SFDA approval for market entry in China and Health Canada for entry to Canada. No animal testing is required before regulatory approval is obtained.

There are various key stages involved on the route to market. Following the study outlined in this proposal, full optimisation and validation of the tests will be required. This will then enable CE Marking, for entry into the European / UK market and FDA Approval for the US. During this process, the technology can be assessed by the National Technology Adoption Centre (NTAC), either directly, or via an Innovation Procurement exercise. In either case, the technology, following due diligence, would be selected, on a call for a 1-year clinical evaluation, after which a NTAC report would be filed on the basis of proof of efficacy and cost saving. At this stage, the technology would be listed as a valid test, recommended to NHS managers, and hopefully the test adopted for clinical use in each hospital. The Technology Advisory Committee is currently being established, to advise on new diagnostics within the NHS. Randox have a distribution network in 130 countries throughout the world, involving 25 direct sales offices and distributors. In addition, the involvement of the urologists in this study allows direct contact with local NHS management which should ease the evaluation of the biochip locally. Challenges involved on the route to market include difficulties because current tenders by hospital trusts may preclude applications involving new technologies; longer term budget planning by the NHS.

### 10. Trial Management

The Trial Steering Committee will have overall responsibility for the trial with appropriate input from the Clinical Committee. At times where additional expertise is required the Steering Committee may call on advice from the Advisory Board members who will have appropriate expertise. Coordination of the analyses and maintenance of the associated databases will be the remit of the Clinical Trials Administrator (CTA) who will report to the Steering Committee quarterly. The Clinical Committee will meet regularly to discuss day-to-day running of the trial (Figure 10).

**Committee Structure.tif**

**Figure 10 Trial Management**

**11. Direct Access to Source Data and Documents**

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| The Investigator(s) will permit trial-related monitoring, audits, REC review, and regulatory inspections (where appropriate) by providing direct access to source data, relevant documents (i.e., patients’ review and recruitment forms, raw data from protein analyses and pathology and cytology diagnostic review reports) and the database. |

**12. Ethics & Regulatory Approvals**

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| The trial will be conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of GCP and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework, as amended in 2006 and any subsequent amendments. The study will comply with all regulations and recommendations.  This protocol and related documents will be submitted for review to the Office for Research Ethics Committees Northern Ireland (ORECNI) at Office Suite 3, 1-4 Haslems Lane, Lisburn, Co Antrim, BT28 1TW. Protocol amendments will be submitted to ORECNI, QUB Research Policy Office and the Belfast Health and Social Care Trust.  Following commencement of the study, annual progress and a final report at conclusion of the trial will be submitted to ORECNI, Randox Laboratories, Director NICTC, Chair ECMC, QUB Research Policy Office and Belfast Health and Social Care Trust. |

### 13. Financial Contribution

We have received a Letter of Offer from Invest Northern Ireland which covers the costs of the HaBio Study. The total grant award to Queen’s University Belfast is £805,188. This grant award is backdated to the beginning of the study.

### 14. Data Handling / Trial documentation

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| The Chief Investigator will act as custodian for the trial data. All trial documentation including consent forms will be filed in locked filing cabinets in accordance with NICTC procedures and practise. Photocopies of the recruitment forms, review forms, pathology diagnostic review and cytology diagnostic review reports will be held in Room G10 in CCRCB in accordance with QUB guidelines for the storage of clinical trial data. There will be no patient identifiers on these photocopies. There will be dates of birth and patient initials on the password protected computer dedicated to the HaBio database which will be situated in room G10 in CCRCB. |

### 15. Dissemination of results

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| Progress and results will be cascaded down at the Annual Meetings for Urology Specialists and participants in the study, at Centre for Cancer Research and Cell Biology, ECMC and NICTC meetings. It is intended that the results of the study will be reported and disseminated at international conferences and in peer-reviewed scientific journals. Alay summary of the results will be written and placed onto the HaBio website (<http://www.qub.ac.uk/sites/habio/>  . |

### 16. Gantt chart



**Gantt Chart.tif17. References**

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### 18 Signatures

*To be signed by Chief Investigator minimum and statistician if applicable.*

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Chief Investigator Date

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