Standardising biosamples for the investigation of *hTERC* and *TERRA* in endometrial cancer pathogenesis

‘Thesis submitted in accordance with the requirements of University of Liverpool for the degree of Doctor in Philosophy’

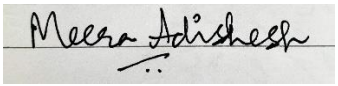
By

Dr Meera Adishesh

Sept 2022

Declaration

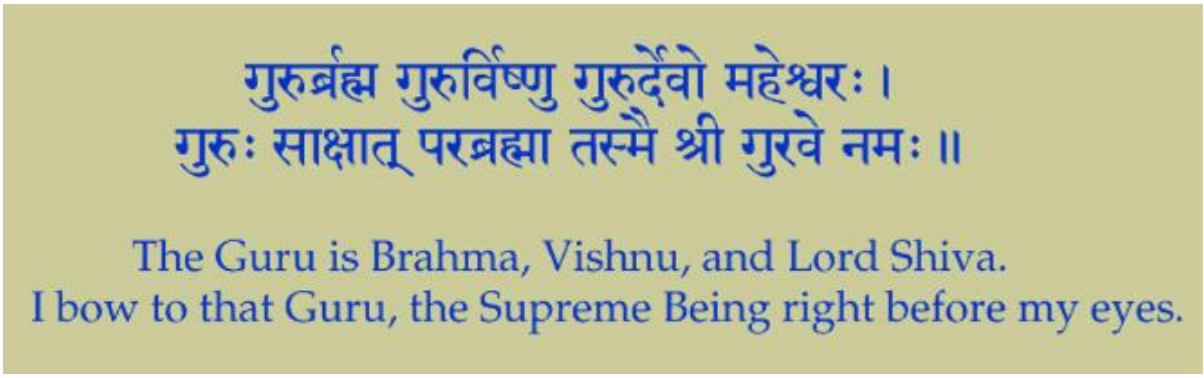
I hereby declare that the work presented in this thesis is my own work and has not been submitted for any other degree.



Dr Meera Adishesh

Table of Contents

Acknowledgments



Sanskrit shloka reproduced from https://in.pinterest.com/pin/359232507758277243/

I am very grateful to my teachers and I salute them.

First and foremost, I would like to thank my supervisor Prof Dharani Hapangama, who has been monumental in developing this project, guiding me through it step by step and finally completing it. She has been a very inspiring person, a visionary and I admire her a lot. During these 7 years of my part time PhD, I have learnt and tried to imbibe many of her great qualities such as dedication, patience, perseverance. I am very grateful to her for giving me the opportunity for obtaining a higher degree and will always remember her for motivating me. I would like to thank my second supervisor, Miss Bridget DeCruze, who has been instrumental in choosing my career path, achieving my dreams and making them a reality. Her kindness and dedication to patients has been a guiding light in my life. I have always benefitted from their valuable suggestions and unwavering support. I am hugely indebted to both of them. Without them, my PhD project would not have been possible.

I am very grateful to Jo Drury, research assistant, for teaching me patiently about all technical aspects used in this thesis and for tolerating my questions all over the last 7 years. I am very thankful to Lisa, Jane, Sarah, Helen for all the technical support and expertise they have. I am very thankful to Chris, who helped me in producing two good quality figures urgently over the last few weeks.

I would like to thank Dr Rafah Alnafakh, who has helped me with cell culturing, hormonal treatment experiments as a part of her project. I would like to thank MReS students Lucy and Shannon for helping me with PCR experiments. I would like to thank other fellow postgraduate degree students Dr Areege Kamal, Dr Nicola Tempest, Dr Alison Maclean for scientific discussions, suggestions and help.

I want to specially thank our collaborators, Dr Gabriele Saretzki, Lecturer in Ageing research, Biosciences Institute and Newcastle University, for helping me to obtain TRAP data and also immensely guide me through *TERRA* work. I also would like to thank Rhiannon Jones and Prof Duncan Baird, Division of Cancer and Genetics, Cardiff University for helping me with STELA experiments. I would like to thank Annabelle Decottignies of Université Catholique de Louvain for advice on *TERRA* qPCR optimisation. I am grateful to all patients who participated and kindly donated tissue towards research and all staff at Liverpool Women's Hospital and Women's health, Institute of Translational medicine, University of Liverpool who helped my project.

I would like to thank the whole Gynaeoncology department at Liverpool Women's Hospital for giving me the opportunity and helping me complete this PhD project. I would like to thank all staff in Gynaecology department who helped me recruit patients for research and during the collection of samples for my project.

I would like to thank my parents-in-law, Mrs Ananthalakshmi Govindan and Mr B.S. Govindan for always believing in me, my capabilities and unconditionally supporting me throughout my life. I would like to thank my parents, Mrs Kathyayini Adishesh and Mr H.R. Adishesh, for always being there for me, for their selflessness and their unconditional support. Without their help and support nothing would have been possible. I would love to thank all my extended family who have always been helpful and supportive.

Last but not the least, I would love to thank the most important people in my life, my daughter, Smruthi, and my husband, Dr Shankar B.G., who are pillars in my life and who keep me grounded to reality. They have sacrificed the most for this PhD to be a reality! Love you Smruthi and thank you for keeping me constantly motivated, tolerating my moaning, waiting for me at home and being this smiling person always. Special thanks to Shankar who is the sole force behind a successful me, thanks for giving me the wings to fly and for always being there during the most difficult times. I would love to thank Zac, my handsome yorkie, for tolerating me being away for so many years and not spending enough time with him.

Acknowledgements

Chapter 3, 4, 5 and 6

Sample collection Meera Adishesh / Alison Maclean / Nic Tempest / Dharani Hapangama

Ishikawa Cell culturing and in vitro hormone treatment Rafah Alnafakh

RNA extraction, cDNA synthesis Meera Adishesh /Lucy Button

qRT PCR Meera Adishesh / Shannon Simon/ Lucy Button

IHC Rafah Alnafakh / Meera Adishesh

TRAP assay Dr Gabriele Saretzski

STELA assay Rhiannon Jones and Prof Duncan Baird

Analysis Meera Adishesh

Chapter 7

Literature review Alison Fyson / Meera Adishesh

Generation of tools Meera Adishesh/Dharani Hapangama

Consensus and statistical analysis Meera Adishesh

Ethics approvals for Chapters 3, 4, 5 and 6

Studies included in this thesis are covered by these ethical approvals. Liverpool and Cambridge Adult Research Ethics Committee granted ethical approval (LREC 09/H1005/55, 11/H1005/4 and CREC 10/H0308/75). We recruited women undergoing hysterectomy to collect endometrial biopsies at Liverpool Women's Hospital between 2016 and 2018 and informed written consent was obtained from all patients.

Sample size calculation

As per the guidance of statistician Dr Steven Lane, as the studies in different chapters in this thesis are preliminary studies, we did not need to perform a formal sample size calculation as we needed a measure of the variability in the outcome and also needed a measure of the expected effect size. We can use the information I have generated from the pilot study to make sample size estimation for future studies.

List of abbreviations

ACTB: beta actin

ALT: Alternative lengthening of telomeres

APBs: ALT associated promyelocytic leukemia nuclear bodies

AR: Androgen receptor

ARID1A: AT-Rich Interaction Domain 1A gene

ATM: ataxia telangiectasia mutated

ATR: ATM and Rad3-related

AU: Arbitrary units

BBMRI: Biobanking and Biomolecular resources Research Infrastructure

BBRB: Biospecimen Research Branch Best Practices for Biospecimen Resources

BIR: Break induced replication

BMA: British Medical Association

BMI: Body mass index

BRCA2: Breast cancer gene 2

BRISQ: Biospecimen Reporting for Improved Study Quality

CA9: Carbonic anhydrase 9

CAB: Cajal body localisation box

cDNA: Complementary DNA

CfWHR: Centre for Women's Health Research

CN: copy number

COC: Combined oral contraceptives

CRUK: Cancer research UK

CT: Computed tomography

CTNNB1: Catenin Beta 1 gene

DDR: DNA damage response

DHEA: dehydroepiandrosterone

DHT: Dihydrotestosterone

DNA: Deoxyribonucleic acid

DM: Diabetes Mellitus

dsRNA: double stranded RNA

E2: Oestrogens/ Oestradiol

EBRT: External beam radiotherapy

EC: endometrial cancer

ECBS: Endometrial cancer biospecimen tool

ECPD: Endometrial cancer patient data collection tool

ECSD: Endometrial cancer surgical data collection tool

ENITEC: European Network of Individualized Treatment in Endometrial Cancer

EORTC: European Organisation for Research and Treatment of Cancer

EPIC: European Prospective Investigation into Cancer

ER: Oestrogen receptor

ERα: Oestrogen receptor Alpha

ERβ: Oestrogen receptor Beta

ERE: Oestrogen responsive element

ESC: Embryonic stem cells

ESMO: European Society of Medical Oncologists

FBXW7: F-Box And WD Repeat Domain Containing 7 gene

FFPE: Formalin fixed paraffin embedded

FGF: Fibroblast growth factor

FIGO: International Federation of Gynaecology and Obstetrics

FNA: Fine needle aspiration

FSH: Follicular stimulating hormone

GnRH: Gonadotrophin releasing hormone

GR: Glucocorticoid receptors

HASTEN : Harmonisation of biobAnking STandards in Endometrial caNcer research

HCC: Hepatocellular carcinoma

HDR: Homology directed repair

hESC: Human embryonic stem cells

HG: High grade

HIF-1: Hypoxia inducible factor 1

HIF-1α: Hypoxia inducible factor 1alpha

HIF-1β: Hypoxia inducible factor 1beta

hnRNPs: Heterogeneous nuclear ribonucleoproteins

HNSCC: Head and neck squamous cell carcinomas

HP1: Heterochromatin protein 1

HRA: Health Research Authority

HRT: Hormone replacement therapy

hTERC: Telomerase RNA

hTERT: human telomerase reverse transcriptase

IARC: International Agency for Research on Cancer

IHC: Immunohistochemistry

IQR: Interquartile range

ISBER: International society for Biological and Environmental Repositories

ISK: Ishikawa

IUS: Intrauterine system

KRAS: Kirsten rat sarcoma virus gene

LESQS: Liverpool endometrial steroid quick score

LG: Low grade

LH: Luteinising hormone

lncRNA: long non-coding RNAs

LNG: Levonorgestrel

LNG-IUS: Levonorgestrel Intrauterine System

LVSI: Lymphovascular space invasion

LTL: Leukocyte telomere length

LUSC: Lung squamous cells cancers

LWH: Liverpool Women's Hospital

LWRTB: Liverpool Women's Research Tissue Bank

m^7G: 7-methylguanosine

MPA: Medroxyprogesterone acetate

mRNA: Messenger RNA

MRC: Medical Research Council

MRI: Magnetic resonance imaging

MSI: Microsatellite instability

NBF: Neutral buffered formalin

NCI: National Cancer Institute

NDTMM: Non-defined TMM mechanism

NGS: Next generation sequencing

NGT: Nominal Group Technique

NHEJ: Non-homologous DNA end joining

NHS: National health service

NIH: National institute of health

NRT: No reverse transcriptase

NSCLC: Non-small cell lung cancers

ORC: origin of recognition complex

OVCARE: Ovarian Cancer Research Program

P4: Progesterone

PCR: Polymerase chain reaction

PCOS: Polycystic ovarian syndrome

P3G: Public population project in genomics

PHDs: Prolyl hydroxylase domain proteins

PI: Proliferative index

PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha gene

PIK3R1: Phosphoinositide-3-Kinase Regulatory Subunit 1 gene

POLE: DNA polymerase epsilon gene

POT1: Protection of telomeres

PP: Proliferative phase

PPIA: Peptidylprolyl Isomerase A

PR: Progesterone receptor

ProMisE: Proactive Molecular Risk Classifier for Endometrial Cancer

PTEN: Phosphatase And Tensin Homolog gene

QC: Quality control

qPCR / qRT-PCR: Quantitative real time polymerase chain reaction

RAM: RAND/UCLA Appropriateness method

RAP1: Repressor/ activator protein 1

RBPs: RNA binding proteins

RCOG: Royal College of Obstetricians and Gynaecologists

RIN: RNA integrity number

RNA: Ribonucleic acid

RNA FISH: RNA fluorescence in situ hybridisation

RPA: Replication protein A

RPH: Royal Preston Hospital

RR: Relative risk

RT-PCR: Real time polymerase chain reaction

sASO-NLS: phosphorothioate antisense oligonucleotides - nuclear localisation signal peptide

SERM: Selective oestrogen receptor modulator

SiRNA: short interfering RNA or silencing RNA

SOP: standard operating procedure

SOP-ECBS: Standard operating procedure for collection, processing and storage of tissue and fluid for endometrial cancer research

SPREC: Standard preanalytical Code

ssDNA: Single stranded DNA

STELA: Single telomere length analysis

TA: Telomerase activity

TBS: Tris-buffered saline

TCAB1: Telomerase cajal body protein 1

TCGA: The Cancer Genome Atlas

TelRNAs: Telomeric RNAs

TERRA: Telomeric repeat containing RNA

TIF: Telomere dysfunction induced foci

TIN2: TRF1 interacting nuclear protein 2

TL: Telomere length

TMG: 2,2,7-trimethylguanosine

TMM: Telomere maintaining mechanism

TNFA: Tumour necrosis factor alpha

TNM staging: tumor (T), nodes (N), and metastases (M) staging system

TP53: Tumour protein P53

TPP1: Tripeptidyl peptidase 1

TRAP: Telomeric repeat amplification protocol

TRF1: Telomeric repeat factor 1

TRF2: Telomeric repeat factor 2

VEGF: Vascular endothelial growth factor

VEGFA: Vascular endothelial growth factor A

WERF EPHect: World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project

WHO: World Health Organisation

Abstract of thesis 'Standardising biosamples for the investigation of *hTERC* and *TERRA* in endometrial cancer pathogenesis' by Dr Meera Adishesh

Endometrial cancer (EC) is the most common gynaecological malignancy and new diagnostic, therapeutic and prognostic strategies are needed to improve the outcomes of millions of women, worldwide. The work presented in my thesis aims to improve the endometrial cancer research, advancing our current knowledge of endometrial carcinogenesis. In the first results chapter, I report the effect of pre analytical variables such as timing of the samples and sampling methods on expressional analysis in the endometrial samples by comparing samples taken before and after hysterectomy. This study has formed the basis for a consistent method used for the sample collection in the subsequent chapters and also highlighted the need for the work described in chapter 7, where harmonisation of EC biosample collection was undertaken.

Further on, to advance our current knowledge about endometrial carcinogenesis by studying the non-coding RNA(Ribonucleic acid)s related to telomerase enzyme. I have demonstrated that *hTERC* levels varied in human endometrium throughout menstrual cycle, highest levels observed in postmenopausal endometrium suggesting constitutive expression of *hTERC*. In EC, *hTERC* levels did decrease, and when compared amongst different grades of cancer, highest levels were detected in grade 3 endometrioid EC.

Although telomeres were considered to be transcriptionally silent until recently, telomeric repeat-containing RNA (*TERRA*) molecules are transcribed from CpG-island containing subtelomeric promotors. Considering the intricate relationship between telomerase, telomeres and endometrial cellular proliferation, I hypothesised that *TERRA*s may have an important role in endometrial carcinogenesis. To test this hypothesis, I examined *TERRA* from *chromosomes 1q-2q-4q-10q-13q-22q,16p* and *20q* in healthy normal, benign proliferative conditions such as endometriosis and EC samples. I demonstrated that *TERRA* levels are dynamic in human endometrium, and significantly lower in ECs.

The dynamic changes in both *hTERC* and *TERRA* levels suggest hormone regulation and therefore I progressed to examine the hormone regulation of *hTERC* and *TERRA* levels both *in vitro* and *in vivo* in the work described in Chapter 6. There were significant changes in the levels of *hTERC* and *TERRA16p* with progesterone and combined O+P treatment. These changes were not replicated with *in vivo* hormonal regulation study, in endometrial samples from women on exogenous hormones such as GnRH analogues and Mirena IUS.

The results from previous chapters identified the importance of standardisation of biospecimen collection and processing. Finally, to achieve this to improve EC research we designed and conducted the HASTEN study (Harmonisation of biobAnking STandards in Endometrial caNcer research). The final tools were accepted by a large multi-disciplinary team and they are published in a well-known peer reviewed journal, where they are freely available to all EC researchers globally.

To conclude, my work has paved way to more detailed understanding of factors affecting EC research, achieving consensus amongst EC researchers about standardising biobanking standards and recognising newer non-coding RNAs to play a role in endometrial carcinogenesis.

Chapter 1: Introduction

Endometrial cancer (EC) is the most common gynaecological malignancy with an increasing incidence and an increase in associated mortality. New diagnostic, therapeutic and prognostic strategies are therefore urgently needed to improve outcomes for millions of women, suffering with EC worldwide. The work presented in this thesis aims to improve EC research, advancing our current knowledge of endometrial carcinogenesis.

Endometrium

Studies of EC require a good understanding of the unique structure and function of healthy human endometrium. Human endometrium is one of the most dynamic somatic organs in the body. It is a complex multicellular tissue, which has two layers, basalis and functionalis that are composed of glands embedded in cellular stroma. The unique quality of the endometrium is its iterative cyclical shedding and regeneration, and this is regulated by ovarian hormones (Hapangama et al., 2015), (Tempest et al., 2020). Human endometrium undergoes growth and differentiation in the first half of the menstrual cycle, then in the absence of conception it undergoes degeneration, shedding and regeneration. At the time of menopause, endometrium becomes quiescent due to the cessation of ovarian hormone production (Kamal et al., 2016a). This thin postmenopausal endometrium can still be regenerated by exogenous ovarian steroid hormones (Ferenczy and Bergeron, 1991), (Ettinger et al., 1997). Thus, it is one of the few human organs, which does not show irreversible age related changes. Although it is a somatic organ, endometrium expresses telomerase activity, which may be the reason for its apparent age defiance (Hapangama et al., 2017).

Changes in endometrium during menstrual cycle

Proliferative phase: As ovarian follicular maturation takes place; proliferative phase refers to the changes in the endometrium corresponding to the increasing estrogen levels produced by the follicular cells the first 14 days of a typical 28 day menstrual cycle. After menstrual shedding, endometrium regenerates fully over day 4-7 of the cycle, with short, straight, and narrow glands and stroma that display mitotic activity. Glands then become elongated, curved, and coiled in the thickened late proliferative endometrium, where spiral arteries elongate to provide adequate blood flow (Monis and Tetrokalashvili, 2021) .

Secretory phase: The latter 14 days of the cycle is also known as luteal phase and is under the influence of both oestrogen and progesterone. In the secretory phase endometrium reaches its maturity, glands become more curled, the glands and arteries begin to entwine, and stroma

becomes oedematous. In the late secretory phase, with the demise of the corpus luteum, progesterone levels plummet, leading to menstrual shedding of the functionalis in absence of pregnancy (Jeanmonod et al., 2021).

Steroidogenesis in women

Steroid hormones are synthesised in organs such as ovary, adrenal cortex, brain and placenta, the number of hormones synthesised depends on the expression of enzymes specific of each organ for example ovaries do not contain 21 α-hydroxylase or 11 α-hydroxylase so are unable to produce glucocorticoids or mineralocorticoids (Holst et al., 2004). Sex steroid hormones can be divided into three groups: Progesterone (21 carbon atoms), androgens (19 carbon atoms) and oestrogens (18 carbon atoms). Steroid hormones synthesised from cholesterol, which is transported to mitochondria and converted to pregnenolone. Pregnenolone is released from mitochondria and can follow two pathways: first Δ^5-hydroxy steroid pathway in adrenal glands which leads to synthesis of dehydroepiandrosterone (DHEA) and androstenediol. Second Δ^4-ketosteroid pathway in corpus luteum granulosa cells, which leads to synthesis of 17 α-hydroxyprogesterone and androstenedione (Figure 1). Progesterone produced in adrenal glands is largely converted to glucocorticoids and androgens (Cable JK, 2021), whereas progesterone produced in the ovaries is carried in blood to exert its effect. Oestrogen is synthesised in pre antral follicles, through effect on theca cells cholesterol is converted androstenedione which is further converted to oestrogens by Follicular Stimulating Hormone (FSH) and also by aromatase enzyme (Kobayashi et al., 1990). Dominant follicle luteinisation occurs due to Luteinising hormone (LH), LDL cholesterol reaching these cells is converted to progesterone by α-hydroxy dehydrogenase (Ravindranath et al., 1992).

Endometrial changes in response to steroid hormones

Endometrium is found to have enzymes capable of synthesising and metabolising steroids (Hausknecht et al., 1982). Steroid hormone action is mainly mediated by receptors; there is a single androgen receptor (AR), two oestrogen receptors (ERα and ERβ). AR are seen in functionalis during proliferative phase and in basalis all throughout the menstrual cycle. AR is downregulated in stromal cells in functionalis during secretory phase, upregulated in epithelial cells when progesterone levels decrease (Marshall et al., 2011). ERα is present in epithelial cells in glands and lumen during proliferative phase and is downregulated in secretory phase. Concentrations of oestrogens (E2) were higher in endometrial tissue than in circulation, and concentrations of E2 were increased in secretory phase compared to proliferative phase

(Huhtinen et al., 2014). The study also reported increased intra tissue levels of androgen precursor DHEA than in the serum, whereas androstenedione and testosterone were lower in endometrium compared to serum and these were not cycle phase dependent (Huhtinen et al., 2014). The hormone receptors are activated by ligand binding, which exert effects involving steroid receptor, dimerising, translocating into the nucleus, binding to the hormone responsive element to initiate co-activators, co-repressors and chromatin remodelling factors (Kamal et al., 2016a) . It is also known that oestrogen induces all endometrial steroid receptor expression, via ERα whilst progesterone downregulates them via progesterone receptor (PR) (Kamal et al., 2016a). Androgens induce their receptors via AR.

17β-estradiol and estrones are main oestrogens which affect the endometrium and exert this via ERα and ERβ. ERα is thought to be essential for fertility and ERβ was mainly thought to be useful in preventing undesired ERα effects of oestrogen. Hapangama et al have reviewed the relevance of ERβ in endometrium and its role in pathological conditions of the endometrium (Hapangama et al., 2015) . They conclude that ERβ has an important role in homeostasis, cell turnover and regeneration in endometrium. ERβ is dynamically expressed in healthy premenopausal endometrium and also in post-menopausal atrophic endometrium (Hapangama et al., 2015).

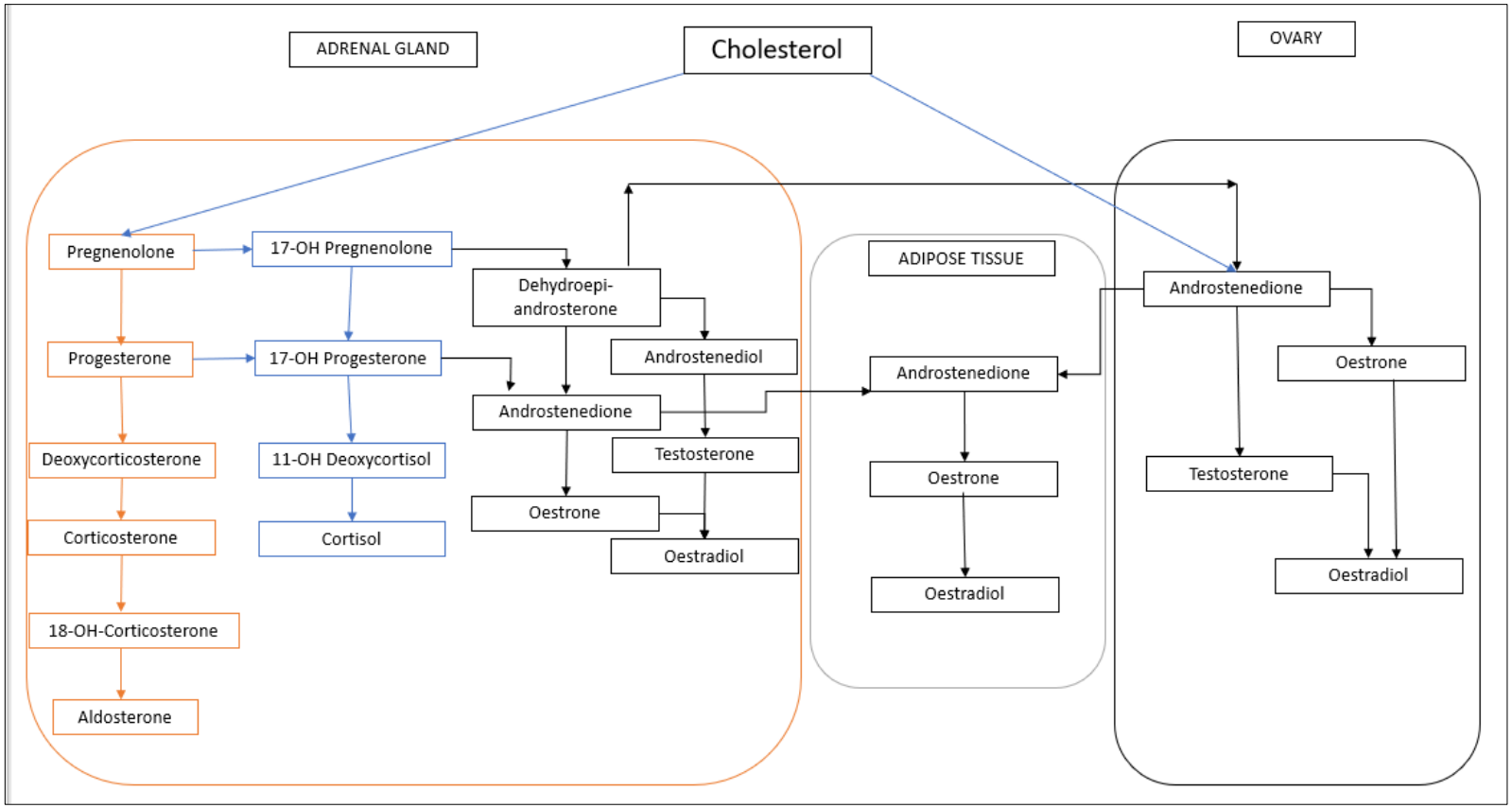
Endometrial cancer

Background and Incidence

EC is the 4th most common cancer in women and overall, most common gynaecological cancer. In the UK, lifetime risk of a woman developing EC is 1:36 (CRUK, 2016 - 2018). In a year, around 9000 new cases were diagnosed in the UK (CRUK, 2016 - 2018). According to the Cancer research UK statistics, this equates to a 65% increase in incidence of EC in the UK compared to the 1970s (CRUK, 2016 - 2018). The National Cancer Intelligence Network statistics highlight that incidence rates have increased by 43% since mid-nineties in UK (NCIN, 2013a).

Lindemann et al estimate the incidence of EC in Norway will increase further in the range of 50-100 % by year 2025 (Lindemann et al., 2010). Based on the data available from 2016, cancer incidence and mortality are projected for 2035 in UK, such that in women uterine cancer will become the commonest cancer (Smittenaar et al., 2016). Along with increase in obesity, tamoxifen use, decline in hysterectomy rates for menorrhagia, and

Figure 1. Diagramatic representation of steroidogenesis in women



longer survival in women, all contribute significantly towards this increasing incidence of EC.

EC accounts for about 3% of all female deaths in the UK (CRUK, 2016-2018a). Furthermore, the mortality rates from this cancer have had an upward trend over the last 20 years, which is expected to continue in the future and project that uterine cancer will be 6th most common cause of cancer mortality in women in 2035 (Smittenaar et al., 2016).

The 2009 Prospective Studies Collaboration reported association of obesity to causes of mortality, and they reported that relative risk of mortality in women with WHO class 3 obesity due to EC was 4.17 (Prospective Studies, 2009).

Prevalence of obesity had increased since 1990s and is estimated that by 2050, 50% of women will be obese in the UK (Tackling obesities, 2007). In UK, about 50% of ECs are attributed to obesity (Reeves et al., 2007). In addition to obesity, metabolic abnormalities caused by diabetes and hypertension work synergistically with obesity to promote EC incidence (Wang et al., 2020), (Crosbie et al., 2022).

Surgery is the mainstay of treatment for early stage EC, and multicentre prospective audit of data for EC in the UK has shown considerable morbidity associated with surgery in these patients due to significant patient and surgical factors (Iyer et al., 2015). Previous studies reveal these patients have higher comorbidities related to being elderly or high body mass index (BMI) (Pierluigi Benedetti et al., 2014), (Everett et al., 2003). Some studies such as Lap2 study showed that obesity is an important barrier for laparoscopic procedures (Walker et al., 2009), (Walker et al., 2012) with significant challenges associated with management of obese patients ranging from ventilatory, anaesthetic issues to limitations in access by surgical instruments and visibility (Gunderson et al., 2014). The burden of caring for obese women is huge from health economics perspective. For example, they require extensive pre-operative investigations, prolonged surgical time, postoperative high dependency care, and specific equipment may also be needed to care for such patients on wards. Therefore, caring for obese patients with EC can also bring challenges which impacts on medical, nursing and psychosocial resources (RCOG, 2021).

All these aspects in EC make it challenging for clinicians to manage and treat patients appropriately, hence it becomes especially important to understand the pathogenesis of the condition to prevent its occurrence or diagnose at early stage.

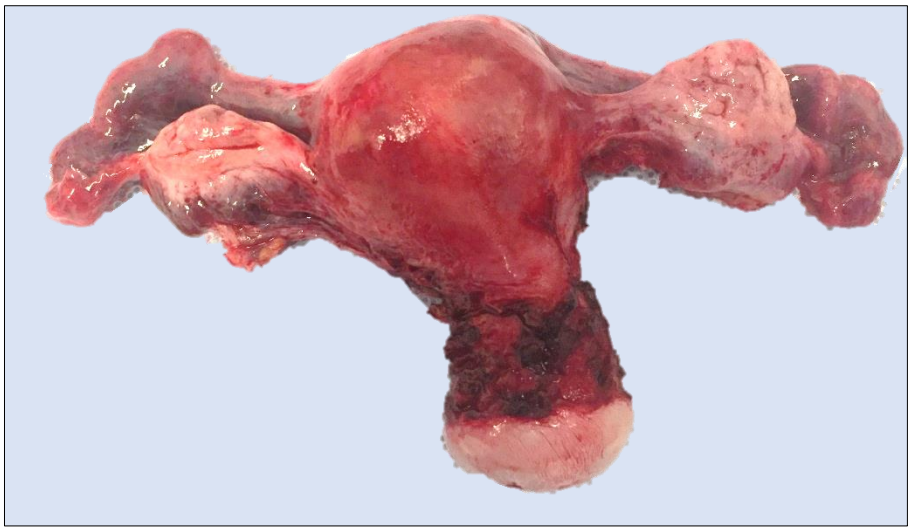


Figure 2.

Photograph of posterior aspect of a normal uterus with fallopian tubes and ovaries Courtesy: Hapangama lab, Department of Women's and Children's Health, Centre for Women's Health Research, Institute of Life Course and Medical Sciences, University of Liverpool, UK.

Classification of EC

There are many ways of classification of EC, and we are yet to reach universal agreement.

1. Type 1 and Type 2 ECs

Prior to 1980's EC was considered to be a single type of malignancy. In 1983, Bokhman described two distinct types of EC, Type 1 and 2.

The specific characteristics of both types are compared in the table below (Bokhman, 1983), (Felix et al., 2010) (Table 1.).

Table 1. Main features and differences between type 1 and type 2 EC

	Type 1	Type 2
Histological type	Endometrioid type – grade 1 and 2	Endometrioid grade 3 and Non Endometrioid (serous or clear cell histology)
Associated with precancer	Yes – endometrial intraepithelial neoplasia	Atrophic endometrium leads to endometrial glandular dysplasia
Incidence	80 – 90% of all ECs	10 – 20% of all ECs
Aetiology	Oestrogen dependent	Non oestrogen dependent – P 53 mutations and chromosomal instability
Age group	Common in pre and perimenopausal women	Mainly postmenopausal
Molecular mechanisms	Deletions in *k-RAS*, *PTEN*, or mismatch repair mechanisms	Mutations or overexpression of *HER2*/*Neu* and *P53*, reduced E-*cadherin* expression, aneuploidy
Mortality and prognosis	Better than type 2	Worse than type 1

2. International FIGO histology grading

In 1988, International Federation of Gynaecology and Obstetrics (FIGO) revised grading recommendations of typical endometrial adenocarcinoma and following validation, was accepted as standard method of grading in 1995 (Zaino et al., 1995), (Scholten et al., 2004) (Table 2).

Table 2. FIGO Grading of EC

Grade 1	non squamous or non-morular solid growth pattern of < 5% or less
Grade 2	non squamous or non-morular solid growth pattern of 6 -50%
Grade 3	non squamous or non-morular solid growth pattern of > 50%

FIGO grading excludes serous or clear cell which are considered high grade.

3. WHO Histopathological (Fanning et al., 1989)

EC is divided into various subtypes depending on cell type by World Health Organisation (WHO) (Table 3).

Table 3. Histopathological types of EC

Epithelial	Mesenchymal	Mixed epithelial and mesenchymal	Other
- Endometrioid adenocarcinoma - Serous endometrial intra-epithelial carcinoma - Serous adenocarcinoma - Mucinous adenocarcinoma - Clear cell adenocarcinoma - Neuroendocrine tumours - Mixed cell adenocarcinoma - Transitional cell carcinoma - Undifferentiated carcinoma - De-differentiated carcinoma	- Leiomyoma - Smooth muscle tumour of uncertain malignant potential - Leiomyosarcoma - Endometrial stromal sarcoma - Miscellaneous	- Carcinosarcoma (malignant mullerian mixed tumour) - Adenomyoma - Adenosarcoma - Adenofibroma	- Gestational trophoblastic disease - Neuroectodermal tumours - Adenomatoid tumours - Germ cell tumours - Lymphoid and myeloid tumours - Lymphomas - Myeloid neoplasms - Secondary tumours

4. Clinical risk classification

According to European Society of Medical Oncologists (ESMO) guidelines FIGO classification are subgrouped into three risk groups (Colombo et al., 2013) (Table 4).

Table 4. ESMO clinical risk classification

Risk classification	Types
Low risk	Stage 1A (grade 1 and 2) with endometrioid type
Intermediate risk	Stage 1A grade 3 with endometrioid type Stage 1B (grade1 and 2) with endometrioid type
High risk	Stage 1B grade 3 with endometrioid type All stages with non-endometrioid type

5. Molecular classification

Molecular classification is reproducible and can be linked with clinical outcomes (Salvesen et al., 2009), (Cancer Genome Atlas Research et al., 2013), (Levine, 2013).

There are different ways of molecular characterisation of EC: The Cancer Genome Atlas (TCGA), Leiden, and Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) molecular classification systems.

TCGA classifies EC into four genomic subgroups (Kandoth et al., 2013), (Murali et al., 2014), (Piulats et al., 2017) (Table 5).

Table 5. Molecular classification of EC

Group	Characteristics	Outcomes
POLE	'Ultra-mutated', very high mutation rate Genes mutated: *POLE* (100%), *PTEN* (94%), *FBXW7* (82%), *PIK3CA* (71%),	Favourable even in high grade tumours PFS good

	PIK3R1 (65%), *ARID1A* (76%), *KRAS* (53%) Mixed MSI (high, low, stable) Copy number aberrations low	
MSI (Microsatellite instability)	'hypermutated', high mutation rate MSI high *PTEN* (88%), *PIK3CA* (54%) Copy number aberrations low	PFS intermediate
CN low (Copy number low)	MSI stable *PTEN* (77%), *CTNNB1* (52%), *PIK3CA* (53%) Low mutation rate Copy number aberrations low	PFS intermediate
CN high (Copy number high)	Copy number aberrations high MSI stable Low mutation rate Genes mutated: *TP53* (92%)	PFS poor

(*POLE*-DNA polymerase epsilon gene, *PTEN*- Phosphatase And Tensin Homolog gene, *FBXW7*-F-Box And WD Repeat Domain Containing 7 gene, *PIK3CA*- Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha gene, *PIK3R1*- Phosphoinositide-3-Kinase Regulatory Subunit 1 gene, *ARID1A*-AT-Rich Interaction Domain 1A gene, *KRAS*- Kirsten rat sarcoma virus gene, MSI-Microsatellite instability, CN- copy number, *CTNNB1*-Catenin Beta 1 gene, *TP53*-Tumour protein P53, PFS-progression free survival)

Staging of EC

FIGO staging (2009) is the commonest system used worldwide.

Another staging system in use is TNM staging (tumor (T), nodes (N), and metastases (M) staging system) (Amant et al., 2018) (Table 6).

74 % women present in stage 1& 18 % women present in advanced stages (3/4) (Di Girolamo et al., 2018), (Office for National Statistics, 2019).

Table 6. FIGO and TNM staging of EC

TNM	FIGO Stage	Description
T1a	1A	Tumour confined to the uterus, no or < 50% myometrial invasion
T1b	1B	Tumour confined to the uterus, > 50% myometrial invasion
T2	2	Tumour invades cervical stroma, not beyond uterus
T3a	3A	Tumour invades serosa or adnexa
T3b	3B	Tumour invades vagina and/or parametrial involvement
	3C1	Pelvic node involvement
	3C2	Para-aortic node involvement
T4	4A	Tumour invasion bladder and/or bowel mucosa
	4B	Distant metastases including abdominal metastases and/or inguinal lymph nodes

Figure 3. Photograph of longitudinal section of uterus showing EC

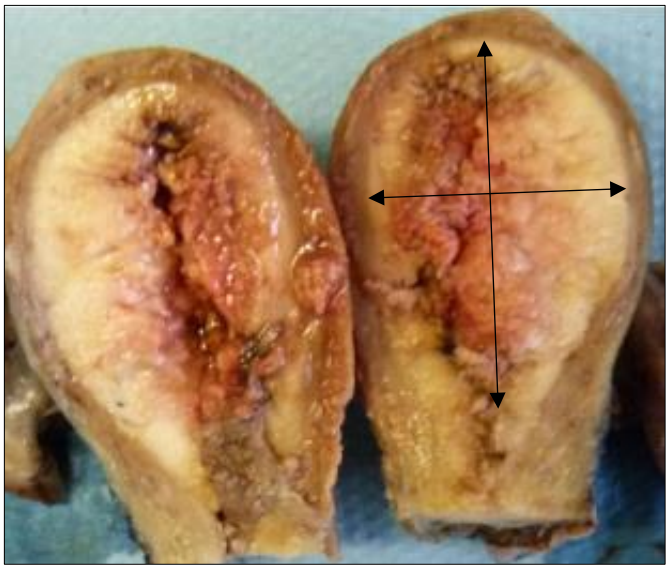
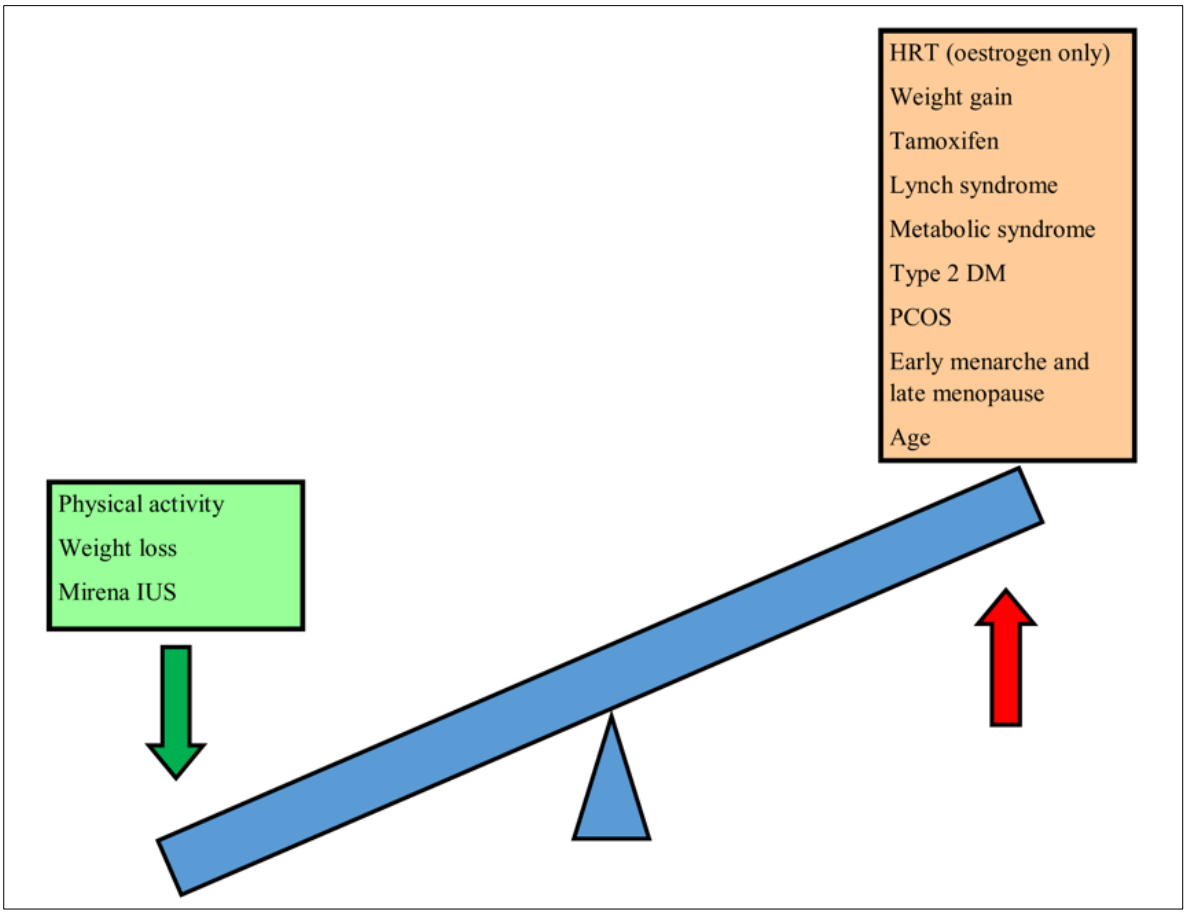


Figure 3.

Photograph of longitudinal section of uterus showing EC (shown by arrow marks) invading into the myometrium

Courtesy: Pathology lab, RPH, Lancashire Teaching University Hospital NHS Trust, Preston.

Risk factors for EC



1. Lifestyle factors:

EC incidence is increasing in parallel with increasing rates of obesity in UK. Obesity causes a metabolic state where the body encounters hyperoestrogenism, inflammation and insulin resistance, which in turn leads to carcinogenesis.

Meta-analysis in 2010 concluded that increased physical activity and decreased sedentary time were associated with decreased risk of EC (Moore et al., 2010). Inactive women who sat down for more than or equal to 9 hours per day have twice the risk of EC compared to women who sat for fewer than 3 hours (RR 2.14: 95% CI 1.48 – 3.10).

Both former smokers and current smokers have reduced incidence of EC compared to non-smokers which is explained by hormonal modulation affecting hormone producing organs like adrenal and ovary (Felix et al., 2014).

2. Demographics:

EC is a disease of postmenopausal women. Age specific incidence rises from age 40–45, then peaks around 70-75 and subsequently drops. Nulliparous women are at higher risk of EC than multiparous women (nulliparous vs parous: HR, 1.42; 95% CI, 1.26-1.60) (Schonfeld et al., 2013). Reduced exposure to oestrogen due to pregnancy and increased progesterone levels in pregnancy seem to be protective factors for multiparous women. A meta-analysis has shown that there is a 4% reduction in EC for every 2-year delay in menarchal age (RR 0.96, 95% CI 0.94 – 0.98) (Gong et al., 2015). Late menopause is also associated with increased EC risk (Ali, 2014).

3. Effect of Medications (contraceptives, hormone replacement therapy (HRT), Tamoxifen):

Use of hormonal contraception is described to be protective to the endometrium (Mueck et al., 2010). The reduction of this risk is proportional to duration of use, every 5 years of use is associated with RR of 0.76 (Collaborative Group in Epidemiological Studies on Endometrial Cancer, 2015). This effect persists for about 30 years and it may be amplified as time progresses (Cook et al., 2014). The effect is not dependent on the types of hormones and their composition however women with higher BMI may need combined oral contraceptives (COC) with highly potent progestin (Maxwell et al., 2006). Levonorgestrel (LNG)- releasing intrauterine system is also associated with reduction in EC, which is more prominent with prolonged use up to 10 years (Soini et al., 2014).

HRT is commonly used by peri and postmenopausal women for management of menopausal symptoms. In a cohort study (Mørch et al., 2016), amongst different types of HRT, Relative risk (RR) of EC with use of oestrogen only products is 2.70 (CI 2.41 – 3.02), whereas the RR with oestrogen and progestin is 1.71 (CI 1.58 – 1.86). Tibolone has a higher RR of 3.56 (CI 2.94 – 4.32) compared to other HRT, which suggests that the progestinic effect of tibolone is weaker compared to continuous combined therapy. In the same study, it was observed that long cyclical combined HRT had a higher RR of 2.89 compared to RR of 2.06 and 1.02 associated with use of cyclic combined and continuous combined respectively. The vaginal and transdermal preparations which are thought to have less systemic side effects also carry a risk of EC (RR of transdermal Oestrogen is 2.77 (2.12 – 3.62) and risk for vaginal Oestrogen preparations is 1.96(1.77 -2.17)). However, the risk of developing type 2 ECs appears to be decreased with continuous combined therapy (0.20 – 1.01) and Oestrogen therapy (0.85 – 2.41) but the results did not reach statistical significance (Mørch et al., 2016).

Tamoxifen is a selective oestrogen receptor modulator (SERM) and used to reduce the risk of breast cancer recurrence and also to reduce the risk of breast cancer in high risk women. Tamoxifen is a non-steroidal anti oestrogen agent. Though it has anti oestrogenic effect on breast tissue, it has a moderate oestrogenic effect on endometrium, hence in standard doses it causes endometrial proliferation and leads to hyperplasia, polyp formation and invasive cancer. Some studies have demonstrated a two to three fold higher RR of EC in women using tamoxifen when compared to age matched population and this increase is dose and time dependent (Sismondi et al., 1994), (Bissett et al., 1994), (Fisher et al., 1994). A systematic review of the effect of tamoxifen on EC shows that in women younger than 50 years, there is no increase in EC risk (RR 1.19, CI 0.53 – 2.65), whereas when commenced in women above 50 years of age, the risk is significantly higher (RR 3.32, CI 1.95 – 5.67) (Iqbal et al., 2012). According to this review, effects of tamoxifen on endometrium is related to circulatory plasma oestradiol. Post menopausal women are at more risk of hyperplasia of endometrium with tamoxifen. Women with persistent ovarian function are at less risk of developing endometrial thickening as tamoxifen, being a selective oestrogen receptor modulator, has an anti oestrogenic effect on endometrium in presence of high oestradiol concentrations (Iqbal et al., 2012).

4. Medical disorders:

Women with polycystic ovary syndrome (PCOS) have a three to four fold increase in incidence of EC, hence their lifetime risk is 9% compared to 3% in the general population (Fearnley et al., 2010), (Haoula et al., 2012), (Gottschau et al., 2015). The endometrium in these women remains in a proliferative state, which is oestrogen mediated due to chronic anovulation (Shao et al., 2014). Hyperandrogenism and peripheral aromatisation of androgens which occurs in adipose tissue and high BMI are all important features of PCOS leading to unopposed oestrogen effect on the endometrium.

Patients with type 2 diabetes are at 62% increased risk of EC, independent of obesity, three main biological pathways are described in different studies such as insulin resistance, hormonal imbalance and systemic inflammation which lead to EC development (Saed et al., 2019), (Shikata et al., 2013), (Njoku et al., 2022a). Women with Parkinson's disease are at 17% higher risk of developing EC than the general population, though the associated pathogenesis is not well understood currently (Ong et al., 2014).

5. Genetics:

Lynch syndrome is an inherited syndrome which is associated with a high risk of colorectal, endometrial, ovarian and urinary tract cancers (Watson et al., 2008), (Crosbie et al., 2022). Lifetime risk of EC in women with Lynch syndrome is about 60% (Dunlop et al., 1997), (Bats et al., 2017), (Ryan et al., 2020). This condition is associated with mutation of mismatch repair gene, involved in DNA mismatch repair (Aarnio et al., 1999) . Defects in mismatch repair genes *hMLH1*, *hMSH2*, *hMSH6*, *hPMS* cause nucleotide repeat variants throughout genome, called microsatellite instability and is hallmark of mismatch repair defects (Meyer, 2009). Hereditary EC in these patients are sentinel cancers, occur in younger women with low BMI, when compared with sporadic tumours (Meyer, 2009). Women with Lynch syndrome who have EC can also have synchronous ovarian cancer (Soliman et al., 2005).

Diagnosis of EC

In postmenopausal women, transvaginal ultrasound is used to detect increased endometrial thickness, with a usual threshold of 5 mm thickness for subsequent endometrial biopsy. In pre menopausal women, endometrial thickness is less specific, as it varies with the menstrual cycle. Outpatient hysteroscopy is the gold standard method when sampling a suspicious lesion of endometrium is indicated . The definite diagnosis is made with histological examination of endometrial sample, which required an invasive procedure. Blind endometrial biopsy can be performed but is associated with 11% chance of failure to detect a cancer due to inadequate sampling and cervical stenosis (van Hanegem et al., 2016). There is ongoing research into other minimally invasive biomarker detection methods, which could detect tumour cells or different proteins in venous blood, uterine lavage, cervico vaginal fluid and urine (Crosbie et al., 2022).

Prognosis of EC

The Cancer Genome Atlas (TCGA) classification subdivided EC into molecular subgroups. Two other models, TransPORTEC and ProMisE, which are not identical to TCGA classification but analogous have been described (Stelloo et al., 2015), (Talhouk et al., 2017). They use formalin fixed paraffin embedded tumour samples rather than fresh frozen tumour samples, therefore have increased clinical applications. These models, can be applied to endometrial biopsy samples, with high interlab concordance rates and, consistent prognostic values (Njoku et al., 2022b), (Stelloo et al., 2014), (DeLair et al., 2017), (Bosse et al., 2018), (Brett et al., 2021).

The TransPORTEC model, divides EC into four molecular subgroups, P53 mutant, MSI high, POLE-mutant, No specific molecular profile (NSMP) (Njoku et al., 2022b) (Table 7). Both

P53 mutant and NSMP groups are associated with high rate of distant metastases and low 5 yr recurrence free survival when compared to POLE mutant and MSI high groups. Similarly, ProMisE model classifies EC patients into MMR-deficient, POLE mutant, P53 mutant, P53 wild type groups. P53 mutant group have worst prognosis with 3-5 fold increased mortality than P53 wild type group. MMR-deficient group have 1.5-2 fold increased mortality compared to P53 wild type group. Best prognosis is seen in women in POLE mutant group.

These molecular subgroups have prognostic and therapeutic implications, P53 mutant cancers are aggressive, will require aggressive / complete surgical treatment and require adjuvant treatment. For patients with P53 wild type, surgical treatment alone may be sufficient treatment as these tumours have low metastatic potential. Those with POLE mutant tumours have a good prognosis and adjuvant treatment may not be required for these patients. MMR-deficient group may have different immunotherapy options as they are highly immunogenic (Marabelle et al., 2020). For women who are considering fertility sparing treatment options, molecular subgroup classification may help us guide treatment decisions (Njoku et al., 2022b).

Management of EC

Staging investigations

Pre-treatment evaluation includes clinical/gynaecological examination, and blood tests such as full blood count, renal function, liver function profile (Colombo et al., 2016). Magnetic resonance imaging (MRI) scan has been proposed to be the best tool to assess cervical involvement and myometrial invasion (Cade et al., 2010), (Colombo et al., 2013). Computed tomography (CT) scan is indicated when distant metastases need to be excluded (Haldorsen and Salvesen, 2016), (Colombo et al., 2016).

	Low risk	Intermediate risk	High-intermediate risk	High risk	Advanced or metastatic
Molecular classification unknown	Stage IA, endometrioid, low-grade, with negative or focal LVSI	Stage IB, endometrioid, low-grade, with negative or focal LVSI Stage IA, endometrioid high-grade, with negative or focal LVSI Stage IA non-endometrioid (serous, clear cell, undifferentiated carcinoma, carcinosarcoma, or mixed) without myometrial invasion	Stage I endometrioid with substantial LVSI, regardless of grade or depth of invasion Stage IB, endometrioid high-grade, regardless of LVSI Stage II endometrioid	Stage III–IVA endometrioid with no residual disease Stage I–IVA non-endometrioid (serous, clear cell, undifferentiated carcinoma, carcinosarcoma, or mixed) with myometrial invasion and no residual disease	Stage II–IVA with residual disease Stage IVB
Molecular classification known*	Stage I–II *POLE*-mutant no residual disease Stage IA, MMRd or NSMP, endometrioid, low-grade, with negative or focal LVSI	Stage IB, MMRd or NSMP, endometrioid, low-grade, with negative or focal LVSI Stage IA, MMRd or NSMP, endometrioid, high-grade, with negative or focal LVSI Stage IA, p53-abnormal, or non-endometroid (serous, clear cell, undifferentiated carcinoma, carcinosarcoma, or mixed), or any combination thereof, without myometrial invasion	Stage I, MMRd or NSMP, endometrioid with substantial LVSI, regardless of grade or depth of invasion Stage IB, MMRd or NSMP, endometrioid high-grade regardless of LVSI Stage II, MMRd or NSMP, endometrioid	Stage III–IVA, MMRd or NSMP, endometrioid with no residual disease Stage I–IVA, MMRd or NSMP, serous, undifferentiated carcinoma, or carcinosarcoma with myometrial invasion and no residual disease Stage I–IVA, p53-abnormal, with myometrial invasion and no residual disease	Stage III–IVA with residual disease of any molecular type Stage IVB of any molecular type

ESGO=European Society of Gynaecological Oncology. ESP=European Society of Pathology. ESTRO=European Society for Radiotherapy and Oncology. LVSI=lymphovascular space invasion. MMRd=mismatch repair deficient. NSMP=non-specific molecular profile. *POLE*=polymerase epsilon. *Insufficient data are available for stage III–IVA *POLE*-mutated endometrial carcinoma and stage I–IVA MMRd or NSMP clear cell carcinoma with myometrial invasion to enable allocation of these patients to a prognostic risk group in the molecular classification. Prospective registries are recommended for these categories.

Table 2: **ESGO–ESP–ESTRO prognostic risk groups defined with and without molecular classification[53]**

Table 7. ESGO-ESP-ESTRO prognostic risk groups "Reprinted from Lancet. 2022 Apr 9, 399(10333), Crosbie EJ, Kitson SJ, McAlpine JN, Mukhopadhyay A, Powell ME, Singh N., Endometrial cancer, 1412-1428, Copyright (2023), with permission from Elsevier "

Factors associated with high risk of recurrence

Patients with extensive lymphovascular space invasion (LVSI) have worse prognosis (Bosse et al., 2015), (Winer et al., 2015). Cancers with histological subtype grade 3 and deep myometrial invasion >/=50% have higher percentage of lymph node metastases hence are at increased risk of recurrence (Colombo et al., 2016). Patients with lymph node metastases, tumour diameter >2 cms usually have advanced stage disease, therefore increased risk of recurrence (Colombo et al., 2016).

Treatment

Minimal access surgical techniques are currently accepted gold standard surgery, and total hysterectomy, bilateral salpingo-oophorectomy, with or without lymphadenectomy is the operation of choice. The LACE trial compared total laparoscopic hysterectomy and total abdominal hysterectomy for patients with stage 1 EC and showed disease free and overall survival and recurrence in both groups to be comparable (Janda et al., 2017). Surgical treatment is associated with complications such as conversion to open operation, venous thromboembolism, organ injury, hernia, lymphoedema, infection, dehiscence and these risks

are higher in women with comorbidities and obesity. The role of systematic pelvic lymphadenectomy is an issue subject to ongoing debate. Numerous studies including a large Italian study and ASTEC trial, showed that in early stage EC, performing systematic lymphadenectomy is not associated with any additional benefit for overall survival or recurrence free survival (Benedetti Panici et al., 2008), (Kitchener et al., 2009). In 2010, SEPAL study suggested that high risk patients benefit from aggressive surgery (Todo et al., 2010). In FIRES study, sentinel lymph nodes in EC detected using indocyanine green (ICG) dye technique found it to be 97.2 % sensitive with false negative rate of 3% (Rossi et al., 2017).

Adjuvant treatment in EC (Colombo et al., 2016)

In low risk EC, no adjuvant treatment is recommended as risk of recurrence with surgery alone is only 5% in these patients (Sorbe et al., 2009). In patients with intermediate risk EC, external beam radiotherapy (EBRT) reduced the risk of pelvic recurrence threefold (14% to 4%), but with no benefit in overall survival. However, this was associated with risk of gastrointestinal toxicity (Kong et al., 2012). Adjuvant brachytherapy is recommended to decrease vaginal recurrence. For younger patients, no adjuvant therapy is an option. For patients with high risk EC, when surgical node staging is negative, adjuvant brachytherapy to decrease vaginal recurrence or no adjuvant therapy is an option. When surgical nodal staging has not been performed, depending on LVSI, either brachytherapy or external beam radiotherapy (EBRT) to the pelvis is recommended to decrease locoregional recurrence.

Stage 2 EC is managed by surgery and adjuvant radiotherapy (limited field EBRT and brachytherapy boost +/- chemotherapy). Patients diagnosed with Stage 3 EC are often treated with EBRT +/- chemotherapy. For patients with advanced or recurrent disease, surgery can be considered if optimal cytoreduction can be achieved. Hormonal therapy is indicated in patients with advanced or recurrent EC, response rates are variable and dependent on hormone receptor status. Progestogens (medroxyprogesterone, MPA 200 mg or MA 160 mg) are generally recommended. Other hormone preparations such as letrozole, anastrozole and fulvestrant can be used (Ma et al., 2004), (Kokka et al., 2010).

Key current challenges and future needs in management of EC

Approximately 10% patients with EC have lymph node metastasis. The presence of pelvic or paraaortic lymph node metastases is an important prognostic factor as associated with poorer outcomes (Reijnen et al., 2020). Routine lymphadenectomy is associated with significant morbidity, hence introduction of sentinel lymph node identification is a less invasive alternative strategy with reduced morbidity. Performing lymph node excision is challenging in obese or

frail patients. Therefore, use of preop risk stratification models to identify those patients at risk of lymph node metastases, can reduce over- or undertreatment. One such model, ENDORISK Bayesian network model, has been developed and validated and it identifies 55% of patients who are at extremely low risk of lymph node metastasis, in whom lymphadenectomy can be omitted. In future, use of such models together with newer molecular markers or biomarkers, could aid individualising clinical decision making in gynaecological oncology (Reijnen et al., 2020).

Risk stratification in EC based on histology and stage remains problematic. In spite of early diagnosis and treatment, 13-17 % women are prone to recurrence of EC which usually occurs within three years of diagnosis (Hutt et al., 2019). The majority of recurrences occur in the low risk category of EC as they have not received adjuvant treatment. Almost 50% of these women develop distant recurrence which leads to poor prognosis (Brennan et al., 2015). Even though we diagnose majority of EC cases at early stage, it is difficult to identify which of these patients will recur hence focus effective individualised adjuvant therapies towards them and avoid over treatment in patients who will not recur and so important to avoid exposing them to short and long term toxicity of adjuvant therapy (Brooks et al., 2019).

High risk EC patients may require multimodal treatment involving both extensive surgery, radiotherapy and chemotherapy. Comorbidities in EC patients, particularly obesity, makes their management more difficult. They face higher morbidity and mortality. The five year net survival is 92% at stage 1 disease, which is drastically reduced to 15% for those with stage 4 disease (CRUK, 2016-2018b).

Molecular classification of EC will allow risk stratification and tailoring of adjuvant treatment decisions based on the individual patient risk scores (Stubert and Gerber, 2016), (Talhouk et al., 2017).

Currently, there are molecular markers which classify EC into risk groups which aid in prognosis but no single biomarker which can aid in diagnosis. Molecular biomarkers are only available in tissues after invasive procedures. We need to study the endometrial carcinogenesis tumour microenvironment to discover new diagnostic, therapeutic and prognostic markers which will guide and supplement the genomic classifications to decide the best individualised cancer treatment plan for EC patients.

Emergence of biospecimens as crucial elements for translational research

History of biospecimen based research started with most biospecimens being procured from corpses thus from the mortuaries. In the 20th century, with the establishment of histological diagnostics in clinical medicine and advent of methods such as paraffin tissue embedding biospecimens were acquired from living donors or surplus material from pathology departments. The current practice of organised biorepositories storing biosamples taken from live donors was only initiated in the 21st century (Sarin, 2006). Human biospecimens are critical substrates for biomedical research, hence efforts must be made for appropriate collection, storage and regulated use of these biosamples for research (Hojat et al., 2019). Precision and personalised medicine is often based on molecular tests that guide the management of patient. This obviously requires high levels of test-accuracy therefore, optimal handling of the biospecimens is vital to link the data generated from them to the patients and their pathology. Accurate, reproducible biomarkers of sufficient quality that can be utilised in clinical practice, requires high quality biosamples procurement, storage and analysis. Estimations suggest that 60-70% of laboratory errors are due to pre analytical factors, such as mishandling during collection, transport, processing and storage of specimens (Lippi et al., 2006), (Lippi et al., 2011), (Carraro et al., 2012), (Lippi et al., 2019). If pre analytical variables that can affect the biospecimens are uncontrolled or undocumented and as a consequence, specimens with unknown or unacceptable quality are included in a research study, that can obviously make the results unreliable and potentially erroneous (Compton et al., 2019).

The regulations based on Human Tissue Act 2004 are applicable to issues of consent, ethical review, and licensing for human tissue samples. Research with human biological materials should have an independent ethical review carried out by a research ethics committee within an academic institute (e.g., university) or within the health service (e.g., the NHS). According to the UK tissue legislation, consent is an integral part for use of human biological material in research (MRC, 2019). Appropriate and comprehensive information must be provided to the patient donors, supporting them on their voluntary decision to donate biosamples for research without any undue coercion or pressure. In addition to this, patients have a right to withdraw from research anytime without that affecting their standard healthcare. Information provided must include information on the process of sample collection, any associated risks, what the samples will be used for and how the results may impact on the patient care in the future. Written information in the form of patient information sheets and consent forms should be based on guidance produced by Health Research Authority (HRA) and Medical Research Council (MRC, 2019). Sample collection is a team effort, involving patients, clinicians, researchers, and biobank staff. For a biospecimen to contribute to a biomarker discovery, proper

collection and storage is obviously crucial. Thus, it is important to co-ordinate work between all members of the research team, who can access the best practice guidelines in all steps of the biospecimen journey from obtaining the patient's consent for donation of the biospecimen till the analysis. Hence it is a general standard agreed by all peer-reviewed, reputed scientific publishers to report on the robustness of this process during publications, for example, of expression of different genes and downstream targets. It is therefore essentially a responsibility of all cancer researchers to collect samples after eliminating variations by following the best practice guidance available, or in the absence of such guidance, to actively produce them.

Quality of Biospecimen as a cause of bias in translational research

Research for biomarkers can be influenced by variations in results for gene expression due to variation in quality, consistency of standards of biospecimen handling (Moore et al., 2009), (Moore et al., 2011a). National Cancer Institute (NCI) has acknowledged that lack of uniformity in quality standards in handling of biospecimens is a roadblock for cancer research. As a result, efforts for standardising the data and the quality of specimens are recognised as an urgent need for future research (Morente et al., 2007), (Yuille et al., 2008), (Vaught and Lockhart, 2012).

1. Factors which influence the usefulness of a biospecimens

Ransohoff and Gourlay (2010) reported that biases introduced prior to the analytical phase of the biospecimens may or may not be recognised. They also recognised that bias can be introduced in the laboratory during sample processing and may not be related to pathogenesis of the disease (Ransohoff and Gourlay, 2010). Their work concluded that biggest flaw in the biomarker discovery is the disparity of specimen groups compared. Lippi et al also found that 60-70% of all preanalytical errors were due to variations in collection and processing of samples (Lippi et al., 2016). Biospecimen handling, may have an obvious effect on the integrity of biospecimens and on downstream analyses (Ellervik and Vaught, 2015) and invalid proteomic analyses and subsequent HER2 clinical assay without controlling the preanalytical variables, is an example of how the use of poor quality biospecimen can be detrimental (Portier et al., 2013).

Life cycle of Biospecimens

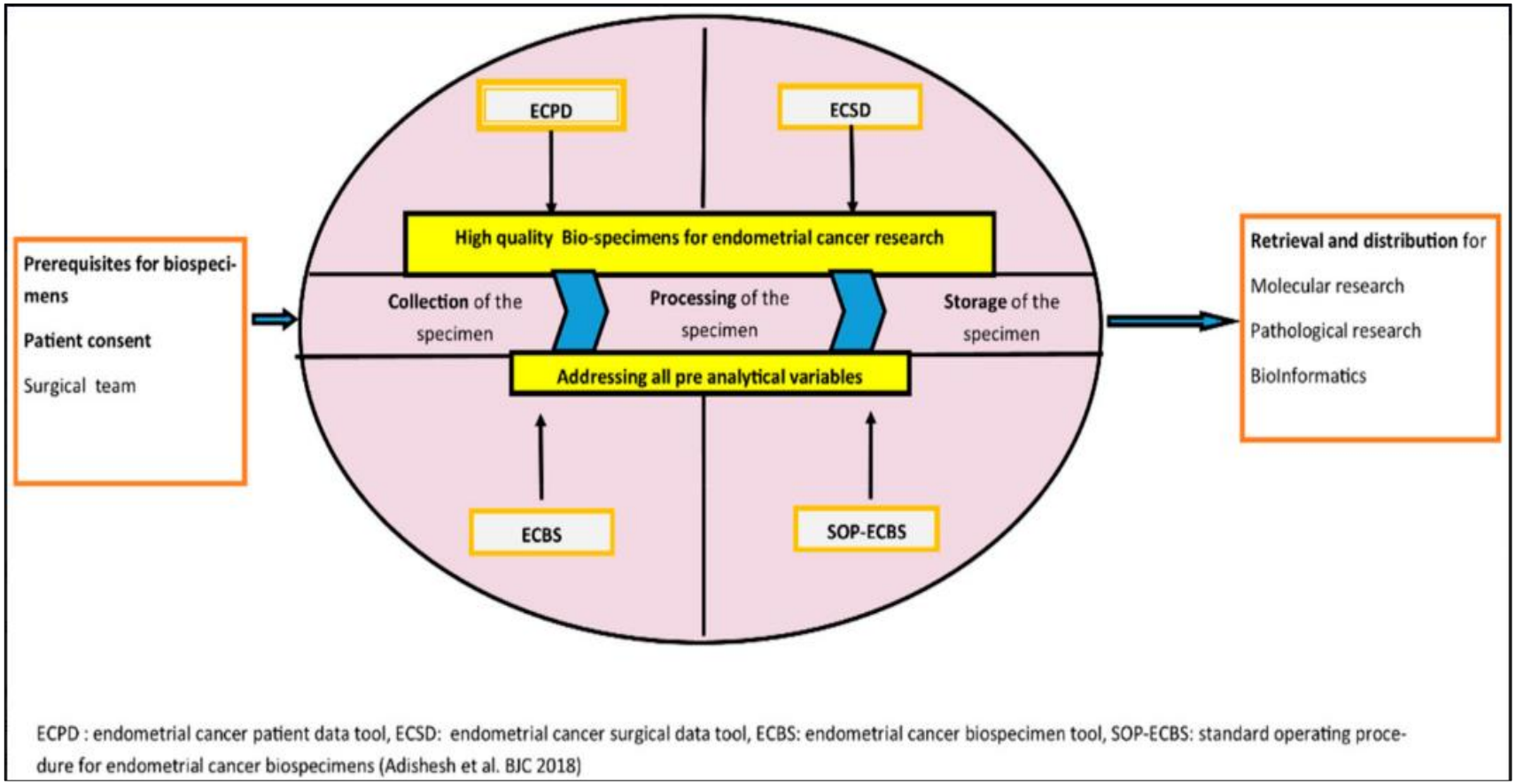


Figure 5.
Diagram of life cycle of biospecimens reproduced from article 'Adishesh, M.; Hapangama, D.K. Enriching Personalized Endometrial Cancer Research with the Harmonization of Biobanking Standards. *Cancers* **2019**, *11*, 1734.'

2. Biobanking standards used for collection, processing, and storage

Biobanks have an important role to play in both formulating and adhering to stringent, explicit standards in the sample collection, processing, and handling of samples. Unfortunately, between biobanks, there are wide variations in biobanking practices, such as the type of samples collected, sample quality, demographic data collected, ethical approval process, consenting patients, sample processing and storage techniques. These can be ameliorated by implementing standard operating procedures that can be regulated and standardised by multidisciplinary teams, including scientists and by conducting research themselves to accurately predict the integrity and quality of the biospecimens (Betsou et al., 2009), (Betsou et al., 2010), (Moore, 2012).

3. Factors affecting the analysis and results

The quality of a sample on the results obtained from it may depend on the type of molecule analysed, analytical method, the specificity, sensitivity, robustness of the method and controlling the pre-analytical variables. Therefore, the researchers need to have a good knowledge of these issues pertinent to their research. Controlling pre-analytical variability is, however, challenging and complex. However, as the quality of data obtained from the sample is directly dependent on variables, it is important to consider using the most appropriate samples and the most robust biomolecule analytical method to obtain useful data, to avoid potentially wasting a lot of time and resources. Pre analytical characterisation, employing multiple techniques to confirm the final data obtained, examining different specimens from the same patient, use of large number of samples from different sources are ways to reduce the biases in the studies. Application of quality management strategies in biobanks and standardising the best practice guidance followed will minimise variations affecting the integrity of the biosamples.

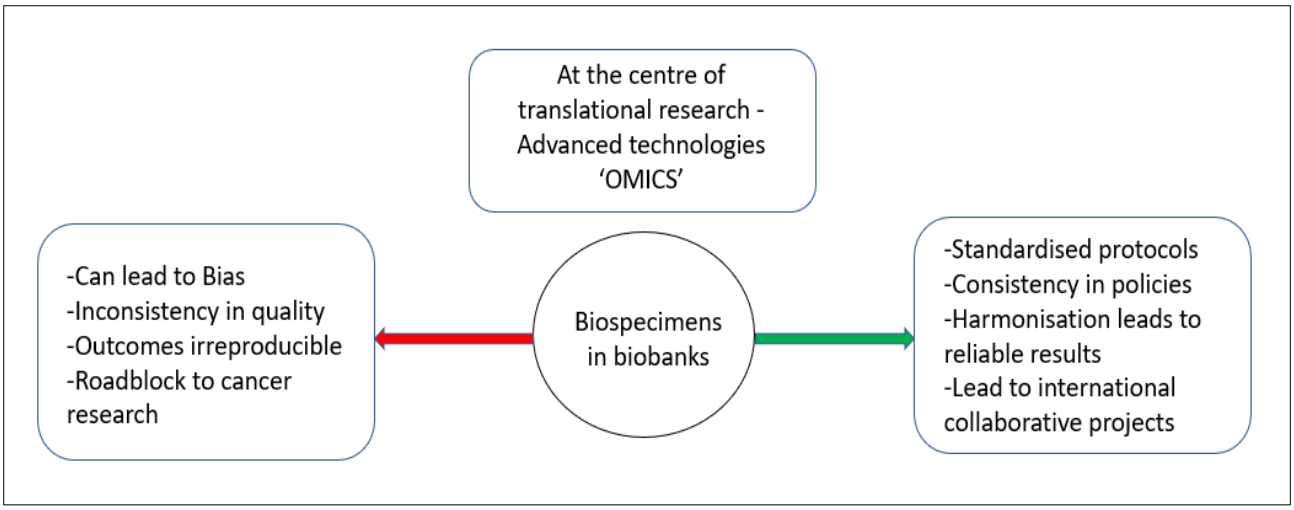


Figure 6. Diagram of implications of the quality of biospecimens on cancer research. The red arrow depicts the negative outcomes of low quality biospecimens. The green arrow depicts the positive outcomes of high quality biospecimens.

International efforts to minimise the pre analytical variables

Some national and international efforts have been drafted to reduce the pre analytical variables, one of them, is the Standard PRE analytical Code (SPREC), which is a seven item code developed by International Society for Biological and Environmental Repositories Biospecimen Science Working Group. These include information about type, collection, handling, storage of the biospecimens, which can be integrated into the current quality management systems used by biobanks (Lehmann et al., 2012). Each sample can be assigned a code, based on its pre analytical characterisation, which further helps to standardise the quality of samples to be used. On the other hand, NCI had developed standard operating procedures (SOPs) for collection, processing, handling, storage of the biospecimens for biomarker discovery and validation (NCI, 2016). Similar guidelines also are laid out by the Biospecimen Reporting for Improved Study Quality (BRISQ), which aims to register all human samples data including the integrity, quality, molecular composition (Moore et al., 2011b). To reduce the inequalities and to standardise the procedures for all biobanks in their network, Spanish biobank Network Biospecimen Working Group conducted an extensive literature review of analytical techniques for samples from colon, breast, kidney, lung, ovary, brain tissues and drafted a proposal for quality assessment of tissues based on type of preservation method and biomolecule on interest (Esteva-Socias et al., 2019). Similarly, The College of

American Pathologists has published guidelines for breast cancer biobanks to standardise and optimise tissue collection, processing, handling, and testing (Hammond et al., 2010).

Which analytical techniques are affected by the variability in the biospecimens?

Tissues with same pathological condition can have different morphological and molecular changes in them and this can have a huge impact of the utility of the tissue samples collected for research (Grizzle et al., 2015). These in addition to the variables introduced due to handling of the biospecimens can adversely affect the downstream applications (Ellervik and Vaught, 2015).

Pre analytical variables may be extremely specific to the type of tissue collected, analytical platform used, gene, transcript, protein affected (Agrawal et al., 2018). The variables also are dependent on the molecules/pathways which are investigated, for example, different proteins may get affected by different storage conditions / processing to a different degree (Agrawal et al., 2018). Hence it is important to identify and validate any biomarkers from different tissues. A simple example of such a situation is the assessment of oestrogen receptor (ER) and progesterone receptor (PR) expression status in breast tumours, which is determined by immunohistochemistry (IHC), and this guides clinicians to provide patients the appropriate treatments. Pathologists in US estimated about 20% of IHC analyses worldwide may be inaccurate and this was partly due to the preanalytical variables (Hammond et al., 2010). Alarmingly, such inaccuracies will result not only in inappropriate treatment but also inaccurate assessment of efficacy of treatment. The pre analytical variables are not just limited to one method, but they can also affect other novel techniques such as next generation sequencing (NGS), where for example, the number of nucleotide variants identified can vary due to delay in fixation, pH of solution etc (Kim et al., 2017). Even the assessment of MSI signals which hold the key in mismatch repair deficient tumours can be affected by tissue preservation methods (Moelans et al., 2011). Agarwal et al have reviewed the factors associated with Formalin fixed paraffin embedded (FFPE) processing and their effect on molecular and proteomic analyses, wherein they also noted that the acceptable duration of cold ischaemia will depend on protein or phosphoprotein of interest and on the characteristics of the biospecimen used (for example type of tissue or tumour, size and method of collection) (Agrawal et al., 2018). Detection of *KRAS* mutation was influenced by the method of tissue acquisition, for example, fine needle aspiration (FNA) missed many whilst the core needle biopsy was more

accurate, as the tumour content in FNA maybe insufficient (Schneider et al., 2015). Success rates for polymerase chain reaction (PCR) were higher in specimens of 3-10 mm size when compared with smaller samples (Neubauer et al., 1992). Preanalytical variables related to clinical medicine may also lead to problems using biospecimens to detect biomarkers, where they can also be related to the preservation methods. For example, the choice of anticoagulant used in collection bottles can lead to increased levels of different markers (Varo et al., 2006), (Azimi-Nezhad et al., 2012), thus, the collection methods must be optimised and validated for a particular marker of interest. Storage of samples and freeze thaw cycling of tissues can vary markers like vascular endothelial growth factor (VEGF), interleukins, tumour necrosis factor alpha (TNFA) as they are sensitive to the thawing process (Lee et al., 2015a), (Agrawal et al., 2018). Duration of storage may impact the stability of the RNA, whereas the plasma stored can be stable for up to 19 months, and longer storage than that can lead to increase in cholesterol, and other chemical changes in the plasma (Pinto et al., 2014), (Glinge et al., 2017), (Pinto et al., 2014).

Types of Pre analytical variables

The preanalytical factors of importance can occur pre or post acquisition of samples and out of these, it is possibly easier and more feasible to control those pertaining to the biospecimen after its acquisition whilst accurate recording of the pre-acquisition variables would be more practical.

Pre analytical variables can be divided into groups based on the cause (Otali et al., 2019):

1) *Inappropriate requests*: When the clinical care and research both need the same tissue, clinical care takes priority especially in small tumours (Otali et al., 2019) . There needs to be good communication between the researcher and the clinician or pathologist collecting the sample, and the biobank. Some requests from the researcher may be too vague, uninformative, or difficult to understand and with limited availability of tissues will then result in improper utilisation of valuable specimens. Requests should match the experimental design and appropriate biospecimens should be collected or effort should be made to obtain them after discussing with the whole team.
2) *Biospecimen sources*: Tissues obtained from living donors and from autopsies or deceased donors, can be used for research and have been used in RNA based studies, reverse transcription polymerase chain reaction (RT-PCR) analysis (van der Linden et al., 2014). Patients who experience shock, vascular ischaemia, also have ischaemic or autolytic tissues hence such tissue is usually not suitable for molecular studies. The

available information from a deceased donor maybe limited and the time frame from death, causes preceding death and tissue collection may vary widely. Considering these variables, tissues from living patient donors is preferred for gene expression studies.

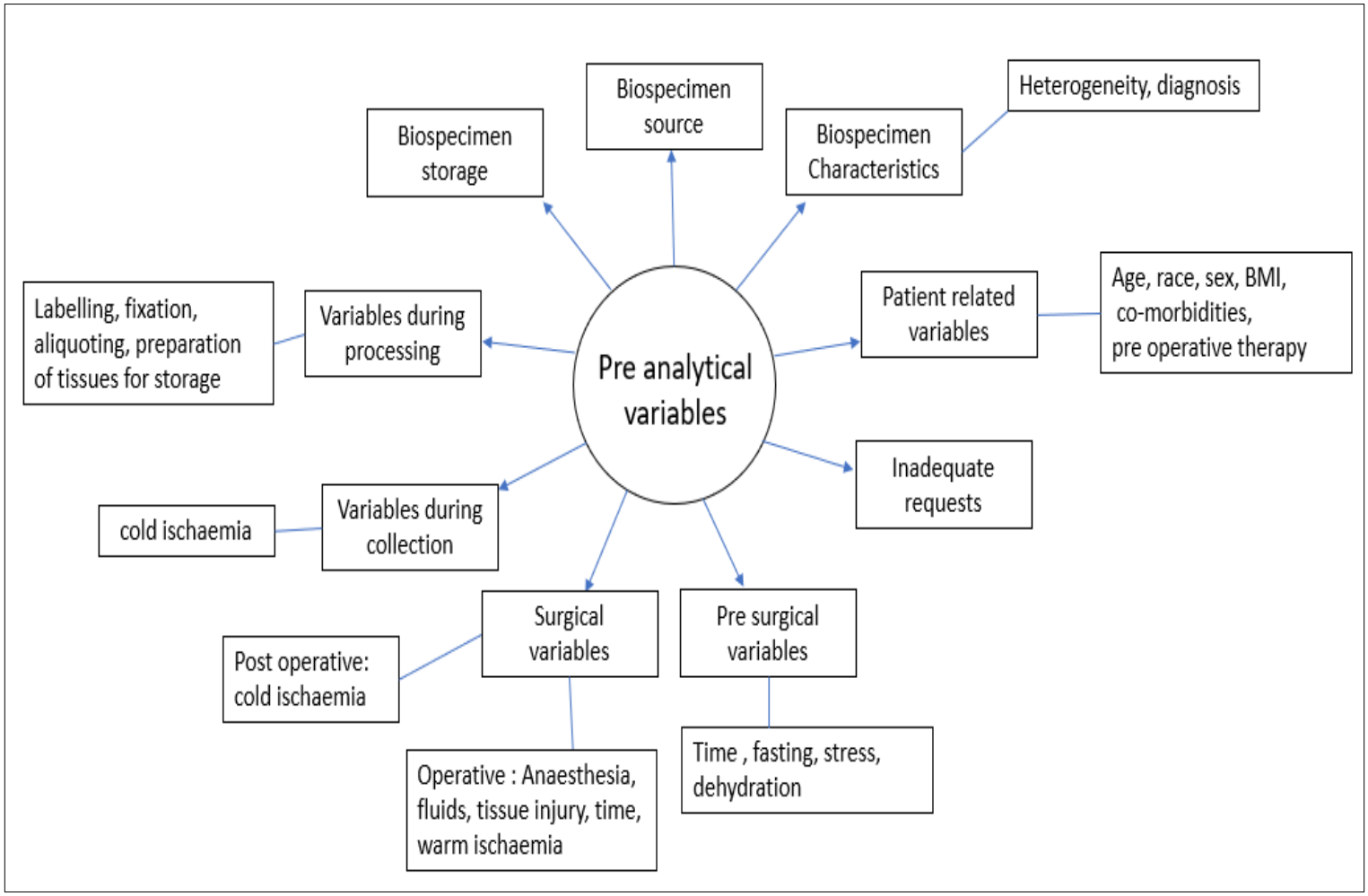
3) *Pathological characteristics of the Biospecimen*: Morphology and molecular characteristics of a disease vary in different patients. Collected tissues may not represent the perceived pathology for which they were collected. Biospecimens collected from cancer patients presumed as cancer may contain only fibrotic or benign lesions and may even contain adjacent normal areas of tissue to the cancer, hence quality control (QC) by an experienced pathologist will be required to evaluate the collected tissue (Grizzle et al., 2015), (Grizzle et al., 2018). Such QC may identify specific biospecimens whose molecular features may vary from that of the known malignant cells, and this may also identify changes associated with neoadjuvant therapy.
4) *Patient related factors*: These factors are not controllable. Comorbidities such as diabetes, cardiovascular, infections and the treatment used for those have an influence on the usefulness of biospecimens. Accurate documentation of these will allow accounting for these in studies.
5) *Pre surgical variables*: During and after the diagnosis of a condition, exposure to different treatments such as chemotherapy, hormonal therapy or radiotherapy may affect the tissues prior to surgery. Exact effects of tissues exposed to such treatments is unknown, but that may cause a lot of cellular or molecular changes therefore need to be considered (Otali et al., 2019).
6) *Surgical variables*: Fasting prior to surgery may cause tissues to be dehydrated and exposed to stress. Exposure to anaesthetic agents, medications, intravenous fluids may have an influence on the stability of biomarkers in the tissues. Interruption of vascular supply to tissues, leads to warm ischaemia, which cannot easily be controlled. Intraoperative use of cautery may cause wider thermal molecular damage to the tissues than a cold knife. After the specimen is removed from the body period of cold ischaemia begins, this can to a certain extent be controlled. Cold and warm ischaemia and their effect on tissues are further discussed in detail later in this chapter.

Some studies have observed the effect of general anaesthetic drugs on blood, serum biomarkers and some anaesthetic drugs influence the tumour biology and thus alter the biomarkers (Braz et al., 2012), (Lennon et al., 2012), (Cruz et al., 2017). Administration

of propofol can affect the cellular viability and apoptosis of circulating CD4 lymphocytes (Braz et al., 2012) . Similarly, opioid therapy can activate the M opioid receptor expression in cancer cells. This may lead to Akt, mTor activation, hence influence the important relevant biomarkers (Lennon et al., 2012). Other anaesthetic drugs such as ketamine, benzodiazepine can also affect the immunosuppression and have immune modulatory effect (Cruz et al., 2017).

7) *Biospecimen collection variables*: Variables at collection mainly depend on resources as to when and where the personnel are able to collect the samples. Variations in specimen collection and storage temperature are detrimental to analysis, thus, it is recommended that biospecimens be maintained at a low temp 4° C during collection, transport, and processing to reduce the effect of cold ischaemia (Otali et al., 2019). Use of optimal temperature is critical for a collected sample as it prevents degradation of the useful content of the sample and preserves the integrity of the sample (Otali et al., 2019). Cryopreservation collection affects the viability and metabolic function of the cells on thawing (Mager et al., 2007). The success of truthful downstream analysis depends on cryopreservation and thawing procedure (Morente et al., 2006).
8) *Processing variables*: Sample processing includes aliquoting tissues into smaller biospecimens, labelling them, entering into databases, and storage. Samples for QC should be obtained during processing and simultaneous confirmation of the pathological diagnosis increases efficiency of the system. Factors such as type of fixative, time in fixative, centrifugation temperature, storage temperature, if dissimilar can affect the tissue molecular morphology (Atkins et al., 2004), (Ergin et al., 2010). Formalin fixation and paraffin embedding of the tissue is one of the commonest and inexpensive method of storing tissues, but these processes have to be optimised and standardised for the tissue to preserve the morphology and molecular integrity. It is recommended that using 10% neutral buffered formalin (NBF) is used to optimally preserve the tissue, but different molecules may need different buffers and fixatives (Atkins et al., 2004). The Paxgene tissue system is another formalin free system, which allows to preserve histology, proteins, antigenicity, and integrity of nucleic acids (Ergin et al., 2010). Moreover, prolonged fixation in Paxgene does not negatively affect the expression of analytes.

Figure 7. Diagram of types of pre analytical variables with examples



9) *Storage of biospecimens*: Storage of specimens depends on the biospecimen type whether it is solid or liquid. Most frozen biospecimens are stored in freezers at -80°C to -130°C (Otali et al., 2019). Long term storage at -20°C results in changes to the molecular composition of tissues after 5-8 months, whereas liquid samples may develop changes in -80°C after 1-2 years (Atherton et al., 2016) . When tissues are stored more than 10 years at -80°C whether in freezer or liquid nitrogen, RNA integrity number (RIN) was low in both methods (Auer et al., 2014), therefore will affect gene expression studies.

   Long term storage of FFPE blocks may serve to preserve tissue morphology but may not preserve complex molecular analytes. Several studies have reported loss of protein expression in tissue sections stored for an extended period of time due to oxidation, denaturation, further degradative modification of tissue biomarkers (Blind et al., 2008). Therefore, the duration of tissue in storage should be considered when using archived samples.

Collection of biospecimens vital for research

As biospecimens are vital for biomarker discovery, attention must be paid to tissue manipulation, both pre and post collection to preserve gene expression and downstream molecular targets. Immediately after the blood supply to an organ is cut off, the tissues become ischaemic and cells are then under stress, which may affect the molecular composition of these cells and thus the downstream analysis. Considering this, I aimed to review different methods that can be used to harvest human endometrial tissue for research.

Methods of Endometrial tissue collection for research

Many methods are described in the literature that can be used to collect human endometrial tissue for research. The main methods include the use of metal or suction (e.g., pipelle sampler) curettage, endometrial cytology, uterine fluid aspirate from an intact uterus whilst a full thickness endometrial wedge biopsy can be obtained from a hysterectomy specimen (Figure 8).

Biopsy by curettage

This is the most widely used, gold standard method for collecting an endometrial biopsy and was first described by Recaimer in 1843. The disadvantages of a blind curettage are that there is a 50% possibility of not successfully obtaining an endometrial sample suitable for histological diagnosis (Stock and Kanbour, 1975), (Al-Talib et al., 2010). As this is an invasive

procedure, associated with the need for cervical dilatation, hence causing significant discomfort, it is not suitable as an outpatient procedure, therefore will need an anaesthetic (Figure 8). This makes it to be an expensive and risky procedure, with complications such as perforation, and cervical trauma (Clark et al., 2002), (Clark and Gupta, 2002).

Figure 8.

Photograph of sharp curette(A), blunt curette (B) and pipelle endometrial sampler (C) used to obtain endometrial biopsies.

Courtesy: Gynaecology theatre suite, Royal Preston Hospital (RPH), Lancashire teaching hospitals NHS Trust, Preston.

Pipelle Endometrial Biopsy

Pipelle biopsy is a suction aspiration method for endometrial sampling developed more recently to be a cheap, cost effective, less invasive procedure that can be used in outpatient setting (Figure 8) (Brand A, 2000), (Vigod and Stewart, 2002), (Abdelazim et al., 2013). The thin plastic tube is inserted into the uterine cavity and the plunger is withdrawn to generate vacuum in the tube which pulls the endometrial tissue into the tube. The plastic tube is removed together with the plunger and biopsy is obtained. Efficacy of collecting tissue is similar to dilatation & curettage, but there are still concerns of adequacy particularly of not able to sample adequate tissue in post-menopausal women and also sampling focal intrauterine lesions. Previous studies have reported that 15-22% of pipelle biopsies will have insufficient tissue to make a histological diagnosis (Brand A, 2000), (Clark et al., 2002).

Endometrial cytology

Use of traditional cervical smear technique was not an efficient technique in identifying endometrial cells to diagnose endometrial pathology. This was due to excess blood, cellular inflammatory infiltrate, and scanty cellularity. Adoption of liquid based cytology has helped in making not only cervical smears easier, but also this method is better at identifying endometrial cellular abnormalities (Wang et al., 2019), (Norimatsu et al., 2009). Endometrial samples can be obtained by intrauterine brushes which are taken from endometrial cavity without anaesthesia and cervical dilatation (Norimatsu et al., 2020), however it is not a very commonly used method in practice in the UK. Cervical pap smears can detect abnormal cells from the endometrium. Around 45% of EC were detected on pap smears and sensitivity is high in patients with non endometrioid ECs and advanced stage (Frias-Gomez et al., 2020). Endometrial liquid based cytology may play an essential role in EC screening (Fambrini et al., 2014). Wang et al have evaluated genetic analyses of DNA recovered by different methods of endometrial cytological sampling, using pap brush versus intrauterine sampling with Tao brush. They detected pap brush detected 81% EC, and Tao brush detected 93% EC samples accurately (Wang et al., 2018).

Wedge biopsy

This technique collects a wedge of tissue from lumen to the myometrial layer, which includes superficial and basal layers of the endometrium as well as the sub-endometrial myometrium from the detached post-hysterectomy uterus (as shown in Figure 9). In cancer research however, this should only be performed by the pathologists, to ensure the clinical diagnosis is not impaired. This may cause a delay in obtaining and processing of the sample and therefore in most units, only paraffin embedded tissue is available for research work.

Uterine fluid aspirates

The technique of uterine lavage was first described in 1957. Aspirating fluid from the uterine cavity and using that to detect RNA or molecular biomarkers is another less invasive method. Initially saline is introduced into uterine cavity then returned by aspiration. Cells are obtained by centrifugation, smeared on slides, then evaluated by cytopathologist (Nair et al., 2016). Some researchers have reported uterine aspirates to be a highly sensitive and specific biosample for identification of EC (de Kroon et al., 2003), (Huang et al., 2007), (Colas et al., 2011), and to represent molecular alterations characterising the primary tumours (Colas et al., 2011), (Muinelo-Romay et al., 2018). However, this method may be associated with a theoretical risk

of pushing the cancer cells into peritoneal cavity from the fimbrial end, hence needs to be analytically and clinically validated in large clinical trials (Muinelo-Romay et al., 2018).

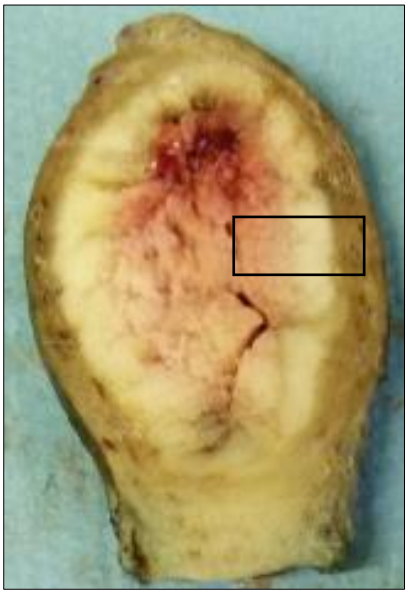


Figure 9. Photograph of longitudinal section of uterus with EC. The rectangle shaped outline shows the pictorial representation of wedge biopsy (endometrium and myometrium).

Courtesy: Pathology department, RPH, Lancashire university teaching hospitals NHS trust, Preston.

Ischaemia – an important pre-analytic variable

Ischaemia is defined as inadequate blood supply to tissues, causing a shortage of oxygen to the tissues particularly due to obstructed blood flow.

Warm Ischaemia

With warm ischaemia, biospecimen is still within the body at body temperature, but with a compromised blood supply. This occurs after clamping the arteries during surgery and can affect the results of different analytes of the tissue that is subsequently collected. During surgery, even after the blood supply to the organ is interrupted, the organ remains at the body temperature, the time duration, from the cessation of the blood supply until the organ is removed from the body, is called warm ischaemia time. During the warm ischaemia, the reduced blood flow to the tissues causes stress to the cells which results in molecular changes, and ischaemia / stress related genes and their related proteins may be upregulated (Schlomm et al., 2008).

The extent of warm ischaemia depends on the procedure and the organ, the surgical approach for example laparoscopic surgeries may double or triple the warm ischaemia time depending on the experience of the surgeon (Ricciardelli et al., 2010). Ischaemia induced metabolic

responses such as posttranslational modification, occur in early stages of loss of blood supply, hypoxia and stress induce degradation of the tissues especially tumour tissue undergoes higher variability and reaction to stress compared to normal tissues (Ma et al., 2012), (David et al., 2014). Studies comparing biopsies before and after clamping major blood vessel have shown many genes, proteins to be altered, amongst them most vulnerable and reactive are phosphorylated proteins and phosphorylation status of the key proteins is significantly altered within a short ischaemic time in both normal and cancer samples (David et al., 2014), (Wolf et al., 2014). Warm ischaemia is dependent on different patient factors such as anatomy, comorbidities, past surgical history, surgical procedure, hence it is hard to minimise or control warm ischaemic time and documentation of this is helpful in understanding how this would have altered the molecular phenotype of the samples.

Cold ischaemia

Cold ischaemia is defined as time taken from removal of tissue from a patient until the tissue is stabilised by chemical fixation or snap freezing. It is perceived that if the cold ischaemia time is longer there is significant molecular degradation of the tissues which results in tissues not reflecting the original physiological state before surgery. Rapid collection and stabilisation of tissues is more expensive. Researchers need to take into consideration that the biases resulting from the variation in the pre analytical variable such as cold ischaemia time are included when evaluating the results of experiments (Atherton et al., 2016).

In some studies, it has been reported that 15 mins after surgery between 1-15% detectable genes and proteins undergo moderate to significant changes from baseline and 30 mins after surgery up to 20% of detectable molecules show changes (Spruessel et al., 2004b), (Juhl, 2010), (Musella et al., 2013), (Grizzle et al., 2016). All factors affecting warm ischaemia also can influence cold ischaemia as well. The cold ischaemia times can be variable for different proteins and markers hence some labs keep biospecimens at 4°C until processing as it has been shown that chilling of samples mitigates the degeneration and will result in better preservative of tissue, stability of the genes and RNA integrity (Apple et al., 2011), (Yildiz-Aktas et al., 2012), (Gündisch et al., 2015).

Due to all above mentioned pre-analytic variations that can affect the final data derived from a biospecimen, strict guidelines should be followed with biospecimen procurement and handling and harmonisation of such guidelines is also imperative for their widespread and effective use.

EC Research Unmet Needs: Improving the utility of research to facilitate rapid translation of research through harmonisation of biobanking standards

High Dimensional Biology: 'Omics' Technologies

Integration of different techniques for detection of genes, messenger RNA (mRNA), proteins and metabolites in a biospecimen in a non-targeted and non-biased manner forms the 'Omics' technologies. These newer techniques are increasingly being used to understand the normal physiological as well as the pathological processes in the humans and these in turn are expected to guide novel biomarker discovery research.

Genomics is one of the 'Omics' technologies and deals with systematic study of an organism's genome. Gene Expression is the process by which the gene is used to synthesise a particular protein which it encodes. This process is dynamic with a wide variability. High quality biospecimens are essential for any 'Omics' studies. To validate and reduce the confounding factors larger sample sizes are a must. Therefore, to have greatest predictive power in a study, it is imperative to have large number of sample sizes, which is only possible by collaborating with different researchers worldwide.

The molecular tailored approach for precision medicine will help increase effectiveness, decrease the toxicity, decrease the cost of care and increase cost effectiveness (Jameson and Longo, 2015). Advances in translational molecular testing in the past few years has decreased costs of these tests significantly and expedited the uptake in cancer treatments which guide clinical decision making and use specific therapies (Compton et al., 2019).

Translational research and role of biobanks

Translational research is defined as "The process of applying ideas, insights and discoveries generated through basic scientific inquiry to the treatment or prevention of human disease" by National Institute of Health, which is simplified as 'Bench to bedside' research (Fang and Casadevall, 2010). Translational research aspires to hasten the process of evolution from the lab to clinical application for the patients who will benefit from the basic science findings. Having a sustainable supply of well-documented and high quality biospecimens is crucial for translational research, therefore, a biobank is a crucial platform where all the biospecimens can be collected and stored (Hewitt and Hainaut, 2011). Disease specific biobanks have a huge influence on detection of new bio-markers, therapeutic targets, in general for research on treatment of particular diseases or cancers (Knoppers et al., 2012). Biobanks are the foundation

for research, are treasured educational sources, bring together all the stakeholders in research and lead in authentication of standards used in pathology. They also play an imperative role in motivating major developments to influence our perception about role of epidemiology, pathogenesis, genetics in EC as novel technological platforms in translational medicine like genomics, proteomics, epigenomics, and metabolomics emerge. These human tissues collected are an indispensable resource which generate molecular data from which targets for diagnosis, treatment and prevention are derived NCI best practice.

Diversity of Biobanks

There are wide dissimilarities in the several biobanking practices such as type of samples collected, sample quality, demographic data collected, ethics, consent, processing techniques and storage workflows, which can generate obstacles for researchers and lead to restrictions in collaborative research projects. The SOPs used in different biobanks vary depending on local resources, hence the quality of sample collection, processing and storage varies widely (NCI, 2011), (NCI, 2016). Genomics studies such as expression microarrays and real time polymerase chain reaction depend on the sample stability and integrity hence even minor changes in collection, processing and storage can significantly modify the scientific results. Implementation of quality management systems in biobanks and standardising the best practice can lead to comparable results, and further collaborative research.

Importance of patient derived biospecimens in cancer research

Individualised medicine in addition to translational research aim to use both clinical and molecular data from patients to develop and validate treatment options with better specificity, to reduce the side effects, to focus on determining disease susceptibility to develop preventative strategies. Human bio-specimens form an essential link between molecular signatures in an individual's specific cancer and diagnostic plus prognostic markers; therefore, treatments can be devised based on the information obtained from the bio-specimens (Moore et al., 2009). In recent years, the 'Omics' revolution has been propelling cancer research forward, and it has given us the prospect of investigating the alternative ways to study tumour biology, heterogeneity, and tumour cell progression (Gambara et al., 2018). Epigenomic distortions together with genetic alterations have a significant impact on cancer initiation and progression (Sharma et al., 2010), (Wild et al., 2013). The fundamental changes in cellular function that are vital in tumorigenesis are influenced by both genetics and environmental factors which can alter the individual's risk of developing cancer. Therefore, advancing our knowledge in these

areas in particular outlines the basis for cancer prevention through directed therapies (Verma et al., 2014). For example, in epigenetic research the commonest types of biospecimens analysed are blood and tissue from the tumours. Biospecimens obtained from patients, cell lines derived from the tumours which retain the phenotype and functional characteristics of the parent tumour are advantageous in a wide variety of research studies since their response to chemotherapeutic agents for example will be more clinically relevant and the data produced will be more representative of the tumour sampled therefore the clinical applicability will be high. This evidently helps to derive direct and swift clinically translatable conclusions, and for customising the therapeutic options for individualist treatment with the maximum effectiveness whilst reducing side effects (Mill and Heijmans, 2013), (Reuben et al., 2015). However, discrepancy in collection, processing and storage of the biospecimens can genuinely hinder this seemingly straightforward process, leading to uncertain molecular nature of the biospecimens and irreproducible results, that impedes development of effective diagnostic and therapeutic translational strategies (Moore et al., 2009).

The internal and external validity of the produced data depends on the use of rigorous standards in collection process of the biospecimens and patient characteristics significant to the cancer type. Deviations in collecting, processing, storing bio-specimens and the associated phenotypic and demographic data make it exceedingly difficult to generalise or to combine data from different studies (Ransohoff and Gourlay, 2010), (Tworoger and Hankinson, 2006). This lack of quality standards and standardisation is roadblock to cancer research and is recognized by the National Cancer Institute. The anomalies and differences in specimens and data collection introduce unalterable bias which are well recognized by many agencies and attempts are being made to overcome this by several international organizations and agencies (Morente et al., 2007), (Yuille et al., 2008), (Vaught and Lockhart, 2012).

Role of harmonisation of biobanking and existing initiatives for harmonisation

'Harmonisation is defined as achieving or improving comparability of similar measures collected by separate databases for different individuals' (NIHR). Harmonisation differs from standardisation as it does not impose a single way, hence is a more flexible way of ensuring effective exchange of information and samples (Fortier et al., 2011) .

The European Prospective Investigation into Cancer (EPIC) study coordinated from the International Agency for Research on Cancer (IARC), and the Telethon Network of Genetic

Biobanks in Italy, are international projects which have ventured into standardising the standard operating procedures, consent, transfer policies and procedures (IARC), (Filocamo et al., 2013) (Table 8). The Wellcome Trust Case Control Consortium identified issues with inconsistent sample quality such as different data from different sources (Wellcome Trust Case Control Consortium, 2007). The Cancer Genome Atlas Project also has recognised this as a serious issue and further on insisted on the high quality and consistency with biospecimens (Waltz, 2007).

The European strategy forum on research infrastructures recognised that major synergy, gain of statistical power and economy of scale is by interlinking, standardising, harmonising or just cross referencing with a large variety of well qualified, existing, up-to-date national resources. This foresaw development of one of the largest health research infrastructure called Biobanking and Biomolecular resources Research Infrastructure (BBMRI) (Yuille et al., 2008).

International biobanking platforms like 'The Marble Arch International Working Group on Biobanking for Biomedical Research' (Meir et al., 2011) and 'ISBER – International society for Biological and Environmental Repositories' are working on standardization of global biobanking (Campbell et al., 2018).

Between 2004 -2007, an investigation launched by NCI to understand the state of resources, quality of biospecimens used in cancer research, lead to detailed best practice guidance for biobanks. The NCI Best Practices has established guiding principles that define gold standard biospecimen resource practices, promotes biospecimen and data quality maintenance, and details the ethical and legal considerations (NIH/NCI, 2006). Though, the adaptation of these guidelines is voluntary, they support optimization of the resources available for cancer research.

These seemingly huge efforts have made many biobanks to individually be well-organised and to have accessible bio-sample repositories, this is not a uniform process. The bank-specific variations that exists are still too large to source samples from all biobanks to a single study. Hence, harmonisation is a necessity to utilise the available resources to their maximum potential.

Existing Guidance/Protocols

Currently there are some established best practice documents from some national institutes such as National Institute of Health/National Cancer Institute's Biorepositories and

Biospecimen Research Branch Best Practices for Biospecimen Resources (BBRB)' (NCI, 2016), 'International Society of Biological and Environmental Repositories Best Practices for Repositories: Collection, Retrieval, and Distribution of Biological Materials for Research' (Campbell et al., 2018), 'World Health Organisation International Agency for Research on Cancer Common Minimum Standards and Protocols for Biological Resource Centers Dedicated to Cancer Research' (Mendy et al., 2017). These policies provide the framework for standardisation to all researchers.

When these guidelines are effectively adapted and applied, they assist to improve the quality of research. Therefore, EC specific standardisation of the collection of biospecimens with distinctive and relevant accompanying clinical data sets is a fundamental unmet need in improving future EC research. This will facilitate future large-scale internationally collaborative research into EC, which could lead to improved biomarker and target treatment discovery. Similar harmonization projects have already been successfully implemented for other gynaecological conditions such as endometriosis - World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project (WERF EPHect), and Ovarian Cancer Research Program (OVCARE) (Wiegand et al., 2010), (Heravi-Moussavi et al., 2012), (Vitonis et al., 2014), (Rahmioglu et al., 2014), (Fassbender et al., 2014).

Key stakeholders in cancer research and need for different tools

Involvement of all key stakeholders is paramount as they are the consumers of the consensus therefore it enhances the ownership and engagement. Progress towards personalised medicine is influential in involving patients in shared decision making and patient empowerment. The advantage of involving patients, who are the most important of all stakeholders has been more recognised recently (Chalmers et al., 2014). This helps us in many ways other than acquiring more accurate and reliable personal, past medical and past surgical data for research. Some examples of information which can be exclusively obtained from patients are current weight, lifestyle data, family history. Personal past medical, surgical history and family history are more likely to be completed accurately by patients themselves rather than the health care professionals.

Healthcare professionals' participation into basic science research is vital. They not only describe, discuss and engage patients for research but also help in addressing the process of practically applying discoveries generated during research. Only clinicians can assist in contributing the surgical information such as details about operation, findings, staging and

follow up data (NCRI, 2012). The involvement of clinicians can bridge the gap between basic and applied research (Kahn et al., 2011).

Pathologists provide the expert advice to regulate the quality of research and are key staff who help us supply the biospecimens required for research. Pathologists are important collaborators, and their participation and contribution will stimulate high quality translational research. As modern tools for research such as expression arrays, new generation sequencing technological advances develop we need pathology contribution to standardise the methodology for every specimen we can use for research hence pathologists can influence developing a standard operating procedure for biospecimen collection, processing and storage.

Researchers and biobank workforce are quintessential staff for specimen processing, storage and data management. The time of processing sample, whether SOP followed, any deviations, reason for deviation from the protocol, all this information is beneficial to obtain consistent information for all patients which will make comparison between groups and individuals possible. It is also vital to have all data stored for the samples collected as it will reduce bias and also be helpful for large scale collaborative studies (Capocasa et al., 2016) .

Methods of harmonisation

Consensus generation methods used in research mainly aim to achieve agreement of opinion on a particular topic especially where published literature is inadequate, and these have been mainly used for problem solving or idea generation (McMillan et al., 2016). Three main consensus generation methods commonly adapted are nominal group technique, consensus development conference and Delphi process (Table 9). The methodologies are generally helpful in overcoming the disadvantages in committee meetings for example domination by one person or group, vested interests. The emphasis of the consensus methods is to evaluate the degree of agreement and to settle disagreement.

Nominal group technique is a structured face to face meeting where the panellists rate, discuss and rerate a number of questions. This has been mainly used in assessing appropriateness of clinical interventions, education and training, practice developments in healthcare setting (Jones and Hunter, 1995).

Table 8. showing national and international efforts of harmonisation, reproduced from the article 'Adishesh, M.; Hapangama, D.K. Enriching Personalized Endometrial Cancer Research with the Harmonization of Biobanking Standards. *Cancers* **2019**, *11*, 1734.'

Year	Project	Role
2003	Public Population Project in Genomics (P3G)	Not for profit international consortium, promoted collaboration between researchers in genomics.
2005	Wellcome Trust Case Control Consortium	UK wide consortium, explored utility, design and analyses of Genome wide association studies.
2005	International Society for Biological and Environmental Repositories (ISBER)	Global forum which addressed the harmonisation of scientific, technical, legal, ethical issues of repositories.
2006	The Cancer Genome Atlas Project	Cancer genomics program, a joint project between National cancer institute and National human genome research institute.
2006	European Human Frozen Tumor Tissue Bank TUBAFROST	Virtual European human frozen tumor tissue bank, has access to high quality tissue collections, which are made available for the researchers.
2006	International Agency of Research on Cancer (IARC)	International project funded by WHO, international collaboration on cancer research for cancer prevention.
2006	First-Generation Guidelines for NCI-Supported Biorepositories	National Cancer Institute (NCI) drafted guidelines to standardize and enhance the quality of research material collected by the repositories.
2007	Biobanking and Biomolecular Resources Infrastructure (BBMRI)	European network, with biobanking focus on human biosamples.
2014	World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project (WERF EPhect)	International working group, which achieved global consensus in standardizing the data collection tools and protocols in endometriosis research.

Consensus development conference have been used for safety, effectiveness and appropriateness of medical care and technology. These are run informally in terms of criteria for generating consensus. The definition of consensus in these conferences is unanimous agreement with the consensus statement. The processes used can affect the value, validity and hence its reproducibility (Halcomb et al., 2008). As in any consensus generation methodology, investment of time is necessary, more so with conferences, as conferences need to be organised and participants have to attend all the meetings for it to be reliable and validated. Hence this method is feasible but more expensive and time consuming. Debates and disagreements during the consensus generation may deviate the attention and focus of the entire group (Halcomb et al., 2008). Some of the well-known examples are those conducted by National institute of health (NIH) (Filipovich et al., 2005) , (Jagasia et al., 2015) and World Endometriosis Research Foundation Endometriosis Phenome And Biobanking Harmonisation Project (Vitonis et al., 2014), (Rahmioglu et al., 2014), (Fassbender et al., 2014).

The Delphi process is also a structured process, but interaction amongst the panel members is through questionnaires and hence anonymity is preserved. In the Delphi process, relevant individuals are invited to provide opinions and invited to participate in different questionnaire rounds. During each round, opinions are grouped together, and questionnaire redrafted till consensus achieved on all topics in the questionnaire. There have been various criticisms about the Delphi process and its validity however there is no clear evidence as to which method is the best. It is sufficient that the reporting study clearly justifies the method used, presentation of findings and its relevance (Jones and Hunter, 1995).

Table 9. Consensus Generation methods

Consensus generation methods	Advantages	Disadvantages
Nominal group technique	-Structured face to face meeting -Panellists rate and rerate the questions	-Time consuming -Disagreements can deviate the focus -Requires skilled facilitator
Consensus development conference	-Face to face -Unanimous agreement of all participants	-Expensive -Time consuming -Debates and disagreements may deviate the focus

Delphi Process	-Structured process -Anonymous questionnaires -Multiple rounds till achieve consensus	-Experts are invited to participate hence biased opinions may be possible -Validity may be questionable

The ultimate use of the gold standard biospecimen procurement is to find new biomarkers that can be used as diagnostic, therapeutic, and prognostic markers for EC. To highlight this aspect, in this thesis, the role of a new biomarker, *TERRA* is discussed.

Endometrial carcinogenesis

Telomere and Telomerase biology in endometrial carcinogenesis

Telomeres are protective caps of the ends of the chromosomes, which contain tandem repeat DNA sequence and specific associated proteins, called shelterins. Their main function is preventing loss of genomic DNA during cell replication (Sandell and Zakian, 1993). When the cells proliferate, there is shortening of telomeres due to end replication problem and cell enters either senescence or crisis phase due to chromosomal instability when the telomeres reach a critical short length. Telomerase is a specialised reverse transcriptase enzyme, which adds repetitive telomeric sequences onto the chromosomal ends (Blackburn et al., 1989), (Greider, 2010) and thus can elongate the telomeres, to maintain telomere lengths in proliferating cells. For this reason, telomerase activation is considered as a critical step in cellular immortality and oncogenesis. Numerous studies have reported the importance of telomeres and role of telomerase in cancer development (Kim et al., 1994), (Hiyama et al., 1996), (Shay and Bacchetti, 1997). When gynaecological cancers are considered, previous studies have demonstrated alteration in telomere lengths in the majority of ECs (Wang et al., 2002b) and high telomerase activity (TA) has been reported to be a common feature of ECs (Kyo et al., 1996), (Kyo et al., 1999a), (Wang et al., 2002b). Therefore, understanding the role of telomeres and telomerase is an important aspect to study in endometrial carcinogenesis.

Telomeres

Telomeres are specialised nucleoprotein complexes at the ends of chromosomes, containing repeated nucleotide sequences (Blackburn and Gall, 1978).

Structure

The telomere complex contains tandemly repeated telomeric DNA (- TTAGGG) and its complementary strand, which is terminated with a short single stranded 3` guanine rich overhang known as G-tail (Royle, 2006), (Blackburn et al., 2015). The G Tail forms a D-loop (displacement), which prevents access to telomerase except in late S phase, when it becomes accessible (Griffith et al., 1999), (de Lange, 2004), (de Lange, 2005), (Palm and de Lange, 2008), (Shay, 2016). The whole telomere forms a duplex structure (T-loop) due to strand invasion from the G tail, which provides telomere capping (Griffith et al., 1999), (Blackburn, 2001). The size of T-loop is proportional to the length of the telomere (Cimino-Reale et al., 2001).

Shelterin complex

Telomeres are associated with a complex of six proteins, which together form shelterin complex and they are: Telomeric repeat factor 1 and 2 (TRF1 and TRF2), Repressor/ activator protein 1 (RAP1). TRF1 interacting nuclear protein 2 (TIN2), Tripeptidyl peptidase 1 (TPP1), Protection of telomeres (POT1) (Liu et al., 2004). TRF 1 and 2 are directly attached to telomeric DNA and POT1 is attached to the G tail, hence these are called telomere DNA binding proteins (Ye et al., 2004). The remaining three proteins bind to these proteins: TIN2 to TRF1, RAP1 to TRF2 and TPP1 to POT1 (de Lange, 2005). In addition to these six proteins, there are many other proteins located at the telomere: for example, NBSI, MRE2, Rad50, tankyrase, PinXI, and Ku. These have a role in DNA damage response (DDR) and also non telomeric functions (Kuimov, 2004), (De Boeck et al., 2009). Shelterin complex is universally expressed and is associated with the telomeres throughout the cell cycle (Royle, 2006), (Takai et al., 2011).

Function of telomeres

- *Prevention of chromosomal DNA identification and breaks* - Both shelterin proteins together with telomeres, protect the chromosomal ends. TRFs and POT1 form T-loop and prevent DDR activation (Palm and de Lange, 2008). TRF2 prevents end to end fusion and POT1 protects the G tail by forming D-loop (Jacob et al., 2007), (Yang, 2008), (Baumann and Price, 2010), (Cesare and Karlseder, 2012).
- *Protect chromosomal ends from degradation by nucleases and end to end fusion* (Xue et al., 2016), (Shay, 2016).
- *Act as sentinels for DNA damage* – Telomeres are more prone to DNA damage compared to genomic DNA due to high guanine content and lack of DNA repair

mechanisms (Wang et al., 2010), (Petersen et al., 1998). These triggers sustained DDR, resulting in cell cycle arrest, and inducing apoptosis or senescence (Shay, 2016), (Blackburn et al., 2015).

- *Recruitment of telomerase* – Shelterin proteins particularly POT1 has dual role in recruiting telomerase as it prevents access to intact telomere complex but after hetero-dimerization with TPP1, it allows telomerase to become active and to extend the 3` overhang in the late S phase (Wang et al., 2007), (Zhang et al., 2013), (Chu et al., 2016).
- *Regulation of gene transcription* – Also participate in regulation of genes which are located nearer are transcribed at a lower rate (Robin et al., 2014).
- *Extra telomeric functions of telomere associated proteins* – The shelterin proteins are associated with regulation of transcription of different genes. RAP1 is associated with regulation of female obesity (Martínez et al., 2013). TIN2 is found in mitochondria and its reduced expression results in inhibition of glycolysis (Chen et al., 2012a).

Telomerase

The main mechanism by which the telomeres are maintained in healthy cells, is the activity of specific/ specialized enzyme, telomerase (Blasco, 2005). However, TA is low or absent in most human somatic cells but present at sufficiently high levels in only specialized cells and cancer cells (Kim et al., 1994), (Shay and Bacchetti, 1997).

Telomerase is a reverse transcriptase (RNA dependent DNA polymerase) that adds G-rich telomeric repeats to G tail using an RNA template (Lingner et al., 1997). Telomerase holoenzyme contains a human reverse transcriptase enzyme TERT (hTERT), human TERC (*hTERC*), the RNA component in each of its dimeric configuration connected by a hinge in the middle (Venteicher et al., 2009), (Blackburn and Collins, 2011) (Figure 10).

Components of telomerase

hTERT is the catalytic component of the telomerase enzyme, and *in vitro*, only hTERT and *hTERC* is sufficient to reconstitute TA (Weinrich et al., 1997) (Figure 10).

Human telomerase reverse transcriptase (*TERT* or *hTERT*)

The catalytic unit of telomerase enzyme and the rate-limiting factor for the enzyme activity is hTERT (Zhou et al., 2006), (Zhang et al., 2013). The gene for hTERT is located on chromosome 5p, has 16 exons and 15 introns and about 35 kb in length (Cong et al., 1999), (Shay and Wright, 2010), (Cong et al., 1999). Whilst there are over 20 spliced variants of

hTERT, only the wild type exhibits reverse transcriptase activity (Hrdličková et al., 2012). hTERT is not only associated with telomere maintenance, but also increases the apoptotic capacity of cells, maintaining pluripotency of stem cells and gene expression regulation (Saretzki, 2014).

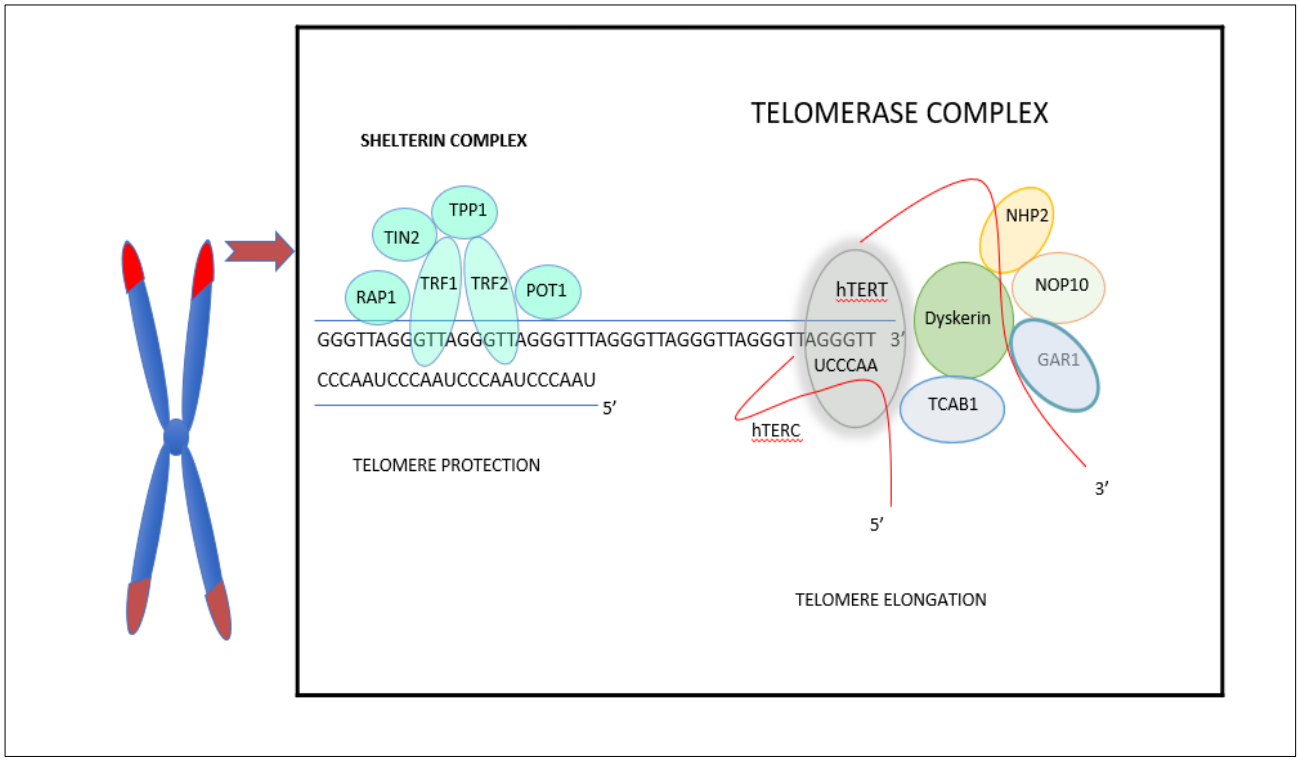


Figure 10. Pictorial representation of the telomerase complex

Human telomeric RNA component (*hTERC* or hTR)

hTERC is a short RNA strand approximately 451 nucleotides in length and its sequence is complementary to telomeric TTAGGG repeat sequence (Theimer and Feigon, 2006). Although species specific in size and sequence, its structure is highly conserved. *hTERC* is a non-coding RNA transcribed by RNA polymerase 2 and its 3` end shares a H/ACA motif with small nucleolar and small cajal body RNAs (snoRNA, scaRNA), which further associates with all four H/ACA RNP components – dyskerin, NOP10, NHP2, GAR1 (Egan and Collins, 2010). Its co-transcriptional association with dyskerin is essential feature for stabilisation, preventing its further cleavage and nuclear retention (Feng et al., 1995), (Fu and Collins, 2003), (Kiss et al., 2010). Mutation in the H/ACA motif area leads to dyskeratosis congenita and variants associated with sequence change outside this motif area do not affect the stability, but in fact

imposes catalytic defects (Fu and Collins, 2003). The binding of *hTERC* to the tetrameric complex of accessory proteins dyskerin, NOP10, NHP2 and NAF1 is vital for TA.

Telomerase associated proteins (Table 10)

Table 10 Telomerase associated proteins

hTERT associated proteins	P23, hsp 90 – ribonucleoprotein assembly Protein14-3-3 regulates apoptosis DHX36 stabilises hTERT VCP like protein regulates telomerase activity Pontin and Reptin facilitate assembly of telomerase	(Xi and Cech, 2014) (Her and Chung, 2012) (Huber et al., 2008)
***hTERC* associated proteins**	H/ACA ribonucleoproteins associated with stability, accumulation, maturation, localisation of *hTERC* Dyskerin stabilises *hTERC* AI and UPI provide accessibility of the telomerase to telomeres Telomerase protein component 1 helps in telomere replication La antigen helps in telomeric homeostasis STAU assists in telomerase processing, localisation, and telomerase assembly TCAB1 (telomerase and cajal body protein1) helps in recruitment of the telomerase holoenzyme to the telomeres	(Wang and Meier, 2004) (Nagata et al., 2008) (Harrington et al., 1997) (Aigner et al., 2003) (Venteicher et al., 2009)

Functions of telomerase

Telomere maintenance - In eukaryotic cells, telomerase elongates the 3’ strand and this counteracts the end replication problem. Telomere lengthening preferentially happens at shortest telomeres to keep telomeres above a critical length, preventing the activation of DDR pathways (Fakhoury et al., 2010), (DSouza et al., 2013) .

Non canonical functions of telomerase component hTERT – hTERT acts as a cofactor in Wnt/β-catenin pathway and hence involved in regulation of Wnt signalling (Park et al.,

2009). hTERT interacts with RNA component of the mitochondrial RNA to form ribonucleoprotein complexes, which produce dsRNA (double stranded), which serve as substrates for SiRNA generation and regulate expression of genes related to stem cell biology (Maida et al., 2009). In addition, TERT participates in improved DNA repair, increased apoptosis resistance, changes in chromatin structure and altered gene expression (Saretzki, 2014).

Telomere maintaining mechanisms (TMM) in cancers

Telomere shortening induces cellular senescence and overcoming that by maintaining telomeres is essential for carcinogenesis. Cancer cells utilise two main pathways to maintain their proliferative potential, resulting in cellular immortalisation; common pathway is the telomerase dependent telomere elongation, based on telomerase enzyme (Figure 12).

(1) ***Telomerase dependent telomere elongation***

Commonly, the telomerase dependent telomere elongation, based on telomerase enzyme and TA, is active in 85% of cancers. This is discussed in detail later in this chapter.

(2) ***Alternative lengthening of telomeres (ALT) pathway***

The other TMM pathway is the telomerase independent, alternative lengthening of telomeres (ALT) pathway (Bryan et al., 1997). ALT occurs in only 10 – 15 % of human cancer cells (Cesare and Reddel, 2010). The hallmark of such cancers are combination of ALT related biomarkers, which are absence of TA, heterogeneity in telomere length, telomere sister-chromatid exchanges, extrachromosomal telomeric repeats or C-circles and ALT associated promyelocytic leukemia protein (PML) bodies (APBs) (Cesare and Reddel, 2010). Detection of C-circles and ALT telomere DNA synthesis in APBs are more robust and advanced assays for ALT (Henson et al., 2009), (Zhang et al., 2019). Recently, it has been understood that there are more than one mechanism of ALT and RAD51 which was originally thought to be essential is now proven to be dispensable. There are three possible mechanisms to ALT, RAD51-dependent recombination, RAD52-dependent break induced replication (BIR) and RAD52-independent BIR (Zhang et al., 2019), (Lee et al., 2021). According to the latest research, a study in yeast cells suggests that as *TERRA* and R loops accumulate at short telomeres and trigger DNA repair (Graf et al., 2017) in addition another study on human tumour cell lines show that RNA endonuclease RNAse H1 regulates the level of R loops and controls recombination, therefore *TERRA* and R-loops may be involved in ALT induction (Arora et al.,

2014). Similarly, it is thought that BRCA2 suppresses ALT by maintaining telomere replication and then by suppressing BIR (Lee et al., 2021). It is also a known fact that majority of BRCA2 mutated cancers also have P53 mutations, hence BRCA 2 deficiency that occurs in conjunction with p53 inactivation may result in ALT and ALT may promote carcinogenesis (Lee et al., 1999), (Bodvarsdottir et al., 2012), (Pompili et al., 2019). Loss or mutation of ATRX or DAXX protein chromatin remodeler complex, and somatic mutation in histone H3.3-ATRX-DAXX chromatin remodelling pathway are all implicated in ALT as well (Heaphy et al., 2011), (Lovejoy et al., 2012), (Schwartzentruber et al., 2012). Accumulation of MRN (MRE11/RAD50/NBS1) complex by sp100 overexpression and depletion of MRN complex both lead to suppression of ALT. In absence of ATRX, MRN relocates to APBs and thereby MRE11/RAD50/NBS1 (MRN) complex is implicated in ALT pathway (Jiang et al., 2005), (Zhong et al., 2007), (Clynes et al., 2015), (Lee et al., 2021). ALT occurs in high frequency in CNS tumours such as astrocytomas, glioblastomas, and in cancers of soft tissue, and bone (Scheel et al., 2001) , (Henson et al., 2005), (Grandin et al., 2019), (Lawlor et al., 2019) .

(3) ***Non-defined TMM mechanism (NDTMM)*** has also been recently reported (Gaspar et al., 2018). In tumours, where both telomerase and ALT were both reported to be absent, the existence of a non-defined TMM (Gaspar et al., 2018) had been proposed. Examples of cancers with neither telomerase nor ALT mechanism, but where telomeres are maintained, include glioblastomas, osteosarcoma, cutaneous melanoma metastases (Royds et al., 2011), (Barthel et al., 2017), (Viceconte et al., 2017). In glioblastomas, NDTMM was accompanying polymorphism of CDKN2A, and was associated with reduced patient survival (Royds et al., 2011). Tumours with NDTMM did not always present with same telomeric features, hence future studies are warranted to elaborate on these mechanisms (Barthel et al., 2017). *TERRA* may play a role in NDTMM.

Epigenetic regulations such as DNA methylation, histone methylation and histone acetylation all affect expression of hTERT and thus appear to have a vital role in telomerase regulation (Lewis and Tollefsbol, 2016).

The mechanisms for the robust maintenance of telomere lengths in tumour cells may be caused by somatic mutations of hTERT promoter (Simon et al., 2015), amplification of hTERT (Visnovsky et al., 2014) and *hTERC* (Liu et al., 2012a), re-arrangement of hTERT gene (Kawashima et al., 2016), germline variants of hTERT gene and its promoter (Baird, 2010), (Chen et al., 2012b), epigenetic changes (Bechter et al., 2002), (Widschwendter et al., 2004),

ALT (Rodriguez et al., 2016), and non-defined TMM (Royds et al., 2011). When the incidence of TMM mechanisms was studied in different cancers in the TCGA cohort, they found that the TERT promotor mutations were most prevalent in brain tumours, hepatocellular carcinoma, skin cancers (melanomas, squamous cell carcinomas, basal cell carcinomas) whereas less prevalent in digestive system, haematopoietic and lymphoid tumours (Barthel et al., 2017). Cervical precancers, lung cancers had high frequency of *hTERC* amplifications (Liu et al., 2012b). ALT is less common, but more prevalent in osteosarcomas, neuroblastomas (Ulaner et al., 2003), (Gaspar et al., 2018). A crucial difference between both pathways is that TA positive cells maintain telomere lengths around 5-10 kb with normal distribution using enzyme telomerase whereas telomeres of ALT cells are long up to 20kb and with significant telomere length (TL) heterogeneity (Sampl et al., 2012).

In female reproductive cancers included in the TCGA cohort, TERT promotor mutation frequency was 21% in clear cell carcinoma of uterus, 11% in uterine endometrial carcinoma. TERT promotor mutations are considered to involve two step tumourigenesis mechanism, where the mutations heal the shortest telomeres in the first phase, and in the second phase, genomic instability arises due to critically short telomeres which induces upregulation of telomerase to sustain cell proliferation (Chiba et al., 2017).

In epithelial cells which have replicative capability, telomere shortening can lead to chromosomal instability by leading to multiple chromosome breakages, fusions, translocations making the chromosomes aneuploid therefore carcinogenesis. One of the mechanisms which stabilise the telomere structure and its length is shelterin complex (de Lange, 2005). This complex protein interactions at the chromosomal ends have a key role in maintaining the telomere length. Telomere lengths are maintained by a negative feedback loop, thus, when telomeres are long, the *cis*-acting mechanism inhibits the telomerase, and this is influenced via shelterin protein, POT1. POT1 can either facilitate or inhibit telomerase binding via binding to the ssDNA at the 3' overhang of the chromosomal ends and burying the base at the 3' end making it inaccessible to the telomerase enzyme (Lei et al., 2005), (Kelleher et al., 2005). POT1 can also inhibit telomerase through its 'steric hindrance' with telomerase enzyme (Lei et al., 2005). RPA (Replication protein A) is another ssDNA binding protein, which has an important role in telomere replication by facilitating telomerase enzyme at the telomeres (Rubtsova et al., 2009), (Luciano et al., 2012), and also recruitment of the ATR-ATRIP protein kinase complex to DNA damage sites and to initiate the checkpoint signalling (Zou and Elledge, 2003), (Haring et al., 2008).

Telomerase and cancers

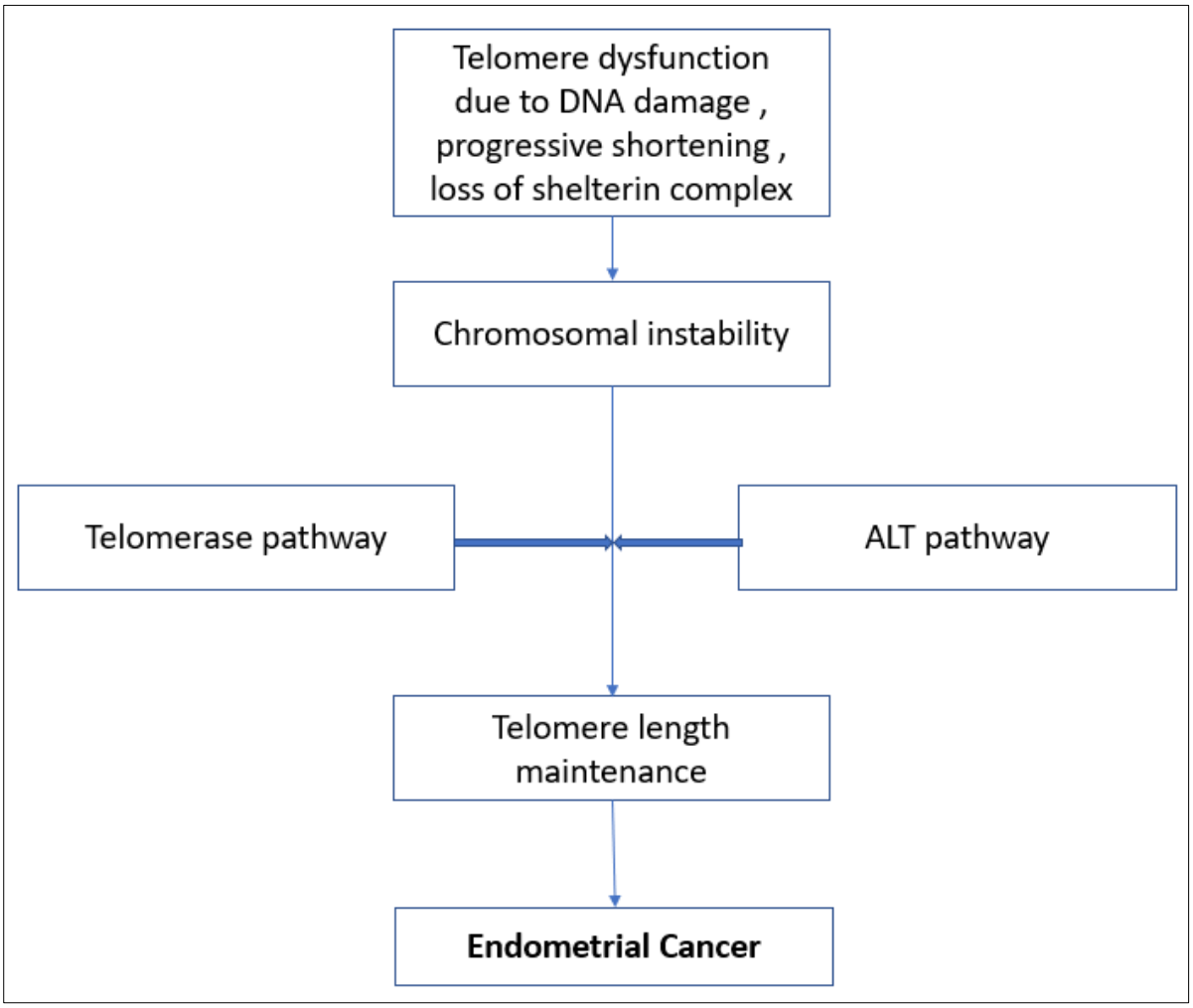
As a general principle, epithelial cancers have high TA compared with normal healthy tissue. For example, TA is found to be higher in pancreatic ductal cell cancers compared with benign tissue or other pancreatic cancers. Gastric cancers with high TA have poorer prognosis (Hiyama and Hiyama, 2004). More than 90% of head and neck squamous cell cancers have high TA (Mao et al., 1996). In colorectal cancers, high TA is associated with poorer prognosis and survival seems to be associated with the level of TA (Tatsumoto et al., 2000). Therefore, taken together, high TA is a common observation in most cancers as per available evidence, as highlighted in many systematic reviews (Shay and Bacchetti, 1997). Mutations of telomerase components that have been reported in many cancer types include activating mutations of the telomerase complex. *hTERC* amplifications were found to be a hallmark of cervical carcinogenesis, and overexpression of *hTERC* is reported in prostate, breast, oral squamous cell carcinomas (Dorji et al., 2015), (Heng et al., 2017), (Zhu et al., 2018), (Baena-Del Valle et al., 2018).

In genome wide association studies (GWAS), variants of chromosome 5p15 which is hTERT gene were identified in bladder, pancreas, brain, prostate, skin, and lung cancers (Rafnar et al., 2009), (Petersen et al., 2010), (Kote-Jarai et al., 2011). hTERT promoter mutations are associated with increased hTERT expression, TA or longer telomeres, have been identified in many cancers such as thyroid, skin, bladder (Heidenreich et al., 2014).

Evidence for altered Telomere/Telomerase Biology in EC

Telomerase is present in human endometrium, despite being a somatic organ, with highest levels reported in the highly mitotic proliferative phase cells (Hapangama et al., 2017) and is likely to be regulated by oestrogen. Two groups, Kyo et al and Saito et al have produced the first reports of high TA in ECs (Kyo et al., 1996), (Saito et al., 1997). Subsequently, many others have reported ECs to have high TA (Ebina et al., 1999), (Maida et al., 2006), (Gul et al., 2013). In a small study, Bonatz et al reported a significant correlation between high TA and the surgical stage and grade of EC (Bonatz et al., 2001). Comparison between hTERT mRNA levels and TA using telomeric repeat amplification protocol (TRAP) assay have shown the levels and activity to be significantly higher in cancer compared with low levels seen in normal non-cancerous endometrium (Lehner et al., 2002).

Figure 12. Chart representing endometrial carcinogenesis process from telomere dysfunction using telomere maintenance mechanisms



Since EC is a hormone responsive cancer, it is important to consider the effect of ovarian hormones on regulation of telomerase. Some *in vitro* studies using EC cell lines, demonstrated that TA levels and hTERT expression to increase by oestrogen via ER alpha dependent and oestrogen responsive element (ERE) dependent effect on hTERT promoter (Boggess et al., 2006). In addition, similar induction of TA with oestrogen via Akt dependent phosphorylation of hTERT was seen in human ovarian cancer cell lines (Kimura et al., 2004). Many driver mutations may affect telomerase components. ARID1A is tumour suppressor protein, frequently mutated in endometrial adenocarcinoma, recently a study has suggested ARID1A negatively regulates hTERT and TA, therefore loss of ARID1A can upregulate the TA (Suryo Rahmanto et al., 2016), (Takeda et al., 2016).

Evidence for Telomere length alterations in EC

The initial evidence for telomere length alterations in EC was reported in 1992, where endometrial adenocarcinomas were found to have shorter telomeres compared with normal tissue (Smith and Yeh, 1992). Later in 2002, Wang et al tried to establish the relationship between TA and telomere lengths in gynaecological cancers, and they found 81% ECs to have TA and 74% of ECs had alterations in telomere lengths (Wang et al., 2002b). There have been further studies, which have compared the epithelial cancer cell telomere length to normal stromal cells, but stromal cells are known to have longer telomeres, hence there is fundamental differences in cell proliferation, TA between these cell types (Akbay et al., 2008), (Hapangama et al., 2017). It was also reported that ECs to show shorter 3' telomeric overhang length, which were even shorter in poorly differentiated or deeply invading tumours, which suggest that these features may have a role in tumour progression (Hashimoto et al., 2005). The TCGA dataset also reported that sarcomas, testicular germ cell tumours, low grade gliomas were associated with longer telomeres, whereas gynaecological malignancies such as cervical cancer and EC had shortest average telomere lengths (Barthel et al., 2017). Mutations of POT1 gene, which is one of the most conserved shelterin proteins were reported in ECs, loss of POT1 may cause inappropriate telomere access to telomerase leading to telomere dysfunction and genetic instability (Flynn et al., 2012), (Liu et al., 2012a). During normal cell proliferation, telomeres shorten with every division and reach a critical length, at which point tumour suppressor checkpoints are triggered to lead the cell into senescence or apoptosis. When tumour suppressor checkpoints are not working, this can lead the cell into crisis phase, where further shortening of telomeres causes telomere dysfunction (Shay and Wright, 2005). Telomere dysfunction can cause genomic instability, for example, end to end fusion of telomeres, which can also lead to cell death. When associated with mutations such as TERT promoter mutations, this can lead to increased TA, hence re-stabilisation of telomeres. Telomere shortening is considered to be one of the early phases of tumourigenesis (Valls et al., 2011), (Roger et al., 2013). The available evidence suggests some cancers such as breast, pancreatic, prostate, colorectal cancers are associated with shorter telomeres (Meeker et al., 2002) , (van Heek et al., 2002), (Meeker et al., 2004), whereas there are further studies for example in hepatocellular carcinomas, that suggest that longer telomeres, increased hTERT expression and higher TRF2 protein to be associated with poorer prognosis (Kim et al., 2013).

In some organs like bladder, oesophagus, stomach, and ovaries, shorter telomeres were significantly associated with carcinogenesis. In contrast to that, studies in ECs did not show any association between relative TL and risk of EC (Wang et al., 2002a), (Maida et al., 2006),

(Prescott et al., 2010). There are contradictory reports of association of longer circulatory leukocyte telomere length (LTL) and significantly increased risk of EC (Sun et al., 2015).

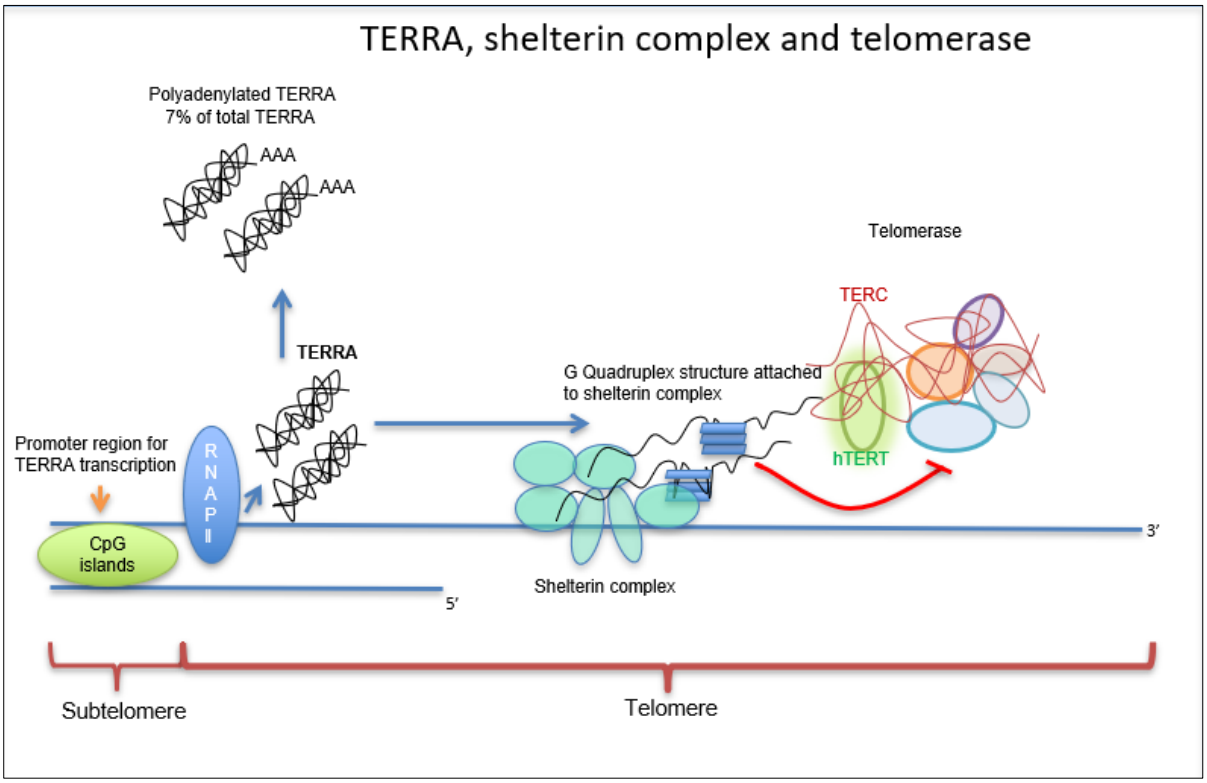
Telomeric repeat containing RNA (*TERRA*)

For many years, the telomeres were considered to be transcriptionally silent, but in 2007, Azzalin et al., demonstrated that they are transcribed into *TERRA* (Azzalin et al., 2007). These long non-coding RNAs are transcribed from subtelomeric CpG-island promoters extending towards telomeric tract and are composed of G-rich UUAGGG repeat sequences, mainly by RNA polymerase II (Azzalin et al., 2007), (Schoeftner and Blasco, 2008). *TERRA*s are expressed in most mammalian tissues.

- ### *TERRA* Structure and associated proteins

  Mammalian *TERRA*s range from 100bp to 9Kb (Azzalin et al., 2007) and their length is reported to be proportional to the length of the transcribed telomere (Arnoult et al., 2012). A small amount of cellular *TERRA*s are bound to telomeres and found associated with transcriptionally silent metaphase chromosomes (Azzalin et al., 2007), (Nergadze et al., 2009), (Porro et al., 2010). The poly (A) tail and 7-methylguanosine (m^7G) cap structure at 5` end of all human *TERRA*s are responsible for stability (Feuerhahn et al., 2010). *TERRA* form G-quadruplexes with shelterin complex protein TRF2 (Biffi et al., 2012) and also co-localise with RAP1 foci (Azzalin et al., 2007) (Figure 13). The *TERRA* content seem to be cell specific, for example around 30% HeLA and primary human lung fibroblasts displayed 3 to 7 *TERRA* foci whereas 80 -100% of human osteosarcoma cells and murine renal cancer cells displayed 20 - 40 foci (Azzalin et al., 2007). *TERRA* levels also fluctuate with the cell cycle phase and subtelomeric promoter methylation inhibits the telomere transcription (Nergadze et al., 2009). There are about 15 different types of proteins which are *TERRA* associated proteins and they have diverse cellular functions. *TERRA* transcription is regulated slightly differently at every chromosome end as there may be some promoters or molecules which may affect certain *TERRA*s more than others for example, TRF1 knockdown has shown to reduce 1q-2q-10q-13q *TERRA* levels but has no effect on 15q *TERRA* (Scheibe et al., 2013).

Figure 13. Diagramatic representation of *TERRA*, shelterin complex and telomerase complex



- **7-methylguanosine cap structure at *TERRA* 5` end**

TERRA is transcribed by RNA polymerase II and hence have 7-methylguanosine (m^7G) cap structure. Some small nuclear RNA's have a 2,2,7-trimethylguanosine (TMG) cap. *TERRA* is found exclusively in the nucleus. Northern blot experiments performed by Porro et al in 2010 confirmed that *TERRA* molecules do not have a TMG cap structure and a large majority of them contain m^7G cap.

- **Poly(A) tail**

About 7% of human *TERRA* molecules are polyadenylated, whereas almost all yeast *TERRA*s carry a poly (A) tail (Azzalin and Lingner, 2008), (Luke et al., 2008). Canonical polyadenylation increases *TERRA* stability, whereas noncanonical poly(A) polymerases trigger efficient decay of the RNAs by recruitment of nuclear exosome complex (Vanácová et al., 2005), (Wyers et al., 2005), (Vanacova and Stefl, 2007). Quantification of the $poly(A)^-$ *TERRA* and $poly(A)^+$ *TERRA* have shown that the half-lives of these are three hours and more than eight hours respectively (Porro et al., 2010). This suggests that the poly(A) tail structure

increases *TERRA* stability and the canonical poly(A) polymerase is responsible for the polyadenylation (Porro et al., 2010).

- **Different *TERRA* subtypes**

Different *TERRA* transcripts were derived about 5 -10kb upstream region of subtelomere from 10 distinct chromosomes (chr 1p, 9p, 12p, 16p, 17p, 19p, Xp,15q, 20q, Xq) and abundance of all of these transcripts increased by 2 to 4 fold with TRF2 depletion (Porro et al., 2014b). In contrast to other long non-coding RNAs, there were no splicing events of *TERRA* transcripts on analysis of the sub telomeric sequence (Porro et al., 2014a). According to the Blasco lab, in humans, out of 18 *TERRA* loci identified to date, only transcripts from 20q and Xp had *TERRA* features (Montero et al., 2016). Deletion of 20q locus resulted in not only significant reduction in *TERRA* levels but also telomere shortening and uncapping, whereas deletion of Xp locus did not decrease the levels. This showed that 20q locus to be considered the most genuine *TERRA* as it resulted in significant change in *TERRA* levels (Montero et al., 2016). Deletions and amplifications of 20q subtelomere have been found to cause different variety of haemotological malignancies and mental retardation. On the contrary, Feretzaki et al, demonstrated by using chromosome-specific subtelomeric primers, that *TERRA* is expressed from a large number of telomeres. The quantity of *TERRA* molecules from each chromosomal end varies within a given cell type. The state of individual telomere will affect the *TERRA* transcription not only in cell types but also amongst telomeres of the same cell (Feretzaki et al., 2019). However, this study could not detect if all telomeres expressed *TERRA* as some subtelomeric sequences were too repetitive to design primers and some *TERRA* transcription started close to the canonical 5'-TTAGGG-3' making the detection by RT- PCR difficult.

- **Regulation of *TERRA***

Levels of *TERRA* are cell cycle regulated and transcription is inhibited by promoter methylation (Nergadze et al., 2009). Most information on *TERRA* regulation comes from studies on yeasts. The transcription is shown to be regulated by telomeric heterochromatin and promoted by TRF1 (Schoeftner and Blasco, 2008). In yeast cells, Rat1, 5`-3` exonuclease negatively regulates *TERRA* levels and this is mediated by RAP1 and its associated proteins like Rif1 and Rif2 (Luke et al., 2008), (Iglesias et al., 2011). TRF4, is an alternative poly (A) polymerase plays a role in degradation of *TERRA* in Rat1 deficient cells. Overexpression of RNAse H, degrades RNA, reduced *TERRA* expression in yeast cells. Although the same regulatory

pathways that are active in eukaryotic cells are expected to be present in upper order mammalian and human cells, further studies are needed to confirm these assumptions in future.

- **Functions of *TERRA* (Figure 14)**

***TERRA* and heterochromatin**

TERRA have been reported to participate in many cellular regulatory functions. They may contribute to telomeric heterochromatin formation via its interaction with proteins such as TRF2, origin of recognition complex (ORC), heterochromatin protein 1(HP1) (Deng et al., 2009) and also by promoting H3K9 trimethylation (Deng et al., 2009), (Arnoult et al., 2012); the regulation of cellular differentiation is thought be secondary to the influence on telomerase and heterochromatin formation. During early expansion of human embryonic stem cells, *TERRA* levels decrease, which in turn causes reduced *TERRA* bound SUV39H1 leading to down regulation of H3K9me3. These processes cause telomeric chromatin remodelling and telomere elongation (Zeng et al., 2017). TRF2-*TERRA*-ORC1 (origin recognition complex – ORC) interactions maintain HP1 and histones at telomeres, which in turn maintains DNA stability (Deng et al., 2009). The main epigenetic difference between telomerase positive cells and ALT cells is that telomerase positive cells have high levels of subtelomeric methylation and display low levels of *TERRA* compared to ALT cells, this indicates that the consolidation of heterochromatin in these cells inhibits telomeric transcription (Ng et al., 2009).

***TERRA* and shelterin proteins**

In human telomeres, loss of TRF1 increases recruitment of telomerase. In both humans and yeasts, RAP1(Rap1 in yeasts) via TRF2 and Taz1 proteins respectively, has a negative regulatory role on telomerase recruitment (Stern and Bryan, 2008), (Wang et al., 2015). *TERRA* associates directly with TRF 1 and 2, which together play a role in telomere protection by blocking ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), Homology directed repair (HDR) and Non-homologous DNA end joining (NHEJ) pathways (Wang et al., 2015). During early to mid S phase, *TERRA* binds to hnRNPs, leads to binding of RPA to ssDNA then release of POT1 to enable telomere extension. When *TERRA* levels decrease, hnRNPs are released which displace RPA from telomeric ssDNA. As *TERRA* reaccumulates in late S phase, hnRNPs binding to ssDNA is antagonised and in presence of hnRNPs POT1 coats the ssDNA displacing the RPA and prevents persistent ATR activation after S phase. This cell cycle regulated *TERRA*, hnRNPs arrangement leads to telomere capping till the next replication forks arrive (Flynn et al., 2011).

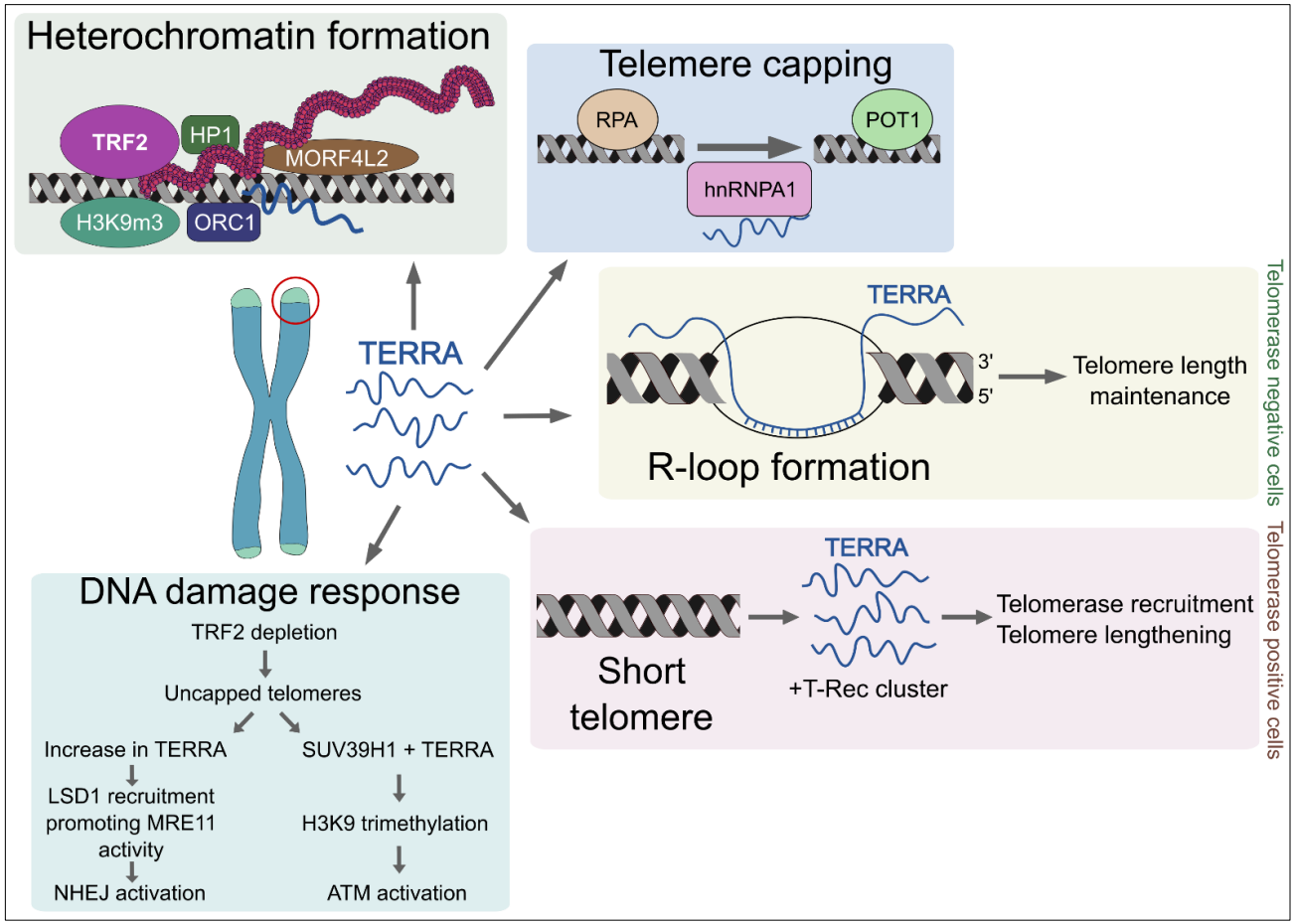
TRF1 is involved in Fibroblast growth factor (FGF) signalling pathway, which plays a major role in regulating human embryonic stem cells (hESC) self-renewal and survival (Zeng et al., 2017). TRF1 is highly expressed in embryonic stem cells (ESCs) compared with somatic cells. TRF1 can be activated by FGF2 signalling pathway. TRF1 binds directly to *TERRA* and TRF1 bound *TERRA* negatively regulates *TERRA* levels during the hESCs establishment. Decreased TERRA levels lead to loss of telomeric chromatin and telomere elongation. This pathway plays an important role in acquisition / maintenance of pluripotency in human embryonic stem cells, which may be involving *TERRA*s (Zeng et al., 2017).

TERRA levels are found to be upregulated in HeLa cells upon TRF2 and TRF1 depletion but not with other shelterin components like POT1, RAP1, TIN2, TPP1 (Porro et al., 2014a). Further on, in cells without telomere protection (TRF2 depleted cells), TERRA increase was found to be due to enhanced transcription rather than improved stability thus maintenance of the transcripts (Porro et al., 2014a). Upregulation of *TERRA* transcripts in TRF2 depleted cells occurs independently of DDR pathways (e.g., P53/ATM /ATR) activation, hence TRF2 is thought to be a direct negative regulator of TERRA transcription (Porro et al., 2014a), (Porro et al., 2014b). Even though both TRF1 and TRF2 suppress *TERRA* transcription, they do so via distinct mechanisms. TRFH domain region on TRF2 was mainly responsible for inhibition of *TERRA* transcription and the same domain is also responsible for preventing ATM pathway activation (Porro et al., 2014a). Therefore, this suggests that *TERRA* may work in parallel to ATM kinase pathway and increase in its transcription may represent an early step in telomeric DDR. *TERRA* increase in TRF2 depleted cells is responsible for increased recruitment of SUV39H1 (histone methyltransferase) to uncapped telomeres, which directly binds to *TERRA*, leads to chromatin reorganisation at dysfunctional telomeres (Porro et al., 2014b).

Formation of RNA-DNA hybrids

TERRA forms RNA-DNA hybrids or R-loops at chromosome ends (Pfeiffer and Lingner, 2012). Accumulation of RNA-DNA hybrids at telomeres, leads to rapid senescence and telomere loss in absence of telomerase and HDR.

Figure 14. Schematic diagram of functions of *TERRA*



Whereas, in absence of telomerase and presence of HDR, there is an increase in *TERRA* levels at shortened telomeres that promotes the telomere elongation by recombination process (Yu et al., 2014), (Balk et al., 2014). At the shortened telomeres, *TERRA* is accumulated in nuclear foci that contain telomerase; thus, these clusters lead to recruitment of telomerase to the telomeres from which they originate in early S phase of cell cycle (Cusanelli et al., 2013). Telomerase preferentially elongates the shortened telomeres and then the *TERRA* levels reduce (Teixeira et al., 2004), (Porro et al., 2010). In yeast cells, accumulation of *TERRA* and R-loops occurs exclusively at short telomeres which contributes to DDR activation and recruitment of Rad51 recombinase (Graf et al., 2017). In mouse cells, *TERRA* acts antagonistic to ATRX, competes with telomeric DNA for ATRX binding, suppresses ATRX localization and ensures telomeric stability. *TERRA* promotes alternative lengthening of telomeres (ALT) by suppressing both TA and ATRX (Chu et al., 2017). There are fundamental differences in yeast and human telomerases, as yeast telomerase is not regulated by *TERRA* (Pfeiffer and Lingner, 2012).

Formation of ribonucleoprotein complex

Studies in mice have shown that RNA binding proteins (RBPs) such as heterogenous ribonucleoproteins are abundantly bound to *TERRA* and have a role in its regulation (López de Silanes et al., 2010). hnRNP A1, A2/B1, H and F in large quantities bind to *TERRA* both *in vivo* and *in vitro*. Each of these hnRNPs have their own individual specialist role as well as common functions, such as maintaining mRNA homeostasis both in nucleus and cytoplasm and interacting with other hnRNPs to form multiple alternatively spliced isoforms. hnRNP F acts as a repressor of *TERRA* levels and partly reduces their stability, whereas hnRNPs M and D block the access of *TERRA* transcripts to telomeres. Cells with shorter telomeres could be rescued on knocking down most of *TERRA* interacting RBPs, which was dependent on presence of telomerase. Downregulation of *TERRA* binding RBPs results in massive mobilization of *TERRA* to telomeres, which in turn protects them from inducing telomere dysfunction induced foci (TIF) (López de Silanes et al., 2010). TA and telomere elongation are regulated via the interaction of hnRNPs and *TERRA* with each other. When hnRNPs are in excess, compared to *TERRA* levels, they inhibit the telomere elongation through biding to DNA substrate, whereas when *TERRA* levels are high, they can bind and inhibit TA. When both hnRNPs and *TERRA*s are in equilibrium, they form inert complexes, which allow telomere extension by telomerase. Therefore, for telomere maintenance and elongation, balanced levels of *TERRA* and hnRNPs are required (Redon et al., 2013).

- **Role of *TERRA* on telomere length**

In yeast cells, RNA exonuclease, Rat1p, degrades *TERRA* and promotes telomere elongation (Luke et al., 2008). When Rat1p is mutated, *TERRA* accumulates and forms a DNA/RNA hybrid, which inhibits telomerase activity and further causes telomere shortening (Luke et al., 2008).

TERRA forms RNA-DNA hybrids or R-loops at chromosome ends (Pfeiffer and Lingner, 2012). Accumulation of RNA-DNA hybrids at telomeres leads to rapid induction of senescence and telomere loss, in absence of telomerase and HDR. However, in the absence of telomerase and in the presence of HDR, there is an increase in *TERRA* levels at short telomeres that promotes the telomere elongation by recombination process (Yu et al., 2014), (Balk et al., 2014). In telomerase proficient cells, at the shortened telomeres, *TERRA* is accumulated in nuclear foci that contain telomerase; thus, these clusters lead to recruitment of telomerase to the telomeres from which they originate in early S phase of cell cycle (Cusanelli et al., 2013). Telomerase then preferentially elongates the shortened telomeres and consequently the *TERRA* levels are reduced with telomere elongation (Teixeira et al., 2004), (Porro et al., 2010).

- **Role of *TERRA*, is it a recruiter or inhibitor of telomerase at the telomere?**

In yeast cells, *TERRA*s were found to sequester and direct telomerase to the telomeres, which needed to be elongated (Cusanelli et al., 2013). In mouse cells, where high levels of telomeric RNAs are associated with longer telomeres, they have been postulated to inhibit telomerase activity by base pairing with *hTERC* at the internal 5'-UUAGGG-3' repeats region that is complementary to the *hTERC* sequence (Schoeftner and Blasco, 2008). This phenomenon was also reported in human 293t cells (cells derived from human embryonic kidney cell line HEK293) where *TERRA*s inhibit telomerase by binding to hTERT (Redon et al., 2010). As mentioned above, in wild type of budding yeast cells, *TERRA*s inhibited telomerase by forming RNA-DNA hybrids (Luke et al., 2008).

During the cell cycle phases, *TERRA* together with hnRNPA1 (heterogenous ribonucleoprotein A1) assist in displacement of RPA and binding of POT1 to the ssDNA, leading to orderly DNA replication and capping (Flynn et al., 2011). In another study, *TERRA* was found to be induced in cells with short telomeres and acted as a scaffold for spatial organisation of the telomerase components forming a *TERRA*-telomerase complex, which helped in recruitment of telomerase

to the telomere of its origin (Cusanelli et al., 2013). Therefore, *TERRA* is proposed to be a recruiter of telomerase enzyme to telomeres and for telomere elongation, rather than being an inhibitor (Cusanelli et al., 2013).

Contrary to the lab based studies using yeast cells, in mouse cells and 293t cells, the *TERRA* like oligonucleotides have been shown to inhibit TA using TRAP and telomerase direct assays (Schoeftner and Blasco, 2008), (Luke et al., 2008), (Redon et al., 2010). In a study using alternative human cellular systems such as tiTEL system, which aimed to determine whether *TERRA* affect TA *in vivo*, demonstrated telomerase led telomere elongation is not affected by the transcription of the telomeres in human cancer cell lines such as HCT116 (human colon adenocarcinoma) and HeLA cells (human cervical cancer) (Farnung et al., 2012). In these cells, it is postulated that shortening of telomeres may not be due to telomerase inhibition, but due to impaired replication due to integrity of the chromosomes affected by high levels of *TERRA*s (Farnung et al., 2012).

- ***TERRA* and carcinogenesis**

TERRA levels have been studied in various cancers, and they have been found to be closely related to tumourigenesis and levels appear to vary between individual cancer types.

Cancers with high *TERRA* levels

TERRA levels in human ovarian normal, cancer (primary and metastatic) samples were compared by Deng et al in their study. They reported that ovarian cancers (both primary and metastatic) had higher levels of *TERRA* compared to normal ovarian tissue (Deng et al., 2012). The same study also compared primary human cancers and matched normal tissue control biopsies from stomach, lung, and colon. This reiterated that *TERRA* levels were higher in cancers compared to matched tissue samples (Deng et al., 2012). *TERRA* levels were analysed for several different telomeres by chromosome specific quantitative reverse transcription PCR (qRT-PCR), however there were no consistency across which telomere *TERRA* was elevated, even if cancers were from the same organ. Therefore, they concluded that *TERRA* expression is increased in different human cancers, and they can be expressed from any chromosome, but this was variable amongst different cells and cancer types (Deng et al., 2012). Recently, Bae et al reported a higher 18p *TERRA* levels in colorectal cancers, and that was associated with long telomere length and seems to be tentatively significant, independent prognostic factor for long term oncologic outcomes (Bae et al., 2019). In a lung cancer study using the TCGA repository, levels of *TERRA* 15q, 1q-2q-10q-13q, and *TERRA 20q* were evaluated in tumour and normal

adjacent tissues of patients with non-small cell lung cancers (NSCLC) (Storti et al., 2020). They also reported an increase in *TERRA* 1q-2q-10q-13q levels and borderline increase in *TERRA 20q* levels in lung squamous cells cancers (LUSC). They finally concluded that telomere maintenance genes were differentially expressed in NSCLC and hence *TERRA* may be a potential biomarker for LUSC (Storti et al., 2020)

Cancers with low *TERRA* levels

Schoeftner and Blasco compared different stages of human cancers in larynx, colon and lymph node with normal tissue, wherein they reported that TelRNAs were significantly downregulated in advanced stages. However, in the information provided in their paper, the results from normal tissue have been compared to low and high grade tumour rather than stages of cancer (Schoeftner and Blasco, 2008). In another study, Sampl et al reported total *TERRA* levels in grade 4 glioblastoma multiforme to be 14-, 31-, 313-fold lower when compared with grade 3, grade 2, and non-malignant tissue respectively (Sampl et al., 2012). Amongst the 2p and 18p specific *TERRA* levels, only 2p *TERRA* levels correlated with tumour grade, hence they concluded that *TERRA* expression in gliomas to be chromosome specific and epigenetically regulated (Sampl et al., 2012). Another study on mice osteoblasts, demonstrated that *TERRA* transcription to be Rb1 dependent, that *TERRA* is upregulated by p53 to protect genomic stability and that Rb1 loss resulted in decrease in *TERRA* transcription, which affects the telomere homeostasis (Tutton et al., 2016), (Gonzalez-Vasconcellos et al., 2017). Other sarcoma type tumours such as Ewing's sarcoma and liposarcoma overexpress the RGG-containing proteins, which were able to interact with G-quadruplex *TERRA* structure (Takahama et al., 2013), (Takahama and Oyoshi, 2013).

It has been recently found that hepatocellular carcinoma (HCC) to be associated with significantly low levels of *TERRA* compared to peritumour tissues (Cao et al., 2020). In HCCs, low *TERRA* levels were associated with significantly poor disease free survival and overall survival than those HCCs that were associated with high *TERRA* levels (Cao et al., 2020). In the same study, *TERRA* levels positively correlated with TRF1, and inversely correlated with TRF2. This study concluded that downregulation of *TERRA* promoted HCC cell growth and metastasis both *in vivo* and *in vitro* due to increased TA and promoted telomere elongation (Cao et al., 2020).

Cancers with variable TERRA levels:

A study in head and neck squamous cell carcinomas (HNSCC) could be grouped into cancers with low *TERRA* and cancers with high/similar levels of *TERRA* when compared to adjacent normal tissue (Vitelli et al., 2018). Tumours with lower *TERRA* levels had worse clinical outcomes, and compared to this group, the tumours with higher levels *of TERRA* had a better prognosis. With these results, the authors felt that *TERRA* levels can be considered to be a potential molecular prognostic marker for HNSCC (Vitelli et al., 2018).

Glioblastoma multiforme tumours with detectable TA and short telomeres had worse prognosis, whereas tumours with low or absent TA in presence of long telomeres (indicating and active ALT mechanism), were associated with a favourable prognosis (Sampl et al., 2012). Detectable TA and low *TERRA* levels predicted a worse survival outcome, but on the other hand, undetectable TA and high *TERRA* levels were associated with the best survival outcome (Sampl et al., 2012), therefore they concluded that, *TERRA* levels together with TA in astrocytomas may have a prognostic significance.

In different types of cervical cancer cell lines, there was an abundance of *TERRA* and their stability was variable amongst different cervical cancer cells. However, there was no correlation of telomere length with *TERRA* levels (Oh et al., 2017).

TERRA levels were high in tumours such as sarcomas and gliomas, that are associated with relative longer TL and the same was also true with somatic alterations in ATRX/ DAXX genes as they have been related to ALT telomere maintenance mechanism (Barthel et al., 2017).

Considering the evidence from different studies, the observed variation at least partly may be due to factors such as the differences in techniques used to assess the *TERRA* expression. They include RNA fluorescence in situ hybridisation (RNA FISH), northern blotting, qRT-PCR, dot blotting. However, it is likely that the *TERRA* expression in human cancers is complex and depends on the cancer type, the telomerase and telomere biology of the cancer types and so some of the patient co-morbidities (Deng et al., 2012).

With this background of *TERRA* in carcinogenesis, I aimed to explore whether *TERRA* was expressed in human endometrium, examined the alteration of *TERRA* levels specific to endometrial carcinogenesis, using patient derived biospecimens.

Chapter 2: Hypotheses

Research Hypotheses considered in this thesis

- Pre analytical variables, such as timing of endometrial samples and method of sampling, change endometrial gene expression analysed by qPCR in EC samples
- Harmonisation of the process of biobanking of biosamples relevant to EC research is possible
- Core component of telomerase enzyme, *hTERC* levels are altered with endometrial carcinogenesis and thus may be a relevant biomarker of EC
- Long noncoding *TERRA* levels composed of subtelomeric and telomeric repeat sequences are also altered with endometrial carcinogenesis and they may be biomarkers of EC
- Since EC is a hormone responsive cancer, endometrial *hTERC* and *TERRA* transcription is hormonally regulated

Chapter 3: Preanalytical variables and their effect on gene expression in endometrial biospecimens

Introduction

With 99,000 new cases per year in Europe (2012) and nearly 10,000 in the United Kingdom alone (2016-2018), EC is the most common gynaecological malignancy. Over the last decade, incidence rates have increased by over 50%, making EC the fourth most common malignancy in females in the UK (CRUK, 2016 - 2018), (Lortet-Tieulent et al., 2018). Survival in EC is 90% in stage 1, thereafter it decreases in every stage to 15% in stage 4 (Office for National Statistics, 2019). Endometrial research is fundamental to discover and improve our understanding of the pathogenesis of EC, particularly with a focus on novel prognostic indicators and therapeutic targets. Biospecimens from patients are essential to conduct translational research to achieve this. Therefore, biobanks play a vital role in EC research for the translation of laboratory scientific findings to clinical practice.

The use of banked human tissue represents an invaluable resource for the understanding of the underlying pathogenesis in EC (Lee et al., 2015b). However, the validity of data generated with patient samples depends greatly upon specimen quality. Many variables influencing human tissue biopsies can result in altered protein and gene expression due to RNA degradation (Jewell et al., 2002). Wide variation in the collection, processing and storage can results in divergent and irreproducible data (Adishesh and Hapangama, 2019). Inequality of biospecimens is one of the biggest flaws in biomarker discovery, and this can lead to bias prior to analysis (Ransohoff and Gourlay, 2010). Harmonisation of biobanking standards is vital to facilitate data sharing, thus expedited advances in research (Sheldon et al., 2011), (Adishesh et al., 2017), (Adishesh and Hapangama, 2019).

Pre-analytical variables are defined as factors which have an effect on tissue samples before analysis, during sample collection, processing, or storage. The understanding of pre-analytical factors is necessary because the molecular composition of tissues may be altered with their variance, ultimately affecting downstream analysis (Grizzle et al., 2016). Numerous studies have reported many factors influencing gene and protein expression data, these are wide ranging and may be biobanking specific or secondary to the patient characteristics (Almeida et al., 2004), (Ma et al., 2012), (Lee et al., 2015b), (Grizzle et al., 2016), (Pedersen et al., 2018).

Warm ischaemia is defined as the time a tissue is at body or room temperature with a compromised blood supply before tissue collection and processing and it is proposed to significantly alter gene and protein expression (Huang et al., 2001), (Schlomm et al., 2008), (Gündisch et al., 2015), (Grizzle et al., 2016), (Pedersen et al., 2018). Post-hysterectomy

endometrial biopsies undergo warm ischaemia as a result of a delay from devascularisation to resection of the uterus and eventual tissue collection.

In this study we report the effect of pre analytical variables such as timing of the sample collection (pre or post hysterectomy) and the different sampling methods (pipelle samples or full-thickness biopsies) on the gene expressional analysis in the endometrial samples. Since, I intended to examine the expression of the mRNA levels for hTERC and TERRA in my PhD project, the effects of the pre-analytic variables considered here on the gene expression levels would inform the best methods of sample collection and sample selection for the studies described later in the thesis.

Therefore, in this study, we examined the expression of three downstream genes regulated by HIF-1. HIF-1 generates an inflammatory response and angiogenesis in response to hypoxia through transcriptional activation of angiogenic genes, such as downstream target and gene of interest, vascular endothelial growth factor A (*VEGFA*) and carbonic anhydrase 9 (*CA9*). *VEGFA* is a dominant inducer of blood vessel growth, mediating angiogenesis from pre-existing vessels (Shweiki et al., 1992). The enzyme *CA9*, is a transmembrane protein induced by hypoxia thus, is also a cellular biomarker of hypoxia, particularly in tumours and one of the most sensitive endogenous sensors of hypoxia inducible factor 1 (HIF-1) activity (Kaluz et al., 2009). Progesterone receptor (*PR*) protein is typically reduced in advanced EC and is associated with poor prognosis (Yang et al., 2014) and has been chosen for analysis because of its prognostic relevance. Furthermore, it also is a hypoxia related gene regulated by HIF1 (Henriquez et al., 2017).

Overall, there are limited studies analysing the effects of pre-analytical variables in patient derived bio-samples and they are particularly unexplored in the endometrium. Patient tissue samples demand high standards of biobanking for their effective use in translational medicine. Stringent and consistent standards for collecting biospecimens are paramount and current practices must be validated. We require a better understanding of pre-analytical variables to meet necessary standards and minimise the effect of specimen handling on expressional analysis. The aim of this study is to examine the effect of pre analytical variables on downstream analysis on benign endometrium and EC samples.

Methods

Patient population

Details of the Ethical approval is described in page 15. Group 1 consisted of 16 women undergoing hysterectomy for benign pathologies (e.g., prolapse, heavy menstrual bleeding,

without known endometrial abnormalities) including 4 myometrial samples and Group 2 included 31 women with a diagnosis of EC undergoing hysterectomy as the surgical treatment, not having had previous chemo/radiotherapy (Table 1). Pre hysterectomy samples were taken using a pipelle endometrial suction curette after induction of anaesthesia in all women. Post hysterectomy samples were collected immediately after the uterus was removed from the patient in theatre. From the surgically removed uterus endometrial samples were collected using two previously described and well-established methods; one using a pipelle in both groups and a further full thickness, post-hysterectomy biopsy was harvested only from women in Group 1 (Table 1). The full thickness biopsy was taken by removing a wedge shaped full thickness section of endometrial mucosa spanning the lumen to the myometrium, including both functional and basal layers of the endometrium.

Table 1. Groups of patients and types of endometrial samples collected

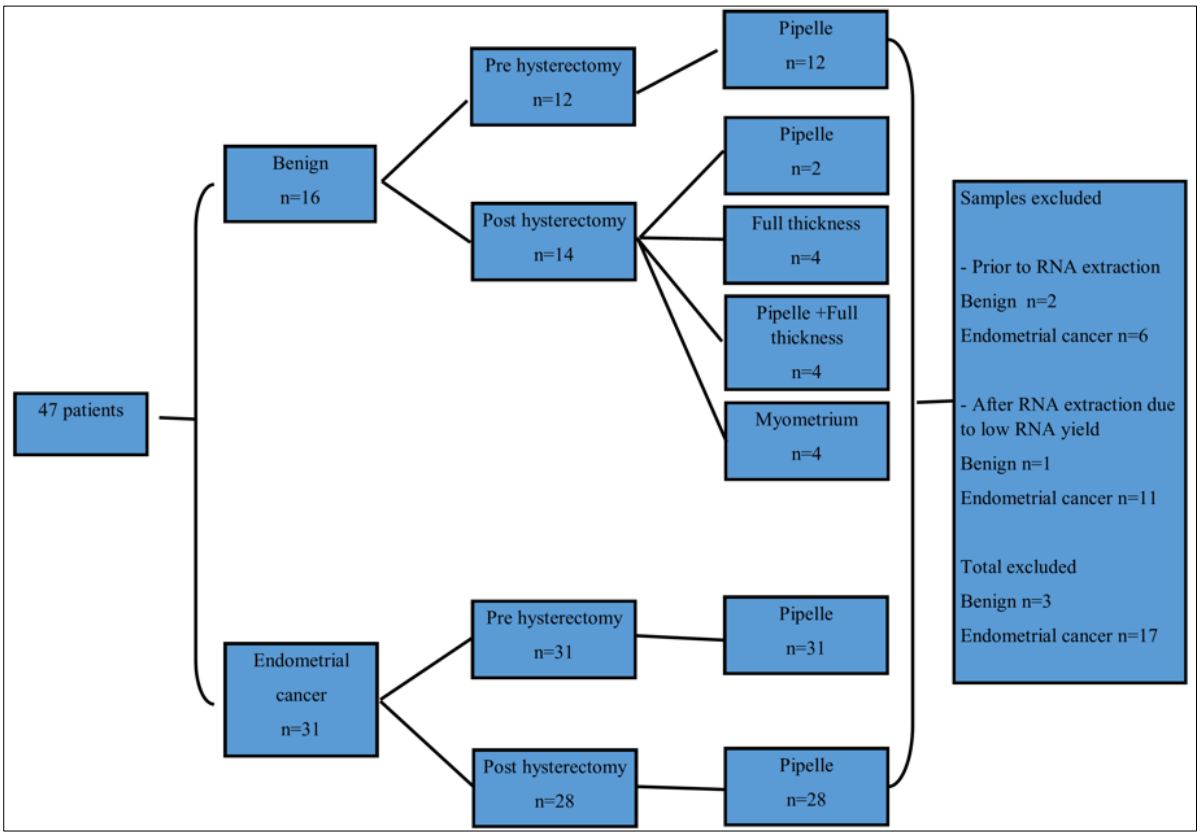
Group 1: Benign pathologies	Pre hysterectomy	Pipelle endometrial samples, n = 12
	Post hysterectomy	Pipelle endometrial samples only, n = 2 Full thickness and pipelle endometrial samples, n = 4 Full thickness endometrial samples only, n = 4 Myometrial samples, n = 4
Group 2: EC	Pre hysterectomy	Pipelle samples, n = 31
	Post hysterectomy	Pipelle samples, n = 28

Out of 12 benign pre hysterectomy samples, two samples were excluded as one of the samples did not have sufficient amount of tissue, but contained mainly blood, and the second sample had poor quality of RNA. Additionally in another recruited woman, we only had the post hysterectomy samples of a pipelle and full thickness biopsy but did not have paired pre hysterectomy pipelle sample. This patients samples were included in the analysis to compare the data derived from matched post hysterectomy samples collected using the 2 different methods. Amongst the 31 EC samples, six were excluded; because four were unpaired (only pre or post hysterectomy samples were obtained), one sample contained complex atypical hyperplasia only, and one pair was removed due to them not being labelled legibly (excluded n=6). Of the remaining 25 EC samples, 11 samples were excluded from PCR analysis as the RNA quality was poor. Therefore, the final analysis includes mRNA levels using qPCR from

9 benign, 14 EC paired samples and four myometrial samples. Details of samples collected, excluded and used for PCR are given in table 2A and 2B.

Following FIGO guidance (Zaino et al., 1995), gynaecological pathologists allocated histological descriptors for EC type and grade. Normal endometrial samples were assigned phases according to histological features and last menstrual date as described by Kamal et al. (Kamal et al., 2016a).

Patient clinico-pathological and demographic details were collected from the patients at the time of recruitment and verified by review of hospital notes and clinical databases. None of the patients received hormonal treatment, chemotherapy, or radiotherapy before surgery. See Table 3 for patient cohorts and demographics.



PCR				
		CA9	VEGFA	PR
Benign	Pre hysterectomy Pipelle	9	9	9
	Post hysterectomy Pipelle	4	2	2
	Post hysterectomy Full thickness	4	4	4
	Post hysterectomy Pipelle + Full thickness	2	4	4
	Myometrium	-	4	4
Endometrial cancer	Pre hysterectomy Pipelle	14	14	14
	Post hysterectomy Pipelle	14	14	14

Table 2B Final number of total samples used for PCR analysis

Table 3 Demographic details of patients included in the study

Sample no	Age	BMI	Hormones	Stage of cycle	PCR
1	43	37	nil	Unknown	Y
2	47	28	nil	Proliferative	Y
3	43	20	nil	Mid cycle	Y
4	37	33	nil	Proliferative	Y
5	40	21	nil	Secretory	Y
6	24	24	nil	Unknown	Y
7	34	32	nil	Unknown	Y
8	34	29	nil	Proliferative	Y
9	40	24	nil	Mid cycle	Y
10	36	31	nil	Proliferative	Y

Sample no	Age	BMI	Type of cancer	Stage of cancer	PCR
1	68	23.9	Endometrioid adenocarcinoma, grade 1	1B	Y
2	79	51.4	Carcinosarcoma	1A	Y

3	76	26.7	Serous carcinoma	1A	Y
4	59	36	Endometrioid adenocarcinoma, grade 1	1A	Y
5	83	-	Carcinosarcoma	1A	Y
6	73	37	Endometrioid adenocarcinoma, grade 1	1B	Y
7	67	23.3	Endometrioid adenocarcinoma, grade 1	1A	Y
8	59	41.9	Endometrioid adenocarcinoma, grade 1	1A	Y
9	73	34	Endometrioid adenocarcinoma, grade 3	1B	Y
10	58	36	Endometrioid adenocarcinoma, grade 1	1B	Y
11	67	28.2	Endometrioid adenocarcinoma, grade 1	1B	Y
12	73	36.3	Endometrioid adenocarcinoma, grade 3	1A	Y
13	74	34	Carcinosarcoma with heterologous differentiation	1B	Y
14	65	25.8	Endometrioid adenocarcinoma, grade 3	1B	Y

Collection and transportation of EC tissue samples

EC tissue samples were collected from patients attending for their primary surgery in theatre, before and after surgery. All EC tissue samples were collected using pipelle sampler. Paired pre and postoperative biopsies were collected from women with a diagnosis of EC immediately before and immediately after the uterus was removed from the patient at hysterectomy and placed in (1) RNA later solution kept on ice and (2) in neutral buffered formalin (NBF) and transferred to the University of Liverpool laboratory at Liverpool Women's Hospital (LWH) using a safe method approved by transport regulations (Recommendations of a United Nations Committee of Experts on the Transport of Dangerous Goods – UN model regulations) immediately for processing.

The pre operative biopsy was fixed immediately after obtaining the sample in the theatre and post operative biopsy sample was obtained as soon as the uterus was removed from the patient and fixed into the above solutions immediately. Immediately following removal the samples were placed in neutral buffered formalin (NBF) or RNA later. RNA later was aspirated prior to storage at -80°C until RNA extraction. Samples in NBF were stored at 4°C for 24 hours and subsequently processed and impregnated in paraffin wax for long term storage at room temperature. The laboratory is situated in the same building as the operating theatres with minimum transport time (typically about 5 minutes on foot).

Tissue processing and storage

After the tissue samples were transported to the laboratory, sample IDs were checked, and the accuracy of labelling of different tubes were re-confirmed by the trained lab personnel

receiving the sample in the lab. The details such as sample ID, date of collection, type of samples and name of the person processing the samples were entered into the sample reception log. Labelled RNA later samples were placed in a refrigerator for 24 hours before the solution was aspirated and cryotube stored in -80°C freezer. The portion of tissue placed in NBF was left for 24 hours in fridge allocated for the sample reception and processed the next day. The freezer log was updated, and location of the tissue noted after each sample was collected and different tubes were stored in -80°C freezer.

RNA extraction and quantification

RNA extraction is an important step in production of complementary DNA (cDNA) for subsequent PCR. RNA can be extracted using multi component kits, which are quick and reliable. Silica-cartridge purification of RNA is the recommended RNA purification method in the gene expression industry. The commonly used TRIzol reagent maintains RNA integrity, disrupts cells, dissolves cellular components, and inhibits RNAses. We used TRIzol Plus RNA Purification Kit (Invitrogen Ltd., Paisley, United Kingdom) following the manufacturer's protocol.

Tissues treated with RNAlater were homogenised using 1 ml of TRIzol. Chloroform (0.2mls) (Sigma, Poole, UK) was added and mixed for 60 sec followed by incubation for 5 min at room temperature. Samples were then centrifugated at 12,000g for 15 min at 4°C. The upper aqueous phase containing RNA was transferred to a new RNAse-free tube. An equal volume of 70% ethanol was then added and vortexed. About 700 µl of RNA/ethanol solution was loaded into a purelink RNA mini kit spin cartridge and centrifuged at 12,000g at room temperature for 30 seconds. The spin cartridge has a silica based membrane to which RNA binds. The flow through was discarded and this step repeated till the whole sample was processed. 700 µl of wash buffer 1 was then added to the cartridge and centrifuged at 12000g for 30 seconds at room temperature. This was then followed by washes with wash buffer 2 and centrifugation to dry the membrane. The spin cartridge was then transferred to new recovery tubes where 30 µl of RNAse free water was added to the centre of the spin cartridge and centrifuged for 2 mins at 12,000g at room temperature. The purified RNA was stored in -80°C freezer.

RNA concentration and purity was determined using NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). A260/A280 ratio of 2.0 was considered as suitable for downstream reactions.

DNAse treatment

This step is to treat the RNA extracted with DNAse enzyme so as to ensure the removal of all genomic DNA. 8µl RNA was treated with 1µl of RQ1 DNAse (DNAse 1 enzyme) in presence of 1 µl 10 X DNAse reaction buffer and was incubated at 37°C for half hour. 1 µl stop buffer (Promega, Hampshire, UK) was added and placed in 65°C for 10 mins to stop the reaction. The 11 µl RNA was then either stored in - 80°C freezer or used for cDNA synthesis.

cDNA synthesis

mRNA isolated was converted to cDNA in a reaction catalysed by reverse transcriptase. The reverse transcriptase enzyme acts on a single strand of mRNA, random hexamers and DNA bases were added to bind to different parts of sequence acting as primer to generate the complementary DNA strand. 1000ng RNA, random hexamer primers and nuclease free water were made up to 8 µl volume in total. This was incubated at 70°C for 5 mins to denature RNA. Further on 10 µl of AMV reaction mix and 2 µl of AMV enzyme mix was added. To the negative control sample, instead of the enzyme mix, 2 µl of nuclease free water was added (no reverse transcriptase (NRT) control). 20 µl mix was then incubated at 25°C for 5 mins, 42°C for one hour. The enzyme was inactivated when incubated at 80°C for 5 mins. This was then diluted with 30 µl of nuclease free water to make 50 µl in total and stored in -20°C freezer till used for PCR.

PCR

The PCR is a commonly used technique for amplification of DNA, cDNA or whole genome. This method was developed by Kary Mullis in 1983. This process uses DNA polymerase enzyme to synthesise complementary new strand of DNA from the substrates. DNA polymerase adds nucleotides to the 3` end on a custom-made oligonucleotide strand when it is annealed to a longer DNA template. The steps in PCR were: 1) Denaturation - DNA template was heated to 94°C. This breaks the weak hydrogen bonds that hold DNA strands together, creating two separate single DNA strands. 2) Annealing – The mixture was cooled to 50-70°C. This allows the primers to bind to their complementary sequence in the template DNA. 3) Extension – The reaction was heated to 60°C, which is the optimal temperature for the DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer using the DNA as template. In one cycle, single DNA strand was amplified to two double-stranded DNA, which are further amplified. As cycles go on, more copies are generated, and the increase is exponential.

qRT-PCR and optimisation

This type of PCR allows both detection and quantification of DNA. The quantity can be absolute number of copies or a relative amount when normalised to a reference gene. The qRT PCR procedure is similar to PCR, the only difference being that the amplified DNA is detected at the end. Products are detected by a nonspecific fluorescent dye such as SYBR Green, that binds to the double stranded DNA. Increase in DNA product leads to increase in fluorescence intensity and is measured each cycle, thus quantifying the amount of DNA produced. All sample preparation is carried out on ice in an RNase/ DNase free area, using DNase/RNase free sterile filter pipette tips. All reagents needed were removed and thawed on ice. Before commencing any work, the PCR hood was cleaned with trigene and ethanol and UV light treated for 10 minutes. In each run, in addition to target samples, a no-template control and a no RT control were also run. To prevent pipetting error, master mixes were produced (containing iTaq, forward, reverse primers and nuclease free water). For each reaction 3 technical replicates were performed to ensure results were reproducible. For every template sample a reference gene primer was run. Housekeeping genes like peptidylprolyl Isomerase A (PPIA), actin were used to ensure the same amount of cDNA is loaded between template samples. 9µl of master mix was added into each well and 1µl of cDNA is added in triplicates into separate wells to make the final volume of 10 µl in each well. For no RT control 1µl of NRT control sample and for no template control 1 µl of nuclease free water were added. After all samples were added into the 96 well plate, it was covered with adhesive plate sealing film. Once centrifuged to make sure the samples are at the bottom of the well, the plate is loaded onto the thermocycler. The reaction was set as per conditions optimised for each target at 40 cycles. There are different methods for analysing the qPCR data, the $\Delta\Delta C_T/\Delta\Delta C_q$ method assumes the primers of the target gene and reference gene are 100% efficient giving a relative quantification. The Pfaffl method uses standard curve to determine the efficiency of the primer. Primers were used with different concentrations of cDNA; a linear graph is produced and the correlation coefficient and efficiency of the primers calculated from the slope ($E=10^{(-1/slope)}$). The last method is the standard curve method which uses same principle as the Pfaffl method but involved doing a standard curve in every reaction. The Pfaffl method was used for all qPCR based work in this study.

Forward and reverse primers and reaction conditions are listed in table 3 and table 4 respectively.

Relative mRNA transcript expression for *VEGFA*, *CA9* and *PGR* was calculated using the ΔΔCT method, normalised to the reference genes, beta actin (ACTB) and PPIA using Bio Rad CFX Manager (Bio-Rad, Hertfordshire, UK).

Gene expression analysis

For gene expression studies, relative quantification is considered to be the best method. $\Delta\Delta C_t$ method is the post popular method used to compare the results of the samples with a calibrator and the geometric mean of the normalisers. Both the sample and calibrator are adjusted in relation to the mean of the normalisers C_t from the same samples. The resulting $\Delta\Delta C_t$ value is incorporated to determine the fold difference in the expression.

$$\text{Fold differences} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta C_t = \Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{calibrator}}$$

$$C_{t\ \text{sample}} = \Delta C_{t\ \text{target}} - \Delta C_{t\ \text{reference}}$$

$$C_{t\ \text{calibrator}} = \Delta C_{t\ \text{target}} - \Delta C_{t\ \text{reference}}$$

Table 3. Primer Information

Target	Primer Sequence/Assay Information		Primer Mixes	Company	Reference
PPIA	Fwd	5'-AGACAAGGTCCCAAAGAC-3'	Separate: fwd and rev	Sigma	(Jacob et al., 2013)
	Rev	5'-ACCACCCTGACACATAAA-3'			
ACTB	Fwd	5'-TGTACGCCCAACACAGTGCTG-3'	Separate: fwd and rev	Sigma	(Arnoult et al., 2012)
	Rev	5'GCTGGAAGGTGGACAGCGA-3'			
PGR	Fwd	5'-CAGTGGGCGTTCCAAATGA-3'	Combined	Sigma	(Henderson et al., 2003)
	Rev	5'-TGGTGGAATCAACTGTATGTCTTGA-3'			
VEGFA	Assay	Assay ID: qHsaCED0043454 RefSeq: NC_000006.11, NG_008732.1, NT_007592.15	Combined	Bio-Rad	Commercially available
CA9	Assay	Assay ID: qHsaCID0017667 RefSeq: NC_000009.11, NG_011620.1, NT_008413.18	Combined	Bio-Rad	Commercially available

Statistical analyses

Data across different groups was analysed using Graph Pad Prism 5 (Graph Pad Prism software, La Jolla California, USA) using non-parametric tests (Mann-Whitney U test and /or Wilcoxon matched paired tests). The criterion for significance was $p \leq 0.05$.

Table 4. qPCR reaction conditions

			Initial Denature	Denature	Anneal/ Extension	Cycles	Extension
Target Gene	**PPIA**		95°C 2min	95°C 5s	60°C 30s	40	
	ACTB		95°C 10 min	98°C 5s + 95°C 10s	60°C 30s	40	
	PGR		95°C 6min	95°C 10s	60°C 20s	40	72°C 30s
	VEGFA		95°C 2min	95°C 5s	60°C 30s	40	
	CA9		95°C 2min	95°C 5s	60°C 30s	40	

Abbreviations: *PPIA*, peptidylprolyl isomerase A; *ACTB*, beta-actin; *PGR*, progesterone receptor; *VEGFA*, vascular endothelial factor A; *CA9*, carbonic anhydrase 9.

Results

VEGFA and *PR* mRNA levels were significantly increased in the endometrial samples taken after hysterectomy

When the pre-hysterectomy endometrial biopsies were compared with the paired post-hysterectomy biopsies (n=23 pairs), the *VEGFA* (P=0.005) and *PR* (P=0.05) mRNA levels were significantly increased in the post hysterectomy samples (Figure 1). This sample set included biopsies from women with and without endometrial pathology. There was no significant difference in *CA9* mRNA level between the pre and post hysterectomy biopsies.

The observed difference in gene expression was apparently specific to endometrial samples from women without EC

To assess if the observed difference in gene expression levels associated with the timing of the endometrial sampling was specific to either benign or EC, the study cohort was split into benign and cancer groups and analysed separately. In the benign group, pre-hysterectomy pipelle endometrial biopsies (n=9) were compared with the paired post-hysterectomy pipelle biopsies (n=6) while the cancer samples, pre and post hysterectomy were all pipelle samples.

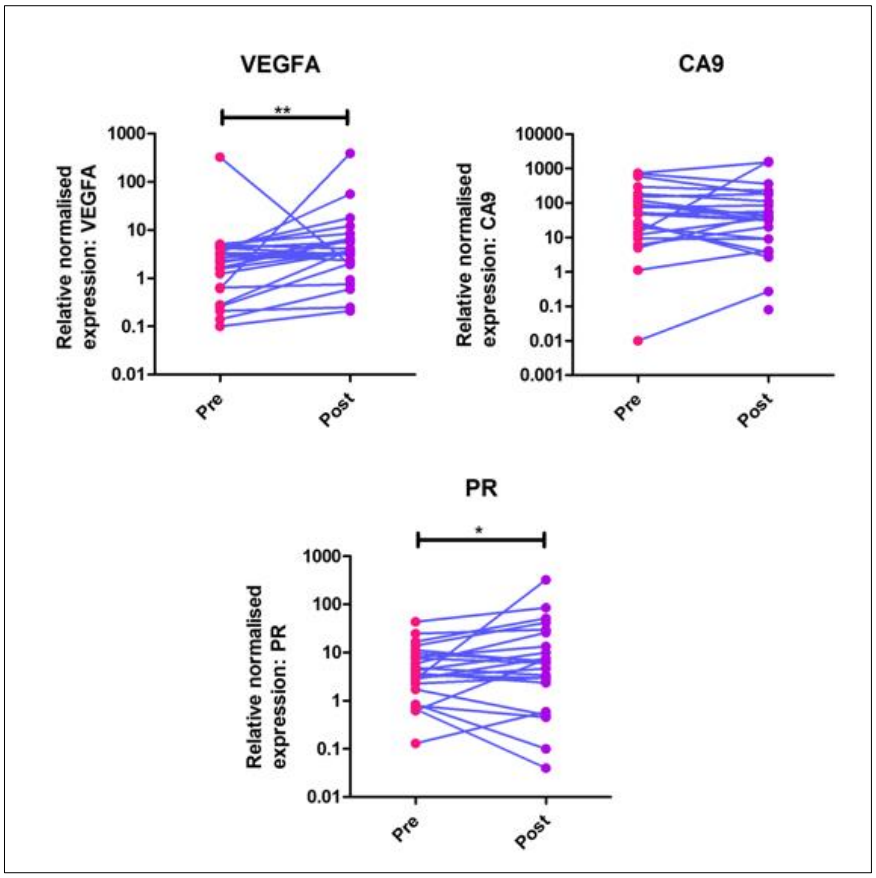


Figure 1. Effect of timing of endometrial samples on *VEGFA*, *CA9* and *PR* expression levels. When pre hysterectomy samples were compared with paired post hysterectomy samples (n=23), there is significant increase in *VEGFA* (**P= 0.005, Wilcoxon matched paired test) and *PR* (*P= 0.05, Wilcoxon matched paired test) levels in post hysterectomy samples whereas no significant difference was observed in *CA9* levels between pre and post hysterectomy samples.

Amongst the benign endometrial samples, there was an apparent but not statistically significant increase in *VEGFA* (P= 0.06) mRNA levels in the post hysterectomy pipelle samples, however, there was limited number of post hysterectomy pipelle samples available for the analysis (Figure 2).

When the EC group was considered, the expression levels of *VEGFA*, *CA9* and *PR* in pre (n=14) and post hysterectomy pipelle (n=14) samples were similar (Figure 3).

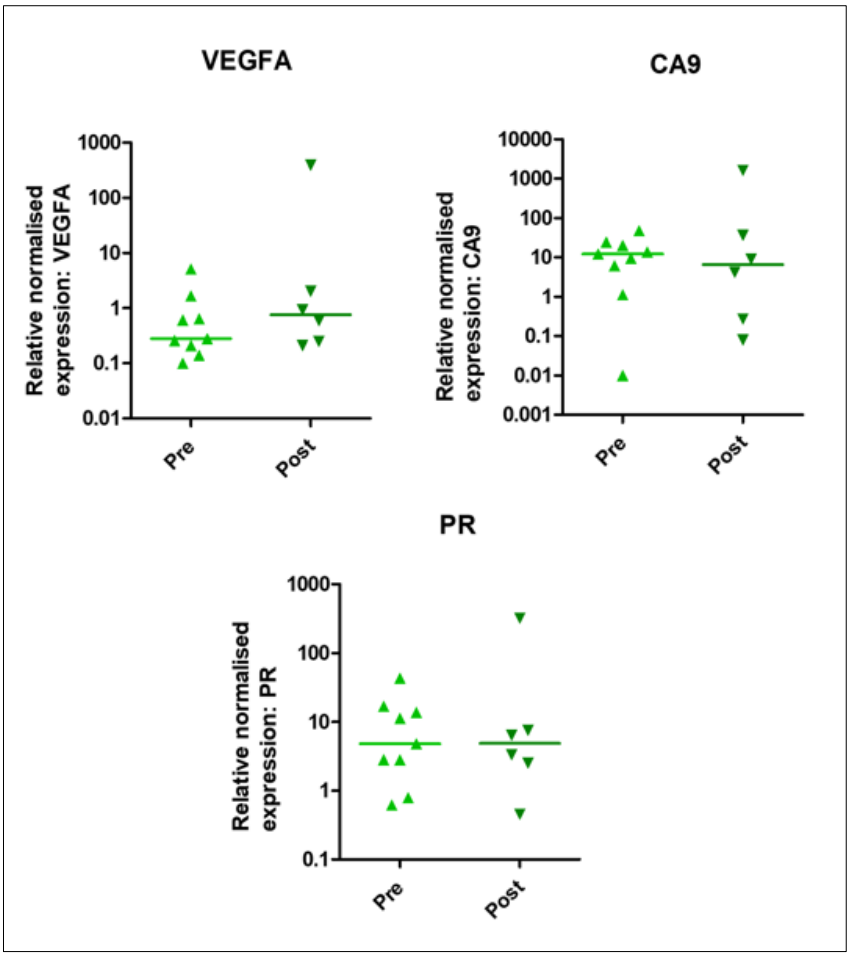


Figure 2. Effect of timing of samples on *VEGFA*, *CA9* and *PR* expression levels in Benign endometrial samples. When pre hysterectomy samples (n=9) were compared with paired post hysterectomy samples (n=6), there was a slight increase in *VEGFA* (P=0.06, Wilcoxon matched paired test), *CA9* (P= 0.19, Wilcoxon matched paired test) and *PR* (P=1, Wilcoxon matched paired test) levels in post hysterectomy samples.

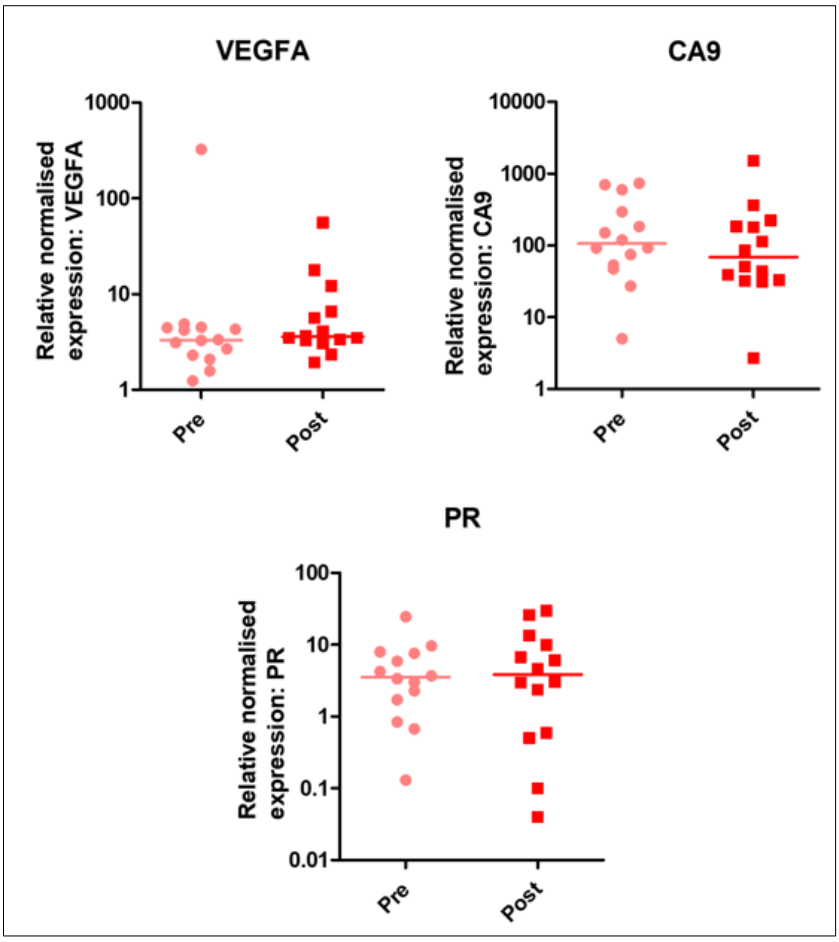


Figure 3. Effect of timing of samples on *VEGFA*, *CA9* and *PR* expression levels in EC samples. When pre hysterectomy samples (n=14) were compared with paired post hysterectomy samples (n=14), there was no significant difference in *VEGFA* (P= 0.09, Wilcoxon matched paired test), *CA9* (P= 0.10, Wilcoxon matched paired test) and *PR* (P= 0.43, Wilcoxon matched paired test) levels in post hysterectomy samples.

Use of different sampling methods in benign endometrial samples shows significantly higher *VEGFA* and *PR* expression in full thickness samples

Comparison of pre hysterectomy endometrial pipelle samples with post hysterectomy full thickness endometrial samples in healthy women with benign gynaecological conditions showed a significant upregulation of *VEGFA* and *PR* (*VEGFA* **P=0.002, *PR* *P=0.02) mRNA levels. Subsequently, we investigated whether this upregulation of *VEGFA* and *PR* could be due to myometrial contamination that is possible in full thickness endometrial samples. A significant increase in *VEGFA* mRNA expression levels were observed in myometrium samples when compared to pre-hysterectomy pipelle samples (*VEGFA* *P=0.01). In contrast, there was no significant difference in myometrial *PR* mRNA expression levels when compared to the pre hysterectomy samples (*PR* P=0.11) (Figure 4).

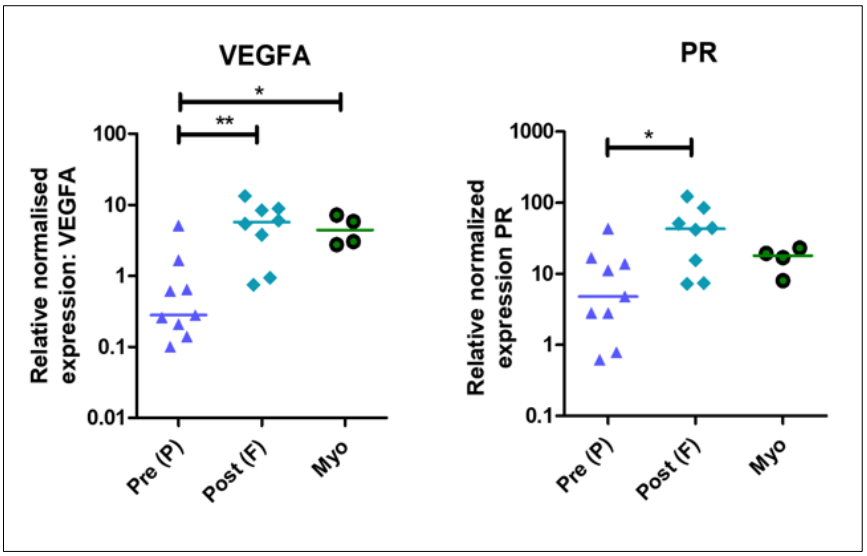


Figure 4. Comparison of *VEGFA* and *PR* expression levels in benign pre hysterectomy pipelle (Pre P), post hysterectomy full thickness (Post F) and myometrial (Myo) samples. *VEGFA* expression significantly high in post hysterectomy full thickness samples and myometrial samples compared to pre hysterectomy pipelle samples (pre hysterectomy pipelle endometrial samples vs post hysterectomy full thickness endometrial samples **P=0.002, Mann Whitney test; pre hysterectomy vs myometrium samples *P=0.01, Mann Whitney test). *PR* expression was significantly high in post hysterectomy full thickness samples but no significant difference in myometrial samples compared to pre hysterectomy pipelle samples (pre hysterectomy pipelle endometrial samples vs post hysterectomy full thickness endometrial samples *P=0.02, Mann Whitney test; pre hysterectomy vs myometrium samples P=0.11, Mann Whitney test).

Testing the hypothesis that EC studies can be affected by imprecise timing of sample collection and variable sampling methods

We examined the effects of preanalytical variables of timing and the endometrial tissue harvesting method on a typical biomarker study examining the differential expression of two biomarkers *VEGFA* and *CA9* in EC, to test the above hypothesis. The data were analysed in the following three possible ways to illustrate how these variables can affect the final results obtained in a typical biomarker study.

Example 1. Differential expression of genes of interest when endometrial samples were collected before hysterectomy (Figure 5)

When *VEGFA*, *CA9* and *PR* mRNA expression levels are measured between benign and cancer samples collected prior to the hysterectomy using pipelle endometrial samplers, a significant upregulation of *VEGFA* and *CA9* levels (*VEGFA* P=0.003, *CA9* P=0.001; Mann Whitney test) were observed in the cancer samples, in comparison with the benign endometrium. There was

a slight downregulation of *PR* mRNA levels however this difference was not statistically significant (P=0.47).

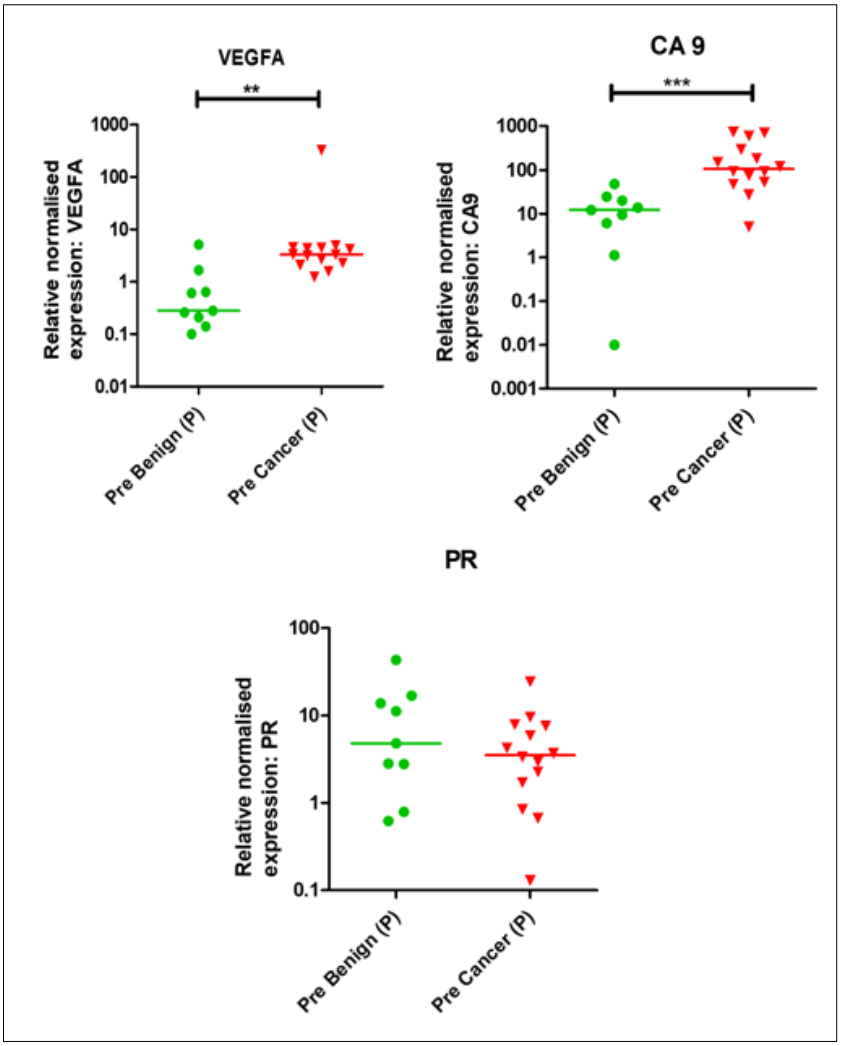


Figure 5. Comparison of effect of timing of samples on *VEGFA*, *CA9* and *PR* mRNA expression levels by qPCR in normal and EC pipelle pre hysterectomy samples. When pre hysterectomy normal pipelle samples (n=9) were compared with pre hysterectomy EC pipelle samples (n=14), there was significant upregulation in *VEGFA* (**P=0.003, Mann Whitney test), *CA9* (***P= 0.001, Mann Whitney test) but no significant drop in *PR* (P=0.47, Mann Whitney test) expression levels in pre hysterectomy cancer samples.

Example 2. When endometrial samples were collected after hysterectomy (Figure 6)

Contrastingly, when pipelle samples were collected from a uterus that was detached from the patient at the end of hysterectomy, there was a significant downregulation in *VEGFA* mRNA levels was observed in cancer samples (P= 0.03, Mann Whitney test) when compared with the

benign endometrial samples. However, there was no significant difference in *CA9* and *PR* levels.

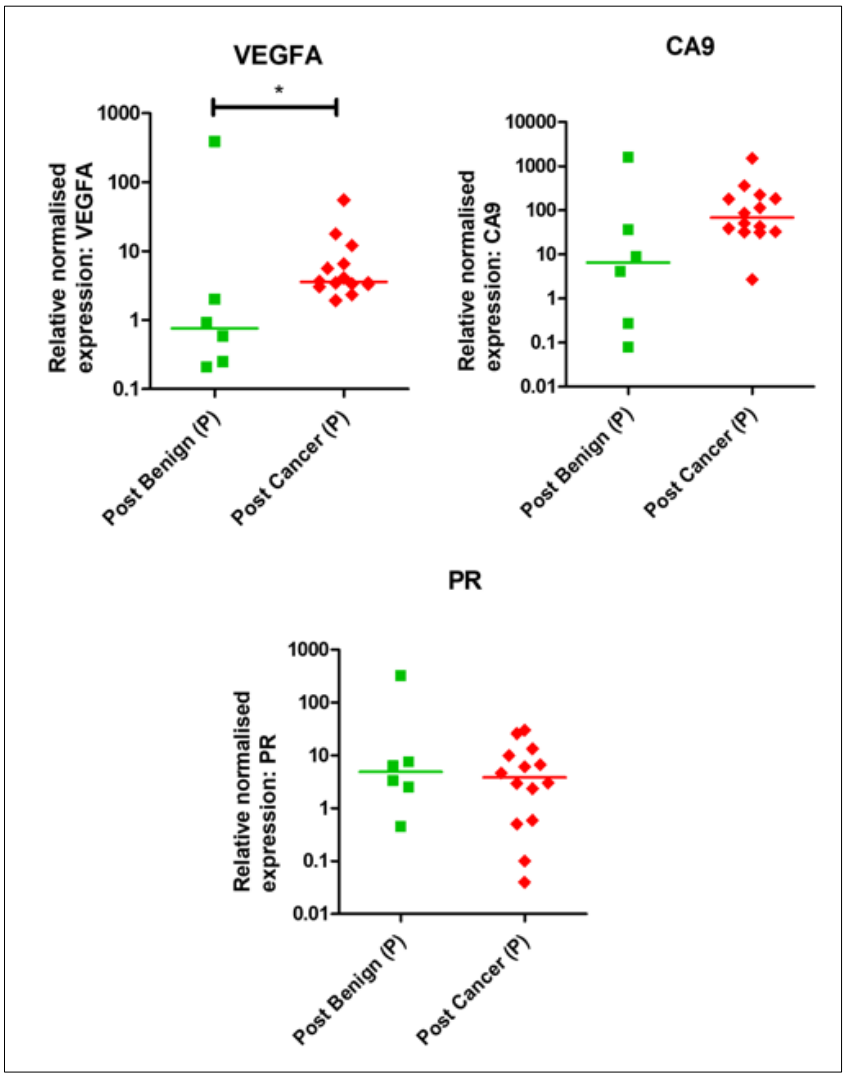


Figure 6. Comparison of effect of timing of samples on *VEGFA*, *CA9* and *PR* expression levels in benign and EC pipelle post hysterectomy samples. When post hysterectomy normal pipelle samples (n=6) were compared with post hysterectomy EC pipelle samples (n=14), there was significant upregulation in *VEGFA* (*P= 0.03, Mann Whitney test) but no significant change in *CA9* (P=0.08, Mann Whitney test) and *PR* (P=0.71, Mann Whitney test) expression levels in post hysterectomy samples.

Example 3. When benign endometrial samples were collected either as full thickness biopsies or as pipelle biopsies after hysterectomy (Figure 7)

In patients with no known endometrial pathology, there were two endometrial tissue sampling methods that could be used, a pipelle sampler or taking a full thickness wedge biopsy. These are commonly utilised methods in benign endometrial research (Armstrong et al., 2017), (Maclean et al., 2020). The uterus should only be opened by a trained pathologist in cases of

suspected or known cancer for clinical staging that has a direct influence on further treatment and predicting prognosis. Therefore, in cancer patients, researchers usually collect a pipelle endometrial sample for immediate placement in RNA later for qPCR studies, preserving the uterus intact for pathological staging. When the full thickness post hysterectomy benign endometrial samples were compared with post hysterectomy cancer samples obtained with the pipelle sampler, there was a significant increase in *CA9* mRNA levels (P= 0.012, Mann Whitney test) and a significant decrease was observed in *PR* levels (P= 0.002, Mann Whitney test).

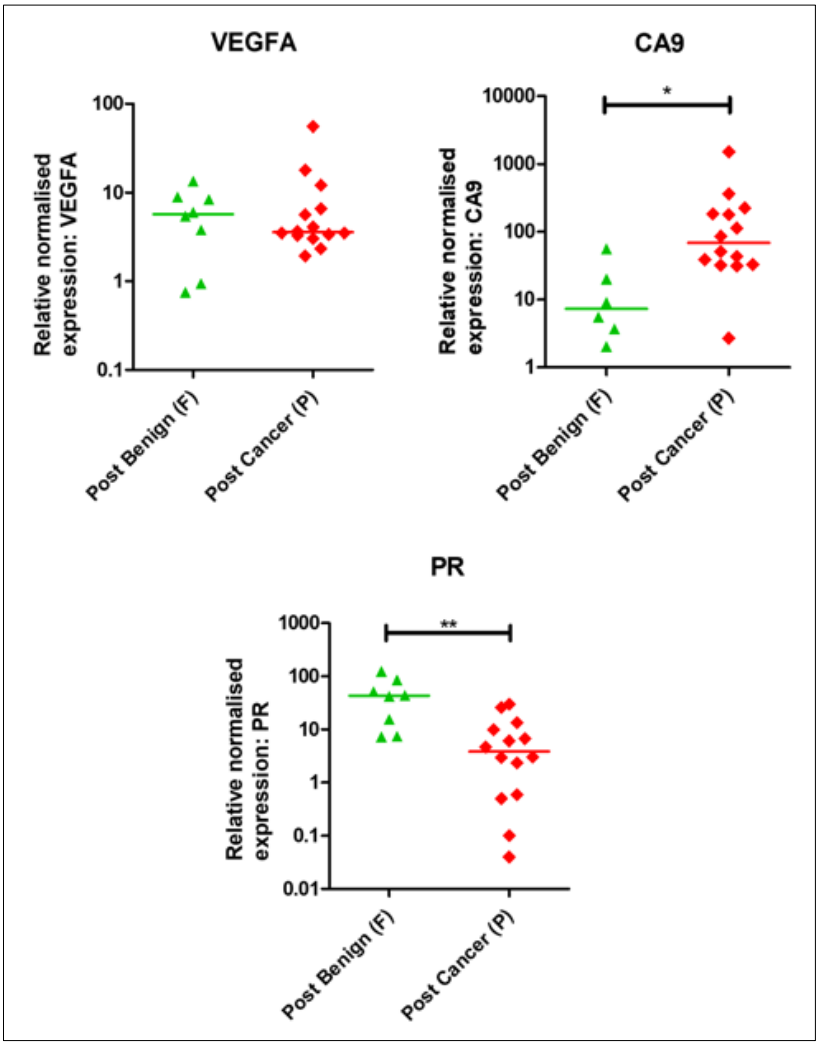


Figure 7. Comparison of effect of timing of samples on *VEGFA*, *CA9* and *PR* mRNA expression levels in normal post hysterectomy full thickness endometrial samples and EC pipelle post hysterectomy samples. When post hysterectomy normal full thickness endometrial samples (n=8) were compared with post hysterectomy EC pipelle samples (n=14), there was significant upregulation in, *CA9* (*P= 0.01, Mann Whitney test) and significant downregulation in *PR* (**P=0.002, Mann Whitney test) but no significant change in *VEGFA* (P=0.76, Mann Whitney test) expression levels in post hysterectomy samples.

PR expression is significantly reduced in high grade cancers independent of the timing of the biopsy

Amongst different grades of ECs, *PR* expression is significantly reduced in high grade cancers (HG) independent of the timing of the biopsies (low grade (LG) vs HG pre hysterectomy *P=0.03; LG vs HG post hysterectomy **P=0.01; Mann Whitney test) (Figure 8).

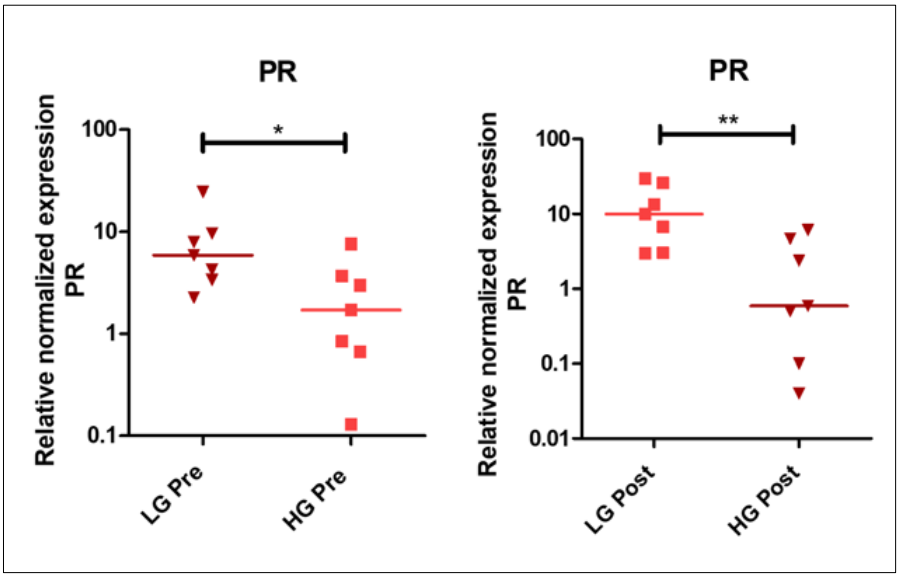


Figure 8. Comparison of *PR* mRNA expression levels in low grade (LG) versus high grade (HG) EC pre and post hysterectomy (Pre and Post) samples. *PR* expression was significantly low in HG pre (n=7) and post (n=7) samples when compared with LG Pre (n=7) and Post (n=7) samples respectively (*PR* Pre LG vs HG *P=0.03, Mann Whitney test; *PR* Post LG vs HG **P=0.01, Mann Whitney test).

Discussion

Traditionally, endometrial samples for research are obtained after hysterectomy. Since some previous reports suggest that warm ischaemia may affect gene expression, this study was designed to examine the effect of preanalytical variables such as timing of endometrial samples either pre/post hysterectomy, and sampling methods in addition to the disease processes on three endometrial bio-markers in human endometrial samples. The samples were collected from women with and without EC , before and after hysterectomy using two different sampling methods. Our data has highlighted important effects of the time and the method employed in harvesting endometrial sample will affect the results, in addition to the difference relevant to EC, i.e., the disease process.

Post-hysterectomy endometrial samples are influenced by warm ischaemia due to the delay between devascularisation through the clamping of both uterine arteries, and subsequent sample collection from the surgically resected uterus, removed from the patient. Several previous studies examining the liver and the prostate have shown that warm ischaemia has a significant impact on different proteins and gene expression (Schlomm et al., 2008), (Gundisch et al., 2012).

When examining the effects of the pre analytical variable of time on expression of the genes of interest, we found that the timing of the biopsy had significant effect on markers, *VEGFA* and *PR* levels. This change seen between pre and post hysterectomy samples is most likely due to effect of warm ischaemia on gene expression. The expression of *VEGFA*, *CA9* and *PR* mRNA levels were detected to be apparently higher in benign post hysterectomy samples and this is possibly due to the influence of warm ischaemia. However, further studies including a larger non-cancerous endometrial sample set is required to confirm this. In EC samples, gene expression of *VEGFA*, *CA9* and *PR* were similar in pre and post hysterectomy samples and we believe this to be related to the hypoxic environment that cancer tissues exist in, due to their unregulated-proliferation and they also constantly outgrow their blood supply. Benign cells are different to cancer cells due to many metabolic pathways being active and the way benign cells handle hypoxia varies from a cancer cell, these fundamental differences are the reason for the variation in results between these samples. Cancers are hypoxic and also have lot of neovascularisation, they exist in a hypoxic environment already prior to hysterectomy, this makes their response different to benign endometrium when vascular supply is interrupted.

Benign endometrial samples could be collected using different methods such as pipelle, or as full thickness biopsy. There was a significant increase in *VEGFA*, *PR* levels in full thickness samples compared with pipelle samples, and this may be due to myometrial contamination in the full thickness samples since our data demonstrated high *VEGFA* levels in myometrium and the levels of full thickness endometrium were similar to the myometrium only biopsy levels. In our unit at Liverpool Women's Hospital, the pathology set up did not allow obtaining full thickness samples from cancer samples as this would incur half hour delay during transport to pathology lab, this delay would invariably result in variation in gene expression levels as already known from previous studies.

Under normoxia, enzymes prolyl hydroxylase domain proteins (PHDs) induce degradation of HIF-1α. Hypoxia inhibits PHDs and stabilizes HIF-1α, which then translocates into the nucleus and dimerizes with constitutively expressed HIF-1β, creating active HIF-1 complex and

triggering the transcription of genes promoting glycolytic metabolism, angiogenesis, and survival (Fan et al., 2014). Tissue hypoxia is a common phenomenon in malignant tumours (Muz et al., 2015), and this may explain the significant change in *VEGFA* and *CA9* levels in cancer samples compared to normal endometrium. This effect was retained with *VEGFA* when biopsies were collected before or after hysterectomy, when the comparison was made with the benign and cancer samples collected at the same time. However, *CA9* levels were only significantly different, in the pre-hysterectomy samples, and their significant difference was lost when the biopsies were obtained from post hysterectomy pipelle samples. This is intriguing and may be due to the effect of warm ischemia on post hysterectomy benign samples. The expression of *PR* did not change much between pre and post hysterectomy samples, with that we can conclude *PR* to be a robust, prognostically relevant marker in EC. *HIF1A* mRNA levels were not examined because *HIF1A* gene is constitutively expressed in order to rapidly respond to hypoxic changes at the protein level. Further work by our group (A. Maclean) examined changes in the protein levels for *HIF1A*, *CA9*, *VEGFA*, and *PR* using immunohistochemistry, to correlate with the gene expression data from my study, and these are included in the recent publication from our group (Maclean et al., 2022).

The change in the gene expression profile seen in our study in EC samples is similar to a previous study by Liu et al. reporting a significant downregulation of gene expression profile with prolonged warm ischaemia in renal cell carcinomas (Liu et al., 2013). Several studies have previously suggested warm ischaemia causing significant changes in gene expression in various cancer tissues such as renal cell carcinomas, colon cancer, prostate cancer (Huang et al., 2001), (Spruessel et al., 2004a), (Lin et al., 2006), (Schlomm et al., 2008). Kitson et al conducted an immunohistochemistry based study examining effect of hypoxia on protein level in EC samples. They concluded that the common biomarkers in EC such as Ki67, hormone receptors are significantly lower in hysterectomy specimen compared to endometrial biopsy performed prior to surgery (Kitson et al., 2019). In my study, I have focussed on gene expression levels in cancer as well as benign endometrial samples. The fact that gene expression is not directly mirrored by protein expression is well established and a prime example of this is HIF1A, where the protein levels are affected rapidly by hypoxia without affecting the gene expression.

It is evident that pre-analytical variables may have a considerable and unpredictable effect on the analysis of many downstream genes, and therefore it is imperative that the researchers take steps to control these and such variables, remain consistent between different groups included

in a single study and/or ideally between studies. To achieve this, patient samples must be collected according to standardised operating procedures, with clearly documented technical/ sampling data in combination with comprehensive clinical data. A lack of complementary information is a recognised limitation of the vast data harboured by the Cancer Genome Atlas, a database hosting mRNA sequencing data for 582 ECs (NIH/NCI, 2006).

Overall, our data proposes that consistent timing preferably at the same time with reference to surgery and similar method of tissue harvesting in EC studies is needed to ensure robust and clinically translatable results. In benign samples, where full thickness samples are routinely taken, there is a risk of myometrial contamination, hence the more appropriate option is to use a method that will only include the endometrium for gene expression studies. Similarly, the timing of the sample collection should be kept the same, either pre or post hysterectomy for all samples collected in a particular study or if that is not feasible, a quality control check of the effect of different time points of tissue procurement should be made considering the genes of interest to control for such bias. Post hysterectomy samples may have more influence from warm ischaemia, therefore, where possible, pre-hysterectomy biopsies are preferred. As a result of conclusion drawn from this study, the work described in chapters 4, 5, and 6 of this thesis, consistently utilised pipelle biopsies obtained after hysterectomy. Therefore, this study has formed the basis for a consistent method used for the sample collection in the subsequent chapters and also highlighted the need for the work described in chapter 7, where harmonisation of EC biosample collection was undertaken.

Limitations and future work

Our study investigated the effect of a few of the potentially possible preanalytical variables on the mRNA expression levels of only three genes. However, there are numerous other factors, which could influence the data generated from tissue samples. In addition to this, the effect of the preanalytical variables on the limited number of genes we have analysed, was variable. Finally, full thickness samples could not be collected from women with a confirmed diagnosis or suspected diagnosis of cancer, as intact uterus is required for histopathological staging. Using novel analytical methods such as spatial transcriptomics, future studies maybe able to analyse the effect of differential timing on the expression levels of multiple genes. This research would also benefit from larger patient sample cohorts to further confirm the effects of patient demographic variability on different gene expression levels. With a limited number of samples in this study, we were also unable to draw comparisons and analyse alternative putative

pre-analytical variables; including grade and stage of cancer, patient co-morbidities and mode of surgery, which may hold some significance.

Conclusion

In this study, we have revealed the effect of pre analytical variables such as timing of samples with regards to hysterectomy, sampling method on the mRNA expression levels of normal endometrial and EC patient samples. Since we report these variable effects on endometrial gene expression, researchers must take caution when analysing data and consider the effects of pre-analytical variables and also understand the limiting nature of data obtained from biosamples due to patient variability. Our results advocate use of a consistent method and time for biopsy collection for all endometrial samples in a study, to avoid misleading expressional analysis. For effective use of biospecimens in research, biorepositories must endeavour to achieve a high quality approach to biospecimen handling and collate comprehensive technical (e.g., sample collection time, and method) and clinical data relevant to the biosample. Biobanking represents an innovative modality with great significance in translational medicine, but this benefit will transpire only when consistent stringent practices are adopted. Furthermore, to remove the potential impact of preanalytical variation on reported data, it is also important that peer reviewers pay attention to how samples were collected in a study.

Chapter 4: Investigation of *hTERC* levels in endometrial proliferative disease to expand our knowledge of telomerase biology in endometrial carcinogenesis

Introduction

Telomeres are nucleoprotein complexes which protect chromosomal ends and they play a vital role in preservation of chromosomes from degradation and fusion (Blackburn and Gall, 1978). Telomerase is the enzyme which maintains the telomere length and consists of three core components: (1) *hTERC* the RNA template for telomere synthesis, (2) *hTERT* the catalytic reverse transcriptase and (3) dyskerin protein (Cohen et al., 2007), (Alnafakh et al., 2021). TA levels are low/undetectable in most somatic cells except in those with self-renewing capability such as human endometrium, germline cells and certain haemopoietic cells (Saretzki, 2018), (Hapangama et al., 2008b). Human endometrium has shown dynamic TA according to the ovarian cycle (Valentijn et al., 2015). Proliferative endometrium has highest TA, whereas postmenopausal endometrium shows lowest levels of TA (Tanaka et al., 1998). TA is elevated in endometrial proliferative pathological conditions such as endometriosis and EC (Valentijn et al., 2015). Most ECs have high TA (Alnafakh et al., 2019).

Endometriosis is a chronic, oestrogen dependent benign gynaecological condition wherein endometrial glands and stroma like tissue are present outside the uterus, leading to bleeding, scarring and inflammatory reaction (Sourial et al., 2014). Since the ectopic endometrial-like tissue exists and grows in foreign (ectopic), not usual environment for endometrial tissue, in the case of endometriosis, it has been proposed to be a benign yet metastatic-like disease. The aetiology of endometriosis is complex, multifactorial with influence of hormonal, genetic, dysfunctional immune system and environmental factors (Sourial et al., 2014). There are no curative treatments for endometriosis, controversies exist as to which is the best treatment. Neither medical or surgical treatments provide long term benefits and remission in disease. There are many studies which demonstrate that eutopic secretory endometrium of women with endometriosis is associated with high TA, *hTERT* expression, with longer mean endometrial telomere lengths (Hapangama et al., 2008b), (Hapangama et al., 2009), (Hapangama et al., 2010), (Valentijn et al., 2013), (Valentijn et al., 2015).

EC is the most common gynaecological malignancy, with around 9400 new cases diagnosed in the UK every year (CRUK, 2016 - 2018). Most (80-90%) of ECs have high TA. EC cells have the ability to invade myometrium / cervix and extra-uterine tissue adjacent to the uterus. These cells migrate and initiate metastatic lesions in distant sites in advanced cancer demonstrating the natural history of malignant metastatic condition, EC.

For *in vitro* TA, *hTERC* and *hTERT* are the only essential components (Hapangama et al., 2017). An active telomerase enzyme can be formed by combining two RNA domains from *hTERC* subunit and *hTERT* protein on oligodeoxynucleotide substrates *in vitro* (Egan and Collins, 2010).

hTERC, telomerase RNA, provides scaffold structure for assembly of telomere complex (Nguyen et al., 2018), and it closely is associated with the reverse transcriptase, hTERT component of telomerase. *hTERC* is expressed constitutively in majority of somatic cells even when telomerase reverse transcriptase (Rubtsova et al., 2018) and its mutations are linked with dysfunctional telomeres such as aplastic anaemia, idiopathic pulmonary fibrosis (Tsakiri et al., 2007), (Trahan and Dragon, 2009). *hTERC* contains 451 nucleotides which form many domains, but only two domains are necessary for telomerase activity to occur (Rubtsova and Dontsova, 2020). *hTERC* length is variable in eukaryotes, structure is conserved (Chen et al., 2000).

Structure of *hTERC*

The four conserved elements in *hTERC* structure are: (1) a pseudoknot domain (CR2/CR3), (2) box H/ACA domain (CR6/CR8), (3) a conserved region 4 - conserved region 5 (CR4/CR5) domain and (4) a CR7 domain (Chen et al., 2000). The pseudoknot domain and distal CR4/5 domain represent the essential regions of *hTERC* to demonstrate TA (Tesmer et al., 1999). The *hTERC* H/ACA region binds with telomerase associated proteins such as dyskerin, NOP10, NHP2, GAR1 and is essential for telomerase biogenesis and RNA stability (Hapangama et al., 2017). In the H/ACA domain, there is another domain, the cajal body localisation box (CAB), which binds with telomerase cajal body protein 1(TCAB1) (Vogan et al., 2016). Therefore, *hTERC* not only provides template for identifying telomere repeat sequence for the telomerase holo-enzyme for its telomere length maintenance function, but also provides motifs which are vital to reconstitute telomerase activity (Cristofari et al., 2007), (Webb and Zakian, 2016). *hTERC* therefore plays essential roles in stability maturation, functional assembly of telomerase holo-enzyme. The template boundary element together with the 3′ end prevents DNA synthesis beyond the template (Feng et al., 1995), (Fu and Collins, 2003), (Kiss et al., 2010).

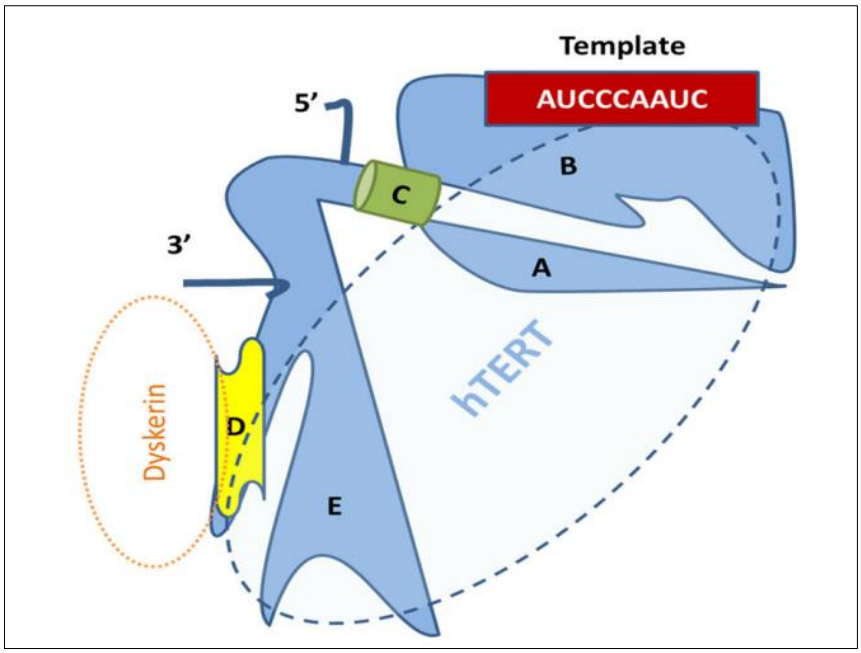


Figure 1. Diagram of the core elements of *hTERC* reproduced as published by Hapangama DK, Kamal A, Saretzki G. Implications of telomeres and telomerase in endometrial pathology. *Hum Reprod Update*. 2017;23(2):166-187. doi:10.1093/humupd/dmw044 : 5′ region containing (A) the pseudoknot domain and (B) RNA template (C) the template boundary element (Theimer and Feigon, 2006). Both A and B domains are important for *in vivo* stability of *hTERC*, and they interact with hTERT. The RNA stabilizing 3′ region contains (D) an H/ACA motif, which interacts with dyskerin or any of the other three H/ACA RNP components (NOP10, NHP2 and GAR1), and (E) trans-activating domain containing CR4/5 C that also binds hTERT (Webb and Zakian, 2016).

Most of the available evidence on the functional roles of TERC are related to TA Studies in transgenic mice models showed increase in mTERC expression following tumourigenesis in mice (Blasco et al., 1996). Upregulation of telomerase RNA component occurs at very early stages of mouse tumourigenesis whereas TA was only detected in end stage tumours (Blasco et al., 1996), hence it can be proposed that TERC activation and expression at early stage is a marker for cell proliferation, independent of telomerase activation at least in mice (Blasco et al., 1996). Further studies in terc-/- mice showed impairment of tumourigenesis process due to overexpression of TERT and also showed delayed wound healing rates (Cayuela et al., 2005). However, mouse have rather long telomeres compared with humans and have a different effect when telomerase function is altered, thus, results from mouse studies may not be directly translatable to humans. In non-small cell lung cancer cell lines, TERC was upregulated and was associated with high TA, compared to the non-cancerous benign lung tissues (Yokoi et al., 2003). *hTERC* gene was located in a more critical region of 3q26 amplicon in these cells (Yokoi et al., 2003). However, there is a reasonable body of evidence suggesting that TERC may also have non-telomerase related direct actions. In different telomerase positive human

colorectal cancer cell line HCT 116, depletion of telomerase RNA leads to effective inhibition of cancer cell growth (Li et al., 2005). *hTERC* knockdown induces global gene expression changes in human colorectal cancer cell line HCT 116, downregulation of genes involved in cell cycle progression, also it results in decreased expression of specific genes coding for proteins for tumour growth, angiogenesis, metastasis (Li et al., 2005) . *hTERC* has multiple binding sites throughout genome, located at Wnt genes and Myc genes, which may have direct effects on transcription to upregulation of the genes related to immune system or indirect effect by attracting *hTERT* (Chu et al., 2011), (Liu et al., 2019). *In vivo* experiments using CD14+ macrophages in patients with type 2 diabetes mellitus (DM) and multiple sclerosis demonstrated *hTERC* to have roles in upregulating the genes related to immune system and those modulating cytokines (Liu et al., 2019). In human breast carcinoma cell lines MCF7, *hTERC* also has been shown to influence cellular signalling systems such as protein kinase ATR (ATM and Rad3 related) (Kedde et al., 2006). In U2OS cells, over-expression of *hTERC*, independent of the TA, caused suppression of ATR kinase activity, which disrupted the cell cycle checkpoint regulation following DNA damage (Kedde et al., 2006). Additionally, *hTERC* is proposed to be involved in activation of DNA-PK, which repairs the double strand breaks by the non-homologous end joining pathway (Lees-Miller and Meek, 2003), (Mahaney et al., 2009), (Ting et al., 2009).

The telomerase RNA was known to be a non-coding RNA, however studies in vertebrates (human, macaca, rabbit, bull, cat, horse and mouse) wild type TERC can be transcribed into protein TERP. The alignment of TERP sequences amongst vertebrates demonstrated 40% or more identity, similar length and amino acid composition. hTERP was detected in telomerase positive cells lines (HEK293T, HT1080 and Jurkat) and absent in telomerase negative cells (VA13 cells). Previously, *hTERC* was reported to prevent apoptosis in human immune cells, since increased levels of *hTERC* resulted in anti-apoptotic defense and this function was independent of telomerase activity (Gazzaniga and Blackburn, 2014). However, Rubstova et al have demonstrated that it is not the level of *hTERC* or TA, but the production of hTERP which protects the cells from drug induced apoptosis as decrease in hTERP levels decreased the cell survival rate (Rubtsova et al., 2018). Therefore, it is proposed that alternative functions of *hTERC* is provided by the protein hTERP but not *hTERC* and hTERP has a role in helping the cells to protect themselves from stress, survive unfavourable conditions (Rubtsova et al., 2018).

The expression of *hTERC* in human endometrium has not been fully elucidated. Therefore, the aim of the work presented in this chapter was to ascertain the *hTERC* levels in normal human

endometrium, across the menstrual cycle and in the endometrium of benign proliferative disease, endometriosis and in malignant EC samples. We standardised biosample collection informed by the work described in chapter 3, to minimise the effect of pre-analytic variables on our results.

Methods

Endometrial tissue samples

Details of Ethical approvals for this study are presented in page 15. Endometrial samples were collected from women without any endometrial pathology, with regular periods and who were not on any hormonal treatment for at least 3 months in proliferative phase (n=6), secretory phase (n=9) and women in postmenopausal period (n=7), as well as from 10 women with surgically diagnosed active, peritoneal endometriosis in the secretory phase of the cycle. 24 endometrial samples from women with a prior diagnosis of EC (Endometrioid (grade-1 n=6, grade-2 n=7, grade-3 n=5 and type 2 ECs n=6) undergoing hysterectomy without receiving any pre-surgical treatment were also collected. The endometrial samples were dated according to histological criteria and patient-claimed last menstrual date. Tumour grade and type was assigned by two experienced gynaecological pathologists according to FIGO guidelines. Four myometrial samples were collected from benign hysterectomy samples near the serosal border to avoid endometrial contamination.

Once collected, endometrial and myometrial samples were divided into three, and immediately placed in i) RNA later for RNA extraction, ii) NBF for paraffin embedding and iii) snap frozen for TRAP assay. Patient clinico-pathological and demographic details were retrieved from the clinical notes and databases (Table 1).

Table 1: Demographic features of study groups

Study groups (n)	***Age (years)**	***BMI (kg /m^2)**
Proliferative phase (7)	43(32-57)	27.8(22-40.5)
Secretory phase (9)	41(21-47)	22.6(18.9-31.6)
Postmenopausal (7)	62(52-85)	24.3(20-39.6)

Secretory phase in women with endometriosis (10)	32.5(25-43)	25.7(17.1-40.6)
Endometrial cancer (24)	67(37-80)	30(23.9-54.4)
Endometrioid Grade 1 (6)	61(46-73)	37.8(28.3-46.1)
Endometrioid Grade 2 (7)	60(37-77)	28.9(25.8-54.4)
Endometrioid Grade 3 (5)	68(60-80)	29.8(23.9-42.7)
Type 2 EC (6)	72.5(60-80)	30.1(24.2-32.9)

*Data expressed as median (range)

Collection, transportation, processing and storage of tissue samples

The protocols followed are described in detail in chapters 3.

RNA extraction and quantification

RNA extraction and quantification is discussed in detail in chapter 3.

cDNA synthesis

mRNA isolated was converted to cDNA in a reaction catalysed by reverse transcriptase as discussed in detail in chapter 3.

q-RT PCR and optimisation

All sample preparation, technique for PCR is as described in chapter 3, apart from the change to amount of cDNA used for the experiments based on our optimisation. Housekeeping genes like PPIA and β-actin were used to ensure the same amount of cDNA was loaded between template samples. 7.5µl of master mix was added into each well and 2.5µl of cDNA was added in triplicates into separate wells to make the final volume of 10 µl in each well. For no RT control, 2.5µl of NRT control sample and for no template control 2.5 µl of nuclease free water were added. For each target and reference, a standard curve was produced and efficiency calculated (Fig 2).

Forward and reverse primers used, and the reaction conditions are listed in Table 2. qPCR conditions are charted in Table 3.

Table 2. Primer sequences used for qPCR amplification

Primer	Sequence	References
TERC	F:5'- GCCTTCCACCGTTCATTCTA-3 R:5'- CCTGAAAGGCCTGAACCTC-3	(Chai et al., 2011)
PPIA	F:5'- AGACAAGGTCCCAAAGAC-3 R:5'- ACCACCCTGACACATAAA-3	(Jacob et al., 2013)
ACTB	F:5'TGTACGCCAACACAGTGCTG-3 R:5'GCTGGAAGGTGGACAGCGA-3	(Wang et al., 2015)

Table 3. qPCR Conditions

		Initial Denature	Denature	Anneal / Extension	Cycles	Efficiency
Target Gene	**PPIA**	95°C 2min	95°C 5s	60°C 30s	40	100.1%
	ACTB	95°C 10min	98°C 5s	95°C 10s 60°C 30s	40	94.6%
	TERC	**95**°C 2min	95°C 5s	60°C 30s	50	86.1%

Abbreviations: *PPIA*, peptidylprolyl isomerase A; *ACTB*, beta-actin

Gene expression and statistical analysis

The Pfaffl method was used to compare the results of relative quantity of the gene of interest in the samples with the geometric mean of the normalisers across those samples.

TRAP

TRAP assay was kindly performed by our collaborator Dr Gabriele Saretzki, Lecturer in Ageing Research, Biosciences Institute and Newcastle University Institute for Ageing, Campus for Ageing and Vitality. TA was measured using TeloTTAGGG TRAP assay (Telomere Repeat Amplification Protocol assay; Roche Diagnostics, Ltd, Burgess Hill, UK) using 1µg of lysate (Valentijn et al., 2015). Optical density was measured as absorbance at 450nm in a Fluostar Omega Plate reader (BMG Labtech) and presented as arbitrary units (AU).

IHC

IHC experiments were performed by Dr Meera Adishesh and Dr Rafah Alnafakh. Antigen retrieval was performed to free the epitopes of the antigen, to improve immunostaining and prevent false negative results which may be caused due to modification of epitopes from the cross links between different proteins (Shi et al., 1991). 3µM FFPE tissue sections were placed in a heated pressurised chamber for 1-3 minutes, with an appropriate optimised buffer. The details of antibody used, concentrations and incubation conditions used are provided in the table 4 below. The sections were incubated in hydrogen peroxide 0.3% in Tris-buffered saline (TBS) (Sigma, Aldrich, Dorset, UK) for 10 min to block endogenous peroxidase. The sections were incubated in a humidified chamber, with anti-human steroid receptor antibodies and Ki67 after blocking unspecific binding with horse serum block for 20 minutes.

Detection was performed using the ImmPRESS polymer-based system (Vector Laboratories, Peterborough, UK) and visualisation was achieved using ImmPACT 3,3'-Diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK) used in accordance with the manufacturer's instructions. One drop of Vector ImmPRESS labelled polymer-horseradish peroxidase matching the primary antibody was added and incubated for 30 minutes followed by DAB substrate for 10 minutes followed by immersion in distilled water for 5 minutes. All steps were performed at room temperature unless stated otherwise. After each step, sections were rinsed in TBS to hold the reaction and eliminate any unbound material to prevent interfering with subsequent steps. Sections were lightly counterstained with Gill 2 Haematoxylin (Thermo Fisher Scientific, Runcorn, UK), dehydrated, cleared and mounted in synthetic resin (Consul Mount, Thermo Fisher Scientific, Runcorn, UK). Negative control and internal positive control were included in each run to assess specificity of the primary antibody and to detect any staining variation between runs respectively. Matching isotype immunoglobulin (0.5µg/ml) replaced the primary antibody in a negative control sample. A specific endometrial tissue sample with positive staining was included as the internal positive control with each staining experiment.

Table 4. Primary antibodies and conditions for IHC

Primary Antibody	Type	Clone	Supplier		Dilution	Incubation Time	Conditions
				HIAR* (min)		Time (hour)	Temp (°C)
ERα	Monoclonal	6F11	Leica[1]	2	1:50	2	18
ERβ	Monoclonal	PPG5/10	Abcam[2]	2	1:50	20	4
PR	Monoclonal	PgR 636	DAKO[3]	2	1:1000	1	18
AR	Monoclonal	AR441	DAKO[3]	2	1:75	20	4
Ki67	Monoclonal	MM1	Leica[1]	2	1:200	20	4
TRF1	Monoclonal	MM1	Santa Cruz[4]	2	1:50	20	4
TRF2	Monoclonal	MM1	Santa Cruz[4]	2	1:200	20	4

*Heat induced antigen retrieval by pressure cooking in citrate buffer pH 6.
[1]Milton Keynes, UK; [2]Cambridge, UK; [3] Ely, Cambridgeshire, UK; [4]Insight Biotech Ltd., Middlesex, UK

Analysis of IHC staining

IHC stained sections were scored by Dr Meera Adishesh and Dr Rafah Alnafakh. The protocol for analysis is as described below. Analysis and scoring were performed using a light microscope (Nikon UK, Surrey, UK). Steroid receptor immunostaining for four steroid receptors was assessed semi-quantitatively using a four-tiered Liverpool endometrial steroid quick score (LESQS) (Kamal et al., 2016b). The final score out of 12 was calculated by multiplying the proportion of positive cells (1-10% =1, 11-20%=2, 21-40%=3 and >40%=4) by the staining intensity categories (0=no staining, 1=weak, 2=moderate and 3=strong). TRF1/2 immunostaining for TRF1 and TRF2 was assessed semi quantitatively by using modified quickscore, by scanning the whole section and estimating the percent of stained proportions of cells with each intensity (0=no staining, 1=weak, 2=moderate and 3=strong). The final score out of 12 was calculated by multiplying the proportion of positive cells (1-25%=1, 26-50%=2, 51-75%=3, >76%=4) by the staining intensity categories (0=no staining, 1=weak, 2=moderate and 3=strong). The Ki67 proliferative index (PI) was evaluated as the percentage of immunopositive cells of any intensity. Epithelial and stromal cell staining was scored separately based on morphological criteria in postmenopausal and malignant endometrium and stratum basalis of healthy proliferative phase (PP) endometrium by two independent observers (myself and Dr Rafah Alnafakh). Consistency and reproducibility if

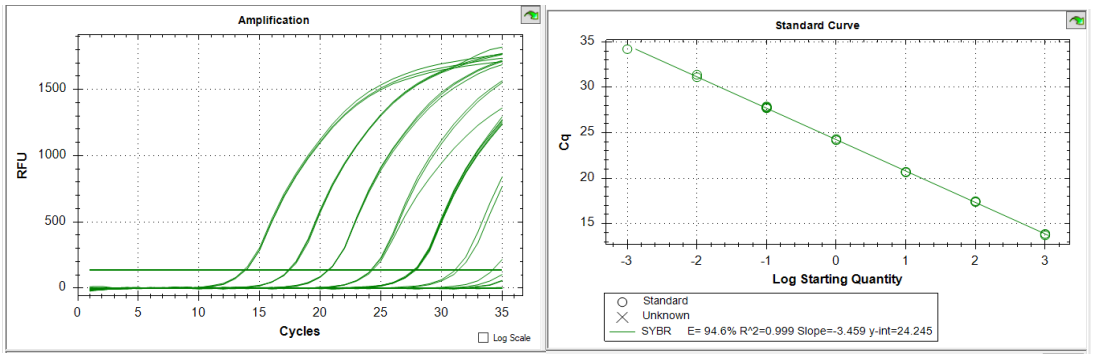
scoring were assessed by randomly revisiting some sections by myself (intraobserver) and with Dr Rafah Alnafakh (interobserver). Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

Statistical analyses

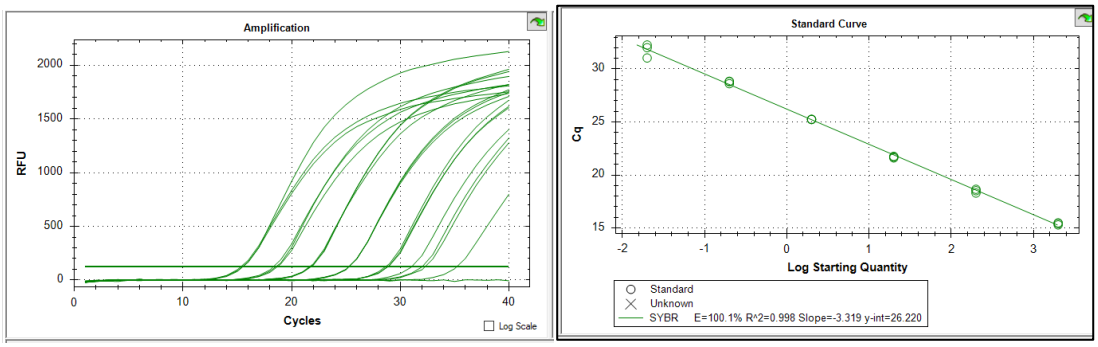
Gene expression data across different groups was analysed using Graph Pad Prism software using non-parametric tests (Mann-Whitney U test and /or Kruskal-Wallis test) or Spearman correlation as appropriate, not assuming Gaussian distribution. The criterion for significance was $p \leq 0.05$.

Standard curve for housekeeping genes and *hTERC*

(A) ACTB



(B) PPIA



(C)*hTERC*

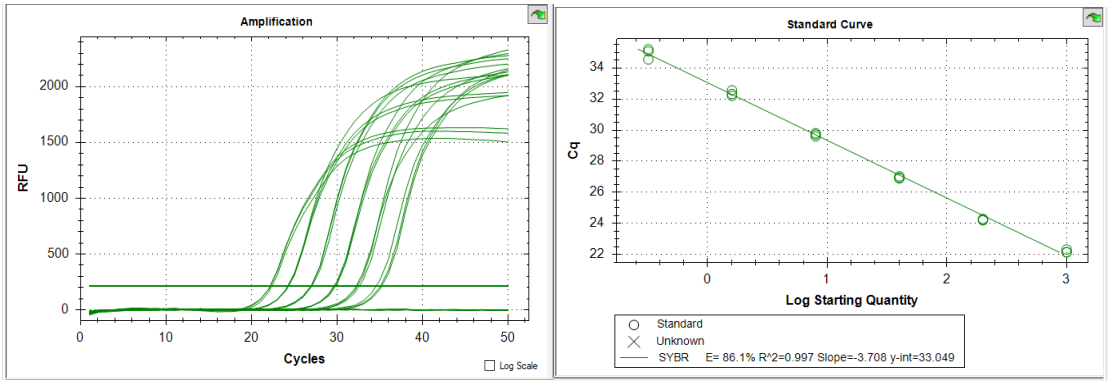


Figure 2. Standard curve for housekeeping genes ACTB (A), PPIA (B) and *hTERC* (C) with efficiencies (ACTB E=94.6%, PPIA E=100.1%, *hTERC* E=86.1%)

Results

hTERC, RNA component of telomerase enzyme, does not show a dynamic change across the menstrual cycle

hTERC was detected in both human endometrium and myometrium at similar levels (Fig.3A). In dissociated fractionated endometrial cells, *hTERC* levels were consistently higher in the stromal fraction (which according to previous reports have a lower TA and longer telomere lengths (Hapangama et al., 2008b) when compared with the endometrial epithelial fraction (Fig.3B). However, *hTERC* levels were apparently high in postmenopausal endometrial samples compared to the premenopausal endometrium (Fig.3C) however this was not statistically significant (P= 0.063, Mann Whitney test).

hTERC levels are significantly higher in endometriosis

When compared with the secretory phase endometrium of healthy women, without endometriosis, significantly higher *hTERC* levels were observed in endometrium from women with endometriosis in the secretory phase of the cycle (P= 0.02, Mann Whitney test, Fig.4).

hTERC levels are significantly higher in grade 3 endometrioid ECs

Compared with the postmenopausal healthy control endometrium, EC samples appeared to have decreased *hTERC* levels, but this observation did not reach statistical significance (P=0.36, Mann Whitney test, Fig 5A). Interestingly, *hTERC* levels increased steadily from grade 1 across to grade 3 of the endometrioid cancers subtype (P= 0.025, Kruskal-Wallis test, Fig 5B) and the lowest *hTERC* levels were observed in type 2, non-endometrioid ECs (P=0.004, Mann Whitney test, Fig 5B).

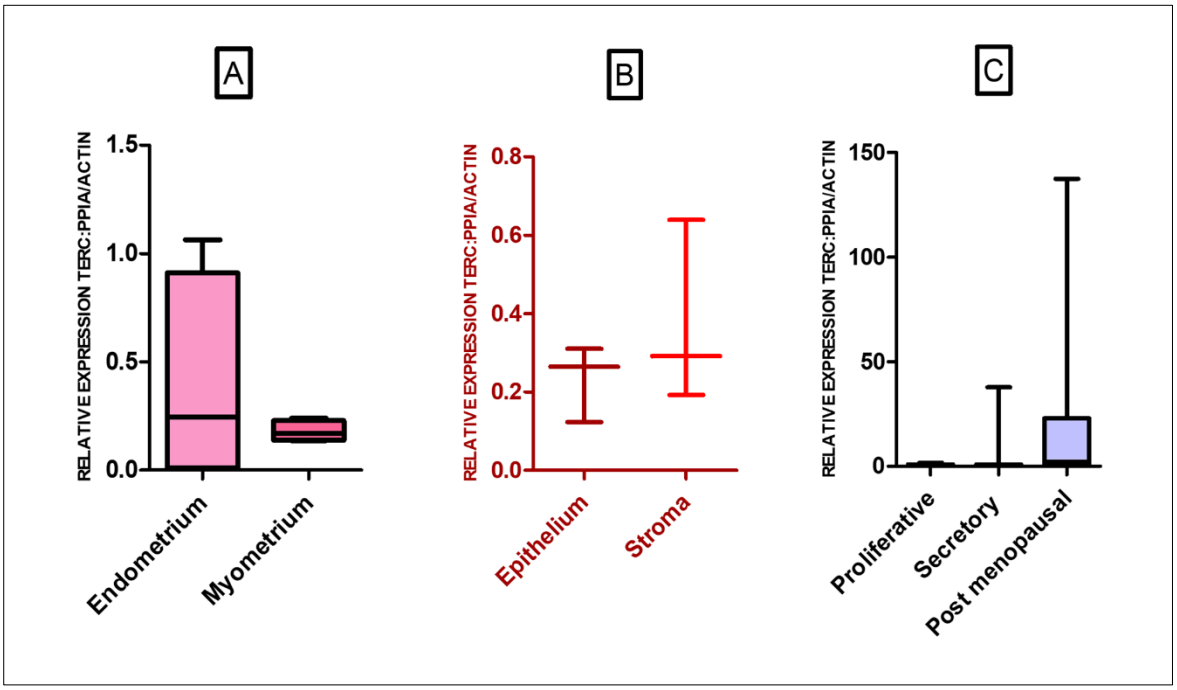


Figure 3. *hTERC* levels in different components of human uterus. (A) *hTERC* levels in human endometrium (n=4) compared to myometrium (n=4) (B) *hTERC* levels were higher in stromal fraction (n=3) compared with the epithelial fraction (n=3) in dissociated fractionated endometrial cells (C) *hTERC* levels across menstrual cycle, higher in postmenopausal endometrium (n=7) compared to proliferative (n=6) and secretory endometrium (n=9), however not statistically significant.

Correlation of *hTERC* levels with telomerase activity, proliferative marker ki67, TRF1, TRF2 immuno-staining scores and steroid receptor quickscores in endometrial samples

In normal and EC samples, *hTERC* RNA levels negatively correlated with LESQS for ERβ (r = -0.42, P=0.01), and TRF1 (r = -0.45, P=0.04) (Table 5). However, they did not correlate with TA levels measured with TRAP assay, other three steroid receptor quickscores we analysed or telomerase associated protein TRF 2. Furthermore, the *hTERC* levels did not correlate with the proliferative marker Ki67.

Discussion

Previous studies have demonstrated high TA and increased hTERT to be a prerequisite of endometrial epithelial proliferation (Tanaka et al., 1998), (Valentijn et al., 2015).

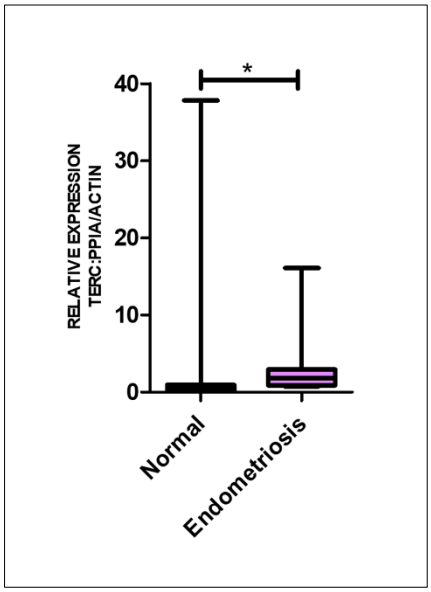


Figure 4. *hTERC* levels in secretory phase endometrium of patients with endometriosis (n=10). When compared to secretory phase endometrium in healthy women (n=9), *hTERC* levels in endometriosis patients were significantly high (P=0.02, Mann Whitney test).

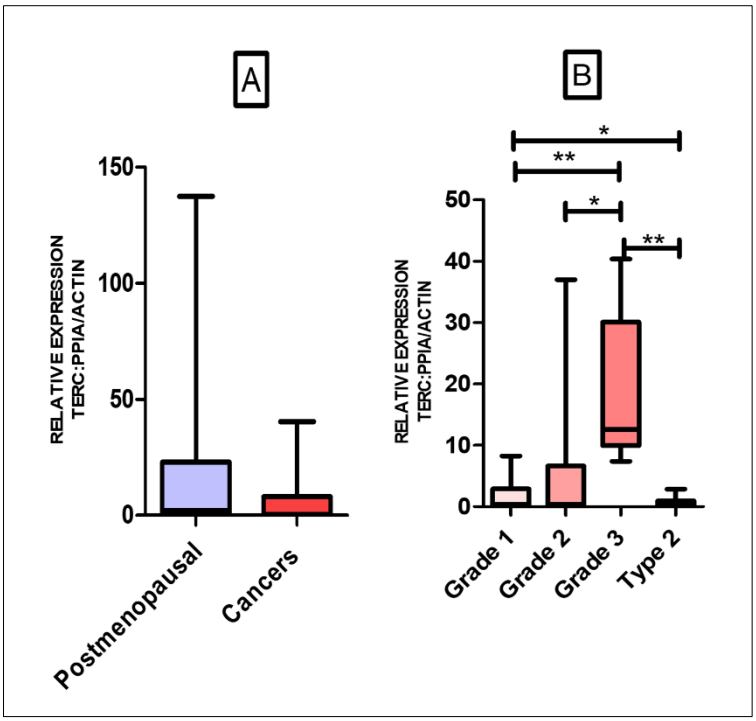


Figure 5. *hTERC* levels in postmenopausal endometrium and EC. (A) When compared to postmenopausal endometrium, *hTERC* levels in EC were low (P=0.36, Mann Whitney test). (B) *hTERC*

levels in EC, showed significant increase from grade 1 to grade 3 endometrioid EC (P=0.025, Kruskal-Wallis test). *hTERC* levels were lowest in type 2 non endometrioid EC (P=0.004, Mann Whitney test).

Correlation of *hTERC*, AR, PR, ERα, Erβ, Ki67, TRF 1, TRF 2 and TRAP in all endometrial samples

		AR	PR	ERα	ERβ	Ki67	TRF1	TRF2	TRAP
hTERC	r	0.148	-0.1022	0.0590	**-0.4239**	-0.0624	**-0.4485**	0.0038	-0.0362
	P	0.4034	0.5715	0.7363	**0.0112**	0.7097	**0.0363**	0.9842	0.7856

Table 5. Correlation of *hTERC* levels with TA, steroid receptors, telomerase associated proteins and proliferative marker. *hTERC* levels negatively correlated with Erβ (r = -0.42, P=0.01) and TRF1 (r = -0.45, P=0.04) whereas there was no correlation seen with other steroid receptors, Ki67 and TA.

This current chapter describes the other main component of telomerase holo-enzyme, *hTERC* in healthy endometrium across all stages of premenopausal menstrual cycle, and in postmenopausal endometrium examining the endogenous hormonal influence. Investigations also extended to include two pathological conditions of the endometrium, endometriosis, a benign proliferative condition and EC, which is the malignant transformation of the endometrium and both these conditions are known to be associated with high TA.

We found no significant alteration of endometrial *hTERC* levels in all phases of pre-menopausal menstrual cycle, suggesting that *hTERC* is constitutively expressed in the endometrium. Although *hTERC* was thought to be non-coding, its translation into hTERP has non canonical functions to regulate the essential processes in cells (Rubtsova et al., 2018). This coding capability may explain the constitutive expression of *hTERC* in somatic cells. This is in line with other studies which have demonstrated that *hTERC* is highly expressed in all tissues including human endometrium (Avilion et al., 1996), (Kyo et al., 1999b), (Lewis and Tollefsbol, 2016). On the contrary, TA is high in premenopausal endometrium and is dynamically regulated throughout the menstrual cycle and with glandular proliferation (Kyo et al., 1997), (Williams et al., 2001), (Hapangama et al., 2008a), (Hapangama et al., 2009), (Valentijn et al., 2015). Proliferating endometrial cells have highest TA, and this is mainly for maintaining and protecting the short telomeres therefore to prevent them from shortening to a critical length (Valentijn et al., 2015). It is known from previous studies, TA and hTERT are dynamically expressed in human endometrium (Hapangama et al., 2008a), (Hapangama et al., 2017). In our study, the endometrial TA and *hTERC* levels did not correlate and some previous

studies have also demonstrated that TA measured by TRAP assay does not necessarily correlate with *hTERC* expression (Ohyashiki et al., 2005), (Avilion et al., 1996). Another observation in our study was TA did not significantly change in the menstrual cycle, this may be due to our small sample size. Therefore, to draw conclusions on which of these two reasons are the cause for the observed results, further adequately powered studies with larger sample size are needed.

Compared to proliferative and secretory endometrium, relatively quiescent postmenopausal endometrium has low TA (Tanaka et al., 1998), (Valentijn et al., 2015). *hTERC* levels although appeared to be higher in postmenopausal samples than in proliferative or secretory endometrium, this was not statistically significant. In agreement, we found no significant correlation with ki67 which is a marker for cellular proliferation.

Eutopic endometrium of women with endometriosis is proposed to be different to those without endometriosis (Hapangama et al., 2010), (Hapangama et al., 2012), (Sourial et al., 2014), (Hapangama et al., 2017), (Lessey and Kim, 2017), (Ahn et al., 2017), (Hapangama et al., 2019). Endometriosis is considered to be a progesterone-resistant disease due to blunted response to progesterone demonstrated in both eutopic and ectopic endometrial tissue (Bulun et al., 2006), (Park et al., 2009). Endometrium in women with endometriosis is suggested to be more proliferative and associated with defective receptivity which is a shift from normal progesterone action (Park et al., 2009), (Hapangama et al., 2012), (Lessey and Kim, 2017). These differences are more exaggerated in the progesterone dominant, secretory phase of the cycle, and that was proposed to be due to the unravelling of relative progesterone resistance (Bulun et al., 2006). This is also thought to be the cause for subfertility often encountered in endometriosis sufferers. Therefore, we examined the secretory phase endometrium in particular from women with endometriosis to ascertain any differences specific to *hTERC* levels.

TA, *hTERT* protein and mRNA levels were reported to be increased in secretory phase endometrium of women with endometriosis (Kim et al., 2007), (Hapangama et al., 2008b). High TA along with the pro proliferative, anti-apoptotic and anti-senescence effect in the secretory endometrium of women with endometriosis, has been suggested to lead to endometriotic lesion formation from deposition of endometrial fragments after retrograde menstruation (Hapangama et al., 2009), (Hapangama et al., 2010).

In the eutopic secretory endometrium of women with endometriosis, *hTERC* levels were significantly increased. Therefore, we postulate that in endometriosis, relative progesterone resistance induces the observed high *hTERC* levels. In a previous study, it has been observed

that decrease in *hTERC* levels reduces the cell proliferation rate without influencing the TA (Li et al., 2005). Overexpression of *hTERC* suggests that *hTERC* may have a role in pro-proliferative effect in human endometrium. Secondly, studies in osteosarcoma cell line U2OS overexpression of *hTERC* levels is associated with upregulation of cytokine expression and increased cytokine secretion, independent of the TA (Liu et al., 2019). If *hTERC* has a similar role in endometrial cells, it may influence the increased inflammatory response seen in women with endometriosis, and also may lead sub-fertility possibly via producing a hostile endometrial environment, preventing embryo implantation (Liu et al., 2019). Another study reported that in peripheral blood mononuclear cells and CD4 T cells, over expression of hTERT protein induced apoptosis that was rescued by over expression of inactive *hTERC* mutants (Gazzaniga and Blackburn, 2014). This cellular protective effect if present in the endometrial cells may play a role in development to ectopic lesions from the shed endometrial cells with high *hTERC*, due to retrograde menstruation. All these above mentioned functions of *hTERC* if present in the endometrial cells that have high *hTERC*, may have a contribution to pathogenesis of endometriosis.

Interestingly in EC, there was no significant difference in endometrial *hTERC* levels when compared with postmenopausal endometrium. This is in keeping with the suggestion of constitutive expression of *hTERC* in endometrium. Analysis of *hTERC* in different types of EC demonstrated that *hTERC* levels were high in grade 3 endometrioid cancers compared to grade 1endometrioid and type 2 ECs. This agrees with well-established fact that different molecular pathways being activated in the different EC subtypes. For example, type 1 cancers frequently contain microsatellite instability and PTEN, PIK3CA, K-RAS, CTNNB1 mutations whereas type 2 ECs commonly exhibit P53 mutations and chromosomal instability (Dedes et al., 2011), (Weigelt and Banerjee, 2012), (Matias-Guiu and Prat, 2013). *hTERC* levels may be relevant to these reported different pathway activation rather than just telomerase associated alterations in cell proliferation. This hypothesis is further supported by the already mentioned lack of correlation found between *hTERC* and Ki67. Ovarian hormones are a well-known regulating factors in EC. Hormones activate various pathways via cognate receptor such as ERβ, and when all endometrial samples were considered together, *hTERC* levels negatively correlated with LESQS for ERβ ($r = -0.42$, $P=0.01$). Previous study on macaque endometrium, shows that oestrogen treatment is associated with increased expression of telomerase RNA in the endometrium (Vidal et al., 2002). Other studies have demonstrated hormonal regulation of TA in different tissues (Misiti et al., 2000). Zhou et al demonstrated that E2 induces TA and

hTERT mRNA expression via ERα dependent manner (Zhang et al., 2013). In our study, *hTERC* did not show any positive correlation with ERα expression. However, as ERβ receptors are known to counteract ERα, this may explain our findings of negative correlation of *hTERC* with ERβ receptors (Bottner et al., 2014), (Hapangama et al., 2015).

Ectopic endometriotic lesions are defined histologically by the inclusion of both endometrial epithelial and stromal like cells (Bulun et al., 2006), (Park et al., 2009). The interaction between different cell types is suggested to play an important role in hormonal regulation especially in steroid receptor expression (Hapangama et al., 2015), (Kamal et al., 2016b). We have used PCR technique in our study to assess the *hTERC* levels in the whole tissue sample, which does not allow to ascertain the exact cellular origin of the *hTERC* levels of the samples analysed. This technique would not allow clarification if a surgically excised endometriotic sample we study may contain the essential cell types or if the exact cells of origin of *hTERC* detected by PCR. Ideally, to study ectopic lesions particular cell types can be extracted/isolated from frozen tissue sections, using techniques such as laser capture microdissection (Maclean et al., 2020). Techniques such as in situ hybridisation, immunohistochemistry or immunofluorescence do not disturb the tissue architecture and directly assess gene or protein expression, may be useful to study endometriotic lesions (Mathew et al., 2016), (Tempest et al., 2018), (Hapangama et al., 2019). During my project, I was not able to check *hTERC* levels in ectopic endometriotic lesions and use the above mentioned techniques, and this is one of the major limitations of my study. As mentioned above, the lack of power with the small sample size of this study that prevent confirmation that lack of correlation we observed between TA and *hTERC*, was a true finding or not is another limitation of our study. However, this study has demonstrated significant changes in *hTERC* levels in pathological conditions of the human endometrium and further studies with suitable techniques and adequate power/ sample size thus are warranted to explore these interesting preliminary data.

Chapter 5: Investigation of *TERRA* levels in endometrial proliferative disease to expand our knowledge of telomerase biology in endometrial carcinogenesis

Introduction

Human endometrium is a dynamic organ which undergoes a repetitive cycle of cell proliferation, differentiation, shedding and regeneration, regulated by ovarian hormones on a monthly basis (Hapangama and Bulmer, 2016). This regenerative potential is retained by the proliferatively quiescent postmenopausal endometrium, even after the cessation of ovarian function (Valentijn et al., 2015). Telomeres are specialized nucleoprotein complexes at the ends of chromosomes, which play an established and essential role in endometrial epithelial proliferation (Hapangama et al., 2017). Telomeric DNA is lost with each round of cell division due to the end replication problem, and unless the lost DNA is replaced by the action of telomerase, telomeres will shorten in proliferating cells (Smogorzewska and de Lange, 2004) Human endometrium demonstrates cyclical TA according to the menstrual cycle, and both *in vivo* and *in vitro* evidence suggests that ovarian hormones regulate endometrial telomerase, telomere lengths and epithelial proliferation (Valentijn et al., 2015), (Hapangama et al., 2017). Common endometrial proliferative diseases such as endometriosis (Hapangama et al., 2017), (Hapangama et al., 2008a), (Hapangama et al., 2008b) and EC are also associated with high TA (Kyo et al., 1996), (Sakamoto et al., 2000), (Lehner et al., 2002), (Saygan-Karamursel et al., 2005).

The telomere complex prevents chromosomal ends from being recognized as DNA damage-foci that can mount a DDR (Griffith et al., 1999), (Yoo and Chung, 2011), (Bodvarsdottir et al., 2012). Unrepaired DNA damage can mediate a permanent cell cycle arrest and eventually result in cellular senescence (Campisi and d'Adda di Fagagna, 2007). Replicative senescence limits the proliferative potential of somatic cells and has therefore been characterized to be a tumour suppressor mechanism. However, specialized cells and cancer cells have sufficiently high TA for telomere maintenance and elongation, thus are senescence-resistant (Kim et al., 1994).

Although telomeres were considered to be transcriptionally silent until recently, *TERRA* molecules are transcribed from CpG-island containing subtelomeric promotors, extending towards the telomeric tract (Schoeftner and Blasco, 2008) (Azzalin et al., 2007). *TERRA* molecules are long non-coding RNAs (lncRNA) located in the nucleus (Azzalin et al., 2007) and they are proportional to telomere lengths in different telomeres (Yehezkel et al., 2008), (Arnoult et al., 2012), (Van Beneden et al., 2013), (Viceconte et al., 2021). *TERRA* have been identified in vertebrates and in several other species such as yeasts and plants (Luke et al., 2008), (Vrbsky et al., 2010). In human cells, 20 *TERRA*-encoding subtelomeres have been

described (Vitelli et al., 2013). Although non-coding, *TERRA*s are involved in the recruitment of telomerase to the telomeres (Smekalova and Baumann, 2013) and they remain partly associated with telomeres establishing the DNA/RNA G-quadruplex structures that protect the single strand DNA (ssDNA)(Griffith et al., 1999). The *TERRA* containing RNA-DNA hybrids at the telomeric end may also have a physiological role in stalling replication forks, hence allowing DNA repair (Aguilera and Gomez-Gonzalez, 2008), (Sollier and Cimprich, 2015). *TERRA* levels are cell specific (Azzalin et al., 2007) and fluctuate with the cell cycle (Porro et al., 2010). The highest *TERRA* molecule levels and lowest TA are detected in early G_1 whilst the opposite is reported in S phase (Zhu et al., 1996), (Porro et al., 2010), (Wang et al., 2015). TERRAs interact with shelterin proteins TRF1 and TRF2 and mediate cellular reprogramming (Marion et al., 2019). Therefore, *TERRA*s are thought to participate in many essential cellular regulatory functions (Schoeftner and Blasco, 2008), (Deng et al., 2009), (Redon et al., 2010), (Arnoult et al., 2012). *TERRA* functions may be relevant to the specific telomere of origin or other telomeres, their levels are inversely correlated with corresponding telomere length (Arnoult et al., 2012), (Montero et al., 2016). *TERRA* are also demonstrated to have a role in recruitment of telomerase to the telomere (Smekalova and Baumann, 2013). On the other hand, by binding to *hTERC*, *hTERT* and by forming RNA-DNA hybrids *TERRA* are also implicated to inhibit telomerase (Luke et al., 2008), (Redon et al., 2010).

Considering the intricate relationship between telomerase, telomeres and endometrial cellular proliferation, (Hapangama et al., 2008a), (Hapangama et al., 2008a), (Hapangama et al., 2009), (Valentijn et al., 2013), (Valentijn et al., 2015), (Hapangama et al., 2017). I hypothesised that *TERRA*s may have an important role in endometrial carcinogenesis. To test this hypothesis, I initially examined collective *TERRA* from chromosomes 1q-2q-4q-10q-13q-22q and 16p in healthy normal, benign proliferative conditions such as endometriosis and EC samples. Out of the 18 *TERRA* loci in human cell transcripts, Montero et al demonstrated that deletion of 20q locus caused decrease in *TERRA* levels (Montero et al., 2016). Therefore, I studied *TERRA* levels from chromosome 20q in the same sample groups. As both pathological conditions benign and malignant display high TA and aberrations under hormonal influence/responsiveness, we aimed to include change in *TERRA* levels across the whole spectrum of pathologies together with TA and mean TL (Redon et al., 2010), (Sourial et al., 2014), (Kamal et al., 2016b).

Methods

Demographics of study groups

Endometrial tissue samples

Details of the Ethical approval are provided in page 15. The sample collection methods, number of samples and demographic details of the patients are described in chapter 4. Patient clinico-pathological and demographic details were retrieved from the clinical notes and databases (as in Table 1 chapter 4).

Collection and transportation of tissue samples, tissue processing and storage

Collection, transportation, processing and storage of samples was as discussed in chapter 3.

RNA extraction and quantification

RNA extraction and quantification is discussed in detail in chapter 3.

cDNA synthesis

mRNA isolated was converted to cDNA in a reaction catalysed by reverse transcriptase as discussed in detail in chapter 3.

Q-RT PCR and optimisation

Sample, equipment preparation and technique used for PCR was carried out as described in chapter 3 and 4 except to the amount of cDNA used in the experiments which is based on our optimisation. 8µl of master mix was added into each well and 2µl of cDNA was added in triplicates into separate wells to make the final volume of 10 µl in each well. For no RT control, 2µl of NRT control sample and for no template control 2µl of nuclease free water were added. The reaction was set as per conditions optimised for each target at 50 cycles. For each target and reference, a standard curve was produced and efficiency generated (Fig 1A, 1B and 1C).

Forward and reverse primers used, and the reaction conditions are listed in Table 2. qPCR conditions are charted in Table 3.

Table 2. Primer sequences used for qPCR amplification

Primer	Sequence	References
TERRA Ch 1q-2q-4q-10q-13q-22q	F:5'GAATCCTGCGCACCGAGAT R:5'CTGCACTTGAACCCTGCAATAC	(Diman et al., 2016)
TERRA Ch16p	F: 5'TGT GTT TCA ACG CTG CAA CTG R: 5'AGT TAG AAC GGT TCA GTG TG	(Wang et al., 2015)
TERRA Ch20q	F:5'GAAGTTGCTGGGTTCTATGG R:5'ATGGTGCAGACACTGTGG	(Montero et al., 2016)

Table 3. qPCR Conditions

		Initial Denature	Denature	Anneal / Extension	Cycles	Efficiency
Target Gene	***TERRA*** *Ch 1q-2q-4q-10q-13q-22q*	95°C 10min	98°C 5s	95°C 10s 60°C 30s	50	101.1%
	TERRA *Ch16p*	95°C 10min	98°C 5s	95°C 10s 60°C 30s	50	95.1%
	TERRA *Ch20q*	95°C 10min	98°C 5s	95°C 10s 60°C 30s	50	105.2%

Gene expression analysis

Gene expression analysis was performed as described in chapter 3.

TRAP assay

TRAP assay was kindly performed by our collaborator Dr Gabriele Saretzki, Lecturer in Ageing Research, Biosciences Institute and Newcastle University Institute for Ageing, Campus for Ageing and Vitality. TRAP assay method is described in chapter 5.

Immunohistochemistry and analysis of IHC staining

IHC experiments and analysis were performed by Dr Meera Adishesh and Dr Rafah Alnafakh as described in chapter 4. 3μM tissue sections were immuno-stained with anti-human steroid receptor antibodies and Ki67 after antigen retrieval at pH6 as previously described. Proliferative index, quickscore and LESQS were obtained for each immunostain for Ki67,

telomerase associated proteins and steroid receptors respectively. Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

Antibody sources, concentrations and incubation conditions are detailed in Table 4 as described in chapter 4.

Single telomere length analysis (STELA)

STELA experiments were kindly performed by collaborators, Rhiannon Jones and Prof Duncan Baird, Division of Cancer and Genetics, Cardiff University, Cardiff. The protocol used was as described below. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Manchester, UK). For telomere length analysis at the XpYp telomere we used the STELA assay, as previously described (Baird et al., 2003), (Lin et al., 2010). Genomic DNA was solubilized, diluted in 10 mmol/l Tric-HCl (pH 7.5) to 10 ng/µl. 10 ng of DNA was further diluted with 1µmol/l Telorette2 linker and 1 mM Tris-HCl to 250pg/µl in a volume of 40µl. Multiple polymerase chain reactions were conducted to test the DNA sample and were cycled in a Tetrad2 thermocycler (BioRad, UK) (22 cycles of 94°C for 15 sec, 65°C for 30 sec, 68°C for 8 min). DNA fragments were resolved by 0.5% TAE agarose gel electrophoresis and detected by southern hybridization. The hybridized fragments were detected using a phosphorimaging with a Typhoon FLA 9500 phosphoimager (GE healthcare, Chalfont St Giles, UK). The molecular weights of the DNA fragments were calculated using the Phoretix ID quantifier (Nonlinear Dynamics, Newcastle Upon Tyne, UK).

Statistical analyses

Gene expression data across different groups was analysed using Graph Pad Prism 5 (Graph Pad Software, Inc., CA, USA) using non-parametric tests (Mann-Whitney U test and /or Kruskal-Wallis test) or Spearman correlation as appropriate, not assuming Gaussian distribution. The criterion for significance was $p \leq 0.05$.

Standard curves

Four to Five point cDNA dilution series spanning across the expected concentration range was used for experimental samples. The results were plotted with the nucleic acid quantity on x-axis and cycle threshold on y axis. The slope of the curve was used to calculate reaction efficiency, for which the acceptable range was 90-110% for all primers used. The acceptable

R2 of the standard curve was around 0.98. Below are the standard curves for *TERRA 1q-2q-4q-10q-13q-22q*, 16p and 20q (Fig 1).

Fig. 1A *TERRA 1q-2q-4q-10q-13q-22q*

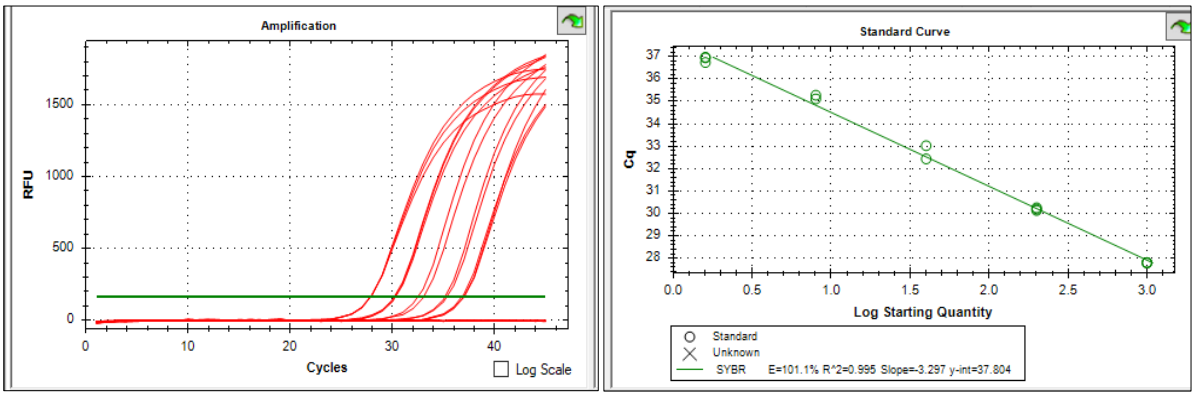


Fig. 1B *TERRA 16p*

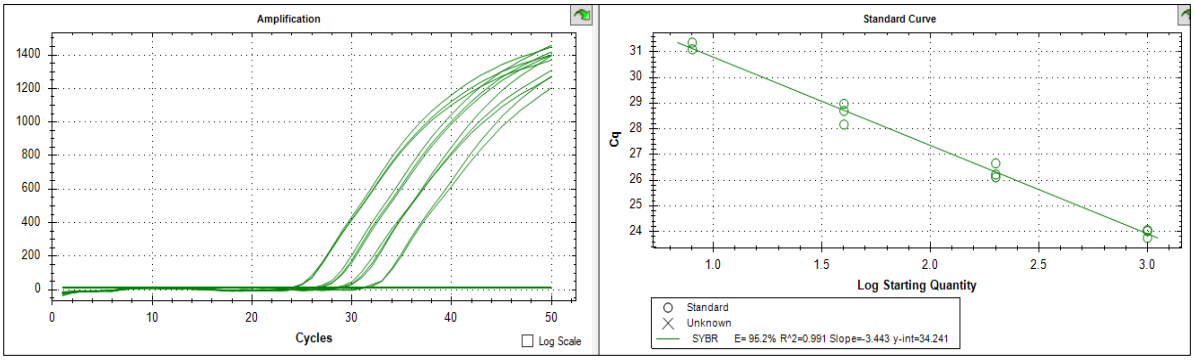


Fig. 1C *TERRA 20q*

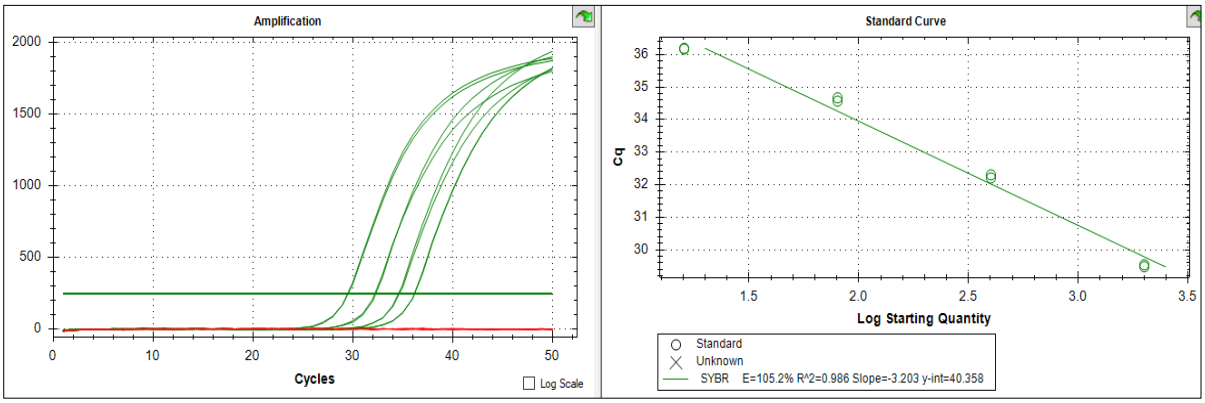


Fig 1. Standard curves for (A)*TERRA 1q-2q-4q-10q-13q-22q* with efficiency 101.1% (B) *TERRA 16p* with efficiency 95.2% (C) *TERRA 20q* with efficiency 105.2%.

Results

TERRA levels show a dynamic pattern in the healthy human endometrium

Both the endometrium and myometrium demonstrated all *TERRA*s examined (chromosomes 1q-2q-4q-10q-13q-22q, 16p and 20q), and myometrial *TERRA* levels were consistent in all samples whereas the endometrial *TERRA* levels of individual samples were spread across a wider range (Fig. 2).

Isolated freshly harvested epithelial and stromal cells also demonstrated all three *TERRA*s, and the *TERRA* levels were consistently higher in the stromal fraction when compared with the endometrial epithelium (Fig.3).

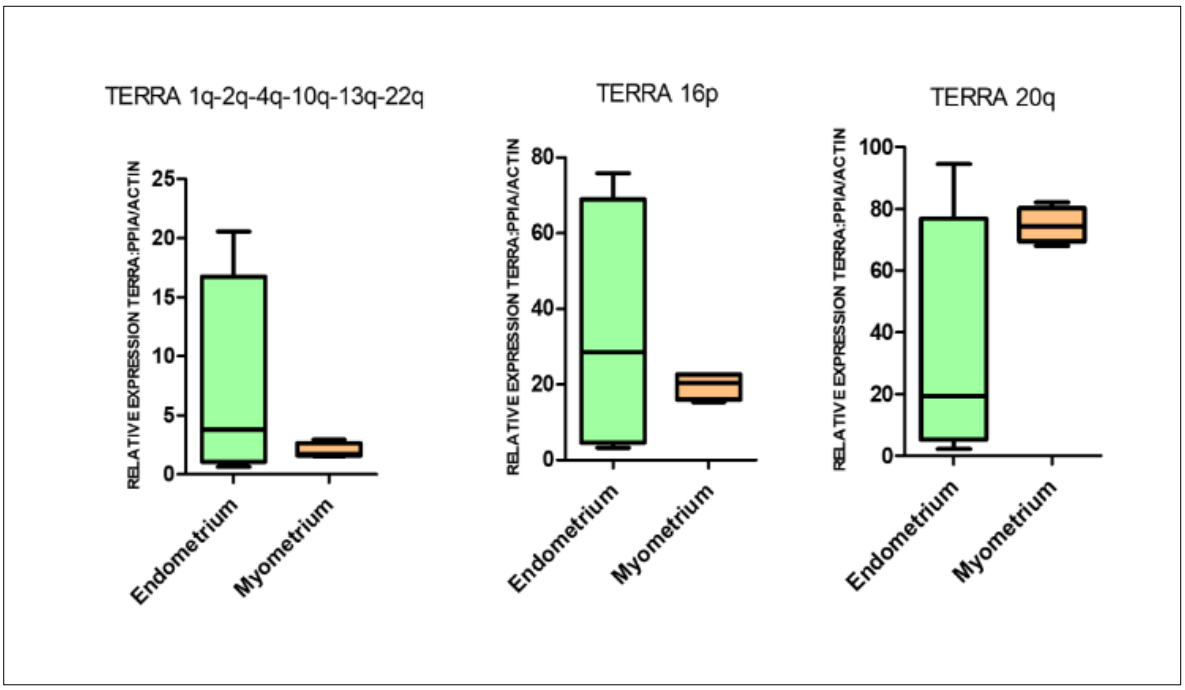


Fig 2. *TERRA* expression in human endometrium (n=4) and myometrium (n=4). *TERRA1q-2q-4q-10q-13q-22q* and *TERRA16p* levels were similar in endometrium and myometrium whereas *TERRA 20q* levels were slightly higher in myometrium however this was not statistically significant (P=0.34; Mann Whitney test)

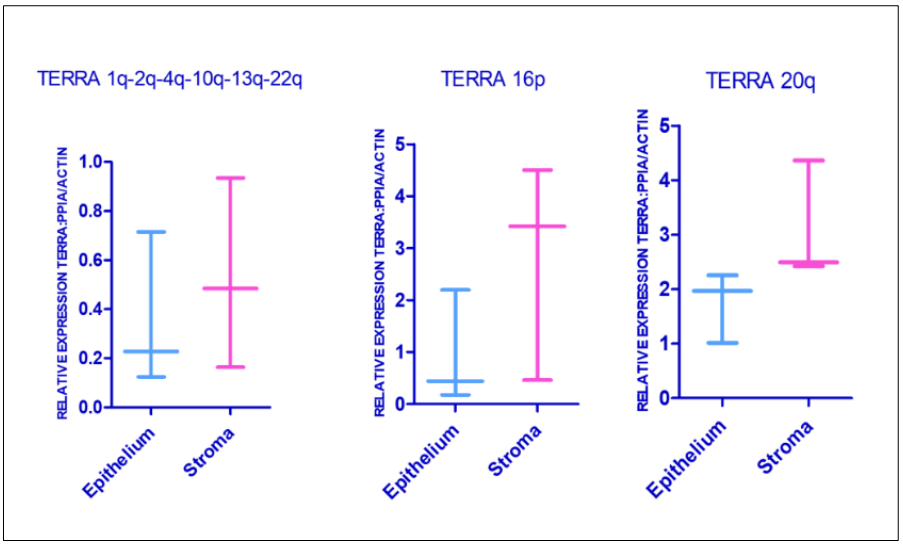


Fig 3. *TERRA* expression in both isolated freshly harvested epithelial and stromal cells. *TERRA 1q-2q-4q-10q-13q-22q*, *TERRA16p* and *TERRA 20q* levels were higher in stromal fraction (n=3) when compared to the epithelial fraction (n=3), however due to the small sample size no statistical analysis was performed.

Healthy human endometrium demonstrated a dynamic pattern of *TERRA* levels in the pre and post-menopausal endometrium (Fig. 4).

Individual *TERRA*s had a unique *TERRA*-specific pattern across the menstrual cycle in the healthy endometrium (Fig. 4). Ch-20q *TERRA* levels were increased in the secretory phase where glandular proliferation is low and the highest levels were observed in the proliferatively quiescent postmenopausal endometrium (P=0.02). The lowest levels were seen in the proliferative phase where cellular proliferative indices were high (Fig.4).

Eutopic endometrium of women with endometriosis showed no significant difference in TERRA levels

We examined the secretory phase endometrium of women with endometriosis, a benign proliferative disease postulated to be associated with progesterone resistance and a well-established high endometrial TA (Hapangama et al., 2008b).

TERRA RNA levels were not significantly different between women with and without endometriosis (*TERRA1q-2q-4q-10q-13q-22q* P=0.07, *TERRA16p* P=0.45, *TERRA 20q* P=0.28; Mann Whitney test) (Fig.5).

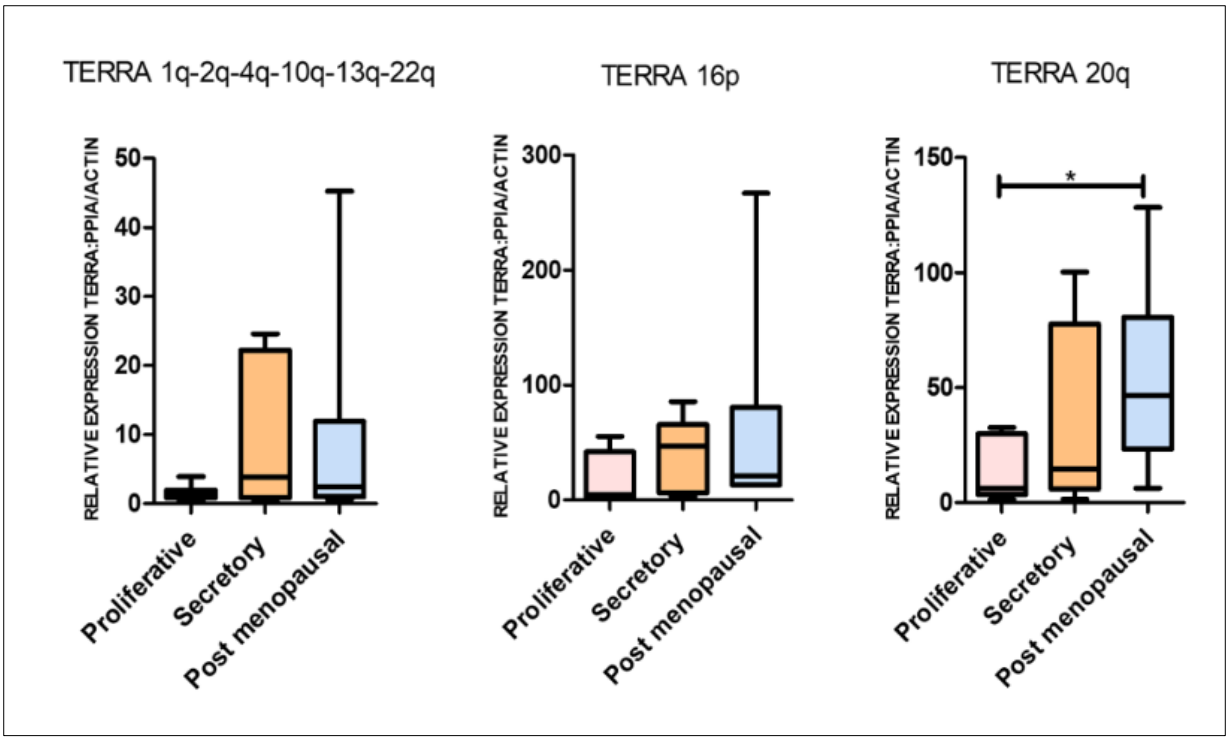


Fig 4. *TERRA* expression in human endometrium across different phases of menstrual cycle and post-menopausal state. Levels of *TERRA1q-2q-4q-10q-13q-22q* and *TERRA16p* were higher in secretory (n=9) phase of menstrual cycle when compared to proliferative phase (n=6) (*TERRA 1q-2q-4q-10q-13q-22q* P=0.23, *TERRA 16p* P=0.09; Mann Whitney test). *TERRA 20q* levels were significantly high in postmenopausal endometrium (n=7) compared to the proliferative phase on menstrual cycle (P=0.02, Mann Whitney test).

Endometrial cancer is associated with a significant decrease in TERRAs

TERRA levels were decreased in EC when compared with the healthy postmenopausal endometrium (Fig.6) and the reduction was significant for both Ch-16p (P=0.002) and Ch-20q (P=0.001) *TERRA*s.

TERRA levels did not change with tumour grades or stages of EC (Fig.7).

Telomerase activity is higher in proliferative phase of menstrual cycle and in ECs

TA is found in the endometrium in spite of it being a somatic organ. It has been previously reported that TA is low in stromal component of the endometrium, compared to the epithelium. During the menstrual phase, higher trend for TA in the proliferative phase compared with the secretory phase (P=0.04, Fig 8) and postmenopausal endometrium (P= 0.24).

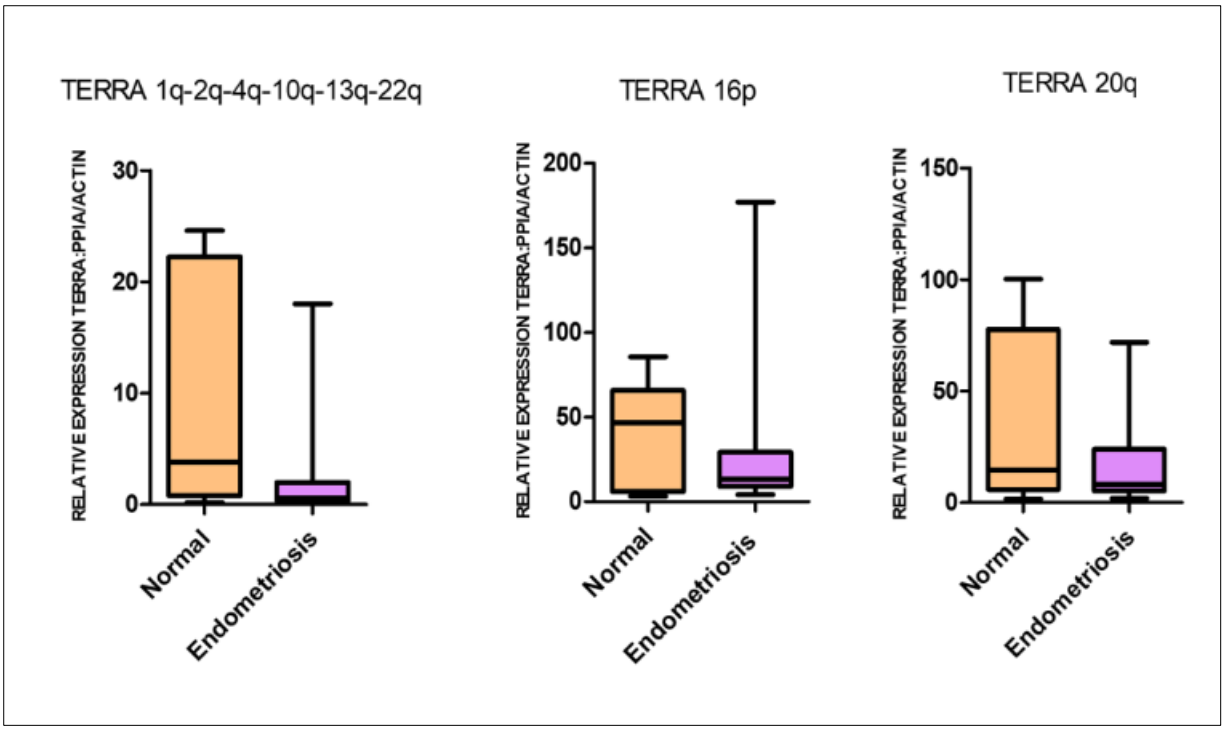


Fig 5. *TERRA* expression in eutopic endometrium of women with endometriosis compared to secretory phase endometrium in healthy women. Levels of *TERRA1q-2q-4q-10q-13q-22q*, *TERRA16p* and *TERRA 20q* in eutopic endometrium (n=10) were similar in secretory phase (n=9) of menstrual cycle (*TERRA1q-2q-4q-10q-13q-22q* P=0.07, *TERRA 16p* P=0.45, *TERRA 20q* P=0.28; Mann Whitney test).

Endometriosis is thought to be a benign proliferative disease associated with high TA. Similarly high TA is seen in ECs compared to postmenopausal phase (P=0.18, Fig.9) whilst no significant change was observed in TA for the different grades (Fig.9).

Endometrial TERRA levels correlated with each other, the proliferative marker Ki67, PR steroid receptors and shelterin protein TRF1 but did not correlate with TA and hTERC

TERRA levels of all samples (healthy and pathological) demonstrated a strong positive correlation with each other (Ch-1q-2q-4q-10q-13q-22q and Ch-16p, r =0.49, P=0.001; Ch-1q-2q-4q-10q-13q-22q and Ch-20q = 0.48, P=0.001; Ch-16p and 20q, r =0.78, P=< 0.0001) but did not significantly correlate with TA. However, when the benign endometrium is considered, Ch-16p *TERRA* levels correlated positively with *hTERC* levels (r =0.32, P=0.03). In all samples, Ki 67 proliferative indices correlated negatively with Ch-16p and Ch-20q *TERRA* (r = -0.35, P=0.03; r = -0.42, P =0.01 respectively).

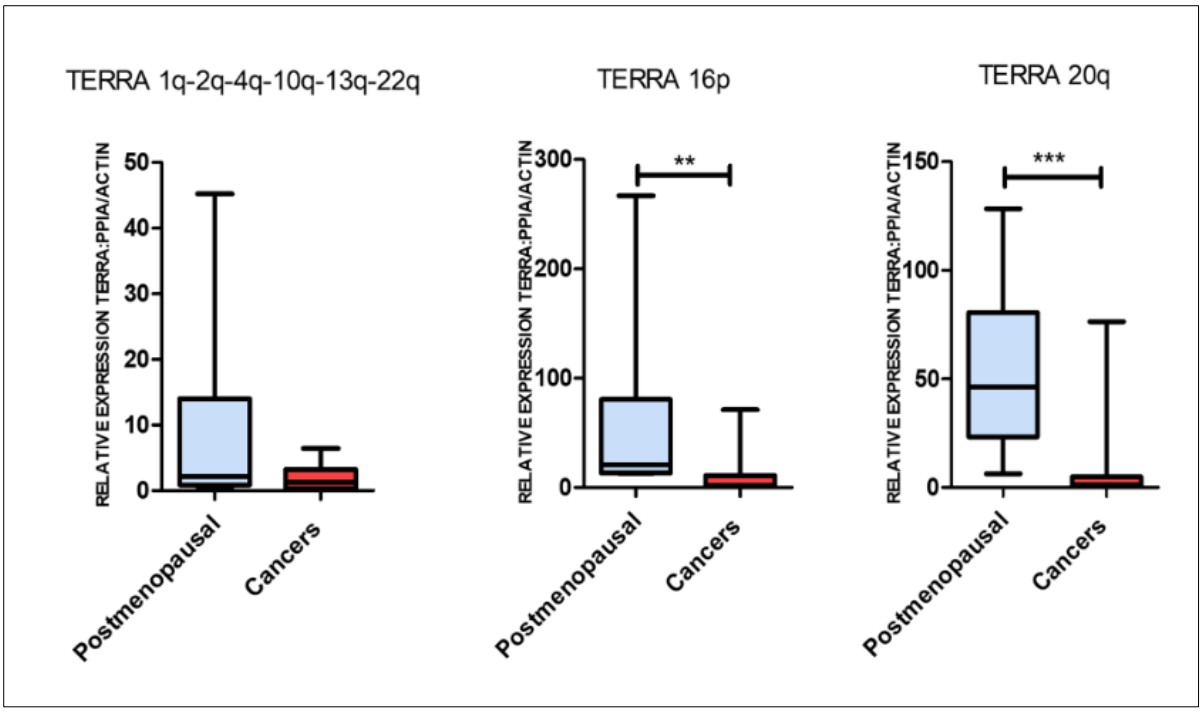


Fig 6. *TERRA* expression in ECs compared to postmenopausal healthy endometrium. Levels of *TERRA16p* and *TERRA 20q* in ECs (n=24) were significantly decreased than in post-menopausal endometrium (n=7) (*TERRA1q-2q-4q-10q-13q-22q* P=0.36, *TERRA 16p* **P=0.002, *TERRA 20q* ***P=0.001; Mann Whitney test).

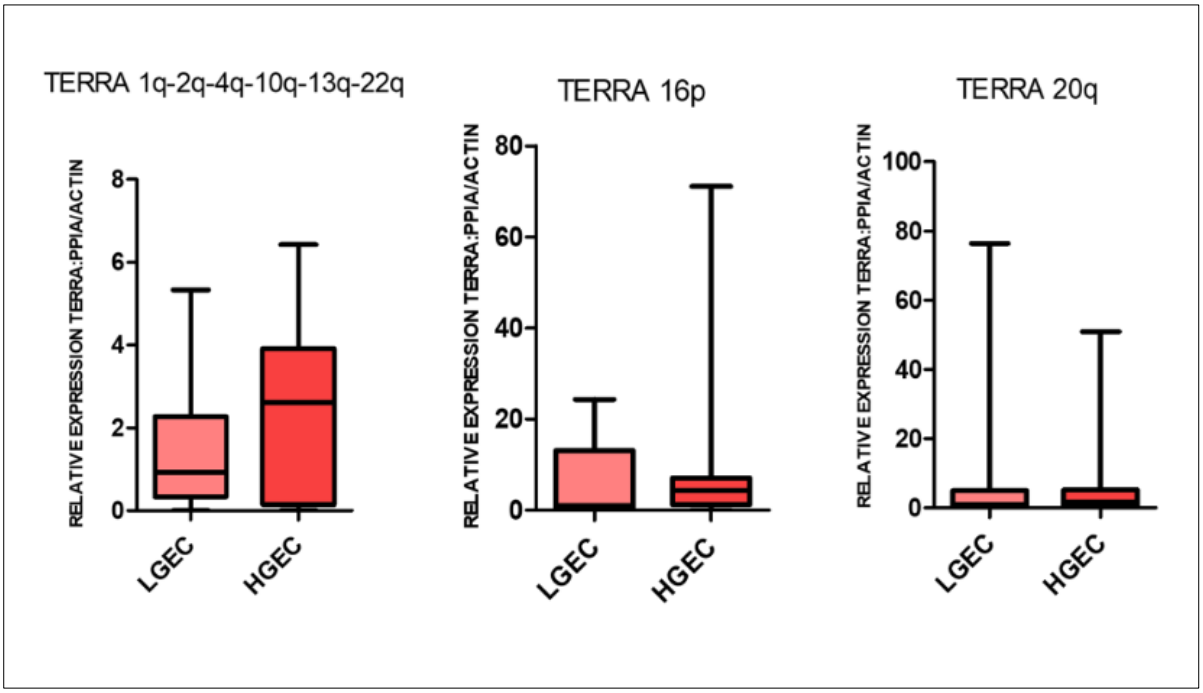


Fig 7. *TERRA* expression in low grade (EAC grade 1 and 2) (n=13) and high grade ECs (EAC grade 3 and type 2 ECs) (n=11). There was no change in levels of *TERRA1q-2q-4q-10q-13q-22q, TERRA16p*

and *TERRA 20q* in different grades of ECs (*TERRA1q-2q-4q-10q-13q-22q* P=0.49, *TERRA 16p* P=0.22, *TERRA 20q* P=0.73; Mann Whitney test).

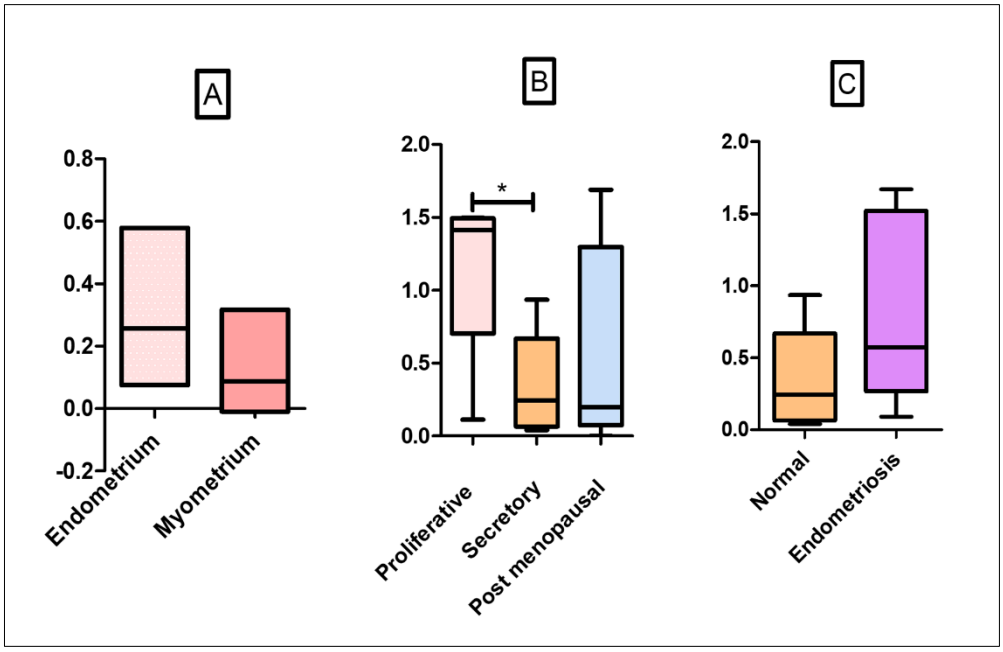


Fig 8. TA in human uterine tissues measured using TRAP. (A) Comparison of TA in endometrium (n=3) and myometrium (n=4), shows TA higher in endometrium (P=0.23; Mann Whitney test). (B)TA across different phases of menstrual cycle and post-menopausal endometrium (n=6). TA significantly high in proliferative phase (n=6) compared to secretory phase (n=6) of menstrual cycle (*P=0.04; Mann Whitney test). (C) TA in secretory endometrium of healthy women (n=6) compared to women with endometriosis (n=10). TA higher in endometrium of patients with endometriosis, however this is not statistically significant (P=0 .18; Mann Whitney test).

Ch-16p *TERRA* correlated negatively with the LESQS scores for PR (r = -0.40, P=0.02). The LESQS for other hormone receptors did not show a significant association with *TERRA* levels (Table 5). Shelterin proteins TRF1/2 are known to interact with *TERRA* and immunostaining and quick-scores for TRF2 positively correlated with Ch-1q-2q-4q-10q-13q-22q , Ch-16p and Ch-20q *TERRA* levels (Ch-1q-2q-4q-10q-13q-22q r =0.49, P=0.007; Ch-16p r =0.61, P=0.001and Ch-20q r =0.53, P=0.003, Table 5).

Telomere length in EC

When STELA results were analysed, there were differences in observed between healthy postmenopausal endometrium and EC samples in both telomere lengths and variance (SD) of the distribution at the XpYp chromosomes.

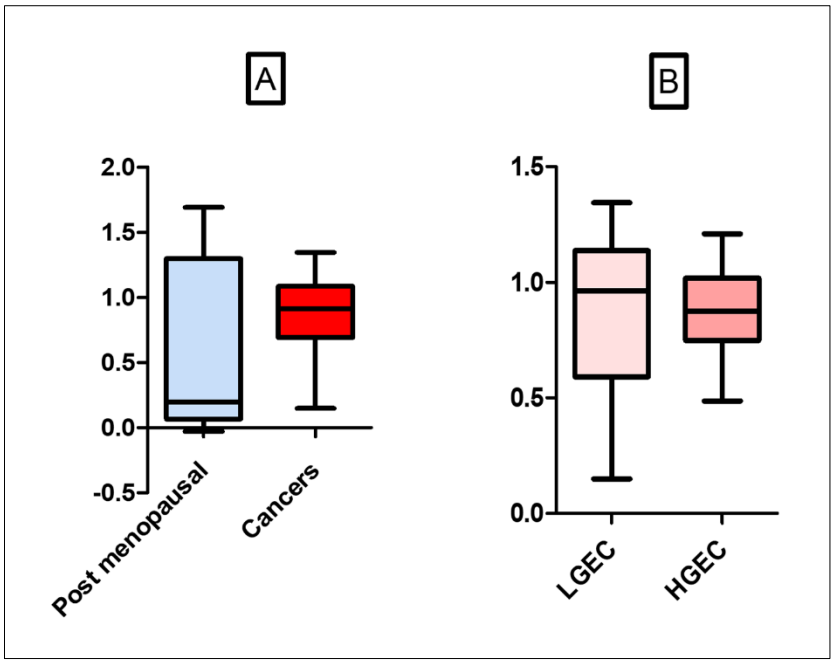


Fig 9. TA in post-menopausal endometrium and ECs. (A) Comparison of TA in post-menopausal endometrium (n=6) and ECs (n=20), shows TA higher in ECs however not statistically significant (P=0.19; Mann Whitney test). (B) In this study, it is noted that there is no change in TA in both low grade (n=10) and high grade ECs (n=10) (P=0 .74; Mann Whitney test).

Postmenopausal endometrial samples demonstrated considerable telomere length heterogeneity with telomeres at XpYp chromosomes ranging in one sample from 1.3kb to 21.7 kb (Fig 10A).

Consistent with previous reports (reviewed in (Alnafakh et al., 2019)), ECs displayed shorter telomeres when compared with healthy postmenopausal endometrium (P=0.002, Fig 10B).

We found a trend towards a decrease of telomere lengths in older women; however, this difference was not significant (Spearman correlation r=0.01, P=0.91, Fig 10C).

Discussion

I report the first comprehensive study on the levels of long non-coding RNAs, *TERRA* in healthy human endometrium in pre and postmenopausal women, and its expression in benign (endometriosis) and malignant (EC) endometrial proliferative pathologies. This study reports levels of three different *TERRA*s in the context of TA, ovarian hormone receptor and shelterin

proteins TRF1/2 expression levels and proliferative indices of the same patient-derived endometrial samples.

All methods available to measure *TERRA* levels have method-specific draw-backs: qPCR may be biased by the production of short transcripts, modified northern blot protocol may measure UUAGG content rather than *TERRA* and RNA-FISH problematic quantification and may under-estimate nuclear *TERRA*. We selected qPCR for its superior quantification utility and reproducibility.

Human endometrium is a unique somatic organ demonstrating dynamic TA, which regulates epithelial proliferation (Hapangama et al., 2008b), (Valentijn et al., 2015), (Hapangama et al., 2017). High TA levels were observed in the epithelial compartment, which has relatively shorter telomere lengths compared with the stromal cells (Valentijn et al., 2015). A paradoxical higher *TERRA* levels were observed in the isolated stromal cells when compared to the endometrial epithelium. In agreement, in benign healthy endometrium, *TERRA* levels appear to negatively correlate with epithelial proliferative indices. During the menstrual cycle, the lowest *TERRA* levels were observed in the proliferative phase, which has been reported to be associated with high TA and relatively longer telomere length (Valentijn et al., 2015). For example, *Ch-20 TERRA* levels were upregulated in the in the secretory phase, where glandular proliferation is low, and the highest levels were observed in the proliferatively quiescent postmenopausal endometrium. Collectively, my data suggest *TERRA* may have an anti-proliferative function in the human endometrium.

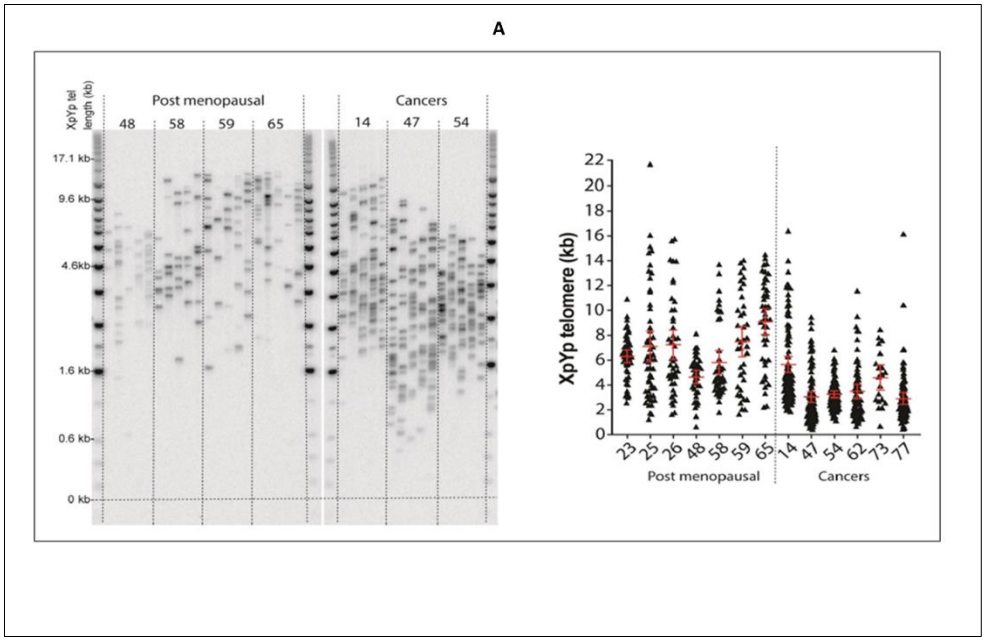
Each of the *TERRA* levels had a unique pattern suggesting *TERRA* specific regulation. Non-replicative cells with shorter telomeres undergo subtelomeric rearrangement during the quiescent phase, and this further results in increased transcription of non-coding *TERRA* agreeing with our data for *Ch-20 TERRA* (Maestroni et al., 2017). Other studies also have demonstrated that quiescent telomerase positive yeast cells have higher *TERRA* levels compared to the replicating cells (Maestroni et al., 2017).

TRF1 and 2 are shelterin proteins which protect the chromosomal ends from fusion and initiation of DNA damage response, help regulate TA through a negative feedback mechanism (van Steensel and de Lange, 1997).

Table 5. Correlation of *TERRA* 1, *TERRA* 16 *TERRA* 20, AR, PR, ERα, Erβ, Ki67, TRF 1, TRF 2, TRAP and *hTERC* in all endometrial samples

		***TERRA*1**	***TERRA*16**	***TERRA*20**	**AR**	**PR**	**ERα**	**ERβ**	**Ki67**	**TRF1**	**TRF2**	**TRAP**	***hTERC***
*TERRA*1	r		**0.4885**	**0.4841**	-0.0816	-0.1068	-0.0579	0.0156	-0.147	0.3324	**0.4884**	-0.2192	-0.0343
	P		**0.0008**	**0.0009**	0.6465	0.5543	0.7411	0.9293	0.3854	0.1307	**0.0072**	0.0953	0.785
*TERRA*16	r	**0.4885**		**0.7792**	-0.2828	**-0.3997**	0.1462	0.1325	**-0.3487**	0.2309	**0.6079**	-0.2362	0.1031
	P	**0.0008**		**< 0.0001**	0.1051	**0.0212**	0.4019	0.4479	**0.0319**	0.3011	**0.0005**	0.0717	0.4027
*TERRA*20	r	**0.4841**	**0.7792**		-0.1727	-0.2094	0.198	0.0831	**-0.4156**	0.34	**0.533**	-0.1509	0.0776
	P	**0.0009**	**< 0.0001**		0.3288	0.2423	0.2543	0.635	**0.0095**	0.1216	**0.0029**	0.254	0.5296

Table 5. Correlation of endometrial *TERRA* levels with each other, *hTERC*, steroid receptors, shelterin proteins and TA. *TERRA* show a strong positive correlation with each other but no significant correlation with TA (*TERRA 16p* and *TERRA 20q*, r =0.78, P=< 0.0001; *TERRA16p* and *TERRA 1q-2q-4q-10q-13q-22q*, r =0.49, P=0.001; *TERRA 20q* and *TERRA 1q-2q-4q-10q-13q-22q* = 0.48, P=0.001). *TERRA 16p* and *20q* negatively correlate with Ki67 (*TERRA 16p*, r = -0.35, P=0.03; *TERRA 20q*, r = -0.42, P =0.01). *TERRA 16p* positively correlated with *hTERC* in benign endometrial samples (r =0.32, P=0.03). Amongst steroid receptors, *TERRA 16p* correlated negatively with LESQS score for PR (r = -0.40, P=0.02). All *TERRA*s positively correlated with TRF2 quick score (ch1q-2q-4q-10q-13q-22q r =0.49, P=0.01; ch16p r =0.61, P=0.001; ch20q r =0.53, P=0.003). (I would like to thank the following collaborators have helped me with obtaining all data for the correlation study: TRAP Dr G Saretzki, TERRA 1q-2q-4q-13q-22q Shannon Simon, TRF1 Dr Lucy Button and steroid receptors Dr Rafah Alnafakh).



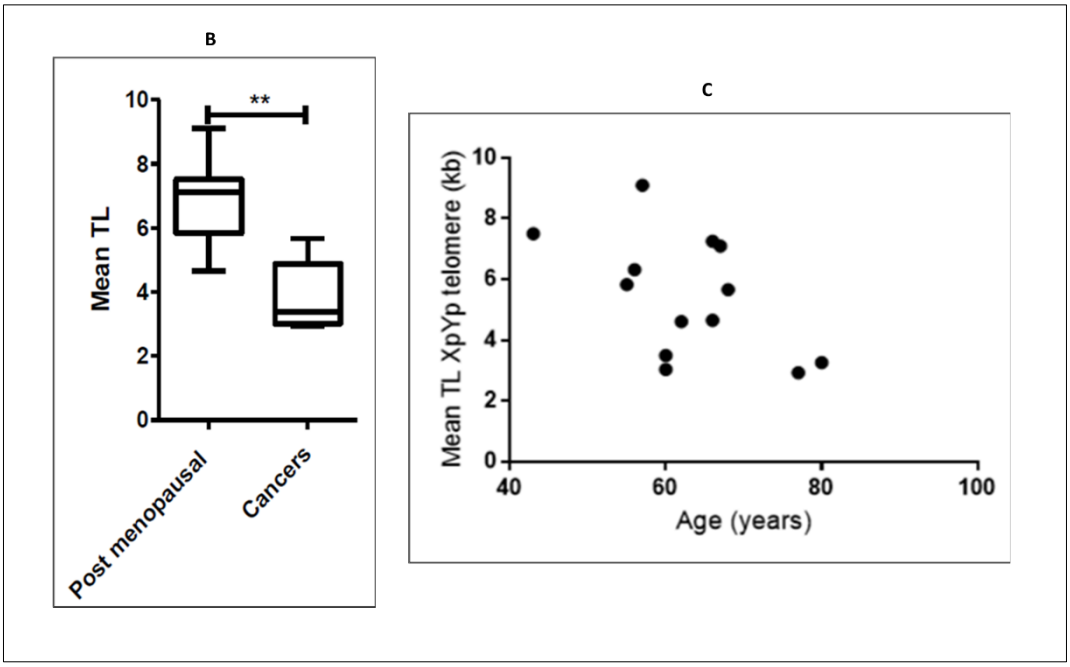


Fig 10. Telomere lengths in post-menopausal endometrium and EC's (A) This graph demonstrates the differences in telomere length heterogeneity and variance (SD) of the distribution at the XpYp chromosomes in post-menopausal endometrium (n=7) and EC's (n=6) (B) EC's demonstrate shorter telomeres compared to post-menopausal endometrium (**P=0.002) (C) Correlation of telomere lengths with age, showed no statistical significance however there was a trend of shorter telomeres in older women (r=0.01, P=0.91). (I would like to thank our collaborators, Dr Rhiannon Jones and Prof Duncan Baird, University of Cardiff for kindly performing the STELA experiments)

The publically available, “The Cancer Genome Atlas” (TCGA) cohort of uterine cancers (TCGA) dataset suggests high expression of TRF1 in ECs to be a poor prognostic indicator (Fig 11). Furthermore, *TERRA*s can bind to other chromosomal locations other than telomeres (Schoeftner and Blasco, 2008) and the interaction between *TERRA* and TRF1/2 ensures that these transcripts remain tethered to these telomeric domains (Deng et al., 2009). In our study, TRF1 protein expression correlated with *Ch-20 TERRA*, and therefore the potential interaction between TRF1 and *Ch-20 TERRA* may have a regulatory effect on other telomeres as well.

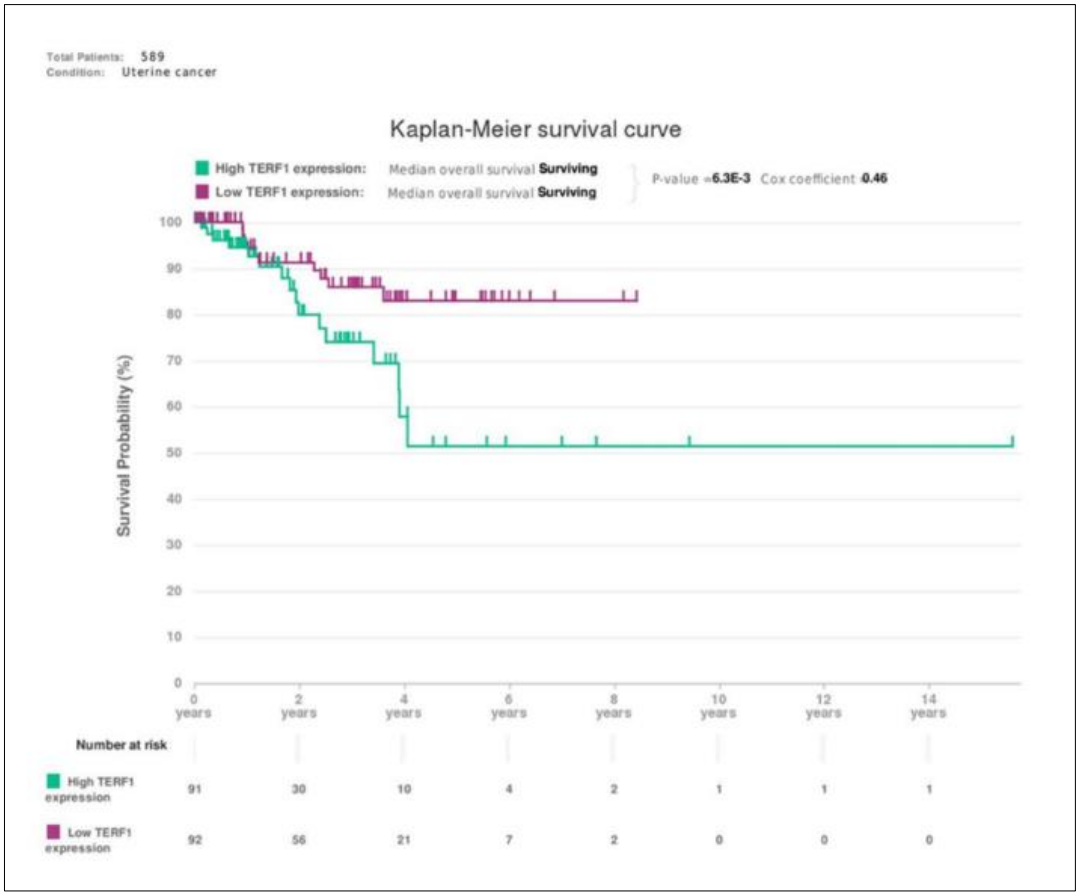


Fig 11. Kaplan-Meier survival curve in TCGA dataset for uterine cancer patient cohort shows that high TRF1 is associated with poor survival.

Endometriosis is known to be associated with progesterone resistance and high TA (Hapangama et al., 2008b). Although *TERRA* levels did not differ in the endometria of women with and without endometriosis, *TERRA* levels were significantly low in ECs with known high TA (Kyo et al., 1999a). Recent study analysing the endometrioid and serous cancer samples from the TCGA dataset, reported EC to be one of the cancers with the shortest mean telomere lengths amongst 31 different cancers (Barthel et al., 2017). In cancers without high TA, longer telomeres associate with high *TERRA* levels. Therefore, we present this interesting observation

in ECs the opposite to be true, with high TA, short telomeres, and reduced *TERRA* levels. The benign endometrial proliferative condition endometriosis which also has high endometrial TA but longer telomeres, does not demonstrate significant alterations in *TERRA* levels. Contrastingly, EC with high TA but shorter telomeres is associated with low *TERRA*, suggesting more complex, pathology-specific, telomere biology. Furthermore, considering the proposed anti-telomerase action of *TERRA*, the reduction of *TERRA* may provide a proliferative advantage for the ECs. This aspect warrants further studies to ascertain the functional role of *TERRA* in the endometrium and in EC.

In conclusion, *TERRA* levels are dynamic in human endometrium, and significantly lower *TERRA* levels are found in ECs. The dynamic changes in *TERRA* levels suggest hormone regulation and therefore I progressed to examine the hormone regulation of *TERRA* levels in the work described in Chapter 6. Since my study has shown *TERRA* levels to be inversely related to the cellular proliferative indices, further studies are needed to determine whether they can be successfully modified and tailored as a therapeutic target in endometrial proliferative conditions.

Chapter 6: Hormonal regulation of *hTERC* and *TERRA* in the endometrium; implication for endometrial carcinogenesis

Introduction

Endometrium is complex, dynamic tissue composed of epithelial (luminal and glandular) and stromal cells, which undergoes monthly growth, differentiation, shedding and regeneration (Hapangama et al., 2015). Endometrium undergoes efficient regeneration, which is mainly due to endometrial stem cells (Tempest et al., 2020). The human endometrium has two distinct layers, superficial stratum functionalis and deeper stratum basalis (Ferenczy and Bergeron, 1991). Stratum functionalis is composed of luminal epithelium, mainly contains glandular epithelium and stroma, and is shed every month (Gargett et al., 2008). The functionalis layer can be divided into two zones, deeper sone contains loosely organized stroma and superficial zone with more compact stroma (Wynn, 1989) and stromal component has fibroblasts and immune cells (Simitsidellis et al., 2018). The stratum basalis mainly consists of endometrial glands and dense stroma. The basalis layer is not shed in the monthly cycle and exists even after menopause as atrophic inactive postmenopausal endometrium (Chhieng and Hui, 2011). The stem cells are proposed to reside in the basalis layer (Valentijn et al., 2013). In addition to the structural difference between the functionalis and basalis, there are functional difference, for example in the hormone responsiveness between the two layers (Padykula et al., 1989). Some studies have discounted the differences between these layers and also the dynamic nature of functionalis (Argenta et al., 2014).

Previously it was thought that endometrial glands were of single and ductular conformation, however, the recent seminal work on 3D architectural organisation of the epithelial compartment has demonstrated that the functionalis glands originate from a complex network of basalis glands (Tempest et al., 2020).

The endometrial proliferation is regulated by ovarian steroid hormones and are directly implicated in endometrial carcinogenesis (Hapangama et al., 2015). Cells in the endometrium respond to ovarian steroid hormones, which coordinate proliferation, differentiation, apoptosis, and recruitment of cells in spatiotemporal manner (Simitsidellis et al., 2018). All steroid receptors, ER, PR, AR, and glucocorticoid receptors (GR) are expressed within human endometrium (Critchley and Saunders, 2009). Oestrogens, progesterone, and androgens are three main ovarian steroid hormones which act on endometrial cells via their cognate receptors (Hapangama and Bulmer, 2016).

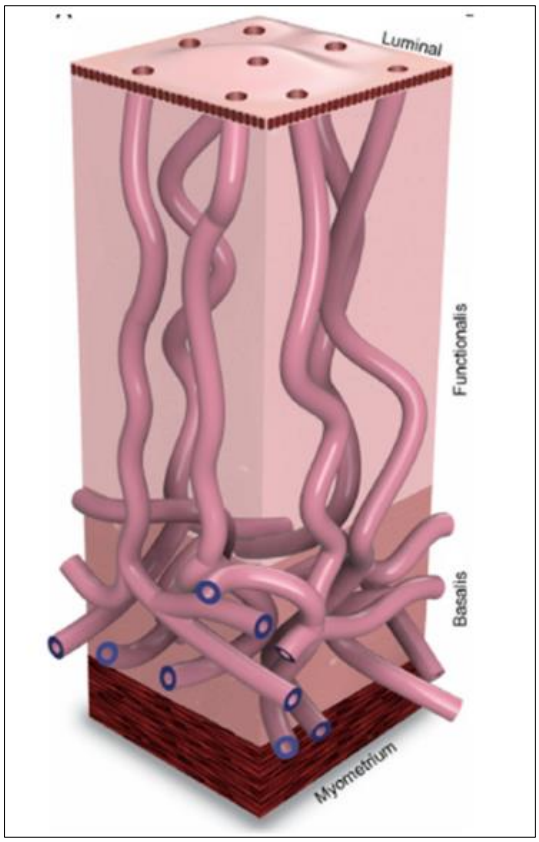


Figure 1. Schematic representation of 3D architectural organisation of endometrial epithelial compartment, demonstrating complex basalis glands running alongside the myometrium reproduced as published by Tempest N, Jansen M, Baker AM, Hill CJ, Hale M, Magee D, Treanor D, Wright NA, Hapangama DK. Histological 3D reconstruction and *in vivo* lineage tracing of the human endometrium. J Pathol. 2020 Aug;251(4):440-451. doi: 10.1002/path.5478. Epub 2020 Jun 30. PMID: 32476144.

17β-oestradiol and oestrones are two main oestrogens, which exert an effect on endometrium (Vani et al., 2008), (Crandall and Barrett-Connor, 2013). Both oestrogen and progesterone exert their functional effects via specific, high affinity, nuclear receptors, which regulate expression of downstream genes (Cheung and Kraus, 2010). The progesterone and oestrogen receptor signalling pathways are regulated in an epithelium and stromal component-specific manner.

There are two isoforms of ER, ERα and Erβ, which are similar in structure however have distinct patterns of expression in different conditions (Hewitt and Korach, 2003). Progesterone receptors (PR) have two isoforms, PR A and PR B, which have identical structure (Jacobsen and Horwitz, 2012). AR is a nuclear receptor which functions as a ligand-activated transcription factor (Lee and Chang, 2003). Testosterone can activate AR directly or by getting converted into 5α-dihydrotestosterone (DHT), which is the more potent agonist at AR (Lee and Chang, 2003). Endometrium is also an organ with intracrine androgen regulation since studies have

demonstrated endometrial expression of enzymes capable of converting precursor steroids to testosterone and DHT (Simitsidellis et al., 2018). Studies on rodent uteri have demonstrated that androgens stimulate growth and differentiation of endometrium and myometrium via AR and this effect is similar to ER (Kamal et al., 2016b). However, the findings of androgen stimulated uterine hypertrophy are not proven in human uteri. There is also complex interaction between coactivators and co repressors with the steroid receptors. Another interesting aspect of steroid receptors is that they interact dynamically with chromatin, regulated by chromatin remodelling, chaperones, binding of other transcription factors (Grontved and Hager, 2012).

Studies have demonstrated that there is cyclical variation in ER and PR expression in endometrium (Mylonas et al., 2007). Human endometrium has strong ER and PR expression during the proliferative phase, which decreases during the secretory phase (Mertens et al., 2001). Proliferative endometrium expresses high levels of steroid receptors in the glandular component as a result of increased oestradiol concentration (Snijders et al., 1992). Mylonas et al (2007) have reported that glandular ERβ expression is highest in proliferative phase and declines throughout the secretory phase of the cycle. They also hypothesise that ERβ distribution, action and transcriptional effect is different to ERα. It is reported that transcription of PR is oestrogen dependent and inhibited by progesterone (Savouret et al., 1994b), (Savouret et al., 1994a). PR expression in glandular component is highest in proliferative phase and decreases after ovulation. In endometrial stroma, no variation in PR expression is observed throughout the menstrual cycle (Snijders et al., 1992). Amongst both isoforms of PR, it is thought that PR A induces cell senescence and PR B induces a secretory phenotype of cells (Dai et al., 2002). AR Positive cells in the functionalis layer during proliferative phase are endometrial fibroblasts, and these are also present in the basalis throughout the cycle (Gibson et al., 2018). In secretory phase, AR is downregulated in stromal component of the functionalis and upregulated when progesterone levels decline in late secretory phase or exposure to progesterone receptor modulator/antagonists (Marshall et al., 2011), (Whitaker et al., 2017). The regulation of AR by androgens is considered to be a feed-forward mechanism of androgen action in the endometrium (Chadha et al., 1994). Current evidence suggests androgens and oestrogens positively regulate AR expression whereas progesterone downregulates AR in the endometrium (Fujimoto et al., 1995), (Mertens et al., 2001), (Burton et al., 2003), (Narvekar et al., 2004).

Postmenopausal endometrium is mainly composed of inactive glands with stroma similar to stratum basalis. Postmenopausal mileu has characteristically absence of oestradiol and progesterone, but presence of oestrone and androgen which may help the maintenance of ER

and PR expression in endometrium (Sivridis and Giatromanolaki, 2004). Kamal et al have reported that postmenopausal endometrium has higher epithelial AR, ERα and similar ERβ and PR expression compared to basalis layer of premenopausal endometrium (Kamal et al., 2016a). Human endometrium undergoes a complex functional change throughout the reproductive period, the processes include proliferation, repair of endometrium, angiogenesis, cell differentiation, matrix remodelling, and all these changes occur as a response to hormones. The common exogenous hormones which are used in gynaecological practice and have a role in regulation of endometrium are oestrogen, progesterone, androgens and GnRH analogues. Oestrogen induces growth of endometrium via cellular proliferation (Groothuis et al., 2007) and oestrogen triggers endometrial angiogenic activity in response to post menstrual hypoxia, by upregulating angiogenic factors such as VEGFA (Punyadeera et al., 2006), (Groothuis et al., 2007). Conversely, progesterone leads to decrease in endometrial cellular mitotic activity (Taraborrelli, 2015) and during the luteal phase, it also prompts extensive vascular development and differentiation of stromal cells into decidual cells (Rider et al., 1998). Short term use of androgens does not stimulate human endometrial proliferation, yet the long-term effect of androgens on the endometrium is not fully clear (Zang et al., 2007).

GnRH agonists have an anti proliferative effect in human endometrium by suppression of ovarian steroid production and by a direct effect on cell apoptosis (Emons et al., 1998). GnRH agonists not only reduce the production of oestrogen but also reduce the metabolism of oestrogen in the endometrium (Ishihara et al., 2003).

In chapters 4 and 5, I demonstrated that *hTERC* and *TERRA* varied throughout the menstrual cycle and changes in levels were noted in proliferative, secretory phases, postmenopausal endometrium. This suggests hormonal regulation. Therefore, to further study the hormonal regulation on *hTERC* and *TERRA* levels, I explored the effect of hormonal treatment on endometrial epithelial cell *hTERC* and *TERRA* levels *in vitro* using an established EC cell line, Ishikawa (ISK) cell model. ISK cells contain all 4 ovarian hormonal receptors and are hormone responsive. Furthermore, to assess the *in vivo* hormonal regulation, *hTERC* and *TERRA* levels in endometrial samples derived from women on exogenous hormones (GnRH analogues simulating hypo-estrogenic status and Levonorgestrel Intrauterine System (LNG-IUS), providing high dose synthetic progestogens) were analysed. GnRH analogues cause an atrophic effect on the endometrium hence mimic the postmenopausal phase, whereas LNG-IUS releases high doses of progestogen in the endometrial cavity and prevent estrogenic action in the endometrium. Both these hormonal agents were chosen due to their protective role in endometrial proliferation and in carcinogenesis. GnRH analogues cause down-regulation of

proliferation, induce apoptosis and are used as a part of fertility preserving therapy in endometrial hyperplasia or early EC (Emons and Grundker, 2021). Progesterone controls oestrogen driven proliferation can promote inhibition of growth and apoptosis (Kim and Chapman-Davis, 2010).

Materials and Methods

Endometrial tissue samples

Details of the Ethical approval are provided in page 15. Endometrial samples were collected from women without any endometrial pathology, with regular periods and who were not on any hormonal treatment for at least 3 months in proliferative phase (n=6), secretory phase (n=9) and women in postmenopausal period (n=7). Women treated with exogenous hormones such as Gonadotropin releasing hormone (GnRH) agonists (n=10), and Levonorgestrel Intra uterine system (LNG-IUS/Mirena, n=11) also donated endometrial samples. The endometrial samples were dated according to histological criteria and patient-claimed last menstrual date.

Once collected, endometrial and myometrial samples were divided into three, and immediately placed in i) RNAlater for RNA extraction, ii) NBF for paraffin embedding and iii) snap frozen for TRAP assay. Three further endometrial samples were also retrieved in collection media as previously described for cell separation (Valentijn et al., 2013). Patient clinico-pathological and demographic details were retrieved from the clinical notes and databases (Table 1).

Table 1. Demographic details of patients included in this study

Study groups (n)	***Age (years)**	***BMI (kg /m^2)**
Proliferative phase (7)	43(32-57)	27.8(22-40.5)
Secretory phase (9)	41(21-47)	22.6(18.9-31.6)
Postmenopausal (7)	62(52-85)	24.3(20-39.6)
Women with Levonorgestrel IUS (11)	34.5(26-47)	25(18.8-33.4)
Women treated with GnRH analogues (10)	41(32-49)	31.1(21.8-35.5)

*Data expressed as median (range)

BMI- body mass index

RNA extraction and Real Time-qPCR

RNA was extracted, quantified and reverse transcribed as previously described in chapter 3 (Mathew et al., 2016). cDNA was amplified using iTaq universal SYBR Green supermix and CFX Connect Real Time System (Bio-Rad, Hertfordshire, UK). Forward and reverse primers used, and the reaction conditions are listed in Table 2 and 3. Relative transcript level was calculated using the ΔΔCT method, normalised to the reference genes, beta actin (ACTB) and peptidylprolyl Isomerase A (PPIA) using Bio Rad CFX Manager (Bio-Rad, Hertfordshire, UK).

TRAP assay

TRAP assay was kindly performed by Dr Gabriele Saretzki, Lecturer in Ageing Research, Biosciences Institute and Newcastle University Institute for Ageing, Campus for Ageing and Vitality, Newcastle. The protocol is as described in chapter 4. TA was measured using TeloTTAGGG TRAP assay (Telomere Repeat Amplification Protocol assay; Roche Diagnostics, Ltd, Burgess Hill, UK) using 1µg of lysate (Valentijn et al., 2015). Amplification product after 30 cycles were measured as absorbance at 450nm in a Fluostar Omega Plate reader (BMG Labtech) and presented as AU.

Table 2. Primer sequences used for qPCR amplification

Primer	Sequence	References
PPIA	F:5'- AGACAAGGTCCCAAAGAC-3 R:5'- ACCACCCTGACACATAAA-3	(Jacob et al., 2013)
ACTB	F:5'TGTACGCCAACACAGTGCTG-3 R:5'GCTGGAAGGTGGACAGCGA-3	(Wang et al., 2015)
TERC	F:5'- GCCTTCCACCGTTCATTCTA-3 R:5'- CCTGAAAGGCCTGAACCTC-3	(Chai et al., 2011)
TERRA Ch 1q-2q-4q-10q-13q-22q	F:5'GAATCCTGCGCACCGAGAT R:5'CTGCACTTGAACCCTGCAATAC	(Diman et al., 2016)
TERRA Ch16p	F: 5'TGT GTT TCA ACG CTG CAA CTG R: 5'AGT TAG AAC GGT TCA GTG TG	(Wang et al., 2015)
TERRA Ch20q	F:5'GAAGTTGCTGGGTTCTATGG R:5'ATGGTGCAGACACTGTGG	(Montero et al., 2016)

Table 3. qPCR Conditions

		Initial Denature	Denature	Anneal / Extension	Cycles	Efficiency
Target Gene	**PPIA**	95°C 2min	95°C 5s	60°C 30s	40	100.1%
	ACTB	95°C 10min	98°C 5s	95°C 10s 60°C 30s	40	94.6%
	TERC	**95**°C 2min	95°C 5s	60°C 30s	50	86.1%
	TERRA1q-2q-4q-10q-13q-22q ***TERRA 16p*** ***TERRA 20q***	95°C 10min	98°C 5s	95°C 10s 60°C 30s	50	101.1% 95.1% 105.2%

Abbreviations: *PPIA*, peptidylprolyl isomerase A; *ACTB*, beta-actin

Cell culture

All cell-culturing procedures were performed under sterile conditions in Biomat^{2}class II hoods (CAS, Manchester, UK) by Dr Rafah Alnafakh. The cell culture protocols available at the Centre for Women's Health Research (CfWHR) laboratories were followed and cell lines mentioned below were cultured at the CfWHR laboratories. Cells were subsequently pelleted for TRAP and RNA extraction for qPCR.

Ishikawa cell line culturing

The culturing experiments were kindly performed by Dr Rafah Alnafakh, PhD student, University of Liverpool. The protocol for ISK cell line culturing used by Dr Rafah Alnafakh, is as described below. The ISK cell line, which was developed from a well-differentiated grade 1 human endometrial adenocarcinoma was obtained from Public Health England (Salisbury, UK). These cells were maintained and grown in a 10 cm culture dish containing MEM/F12, 10% FBS supplemented with L-glutamine and antibiotic penicillin / streptomycin. Cells were incubated at 37°C under 5% CO2 in humidified atmosphere. The medium was changed 24 hours after seeding and renewed at intervals of 48 hours until cell culture achieved sub confluence status. Cells were passaged at 1:5 dilutions using trypsin, the 3rd to 12th passages

were used for the experiments. The cells were prepared for steroid treatment by preconditioning in DMEM/F12 (phenol-free, Life Technologies, UK) supplemented with antibiotic and 2% charcoal-stripped FBS for 48 hours prior to the experiment.

In vitro hormone treatment

The protocol for *in vitro* treatment of cells used by Dr Rafah Alnafakh, PhD student, University of Liverpool is as described below. Cultured ISK cells were maintained in Dulbecco modified Eagle medium/F12 medium supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO2 atmosphere. ISK cells were allowed to grow in DMEM/F12 (phenol-red free; Life Technologies, Paisley, UK) with 2% (v/v) charcoal-stripped FBS for 48 hours prior to the hormonal treatment. The cells were treated with 17β oestradiol (E2; 10^{-8} M), progesterone (P4; 10^{-6} M) (progesterone; Merck, Nottingham, UK) and 5α dihydrotestosterone (DHT; 10^{-6} and 10^{-8} M) individually and in combination (E2+P4) for 12-72 hours. The experiments using DHT tested two concentrations, (1) DHT 10^{-8} M, the physiological dose and 10^{-6} M supraphysiological dose were examined at 0, 12, 48 hours.
The medium containing progesterone was changed twice daily. All cell culturing procedures were accomplished under sterile conditions in BioMat2 Class II hoods (CAS, Manchester, UK). All cell culture reagents and steroid hormones were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise. Cells were subsequently pelleted for TRAP and RNA extraction for qPCR.

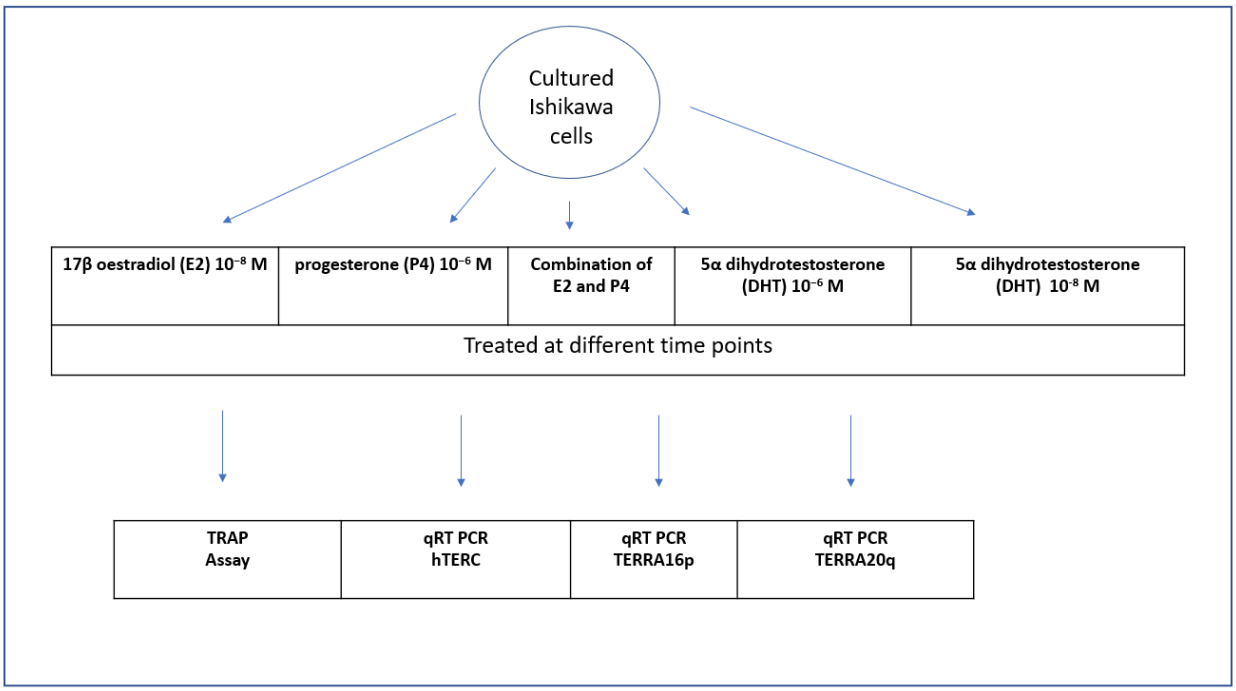


Figure 2. Diagramatic representation of cell culture and hormonal treatment experiments and methods used.

Results

Telomerase activity has variable response to different hormones

The effect of the 3 main ovarian hormone treatments were tested in the same *in vitro* model using the hormone responsive endometrial epithelial cell line, Ishikawa. Ishikawa cells grown in 2D were tested with (1) E2, (2) P4 (3) E2+P4 in combination and (4) DHT 10^{-6} M and (5) DHT 10^{-8} M.

TRAP data from *in vitro* studies on endometrial epithelial cell line, ISK, demonstrated a slight increase and decrease in TA in response to E2 at 12 and 48 hours respectively. TA levels decreased with P4 and combination of E2+P4 at both 12 and 72 hours however, none of these changes were statistically significant (Fig 3.2 A and Fig 3.3 A). With both DHT 10^{-6} and DHT 10^{-8}, TA increased initially at 12 hours then decreased at 48 hours (Fig 3.1 A, Fig 3.4 A and Fig 3.5 A).

Progesterone increase hTERC levels in Ishikawa cells In vitro

hTERC levels appear to increase with E2 (Fig 3.1 B), E2+P4 (Fig 3.3 B) and P4 (Fig 3.2 B) treatment at 0,12,72 hours however, significant increase was only seen 72 hours after the treatment with P4 and E2+P4 combination treatment {E2; P= 0.72, Kruskal-Wallis test, E2+P4; P=0.002, Kruskal-Wallis test , P4; P=0.011, Kruskal-Wallis test}.

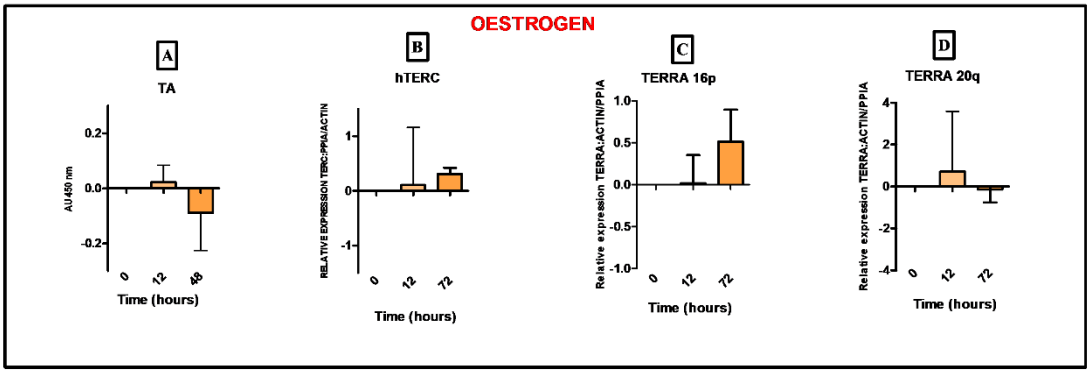
Although there was a trend of increased *hTERC* levels after 10^{-6} M DHT treatment at 48 hours (Fig 3.4 B), in the cells treated with 10^{-8}M, there was an apparent decrease from 12h but the effect appears to wear off by 48h (Fig 3.5 B). None of these differences reached statistical significance. Unfortunately, DHT experiments using both doses of the hormone did not include assessment beyond 48 hours of treatment unlike with the E2 and P experiments. Therefore, the effect of DHT treatment on the *hTERC* levels on Ishikawa cells is not complete comparative to the other experiments with E2 and P4 hormones.

TERRA levels in Ishikawa cells after hormone treatment in vitro suggests a variable cell specific effect.

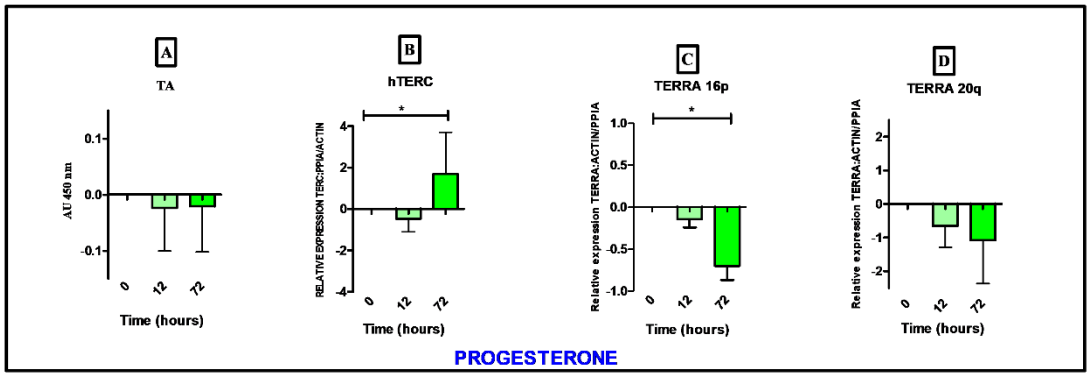
TERRA 16p levels appear to increase with E2 (Fig 3.1 C), but they decreased with E2+P4 (Fig 3.3 C) and P4 (Fig 3.2 C) treatment at 0,12,72 hours however, significant increase was only

seen 72 hours after the treatment with progesterone alone and both E2+P4 in combination {E2;P= 0.72, Kruskal-Wallis test, E2+P4; P= 0.04, Kruskal-Wallis test , P4; P= 0.001, Kruskal-Wallis test}.

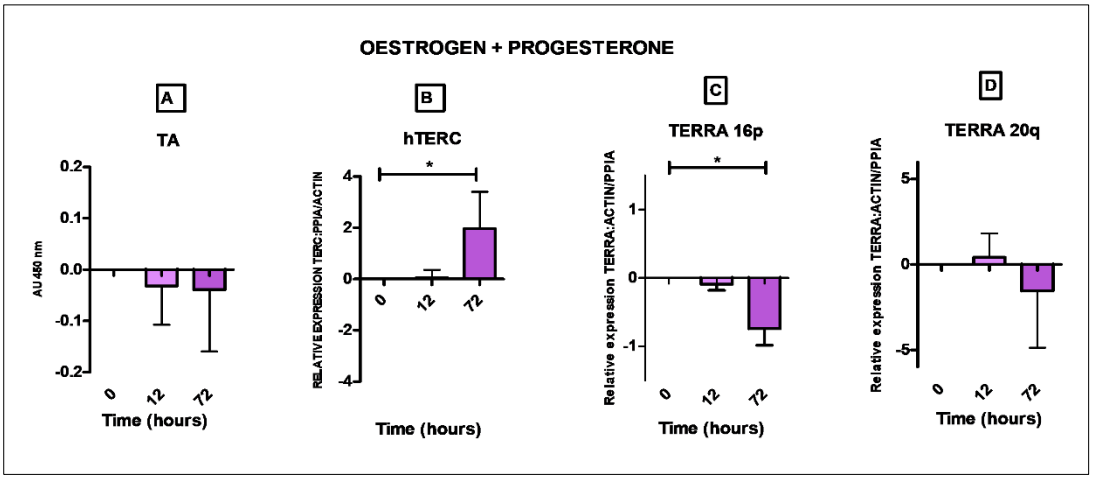
TERRA 20q appear to have variable response to all hormones, without any significant trend in the levels {E2;P= 0.97, Kruskal-Wallis test, E2+P4; P= 0.51, Kruskal-Wallis test , P4; P= 0.84, Kruskal-Wallis test} (Figure 3.1 D to Figure 3.3 D). When treated with lower or higher concentration of DHT, there was variable response in *TERRA (16p and 20q)* levels to both 10^{-6} and 10^{-8} concentrations {DHT 10^{-6} *TERRA 16 p* P=0.86, *TERRA 20q* P=0.79 and DHT 10^{-8} *TERRA 16 p* P=0.26, *TERRA 20q* P=0.16} (Fig 3.4 C&D, Fig 3.5 C&D).



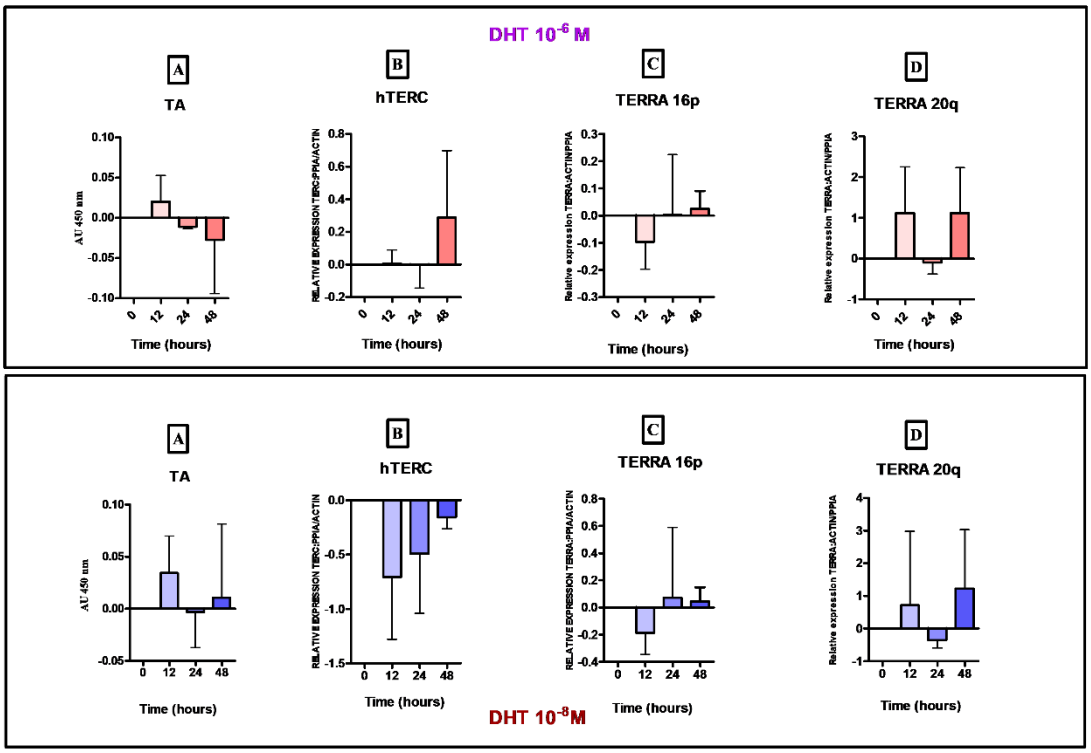
3.1



3.2



3.3



3.4

3.5

Fig 3. TA, *hTERC*, *TERRA 16p* and *TERRA 20q* levels in Ishikawa cells after hormone treatment (n=3 in each hormone treatment group). (3.1) TA levels showed a slight increase and decrease in TA in response to E2 at 12 and 48 hours respectively. *hTERC* levels appear to increase with E2 treatment at 0,12,72 hours without statistically significant change. *TERRA 16p* levels appear to increase with E2 treatment at 0,12,72 hours however, *TERRA 20q* appear to have variable response, without any significant trend in the levels. (3.2) TA levels decreased and *hTERC* levels appear to increase with P4 at 0,12,72 hours. *TERRA 16p* levels decreased with P4 treatment at 0,12,72 hours. *TERRA 20q* appear to have variable response, without any significant change. Significant increase was only seen 72 hours in *hTERC* levels and *TERRA 16p* levels after the treatment with P4 (*hTERC* ; P=0.011, Kruskal-Wallis test, *TERRA 16p*; P= 0.001, Kruskal-Wallis test). (3.3) TA levels decreased and *hTERC* levels increased with combination treatment of E2+P4 at both 12 and 72 hours however, none of these changes were statistically significant. *hTERC* levels appear to increase with E2+P4 at 0, 12, 72 hours. *TERRA 16p* levels decreased with E2+P4 treatment at 0,12,72 hour. Significant increase was only seen in *hTERC* levels and *TERRA 16p* levels 72 hours after the treatment with E2+P4 in combination (*hTERC*; P=0.002, Kruskal-Wallis test, *TERRA 16p*; P= 0.04, Kruskal-Wallis test) . *TERRA 20q* appear to have variable response to combination treatment. (3.4) and (3.5) With both DHT 10^{-6} and DHT 10^{-8}, TA increased initially at 12 hours then decreased at 48 hours. There was a trend of increased *hTERC* levels after 10^{-6} M DHT treatment at 48 hours, in the cells treated with 10^{-8}M, there was an apparent decrease from 12h but the effect appears to wear off by 48h. When treated with lower or higher concentration of DHT, there was variable response in *TERRA (16p and 20q)* levels to both 10^{-6} and 10^{-8} concentrations.

TA did not significantly change in endometrium from women who were treated with exogenous hormones

There was no significant difference in endometrial TA when treated by exogenous hormones (progestogens and GnRH) (Fig 4). It was noted that TA is slightly higher in progestogens group compared GnRH group though these are not significant. TA being lower in GnRH group is most likely due least proliferation of endometrium during treatment with GnRH analogues which induce atrophic changes.

hTERC levels did not significantly change in endometrium from women who were treated with exogenous hormones

hTERC levels did not show a significant difference in endometrium exposed to exogenous hormones such as progestogens or GnRH (hypo oestrogenic) (Fig 5). Levels of *hTERC* in endometrium treated with exogenous hormones was lower than postmenopausal endometrium but was not statistically significant.

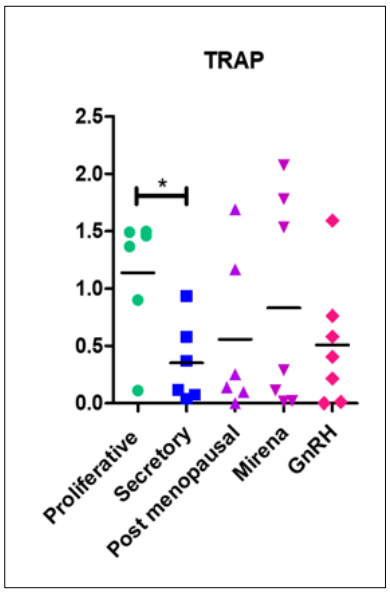


Fig. 4 TA in human endometrium across the menstrual cycle compared with women treated with exogenous hormones. TA did not show any significant change in endometria of women treated with exogenous hormones LNG-IUS (Mirena) (n=11) and GnRH (n=10). TA low in GnRH group.

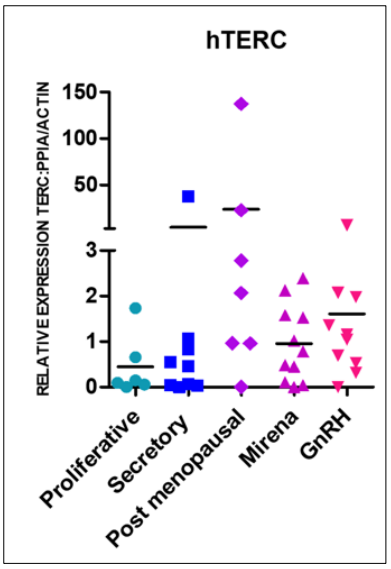


Fig. 5 *hTERC* levels in human endometrium across the menstrual cycle compared with women treated with exogenous hormones. *hTERC* did not show any significant change in endometria of women treated with exogenous hormones LNG-IUS (Mirena) (n=11) and GnRH (n=10)

Exogenous hormonal treatment of endometrium suggests a complex cell specific effect on TERRA levels

Endometrium from women on GnRH (hypo-oestrogenic) treatment demonstrated high *TERRA* levels compared to premenopausal endometrium, and this difference was significant for *TERRA 20q* (P=<0.01, Fig 6C). The progestogen (LNG-IUS)-treated endometrial *TERRA* levels did not show a statistically significant difference when compared with endometrium from women not on hormone treatments (Fig 6 A, B, C).

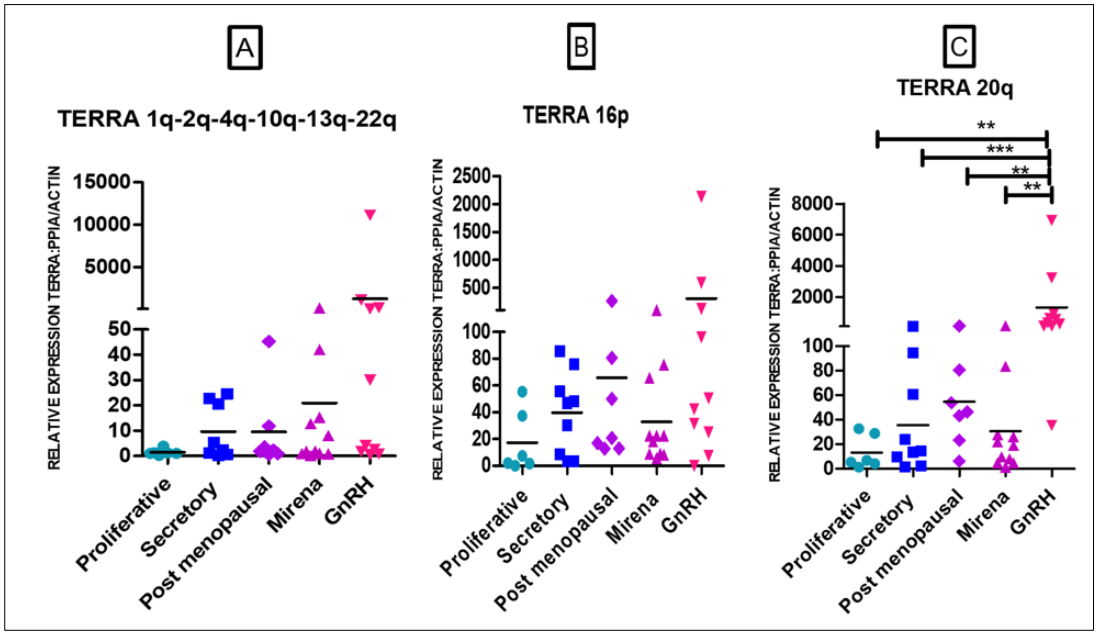


Fig 6

Figure 6. *TERRA* levels in human endometrium treated with exogenous hormonal treatment (LNG-IUS (n=11) and GnRH agonists treatment (n=10)). Significant increase in *TERRA 20q* levels in endometrium treated with GnRH agonists (P=<0.01; Mann Whitney test).

Discussion

Human endometrium is the primary target organ for ovarian hormones. Endometrial epithelial cells can respond to the monthly menstrual change in hormones, since they have oestrogen, progesterone and androgen receptors. It has been reported that the gene expressions in endometrium change in response to steroid hormones and genome wide DNA methylation profiling of endometrium had shown dynamic DNA methylation patterns, interplay between steroid hormone and these methylome dynamics appear to regulate the endometrial function. Therefore, abnormalities in these may result in endometrial dysfunction and disorders (Houshdaran et al., 2014). Oestrogen, progesterone and androgen receptors are classical nuclear steroid receptors and their differential expression, in different cells and physiological states, contribute to differential effects of the steroids. The pleiotropic effect of steroid hormones is due to expression and activity of the co-activators and co-repressors, and this can be different in various mammalian species (Young, 2013). Mechanisms through which oestrogen and progesterone work is complex, and cell type specific effect of these hormones is dependent on expression of receptors, chaperones, co-regulators and chromatin structure (Young, 2013).

Oestrogen *in vitro* did not show significant effect on TA, *hTERC* and *TERRA* levels. However when the endometrium of women in different phases of menstrual cycle and in women treated with exogenous hormones were considered, higher TA levels in proliferative phase samples confirm previous reports of effect of oestrogen induction of TA. Postmenopausal phase is a physiological hypooestrogenic phase, whereas GnRH treatment creates a pharmacological hypooestrogenic state. GnRH treatment was associated with high endometrial *TERRA 20q* levels and was associated with low TA. There was no difference in *hTERC* levels, but a low oestrogenic state may induce *TERRA 20q* levels. GnRH and postmenopausal state may not be the same, as effect of GnRH on endometrial stroma and various other hormones such as androgens can change with age. In premenopausal women, the endometrium is influenced by lot of hormones such as androgens, oestrogen, progesterone etc, whereas postmenopausal endometrium is quiescent and postmenopausal period is associated with almost complete cessation of ovarian hormone production.

Progesterone *in vitro* has shown an increase in *hTERC* levels, a decrease in *TERRA16p* and a decrease in *TERRA 20q* levels. However *in vivo* treatment with progesterone did not demonstrate any significant effect on either *hTERC* or *TERRA* levels in the endometrium. Progesterone's direct effect that we observed on Ishikawa cells in *hTERC* and *TERRA* levels

in vitro, was observed in a single cell type cultured in 2D system. However, *in vivo* treatment is rather different, the human endometrium contains multiple cell types including epithelial and stromal cells as well as leucocytes and vascular cells, existing in 3D configuration. I therefore propose further studies using culture models including all different cell types in the endometrium using 3D model systems to examine the clinically relatable effects of hormones on the endometrium, to make it more relevant to physiological conditions.

Combination of oestradiol and progesterone *in vitro* showed an increase in *hTERC* and decrease in *TERRA (16p and 20q)* levels, similar to the observation with progesterone only treatment. This suggests that even without exogenous addition of oestrogen, the ISK cells exist in an oestrogen driven status, and this can be counteracted by adding progesterone.

Interestingly, *TERRA 20q* levels were high in GnRH treated endometrium and future studies examining the functional effect of *TERRA 20q* needs to focus on understanding the role of GnRH in regulating those functions. GnRH causes atrophy of endometrial functionalis hence the persistent basalis layer of the endometrium may be preferentially sampled in these women. We already know from previous studies that basalis layer has high TA (Valentijn et al., 2013). This suggest that different endometrial layers (functionalis / basalis) may have correspondingly different *TERRA* levels. Furthermore, contamination by myometrial cells in an atrophic endometrial sample is also possible and need to be examined in future studies.

Another limitation of this study is the *in vitro* model used a 2D single cell type culture model which cannot easily be compared with the *in vivo* effects seen in the endometrium. For example the anti-proliferative effect of progesterone on the endometrial glands is thought to be an indirect effect via the stromal cells (Li et al., 2011), (Valentijn et al., 2015). Our *in vitro* mono-culture system contained only epithelial cells derived from an endometrial adenocarcinoma, therefore it may not replicate the complex epithelial/stromal interplay thus the physiological effect of the hormone.

In conclusion, there appear to be a hormonal regulation of levels of *hTERC* and *TERRA* in the endometrial cells, however, further studies are required to understand the clinically translatable effects of each of these hormones on the endometrial *hTERC* and *TERRAs* to clarify if they have any clinical therapeutic utility in EC.

Chapter 7: Harmonisation of biobanking standards in endometrial cancer research

Introduction

EC is the most common gynaecological cancer in the developed world and is the fourth most common cancer in women (Ferlay et al., 2015). In the UK between 2017-2019, nearly 7 women died of and 27 women were diagnosed with EC in the UK every day; with 9703 new cases and approximately 2500 deaths reported in a year (CRUK, 2016 - 2018). It has been reported that the incidence rate of EC is increasing rapidly and is estimated to increase by 50 -100% by 2025 (Lindemann et al., 2010). There has been a significant increase in incidence of uterine cancer in the UK by 59% since 1990's (CRUK, 2016 - 2018). The mortality rates have increased by 24% in the UK over the last 10 years (CRUK, 2016-2018a). The mortality rates are predicted to rise by 19% by 2035 (CRUK, 2016-2018a). In early stages the prognosis of EC is good with 5 year survival rate is 92% however reduces to 15% in advanced stages (CRUK, 2016 - 2018). Finding new prevention, diagnostic, prognostic and therapeutic targets are urgently required to reduce the high mortality and morbidity rates associated with EC. Although traditionally, researchers have used animal models and cell lines to study cancer, they rarely simulate the *in vivo* environment in humans, thus human tissue samples are critical for EC research. The research methods such as immunohistochemistry, based on formalin fixed paraffin embedded tissue allowed study of a limited number of proteins only. 21st century has seen evolution of molecular oncology by understanding tumour biology, molecular characterisation of tumour pathways and processes, development of targeted therapies. Therefore, tissue samples stored in biobanks, collected from a wide range of different patients, including for example fresh frozen tissue, urine, blood or saliva, play a vital role in providing unique and essential resource for clinically relevant scientific studies and also aid to the rapid translation into clinical practice. Biospecimens are at the centre of the personalised medicine evolutionary wheel.

Biospecimens stored in biobanks allow exploration of multiple aspects of endometrial carcinogenesis using novel technological platforms in genomics, proteomics, epigenomics, and metabolomics that can be collectively and simultaneously applied to the patient samples in order to efficiently utilise the resources. This all-encompassing approach is expected to substantially reduce the time taken for new basic scientific discoveries to translate as new treatments as well as allowing the samples donated by patients to be fully utilised. In the previous chapter, I have demonstrated how timing of endometrial biopsies in the context of hysterectomy and the method of sampling may affect results obtained from them in chapter 3. This highlights how simple deviations in methodology may affect the final results in a study.

The success of biobanks depends on internal and external validity of the generated data from the biospecimens which depends mainly on their quality, which is in turn dependent on the use of standardised protocols and policies in biospecimen collection and demographic data associated with it. Variations in biobanking processes such as collecting, processing, storing different bio-specimens and the accompanying phenotypic data make it particularly challenging to extrapolate or to merge data from different studies (Ransohoff and Gourlay, 2010) (Tworoger and Hankinson, 2006). The lack of standards and uniformity is considered as a roadblock in cancer research by the NCI (NCI, 2011). The irreversible bias introduced by the dissimilarities in specimens and data collection are well recognized and many efforts are being made by international organizations such as NCI, European Organisation for Research and Treatment of Cancer (EORTC),and the UK biobank (Morente et al., 2006), (Morente et al., 2007), (Yuille et al., 2008), (Peakman and Elliott, 2008), (Vaught and Lockhart, 2012).

The NCI best practice guidance for biobanks for cancer research, supports optimisation of the resources, advocates the policy for minimising the conditions which could critically affect the results and quality control or assurance which in turn helps in managing the resource aptly (NCI, 2011). This guidance has been effective in raising the awareness and quality of research involving bio specimens.

Although this is an important issue, many parameters of interest, including choice of biospecimens, most robust techniques required and clinical data, are cancer-type specific. Thus, universal biobanking standards are not necessarily applicable to every cancer-type and should be adapted to a specific disease. The importance of cancer specific harmonisation of biobanking standards is emphasised by the cancer genome atlas (Kandoth et al., 2013), which contains around 532 EC samples with RNA sequencing, copy number variation, proteomic, mutation and microarray data. However, the limited clinical data associated with most of these samples and datasets severely affects the ability of researchers to draw clinically applicable information.

Therefore, EC specific standardisation of the collection of biospecimens with relevant accompanying clinical data sets is a fundamental unmet need in improving future EC research. We believe this will promote future large-scale international collaborative research into EC, which could steer to a better quality biomarker or treatment breakthrough. Similar harmonisation projects have been effectively implemented for other conditions such as endometriosis - WERF EPHect, and OVCARE (Wiegand et al., 2010), (Heravi-Moussavi et al., 2012), (Vitonis et al., 2014), (Rahmioglu et al., 2014), (Fassbender et al., 2014).

With this background and considering the finding of the work described in the previous chapter (Chapter 3), we commenced our study (Harmonisation of biobAnking STandards in Endometrial caNcer research - HASTEN) to achieve consensus amongst EC researchers; to standardise the collection, processing and storage of all bio specimens, and the accompanying clinical data for EC research owing to a co-operative effort of patients, researchers, surgeons, pathologists, and the biobank staff. We proposed to develop standard operative procedure tools which will be regularly updated and freely available for future researchers in EC.

Consensus generation processes

There are four formal consensus generation methods described in the literature (Nair et al., 2011): 1) Delphi method, 2) Nominal Group Technique (NGT), 3) RAND/UCLA Appropriateness method (RAM), 4) NIH consensus development conference methodology. Following one method is preferable, nevertheless, a combination of two methods can also be used, which is referred to be a 'modified' consensus method. The table below shows the important differences between different methods (Murphy et al., 1998).

Consensus development method	Mailed Questionnaires	Private decisions elicited	Formal feedback of group choices	Face to face contact	Interaction structured	Aggregation method
Informal	No	No	No	Yes	No	Implicit
Delphi method	Yes	Yes	Yes	No	Yes	Explicit
NGT	No	Yes	Yes	Yes	Yes	Explicit
Consensus development conference	No	No	No	Yes	No	Implicit

Methods

Generation of the initial tools

Literature search

We performed a systematic review of the literature using the key words 'endometrial cancer', 'risk factors' 'age of presentation', 'parity', 'menopausal status', 'metformin', 'progestogens or Mirena', 'hormone replacement therapy', 'polycystic ovary syndrome', 'tamoxifen', 'bowel cancer', 'colorectal cancer', 'breast cancer', 'diabetes', 'hypertension', 'ethnicity',

‘anthropometric assessment’, ‘smoking’, ‘standard operating procedure’, ‘endometrium’, ‘blood or plasma or serum’, ‘saliva’, ‘urine’, ‘endometrial fluid’, ‘peritoneal fluid’, ‘biobank best practices’, ‘histopathology markers’, ‘outcomes’, ‘biomarkers’, ‘Laboratory processing procedures of tissue, blood and body fluids’, ‘biobanking standards’, ‘SOPs for collection of tissue, fluids, blood, saliva, urine’ in Scopus, Discover and Pubmed databases. The literature search was limited to studies published in the last 10 years. Out of 3,464 papers identified in the initial search, 413 papers were selected for further detailed scrutiny based on the following inclusion criteria:

1. Papers which investigated how the aforementioned factors affect an individual’s risk of developing EC.
2. Publications which proposed SOPs or best practices for the collection, storage and processing of the different tissues or fluids.
3. Papers in English language only.
4. Papers available as full text via all available resources to the authors (e.g., online resources or library facilities at LWH / University of Liverpool, British Medical Association (BMA) or Royal College of Obstetricians and Gynaecologists (RCOG).

   We further conducted manual searches for the relevant manuscripts referenced in these selected papers and the relevant guidelines from the large bio repositories.

Further development of the tools

1st Local consultation

The local team at Liverpool, comprising of four members of surgical gynaecological oncology team, four Macmillan clinical cancer nurse specialists, two clinical academics with an interest in EC research, two pathologists, two biobank staff members and a medical student, developed the three forms (Patient data collection tool, surgical data collection tool, biospecimen form) and a SOP. These forms and the SOP were based on; a) the information gathered in the literature search; b) by considering the forms that were already in use in Liverpool Women’s Research Tissue Bank (LWRTB) to collect biospecimens and data in EC research studies. LWH is a tertiary referral regional cancer center for gynaecological cancers and is part of the Cheshire and Merseyside strategic clinical networks which serves a population of 2.4 million. The age standardised incidence rate of EC in the Merseyside and Cheshire cancer network is 18.3 per 100,000 female members of the population (NCIN, 2013b). c) Standard operating

procedures developed by the National Institutes of Health, Human Endometrial Tissue and DNA Bank for the collection, transport, storage of human endometrial tissue and blood samples of women undergoing endometrial biopsy or hysterectomy for non-malignant indications (Sheldon et al., 2011). d) sample handling and storage protocol published by the UK Biobank to collect urine and blood samples (Elliott and Peakman, 2008). The forms were revised and amended based on the local consultation.

2nd Regional / National consultation

The modified versions of the three forms and the SOP mentioned above were disseminated amongst three regional and eight national research centres involved in EC research in the UK and forms were revised assimilating their feedback and two different tools, a minimal and a standard tool were devised. This pragmatic and inclusive approach provides guidance for collecting either a minimal or the ideal "standard" datasets considering the available resources.

3rd European Consultation

The modified forms were then circulated to all members of the European Network of Individualized Treatment in Endometrial Cancer (ENITEC) and were further revised according to feedback received. The revised tools were presented at the annual ENITEC face to face meeting in June 2016, where the minimal form was unanimously approved by all 47 attendees. Some further modifications were suggested for the standard tool thus was revised accordingly and the revised forms were re-populated to all participated in the consultations rounds 1-3 to obtain their final approval.

Consensus generation

A modified Delphi system was used to generate consensus regarding the final adapted tools. For this, the forms were disseminated to a group of selected panel members of representing all relevant groups of individuals (patients, gynaecological oncologists, researchers, pathologists and biobank staff, randomly selected from the participants of the consultation, n=40) to evaluate and score the tools using a score sheet recording their concordance.

Statistical Analysis

The consensus round to obtain general agreement amongst panel members was quantified employing a modified Delphi technique and we have reported median with inter quartile range and also percentages for each category of the Likert scale. A 9 point Likert scale was used

except for the patient data tool where the scale was reduced to 5 points to reduce complexity for patient usability.

Results

Endometrial Cancer Patient Data (ECPD) Collection Tool (Appendix VI (1))

An ECPD was devised to capture vital demographic variables that are directly relevant to EC research that can only be accurately recalled by the patient herself. Many risk factors for EC such as the age of presentation, the postmenopausal status, PCOS (Fearnley et al., 2010), nulliparity (Schonfeld et al., 2013), early age of menarche (Gong et al., 2015), family history of hereditary Lynch syndrome related cancers (Boilesen et al., 2008), past history of Lynch Syndrome related cancers, medical conditions such diabetes (Friberg et al., 2007), previous use of tamoxifen (Bergman et al., 2000), HRT (Beral et al., 2005) and exercise habits were included in the tool. Some other factors with inconclusive links to EC at present such as smoking (Lindemann et al., 2010) were also included in anticipation of their confirmation in appropriate future studies.

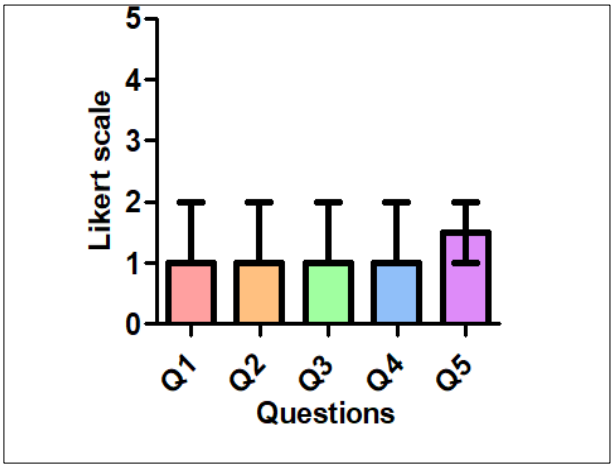


Figure 1A

Statistical analysis of the ECPD tool

(5 point Likert scale: 1-strongly agree, 2-agree, 3 -undecided, 4-disagree, 5-strongly disagree)

This figure is reproduced as published by Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493.

Table 1. Final results of consensus for Endometrial Cancer Patient Data (ECPD) Tool using 5 point likert scale (IQR – Interquartile range, 5 point likert scale: 1-Strongly agree, 2-Agree, 3-Undecided, 4-Disagree, 5-Strongly disagree, n =10). This table is reproduced as published in supplementary tables in Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493

Statements in the score sheet for patients	Score Median (IQR)	Percentages of responses (%)				
		1	2	3	4	5
The information asked in personal history is easy to fill.	1 (1-2)	60	40	0	0	0
The questions in medical history section are easy to understand and fill.	1 (1-2)	60	40	0	0	0
The questions in past history are easy to understand and fill.	1 (1-2)	60	40	0	0	0
The questions in social history section are easy to understand and fill.	1 (1-2)	60	30	10	0	0
Overall, the form is easy to understand and does not take much time to fill it.	1.5 (1-2)	50	50	0	0	0

Figure 1A and Table 1 illustrate the outcome of consensus and the final tool approved by the panellists. Score for each question in ECPD was obtained using the Likert scale which assesses the suitability and usability.

Amongst the panel members, only 2% were undecided on the clarity of the questions in social history section and overall, 98% patients agreed that tool was easy to use (Figure 2).

Endometrial Cancer Surgical Data (ECSD) Collection Tool (Appendix VI (2) and (3))

The ECSD included relevant demographic, histological and pre/post-operative features. Demographic features such as BMI was for its universal use and reproducibility. In a recent study (Painter et al., 2016), BMI was found to be a causal factor and was associated with EC compared with waist to hip ratio. All anthropometric assessments (BMI, waist to hip ratio,

waist and hip circumferences) are strongly associated with increased risk of EC (Friedenreich et al., 2007), accurate data on waist to hip ratio or waist circumferences is unlikely to be universally feasible using the same reference points as healthcare team. The preoperative imaging details help to assess the spread of cancer locally and rule out distant metastases. It is important to document the biopsy results as discordance between endometrial biopsy and final histology results has been shown to be associated with poorer survival outcome (Werner et al., 2013). Findings relevant to surgical staging also include operative findings, final histopathologic details, which is important when correlating with outcomes. It is important to note the immunohistochemical biomarkers and prognostic biomarkers that are correlated with clinical outcome (Li M, 2013). Standard information collected together with bio-samples will naturally boost the internal and external validity of the biosample quality and the accuracy of the data generated by using them. In addition, it is important to collect patient follow up data including the accurately documented cause of death, which should also be updated regularly until the completion of the standardised follow up period (either 3 years (minimum) or 5 years), in UK. The final form is arranged where information collection is separated in to 3 sections –

1. Surgical data - completed at the time sample collection
2. Histopathology details - completed after final staging results
3. Outcome data -completed during follow up and final at the end of follow up

The results of final consensus are as shown in table 2, wherein we have calculated the median with interquartile range. There was a high level of concurrence amongst the panellists for all sections, except that some considered sections on the history, antecedent biopsy details and sample collection details to be not relevant (Figure 1B). Overall, 96.25 % of panel members agreed on different aspects of the tool (Figure 2).

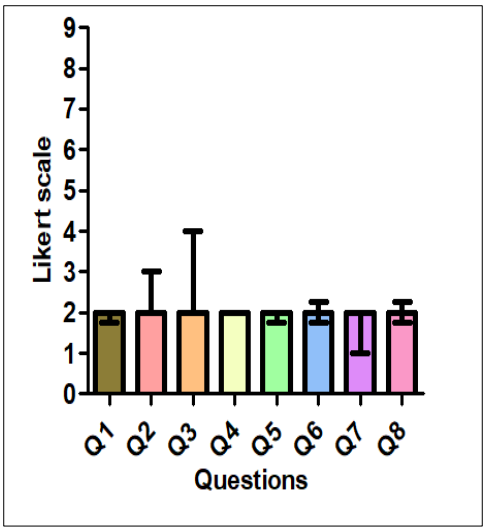


Figure 1B

Statistical analysis of ECSD tool

(9 point likert scale: 1-Strongly agree, 2-Agree, 3-Moderately agree, 4-Mildly agree, 5-Undecided, 6-Mildly disagree, 7-Disagree, 8-Moderately disagree, 9-Strongly disagree)

This figure is reproduced as published by Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493.

Endometrial Cancer Biospecimen (ECBS) tool (Appendix VI (4))

Disparities in the collection methods and biobanking conditions (processing and storage) may modify the molecular composition, expression and stability of biomarker profile (Zander et al., 2014), thus uniformity and strict practices according to standard operating procedures is vital (Moore et al., 2009). Therefore, biobank staff with applied experience and knowledge on clinical biobanking were involved in devising, amending, and attaining final consensus on the biospecimen tool. Only a few respondents felt that the tissue processing (both uterine and extra-uterine) section of the form was difficult to understand, whilst all respondents agreed on the significance and transparency of all other sections. Overall, there was a 94% level of agreement on the different aspects of this tool. The detailed results were as shown in table 3, Figure 1C and Figure 2.

Standard operating procedure for collection, processing and storage of tissue and fluid for endometrial cancer research (SOP-ECBS) (Appendix VI (5))

Different tissues such as endometrium, myometrium, extrauterine tissues and body fluid types are studied in EC research. The investigations of such bio-specimens may involve extraction of protein, RNA and DNA to be assessed using techniques such as proteomics, genomics and metabolomics. I devised the final SOP after amalgamating a number of separate, detailed methodological protocols (for example, for centrifugation, filtration, addition of preservatives, as well as storage temperatures). Availability of this information from a biobank will allow the scientists to precisely interpret their data; for example, to examine the metabolic profile of

samples such as blood, tissue, endometrial fluid or aspirate and detect disease specific changes with confidence especially when collaborating with other centres (Assfalg et al., 2008), (Bernini et al., 2009). Studies examining hormones in blood specimens are of major relevance to the endometrium and some have studied by utilising non-invasive specimens including saliva and urine (Shirtcliff et al., 2001). Non-invasive tests are patient friendly and of a particular interest in clinical research and future work is predicted to focus more on them.

Table 2. Outcome of the final round of consensus for Endometrial Cancer Surgical Data (ECSD) Tool using 9 point likert scale (IQR – interquartile range, 9 point likert scale: 1-Strongly agree, 2-Agree, 3-Moderately agree, 4-Mildly agree, 5-Undecided, 6-Mildly disagree, 7-Disagree , 8-Moderately disagree, 9-Strongly disagree, n =10). This table is reproduced as published in supplementary tables in Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493

Questions in the score sheet for gynaeoncologists	**Score Median (IQR)**	**Percentages of responses (%)**								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Is the general information about patient relevant?	2 (1.75-2)	20	80	0	0	0	0	0	0	0
Is the section on history relevant?	2 (2-3)	10	60	20	0	0	0	0	10	0
Are the Imaging details relevant and sufficient?	2 (2-4)	10	80	0	30	0	0	0	0	0
Are Antecedent biopsy details relevant?	2 (2-2)	10	80	0	0	0	0	0	0	10
Is the Operative findings section relevant?	2 (1.75-2)	20	80	0	0	0	0	0	0	0
Is the Histopathology type details section relevant and sufficient?	2 (1.75-2.25)	20	60	10	10	0	0	0	0	0
Is the Sample collection details section easy to complete?	2 (1-2)	40	50	0	0	0	0	0	0	10

Are Outcome details relevant?	2 (1.75-2.25)	20	60	10	10	0	0	0	0	0

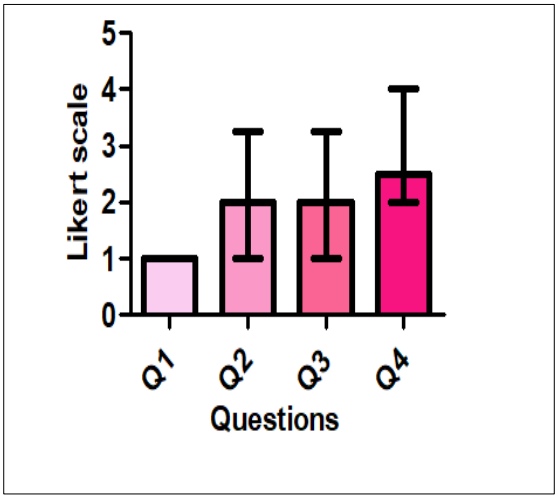


Figure 1C

Statistical analysis of ECBS

This figure is reproduced as published by Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493.

Table 3. Outcome of the final round of consensus for Endometrial Cancer Biospecimen (ECBS) Tool using 9 point likert scale (IQR – interquartile range, 9 point likert scale: 1-Strongly agree, 2-Agree, 3-Moderately agree, 4-Mildly agree, 5-Undecided, 6-Mildly disagree, 7-Disagree, 8-Moderately disagree, 9-Strongly disagree n =10). This table is reproduced as published in supplementary tables in Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493

Questions in the score sheet for biobank staff	**Score Median (IQR)**	**Percentages of responses (%)**								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample ID – Is this relevant?	1 (1-1)	90	10	0	0	0	0	0	0	0
Methods of tissue processing (Endometrium) – Is this section easy to understand?	2 (1 -3.25)	40	30	10	10	0	0	0	10	0
Methods of tissue processing (Extra uterine tissue) – Is this section easy to understand?	2 (1-3.25)	40	30	10	10	0	0	0	10	0

Methods of fluid processing (Endometrial/Peritoneal/ Blood/Saliva/Urine) - Is this section easy to understand?	2.5 (2-4)	10	40	10	40	0	0	0	0	0

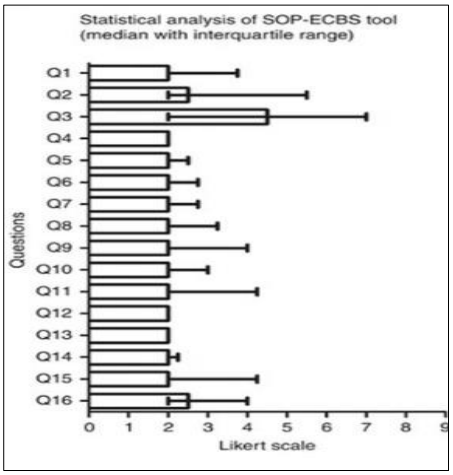


Figure 1D

Statistical analysis of SOP-ECBS

This figure is reproduced as published by Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493.

Figure 2

Overall scores of agreements by different panels for different tools using modified delphi technique (GO- gynaeoncologists, PT- patients, BS- biobank staff, PATH- pathologists)

This figure is reproduced as published by Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493.

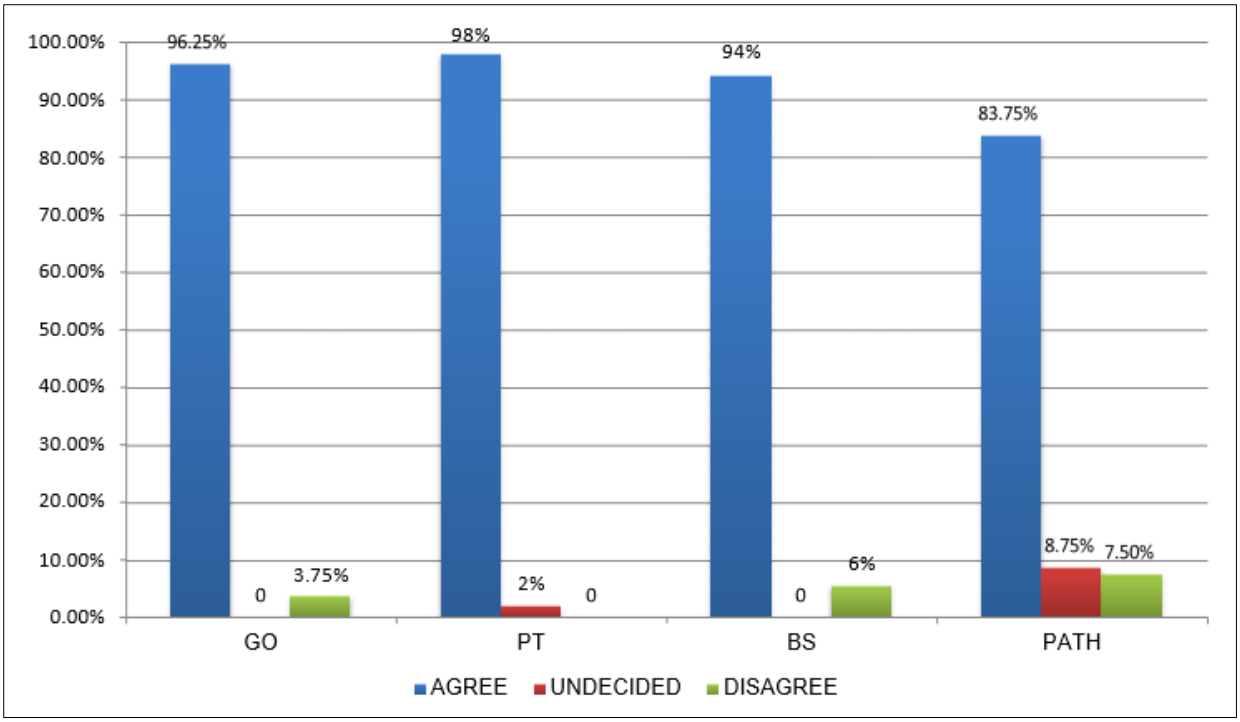


Table 4. **Outcome of the final round of consensus for Standard operating procedure (SOP-ECBS) Tool using 9 point likert scale (IQR – interquartile range, 9 point likert scale: Strongly agree, Agree, Moderately agree, Mildly agree, Undecided, Mildly disagree, Disagree, Strongly disagree n =10).** This table is reproduced as published in supplementary tables in Adishesh et al, Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 2017 Aug 8;117(4):485-493

Questions in the score sheet for pathologists	Score Median (IQR)	Percentages of responses (%)								
		Strongly agree	Agree	Moderately agree	Mildly agree	Undecided	Mildly disagree	Moderately disagree	Disagree	Strongly disagree

Is section: Processing and storage materials, relevant and easy to understand?	2 (2 - 3.75)	10	50	20	0	0	20	0	0	0
Is section: Collection – Tissue, relevant and easy to understand?	2.5 (2 -5.5)	10	40	10	10	10	0	20	0	0
Is section: Collection – Blood, relevant and easy to understand?	4.5 (2 -7)	10	30	0	10	10	10	30	0	0
Is section: Collection – Urine, relevant and easy to understand?	2 (2-2)	10	80	10	0	0	0	0	0	0
Is section: Collection – Saliva, relevant and easy to understand?	2 (2-2.5)	10	70	0	10	10	0	0	0	0
Is section: Collection – Peritoneal fluid, relevant and easy to understand?	2 (2-2.75)	0	80	0	0	20	0	0	0	0
Is section: Collection – Endometrial fluid / uterine aspirates, relevant and easy to understand?	2 (2-2.75)	0	80	0	0	20	0	0	0	0
Is section: Sample processing – Tissue, relevant and easy to understand?	2 (2-3.25)	0	70	10	10	10	0	0	0	0
Is section: Sample processing – Blood, relevant and easy to understand?	2 (2-4)	0	60	10	20	10	0	0	0	0
Is section: Sample processing – Urine, relevant and easy to understand?	2 (2-3)	0	70	20	0	0	10	0	0	0
Is section: Sample processing – Saliva, relevant and easy to understand?	2 (2-4.25)	0	60	10	10	10	10	0	0	0
Is section: Sample processing – Peritoneal	2 (2-2)	10	80	0	0	10	0	0	0	0

fluid, relevant and easy to understand?										
Is section: Sample processing – Endometrial fluid / uterine aspirates, relevant and easy to understand?	2 (2-2)	10	80	0	0	10	0	0	0	0
Is section: Storage and data recording, relevant and easy to understand?	2 (2-2.25)	10	70	10	0	0	10	0	0	0
Is section: Freezer check, relevant and easy to understand?	2 (2-4.25)	0	60	10	10	10	10	0	0	0
Is section: Checklist, relevant and easy to understand?	2.5 (2-4)	0	50	10	30	10	0	0	0	0

The outcome details of the final round of consensus regarding the SOP-ECBS are as presented in table 4 and Figure 1D. There was general agreement on the user friendliness and relevance of the tool. Few panellists responded that tissue and blood collection details could be modified further for clarity. Overall, 83.75% of panellists agreed, 8.75% were undecided and 7.5% disagreed with different sections of this tool (Figure 2).

Discussion

We have modelled evidence-based standard data collection forms ECPD, ECSD (minimal), ECSD (Standard) and an SOP-ECBS with comprehensive contribution and agreement of all stakeholders in EC cancer biobanking. The final tools were accepted by a large multi-disciplinary team and after reaching consensus (Figure 2), they are published in a well-known peer reviewed journal, where they are freely available to all EC researchers globally. These tools provide a means to reduce confounding factors in the collected data and aid larger multicentre collaborative studies.

Our choice of information to be collected by researchers was based on critical appraisal of the best available evidence. If no published evidence existed, consultation of the experts' opinion and the SOPs of the larger biobanks were considered, one such example is centrifugation speed in processing blood.

We have used a modified Delphi technique, with variations from the standard technique, where multiple rounds of feedbacks are obtained from the same panel to reassess or reconsider initial judgment, for participant anonymity, controlled feedback and statistical analysis to interpret data between the rounds. Previously, similar variations to original Delphi system, for example restricting the ability of the experts to respond to the original question, alterations in the expert groups, as well as changing the end point have been employed in studies (Thompson, 2009).

Repeated use of a same panel was unjustifiable for our research aims for the following reasons. My endeavour was to generate separate forms for varied end points e.g., patient data collection, surgical data collection, tissue processing information and the standard operative procedures. These clearly required panel members of disparate backgrounds, with different fields of expertise.

The main difference from the standard Delphi technique was the number of consultation rounds and the end point. Our first two rounds were descriptive in order to create opinions and ideas from different expert panel members. We included their feedback to generate the finalised forms and SOP. During final round of the consensus, our panel members included stakeholders representing all previous panels to whom we distributed a score sheet to each of the panellists along with the forms to gauge their agreement with the final tools. The high percentage of agreement observed with the statistical analysis of data obtained from the third and final round, excluded the need for any further consensus rounds.

Since more thorough, standardised surgical data collection will allow a complete assessment of the relationship between the surgical phenotypical data with the outcomes of treatments, we strongly recommend the use of the standard rather than the minimal ECSD tool. However, if the collection or quality of the data or specimens cannot be assured, the minimal set should be employed. We plan to update these tools in the future through information obtained by feedback and review of future literature. Future deliberations in the context of our initiatives include generating an internationally funded web based central database system allowing voluntary deposition of the information on all biospecimens collected by EC researchers worldwide, which will be easily accessible to all. This approach will diminish costs and time

consumed for the individual research groups whilst expanding the credibility of the data produced and will offer an apparent, collective platform for newer collaborations.

Chapter 8: Discussion

EC is the sixth most common cancer in women worldwide and the most common amongst all gynaecological cancers. Globally, there were 382,069 new cases and 89,929 estimated deaths reported in 2018 due to EC (Bray et al., 2018). According to US statistics, unlike any other malignancy, the mortality rate due to EC is also increasing (ACS). Similarly, EC incidence in the UK has also increased by 50% since the nineties and the reasons for this is thought to be the increase in obesity, longevity and the use of adjuvant tamoxifen for breast cancer (Evans et al., 2011). Over the next five years, the incidence and the death rate are expected to rise approximately by 20% and 17% respectively (Bray et al., 2018).

Understanding the pathogenesis and molecular genesis of EC, will help to influence the rising rates of EC and EC related deaths. For example, mortality rate can be reduced with the availability of good prognostic markers predicting which cancers would recur, and thus treatments can be directed towards those patients. Finding early diagnostic tests and developing an understanding about the pathogenesis of EC were amongst the top research priorities identified in the James Lind Alliance Priority Setting Partnership exercises (Badrick et al., 2019), (Wan et al., 2016). Prevention as well as better personalised treatments for EC are the desires of patients and all stakeholders providing care for women with EC. Translational research underpins these requirements and it invariably requires the use of patient derived biospecimens.

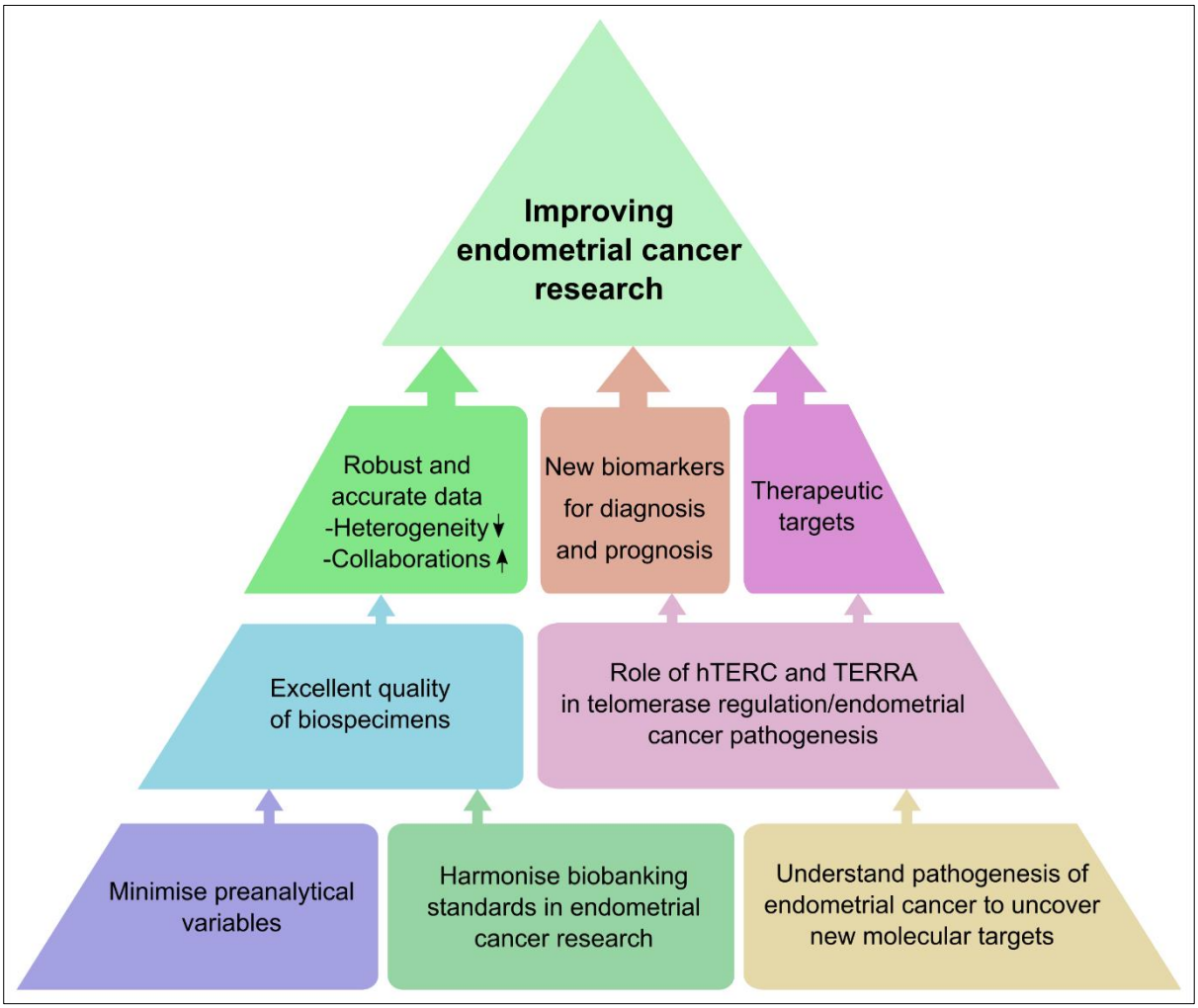
Considering the above requirements, this PhD project focussed on the following three unmet needs in EC research (Figure 1):

(1) to assess the effects of preanalytical variables on the downstream analysis results of patient derived endometrial biopsies,

(2) to harmonise the process of biobanking of biosamples relevant to EC research, and

(3) to uncover new specific molecular targets, which could assist in better understanding of pathogenesis, and consequently to facilitate formulation of future therapy.

Figure 1 shows diagramatic representation of three main goals of this thesis in improving EC research (I thank Christopher Hill, University of Liverpool for helping me to create the diagram)



Although it is expected that preanalytical variables may affect the data generated from endometrial biospecimens, this important aspect has not been previously explored in EC research. The reliability of molecular data derived from the patient-derived biospecimens is dependent on the quality of biospecimens. When quality of biospecimens is altered with multiple pre-analytic variables during the sample procurement, they can in turn affect the data generated and thus, ultimately patient's management in personalised data driven treatment pathways (Compton et al., 2019). Traditional molecular tests for EC were conducted mainly on formalin fixed paraffin embedded tissue specimens and on blood/plasma specimens, With the emergence of newer technologies such as 'omics', and next generation RNA sequencing, there is a demand for newer biomarkers using different biosamples and new high throughput analytic methods. Endometrial biopsies that are suitably collected and preserved are one of the

most important biosamples to be used in these molecular analytes, and due to their close proximity to cancer (directly sampling cancer) when compared with other biosamples such as blood or urine, stakes for generating accurate results from them are expected to be higher. The focus is on accuracy of molecular tests, which impact patient management is justified since false positive or negative results may have significant consequences for the patient. Considering this, the first objective that was realised in this thesis was to examine how several selected preanalytical variables such as timing of the biopsy (pre-/post- hysterectomy) or the method employed to collect the biopsy, may affect the data generated from biospecimens in a typical study using EC biopsies.

Pre analytical variables affected data generated from human endometrial biosamples

In paired endometrial biospecimens from the same woman, taken before and after hysterectomy using different collection methods (pipelle or full thickness biopsy), we assess if those pre analytical variables significantly affected subsequent molecular data generated from them. Both benign endometrium and EC samples were included in this study to examine if there were pathology specific differences were also present. Post hysterectomy specimens are exposed to warm ischaemia due to devascularisation of the uterus thus we chose to examine three downstream genes relevant to hypoxia, which maybe likely to be affected. Following hysterectomy, endometrial specimens were obtained using a pipelle sampler in cancer patients and using both pipelle and full thickness wedge biopsies from women without EC. The timing of endometrial biopsy collection significantly altered the expression of some hypoxia related genes. The RNA expression levels of VEGFA, PR were significantly increased in post hysterectomy samples but no significant change was observed in CA9 expression between pre/post hysterectomy samples. Variability in method of collection of endometrial biopsy also significantly altered the expression of some genes in benign samples. VEGFA and PR RNA expression levels were higher in full thickness samples compared with the pipelle samples. No change was seen in CA9 gene expression levels between samples collected using the two different methods. In cancer samples, only timing of the biopsy was assessed and no significant differences were detected between pre and post hysterectomy biopsies with regards to the levels of three genes tested. Since cancers are proposed to have a more hypoxic environment, we believe that was the reason for the observed molecular data in cancers. The cancers already have high levels of hypoxic genes expressed, thus warm hypoxia may not incur an additional

hypoxic burden on these samples. My results clearly highlight the important fact that all samples used in a study should be treated in a uniform manner to reduce the possible influence from pre-analytic variables on the molecular data generated from them. Research study design, sample collection should be evaluated objectively prior to the specific molecular analytic method is employed in a study, prior to commencement of sample collection in order to generate credible data. The endometrial biosamples collected blindly using a sampler may contain tissue which is from the adjacent normal endometrium, hyperplasia or frank cancer. Furthermore, the samples may also be full of blood clots or mucus. The potential cross contamination of the sample with these different material is an important bias when used without assessment in studies using methods such as qPCR. When the whole biopsy is considered as a whole, for example when a full thickness endometrial sample is used to extract RNA and qPCR/western blotting methods are used to generate data, this would not take into account the contribution from myometrial cells. Techniques such as single cell isolation, Laser capture and dissection, may be better at obtaining specific cell types relevant to a particular pathology from a whole biopsy to reduce such contamination bias. Chapter 3 of this thesis provide robust data, demonstrating that the effect on the levels of gene expression by the preanalytical variables examined is not uniform, but only some genes are affected, therefore, generation of spurious data from a study can be prevented by standardised sample collection.

Understanding carcinogenesis in the endometrium to discovery of new therapeutic targets

The main interests of this thesis is to find clinically useful diagnostic or prognostic tests in EC. This is also an important area for all EC researchers in general. One of the top ten research questions identified in JLA PSP in EC research, has acknowledged that there is a need for biomarkers which can predict survival and recurrence of the disease (Wan et al., 2016). The available histological diagnosis is dependent on symptoms such as post-menopausal bleeding (Clarke et al., 2018) and it would trigger the use multi modal investigations to diagnose the presence or absence of EC accurately (Dueholm et al., 2019). Even though there are many advances in EC classification with the genomic testing and through the analysis of TCGA dataset, risk prediction models may be further improved by including reliable biomarkers (Fortner et al., 2017). Recently, there has been a focus on developing risk stratification models specific to EC. For example, Markov modelling of serum screening used a biomarker panel, which has been shown to be cost effective in obese women of 45-80 years of age (Havrilesky

et al., 2009). Also, another pan cancer panel has been described, but its role in EC screening is yet to be clarified (Cohen et al., 2018). Therefore, the final unmet need in EC research I have focussed on in this thesis, is discovering biomarkers which can predict the development of EC which may be a therapeutic target.

Cellular proliferation and self-renewal are hallmarks of cancer, and these processers are regulated by telomerase enzyme activation (Hanahan and Weinberg, 2011). Various genetic and epigenetic mechanisms may be associated with telomerase reactivation in EC, and more importantly, these associations may have diagnostic and prognostic relevance (Leao et al., 2018). It is already known that human endometrium is a somatic organ with dynamic telomerase activity during the menstrual cycle, and telomerase plays an important role in endometrial regeneration. Telomerase drives epithelial cell proliferation in human endometrium, and aberrant cellular proliferation is cardinal feature of carcinogenesis. Benign proliferative metastatic condition, endometriosis also have high TA. Most of the components of telomerase have been examined in the endometrium and in endometrial pathologies such as endometriosis and EC. Telomerase activity is very low in post-menopausal endometrium (Tanaka et al., 1998), but is high in ECs (Kyo et al., 1999a), (Saito et al., 1997), (Maida et al., 2002). Therefore, factors which lead to dysregulation of telomerase enzyme in postmenopausal endometrium is likely to initiate EC. Endometrium is the primary target organ for ovarian hormonal action, and EC is a hormone responsive cancer, therefore the telomerase biology of post-menopausal endometrium that is under complex hormonal interactions may also have a role in endometrial carcinogenesis. Telomeres shortened to a critical length, will lead the cells into either apoptosis or senescence pathways. Senescence is irreversible growth arrest of cells with particular morphological alterations (Kuilman et al., 2010). Cells deficient of telomerase can overcome senescence by expressing certain viral oncoproteins, then continue proliferation but they will then accumulate chromosomal instability. High telomerase activity has a role in ascertaining genome stability by preventing telomere attrition. Telomerase interacts with different cellular pathways such as ATM/ATR pathway, WnT pathway for telomere maintenance (Diala et al., 2013), (Tong et al., 2015). Alterations in the intricate relationship between telomeres and telomerase enzyme can lead to cellular functional disruption and trigger the carcinogenesis process. ECs are associated with high telomerase activity, which maintains the short telomeres. Further knowledge of the changes in the telomerase components and telomere transcripts may improve our understanding of the pathophysiological processes leading to EC.

hTERC expression levels in EC

Telomerase enzyme has two essential components, *hTERC*, telomeric RNA and reverse transcriptase enzyme hTERT. Although there are many studies that have explored the role of hTERT, in the human endometrium and EC, only a few studies have explored *hTERC* in human endometrium and in ECs. Kyo et al have studied the hTERT and *hTERC* expression in human endometrium, both in healthy and EC samples. In their study, *hTERC* was expressed in all normal endometrium and there was no correlation between *hTERC* and telomerase activity (Kyo et al., 1999b). In agreement with their study, in our cohort, *hTERC* was constitutively expressed throughout the menstrual cycle. *hTERC* levels, however, were altered in both proliferative endometrial disorders we examined, endometriosis and ECs. Interestingly, endometrial *hTERC* levels did not correlate with TA. This discordance of *hTERC* levels with the TA may also be due to the limited sample set included in this study. Another reason for the non-correlation may be due to heterogeneity of tumour cells in the specimens used for *hTERC* levels and TA despite them being derived from the same sample. Alternatively, as suggested in Kyo et al, it may be due to critical balance of expression of each subunit of telomerase, *hTERC* and hTERT levels to express the enzymatic activity of telomerase. Among EC subtypes, we observed that *hTERC* levels to be significantly raised in grade 3 ECs, compared to other types of cancer. TA levels are high in all ECs, both *hTERC* and hTERT are essential for telomerase activity. Therefore, at least in grade 3 ECs, interference with expression of *hTERC* may be used as a therapeutic target. Although there was no correlation between TA and *hTERC* levels, *in vitro* studies have demonstrated that inhibition of TA was possible by phosphorothioate antisense oligonucleotides - nuclear localisation signal peptide (sASO-NLS) conjugates targeting *hTERC* (Diala et al., 2020). Future studies could explore whether targeting *hTERC* in grade 3 ECs would inhibit TA sufficiently to alter EC cell proliferation, thereby assessing the therapeutic potential for that approach in this aggressive cancer subtype EC. Furthermore, the future studies may also explore the diagnostic and functional importance of the significantly reduced *hTERC* levels in endometriosis. Since endometriosis is a condition that is associated with severely delayed diagnosis, and diagnosis often requires invasive surgery (diagnostic laparoscopy), assessment of *hTERC* levels in an easily obtainable outpatient endometrial pipelle biopsy is an attractive option.

TERRA in endometrial carcinogenesis

Telomeres are ends of chromosomes, which protect the chromosomes from shortening at every cell division. Telomeres were thought to be transcriptionally silent. But a new class of long non-coding RNAs, called *TERRA* are transcribed from telomeres and *TERRA*s have also been found to participate in telomere maintenance and they were shown to have a role in genome stability. Several PCR based studies have shown that *TERRA*s are expressed from multiple chromosomal ends in humans (Feretzaki and Lingner, 2017), (Diman and Decottignies, 2018). In my study, *TERRA*s from eight different chromosomes *Ch 1q-2q-4q-10q-22q, Ch 16p* and *Ch 20q* were studied in healthy endometrium, endometrium from benign proliferative condition, endometriosis and in EC samples. In this context, I found that *TERRA*s were dynamically expressed throughout menstrual cycle. *TERRA* levels were significantly low in ECs compared with healthy postmenopausal endometrial samples and the levels correlated negatively with the proliferation marker Ki67. These findings may suggest *TERRA*s to be associated with endometrial carcinogenesis. Kamal et al., have demonstrated in their study that AR expression in EC negatively correlated with Ki 67. This study also demonstrated loss of AR correlated with unfavourable clinicopathological factors which predicted poor clinical outcome (Kamal et al., 2016b). Previous other studies have demonstrated strong association of Ki67 score with known pathological variables and high Ki67 score to be associated with poor cancer outcomes (Kitson et al., 2017). However, no correlation was found between *TERRA* and telomerase activity, but this may be due to the small sample size included in this study. In ECs, we found high telomerase activity, shorter telomere lengths and low *TERRA* levels. This may suggest that *TERRA*s may have an inhibitory role on telomerase enzyme. As this was a proof of principle study, I conclude that sufficiently powered future study may help us to explore the true relationship between telomerase and *TERRA.*

In some other cancer studies, *TERRA* levels have been associated with prognostic significance. For example, when HNSCC tumours were divided into two groups, dependent on *TERRA* levels, patients with high *TERRA* tumours survived longer compared with patients with low *TERRA* level tumours (Vitelli et al., 2018). In colorectal cancer, *TERRA* is speculated to be an independent prognostic factor for long term outcomes. In this preliminary study, however such correlation was not possible due to the small sample size.

TERRA levels may be relevant to the pathway cancers use for telomere maintenance, either an active telomerase pathway or ALT pathway. Depending on the pathway which is active in a particular cancer, *TERRA* may act as a tumour suppressor or oncogenic stimulus respectively (Gala and Khattar, 2021). In that context, *TERRA* can be considered as a therapeutic target and

its levels can be modified depending on the telomerase maintenance pathway active in carcinogenesis process. In telomerase positive cells, treatment with medications such as 5-azacytidine may increase the accumulation of *TERRA*, thereby reduction in telomerase activity and decreased proliferation of cells. The results generated by the preliminary study included in Chapter 6 of this thesis describe the expression of *TERRA*s at 8 different chromosomes in the human endometrium, thus paves the way for future studies to test the possible roles for *TERRA*s in endometrial carcinogenesis.

Hormonal regulation of *hTERC* and *TERRA*s in the endometrium; implication for endometrial carcinogenesis

EC is a hormone responsive cancer. Hormone regulation of any potential biomarker or a therapeutic target is important to understand its role in the pathogenesis of EC. In this context, the final results chapter in this thesis describe the experimental work, which examined the hormone regulation of *hTERC* and *TERRA*. The data generated expands our understanding of the effect of hormonal drive on the biomarkers as well as the effect of hormonal anti-cancer treatment on these markers. Unopposed oestrogen due to factors such as obesity, diabetes, polycystic ovarian syndrome, nulliparity, hormone replacement and tamoxifen therapy can lead to increased mitotic activity in endometrial cells (Pua and Chuang, 2016). From previous studies, there is *in vitro* and *in vivo* evidence that oestrogens, appear to induce expression TA in the endometrium (Kyo et al., 1999b). In other cell types, progesterone and androgens also have shown to regulate telomerase (Tomlinson et al., 2006), (Calado et al., 2009), (Valentijn et al., 2015). Previous studies have also demonstrated that oestrogen regulates *hTERT* expression level and telomerase activity via MAPK pathway and progesterone suppresses the oestrogen induced *hTERT* mRNA expression in PR positive EC cells lines (Bae-Jump et al., 2006), (Zhou et al., 2013). However, there are no studies in *hTERC* or *TERRA* and its regulation by hormones. The study presented in Chapter 7 of this thesis has demonstrated that progesterone significantly alters levels of *hTERC* and *TERRA 16p* at 72 hours after *in vitro* exposure to hormones. *In vivo* effect of hormones on *hTERC* and *TERRA* levels were examined in endometrial samples collected from women on two most commonly used hormonal treatments in gynaecological conditions. A significantly high level of *TERRA*s were detected in endometrium of GnRH treated women which mimics the postmenopausal endometrial phase. It is of note that in the endometrium, hormonal regulation is complex, for example, the epithelial anti-proliferative effect of progesterone is exerted via the endometrial stromal cells. In the studies described in

Chapter 7, an epithelial monolayer culture system was employed. Therefore, this may have limited ability to explore the true hormonal regulation of *TERRA*s and *hTERC* in the EC cells. Collectively however, the data presented in Chapter 7 does suggest that both *hTERC* and *TERRA* are regulated by ovarian hormones. However, further studies are required with more biological replicates of the *in vitro* experiments and possibly utilising a epithelial and stromal cell co-culture system in a more physiologically relevant 3D model to confirm the true extent of such hormone regulation in future.

Harmonisation of biobanking standards for EC research

Considering data produced in Chapter 7 of this thesis, it was obvious that harmonisation of biosamples would be essential to ensure EC research studies produce robust translatable results. With the advances in technologies in translational research, using human biospecimens has become vital to discovering causation of cancer, determining the progression of cancer and resistance or response to treatment (Riegman et al., 2006). To benefit from the molecular and genetic cancer research data for personalised and precision medicine, collecting, storing, and analysing many human biospecimens is necessary. To participate in large scale studies, it is important for biobanks to store large amounts of high-quality biospecimens for example in TCGA project. The most important aspect of biobanking is to ensure the quality of biospecimens. For that biobanking practices have to be standardised, with sufficient quality control and regulations in place to ensure the highest operational standards. Heterogeneity in collection, processing and storage of biospecimens can hamper the process and results generated from samples of questionable integrity will obviously impede development of diagnostic and therapeutic targets (Moore et al., 2009). NCI recognised non-uniformity and inconsistent standards used in biospecimens handling to be a roadblock to cancer research (Morente et al., 2007), (Vaught and Lockhart, 2012); Pan European biobank network, BBMRI, had been established in Europe to help build a suitable integrate infrastructures (Yuille et al., 2008) and harmonisation of the biospecimen collection. When discrepancies exist between biobanks merging data across different sites or studies, many challenges present themselves. In the context of this background, the study presented in Chapter 7 of this thesis describes the approach we took to harmonise biobanking of biosamples used in EC research. This required participation of all stakeholders, including patients, surgeons, pathologists, researchers and biobank staff. Involvement of patients is increasingly recognised to procure accurate and reliable personal, past medical surgical data for research (Chalmers et al., 2014). Developing patient questionnaires that are acceptable, user friendly, and clear is also essential to this

process. Healthcare professionals are instrumental in transferring the data from research labs into clinical practice, they can convey the relevance of the research to patients and recruit patients to trials. For these reasons, members of the clinical team providing EC care are well placed to accurately extract all relevant information regarding the investigations, surgical findings, staging and follow up data (Kahn et al., 2011), (NCRI, 2012). Pathologists are key members of the biobanking process, who are quality controllers, and they can contribute towards robust SOPs for biospecimen collection, processing and storage (NCRI, 2012). Last but not least, researchers and biobank personnel work in processing, storing and data management. They are well versed with details of processing samples, noting any deviations from the standard protocol thus can input valuable information essential for assignment of specimen quality (Capocasa et al., 2016).

Universal biobanking standards are not applicable to each disease or cancer type, hence should be adapted to each cancer type of interest. The importance of cancer specific harmonisation is highlighted by the TCGA which contains over 532 uterine cancer samples with RNA sequencing, copy number alteration and mutation data (Kandoth et al., 2013). The limited clinical data accompanying these samples has affected the ability of this dataset to draw clinically applicable conclusions. HASTEN study described in Chapter 4 of this thesis, therefore, focusses on another fundamental, yet unmet need in EC research, to standardise EC specific collection of biospecimens with relevant clinical data. This effort has facilitated researchers to conduct large scale, international collaborative EC research studies leading to improve biomarker and target treatment discoveries. After initial literature search, appraising available evidence, four tools that can be used in biosample procurement were devised. Local, regional and European consensus was obtained using a comprehensive consultation process and final consensus was obtained by following a modified Delphi system, which included panel members representing all stakeholders in EC research. The final tools are now available freely for all EC researchers via open access publication and they include:

(1) ECPD,

(2) ECSD,

(3) ECBS and

(4) standard operating procedure for collection, processing and storage of tissue or fluid for EC research.

I anticipate that harmonisation of biobanking standards is the first step towards high quality standardised large scale global collaborative projects, which will benefit EC research and translatability of research data into patient management in the future. Although my study had been published in 2017, making these tools available freely to the researchers worldwide, only 8 citations of the original paper and 12 citations of the review paper has been documented to date. Both papers exceeded the citation index of the published journals, yet, we expected the citations to be more, reflecting the adaptation of these tools. The difficulty in fully adapting the minimal standards we have suggested may be a reason for this observation of less than expected citation numbers. Our suggested minimal requirements may remove the possibility of using already archived samples from biobanks that are available to researchers in their studies. Most larger studies containing big sample sizes traditionally use many biobanks with already biobanked samples that are collected over several years. Most of these samples would not have been collected according to the minimal standards described by our work. This is particularly true when follow up data, which is important for oncological outcomes are considered. Therefore, although our work may have been appreciated and as we understand many are trying to use them for prospective studies, researchers may not be able to fully adapt these tools for practical reasons. Therefore, it will take a considerably long time for our tools to be adapted and citation numbers to reflect that adaptation. Appreciation by funding bodies and publishers of this cardinal need for accurate biosample procurement to generate clinically transferable data and provision of resources will be needed to adapt our tools fully. Furthermore, mere citation numbers are unlikely to fully expose the issues around such adaptation.

Future directives

The results generated during this PhD studentship and the studies presented in this thesis provide several future avenues of research to improve our current understanding of EC.

Future studies examining gene expression of EC samples should consider preanalytical variables that can produce spurious results. The pre-analytic variables to consider should include methods of processing, timing of processing, duration of storage of biosamples as well. Importantly, there are some genes that are resistant to these variables while other change. Therefore, unless strict standardised methods are implemented in sample handling, testing one gene or a few for their change in the context of the presumed variable sample handling will not give information relevant to all genes.

Considering the novel data generated in this PhD studentship on endometrial *hTERC* and *TERRA*s, much work is left to be done. Since this work has demonstrated *TERRA*s to be expressed in the human endometrium and for them to be differentially expressed in ECs, further research is warranted to further our understanding of this long non-coding RNA's in EC. This observational study warrants further functional studies to explore their possible functional role in cell fate regulation and their involvement in important telomerase biology. The effect of chemotherapeutic agents on *hTERC* and *TERRA* levels, and manipulation of the *hTERC* and *TERRA* expression levels and assessing their functional relevance on cell proliferation and tumourigenesis in laboratory and in subsequent pre-clinical animal models, may test the hypothesis I have generated in here; that *TERRA*s and *hTERC* have a role in endometrial carcinogenesis. Studying *hTERC* and *TERRA* levels in relation to the new molecular classification of EC is also important. These transcripts should be assessed in pre-cancerous endometrial hyperplasia with atypia, since studies in cervical cancer have shown that extra copies of *hTERC* were found in higher grade/high risk lesions developing into cervical cancer (Jiang et al., 2010). Furthermore, exploring *TERRA* levels in blood of EC patients may allow examining the existence of any correlation between blood levels and tumour levels of *TERRA*, which may facilitate testing of its diagnostic potential.

The tools generated as described in Chapter 7 to harmonise biobanking of patient derived biosamples in EC will be used by all endometrial researchers in the future. The quality and utility of the biosamples banked before and after their utility can be assessed in future studies. Similar pathway can also be adapted for biobanking other gynaecological cancer biosamples.

Conclusion

This PhD studentship, has demonstrated the effects of several preanalytical variables on a selected downstream gene targets in endometrial tissue samples. In order to prevent such undesirable effects on bio-samples banked in EC research, standardisation tools were developed to facilitate harmonisation of biosample collection that is made freely available to EC researchers worldwide. This is expected to improve EC research and support identification of potential robust biomarkers. Finally, the expression of *TERRA*s and *hTERC* had been examined in human endometrial tissue, to determine their potential role in endometrial carcinogenesis and potential future directions of study into these transcripts have been identified. The work presented in this thesis therefore has contributed to expand the current

understanding of endometrial carcinogenesis, provided information and means to direct and improve future EC research.

References :

AARNIO, M., SANKILA, R., PUKKALA, E., SALOVAARA, R., AALTONEN, L. A., DE LA CHAPELLE, A., PELTOMÄKI, P., MECKLIN, J.-P. & JÄRVINEN, H. J. 1999. Cancer risk in mutation carriers of DNA-mismatch-repair genes. *International Journal of Cancer,* 81**,** 214-218.

ABDELAZIM, I. A., ABOELEZZ, A. & ABDULKAREEM, A. F. 2013. Pipelle endometrial sampling versus conventional dilatation & curettage in patients with abnormal uterine bleeding. *J Turk Ger Gynecol Assoc.*

ADISHESH, M., FYSON, A., DECRUZE, S. B., KIRWAN, J., CONSORTIUM, E., WERNER, H. M. J. & HAPANGAMA, D. K. 2017. Harmonisation of biobanking standards in endometrial cancer research. *Br J Cancer,* 117**,** 485-493.

ADISHESH, M. & HAPANGAMA, D. K. 2019. Enriching Personalized Endometrial Cancer Research with the Harmonization of Biobanking Standards. *Cancers (Basel),* 11.

AGRAWAL, L., ENGEL, K. B., GREYTAK, S. R. & MOORE, H. M. 2018. Understanding preanalytical variables and their effects on clinical biomarkers of oncology and immunotherapy. *Semin Cancer Biol,* 52**,** 26-38.

AGUILERA, A. & GOMEZ-GONZALEZ, B. 2008. Genome instability: a mechanistic view of its causes and consequences. *Nat Rev Genet,* 9**,** 204-17.

AHN, S. H., SINGH, V. & TAYADE, C. 2017. Biomarkers in endometriosis: challenges and opportunities. *Fertil Steril,* 107**,** 523-532.

AIGNER, S., POSTBERG, J., LIPPS, H. J. & CECH, T. R. 2003. The Euplotes La motif protein p43 has properties of a telomerase-specific subunit. *Biochemistry,* 42**,** 5736-47.

AKBAY, E. A., CONTRERAS, C. M., PERERA, S. A., SULLIVAN, J. P., BROADDUS, R. R., SCHORGE, J. O., ASHFAQ, R., SABOORIAN, H., WONG, K. K. & CASTRILLON, D. H. 2008. Differential roles of telomere attrition in type I and II endometrial carcinogenesis. *Am J Pathol,* 173**,** 536-44.

AL-TALIB, A., NEZHAT, F. & TULANDI, T. 2010. The role of hysteroscopy in diagnosis and management of endometrial cancer. *Gynecological Surgery,* 7**,** 211-216.

ALI, A. T. 2014. Reproductive factors and the risk of endometrial cancer. *Int J Gynecol Cancer,* 24**,** 384-93.

ALMEIDA, A., PAUL THIERY, J., MAGDELENAT, H. & RADVANYI, F. 2004. Gene expression analysis by real-time reverse transcription polymerase chain reaction: influence of tissue handling. *Anal Biochem,* 328**,** 101-8.

ALNAFAKH, R., SARETZKI, G., MIDGLEY, A., FLYNN, J., KAMAL, A. M., DOBSON, L., NATARAJAN, P., STRINGFELLOW, H., MARTIN-HIRSCH, P., DECRUZE, S. B., COUPLAND, S. E. & HAPANGAMA, D. K. 2021. Aberrant Dyskerin Expression Is Related to Proliferation and Poor Survival in Endometrial Cancer. *Cancers (Basel),* 13.

ALNAFAKH, R. A. A., ADISHESH, M., BUTTON, L., SARETZKI, G. & HAPANGAMA, D. K. 2019. Telomerase and Telomeres in Endometrial Cancer. *Front Oncol,* 9**,** 344.

AMANT, F., MIRZA, M. R., KOSKAS, M. & CREUTZBERG 2018. Cancer of the corpus uteri. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics,* 143 Suppl 2.

APPLE, S., PUCCI, R., LOWE, A. C., SHINTAKU, I., SHAPOURIFAR-TEHRANI, S. & MOATAMED, N. 2011. The effect of delay in fixation, different fixatives, and duration of fixation in estrogen and progesterone receptor results in breast carcinoma. *Am J Clin Pathol,* 135**,** 592-8.

ARGENTA, P., SVENDSEN, C., ELISHAEV, E., GLOYESKE, N., GELLER, M. A., EDWARDS, R. P. & LINKOV, F. 2014. Hormone receptor expression patterns in the endometrium of asymptomatic morbidly obese women before and after bariatric surgery. *Gynecol Oncol,* 133**,** 78-82.

ARMSTRONG, G. M., MAYBIN, J. A., MURRAY, A. A., NICOL, M., WALKER, C., SAUNDERS, P. T. K., ROSSI, A. G. & CRITCHLEY, H. 2017. Endometrial apoptosis and neutrophil infiltration during menstruation exhibits spatial and temporal dynamics that are recapitulated in a mouse model. *Scientific reports,* 7.

ARNOULT, N., VAN BENEDEN, A. & DECOTTIGNIES, A. 2012. Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1α. *Nat Struct Mol Biol,* 19**,** 948-56.

ARORA, R., LEE, Y., WISCHNEWSKI, H., BRUN, C. M., SCHWARZ, T. & AZZALIN, C. M. 2014. RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. *Nat Commun,* 5**,** 5220.

ASSFALG, M., BERTINI, I., COLANGIULI, D., LUCHINAT, C., SCHAFER, H., SCHUTZ, B. & SPRAUL, M. 2008. Evidence of different metabolic phenotypes in humans. *Proc Natl Acad Sci U S A,* 105**,** 1420-4.

ATHERTON, D. S., SEXTON, K. C., OTALI, D., BELL, W. C. & GRIZZLE, W. E. 2016. Factors Affecting the Use of Human Tissues in Biomedical Research: Implications in the Design and Operation of a Biorepository. *Methods Mol Biol,* 1381**,** 1-38.

ATKINS, D., REIFFEN, K.-A., TEGTMEIER, C. L., WINTHER, H., BONATO, M. S. & STÖRKEL, S. 2004. Immunohistochemical Detection of EGFR in Paraffin-embedded Tumor Tissues:Variation in Staining Intensity Due to Choice of Fixative and Storage Time of Tissue Sections. *Journal of Histochemistry & Cytochemistry,* 52**,** 893-901.

AUER, H., MOBLEY, J. A., AYERS, L. W., BOWEN, J., CHUAQUI, R. F., JOHNSON, L. A., LIVOLSI, V. A., LUBENSKY, I. A., MCGARVEY, D., MONOVICH, L. C., MOSKALUK, C. A., RUMPEL, C. A., SEXTON, K. C., WASHINGTON, M. K., WILES, K. R., GRIZZLE, W. E. & RAMIREZ, N. C. 2014. The effects of frozen tissue storage conditions on the integrity of RNA and protein. *Biotech Histochem,* 89**,** 518-28.

AVILION, A. A., PIATYSZEK, M. A., GUPTA, J., SHAY, J. W., BACCHETTI, S. & GREIDER, C. W. 1996. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res,* 56**,** 645-50.

AZIMI-NEZHAD, M., LAMBERT, D., OTTONE, C., PERRIN, C., CHAPEL, C., GAILLARD, G., PFISTER, M., MASSON, C., TABONE, E., BETSOU, F., MEYRONET, D., UNGEHEUER, M. N. & SIEST, S. V. 2012. Influence of pre-analytical variables on VEGF gene expression and circulating protein concentrations. *Biopreserv Biobank,* 10**,** 454-61.

AZZALIN, C. M. & LINGNER, J. 2008. Telomeres: the silence is broken. *Cell Cycle,* 7**,** 1161-5.

AZZALIN, C. M., REICHENBACH, P., KHORIAULI, L., GIULOTTO, E. & LINGNER, J. 2007. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science,* 318**,** 798-801.

BADRICK, E., CRESSWELL, K., ELLIS, P., DETECTING CANCER EARLY PRIORITY SETTING PARTNERSHIP STEERING, G., RENEHAN, A. G. & CROSBIE, E. J. 2019. Top ten research priorities for detecting cancer early. *Lancet Public Health,* 4**,** e551.

BAE-JUMP, V. L., ZHOU, C., GEHRIG, P. A. & BOGGESS, J. F. 2006. Progesterone regulation of hTERT transcription in human endometrial cancer cells. *Cancer Research,* 66**,** 1255-1255.

BAE, S. U., PARK, W. J., JEONG, W. K., BAEK, S. K., LEE, H. W. & LEE, J. H. 2019. Prognostic impact of telomeric repeat-containing RNA expression on long-term oncologic outcomes in colorectal cancer. *Medicine (Baltimore),* 98**,** e14932.

BAENA-DEL VALLE, J. A., ZHENG, Q., ESOPI, D. M., RUBENSTEIN, M., HUBBARD, G. K., MONCALIANO, M. C., HRUSZKEWYCZ, A., VAGHASIA, A., YEGNASUBRAMANIAN, S., WHEELAN, S. J., MEEKER, A. K., HEAPHY, C. M., GRAHAM, M. K. & DE MARZO, A. M. 2018. MYC drives overexpression of telomerase RNA (hTR/TERC) in prostate cancer. *J Pathol,* 244**,** 11-24.

BAIRD, D. M. 2010. Variation at the TERT locus and predisposition for cancer. *Expert Reviews in Molecular Medicine,* 12**,** e16.

BAIRD, D. M., ROWSON, J., WYNFORD-THOMAS, D. & KIPLING, D. 2003. Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat Genet,* 33**,** 203-7.

BALK, B., DEES, M., BENDER, K. & LUKE, B. 2014. The differential processing of telomeres in response to increased telomeric transcription and RNA-DNA hybrid accumulation. *RNA Biol,* 11**,** 95-100.

BARTHEL, F. P., WEI, W., TANG, M., MARTINEZ-LEDESMA, E., HU, X., AMIN, S. B., AKDEMIR, K. C., SETH, S., SONG, X., WANG, Q., LICHTENBERG, T., HU, J., ZHANG, J., ZHENG, S. & VERHAAK, R. G. 2017. Systematic analysis of telomere length and somatic alterations in 31 cancer types. *Nat Genet,* 49**,** 349-357.

BATS, A. S., ROSSI, L., LE FRERE-BELDA, M. A., NARJOZ, C., COURNOU, C., GOSSET, M., NGO, C., DELOMENIE, M., NOS, C., BLONS, H., LAURENT-PUIG, P. & LECURU, F. 2017. [Lynch syndrome and endometrial cancer]. *Bull Cancer,* 104**,** 1013-1021.

BAUMANN, P. & PRICE, C. 2010. Pot1 and telomere maintenance. *FEBS Letters,* 584**,** 3779-3784.

BECHTER, O. E., EISTERER, W., DLASKA, M., KÜHR, T. & THALER, J. 2002. CpG island methylation of the hTERT promoter is associated with lower telomerase activity in B-cell lymphocytic leukemia. *Exp Hematol,* 30**,** 26-33.

BENEDETTI PANICI, P., BASILE, S., MANESCHI, F., ALBERTO LISSONI, A., SIGNORELLI, M., SCAMBIA, G., ANGIOLI, R., TATEO, S., MANGILI, G., KATSAROS, D., GAROZZO, G., CAMPAGNUTTA, E., DONADELLO, N., GREGGI, S., MELPIGNANO, M., RASPAGLIESI, F., RAGNI, N., CORMIO, G., GRASSI, R., FRANCHI, M., GIANNARELLI, D., FOSSATI, R., TORRI, V., AMOROSO, M., CROCÈ, C. & MANGIONI, C. 2008. Systematic pelvic lymphadenectomy vs. no lymphadenectomy in early-stage endometrial carcinoma: randomized clinical trial. *J Natl Cancer Inst,* 100**,** 1707-16.

BERAL, V., BULL, D., REEVES, G. & MILLION WOMEN STUDY, C. 2005. Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet,* 365**,** 1543-51.

BERGMAN, L., BEELEN, M. L., GALLEE, M. P., HOLLEMA, H., BENRAADT, J. & VAN LEEUWEN, F. E. 2000. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. *Lancet,* 356**,** 881-7.

BERNINI, P., BERTINI, I., LUCHINAT, C., NEPI, S., SACCENTI, E., SCHAFER, H., SCHUTZ, B., SPRAUL, M. & TENORI, L. 2009. Individual human phenotypes in metabolic space and time. *J Proteome Res,* 8**,** 4264-71.

BETSOU, F., BEAUDEUX, J. L., BERTHELAIX, A., BORG, J., DUPUY, A., LIZARD, G., PEOC'H, K., QUILLARD, M. & LEHMANN, S. 2009. ["Standard" protocol for evaluating the impact of preanalytical variables on peptidic and proteic analytes and standard coding of preanalytical procedures]. *Ann Biol Clin (Paris),* 67**,** 641-9.

BETSOU, F., LEHMANN, S., ASHTON, G., BARNES, M., BENSON, E. E., COPPOLA, D., DESOUZA, Y., ELIASON, J., GLAZER, B., GUADAGNI, F., HARDING, K., HORSFALL, D. J., KLEEBERGER, C., NANNI, U., PRASAD, A., SHEA, K., SKUBITZ, A., SOMIARI, S. & GUNTER, E. 2010. Standard preanalytical coding for biospecimens: defining the sample PREanalytical code. *Cancer Epidemiol Biomarkers Prev,* 19**,** 1004-11.

BIFFI, G., TANNAHILL, D. & BALASUBRAMANIAN, S. 2012. An Intramolecular G-Quadruplex Structure Is Required for Binding of Telomeric Repeat-Containing RNA to the Telomeric Protein TRF2. *Journal of the American Chemical Society,* 134**,** 11974-11976.

BISSETT, D., DAVIS, J. A. & GEORGE, W. D. 1994. Gynaecological monitoring during tamoxifen therapy. *Lancet,* 344**,** 1244.

BLACKBURN, E. H. 2001. Switching and Signaling at the Telomere. *Cell,* 106**,** 661-673.

BLACKBURN, E. H. & COLLINS, K. 2011. Telomerase: an RNP enzyme synthesizes DNA. *Cold Spring Harb Perspect Biol,* 3.

BLACKBURN, E. H., EPEL, E. S. & LIN, J. 2015. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. *Science,* 350**,** 1193-8.

BLACKBURN, E. H. & GALL, J. G. 1978. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *J Mol Biol,* 120**,** 33-53.

BLACKBURN, E. H., GREIDER, C. W., HENDERSON, E., LEE, M. S., SHAMPAY, J. & SHIPPEN-LENTZ, D. 1989. Recognition and elongation of telomeres by telomerase. *Genome,* 31**,** 553-60.

BLASCO, M. A. 2005. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet,* 6**,** 611-22.
BLASCO, M. A., RIZEN, M., GREIDER, C. W. & HANAHAN, D. 1996. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nat Genet,* 12**,** 200-4.
BLIND, C., KOEPENIK, A., PACYNA-GENGELBACH, M., FERNAHL, G., DEUTSCHMANN, N., DIETEL, M., KRENN, V. & PETERSEN, I. 2008. Antigenicity testing by immunohistochemistry after tissue oxidation. *J Clin Pathol,* 61**,** 79-83.
BODVARSDOTTIR, S. K., STEINARSDOTTIR, M., BJARNASON, H. & EYFJORD, J. E. 2012. Dysfunctional telomeres in human BRCA2 mutated breast tumors and cell lines. *Mutat Res,* 729**,** 90-9.
BOGGESS, J. F., ZHOU, C., BAE-JUMP, V. L., GEHRIG, P. A. & WHANG, Y. E. 2006. Estrogen-receptor-dependent regulation of telomerase activity in human endometrial cancer cell lines. *Gynecol Oncol,* 103**,** 417-24.
BOILESEN, A. E., BISGAARD, M. L. & BERNSTEIN, I. 2008. Risk of gynecologic cancers in Danish hereditary non-polyposis colorectal cancer families. *Acta Obstet Gynecol Scand,* 87**,** 1129-35.
BOKHMAN 1983. Two pathogenetic types of endometrial carcinoma. *Gynecologic oncology,* 15.
BONATZ, G., FRAHM, S. O., KLAPPER, W., HELFENSTEIN, A., HEIDORN, K., JONAT, W., KRUPP, G., PARWARESCH, R. & RUDOLPH, P. 2001. High telomerase activity is associated with cell cycle deregulation and rapid progression in endometrioid adenocarcinoma of the uterus. *Hum Pathol,* 32**,** 605-14.
BOSSE, T., NOUT, R. A., MCALPINE, J. N., MCCONECHY, M. K., BRITTON, H., HUSSEIN, Y. R., GONZALEZ, C., GANESAN, R., STEELE, J. C., HARRISON, B. T., OLIVA, E., VIDAL, A., MATIAS-GUIU, X., ABU-RUSTUM, N. R., LEVINE, D. A., GILKS, C. B. & SOSLOW, R. A. 2018. Molecular Classification of Grade 3 Endometrioid Endometrial Cancers Identifies Distinct Prognostic Subgroups. *Am J Surg Pathol,* 42**,** 561-568.
BOSSE, T., PETERS, E. E., CREUTZBERG, C. L., JÜRGENLIEMK-SCHULZ, I. M., JOBSEN, J. J., MENS, J. W., LUTGENS, L. C., VAN DER STEEN-BANASIK, E. M., SMIT, V. T. & NOUT, R. A. 2015. Substantial lymph-vascular space invasion (LVSI) is a significant risk factor for recurrence in endometrial cancer--A pooled analysis of PORTEC 1 and 2 trials. *Eur J Cancer,* 51**,** 1742-50.
BOTTNER, M., THELEN, P. & JARRY, H. 2014. Estrogen receptor beta: tissue distribution and the still largely enigmatic physiological function. *J Steroid Biochem Mol Biol,* 139**,** 245-51.
BRAND A, D.-L. J. E. T. G. P. M. 2000. *Diagnosis of endometrial cancer in women in women with abnormal vaginal bleeding. SOGC Clinical Practice Guidelines* [Online]. J Soc Obst & Gynae Can. Available: https://poliklinika-harni.hr/images/uploads/217/dijagnoza-karcinoma-endometrija.pdf [Accessed].
BRAY, F., FERLAY, J., SOERJOMATARAM, I., SIEGEL, R. L., TORRE, L. A. & JEMAL, A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin,* 68**,** 394-424.
BRAZ, M. G., BRAZ, L. G., MAZOTI, M. A., PINOTTI, M. F., PARDINI, M. I., BRAZ, J. R. & SALVADORI, D. M. 2012. Lower levels of oxidative DNA damage and apoptosis in lymphocytes from patients undergoing surgery with propofol anesthesia. *Environ Mol Mutagen,* 53**,** 70-7.
BRENNAN, D. J., HACKETHAL, A., MANN, K. P., MUTZ-DEHBALAIE, I., FIEGL, H., MARTH, C. & OBERMAIR, A. 2015. Serum HE4 detects recurrent endometrial cancer in patients undergoing routine clinical surveillance. *BMC Cancer,* 15**,** 33.
BRETT, M. A., ATENAFU, E. G., SINGH, N., GHATAGE, P., CLARKE, B. A., NELSON, G. S., BERNARDINI, M. Q. & KOBEL, M. 2021. Equivalent Survival of p53 Mutated Endometrial Endometrioid Carcinoma Grade 3 and Endometrial Serous Carcinoma. *Int J Gynecol Pathol,* 40**,** 116-123.
BROOKS, R. A., FLEMING, G. F., LASTRA, R. R., LEE, N. K., MORONEY, J. W., SON, C. H., TATEBE, K. & VENERIS, J. L. 2019. Current recommendations and recent progress in endometrial cancer. *CA Cancer J Clin,* 69**,** 258-279.

BRYAN, T. M., ENGLEZOU, A., DALLA-POZZA, L., DUNHAM, M. A. & REDDEL, R. R. 1997. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med,* 3**,** 1271-4.

BULUN, S. E., CHENG, Y. H., YIN, P., IMIR, G., UTSUNOMIYA, H., ATTAR, E., INNES, J. & JULIE KIM, J. 2006. Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol Cell Endocrinol,* 248**,** 94-103.

BURTON, K. A., HENDERSON, T. A., HILLIER, S. G., MASON, J. I., HABIB, F., BRENNER, R. M. & CRITCHLEY, H. O. 2003. Local levonorgestrel regulation of androgen receptor and 17beta-hydroxysteroid dehydrogenase type 2 expression in human endometrium. *Hum Reprod,* 18**,** 2610-7.

CABLE JK, G. M. 2021. *Progesterone Physiology,* StatPearls [Internet]. Treasure Island (FL): StatPearls .

CADE, T. J., QUINN, M. A., MCNALLY, O. M., NEESHAM, D., PYMAN, J. & DOBROTWIR, A. 2010. Predictive value of magnetic resonance imaging in assessing myometrial invasion in endometrial cancer: is radiological staging sufficient for planning conservative treatment? *Int J Gynecol Cancer,* 20**,** 1166-9.

CALADO, R. T., YEWDELL, W. T., WILKERSON, K. L., REGAL, J. A., KAJIGAYA, S., STRATAKIS, C. A. & YOUNG, N. S. 2009. Sex hormones, acting on the TERT gene, increase telomerase activity in human primary hematopoietic cells. *Blood,* 114**,** 2236-43.

CAMPBELL, L. D., ASTRIN, J. J., DESOUZA, Y., GIRI, J., PATEL, A. A., RAWLEY-PAYNE, M., RUSH, A. & SIEFFERT, N. 2018. The 2018 Revision of the ISBER Best Practices: Summary of Changes and the Editorial Team's Development Process. *Biopreserv Biobank,* 16**,** 3-6.

CAMPISI, J. & D'ADDA DI FAGAGNA, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol,* 8**,** 729-40.

CANCER GENOME ATLAS RESEARCH, N., KANDOTH, C., SCHULTZ, N., CHERNIACK, A. D., AKBANI, R., LIU, Y., SHEN, H., ROBERTSON, A. G., PASHTAN, I., SHEN, R., BENZ, C. C., YAU, C., LAIRD, P. W., DING, L., ZHANG, W., MILLS, G. B., KUCHERLAPATI, R., MARDIS, E. R. & LEVINE, D. A. 2013. Integrated genomic characterization of endometrial carcinoma. *Nature,* 497**,** 67-73.

CAO, H., ZHAI, Y., JI, X., WANG, Y., ZHAO, J., XING, J., AN, J. & REN, T. 2020. Noncoding telomeric repeat-containing RNA inhibits the progression of hepatocellular carcinoma by regulating telomerase-mediated telomere length. *Cancer Sci,* 111**,** 2789-2802.

CAPOCASA, M., ANAGNOSTOU, P., D'ABRAMO, F., MATTEUCCI, G., DOMINICI, V., DESTRO BISOL, G. & RUFO, F. 2016. Samples and data accessibility in research biobanks: an explorative survey. *PeerJ,* 4**,** e1613.

CARRARO, P., ZAGO, T. & PLEBANI, M. 2012. Exploring the initial steps of the testing process: frequency and nature of pre-preanalytic errors. *Clin Chem,* 58**,** 638-42.

CAYUELA, M. L., FLORES, J. M. & BLASCO, M. A. 2005. The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression. *EMBO reports,* 6.

CESARE, A. J. & KARLSEDER, J. 2012. A three-state model of telomere control over human proliferative boundaries. *Current opinion in cell biology,* 24**,** 731-738.

CESARE, A. J. & REDDEL, R. R. 2010. Alternative lengthening of telomeres: models, mechanisms and implications. *Nature Reviews Genetics,* 11**,** 319-330.

CHADHA, S., PACHE, T. D., HUIKESHOVEN, J. M., BRINKMANN, A. O. & VAN DER KWAST, T. H. 1994. Androgen receptor expression in human ovarian and uterine tissue of long-term androgen-treated transsexual women. *Human pathology,* 25.

CHAI, J. H., ZHANG, Y., TAN, W. H., CHNG, W. J., LI, B. & WANG 2011. Regulation of hTERT by BCR-ABL at multiple levels in K562 cells. *BMC cancer,* 11.

CHALMERS, I., BRACKEN, M. B., DJULBEGOVIC, B., GARATTINI, S., GRANT, J., GÜLMEZOGLU, A. M., HOWELLS, D. W., IOANNIDIS, J. P. & OLIVER, S. 2014. How to increase value and reduce waste when research priorities are set. *Lancet,* 383**,** 156-65.

CHEN, J. L., BLASCO, M. A. & GREIDER, C. W. 2000. Secondary structure of vertebrate telomerase RNA. *Cell,* 100**,** 503-14.

CHEN, L. Y., ZHANG, Y., ZHANG, Q., LI, H., LUO, Z., FANG, H., KIM, S. H., QIN, L., YOTNDA, P., XU, J., TU, B. P., BAI, Y. & SONGYANG, Z. 2012a. Mitochondrial localization of telomeric protein TIN2 links telomere regulation to metabolic control. *Mol Cell,* 47**,** 839-50.

CHEN, X. F., CAI, S., CHEN, Q. G., NI, Z. H., TANG, J. H., XU, D. W. & WANG, X. B. 2012b. Multiple variants of TERT and CLPTM1L constitute risk factors for lung adenocarcinoma. *Genet Mol Res,* 11**,** 370-8.

CHEUNG, E. & KRAUS, W. L. 2010. Genomic analyses of hormone signaling and gene regulation. *Annu Rev Physiol,* 72**,** 191-218.

CHHIENG, D. & HUI, P. 2011. Normal Histology of Female Genital Organs. *In:* CHHIENG, D. & HUI, P. (eds.) *Cytology and Surgical Pathology of Gynecologic Neoplasms.* Totowa, NJ: Humana Press.

CHIBA, K., LORBEER, F. K., SHAIN, A. H., MCSWIGGEN, D. T., SCHRUF, E., OH, A., RYU, J., DARZACQ, X., BASTIAN, B. C. & HOCKEMEYER, D. 2017. Mutations in the promoter of the telomerase gene TERT contribute to tumorigenesis by a two-step mechanism. *Science,* 357**,** 1416-1420.

CHU, C., QU, K., ZHONG, F. L., ARTANDI, S. E. & CHANG, H. Y. 2011. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol Cell,* 44**,** 667-78.

CHU, H. P., CIFUENTES-ROJAS, C., KESNER, B., AEBY, E., LEE, H. G., WEI, C., OH, H. J., BOUKHALI, M., HAAS, W. & LEE, J. T. 2017. TERRA RNA Antagonizes ATRX and Protects Telomeres. *Cell,* 170**,** 86-101.e16.

CHU, T. W., D'SOUZA, Y. & AUTEXIER, C. 2016. The Insertion in Fingers Domain in Human Telomerase Can Mediate Enzyme Processivity and Telomerase Recruitment to Telomeres in a TPP1-Dependent Manner. *Mol Cell Biol,* 36**,** 210-22.

CIMINO-REALE, G., PASCALE, E., BATTILORO, E., STARACE, G., VERNA, R. & D'AMBROSIO, E. 2001. The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res,* 29**,** E35.

CLARK, T. J. & GUPTA, J. K. 2002. Outpatient hysteroscopy. *The Obstetrician & Gynaecologist,* 4**,** 217-221.

CLARK, T. J., MANN, C. H., SHAH, N., KHAN, K. S., SONG, F. & GUPTA, J. K. 2002. Accuracy of outpatient endometrial biopsy in the diagnosis of endometrial cancer: a systematic quantitative review. *Bjog,* 109**,** 313-21.

CLARKE, M. A., LONG, B. J., DEL MAR MORILLO, A., ARBYN, M., BAKKUM-GAMEZ, J. N. & WENTZENSEN, N. 2018. Association of Endometrial Cancer Risk With Postmenopausal Bleeding in Women: A Systematic Review and Meta-analysis. *JAMA Intern Med,* 178**,** 1210-1222.

CLYNES, D., JELINSKA, C., XELLA, B., AYYUB, H., SCOTT, C., MITSON, M., TAYLOR, S., HIGGS, D. R. & GIBBONS, R. J. 2015. Suppression of the alternative lengthening of telomere pathway by the chromatin remodelling factor ATRX. *Nat Commun,* 6**,** 7538.

COHEN, J. D., LI, L., WANG, Y., THOBURN, C., AFSARI, B., DANILOVA, L., DOUVILLE, C., JAVED, A. A., WONG, F., MATTOX, A., HRUBAN, R. H., WOLFGANG, C. L., GOGGINS, M. G., DAL MOLIN, M., WANG, T. L., RODEN, R., KLEIN, A. P., PTAK, J., DOBBYN, L., SCHAEFER, J., SILLIMAN, N., POPOLI, M., VOGELSTEIN, J. T., BROWNE, J. D., SCHOEN, R. E., BRAND, R. E., TIE, J., GIBBS, P., WONG, H. L., MANSFIELD, A. S., JEN, J., HANASH, S. M., FALCONI, M., ALLEN, P. J., ZHOU, S., BETTEGOWDA, C., DIAZ, L. A., JR., TOMASETTI, C., KINZLER, K. W., VOGELSTEIN, B., LENNON, A. M. & PAPADOPOULOS, N. 2018. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science,* 359**,** 926-930.

COHEN, S. B., GRAHAM, M. E., LOVRECZ, G. O., BACHE, N., ROBINSON, P. J. & REDDEL, R. R. 2007. Protein composition of catalytically active human telomerase from immortal cells. *Science,* 315**,** 1850-3.

COLAS, E., PEREZ, C., CABRERA, S., PEDROLA, N., MONGE, M., CASTELLVI, J., EYZAGUIRRE, F., GREGORIO, J., RUIZ, A., LLAURADO, M., RIGAU, M., GARCIA, M., ERTEKIN, T., MONTES, M., LOPEZ-LOPEZ, R., CARRERAS, R., XERCAVINS, J., ORTEGA, A., MAES, T., ROSELL, E., DOLL, A., ABAL, M., REVENTOS, J. & GIL-MORENO, A. 2011. Molecular markers of endometrial carcinoma detected in uterine aspirates. *Int J Cancer,* 129**,** 2435-44.

COLLABORATIVE GROUP IN EPIDEMIOLOGICAL STUDIES ON ENDOMETRIAL CANCER, C. 2015. Endometrial cancer and oral contraceptives: an individual participant meta-analysis of 27 276 women with endometrial cancer from 36 epidemiological studies. *The Lancet Oncology,* 16**,** 1061-1070.

COLOMBO, N., CREUTZBERG, C., AMANT, F., BOSSE, T., GONZÁLEZ-MARTÍN, A., LEDERMANN, J., MARTH, C., NOUT, R., QUERLEU, D., MIRZA, M. R. & SESSA, C. 2016. ESMO-ESGO-ESTRO Consensus Conference on Endometrial Cancer: diagnosis, treatment and follow-up. *Ann Oncol,* 27**,** 16-41.

COLOMBO, N., PRETI, E., LANDONI, F., CARINELLI, S., COLOMBO, A., MARINI, C. & SESSA, C. 2013. Endometrial cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology,* 24 Suppl 6.

COMPTON, C. C., ROBB, J. A., ANDERSON, M. W., BERRY, A. B., BIRDSONG, G. G., BLOOM, K. J., BRANTON, P. A., CROTHERS, J. W., CUSHMAN-VOKOUN, A. M., HICKS, D. G., KHOURY, J. D., LASER, J., MARSHALL, C. B., MISIALEK, M. J., NATALE, K. E., NOWAK, J. A., OLSON, D., PFEIFER, J. D., SCHADE, A., VANCE, G. H., WALK, E. E. & YOHE, S. L. 2019. Preanalytics and Precision Pathology: Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine. *Arch Pathol Lab Med,* 143**,** 1346-1363.

CONG, Y. S., WEN, J. & BACCHETTI, S. 1999. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. *Hum Mol Genet,* 8**,** 137-42.

COOK, L. S., DONG, Y., ROUND, P., HUANG, X., MAGLIOCCO, A. M. & FRIEDENREICH, C. M. 2014. Hormone contraception before the first birth and endometrial cancer risk. *Cancer Epidemiol Biomarkers Prev,* 23**,** 356-61.

CRANDALL, C. J. & BARRETT-CONNOR, E. 2013. Endogenous sex steroid levels and cardiovascular disease in relation to the menopause: a systematic review. *Endocrinol Metab Clin North Am,* 42**,** 227-53.

CRISTOFARI, G., ADOLF, E., REICHENBACH, P., SIKORA, K., TERNS, R. M., TERNS, M. P. & LINGNER, J. 2007. Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. *Mol Cell,* 27**,** 882-9.

CRITCHLEY, H. O. & SAUNDERS, P. T. 2009. Hormone receptor dynamics in a receptive human endometrium. *Reprod Sci,* 16**,** 191-9.

CROSBIE, E. J., KITSON, S. J., MCALPINE, J. N., MUKHOPADHYAY, A., POWELL, M. E. & SINGH, N. 2022. Endometrial cancer. *The Lancet,* 399**,** 1412-1428.

CRUK. 2016-2018a. *https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer/mortality* [Online]. CRUK website. Available: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer/mortality [Accessed 3 rd February 2020].

CRUK. 2016-2018b. *Survival for womb (uterine) cancer | Cancer Research UK* [Online]. Available: https://www.cancerresearchuk.org/about-cancer/womb-cancer/survival [Accessed 4th sept 2022].

CRUK. 2016 - 2018. *Uterine cancer statistics* [Online]. Available: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer [Accessed 5th June 2022].

CRUZ, F. F., ROCCO, P. R. M. & PELOSI, P. 2017. Anti-inflammatory properties of anesthetic agents. *Critical Care,* 21**,** 67.

CUSANELLI, E., ROMERO, C. A. & CHARTRAND, P. 2013. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. *Mol Cell,* 51**,** 780-91.

DAI, D., WOLF, D. M., LITMAN, E. S., WHITE, M. J. & LESLIE, K. K. 2002. Progesterone inhibits human endometrial cancer cell growth and invasiveness: down-regulation of cellular adhesion molecules through progesterone B receptors. *Cancer research,* 62.

DAVID, K. A., UNGER, F. T., UHLIG, P., JUHL, H., MOORE, H. M., COMPTON, C., NASHAN, B., DÖRNER, A., DE WEERTH, A. & ZORNIG, C. 2014. Surgical procedures and postsurgical tissue processing significantly affect expression of genes and EGFR-pathway proteins in colorectal cancer tissue. *Oncotarget,* 5**,** 11017-28.

DE BOECK, G., FORSYTH, R. G., PRAET, M. & HOGENDOORN, P. C. 2009. Telomere-associated proteins: cross-talk between telomere maintenance and telomere-lengthening mechanisms. *The Journal of Pathology,* 217**,** 327-344.

DE KROON, C. D., DE BOCK, G. H., DIEBEN, S. W. & JANSEN, F. W. 2003. Saline contrast hysterosonography in abnormal uterine bleeding: a systematic review and meta-analysis. *Bjog,* 110**,** 938-47.

DE LANGE, T. 2004. T-loops and the origin of telomeres. *Nat Rev Mol Cell Biol,* 5**,** 323-9.

DE LANGE, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev,* 19**,** 2100-10.

DEDES, K. J., WETTERSKOG, D., ASHWORTH, A., KAYE, S. B. & REIS-FILHO, J. S. 2011. Emerging therapeutic targets in endometrial cancer. *Nat Rev Clin Oncol,* 8**,** 261-71.

DELAIR, D. F., BURKE, K. A., SELENICA, P., LIM, R. S., SCOTT, S. N., MIDDHA, S., MOHANTY, A. S., CHENG, D. T., BERGER, M. F., SOSLOW, R. A. & WEIGELT, B. 2017. The genetic landscape of endometrial clear cell carcinomas. *J Pathol,* 243**,** 230-241.

DENG, Z., NORSEEN, J., WIEDMER, A., RIETHMAN, H. & LIEBERMAN, P. M. 2009. TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. *Mol Cell,* 35**,** 403-13.

DENG, Z., WANG, Z., XIANG, C., MOLCZAN, A., BAUBET, V., CONEJO-GARCIA, J., XU, X., LIEBERMAN, P. M. & DAHMANE, N. 2012. Formation of telomeric repeat-containing RNA (TERRA) foci in highly proliferating mouse cerebellar neuronal progenitors and medulloblastoma. *J Cell Sci,* 125**,** 4383-94.

DI GIROLAMO, C., WALTERS, S., BENITEZ MAJANO, S., RACHET, B., COLEMAN, M. P., NJAGI, E. N. & MORRIS, M. 2018. Characteristics of patients with missing information on stage: a population-based study of patients diagnosed with colon, lung or breast cancer in England in 2013. *BMC Cancer,* 18**,** 492.

DIALA, I., SHIOHAMA, Y., FUJITA, T., KOTAKE, Y., DEMONACOS, C., KRSTIC-DEMONACOS, M., LEVA, G. D. & FUJII, M. 2020. Telomerase inhibition, telomere attrition and proliferation arrest of cancer cells induced by phosphorothioate ASO-NLS conjugates targeting hTERC and siRNAs targeting hTERT. *Nucleosides Nucleotides Nucleic Acids,* 39**,** 407-425.

DIALA, I., WAGNER, N., MAGDINIER, F., SHKRELI, M., SIRAKOV, M., BAUWENS, S., SCHLUTH-BOLARD, C., SIMONET, T., RENAULT, V. M., YE, J., DJERBI, A., PINEAU, P., CHOI, J., ARTANDI, S., DEJEAN, A., PLATEROTI, M. & GILSON, E. 2013. Telomere protection and TRF2 expression are enhanced by the canonical Wnt signalling pathway. *EMBO Rep.*

DIMAN, A., BOROS, J., POULAIN, F., RODRIGUEZ, J., PURNELLE, M., EPISKOPOU, H., BERTRAND, L., FRANCAUX, M., DELDICQUE, L. & DECOTTIGNIES, A. 2016. Nuclear respiratory factor 1 and endurance exercise promote human telomere transcription. *Sci Adv,* 2**,** e1600031.

DIMAN, A. & DECOTTIGNIES, A. 2018. Genomic origin and nuclear localization of TERRA telomeric repeat-containing RNA: from Darkness to Dawn. *FEBS J,* 285**,** 1389-1398.

DORJI, T., MONTI, V., FELLEGARA, G., GABBA, S., GRAZIOLI, V., REPETTI, E., MARCIALIS, C., PELUSO, S., DI RUZZA, D., NERI, F. & FOSCHINI, M. P. 2015. Gain of hTERC: a genetic marker of malignancy in oral potentially malignant lesions. *Hum Pathol,* 46**,** 1275-81.

DSOUZA, Y., CHU, T. W. & AUTEXIER, C. 2013. A translocation-defective telomerase with low levels of activity and processivity stabilizes short telomeres and confers immortalization. *Molecular biology of the cell,* 24**,** 1469-1479.

DUEHOLM, M., HJORTH, I. M. D., DAHL, K., HANSEN, E. S. & ORTOFT, G. 2019. Ultrasound Scoring of Endometrial Pattern for Fast-track Identification or Exclusion of Endometrial Cancer in Women with Postmenopausal Bleeding. *J Minim Invasive Gynecol,* 26**,** 516-525.

DUNLOP, M. G., FARRINGTON, S. M., CAROTHERS, A. D., WYLLIE, A. H., SHARP, L., BURN, J., LIU, B., KINZLER, K. W. & VOGELSTEIN, B. 1997. Cancer risk associated with germline DNA mismatch repair gene mutations. *Hum Mol Genet,* 6**,** 105-10.

EBINA, Y., YAMADA, H., FUJINO, T., FURUTA, I., SAKURAGI, N., YAMAMOTO, R., KATOH, M., OSHIMURA, M. & FUJIMOTO, S. 1999. Telomerase activity correlates with histo-pathological factors in uterine endometrial carcinoma. *Int J Cancer,* 84**,** 529-32.

EGAN, E. D. & COLLINS, K. 2010. Specificity and stoichiometry of subunit interactions in the human telomerase holoenzyme assembled in vivo. *Mol Cell Biol,* 30**,** 2775-86.

ELLERVIK, C. & VAUGHT, J. 2015. Preanalytical variables affecting the integrity of human biospecimens in biobanking. *Clin Chem,* 61**,** 914-34.

ELLIOTT, P. & PEAKMAN, T. C. 2008. The UK Biobank sample handling and storage protocol for the collection, processing and archiving of human blood and urine. *International journal of epidemiology,* 37.

EMONS, G. & GRUNDKER, C. 2021. The Role of Gonadotropin-Releasing Hormone (GnRH) in Endometrial Cancer. *Cells,* 10.

EMONS, G., MULLER, V., ORTMANN, O. & SCHULZ, K. D. 1998. Effects of LHRH-analogues on mitogenic signal transduction in cancer cells. *J Steroid Biochem Mol Biol,* 65**,** 199-206.

ERGIN, B., MEDING, S., LANGER, R., KAP, M., VIERTLER, C., SCHOTT, C., FERCH, U., RIEGMAN, P., ZATLOUKAL, K., WALCH, A. & BECKER, K.-F. 2010. Proteomic Analysis of PAXgene-Fixed Tissues. *Journal of Proteome Research,* 9**,** 5188-5196.

ESTEVA-SOCIAS, M., ARTIGA, M. J., BAHAMONDE, O., BELAR, O., BERMUDO, R., CASTRO, E., ESCÁMEZ, T., FRAGA, M., JAUREGUI-MOSQUERA, L., NOVOA, I., PEIRÓ-CHOVA, L., REJÓN, J. D., RUIZ-MIRÓ, M., VIEIRO-BALO, P., VILLAR-CAMPO, V., ZAZO, S., RÁBANO, A. & VILLENA, C. 2019. In search of an evidence-based strategy for quality assessment of human tissue samples: report of the tissue Biospecimen Research Working Group of the Spanish Biobank Network. *J Transl Med,* 17**,** 370.

ETTINGER, B., BAINTON, L., UPMALIS, D. H., CITRON, J. T. & VANGESSEL, A. 1997. Comparison of endometrial growth produced by unopposed conjugated estrogens or by micronized estradiol in postmenopausal women. *Am J Obstet Gynecol,* 176**,** 112-7.

EVANS, T., SANY, O., PEARMAIN, P., GANESAN, R., BLANN, A. & SUNDAR, S. 2011. Differential trends in the rising incidence of endometrial cancer by type: data from a UK population-based registry from 1994 to 2006. *Br J Cancer,* 104**,** 1505-10.

EVERETT, E., EVERETT@U.WASHINGTON.EDU, UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., TAMIMI, H., UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., GREER, B., UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., SWISHER, E., UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., PALEY, P., UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., MANDEL, L., UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A., GOFF, B. & UNIVERSITY OF WASHINGTON MEDICAL CENTER, S. W. A. U. S. A. 2003. The effect of body mass index on clinical/pathologic features, surgical morbidity, and outcome in patients with endometrial cancer. *Gynecologic Oncology,* 90**,** 150-157.

FAKHOURY, J., MARIE-EGYPTIENNE, D. T., LONDOÑO-VALLEJO, J.-A. & AUTEXIER, C. 2010. Telomeric function of mammalian telomerases at short telomeres. *Journal of Cell Science,* 123**,** 1693-1704.

FAMBRINI, M., SORBI, F., SISTI, G., CIONI, R., TURRINI, I., TADDEI, G. & GUASCHINO, S. 2014. Endometrial carcinoma in high-risk populations: is it time to consider a screening policy? *Cytopathology,* 25**,** 71-7.

FAN, L., LI, J., YU, Z., DANG, X. & WANG, K. 2014. The hypoxia-inducible factor pathway, prolyl hydroxylase domain protein inhibitors, and their roles in bone repair and regeneration. *Biomed Res Int,* 2014**,** 239356.

FANG, F. C. & CASADEVALL, A. 2010. Lost in translation--basic science in the era of translational research. *Infect Immun,* 78**,** 563-6.

FANNING, J., EVANS, M. C., PETERS, A. J., SAMUEL, M., HARMON, E. R. & BATES, J. 1989. Endometrial adenocarcinoma histologic subtypes: clinical and pathologic profile. *Gynecologic oncology,* 32.

FARNUNG, B. O., BRUN, C. M., ARORA, R., LORENZI, L. E. & AZZALIN, C. M. 2012. Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. *PLoS One,* 7**,** e35714.

FASSBENDER, A., RAHMIOGLU, N., VITONIS, A. F., VIGANO, P., GIUDICE, L. C., D'HOOGHE, T. M., HUMMELSHOJ, L., ADAMSON, G. D., BECKER, C. M., MISSMER, S. A., ZONDERVAN, K. T. & GROUP, W. E. W. 2014. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. *Fertil Steril,* 102**,** 1244-53.

FEARNLEY, E. J., MARQUART, L., SPURDLE, A. B., WEINSTEIN, P., WEBB, P. M., THE AUSTRALIAN OVARIAN CANCER STUDY, G. & THE AUSTRALIAN NATIONAL ENDOMETRIAL CANCER STUDY, G. 2010. Polycystic ovary syndrome increases the risk of endometrial cancer in women aged less than 50 years: an Australian case–control study. *Cancer Causes & Control,* 21**,** 2303-2308.

FELIX, A. S., WEISSFELD, J. L., STONE, R. A., BOWSER, R., CHIVUKULA, M., EDWARDS, R. P. & LINKOV, F. 2010. Factors associated with Type I and Type II endometrial cancer. *Cancer Causes Control,* 21**,** 1851-6.

FELIX, A. S., YANG, H. P., GIERACH, G. L., PARK, Y. & BRINTON, L. A. 2014. Cigarette smoking and endometrial carcinoma risk: the role of effect modification and tumor heterogeneity. *Cancer Causes Control,* 25**,** 479-89.

FENG, J., FUNK, W. D., WANG, S. S., WEINRICH, S. L., AVILION, A. A., CHIU, C. P., ADAMS, R. R., CHANG, E., ALLSOPP, R. C., YU, J. & ET AL. 1995. The RNA component of human telomerase. *Science,* 269**,** 1236-41.

FERENCZY, A. & BERGERON, C. 1991. Histology of the human endometrium: from birth to senescence. *Ann N Y Acad Sci,* 622**,** 6-27.

FERETZAKI, M. & LINGNER, J. 2017. A practical qPCR approach to detect TERRA, the elusive telomeric repeat-containing RNA. *Methods,* 114**,** 39-45.

FERETZAKI, M., RENCK NUNES, P. & LINGNER, J. 2019. Expression and differential regulation of human TERRA at several chromosome ends. *Rna,* 25**,** 1470-1480.

FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D. M., FORMAN, D. & BRAY, F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer,* 136**,** E359-86.

FEUERHAHN, S., IGLESIAS, N., PANZA, A., PORRO, A. & LINGNER, J. 2010. TERRA biogenesis, turnover and implications for function. *FEBS Lett,* 584**,** 3812-8.

FILIPOVICH, A. H., WEISDORF, D., PAVLETIC, S., SOCIE, G., WINGARD, J. R., LEE, S. J., MARTIN, P., CHIEN, J., PRZEPIORKA, D., COURIEL, D., COWEN, E. W., DINNDORF, P., FARRELL, A., HARTZMAN, R., HENSLEE-DOWNEY, J., JACOBSOHN, D., MCDONALD, G., MITTLEMAN, B., RIZZO, J. D., ROBINSON, M., SCHUBERT, M., SCHULTZ, K., SHULMAN, H., TURNER, M., VOGELSANG, G. & FLOWERS, M. E. 2005. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant,* 11**,** 945-56.

FILOCAMO, M., BALDO, C., GOLDWURM, S., RENIERI, A., ANGELINI, C., MOGGIO, M., MORA, M., MERLA, G., POLITANO, L., GARAVAGLIA, B., CASARETO, L., BRICARELLI, F. D. & FOR TELETHON NETWORK OF GENETIC BIOBANKS, S. 2013. Telethon Network of Genetic Biobanks: a key service for diagnosis and research on rare diseases. *Orphanet Journal of Rare Diseases,* 8**,** 129.

FISHER, B., COSTANTINO, J. P., REDMOND, C. K., FISHER, E. R., WICKERHAM, D. L. & CRONIN, W. M. 1994. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst,* 86**,** 527-37.

FLYNN, R. L., CENTORE, R. C., O'SULLIVAN, R. J., RAI, R., TSE, A., SONGYANG, Z., CHANG, S., KARLSEDER, J. & ZOU, L. 2011. TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA. *Nature,* 471**,** 532-6.

FLYNN, R. L., CHANG, S. & ZOU, L. 2012. RPA and POT1: friends or foes at telomeres? *Cell Cycle,* 11**,** 652-7.

FORTIER, I., DOIRON, D., BURTON, P. & RAINA, P. 2011. Invited commentary: consolidating data harmonization--how to obtain quality and applicability? *Am J Epidemiol,* 174**,** 261-4; author reply 265-6.

FORTNER, R. T., HUSING, A., KUHN, T., KONAR, M., OVERVAD, K., TJONNELAND, A., HANSEN, L., BOUTRON-RUAULT, M. C., SEVERI, G., FOURNIER, A., BOEING, H., TRICHOPOULOU, A., BENETOU, V., ORFANOS, P., MASALA, G., AGNOLI, C., MATTIELLO, A., TUMINO, R., SACERDOTE, C., BUENO-DE-MESQUITA, H. B., PEETERS, P. H., WEIDERPASS, E., GRAM, I. T., GAVRILYUK, O., QUIROS, J. R., MARIA HUERTA, J., ARDANAZ, E., LARRANAGA, N., LUJAN-BARROSO, L., SANCHEZ-CANTALEJO, E., BUTT, S. T., BORGQUIST, S., IDAHL, A., LUNDIN, E., KHAW, K. T., ALLEN, N. E., RINALDI, S., DOSSUS, L., GUNTER, M., MERRITT, M. A., TZOULAKI, I., RIBOLI, E. & KAAKS, R. 2017. Endometrial cancer risk prediction including serum-based biomarkers: results from the EPIC cohort. *Int J Cancer,* 140**,** 1317-1323.

FRIAS-GOMEZ, J., BENAVENTE, Y., PONCE, J., BRUNET, J., IBANEZ, R., PEREMIQUEL-TRILLAS, P., BAIXERAS, N., ZANCA, A., PIULATS, J. M., AYTES, A., MATIAS-GUIU, X., BOSCH, F. X., DE SANJOSE, S., ALEMANY, L., COSTAS, L. & SCREENWIDE, T. 2020. Sensitivity of cervico-vaginal cytology in endometrial carcinoma: A systematic review and meta-analysis. *Cancer Cytopathol,* 128**,** 792-802.

FRIBERG, E., ORSINI, N., MANTZOROS, C. S. & WOLK, A. 2007. Diabetes mellitus and risk of endometrial cancer: a meta-analysis. *Diabetologia,* 50**,** 1365-74.

FRIEDENREICH, C., CUST, A., LAHMANN, P. H., STEINDORF, K., BOUTRON-RUAULT, M. C., CLAVEL-CHAPELON, F., MESRINE, S., LINSEISEN, J., ROHRMANN, S., BOEING, H., PISCHON, T., TJØNNELAND, A., HALKJAER, J., OVERVAD, K., MENDEZ, M., REDONDO, M. L., GARCIA, C. M., LARRAÑAGA, N., TORMO, M. J., GURREA, A. B., BINGHAM, S., KHAW, K. T., ALLEN, N., KEY, T., TRICHOPOULOU, A., VASILOPOULOU, E., TRICHOPOULOS, D., PALA, V., PALLI, D., TUMINO, R., MATTIELLO, A., VINEIS, P., BUENO-DE-MESQUITA, H. B., PEETERS, P. H., BERGLUND, G., MANJER, J., LUNDIN, E., LUKANOVA, A., SLIMANI, N., JENAB, M., KAAKS, R., RIBOLI, E., C., F., CUST, A., LAHMANN, P. H., STEINDORF, K., BOUTRON-RUAULT, M. C., CLAVEL-CHAPELON, F., MESRINE, S., LINSEISEN, J., ROHRMANN, S., BOEING, H., PISCHON, T., TJØNNELAND, A., HALKJAER, J., OVERVAD, K. & MENDEZ 2007. Anthropometric factors and risk of endometrial cancer: the European prospective investigation into cancer and nutrition. *Cancer causes & control : CCC,* 18.

FU, D. & COLLINS, K. 2003. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol Cell,* 11**,** 1361-72.

FUJIMOTO, J., NISHIGAKI, M., HORI, M., ICHIGO, S., MORISHITA, S. & TAMAYA, T. 1995. Effects of Estradiol and Testosterone on the Synthesis, Expression and Degradation of Androgen Receptor in Human Uterine Endometrial Fibroblasts. *J Biomed Sci,* 2**,** 160-165.

GALA, K. & KHATTAR, E. 2021. Long non-coding RNAs at work on telomeres: Functions and implications in cancer therapy. *Cancer letters,* 502.

GAMBARA, G., GAEBLER, M., KEILHOLZ, U., REGENBRECHT, C. R. A. & SILVESTRI, A. 2018. From Chemotherapy to Combined Targeted Therapeutics: In Vitro and in Vivo Models to Decipher Intra-tumor Heterogeneity. *Front Pharmacol,* 9**,** 77.

GARGETT, C. E., CHAN, R. W. & SCHWAB, K. E. 2008. Hormone and growth factor signaling in endometrial renewal: role of stem/progenitor cells. *Mol Cell Endocrinol,* 288**,** 22-9.

GASPAR, T. B., SÁ, A., LOPES, J. M., SOBRINHO-SIMÕES, M., SOARES, P. & VINAGRE, J. 2018. Telomere Maintenance Mechanisms in Cancer. *Genes (Basel),* 9.

GAZZANIGA, F. S. & BLACKBURN, E. H. 2014. An antiapoptotic role for telomerase RNA in human immune cells independent of telomere integrity or telomerase enzymatic activity. *Blood,* 124**,** 3675-84.

GIBSON, D. A., SIMITSIDELLIS, I., COLLINS, F. & SAUNDERS, P. T. K. 2018. Endometrial Intracrinology: Oestrogens, Androgens and Endometrial Disorders. *Int J Mol Sci,* 19.

GLINGE, C., CLAUSS, S., BODDUM, K., JABBARI, R., JABBARI, J., RISGAARD, B., TOMSITS, P., HILDEBRAND, B., KÄÄB, S., WAKILI, R., JESPERSEN, T. & TFELT-HANSEN, J. 2017. Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations. *PLoS One,* 12**,** e0167969.

GONG, T. T., WANG, Y. L. & MA, X. X. 2015. Age at menarche and endometrial cancer risk: a dose-response meta-analysis of prospective studies. *Scientific reports,* 5.

GONZALEZ-VASCONCELLOS, I., SCHNEIDER, R., ANASTASOV, N., ALONSO-RODRIGUEZ, S., SANLI-BONAZZI, B., FERNÁNDEZ, J. L. & ATKINSON, M. J. 2017. The Rb1 tumour suppressor gene modifies telomeric chromatin architecture by regulating TERRA expression. *Sci Rep,* 7**,** 42056.

GOTTSCHAU, M., KJAER, S. K., JENSEN, A., MUNK, C. & MELLEMKJAER, L. 2015. Risk of cancer among women with polycystic ovary syndrome: A Danish cohort study. *Gynecologic Oncology,* 136**,** 99-103.

GRAF, M., BONETTI, D., LOCKHART, A., SERHAL, K., KELLNER, V., MAICHER, A., JOLIVET, P., TEIXEIRA, M. T. & LUKE, B. 2017. Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle. *Cell,* 170**,** 72-85.e14.

GRANDIN, N., PEREIRA, B., COHEN, C., BILLARD, P., DEHAIS, C., CARPENTIER, C., IDBAIH, A., BIELLE, F., DUCRAY, F., FIGARELLA-BRANGER, D., DELATTRE, J. Y., SANSON, M., LOMONTE, P., PONCET, D., VERRELLE, P. & CHARBONNEAU, M. 2019. The level of activity of the alternative lengthening of telomeres correlates with patient age in IDH-mutant ATRX-loss-of-expression anaplastic astrocytomas. *Acta Neuropathol Commun,* 7**,** 175.

GREIDER, C. W. 2010. Telomerase discovery: the excitement of putting together pieces of the puzzle (Nobel lecture). *Angew Chem Int Ed Engl,* 49**,** 7422-39.

GRIFFITH, J. D., COMEAU, L., ROSENFIELD, S., STANSEL, R. M., BIANCHI, A., MOSS, H. & DE LANGE, T. 1999. Mammalian telomeres end in a large duplex loop. *Cell,* 97**,** 503-14.

GRIZZLE, W. E., GUNTER, E. W., SEXTON, K. C. & BELL, W. C. 2015. Quality management of biorepositories. *Biopreserv Biobank,* 13**,** 183-94.

GRIZZLE, W. E., OTALI, D., SEXTON, K. C. & ATHERTON, D. S. 2016. Effects of Cold Ischemia on Gene Expression: A Review and Commentary. *Biopreserv Biobank,* 14**,** 548-558.

GRIZZLE, W. E., SEXTON, K. C., MCGARVEY, D., MENCHHOFEN, Z. V. & LIVOLSI, V. 2018. Lessons Learned During Three Decades of Operations of Two Prospective Bioresources. *Biopreserv Biobank,* 16**,** 483-492.

GRONTVED, L. & HAGER, G. L. 2012. Impact of chromatin structure on PR signaling: transition from local to global analysis. *Mol Cell Endocrinol,* 357**,** 30-6.

GROOTHUIS, P. G., DASSEN, H. H., ROMANO, A. & PUNYADEERA 2007. Estrogen and the endometrium: lessons learned from gene expression profiling in rodents and human. *Human reproduction update,* 13.

GUL, I., DUNDAR, O., BODUR, S., TUNCA, Y. & TUTUNCU, L. 2013. The status of telomerase enzyme activity in benign and malignant gynaecologic pathologies. *Balkan Med J,* 30**,** 287-92.

GUNDERSON, C. C., JAVA, J., MOORE, K. N. & WALKER, J. L. 2014. The impact of obesity on surgical staging, complications, and survival with uterine cancer: a Gynecologic Oncology Group LAP2 ancillary data study. *Gynecol Oncol,* 133**,** 23-7.

GÜNDISCH, S., ANNARATONE, L., BEESE, C., DRECOL, E., MARCHIÒ, C., QUAGLINO, E., SAPINO, A., BECKER, K. F. & BUSSOLATI, G. 2015. Critical roles of specimen type and temperature before and during fixation in the detection of phosphoproteins in breast cancer tissues. *Lab Invest,* 95**,** 561-71.

GUNDISCH, S., HAUCK, S., SARIOGLU, H., SCHOTT, C., VIERTLER, C., KAP, M., SCHUSTER, T., REISCHAUER, B., ROSENBERG, R., VERHOEF, C., MISCHINGER, H. J., RIEGMAN, P., ZATLOUKAL, K. & BECKER, K. F. 2012. Variability of protein and phosphoprotein levels in clinical tissue specimens during the preanalytical phase. *J Proteome Res,* 11**,** 5748-62.

HALCOMB, E., DAVIDSON, P. & HARDAKER, L. 2008. Using the consensus development conference method in healthcare research. *Nurse Res,* 16**,** 56-71.

HALDORSEN, I. S. & SALVESEN, H. B. 2016. What Is the Best Preoperative Imaging for Endometrial Cancer? *Curr Oncol Rep,* 18**,** 25.

HAMMOND, M. E., HAYES, D. F., DOWSETT, M., ALLRED, D. C., HAGERTY, K. L., BADVE, S., FITZGIBBONS, P. L., FRANCIS, G., GOLDSTEIN, N. S., HAYES, M., HICKS, D. G., LESTER, S., LOVE, R., MANGU, P. B., MCSHANE, L., MILLER, K., OSBORNE, C. K., PAIK, S., PERLMUTTER, J., RHODES, A., SASANO, H., SCHWARTZ, J. N., SWEEP, F. C., TAUBE, S., TORLAKOVIC, E. E., VALENSTEIN, P., VIALE, G., VISSCHER, D., WHEELER, T., WILLIAMS, R. B., WITTLIFF, J. L. & WOLFF, A. C. 2010. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol,* 28**,** 2784-95.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HAOULA, Z., SALMAN, M. & ATIOMO 2012. Evaluating the association between endometrial cancer and polycystic ovary syndrome. *Human reproduction (Oxford, England),* 27.

HAPANGAMA, D. K. & BULMER, J. N. 2016. Pathophysiology of heavy menstrual bleeding. *Womens Health (Lond),* 12**,** 3-13.

HAPANGAMA, D. K., DRURY, J., DA SILVA, L., AL-LAMEE, H., EARP, A., VALENTIJN, A. J., EDIRISINGHE, D. P., MURRAY, P. A., FAZLEABAS, A. T. & GARGETT, C. E. 2019. Abnormally located SSEA1+/SOX9+ endometrial epithelial cells with a basalis-like phenotype in the eutopic functionalis layer may play a role in the pathogenesis of endometriosis. *Hum Reprod,* 34**,** 56-68.

HAPANGAMA, D. K., KAMAL, A. & SARETZKI, G. 2017. Implications of telomeres and telomerase in endometrial pathology. *Hum Reprod Update,* 23**,** 166-187.

HAPANGAMA, D. K., KAMAL, A. M. & BULMER, J. N. 2015. Estrogen receptor beta: the guardian of the endometrium. *Hum Reprod Update,* 21**,** 174-93.

HAPANGAMA, D. K., RAJU, R. S., VALENTIJN, A. J., BARRACLOUGH, D., HART, A., TURNER, M. A., PLATT-HIGGINS, A., BARRACLOUGH, R. & RUDLAND, P. S. 2012. Aberrant expression of metastasis-inducing proteins in ectopic and matched eutopic endometrium of women with endometriosis: implications for the pathogenesis of endometriosis. *Hum Reprod,* 27**,** 394-407.

HAPANGAMA, D. K., TURNER, M. A., DRURY, J., HEATHCOTE, L., AFSHAR, Y., MAVROGIANIS, P. A. & FAZLEABAS, A. T. 2010. Aberrant expression of regulators of cell-fate found in eutopic endometrium is found in matched ectopic endometrium among women and in a baboon model of endometriosis. *Hum Reprod,* 25**,** 2840-50.

HAPANGAMA, D. K., TURNER, M. A., DRURY, J. A., MARTIN-RUIZ, C., VON ZGLINICKI, T., FARQUHARSON, R. G. & QUENBY, S. 2008a. Endometrial telomerase shows specific

expression patterns in different types of reproductive failure. *Reprod Biomed Online,* 17**,** 416-24.
HAPANGAMA, D. K., TURNER, M. A., DRURY, J. A., QUENBY, S., HART, A., MADDICK, M., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2009. Sustained replication in endometrium of women with endometriosis occurs without evoking a DNA damage response. *Hum Reprod,* 24**,** 687-96.
HAPANGAMA, D. K., TURNER, M. A., DRURY, J. A., QUENBY, S., SARETZKI, G., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2008b. Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length. *Hum Reprod,* 23**,** 1511-9.
HARING, S. J., MASON, A. C., BINZ, S. K. & WOLD, M. S. 2008. Cellular functions of human RPA1. Multiple roles of domains in replication, repair, and checkpoints. *J Biol Chem,* 283**,** 19095-111.
HARRINGTON, L., MCPHAIL, T., MAR, V., ZHOU, W., OULTON, R., BASS, M. B., ARRUDA, I. & ROBINSON, M. O. 1997. A mammalian telomerase-associated protein. *Science,* 275**,** 973-7.
HASHIMOTO, M., KYO, S., MASUTOMI, K., MAIDA, Y., SAKAGUCHI, J., MIZUMOTO, Y., NAKAMURA, M., HAHN, W. C. & INOUE, M. 2005. Analysis of telomeric single-strand overhang length in human endometrial cancers. *FEBS Lett,* 579**,** 2959-64.
HAUSKNECHT, LOPEZ DE LA OSA, E. & GURPIDE 1982. In vitro metabolism of C19 steroids in human endometrium. *Journal of steroid biochemistry,* 17.
HAVRILESKY, L. J., MAXWELL, G. L. & MYERS, E. R. 2009. Cost-effectiveness analysis of annual screening strategies for endometrial cancer. *Am J Obstet Gynecol,* 200**,** 640 e1-8.
HEAPHY, C. M., DE WILDE, R. F., JIAO, Y., KLEIN, A. P., EDIL, B. H., SHI, C., BETTEGOWDA, C., RODRIGUEZ, F. J., EBERHART, C. G., HEBBAR, S., OFFERHAUS, G. J., MCLENDON, R., RASHEED, B. A., HE, Y., YAN, H., BIGNER, D. D., OBA-SHINJO, S. M., MARIE, S. K., RIGGINS, G. J., KINZLER, K. W., VOGELSTEIN, B., HRUBAN, R. H., MAITRA, A., PAPADOPOULOS, N. & MEEKER, A. K. 2011. Altered telomeres in tumors with ATRX and DAXX mutations. *Science,* 333**,** 425.
HEIDENREICH, B., RACHAKONDA, P. S., HEMMINKI, K. & KUMAR, R. 2014. TERT promoter mutations in cancer development. *Curr Opin Genet Dev,* 24**,** 30-7.
HENDERSON, T. A., SAUNDERS, P. T., MOFFETT-KING, A., GROOME, N. P. & CRITCHLEY, H. O. 2003. Steroid receptor expression in uterine natural killer cells. *J Clin Endocrinol Metab,* 88**,** 440-9.
HENG, J., ZHANG, F., GUO, X., TANG, L., PENG, L., LUO, X., XU, X., WANG, S., DAI, L. & WANG, J. 2017. Integrated analysis of promoter methylation and expression of telomere related genes in breast cancer. *Oncotarget,* 8**,** 25442-25454.
HENRIQUEZ, S., KOHEN, P., MUNOZ, A., GODOY, A., ORGE, F., STRAUSS, J. F., 3RD & DEVOTO, L. 2017. In-vitro study of gonadotrophin signaling pathways in human granulosa cells in relation to progesterone receptor expression. *Reprod Biomed Online,* 35**,** 363-371.
HENSON, J. D., CAO, Y., HUSCHTSCHA, L. I., CHANG, A. C., AU, A. Y., PICKETT, H. A. & REDDEL, R. R. 2009. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. *Nat Biotechnol,* 27**,** 1181-5.
HENSON, J. D., HANNAY, J. A., MCCARTHY, S. W., ROYDS, J. A., YEAGER, T. R., ROBINSON, R. A., WHARTON, S. B., JELLINEK, D. A., ARBUCKLE, S. M., YOO, J., ROBINSON, B. G., LEAROYD, D. L., STALLEY, P. D., BONAR, S. F., YU, D., POLLOCK, R. E. & REDDEL, R. R. 2005. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. *Clin Cancer Res,* 11**,** 217-25.
HER, J. & CHUNG, I. K. 2012. The AAA-ATPase NVL2 is a telomerase component essential for holoenzyme assembly. *Biochemical and Biophysical Research Communications,* 417**,** 1086-1092.
HERAVI-MOUSSAVI, A., ANGLESIO, M. S., CHENG, S. W., SENZ, J., YANG, W., PRENTICE, L., FEJES, A. P., CHOW, C., TONE, A., KALLOGER, S. E., HAMEL, N., ROTH, A., HA, G., WAN, A. N., MAINES-BANDIERA, S., SALAMANCA, C., PASINI, B., CLARKE, B. A., LEE, A. F., LEE, C. H., ZHAO, C., YOUNG, R. H., APARICIO, S. A., SORENSEN, P. H., WOO, M. M., BOYD, N., JONES, S. J., HIRST, M., MARRA, M. A., GILKS, B., SHAH, S. P., FOULKES, W. D., MORIN, G. B. & HUNTSMAN, D. G.

2012. Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N Engl J Med,* 366**,** 234-42.
HEWITT, R. & HAINAUT, P. 2011. Biobanking in a Fast Moving World: An International Perspective. *JNCI Monographs,* 2011**,** 50-51.
HEWITT, S. C. & KORACH, K. S. 2003. Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction,* 125**,** 143-9.
HIYAMA, E., GOLLAHON, L., KATAOKA, T., KUROI, K., YOKOYAMA, T., GAZDAR, A. F., HIYAMA, K., PIATYSZEK, M. A. & SHAY, J. W. 1996. Telomerase activity in human breast tumors. *J Natl Cancer Inst,* 88**,** 116-22.
HIYAMA, E. & HIYAMA, K. 2004. Telomerase detection in the diagnosis and prognosis of cancer. *Cytotechnology,* 45**,** 61-74.
HOJAT, A., WEI, B., OLSON, M. G., MAO, Q. & YONG, W. H. 2019. Procurement and Storage of Surgical Biospecimens. *Methods Mol Biol,* 1897**,** 65-76.
HOLST, J. P., SOLDIN, O. P., GUO, T. & SOLDIN, S. J. 2004. Steroid hormones: relevance and measurement in the clinical laboratory. *Clin Lab Med,* 24**,** 105-18.
HOUSHDARAN, S., ZELENKO, Z., IRWIN, J. C. & GIUDICE, L. C. 2014. Human endometrial DNA methylome is cycle-dependent and is associated with gene expression regulation. *Molecular endocrinology (Baltimore, Md.),* 28.
HRDLIČKOVÁ, R., NEHYBA, J. & BOSE, H. R. 2012. Alternatively Spliced Telomerase Reverse Transcriptase Variants Lacking Telomerase Activity Stimulate Cell Proliferation. *Molecular and Cellular Biology,* 32**,** 4283-4296.
HUANG, G. S., GEBB, J. S., EINSTEIN, M. H., SHAHABI, S., NOVETSKY, A. P. & GOLDBERG, G. L. 2007. Accuracy of preoperative endometrial sampling for the detection of high-grade endometrial tumors. *Am J Obstet Gynecol,* 196**,** 243.e1-5.
HUANG, J., QI, R., QUACKENBUSH, J., DAUWAY, E., LAZARIDIS, E. & YEATMAN, T. 2001. Effects of ischemia on gene expression. *J Surg Res,* 99**,** 222-7.
HUBER, O., MÉNARD, L., HAURIE, V., NICOU, A., TARAS, D. & ROSENBAUM, J. 2008. Pontin and reptin, two related ATPases with multiple roles in cancer. *Cancer Res,* 68**,** 6873-6.
HUHTINEN, SALONIEMI-HEINONEN, T., KESKI-RAHKONEN, P., DESAI, R., LAAJALA, D., STÅHLE, M., HÄKKINEN, M. R., AWOSANYA, M., SUVITIE, P., KUJARI, H., AITTOKALLIO, T., HANDELSMAN, D. J., AURIOLA, S., PERHEENTUPA, A. & POUTANEN 2014. Intra-tissue steroid profiling indicates differential progesterone and testosterone metabolism in the endometrium and endometriosis lesions. *The Journal of clinical endocrinology and metabolism,* 99.
HUTT, S., TAILOR, A., ELLIS, P., MICHAEL, A., BUTLER-MANUEL, S. & CHATTERJEE, J. 2019. The role of biomarkers in endometrial cancer and hyperplasia: a literature review. *Acta Oncol,* 58**,** 342-352.
IARC. *EPIC Study: https://epic.iarc.fr/about/biologicalbank.php* [Online]. Available: https://epic.iarc.fr/about/biologicalbank.php [Accessed 20 th July 2022].
IGLESIAS, N., REDON, S., PFEIFFER, V., DEES, M., LINGNER, J. & LUKE, B. 2011. Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast. *EMBO Rep,* 12**,** 587-93.
IQBAL, J., GINSBURG, O. M., WIJERATNE, T. D., HOWELL, A., EVANS, G., SESTAK, I. & NAROD, S. A. 2012. Endometrial cancer and venous thromboembolism in women under age 50 who take tamoxifen for prevention of breast cancer: a systematic review. *Cancer Treat Rev,* 38**,** 318-28.
ISHIHARA, H., KITAWAKI, J., KADO, N., KOSHIBA, H., FUSHIKI, S. & HONJO, H. 2003. Gonadotropin-releasing hormone agonist and danazol normalize aromatase cytochrome P450 expression in eutopic endometrium from women with endometriosis, adenomyosis, or leiomyomas. *Fertil Steril,* 79 Suppl 1**,** 735-42.
IYER, R., GENTRY-MAHARAJ, A., NORDIN, A., BURNELL, M., LISTON, R., MANCHANDA, R., DAS, N., DESAI, R., GORNALL, R., BEARDMORE-GRAY, A., NEVIN, J., HILLABY, K., LEESON, S., LINDER,

A., LOPES, A., MEECHAN, D., MOULD, T., VARKEY, S., OLAITAN, A., RUFFORD, B., RYAN, A., SHANBHAG, S., THACKERAY, A., WOOD, N., REYNOLDS, K. & MENON, U. 2015. Predictors of complications in gynaecological oncological surgery: a prospective multicentre study (UKGOSOC-UK gynaecological oncology surgical outcomes and complications). *Br J Cancer,* 112**,** 475-84.

JACOB, F., GUERTLER, R., NAIM, S., NIXDORF, S., FEDIER, A., HACKER, N. F. & HEINZELMANN, S. 2013. Careful selection of reference genes is required for reliable performance of RT-qPCR in human normal and cancer cell lines. *PloS one,* 8.

JACOB, N. K., LESCASSE, R., LINGER, B. R. & PRICE, C. M. 2007. Tetrahymena POT1a regulates telomere length and prevents activation of a cell cycle checkpoint. *Molecular and cellular biology,* 27**,** 1592-1601.

JACOBSEN, B. M. & HORWITZ, K. B. 2012. Progesterone receptors, their isoforms and progesterone regulated transcription. *Molecular and cellular endocrinology,* 357.

JAGASIA, M. H., GREINIX, H. T., ARORA, M., WILLIAMS, K. M., WOLFF, D., COWEN, E. W., PALMER, J., WEISDORF, D., TREISTER, N. S., CHENG, G. S., KERR, H., STRATTON, P., DUARTE, R. F., MCDONALD, G. B., INAMOTO, Y., VIGORITO, A., ARAI, S., DATILES, M. B., JACOBSOHN, D., HELLER, T., KITKO, C. L., MITCHELL, S. A., MARTIN, P. J., SHULMAN, H., WU, R. S., CUTLER, C. S., VOGELSANG, G. B., LEE, S. J., PAVLETIC, S. Z. & FLOWERS, M. E. 2015. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant,* 21**,** 389-401.e1.

JAMESON, J. L. & LONGO, D. L. 2015. Precision medicine--personalized, problematic, and promising. *N Engl J Med,* 372**,** 2229-34.

JANDA, M., GEBSKI, V., DAVIES, L. C., FORDER, P., BRAND, A., HOGG, R., JOBLING, T. W., LAND, R., MANOLITSAS, T., NASCIMENTO, M., NEESHAM, D., NICKLIN, J. L., OEHLER, M. K., OTTON, G., PERRIN, L., SALFINGER, S., HAMMOND, I., LEUNG, Y., SYKES, P., NGAN, H., GARRETT, A., LANEY, M., NG, T. Y., TAM, K., CHAN, K., WREDE, C. D., PATHER, S., SIMCOCK, B., FARRELL, R., ROBERTSON, G., WALKER, G., ARMFIELD, N. R., GRAVES, N., MCCARTNEY, A. J. & OBERMAIR, A. 2017. Effect of Total Laparoscopic Hysterectomy vs Total Abdominal Hysterectomy on Disease-Free Survival Among Women With Stage I Endometrial Cancer: A Randomized Clinical Trial. *Jama,* 317**,** 1224-1233.

JEANMONOD, D. K. T., HAJIRA, B. & REBECCA 2021. *Physiology, Menstrual Cycle*, StatPearls Publishing.

JEWELL, S. D., SRINIVASAN, M., MCCART, L. M., WILLIAMS, N., GRIZZLE, W. H., LIVOLSI, V., MACLENNAN, G. & SEDMAK, D. D. 2002. Analysis of the molecular quality of human tissues: an experience from the Cooperative Human Tissue Network. *Am J Clin Pathol,* 118**,** 733-41.

JIANG, J., WEI, L. H., LI, Y. L., WU, R. F., XIE, X., FENG, Y. J., ZHANG, G., ZHAO, C., ZHAO, Y. & CHEN, Z. 2010. Detection of TERC amplification in cervical epithelial cells for the diagnosis of high-grade cervical lesions and invasive cancer: a multicenter study in China. *J Mol Diagn,* 12**,** 808-17.

JIANG, W. Q., ZHONG, Z. H., HENSON, J. D., NEUMANN, A. A., CHANG, A. C. & REDDEL, R. R. 2005. Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. *Mol Cell Biol,* 25**,** 2708-21.

JONES, J. & HUNTER, D. 1995. Consensus methods for medical and health services research. *Bmj,* 311**,** 376-80.

JUHL, H. 2010. Preanalytical aspects: a neglected issue. *Scand J Clin Lab Invest Suppl,* 242**,** 63-5.

KAHN, K., RYAN, G., BECKETT, M., TAYLOR, S., BERREBI, C., CHO, M., QUITER, E., FREMONT, A. & PINCUS, H. 2011. Bridging the gap between basic science and clinical practice: a role for community clinicians. *Implementation Science,* 6**,** 34.

KALUZ, S., KALUZOVÁ, M., LIAO, S. Y., LERMAN, M. & STANBRIDGE, E. 2009. Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? *Biochimica et biophysica acta,* 1795.

KAMAL, A., TEMPEST, N., PARKES, C., ALNAFAKH, R., MAKRYDIMA, S., ADISHESH, M. & HAPANGAMA, D. K. 2016a. Hormones and endometrial carcinogenesis. *Horm Mol Biol Clin Investig,* 25**,** 129-48.

KAMAL, A. M., BULMER, J. N., DECRUZE, S. B., STRINGFELLOW, H. F., MARTIN-HIRSCH, P. & HAPANGAMA, D. K. 2016b. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. *Br J Cancer,* 114**,** 688-96.

KANDOTH, C., SCHULTZ, N., CHERNIACK, A. D., AKBANI, R., LIU, Y., SHEN, H., ROBERTSON, A. G., PASHTAN, I., SHEN, R., BENZ, C. C., YAU, C., LAIRD, P. W., DING, L., ZHANG, W., MILLS, G. B., KUCHERLAPATI, R., MARDIS, E. R. & LEVINE, D. A. 2013. Integrated genomic characterization of endometrial carcinoma. *Nature,* 497**,** 67-73.

KAWASHIMA, M., KOJIMA, M., UEDA, Y., KURIHARA, S. & HIYAMA, E. 2016. Telomere biology including TERT rearrangements in neuroblastoma: a useful indicator for surgical treatments. *J Pediatr Surg,* 51**,** 2080-2085.

KEDDE, M., LE SAGE, C., DUURSMA, A., ZLOTORYNSKI, E., VAN LEEUWEN, B., NIJKAMP, W., BEIJERSBERGEN, R. & AGAMI, R. 2006. Telomerase-independent regulation of ATR by human telomerase RNA. *J Biol Chem,* 281**,** 40503-14.

KELLEHER, C., KURTH, I. & LINGNER, J. 2005. Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol Cell Biol,* 25**,** 808-18.

KIM, C. M., OH, Y. J., CHO, S. H., CHUNG, D. J., HWANG, J. Y., PARK, K. H., CHO, D. J., CHOI, Y. M. & LEE, B. S. 2007. Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis. *Hum Reprod,* 22**,** 843-9.

KIM, H., YOO, J. E., CHO, J. Y., OH, B. K., YOON, Y. S., HAN, H. S., LEE, H. S., JANG, J. J., JEONG, S. H., KIM, J. W. & PARK, Y. N. 2013. Telomere length, TERT and shelterin complex proteins in hepatocellular carcinomas expressing "stemness"-related markers. *J Hepatol,* 59**,** 746-52.

KIM, J. J. & CHAPMAN-DAVIS, E. 2010. Role of progesterone in endometrial cancer. *Semin Reprod Med,* 28**,** 81-90.

KIM, N. W., PIATYSZEK, M. A., PROWSE, K. R., HARLEY, C. B., WEST, M. D., HO, P. L., COVIELLO, G. M., WRIGHT, W. E., WEINRICH, S. L. & SHAY, J. W. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science,* 266**,** 2011-5.

KIM, S., PARK, C., JI, Y., KIM, D. G., BAE, H., VAN VRANCKEN, M., KIM, D. H. & KIM, K. M. 2017. Deamination Effects in Formalin-Fixed, Paraffin-Embedded Tissue Samples in the Era of Precision Medicine. *J Mol Diagn,* 19**,** 137-146.

KIMURA, A., OHMICHI, M., KAWAGOE, J., KYO, S., MABUCHI, S., TAKAHASHI, T., OHSHIMA, C., ARIMOTO-ISHIDA, E., NISHIO, Y., INOUE, M., KURACHI, H., TASAKA, K. & MURATA, Y. 2004. Induction of hTERT expression and phosphorylation by estrogen via Akt cascade in human ovarian cancer cell lines. *Oncogene,* 23**,** 4505-15.

KISS, T., FAYET-LEBARON, E. & JÁDY, B. E. 2010. Box H/ACA small ribonucleoproteins. *Mol Cell,* 37**,** 597-606.

KITCHENER, H., SWART, A. M., QIAN, Q., AMOS, C. & PARMAR, M. K. 2009. Efficacy of systematic pelvic lymphadenectomy in endometrial cancer (MRC ASTEC trial): a randomised study. *Lancet,* 373**,** 125-36.

KITSON, S., SIVALINGAM, V. N., BOLTON, J., MCVEY, R., NICKKHO-AMIRY, M., POWELL, M. E., LEARY, A., NIJMAN, H. W., NOUT, R. A., BOSSE, T., RENEHAN, A. G., KITCHENER, H. C., EDMONDSON, R. J. & CROSBIE, E. J. 2017. Ki-67 in endometrial cancer: scoring optimization and prognostic relevance for window studies. *Mod Pathol,* 30**,** 459-468.

KITSON, S. J., MASKELL, Z., SIVALINGAM, V. N., SHAW, J. & CROSBIE, E. J. 2019. Optimization of Window Study Endpoints in Endometrial Cancer. *Front Oncol,* 9**,** 428.

KNOPPERS, B. M., ZAWATI, M. H. & KIRBY, E. S. 2012. Sampling populations of humans across the world: ELSI issues. *Annu Rev Genomics Hum Genet,* 13**,** 395-413.

KOBAYASHI, M., NAKANO, R. & OOSHIMA, A. 1990. Immunohistochemical localization of pituitary gonadotrophins and gonadal steroids confirms the 'two-cell, two-gonadotrophin' hypothesis of steroidogenesis in the human ovary. *J Endocrinol,* 126**,** 483-8.

KOKKA, F., BROCKBANK, E., ORAM, D., GALLAGHER, C. & BRYANT, A. 2010. Hormonal therapy in advanced or recurrent endometrial cancer. *Cochrane Database Syst Rev***,** Cd007926.

KONG, A., JOHNSON, N., KITCHENER, H. C. & LAWRIE, T. A. 2012. Adjuvant radiotherapy for stage I endometrial cancer. *Cochrane Database Syst Rev,* 2012**,** Cd003916.

KOTE-JARAI, Z., OLAMA, A. A., GILES, G. G., SEVERI, G., SCHLEUTKER, J., WEISCHER, M., CAMPA, D., RIBOLI, E., KEY, T., GRONBERG, H., HUNTER, D. J., KRAFT, P., THUN, M. J., INGLES, S., CHANOCK, S., ALBANES, D., HAYES, R. B., NEAL, D. E., HAMDY, F. C., DONOVAN, J. L., PHAROAH, P., SCHUMACHER, F., HENDERSON, B. E., STANFORD, J. L., OSTRANDER, E. A., SORENSEN, K. D., DORK, T., ANDRIOLE, G., DICKINSON, J. L., CYBULSKI, C., LUBINSKI, J., SPURDLE, A., CLEMENTS, J. A., CHAMBERS, S., AITKEN, J., GARDINER, R. A., THIBODEAU, S. N., SCHAID, D., JOHN, E. M., MAIER, C., VOGEL, W., COONEY, K. A., PARK, J. Y., CANNON-ALBRIGHT, L., BRENNER, H., HABUCHI, T., ZHANG, H. W., LU, Y. J., KANEVA, R., MUIR, K., BENLLOCH, S., LEONGAMORNLERT, D. A., SAUNDERS, E. J., TYMRAKIEWICZ, M., MAHMUD, N., GUY, M., O'BRIEN, L. T., WILKINSON, R. A., HALL, A. L., SAWYER, E. J., DADAEV, T., MORRISON, J., DEARNALEY, D. P., HORWICH, A., HUDDART, R. A., KHOO, V. S., PARKER, C. C., VAN AS, N., WOODHOUSE, C. J., THOMPSON, A., CHRISTMAS, T., OGDEN, C., COOPER, C. S., LOPHATONANON, A., SOUTHEY, M. C., HOPPER, J. L., ENGLISH, D. R., WAHLFORS, T., TAMMELA, T. L., KLARSKOV, P., NORDESTGAARD, B. G., RODER, M. A., TYBJAERG-HANSEN, A., BOJESEN, S. E., TRAVIS, R., CANZIAN, F., KAAKS, R., WIKLUND, F., ALY, M., LINDSTROM, S., DIVER, W. R., GAPSTUR, S., STERN, M. C., CORRAL, R., VIRTAMO, J., COX, A., HAIMAN, C. A., LE MARCHAND, L., FITZGERALD, L., KOLB, S., et al. 2011. Seven prostate cancer susceptibility loci identified by a multi-stage genome-wide association study. *Nat Genet,* 43**,** 785-91.

KUILMAN, T., MICHALOGLOU, C., MOOI, W. J. & PEEPER, D. S. 2010. The essence of senescence. *Genes Dev,* 24**,** 2463-79.

KUIMOV, A. N. 2004. Polypeptide components of telomere nucleoprotein complex. *Biochemistry (Mosc),* 69**,** 117-29.

KYO, S., KANAYA, T., ISHIKAWA, H., UENO, H. & INOUE, M. 1996. Telomerase activity in gynecological tumors. *Clin Cancer Res,* 2**,** 2023-8.

KYO, S., KANAYA, T., TAKAKURA, M., TANAKA, M. & INOUE, M. 1999a. Human telomerase reverse transcriptase as a critical determinant of telomerase activity in normal and malignant endometrial tissues. *Int J Cancer,* 80**,** 60-3.

KYO, S., TAKAKURA, M., KANAYA, T., ZHUO, W., FUJIMOTO, K., NISHIO, Y., ORIMO, A. & INOUE, M. 1999b. Estrogen activates telomerase. *Cancer Res,* 59**,** 5917-21.

KYO, S., TAKAKURA, M., KOHAMA, T. & INOUE, M. 1997. Telomerase activity in human endometrium. *Cancer Res,* 57**,** 610-4.

LAWLOR, R. T., VERONESE, N., PEA, A., NOTTEGAR, A., SMITH, L., PILATI, C., DEMURTAS, J., FASSAN, M., CHENG, L. & LUCHINI, C. 2019. Alternative lengthening of telomeres (ALT) influences survival in soft tissue sarcomas: a systematic review with meta-analysis. *BMC Cancer,* 19**,** 232.

LEAO, R., APOLONIO, J. D., LEE, D., FIGUEIREDO, A., TABORI, U. & CASTELO-BRANCO, P. 2018. Mechanisms of human telomerase reverse transcriptase (hTERT) regulation: clinical impacts in cancer. *J Biomed Sci,* 25**,** 22.

LEE, H., TRAINER, A. H., FRIEDMAN, L. S., THISTLETHWAITE, F. C., EVANS, M. J., PONDER, B. A. & VENKITARAMAN, A. R. 1999. Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, Brca2. *Mol Cell,* 4**,** 1-10.

LEE, H. J. & CHANG, C. 2003. Recent advances in androgen receptor action. *Cell Mol Life Sci,* 60**,** 1613-22.

LEE, J. E., KIM, S. Y. & SHIN, S. Y. 2015a. Effect of Repeated Freezing and Thawing on Biomarker Stability in Plasma and Serum Samples. *Osong Public Health Res Perspect,* 6**,** 357-62.

LEE, J. J., LEE, J. & LEE, H. 2021. Alternative paths to telomere elongation. *Semin Cell Dev Biol,* 113**,** 88-96.

LEE, S. M., SCHELCHER, C., THASLER, R., SCHIERGENS, T. S. & THASLER, W. E. 2015b. Pre-Analytical Determination of the Effect of Extended Warm or Cold Ischaemia on RNA Stability in the Human Ileum Mucosa. *PLoS One,* 10**,** e0138214.

LEES-MILLER, S. P. & MEEK, K. 2003. Repair of DNA double strand breaks by non-homologous end joining. *Biochimie,* 85**,** 1161-73.

LEHMANN, S., GUADAGNI, F., MOORE, H., ASHTON, G., BARNES, M., BENSON, E., CLEMENTS, J., KOPPANDI, I., COPPOLA, D., DEMIROGLU, S. Y., DESOUZA, Y., DE WILDE, A., DUKER, J., ELIASON, J., GLAZER, B., HARDING, K., JEON, J. P., KESSLER, J., KOKKAT, T., NANNI, U., SHEA, K., SKUBITZ, A., SOMIARI, S., TYBRING, G., GUNTER, E. & BETSOU, F. 2012. Standard preanalytical coding for biospecimens: review and implementation of the Sample PREanalytical Code (SPREC). *Biopreserv Biobank,* 10**,** 366-74.

LEHNER, R., ENOMOTO, T., MCGREGOR, J. A., SHROYER, A. L., HAUGEN, B. R., PUGAZHENTHI, U. & SHROYER, K. R. 2002. Quantitative analysis of telomerase hTERT mRNA and telomerase activity in endometrioid adenocarcinoma and in normal endometrium. *Gynecol Oncol,* 84**,** 120-5.

LEI, M., ZAUG, A. J., PODELL, E. R. & CECH, T. R. 2005. Switching human telomerase on and off with hPOT1 protein in vitro. *J Biol Chem,* 280**,** 20449-56.

LENNON, F. E., MIRZAPOIAZOVA, T., MAMBETSARIEV, B., SALGIA, R., MOSS, J. & SINGLETON, P. A. 2012. Overexpression of the μ-opioid receptor in human non-small cell lung cancer promotes Akt and mTOR activation, tumor growth, and metastasis. *Anesthesiology,* 116**,** 857-67.

LESSEY, B. A. & KIM, J. J. 2017. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. *Fertil Steril,* 108**,** 19-27.

LEVINE, D. A. 2013. Integrated genomic characterization of endometrial carcinoma. *Nature,* 497**,** 67-73.

LEWIS, K. A. & TOLLEFSBOL, T. O. 2016. Regulation of the Telomerase Reverse Transcriptase Subunit through Epigenetic Mechanisms. *Front Genet,* 7**,** 83.

LI M, Z. L., QI W, SHEN D, LI X, WANG J, WEI L 2013. Clinical implications and prognostic value of five biomarkers in endometrial carcinoma. *The Chinese-German Journal of Clinical Oncology,* 12**,** 586-591.

LI, Q., KANNAN, A., DEMAYO, F. J., LYDON, J. P., COOKE, P. S., YAMAGISHI, H., SRIVASTAVA, D., BAGCHI, M. K. & BAGCHI, I. C. 2011. The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science (New York, N.y.),* 331**,** 912-916.

LI, S., CROTHERS, J., HAQQ, C. M. & BLACKBURN, E. H. 2005. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *J Biol Chem,* 280**,** 23709-17.

LIN, D. W., COLEMAN, I. M., HAWLEY, S., HUANG, C. Y., DUMPIT, R., GIFFORD, D., KEZELE, P., HUNG, H., KNUDSEN, B. S., KRISTAL, A. R. & NELSON, P. S. 2006. Influence of surgical manipulation on prostate gene expression: implications for molecular correlates of treatment effects and disease prognosis. *J Clin Oncol,* 24**,** 3763-70.

LIN, T. T., LETSOLO, B. T., JONES, R. E., ROWSON, J., PRATT, G., HEWAMANA, S., FEGAN, C., PEPPER, C. & BAIRD, D. M. 2010. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. *Blood,* 116**,** 1899-907.

LINDEMANN, ESKILD, A., VATTEN, L. J. & BRAY 2010. Endometrial cancer incidence trends in Norway during 1953-2007 and predictions for 2008-2027. *International journal of cancer,* 127.

LINGNER, J., HUGHES, T. R., SHEVCHENKO, A., MANN, M., LUNDBLAD, V. & CECH, T. R. 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science,* 276**,** 561-7.

LIPPI, G., BOWEN, R. & ADCOCK, D. M. 2016. Re-engineering laboratory diagnostics for preventing preanalytical errors. *Clin Biochem,* 49**,** 1313-1314.

LIPPI, G., CHANCE, J. J., CHURCH, S., DAZZI, P., FONTANA, R., GIAVARINA, D., GRANKVIST, K., HUISMAN, W., KOURI, T., PALICKA, V., PLEBANI, M., PURO, V., SALVAGNO, G. L., SANDBERG, S., SIKARIS, K., WATSON, I., STANKOVIC, A. K. & SIMUNDIC, A. M. 2011. Preanalytical quality improvement: from dream to reality. *Clin Chem Lab Med,* 49**,** 1113-26.

LIPPI, G., GUIDI, G. C., MATTIUZZI, C. & PLEBANI, M. 2006. Preanalytical variability: the dark side of the moon in laboratory testing. *Clin Chem Lab Med,* 44**,** 358-65.

LIPPI, G., VON MEYER, A., CADAMURO, J. & SIMUNDIC, A. M. 2019. Blood sample quality. *Diagnosis (Berl),* 6**,** 25-31.

LIU, D., O'CONNOR, M. S., QIN, J. & SONGYANG, Z. 2004. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem,* 279**,** 51338-42.

LIU, F., PU, X. Y., HUANG, S. G., XIANG, G. M., JIANG, D. N., HOU, G. & HUANG, D. N. 2012a. Expression of hPOT1 in HeLa cells and the probability of gene variation of hpot1 Exon14 in endometrial cancer are much higher than in other cancers. *Asian Pac J Cancer Prev,* 13**,** 5659-63.

LIU, H., LIU, S., WANG, H., XIE, X., CHEN, X., ZHANG, X. & ZHANG, Y. 2012b. Genomic amplification of the human telomerase gene (hTERC) associated with human papillomavirus is related to the progression of uterine cervical dysplasia to invasive cancer. *Diagn Pathol,* 7**,** 147.

LIU, H., YANG, Y., GE, Y., LIU, J. & ZHAO, Y. 2019. TERC promotes cellular inflammatory response independent of telomerase. *Nucleic Acids Res,* 47**,** 8084-8095.

LIU, N. W., SANFORD, T., SRINIVASAN, R., LIU, J. L., KHURANA, K., APRELIKOVA, O., VALERO, V., BECHERT, C., WORRELL, R., PINTO, P. A., YANG, Y., MERINO, M., LINEHAN, W. M. & BRATSLAVSKY, G. 2013. Impact of ischemia and procurement conditions on gene expression in renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 19**,** 42-49.

LÓPEZ DE SILANES, I., STAGNO D'ALCONTRES, M. & BLASCO, M. A. 2010. TERRA transcripts are bound by a complex array of RNA-binding proteins. *Nat Commun,* 1**,** 33.

LORTET-TIEULENT, J., FERLAY, J., BRAY, F. & JEMAL, A. 2018. International Patterns and Trends in Endometrial Cancer Incidence, 1978-2013. *J Natl Cancer Inst,* 110**,** 354-361.

LOVEJOY, C. A., LI, W., REISENWEBER, S., THONGTHIP, S., BRUNO, J., DE LANGE, T., DE, S., PETRINI, J. H., SUNG, P. A., JASIN, M., ROSENBLUH, J., ZWANG, Y., WEIR, B. A., HATTON, C., IVANOVA, E., MACCONAILL, L., HANNA, M., HAHN, W. C., LUE, N. F., REDDEL, R. R., JIAO, Y., KINZLER, K., VOGELSTEIN, B., PAPADOPOULOS, N. & MEEKER, A. K. 2012. Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. *PLoS Genet,* 8**,** e1002772.

LUCIANO, P., COULON, S., FAURE, V., CORDA, Y., BOS, J., BRILL, S. J., GILSON, E., SIMON, M.-N. & GÉLI, V. 2012. RPA facilitates telomerase activity at chromosome ends in budding and fission yeasts. *The EMBO Journal,* 31**,** 2034-2046.

LUKE, B., PANZA, A., REDON, S., IGLESIAS, N., LI, Z. & LINGNER, J. 2008. The Rat1p 5' to 3' exonuclease degrades telomeric repeat-containing RNA and promotes telomere elongation in Saccharomyces cerevisiae. *Mol Cell,* 32**,** 465-77.

MA, B. B., OZA, A., EISENHAUER, E., STANIMIR, G., CAREY, M., CHAPMAN, W., LATTA, E., SIDHU, K., POWERS, J., WALSH, W. & FYLES, A. 2004. The activity of letrozole in patients with advanced

or recurrent endometrial cancer and correlation with biological markers--a study of the National Cancer Institute of Canada Clinical Trials Group. *Int J Gynecol Cancer,* 14**,** 650-8.
MA, Y., DAI, H. & KONG, X. 2012. Impact of warm ischemia on gene expression analysis in surgically removed biosamples. *Anal Biochem,* 423**,** 229-35.
MACLEAN, A., ADISHESH, M., BUTTON, L., RICHARDS, L., ALNAFAKH, R., NEWTON, E., DRURY, J. & HAPANGAMA, D. K. 2022. The effect of pre-analytical variables on downstream application and data analysis of human endometrial biopsies. *Hum Reprod Open,* 2022**,** hoac026.
MACLEAN, A., KAMAL, A., ADISHESH, M., ALNAFAKH, R., TEMPEST, N. & HAPANGAMA, D. K. 2020. Human Uterine Biopsy: Research Value and Common Pitfalls. *Int J Reprod Med,* 2020**,** 9275360.
MAESTRONI, L., AUDRY, J., MATMATI, S., ARCANGIOLI, B., GELI, V. & COULON, S. 2017. Eroded telomeres are rearranged in quiescent fission yeast cells through duplications of subtelomeric sequences. *Nat Commun,* 8**,** 1684.
MAGER, S. R., OOMEN, M. H., MORENTE, M. M., RATCLIFFE, C., KNOX, K., KERR, D. J., PEZZELLA, F. & RIEGMAN, P. H. 2007. Standard operating procedure for the collection of fresh frozen tissue samples. *Eur J Cancer,* 43**,** 828-34.
MAHANEY, B. L., MEEK, K. & LEES-MILLER, S. P. 2009. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochem J,* 417**,** 639-50.
MAIDA, Y., KYO, S., FORSYTH, N., TAKAKURA, M., SAKAGUCHI, J., MIZUMOTO, Y., HASHIMOTO, M., NAKAMURA, M., NAKAO, S. & INOUE, M. 2006. Distinct telomere length regulation in premalignant cervical and endometrial lesions: implications for the roles of telomeres in uterine carcinogenesis. *The Journal of Pathology,* 210**,** 214-223.
MAIDA, Y., KYO, S., KANAYA, T., WANG, Z., TANAKA, M., YATABE, N., NAKAMURA, M. & INOUE, M. 2002. Is the telomerase assay useful for screening of endometrial lesions? *Int J Cancer,* 100**,** 714-8.
MAIDA, Y., YASUKAWA, M., FURUUCHI, M., LASSMANN, T., POSSEMATO, R., OKAMOTO, N., KASIM, V., HAYASHIZAKI, Y., HAHN, W. C. & MASUTOMI, K. 2009. An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature,* 461**,** 230-5.
MAO, L., EL-NAGGAR, A. K., FAN, Y. H., LEE, J. S., LIPPMAN, S. M., KAYSER, S., LOTAN, R. & HONG, W. K. 1996. Telomerase activity in head and neck squamous cell carcinoma and adjacent tissues. *Cancer Res,* 56**,** 5600-4.
MARABELLE, A., LE, D. T., ASCIERTO, P. A., DI GIACOMO, A. M., DE JESUS-ACOSTA, A., DELORD, J. P., GEVA, R., GOTTFRIED, M., PENEL, N., HANSEN, A. R., PIHA-PAUL, S. A., DOI, T., GAO, B., CHUNG, H. C., LOPEZ-MARTIN, J., BANG, Y. J., FROMMER, R. S., SHAH, M., GHORI, R., JOE, A. K., PRUITT, S. K. & DIAZ, L. A., JR. 2020. Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. *J Clin Oncol,* 38**,** 1-10.
MARION, R. M., MONTERO, J. J., LOPEZ DE SILANES, I., GRANA-CASTRO, O., MARTINEZ, P., SCHOEFTNER, S., PALACIOS-FABREGA, J. A. & BLASCO, M. A. 2019. TERRA regulate the transcriptional landscape of pluripotent cells through TRF1-dependent recruitment of PRC2. *Elife,* 8.
MARSHALL, LOWREY, J., MACPHERSON, S., MAYBIN, J. A., COLLINS, F., CRITCHLEY, H. O. & SAUNDERS 2011. In silico analysis identifies a novel role for androgens in the regulation of human endometrial apoptosis. *The Journal of clinical endocrinology and metabolism,* 96.
MARTÍNEZ, P., GÓMEZ-LÓPEZ, G., GARCÍA, F., MERCKEN, E., MITCHELL, S., FLORES, J. M., DE CABO, R. & BLASCO, M. A. 2013. RAP1 protects from obesity through its extratelomeric role regulating gene expression. *Cell Rep,* 3**,** 2059-74.
MATHEW, D., DRURY, J. A., VALENTIJN, A. J., VASIEVA, O. & HAPANGAMA, D. K. 2016. In silico, in vitro and in vivo analysis identifies a potential role for steroid hormone regulation of FOXD3 in endometriosis-associated genes. *Hum Reprod,* 31**,** 345-54.

MATIAS-GUIU, X. & PRAT, J. 2013. Molecular pathology of endometrial carcinoma. *Histopathology,* 62**,** 111-23.

MAXWELL, G. L., SCHILDKRAUT, J. M., CALINGAERT, B., RISINGER, J. I., DAINTY, L., MARCHBANKS, P. A., BERCHUCK, A., BARRETT, J. C. & RODRIGUEZ, G. C. 2006. Progestin and estrogen potency of combination oral contraceptives and endometrial cancer risk. *Gynecol Oncol,* 103**,** 535-40.

MCMILLAN, S. S., KING, M. & TULLY, M. P. 2016. How to use the nominal group and Delphi techniques. *Int J Clin Pharm,* 38**,** 655-62.

MEEKER, A. K., HICKS, J. L., GABRIELSON, E., STRAUSS, W. M., DE MARZO, A. M. & ARGANI, P. 2004. Telomere Shortening Occurs in Subsets of Normal Breast Epithelium as well as in Situ and Invasive Carcinoma. *Am J Pathol,* 164**,** 925-35.

MEEKER, A. K., HICKS, J. L., PLATZ, E. A., MARCH, G. E., BENNETT, C. J., DELANNOY, M. J. & DE MARZO, A. M. 2002. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res,* 62**,** 6405-9.

MEIR, K., GAFFNEY, E. F., SIMEON-DUBACH, D., RAVID, R., WATSON, P. H., SCHACTER, B., MORENTE & THE MARBLE ARCH INTERNATIONAL WORKING GROUP ON BIOBANKING, M. M. 2011. The human face of biobank networks for translational research. *Biopreserv Biobank,* 9**,** 279-85.

MENDY, M., ELODIE, C., RITA, T. L., JESSICA, W. & CHRISTOPHER, P. W. 2017. Common Minimum Technical Standards and Protocols for Biobanks Dedicated to Cancer Research. International Agency for Research on Cancer.

MERTENS, H. J., HEINEMAN, M. J., THEUNISSEN, P. H., DE JONG, F. H. & EVERS, J. L. 2001. Androgen, estrogen and progesterone receptor expression in the human uterus during the menstrual cycle. *European journal of obstetrics, gynecology, and reproductive biology,* 98.

MEYER, J. 2009. *Pharmacogenomic profiling and clarification of the role of the mismatch repair genes in response to the chemotherapeutic agent 5-Fluorouracil in a South African colorectal cancer cohort.* University of Cape Town.

MILL, J. & HEIJMANS, B. T. 2013. From promises to practical strategies in epigenetic epidemiology. *Nat Rev Genet,* 14**,** 585-94.

MISITI, S., NANNI, S., FONTEMAGGI, G., CONG, Y. S., WEN, J., HIRTE, H. W., PIAGGIO, G., SACCHI, A., PONTECORVI, A., BACCHETTI, S. & FARSETTI, A. 2000. Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells. *Mol Cell Biol,* 20**,** 3764-71.

MOELANS, C. B., OOSTENRIJK, D., MOONS, M. J. & VAN DIEST, P. J. 2011. Formaldehyde substitute fixatives: effects on nucleic acid preservation. *J Clin Pathol,* 64**,** 960-7.

MONIS, C. N. & TETROKALASHVILI, M. 2021. Menstrual Cycle Proliferative And Follicular Phase. *StatPearls.* Treasure Island (FL).

MONTERO, J. J., LÓPEZ DE SILANES, I., GRAÑA, O. & BLASCO, M. A. 2016. Telomeric RNAs are essential to maintain telomeres. *Nat Commun,* 7**,** 12534.

MOORE, H. M. 2012. The NCI Biospecimen Research Network. *Biotechnic & Histochemistry,* 87**,** 18-23.

MOORE, H. M., COMPTON, C. C., LIM, M. D., VAUGHT, J., CHRISTIANSEN, K. N. & ALPER, J. 2009. 2009 Biospecimen research network symposium: advancing cancer research through biospecimen science. *Cancer Res,* 69**,** 6770-2.

MOORE, H. M., KELLY, A., JEWELL, S. D., MCSHANE, L. M., CLARK, D. P., GREENSPAN, R., HAINAUT, P., HAYES, D. F., KIM, P., MANSFIELD, E., POTAPOVA, O., RIEGMAN, P., RUBINSTEIN, Y., SEIJO, E., SOMIARI, S., WATSON, P., WEIER, H. U., ZHU, C. & VAUGHT, J. 2011a. Biospecimen Reporting for Improved Study Quality. *Biopreserv Biobank,* 9**,** 57-70.

MOORE, H. M., KELLY, A. B., JEWELL, S. D., MCSHANE, L. M., CLARK, D. P., GREENSPAN, R., HAYES, D. F., HAINAUT, P., KIM, P., MANSFIELD, E., POTAPOVA, O., RIEGMAN, P., RUBINSTEIN, Y., SEIJO, E., SOMIARI, S., WATSON, P., WEIER, H. U., ZHU, C. & VAUGHT, J. 2011b. Biospecimen reporting for improved study quality (BRISQ). *J Proteome Res,* 10**,** 3429-38.

MOORE, S. C., GIERACH, G. L., SCHATZKIN, A. & MATTHEWS, C. E. 2010. Physical activity, sedentary behaviours, and the prevention of endometrial cancer. *Br J Cancer,* 103**,** 933-8.

MØRCH, L. S., KJAER, S. K., KEIDING, N., LØKKEGAARD, E. & LIDEGAARD, Ø. 2016. The influence of hormone therapies on type I and II endometrial cancer: A nationwide cohort study. *Int J Cancer,* 138**,** 1506-15.

MORENTE, M. M., DE ALAVA, E. & FERNANDEZ, P. L. 2007. Tumour banking: the Spanish design. *Pathobiology,* 74**,** 245-50.

MORENTE, M. M., MAGER, R., ALONSO, S., PEZZELLA, F., SPATZ, A., KNOX, K., KERR, D., DINJENS, W. N., OOSTERHUIS, J. W., LAM, K. H., OOMEN, M. H., VAN DAMME, B., VAN DE VIJVER, M., VAN BOVEN, H., KERJASCHKI, D., PAMMER, J., LOPEZ-GUERRERO, J. A., LLOMBART BOSCH, A., CARBONE, A., GLOGHINI, A., TEODOROVIC, I., ISABELLE, M., PASSIOUKOV, A., LEJEUNE, S., THERASSE, P., VAN VEEN, E. B., RATCLIFFE, C. & RIEGMAN, P. H. 2006. TuBaFrost 2: Standardising tissue collection and quality control procedures for a European virtual frozen tissue bank network. *Eur J Cancer,* 42**,** 2684-91.

MRC. 2019. *Human tissue and biological samples for use in research* [Online]. @UKRI_News. Available: https://www.ukri.org/publications/human-tissue-and-biological-samples-for-use-in-research/ [Accessed 4 th sept 2022].

MUECK, A. O., SEEGER, H. & RABE, T. 2010. Hormonal contraception and risk of endometrial cancer: a systematic review. *Endocr Relat Cancer,* 17**,** R263-71.

MUINELO-ROMAY, L., CASAS-AROZAMENA, C. & ABAL, M. 2018. Liquid Biopsy in Endometrial Cancer: New Opportunities for Personalized Oncology. *Int J Mol Sci,* 19.

MURALI, R., SOSLOW, R. A. & WEIGELT 2014. Classification of endometrial carcinoma: more than two types. *The Lancet. Oncology,* 15.

MURPHY, M. K., BLACK, N. A., LAMPING, D. L., MCKEE, C. M., SANDERSON, C. F., ASKHAM, J. & MARTEAU, T. 1998. Consensus development methods, and their use in clinical guideline development. *Health Technol Assess,* 2**,** i-iv, 1-88.

MUSELLA, V., VERDERIO, P., REID, J. F., PIZZAMIGLIO, S., GARIBOLDI, M., CALLARI, M., MILIONE, M., DE CECCO, L., VENERONI, S., PIEROTTI, M. A. & DAIDONE, M. G. 2013. Effects of warm ischemic time on gene expression profiling in colorectal cancer tissues and normal mucosa. *PLoS One,* 8**,** e53406.

MUZ, B., DE LA PUENTE, P., AZAB, F. & AZAB, A. K. 2015. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl),* 3**,** 83-92.

MYLONAS, I., JESCHKE, U., SHABANI, N., KUHN, C., KUNZE, S., DIAN, D., FRIEDL, C., KUPKA, M. S. & FRIESE, K. 2007. Steroid receptors ERalpha, ERbeta, PR-A and PR-B are differentially expressed in normal and atrophic human endometrium. *Histol Histopathol,* 22**,** 169-76.

NAGATA, T., TAKADA, Y., ONO, A., NAGATA, K., KONISHI, Y., NUKINA, T., ONO, M., MATSUGAMI, A., FURUKAWA, A., FUJIMOTO, N., FUKUDA, H., NAKAGAMA, H. & KATAHIRA, M. 2008. Elucidation of the mode of interaction in the UP1-telomerase RNA-telomeric DNA ternary complex which serves to recruit telomerase to telomeric DNA and to enhance the telomerase activity. *Nucleic Acids Res,* 36**,** 6816-24.

NAIR, N., CAMACHO-VANEGAS, O., RYKUNOV, D., DASHKOFF, M., CAMACHO, S. C., SCHUMACHER, C. A., IRISH, J. C., HARKINS, T. T., FREEMAN, E., GARCIA, I., PEREIRA, E., KENDALL, S., BELFER, R., KALIR, T., SEBRA, R., REVA, B., DOTTINO, P. & MARTIGNETTI, J. A. 2016. Genomic Analysis of Uterine Lavage Fluid Detects Early Endometrial Cancers and Reveals a Prevalent Landscape of Driver Mutations in Women without Histopathologic Evidence of Cancer: A Prospective Cross-Sectional Study. *PLoS Med,* 13**,** e1002206.

NAIR, R., AGGARWAL, R. & KHANNA, D. 2011. Methods of formal consensus in classification/diagnostic criteria and guideline development. *Semin Arthritis Rheum,* 41**,** 95-105.

NARVEKAR, N., CAMERON, S., CRITCHLEY, H. O., LIN, S., CHENG, L. & BAIRD, D. T. 2004. Low-dose mifepristone inhibits endometrial proliferation and up-regulates androgen receptor. *The Journal of clinical endocrinology and metabolism,* 89.

NCI. 2011. *NCI Best Practices for Biospecimen Resources* [Online]. Available: https://biospecimens.cancer.gov/bestpractices/2011-NCIBestPractices.pdf [Accessed 8th June 2022].
NCI. 2016. *NCI Best Practices for Biospecimen Resources* [Online]. Available: https://biospecimens.cancer.gov/bestpractices/2016-NCIBestPractices.pdf [Accessed 8th June 2022].
NCIN. 2013a. *Outline of Uterine Cancer in the United Kingdom: Incidence, Mortality and Survival* [Online]. NCIN. [Accessed 26th June 2021].
NCIN 2013b. Outline of Uterine Cancer in the United Kingdom: Incidence, Mortality and Survival. NCIN.
NCRI 2012. Challenges and opportunities in surgical cancer research in the UK. NCRI October 2012.
NERGADZE, S. G., FARNUNG, B. O., WISCHNEWSKI, H., KHORIAULI, L., VITELLI, V., CHAWLA, R., GIULOTTO, E. & AZZALIN, C. M. 2009. CpG-island promoters drive transcription of human telomeres. *RNA,* 15**,** 2186-94.
NEUBAUER, A., NEUBAUER, B., HE, M., EFFERT, P., IGLEHART, D., FRYE, R. A. & LIU, E. 1992. Analysis of gene amplification in archival tissue by differential polymerase chain reaction. *Oncogene,* 7**,** 1019-25.
NG, L. J., CROPLEY, J. E., PICKETT, H. A., REDDEL, R. R. & SUTER, C. M. 2009. Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. *Nucleic Acids Res,* 37**,** 1152-9.
NGUYEN, T. H. D., TAM, J., WU, R. A., GREBER, B. J., TOSO, D., NOGALES, E. & COLLINS, K. 2018. Cryo-EM structure of substrate-bound human telomerase holoenzyme. *Nature,* 557**,** 190-195.
NIH/NCI. 2006. *First-Generation Guidelines for NCI-Supported Biorepositories : https://www.federalregister.gov/documents/2006/04/28/06-3997/first-generationguidelines- for-nci-supported-biorepositories* [Online]. Available: https://www.federalregister.gov/documents/2006/04/28/06-3997/first-generation-guidelines-for-nci-supported-biorepositories [Accessed 29 th sept 2019].
NIHR. *DAPA Measurement Toolkit : https://dapa-toolkit.mrc.ac.uk/concepts/harmonisation* [Online]. Available: https://dapa-toolkit.mrc.ac.uk/concepts/harmonisation [Accessed 2022 July].
NJOKU, K., AGNEW, H. J. & CROSBIE, E. J. 2022a. Impact of Type 2 Diabetes Mellitus on Endometrial Cancer Survival: A Prospective Database Analysis. *Front Oncol,* 12**,** 899262.
NJOKU, K., BARR, C. E. & CROSBIE, E. J. 2022b. Current and Emerging Prognostic Biomarkers in Endometrial Cancer. *Front Oncol,* 12**,** 890908.
NORIMATSU, Y., KOUDA, H., KOBAYASHI, T. K., SHIMIZU, K., YANOH, K., TSUKAYAMA, C., MIYAKE, Y. & OHNO, E. 2009. Utility of liquid-based cytology in endometrial pathology: diagnosis of endometrial carcinoma. *Cytopathology,* 20**,** 395-402.
NORIMATSU, Y., YANOH, K., HIRAI, Y., KUROKAWA, T., KOBAYASHI, T. K. & FULCINITI, F. 2020. A Diagnostic Approach to Endometrial Cytology by Means of Liquid-Based Preparations. *Acta Cytol,* 64**,** 195-207.
OFFICE FOR NATIONAL STATISTICS, C. S. B. S. A. D. F. E. 2019. *Cancer survival in England - Office for National Statistics* [Online]. Public Health England: National Cancer Registration and Analysis Service, Office for National Statistics. Available: https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancersurvivalinengland/nationalestimatesforpatientsfollowedupto2017 [Accessed 5 th June 2022].
OH, B. K., KEO, P., BAE, J., KO, J. H. & CHOI, J. S. 2017. Variable TERRA abundance and stability in cervical cancer cells. *Int J Mol Med,* 39**,** 1597-1604.
OHYASHIKI, J. H., HISATOMI, H., NAGAO, K., HONDA, S., TAKAKU, T., ZHANG, Y., SASHIDA, G. & OHYASHIKI 2005. Quantitative relationship between functionally active telomerase and major telomerase components (hTERT and hTR) in acute leukaemia cells. *British journal of cancer,* 92.

ONG, E. L., GOLDACRE, R. & GOLDACRE, M. 2014. Differential risks of cancer types in people with Parkinsons disease: A national record-linkage study. *European Journal of Cancer,* 50**,** 2456-2462.

OTALI, D., AL DIFFALHA, S. & GRIZZLE, W. E. 2019. Biological, Medical, and Other Tissue Variables Affecting Biospecimen Utilization. *Biopreserv Biobank,* 17**,** 258-263.

PADYKULA, H. A., COLES, L. G., OKULICZ, W. C., RAPAPORT, S. I., MCCRACKEN, J. A., KING, N. W., JR., LONGCOPE, C. & KAISERMAN-ABRAMOF, I. R. 1989. The basalis of the primate endometrium: a bifunctional germinal compartment. *Biol Reprod,* 40**,** 681-90.

PAINTER, J. N., O'MARA, T. A., MARQUART, L., WEBB, P. M., ATTIA, J., MEDLAND, S. E., CHENG, T., DENNIS, J., HOLLIDAY, E. G., MCEVOY, M., SCOTT, R. J., AHMED, S., HEALEY, C. S., SHAH, M., GORMAN, M., MARTIN, L., HODGSON, S. V., BECKMANN, M. W., EKICI, A. B., FASCHING, P. A., HEIN, A., RÜBNER, M., CZENE, K., DARABI, H., HALL, P., LI, J., DÖRK, T., DÜRST, M., HILLEMANNS, P., RUNNEBAUM, I. B., AMANT, F., ANNIBALI, D., DEPREEUW, J., LAMBRECHTS, D., NEVEN, P., CUNNINGHAM, J. M., DOWDY, S. C., GOODE, E. L., FRIDLEY, B. L., WINHAM, S. J., NJØLSTAD, T. S., SALVESEN, H. B., TROVIK, J., WERNER, H. M., ASHTON, K. A., OTTON, G., PROIETTO, A., MINTS, M., THAM, E., BOLLA, M. K., MICHAILIDOU, K., WANG, Q., TYRER, J. P., HOPPER, J. L., PETO, J., SWERDLOW, A. J., BURWINKEL, B., BRENNER, H., MEINDL, A., BRAUCH, H., LINDBLOM, A., CHANG-CLAUDE, J., COUCH, F. J., GILES, G. G., KRISTENSEN, V. N., COX, A., PHAROAH, P. D., TOMLINSON, I., DUNNING, A. M., EASTON, D. F., THOMPSON, D. J., SPURDLE, A. B., N., P. J., O'MARA, T. A., MARQUART, L., WEBB, P. M., ATTIA, J., MEDLAND, S. E., CHENG, T., DENNIS, J., HOLLIDAY, E. G., MCEVOY, M., SCOTT, R. J., AHMED, S., HEALEY, C. S., SHAH, M. & GORMAN 2016. Genetic Risk Score Mendelian Randomization Shows that Obesity Measured as Body Mass Index, but not Waist:Hip Ratio, Is Causal for Endometrial Cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* 25.

PALM, W. & DE LANGE, T. 2008. How shelterin protects mammalian telomeres. *Annu Rev Genet,* 42**,** 301-34.

PARK, J. I., VENTEICHER, A. S., HONG, J. Y., CHOI, J., JUN, S., SHKRELI, M., CHANG, W., MENG, Z., CHEUNG, P., JI, H., MCLAUGHLIN, M., VEENSTRA, T. D., NUSSE, R., MCCREA, P. D. & ARTANDI, S. E. 2009. Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature,* 460**,** 66-72.

PEAKMAN, T. C. & ELLIOTT, P. 2008. The UK Biobank sample handling and storage validation studies. *Int J Epidemiol,* 37 Suppl 1**,** i2-6.

PEDERSEN, I. S., THOMASSEN, M., TAN, Q., KRUSE, T., THORLACIUS-USSING, O., GARNE, J. P. & KRARUP, H. B. 2018. Differential effect of surgical manipulation on gene expression in normal breast tissue and breast tumor tissue. *Mol Med,* 24**,** 57.

PETERSEN, G. M., AMUNDADOTTIR, L., FUCHS, C. S., KRAFT, P., STOLZENBERG-SOLOMON, R. Z., JACOBS, K. B., ARSLAN, A. A., BUENO-DE-MESQUITA, H. B., GALLINGER, S., GROSS, M., HELZLSOUER, K., HOLLY, E. A., JACOBS, E. J., KLEIN, A. P., LACROIX, A., LI, D., MANDELSON, M. T., OLSON, S. H., RISCH, H. A., ZHENG, W., ALBANES, D., BAMLET, W. R., BERG, C. D., BOUTRON-RUAULT, M. C., BURING, J. E., BRACCI, P. M., CANZIAN, F., CLIPP, S., COTTERCHIO, M., DE ANDRADE, M., DUELL, E. J., GAZIANO, J. M., GIOVANNUCCI, E. L., GOGGINS, M., HALLMANS, G., HANKINSON, S. E., HASSAN, M., HOWARD, B., HUNTER, D. J., HUTCHINSON, A., JENAB, M., KAAKS, R., KOOPERBERG, C., KROGH, V., KURTZ, R. C., LYNCH, S. M., MCWILLIAMS, R. R., MENDELSOHN, J. B., MICHAUD, D. S., PARIKH, H., PATEL, A. V., PEETERS, P. H., RAJKOVIC, A., RIBOLI, E., RODRIGUEZ, L., SEMINARA, D., SHU, X. O., THOMAS, G., TJONNELAND, A., TOBIAS, G. S., TRICHOPOULOS, D., VAN DEN EEDEN, S. K., VIRTAMO, J., WACTAWSKI-WENDE, J., WANG, Z., WOLPIN, B. M., YU, H., YU, K., ZELENIUCH-JACQUOTTE, A., FRAUMENI, J. F., JR., HOOVER, R. N., HARTGE, P. & CHANOCK, S. J. 2010. A genome-wide

association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet,* 42**,** 224-8.

PETERSEN, S., SARETZKI, G. & VON ZGLINICKI, T. 1998. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res,* 239**,** 152-60.

PFEIFFER, V. & LINGNER, J. 2012. TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends. *PLoS genetics,* 8**,** e1002747-e1002747.

PIERLUIGI BENEDETTI, P., DEPARTMENT OF, O., GYNECOLOGY, S. U. H. R. I., STEFANO, B., S.BASILE@AO, P. T. I., DEPARTMENT OF, O., GYNECOLOGY, A. O.-U. P. P. I., MARIA GIOVANNA, S., DEPARTMENT OF, O., GYNECOLOGY, A. O.-U. P. P. I., VIOLANTE DI, D., DEPARTMENT OF, O., GYNECOLOGY, S. U. H. R. I., CLAUDIA, M., DEPARTMENT OF, O., GYNECOLOGY, S. U. H. R. I., GIORGIA, P., DEPARTMENT OF, O., GYNECOLOGY, S. U. H. R. I., ANTONIO, P., DEPARTMENT OF, O., GYNECOLOGY, S. U. H. R. I., ALESSANDRA, P., DEPARTMENT OF, O., GYNECOLOGY, A. O.-U. P. P. I., FRANCESCO, M., DEPARTMENT OF, O., GYNECOLOGY, S. M. G. H. L. I., ANDREA ALBERTO, L., DEPARTMENT OF, O., GYNECOLOGY, S. G. H. M. I., MAURO, S., DEPARTMENT OF, O., GYNECOLOGY, S. G. H. M. I., GIOVANNI, S., DEPARTMENT OF, O., GYNECOLOGY, G. H. R. I., SAVERIO, T., DEPARTMENT OF, O., GYNECOLOGY, F. P. S. M. P. M. I., GIORGIA, M., DEPARTMENT OF, O., GYNECOLOGY, S. R. H. M. I., DIONYSSIOS, K., DEPARTMENT OF, O., GYNECOLOGY, S. A. H. T. I., ELIO, C., DEPARTMENT OF, O., GYNECOLOGY, A. H. A. I., NICOLETTA, D., DEPARTMENT OF, O., GYNECOLOGY, U. O. V. V. I., STEFANO, G., DEPARTMENT OF, O., GYNECOLOGY, I. N. T. F. G. P. N. I., MAURO, M., DEPARTMENT OF, O., GYNECOLOGY, P. U. H. P. I., FRANCESCO, R., DEPARTMENT OF, O., GYNECOLOGY, I. N. T. M. I., GENNARO, C., DEPARTMENT OF, O., GYNECOLOGY, U. O. B. B. I., ROBERTO, G., DEPARTMENT OF, O., GYNECOLOGY, T. H. T. I., MASSIMO, F., DEPARTMENT OF, O., GYNECOLOGY, U. O. V. V. I., DIANA, G., DEPARTMENT OF, O., GYNECOLOGY, I. I. R. E. R. I., ROLDANO, F., DEPARTMENT OF, O., GYNECOLOGY, M. N. I. M. I., VALTER, T., DEPARTMENT OF, O., GYNECOLOGY, M. N. I. M. I., CLARA, C., DEPARTMENT OF, O., GYNECOLOGY, N. W. S. H., OBSTETRICS, C. N. C., COSTANTINO, M., DEPARTMENT OF, O. & GYNECOLOGY, S. G. H. M. I. 2014. Secondary analyses from a randomized clinical trial: age as the key prognostic factor in endometrial carcinoma. *American Journal of Obstetrics & Gynecology,* 210**,** 363.e1-363.e10.

PINTO, J., DOMINGUES, M. R., GALHANO, E., PITA, C., ALMEIDA MDO, C., CARREIRA, I. M. & GIL, A. M. 2014. Human plasma stability during handling and storage: impact on NMR metabolomics. *Analyst,* 139**,** 1168-77.

PIULATS, J. M., GUERRA, E., GIL-MARTÍN, M., ROMAN-CANAL, B., GATIUS, S., SANZ-PAMPLONA, R., VELASCO, A., VIDAL, A. & MATIAS, G. 2017. Molecular approaches for classifying endometrial carcinoma. *Gynecologic oncology,* 145.

POMPILI, L., MARESCA, C., DELLO STRITTO, A., BIROCCIO, A. & SALVATI, E. 2019. BRCA2 Deletion Induces Alternative Lengthening of Telomeres in Telomerase Positive Colon Cancer Cells. *Genes (Basel),* 10.

PORRO, A., FEUERHAHN, S., DELAFONTAINE, J., RIETHMAN, H., ROUGEMONT, J. & LINGNER, J. 2014a. Functional characterization of the TERRA transcriptome at damaged telomeres. *Nature Communications,* 5**,** 5379.

PORRO, A., FEUERHAHN, S. & LINGNER, J. 2014b. TERRA-reinforced association of LSD1 with MRE11 promotes processing of uncapped telomeres. *Cell Rep,* 6**,** 765-76.

PORRO, A., FEUERHAHN, S., REICHENBACH, P. & LINGNER, J. 2010. Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways. *Mol Cell Biol,* 30**,** 4808-17.

PORTIER, B. P., WANG, Z., DOWNS-KELLY, E., ROWE, J. J., PATIL, D., LANIGAN, C., BUDD, G. T., HICKS, D. G., RIMM, D. L. & TUBBS, R. R. 2013. Delay to formalin fixation 'cold ischemia time': effect on ERBB2 detection by in-situ hybridization and immunohistochemistry. *Mod Pathol,* 26**,** 1-9.

PRESCOTT, J., MCGRATH, M., LEE, I.-M., BURING, J. E. & DE VIVO, I. 2010. Telomere length and genetic analyses in population-based studies of endometrial cancer risk. *Cancer,* 116**,** 4275-4282.

PROSPECTIVE STUDIES, C. 2009. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *The Lancet,* 373**,** 1083-1096.

PUA, T. & CHUANG, L. 2016. *Endocrinology of Patients with Endometrial Cancer | GLOWM* [Online]. Available: http://www.glowm.com/section-view/heading/Endocrinology of Patients with Endometrial Cancer/item/237 [Accessed 9th June 2022].

PUNYADEERA, C., THIJSSEN, V. L., TCHAIKOVSKI, S., KAMPS, R., DELVOUX, B., DUNSELMAN, G. A., DE GOEIJ, A. F., GRIFFIOEN, A. W. & GROOTHUIS, P. G. 2006. Expression and regulation of vascular endothelial growth factor ligands and receptors during menstruation and post-menstrual repair of human endometrium. *Mol Hum Reprod,* 12**,** 367-75.

RAFNAR, T., SULEM, P., STACEY, S. N., GELLER, F., GUDMUNDSSON, J., SIGURDSSON, A., JAKOBSDOTTIR, M., HELGADOTTIR, H., THORLACIUS, S., ABEN, K. K., BLONDAL, T., THORGEIRSSON, T. E., THORLEIFSSON, G., KRISTJANSSON, K., THORISDOTTIR, K., RAGNARSSON, R., SIGURGEIRSSON, B., SKULADOTTIR, H., GUDBJARTSSON, T., ISAKSSON, H. J., EINARSSON, G. V., BENEDIKTSDOTTIR, K. R., AGNARSSON, B. A., OLAFSSON, K., SALVARSDOTTIR, A., BJARNASON, H., ASGEIRSDOTTIR, M., KRISTINSSON, K. T., MATTHIASDOTTIR, S., SVEINSDOTTIR, S. G., POLIDORO, S., HOIOM, V., BOTELLA-ESTRADA, R., HEMMINKI, K., RUDNAI, P., BISHOP, D. T., CAMPAGNA, M., KELLEN, E., ZEEGERS, M. P., DE VERDIER, P., FERRER, A., ISLA, D., VIDAL, M. J., ANDRES, R., SAEZ, B., JUBERIAS, P., BANZO, J., NAVARRETE, S., TRES, A., KAN, D., LINDBLOM, A., GURZAU, E., KOPPOVA, K., DE VEGT, F., SCHALKEN, J. A., VAN DER HEIJDEN, H. F., SMIT, H. J., TERMEER, R. A., OOSTERWIJK, E., VAN HOOIJ, O., NAGORE, E., PORRU, S., STEINECK, G., HANSSON, J., BUNTINX, F., CATALONA, W. J., MATULLO, G., VINEIS, P., KILTIE, A. E., MAYORDOMO, J. I., KUMAR, R., KIEMENEY, L. A., FRIGGE, M. L., JONSSON, T., SAEMUNDSSON, H., BARKARDOTTIR, R. B., JONSSON, E., JONSSON, S., OLAFSSON, J. H., GULCHER, J. R., MASSON, G., GUDBJARTSSON, D. F., KONG, A., THORSTEINSDOTTIR, U. & STEFANSSON, K. 2009. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet,* 41**,** 221-7.

RAHMIOGLU, N., FASSBENDER, A., VITONIS, A. F., TWOROGER, S. S., HUMMELSHOJ, L., D'HOOGHE, T. M., ADAMSON, G. D., GIUDICE, L. C., BECKER, C. M., ZONDERVAN, K. T., MISSMER, S. A. & GROUP, W. E. W. 2014. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil Steril,* 102**,** 1233-43.

RANSOHOFF, D. F. & GOURLAY, M. L. 2010. Sources of bias in specimens for research about molecular markers for cancer. *J Clin Oncol,* 28**,** 698-704.

RAVINDRANATH, N., LITTLE-IHRIG, L., BENYO, D. F. & ZELEZNIK, A. J. 1992. Role of luteinizing hormone in the expression of cholesterol side-chain cleavage cytochrome P450 and 3 beta-hydroxysteroid dehydrogenase, delta 5-4 isomerase messenger ribonucleic acids in the primate corpus luteum. *Endocrinology,* 131**,** 2065-70.

RCOG. 2021. *Endometrial Cancer in Obese Women (Scientific Impact Paper No. 32)* [Online]. Available: https://www.rcog.org.uk/en/guidelines-research-services/guidelines/sip32/ [Accessed 2nd July 2021].

REDON, S., REICHENBACH, P. & LINGNER, J. 2010. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res,* 38**,** 5797-806.

REDON, S., ZEMP, I. & LINGNER, J. 2013. A three-state model for the regulation of telomerase by TERRA and hnRNPA1. *Nucleic Acids Res,* 41**,** 9117-28.

REEVES, PIRIE, K., BERAL, V., GREEN, J., SPENCER, E. & BULL, D. 2007. Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. *BMJ (Clinical research ed.) 10.1136/bmj.39367.495995.AE,* 335.

REIJNEN, C., GOGOU, E., VISSER, N. C. M., ENGERUD, H., RAMJITH, J., VAN DER PUTTEN, L. J. M., VAN DE VIJVER, K., SANTACANA, M., BRONSERT, P., BULTEN, J., HIRSCHFELD, M., COLAS, E., GIL-MORENO, A., REQUES, A., MANCEBO, G., KRAKSTAD, C., TROVIK, J., HALDORSEN, I. S., HUVILA, J., KOSKAS, M., WEINBERGER, V., BEDNARIKOVA, M., HAUSNEROVA, J., VAN DER WURFF, A. A. M., MATIAS-GUIU, X., AMANT, F., MASSUGER, L., SNIJDERS, M., KÜSTERS-VANDEVELDE, H. V. N., LUCAS, P. J. F. & PIJNENBORG, J. M. A. 2020. Preoperative risk stratification in endometrial cancer (ENDORISK) by a Bayesian network model: A development and validation study. *PLoS Med,* 17**,** e1003111.

REUBEN, A., GOPALAKRISHNAN, V., WAGNER, H. E., SPENCER, C. N., AUSTIN-BRENEMAN, J., JIANG, H., COOPER, Z. A. & WARGO, J. A. 2015. Working with Human Tissues for Translational Cancer Research. *J Vis Exp. 2015 Nov 26;(105):53189. doi: 10.3791/53189.*

RICCIARDELLI, C., BIANCO-MIOTTO, T., JINDAL, S., DODD, T. J., COHEN, P. A., MARSHALL, V. R., SUTHERLAND, P. D., SAMARATUNGA, H., KENCH, J. G., DONG, Y., WANG, H., CLEMENTS, J. A., RISBRIDGER, G. P., SUTHERLAND, R. L., TILLEY, W. D. & HORSFALL, D. J. 2010. Comparative biomarker expression and RNA integrity in biospecimens derived from radical retropubic and robot-assisted laparoscopic prostatectomies. *Cancer Epidemiol Biomarkers Prev,* 19**,** 1755-65.

RIDER, V., KIMLER, B. F. & JUSTICE, W. M. 1998. Progesterone-growth factor interactions in uterine stromal cells. *Biol Reprod,* 59**,** 464-9.

RIEGMAN, P. H., OOMEN, M. H., DINJENS, W. N., OOSTERHUIS, J. W., LAM, K. H., SPATZ, A., RATCLIFFE, C., KNOX, K., MAGER, R., KERR, D., PEZZELLA, F., VAN DAMME, B., VAN DE VIJVER, M., VAN BOVEN, H., MORENTE, M. M., ALONSO, S., KERJASCHKI, D., PAMMER, J., LOPEZ-GUERRERO, J. A., LLOMBART-BOSCH, A., CARBONE, A., GLOGHINI, A., TEODOROVIC, I., ISABELLE, M., PASSIOUKOV, A., LEJEUNE, S., THERASSE, P. & VAN VEEN, E. B. 2006. TuBaFrost: European virtual tumor tissue banking. *Adv Exp Med Biol,* 587**,** 65-74.

ROBIN, J. D., LUDLOW, A. T., BATTEN, K., MAGDINIER, F., STADLER, G., WAGNER, K. R., SHAY, J. W. & WRIGHT, W. E. 2014. Telomere position effect: regulation of gene expression with progressive telomere shortening over long distances. *Genes Dev,* 28**,** 2464-76.

RODRIGUEZ, F. J., VIZCAINO, M. A., BLAKELEY, J. & HEAPHY, C. M. 2016. Frequent alternative lengthening of telomeres and ATRX loss in adult NF1-associated diffuse and high-grade astrocytomas. *Acta Neuropathol,* 132**,** 761-763.

ROGER, L., JONES, R. E., HEPPEL, N. H., WILLIAMS, G. T., SAMPSON, J. R. & BAIRD, D. M. 2013. Extensive telomere erosion in the initiation of colorectal adenomas and its association with chromosomal instability. *J Natl Cancer Inst,* 105**,** 1202-11.

ROSSI, E. C., KOWALSKI, L. D., SCALICI, J., CANTRELL, L., SCHULER, K., HANNA, R. K., METHOD, M., ADE, M., IVANOVA, A. & BOGGESS, J. F. 2017. A comparison of sentinel lymph node biopsy to lymphadenectomy for endometrial cancer staging (FIRES trial): a multicentre, prospective, cohort study. *The Lancet Oncology,* 18**,** 384-392.

ROYDS, J. A., AL NADAF, S., WILES, A. K., CHEN, Y. J., AHN, A., SHAW, A., BOWIE, S., LAM, F., BAGULEY, B. C., BRAITHWAITE, A. W., MACFARLANE, M. R., HUNG, N. A. & SLATTER, T. L. 2011. The CDKN2A G500 allele is more frequent in GBM patients with no defined telomere maintenance mechanism tumors and is associated with poorer survival. *PLoS One,* 6**,** e26737.

ROYLE, N. J. 2006. *Telomeres, 2nd Edition. Edited by T. de Lange, V. Lundblad and E. Blackburn. Cold Spring Harbor Laboratory Press. 2006. 576 Pages. ISBN 0-87969-734-2.* .

RUBTSOVA, M. & DONTSOVA, O. 2020. Human Telomerase RNA: Telomerase Component or More? *Biomolecules. 2020 Jun 6;10(6). pii: biom10060873. doi: 10.3390/biom10060873.,* 10.

RUBTSOVA, M., NARAYKINA, Y., VASILKOVA, D., MEERSON, M., ZVEREVA, M., PRASSOLOV, V., LAZAREV, V., MANUVERA, V., KOVALCHUK, S., ANIKANOV, N., BUTENKO, I., POBEGUTS, O., GOVORUN, V. & DONTSOVA 2018. Protein encoded in human telomerase RNA is involved in cell protective pathways. *Nucleic acids research, 10.1093/nar/gky705,* 46.

RUBTSOVA, M. P., SKVORTSOV, D. A., PETRUSEVA, I. O., LAVRIK, O. I., SPIRIN, P. V., PRASOLOV, V. S., KISSELJOV, F. L. & DONTSOVA, O. A. 2009. Replication protein A modulates the activity of human telomerase in vitro. *Biochemistry (Mosc),* 74**,** 92-6.

RYAN, N. A. J., MCMAHON, R., TOBI, S., SNOWSILL, T., ESQUIBEL, S., WALLACE, A. J., BUNSTONE, S., BOWERS, N., MOSNEAG, I. E., KITSON, S. J., O'FLYNN, H., RAMCHANDER, N. C., SIVALINGAM, V. N., FRAYLING, I. M., BOLTON, J., MCVEY, R. J., EVANS, D. G. & CROSBIE, E. J. 2020. The proportion of endometrial tumours associated with Lynch syndrome (PETALS): A prospective cross-sectional study. *PLoS Med,* 17**,** e1003263.

SAED, L., VARSE, F., BARADARAN, H. R., MORADI, Y., KHATERI, S., FRIBERG, E., KHAZAEI, Z., GHARAHJEH, S., TEHRANI, S., SIOOFY-KHOJINE, A. B. & NAJMI, Z. 2019. The effect of diabetes on the risk of endometrial Cancer: an updated a systematic review and meta-analysis. *BMC Cancer,* 19**,** 527.

SAITO, T., SCHNEIDER, A., MARTEL, N., MIZUMOTO, H., BULGAY-MOERSCHEL, M., KUDO, R. & NAKAZAWA, H. 1997. Proliferation-associated regulation of telomerase activity in human endometrium and its potential implication in early cancer diagnosis. *Biochem Biophys Res Commun,* 231**,** 610-4.

SAKAMOTO, M., TOYOIZUMI, T., KIKUCHI, Y., OKAMOTO, A., NAKAYAMA, H., AOKI, D., YAMAMOTO, K., HATA, H., SUGISHITA, T. & TENJIN, Y. 2000. Telomerase activity in gynecological tumors. *Oncol Rep,* 7**,** 1003-9.

SALVESEN, H. B., CARTER, S. L., MANNELQVIST, M., DUTT, A., GETZ, G., STEFANSSON, I. M., RAEDER, M. B., SOS, M. L., ENGELSEN, I. B., TROVIK, J., WIK, E., GREULICH, H., BO, T. H., JONASSEN, I., THOMAS, R. K., ZANDER, T., GARRAWAY, L. A., OYAN, A. M., SELLERS, W. R., KALLAND, K. H., MEYERSON, M., AKSLEN, L. A. & BEROUKHIM, R. 2009. Integrated genomic profiling of endometrial carcinoma associates aggressive tumors with indicators of PI3 kinase activation. *Proc Natl Acad Sci U S A,* 106**,** 4834-9.

SAMPL, S., PRAMHAS, S., STERN, C., PREUSSER, M., MAROSI, C. & HOLZMANN, K. 2012. Expression of Telomeres in Astrocytoma WHO Grade 2 to 4: TERRA Level Correlates with Telomere Length, Telomerase Activity, and Advanced Clinical Grade. *Translational Oncology,* 5**,** 56-IN4.

SANDELL, L. L. & ZAKIAN, V. A. 1993. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell,* 75**,** 729-39.

SARETZKI, G. 2014. Extra-telomeric functions of human telomerase: cancer, mitochondria and oxidative stress. *Curr Pharm Des,* 20**,** 6386-403.

SARETZKI, G. 2018. Telomeres, Telomerase and Ageing. *Subcell Biochem,* 90**,** 221-308.

SARIN, R. 2006. The journey of biospecimens: mortuaries to biorepositories. *Journal of Cancer Research and Therapeutics,* 2**,** 3-4.

SAVOURET, J. F., CHAUCHEREAU, A., MISRAHI, M., LESCOP, P., MANTEL, A., BAILLY, A. & MILGROM, E. 1994a. The progesterone receptor. Biological effects of progestins and antiprogestins. *Hum Reprod,* 9 Suppl 1**,** 7-11.

SAVOURET, J. F., RAUCH, M., REDEUILH, G., SAR, S., CHAUCHEREAU, A., WOODRUFF, K., PARKER, M. G. & MILGROM 1994b. Interplay between estrogens, progestins, retinoic acid and AP-1 on a single regulatory site in the progesterone receptor gene. *The Journal of biological chemistry,* 269.

SAYGAN-KARAMURSEL, B., DIKMEN, G., DOGAN, P., AKSU, T., GUVEN, S. & AYHAN, A. 2005. Quantitative telomerase activity in malignant, benign and normal gynecological tissues. *Eur J Gynaecol Oncol,* 26**,** 83-6.

SCHEEL, C., SCHAEFER, K. L., JAUCH, A., KELLER, M., WAI, D., BRINKSCHMIDT, C., VAN VALEN, F., BOECKER, W., DOCKHORN-DWORNICZAK, B. & POREMBA, C. 2001. Alternative lengthening of telomeres is associated with chromosomal instability in osteosarcomas. *Oncogene,* 20**,** 3835-44.

SCHEIBE, M., ARNOULT, N., KAPPEI, D., BUCHHOLZ, F., DECOTTIGNIES, A., BUTTER, F. & MANN, M. 2013. Quantitative interaction screen of telomeric repeat-containing RNA reveals novel TERRA regulators. *Genome Res,* 23**,** 2149-57.

SCHLOMM, T., NAKEL, E., LUBKE, A., BUNESS, A., CHUN, F. K., STEUBER, T., GRAEFEN, M., SIMON, R., SAUTER, G., POUSTKA, A., HULAND, H., ERBERSDOBLER, A., SULTMANN, H. & HELLWINKEL, O. J. 2008. Marked gene transcript level alterations occur early during radical prostatectomy. *Eur Urol,* 53**,** 333-44.

SCHNEIDER, F., SMITH, M. A., LANE, M. C., PANTANOWITZ, L., DACIC, S. & OHORI, N. P. 2015. Adequacy of core needle biopsy specimens and fine-needle aspirates for molecular testing of lung adenocarcinomas. *Am J Clin Pathol,* 143**,** 193-200;.

SCHOEFTNER, S. & BLASCO, M. A. 2008. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol,* 10**,** 228-36.

SCHOLTEN, A. N., SMIT, V. T., BEERMAN, H., VAN PUTTEN, W. L. & CREUTZBERG 2004. Prognostic significance and interobserver variability of histologic grading systems for endometrial carcinoma. *Cancer,* 100.

SCHONFELD, S. J., HARTGE, P., PFEIFFER, R. M., FREEDMAN, D. M., GREENLEE, R. T., LINET, M. S., PARK, Y., SCHAIRER, C., VISVANATHAN, K. & LACEY, J. V., JR. 2013. An aggregated analysis of hormonal factors and endometrial cancer risk by parity. *Cancer,* 119**,** 1393-401.

SCHWARTZENTRUBER, J., KORSHUNOV, A., LIU, X. Y., JONES, D. T., PFAFF, E., JACOB, K., STURM, D., FONTEBASSO, A. M., QUANG, D. A., TÖNJES, M., HOVESTADT, V., ALBRECHT, S., KOOL, M., NANTEL, A., KONERMANN, C., LINDROTH, A., JÄGER, N., RAUSCH, T., RYZHOVA, M., KORBEL, J. O., HIELSCHER, T., HAUSER, P., GARAMI, M., KLEKNER, A., BOGNAR, L., EBINGER, M., SCHUHMANN, M. U., SCHEURLEN, W., PEKRUN, A., FRÜHWALD, M. C., ROGGENDORF, W., KRAMM, C., DÜRKEN, M., ATKINSON, J., LEPAGE, P., MONTPETIT, A., ZAKRZEWSKA, M., ZAKRZEWSKI, K., LIBERSKI, P. P., DONG, Z., SIEGEL, P., KULOZIK, A. E., ZAPATKA, M., GUHA, A., MALKIN, D., FELSBERG, J., REIFENBERGER, G., VON DEIMLING, A., ICHIMURA, K., COLLINS, V. P., WITT, H., MILDE, T., WITT, O., ZHANG, C., CASTELO-BRANCO, P., LICHTER, P., FAURY, D., TABORI, U., PLASS, C., MAJEWSKI, J., PFISTER, S. M. & JABADO, N. 2012. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature,* 482**,** 226-31.

SHAO, R., LI, X., FENG, Y., LIN, J. F. & BILLIG, H. 2014. Direct effects of metformin in the endometrium: a hypothetical mechanism for the treatment of women with PCOS and endometrial carcinoma. *J Exp Clin Cancer Res,* 33**,** 41.

SHARMA, S., KELLY, T. K. & JONES, P. A. 2010. Epigenetics in cancer. *Carcinogenesis,* 31**,** 27-36.

SHAY, J. W. 2016. Role of Telomeres and Telomerase in Aging and Cancer. *Cancer Discov,* 6**,** 584-93.

SHAY, J. W. & BACCHETTI, S. 1997. A survey of telomerase activity in human cancer. *Eur J Cancer,* 33**,** 787-91.

SHAY, J. W. & WRIGHT, W. E. 2005. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis,* 26**,** 867-74.

SHAY, J. W. & WRIGHT, W. E. 2010. Telomeres and telomerase in normal and cancer stem cells. *FEBS Lett,* 584**,** 3819-25.

SHELDON, E., VO, K. C., MCINTIRE, R. A., AGHAJANOVA, L., ZELENKO, Z., IRWIN, J. C. & GIUDICE, L. C. 2011. Biobanking human endometrial tissue and blood specimens: standard operating procedure and importance to reproductive biology research and diagnostic development. *Fertil Steril,* 95**,** 2120-2, 2122 e1-12.

SHI, S. R., KEY, M. E. & KALRA, K. L. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem,* 39**,** 741-8.

SHIKATA, K., NINOMIYA, T. & KIYOHARA, Y. 2013. Diabetes mellitus and cancer risk: review of the epidemiological evidence. *Cancer Sci,* 104**,** 9-14.

SHIRTCLIFF, E. A., GRANGER, D. A., SCHWARTZ, E. & CURRAN, M. J. 2001. Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology, 10.1016/s0306-4530(00)00042-1,* 26.

SHWEIKI, D., ITIN, A., SOFFER, D. & KESHET 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature,* 359, 10.1038/359843a0.

SIMITSIDELLIS, I., SAUNDERS, P. T. K. & GIBSON, D. A. 2018. Androgens and endometrium: New insights and new targets. *Mol Cell Endocrinol,* 465**,** 48-60.

SIMON, M., HOSEN, I., GOUSIAS, K., RACHAKONDA, S., HEIDENREICH, B., GESSI, M., SCHRAMM, J., HEMMINKI, K., WAHA, A. & KUMAR, R. 2015. TERT promoter mutations: a novel independent prognostic factor in primary glioblastomas. *Neuro Oncol,* 17**,** 45-52.

SISMONDI, P., BIGLIA, N., VOLPI, E., GIAI, M. & DE GRANDIS, T. 1994. Tamoxifen and endometrial cancer. *Ann N Y Acad Sci,* 734**,** 310-21.

SIVRIDIS, E. & GIATROMANOLAKI, A. 2004. Proliferative activity in postmenopausal endometrium: the lurking potential for giving rise to an endometrial adenocarcinoma. *J Clin Pathol,* 57**,** 840-4.

SMEKALOVA, E. & BAUMANN, P. 2013. TERRA -a calling card for telomerase. *Mol Cell,* 51**,** 703-4.

SMITH, J. K. & YEH, G. 1992. Telomere reduction in endometrial adenocarcinoma. *Am J Obstet Gynecol,* 167**,** 1883-7.

SMITTENAAR, PETERSEN, K. A., STEWART, K. & MOITT 2016. Cancer incidence and mortality projections in the UK until 2035. *British journal of cancer,* 115.

SMOGORZEWSKA, A. & DE LANGE, T. 2004. Regulation of telomerase by telomeric proteins. *Annu Rev Biochem,* 73**,** 177-208.

SNIJDERS, M. P., DE GOEIJ, A. F., KOUDSTAAL, J., THUNNISSEN, E. B., DE HAAN, J. & BOSMAN, F. T. 1992. Oestrogen and progesterone receptor immunocytochemistry in human hyperplastic and neoplastic endometrium. *J Pathol,* 166**,** 171-7.

SOINI, T., HURSKAINEN, R., GRÉNMAN, S., MÄENPÄÄ, J., PAAVONEN, J. & PUKKALA, E. 2014. Cancer risk in women using the levonorgestrel-releasing intrauterine system in Finland. *Obstet Gynecol,* 124**,** 292-299.

SOLIMAN, P. T., BROADDUS, R. R., SCHMELER, K. M., DANIELS, M. S., GONZALEZ, D., SLOMOVITZ, B. M., GERSHENSON, D. M. & LU, K. H. 2005. Women with synchronous primary cancers of the endometrium and ovary: do they have Lynch syndrome? *J Clin Oncol,* 23**,** 9344-50.

SOLLIER, J. & CIMPRICH, K. A. 2015. Breaking bad: R-loops and genome integrity. *Trends Cell Biol,* 25**,** 514-22.

SORBE, B., NORDSTRÖM, B., MÄENPÄÄ, J., KUHELJ, J., KUHELJ, D., OKKAN, S., DELALOYE, J. F. & FRANKENDAL, B. 2009. Intravaginal brachytherapy in FIGO stage I low-risk endometrial cancer: a controlled randomized study. *Int J Gynecol Cancer,* 19**,** 873-8.

SOURIAL, S., TEMPEST, N. & HAPANGAMA, D. K. 2014. Theories on the pathogenesis of endometriosis. *Int J Reprod Med,* 2014**,** 179515.

SPRUESSEL, A., STEIMANN, G., JUNG, M., LEE, S. A., CARR, T., FENTZ, A. K., SPANGENBERG, J., ZORNIG, C., JUHL, H. H. & DAVID, K. 2004a. Tissue ischemia time affects gene and protein expression patterns within minutes following surgical tumor excision. *BioTechniques,* 36.

SPRUESSEL, A., STEIMANN, G., JUNG, M., LEE, S. A., CARR, T., FENTZ, A. K., SPANGENBERG, J., ZORNIG, C., JUHL, H. H. & DAVID, K. A. 2004b. Tissue ischemia time affects gene and protein expression patterns within minutes following surgical tumor excision. *Biotechniques,* 36**,** 1030-7.

STELLOO, E., BOSSE, T., NOUT, R. A., MACKAY, H. J., CHURCH, D. N., NIJMAN, H. W., LEARY, A., EDMONDSON, R. J., POWELL, M. E., CROSBIE, E. J., KITCHENER, H. C., MILESHKIN, L., POLLOCK, P. M., SMIT, V. T. & CREUTZBERG, C. L. 2015. Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; a TransPORTEC initiative. *Mod Pathol,* 28**,** 836-44.

STELLOO, E., NOUT, R. A., NAVES, L. C., TER HAAR, N. T., CREUTZBERG, C. L., SMIT, V. T. & BOSSE 2014. High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial carcinoma. *Gynecologic oncology,* 133.

STERN, J. L. & BRYAN, T. M. 2008. Telomerase recruitment to telomeres. *Cytogenet Genome Res,* 122**,** 243-54.

STOCK, R. J. & KANBOUR, A. 1975. Prehysterectomy curettage. *Obstet Gynecol,* 45**,** 537-41.

STORTI, C. B., DE OLIVEIRA, R. A., DE CARVALHO, M., HASIMOTO, E. N., CATANEO, D. C., CATANEO, A. J. M., DE FAVERI, J., VASCONCELOS, E. J. R., DOS REIS, P. P. & CANO, M. I. N. 2020. Telomere-associated genes and telomeric lncRNAs are biomarker candidates in lung squamous cell carcinoma (LUSC). *Exp Mol Pathol,* 112**,** 104354.

STUBERT, J. & GERBER, B. 2016. Current Issues in the Diagnosis and Treatment of Endometrial Carcinoma. *Geburtshilfe Frauenheilkd,* 76**,** 170-175.

SUN, Y., ZHANG, L., ZHAO, L., WU, X. & GU, J. 2015. Association of leukocyte telomere length in peripheral blood leukocytes with endometrial cancer risk in Caucasian Americans. *Carcinogenesis,* 36**,** 1327-32.

SURYO RAHMANTO, Y., JUNG, J. G., WU, R. C., KOBAYASHI, Y., HEAPHY, C. M., MEEKER, A. K., WANG, T. L. & SHIH IE, M. 2016. Inactivating ARID1A Tumor Suppressor Enhances TERT Transcription and Maintains Telomere Length in Cancer Cells. *J Biol Chem,* 291**,** 9690-9.

TACKLING OBESITIES. 2007. *https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/295684/07-926A2-obesity-international.pdf* [Online]. [Accessed 2nd July 2021].

TAKAHAMA, K. & OYOSHI, T. 2013. Specific binding of modified RGG domain in TLS/FUS to G-quadruplex RNA: tyrosines in RGG domain recognize 2'-OH of the riboses of loops in G-quadruplex. *J Am Chem Soc,* 135**,** 18016-9.

TAKAHAMA, K., TAKADA, A., TADA, S., SHIMIZU, M., SAYAMA, K., KUROKAWA, R. & OYOSHI, T. 2013. Regulation of telomere length by G-quadruplex telomere DNA- and TERRA-binding protein TLS/FUS. *Chem Biol,* 20**,** 341-50.

TAKAI, K. K., KIBE, T., DONIGIAN, J. R., FRESCAS, D. & DE LANGE, T. 2011. Telomere protection by TPP1/POT1 requires tethering to TIN2. *Mol Cell,* 44**,** 647-59.

TAKEDA, T., BANNO, K., OKAWA, R., YANOKURA, M., IIJIMA, M., IRIE-KUNITOMI, H., NAKAMURA, K., IIDA, M., ADACHI, M., UMENE, K., NOGAMI, Y., MASUDA, K., KOBAYASHI, Y., TOMINAGA, E. & AOKI, D. 2016. ARID1A gene mutation in ovarian and endometrial cancers (Review). *Oncol Rep,* 35**,** 607-13.

TALHOUK, A., MCCONECHY, M. K., LEUNG, S., YANG, W., LUM, A., SENZ, J., BOYD, N., PIKE, J., ANGLESIO, M., KWON, J. S., KARNEZIS, A. N., HUNTSMAN, D. G., GILKS, C. B. & MCALPINE, J. N. 2017. Confirmation of ProMisE: A simple, genomics-based clinical classifier for endometrial cancer. *Cancer,* 123**,** 802-813.

TANAKA, M., KYO, S., TAKAKURA, M., KANAYA, T., SAGAWA, T., YAMASHITA, K., OKADA, Y., HIYAMA, E. & INOUE, M. 1998. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. *Am J Pathol,* 153**,** 1985-91.

TARABORRELLI, S. 2015. Physiology, production and action of progesterone. *Acta Obstet Gynecol Scand,* 94 Suppl 161**,** 8-16.

TATSUMOTO, N., HIYAMA, E., MURAKAMI, Y., IMAMURA, Y., SHAY, J. W., MATSUURA, Y. & YOKOYAMA, T. 2000. High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res,* 6**,** 2696-701.

TEIXEIRA, M. T., ARNERIC, M., SPERISEN, P. & LINGNER, J. 2004. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. *Cell,* 117**,** 323-35.

TEMPEST, N., BAKER, A. M., WRIGHT, N. A. & HAPANGAMA, D. K. 2018. Does human endometrial LGR5 gene expression suggest the existence of another hormonally regulated epithelial stem cell niche? *Hum Reprod,* 33**,** 1052-1062.

TEMPEST, N., JANSEN, M., BAKER, A. M., HILL, C. J., HALE, M., MAGEE, D., TREANOR, D., WRIGHT, N. A. & HAPANGAMA, D. K. 2020. Histological 3D reconstruction and in vivo lineage tracing of the human endometrium. *J Pathol,* 251**,** 440-451.

TESMER, V. M., FORD, L. P., HOLT, S. E., FRANK, B. C., YI, X., AISNER, D. L., OUELLETTE, M., SHAY, J. W. & WRIGHT, W. E. 1999. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. *Mol Cell Biol,* 19**,** 6207-16.

THEIMER, C. A. & FEIGON, J. 2006. Structure and function of telomerase RNA. *Curr Opin Struct Biol,* 16**,** 307-18.

THOMPSON, M. 2009. Considering the implication of variations within Delphi research. *Family practice,* 26.

TING, N. S., POHORELIC, B., YU, Y., LEES-MILLER, S. P. & BEATTIE, T. L. 2009. The human telomerase RNA component, hTR, activates the DNA-dependent protein kinase to phosphorylate heterogeneous nuclear ribonucleoprotein A1. *Nucleic Acids Res,* 37**,** 6105-15.

TODO, Y., KATO, H., KANEUCHI, M., WATARI, H., TAKEDA, M. & SAKURAGI, N. 2010. Survival effect of para-aortic lymphadenectomy in endometrial cancer (SEPAL study): a retrospective cohort analysis. *Lancet,* 375**,** 1165-72.

TOMLINSON, R. L., ZIEGLER, T. D., SUPAKORNDEJ, T., TERNS, R. M. & TERNS, M. P. 2006. Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol Biol Cell,* 17**,** 955-65.

TONG, A. S., STERN, J. L., SFEIR, A., KARTAWINATA, M., DE LANGE, T., ZHU, X. D. & BRYAN, T. M. 2015. ATM and ATR Signaling Regulate the Recruitment of Human Telomerase to Telomeres. *Cell Rep,* 13**,** 1633-46.

TRAHAN, C. & DRAGON 2009. Dyskeratosis congenita mutations in the H/ACA domain of human telomerase RNA affect its assembly into a pre-RNP. *RNA (New York, N.Y.),* 15.

TSAKIRI, K. D., CRONKHITE, J. T., KUAN, P. J., XING, C., RAGHU, G., WEISSLER, J. C., ROSENBLATT, R. L., SHAY, J. W. & GARCIA, C. K. 2007. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci U S A,* 104**,** 7552-7.

TUTTON, S., AZZAM, G. A., STONG, N., VLADIMIROVA, O., WIEDMER, A., MONTEITH, J. A., BEISHLINE, K., WANG, Z., DENG, Z., RIETHMAN, H., MCMAHON, S. B., MURPHY, M. & LIEBERMAN, P. M. 2016. Subtelomeric p53 binding prevents accumulation of DNA damage at human telomeres. *Embo j,* 35**,** 193-207.

TWOROGER, S. S. & HANKINSON, S. E. 2006. Use of biomarkers in epidemiologic studies: minimizing the influence of measurement error in the study design and analysis. *Cancer causes & control : CCC,* 17.

ULANER, G. A., HUANG, H. Y., OTERO, J., ZHAO, Z., BEN-PORAT, L., SATAGOPAN, J. M., GORLICK, R., MEYERS, P., HEALEY, J. H., HUVOS, A. G., HOFFMAN, A. R. & LADANYI, M. 2003. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. *Cancer Res,* 63**,** 1759-63.

VALENTIJN, A. J., PALIAL, K., AL-LAMEE, H., TEMPEST, N., DRURY, J., VON ZGLINICKI, T., SARETZKI, G., MURRAY, P., GARGETT, C. E. & HAPANGAMA, D. K. 2013. SSEA-1 isolates human endometrial basal glandular epithelial cells: phenotypic and functional characterization and implications in the pathogenesis of endometriosis. *Hum Reprod,* 28**,** 2695-708.

VALENTIJN, A. J., SARETZKI, G., TEMPEST, N., CRITCHLEY, H. O. & HAPANGAMA, D. K. 2015. Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis. *Hum Reprod,* 30**,** 2816-28.

VALLS, C., PINOL, C., RENE, J. M., BUENESTADO, J. & VINAS, J. 2011. Telomere length is a prognostic factor for overall survival in colorectal cancer. *Colorectal Dis,* 13**,** 1265-72.

VAN BENEDEN, A., ARNOULT, N. & DECOTTIGNIES, A. 2013. Telomeric RNA expression: length matters. *Front Oncol,* 3**,** 178.
VAN DER LINDEN, A., BLOKKER, B. M., KAP, M., WEUSTINK, A. C., ROBERTUS, J. L., RIEGMAN, P. H. & OOSTERHUIS, J. W. 2014. Post-mortem tissue biopsies obtained at minimally invasive autopsy: an RNA-quality analysis. *PLoS One,* 9**,** e115675.
VAN HANEGEM, N., PRINS, M. M., BONGERS, M. Y., OPMEER, B. C., SAHOTA, D. S., MOL, B. W. & TIMMERMANS, A. 2016. The accuracy of endometrial sampling in women with postmenopausal bleeding: a systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol,* 197**,** 147-55.
VAN HEEK, N. T., MEEKER, A. K., KERN, S. E., YEO, C. J., LILLEMOE, K. D., CAMERON, J. L., OFFERHAUS, G. J., HICKS, J. L., WILENTZ, R. E., GOGGINS, M. G., DE MARZO, A. M., HRUBAN, R. H. & MAITRA, A. 2002. Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia. *Am J Pathol,* 161**,** 1541-7.
VAN STEENSEL, B. & DE LANGE, T. 1997. Control of telomere length by the human telomeric protein TRF1. *Nature,* 385**,** 740-3.
VANACOVA, S. & STEFL, R. 2007. The exosome and RNA quality control in the nucleus. *EMBO Rep,* 8**,** 651-7.
VANÁCOVÁ, S., WOLF, J., MARTIN, G., BLANK, D., DETTWILER, S., FRIEDLEIN, A., LANGEN, H., KEITH, G. & KELLER, W. 2005. A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol,* 3**,** e189.
VANI, S., CRITCHLEY, H. O., FRASER, I. S. & HICKEY 2008. Endometrial expression of steroid receptors in postmenopausal hormone replacement therapy users: relationship to bleeding patterns. *The journal of family planning and reproductive health care*.
VARO, N., NUZZO, R., NATAL, C., LIBBY, P. & SCHÖNBECK, U. 2006. Influence of pre-analytical and analytical factors on soluble CD40L measurements. *Clin Sci (Lond),* 111**,** 341-7.
VAUGHT, J. & LOCKHART, N. C. 2012. The evolution of biobanking best practices. *Clin Chim Acta,* 413**,** 1569-75.
VENTEICHER, A. S., ABREU, E. B., MENG, Z., MCCANN, K. E., TERNS, R. M., VEENSTRA, T. D., TERNS, M. P. & ARTANDI, S. E. 2009. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. *Science,* 323**,** 644-8.
VERMA, M., ROGERS, S., DIVI, R. L., SCHULLY, S. D., NELSON, S., JOSEPH SU, L., ROSS, S. A., PILCH, S., WINN, D. M. & KHOURY, M. J. 2014. Epigenetic research in cancer epidemiology: trends, opportunities, and challenges. *Cancer Epidemiol Biomarkers Prev,* 23**,** 223-33.
VICECONTE, N., DHEUR, M. S., MAJEROVA, E., PIERREUX, C. E., BAURAIN, J. F., VAN BAREN, N. & DECOTTIGNIES, A. 2017. Highly Aggressive Metastatic Melanoma Cells Unable to Maintain Telomere Length. *Cell Rep,* 19**,** 2529-2543.
VICECONTE, N., LORIOT, A., LONA ABREU, P., SCHEIBE, M., FRADERA SOLA, A., BUTTER, F., DE SMET, C., AZZALIN, C. M., ARNOULT, N. & DECOTTIGNIES, A. 2021. PAR-TERRA is the main contributor to telomeric repeat-containing RNA transcripts in normal and cancer mouse cells. *Rna,* 27**,** 106-121.
VIDAL, J. D., REGISTER, T. C., GUPTA, M. & CLINE, J. M. 2002. Estrogen replacement therapy induces telomerase RNA expression in the macaque endometrium. *Fertil Steril,* 77**,** 601-8.
VIGOD, S. N. & STEWART, D. E. 2002. Management of abnormal uterine bleeding by northern, rural and isolated primary care physicians: PART I - How are we doing? *BMC Womens Health,* 2**,** 10.
VISNOVSKY, J., KUDELA, E., FARKASOVA, A., BALHAREK, T., KRKOSKA, M. & DANKO, J. 2014. Amplification of TERT and TERC genes in cervical intraepithelial neoplasia and cervical cancer. *Neuro Endocrinol Lett,* 35**,** 518-22.
VITELLI, V., FALVO, P., KHORIAULI, L., SMIRNOVA, A., GAMBA, R., SANTAGOSTINO, M., NERGADZE, S. G. & GIULOTTO, E. 2013. More on the Lack of Correlation between Terra Expression and Telomere Length. *Front Oncol,* 3**,** 245.

VITELLI, V., FALVO, P., S, G. N., SANTAGOSTINO, M., KHORIAULI, L., PELLANDA, P., BERTINO, G., OCCHINI, A., BENAZZO, M., MORBINI, P., PAULLI, M., PORTA, C. & GIULOTTO, E. 2018. Telomeric Repeat-Containing RNAs (TERRA) Decrease in Squamous Cell Carcinoma of the Head and Neck Is Associated with Worsened Clinical Outcome. *Int J Mol Sci*.

VITONIS, A. F., VINCENT, K., RAHMIOGLU, N., FASSBENDER, A., BUCK LOUIS, G. M., HUMMELSHOJ, L., GIUDICE, L. C., STRATTON, P., ADAMSON, G. D., BECKER, C. M., ZONDERVAN, K. T., MISSMER, S. A. & GROUP, W. E. W. 2014. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: II. Clinical and covariate phenotype data collection in endometriosis research. *Fertil Steril,* 102**,** 1223-32.

VOGAN, J. M., ZHANG, X., YOUMANS, D. T., REGALADO, S. G., JOHNSON, J. Z., HOCKEMEYER, D. & COLLINS, K. 2016. Minimized human telomerase maintains telomeres and resolves endogenous roles of H/ACA proteins, TCAB1, and Cajal bodies. *Elife,* 5.

VRBSKY, J., AKIMCHEVA, S., WATSON, J. M., TURNER, T. L., DAXINGER, L., VYSKOT, B., AUFSATZ, W. & RIHA, K. 2010. siRNA-mediated methylation of Arabidopsis telomeres. *PLoS Genet,* 6**,** e1000986.

WALKER, J. L., PIEDMONTE, M. R., SPIRTOS, N. M., EISENKOP, S. M., SCHLAERTH, J. B., MANNEL, R. S., BARAKAT, R., PEARL, M. L. & SHARMA 2012. Recurrence and survival after random assignment to laparoscopy versus laparotomy for comprehensive surgical staging of uterine cancer: Gynecologic Oncology Group LAP2 Study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*.

WALKER, J. L., PIEDMONTE, M. R., SPIRTOS, N. M., EISENKOP, S. M., SCHLAERTH, J. B., MANNEL, R. S., SPIEGEL, G., BARAKAT, R., PEARL, M. L. & SHARMA 2009. Laparoscopy compared with laparotomy for comprehensive surgical staging of uterine cancer: Gynecologic Oncology Group Study LAP2. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*.

WALTZ, E. 2007. Pricey cancer genome project struggles with sample shortage. *Nat Med,* 13**,** 391.

WAN, Y. L., BEVERLEY-STEVENSON, R., CARLISLE, D., CLARKE, S., EDMONDSON, R. J., GLOVER, S., HOLLAND, J., HUGHES, C., KITCHENER, H. C., KITSON, S., MILES, T., MORLEY, R., MORRISON, J., NELSON, L., POWELL, M., SADLER, L., TOMLINSON, A., TYLKO-HILL, K., WHITCOMBE, J. & CROSBIE, E. J. 2016. Working together to shape the endometrial cancer research agenda: The top ten unanswered research questions. *Gynecol Oncol,* 143**,** 287-293.

WANG, DU, Z. H., QIAO, J. M. & GAO 2020. Association between metabolic syndrome and endometrial cancer risk: a systematic review and meta-analysis of observational studies. *Aging*.

WANG, C. & MEIER, U. T. 2004. Architecture and assembly of mammalian H/ACA small nucleolar and telomerase ribonucleoproteins. *Embo j,* 23**,** 1857-67.

WANG, C., ZHAO, L. & LU, S. 2015. Role of TERRA in the regulation of telomere length. *Int J Biol Sci,* 11**,** 316-23.

WANG, F., PODELL, E. R., ZAUG, A. J., YANG, Y., BACIU, P., CECH, T. R. & LEI, M. 2007. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature,* 445**,** 506-10.

WANG, Q., WANG, Q., ZHAO, L., HAN, L., SUN, C., MA, S., HOU, H., SONG, Q. & LI, Q. 2019. Endometrial Cytology as a Method to Improve the Accuracy of Diagnosis of Endometrial Cancer: Case Report and Meta-Analysis. *Front Oncol,* 9**,** 256.

WANG, S.-J., SAKAMOTO, T., YASUDA, S.-I., FUKASAWA, I., OTA, Y., HAYASHI, M., OKURA, T., ZHENG, J.-H. & INABA, N. 2002a. The Relationship between Telomere Length and Telomerase Activity in Gynecologic Cancers. *Gynecologic Oncology,* 84**,** 81-84.

WANG, S. J., SAKAMOTO, T., YASUDA SI, S., FUKASAWA, I., OTA, Y., HAYASHI, M., OKURA, T., ZHENG, J. H. & INABA, N. 2002b. The relationship between telomere length and telomerase activity in gynecologic cancers. *Gynecol Oncol,* 84**,** 81-4.

WANG, Y., LI, L., DOUVILLE, C., COHEN, J. D., YEN, T. T., KINDE, I., SUNDFELT, K., KJAER, S. K., HRUBAN, R. H., SHIH, I. M., WANG, T. L., KURMAN, R. J., SPRINGER, S., PTAK, J., POPOLI, M.,

SCHAEFER, J., SILLIMAN, N., DOBBYN, L., TANNER, E. J., ANGARITA, A., LYCKE, M., JOCHUMSEN, K., AFSARI, B., DANILOVA, L., LEVINE, D. A., JARDON, K., ZENG, X., ARSENEAU, J., FU, L., DIAZ, L. A., JR., KARCHIN, R., TOMASETTI, C., KINZLER, K. W., VOGELSTEIN, B., FADER, A. N., GILBERT, L. & PAPADOPOULOS, N. 2018. Evaluation of liquid from the Papanicolaou test and other liquid biopsies for the detection of endometrial and ovarian cancers. *Sci Transl Med,* 10.

WANG, Z., RHEE, D. B., LU, J., BOHR, C. T., ZHOU, F., VALLABHANENI, H., DE SOUZA-PINTO, N. C. & LIU, Y. 2010. Characterization of oxidative guanine damage and repair in mammalian telomeres. *PLoS genetics,* 6**,** e1000951-e1000951.

WATSON, P., VASEN, H. F. A., MECKLIN, J. P., BERNSTEIN, I., AARNIO, M., JÄRVINEN, H. J., MYRHØJ, T., SUNDE, L., WIJNEN, J. T. & LYNCH, H. T. 2008. The risk of extra-colonic, extra-endometrial cancer in the Lynch syndrome. *Int J Cancer,* 123**,** 444-449.

WEBB, C. J. & ZAKIAN, V. A. 2016. Telomerase RNA is more than a DNA template. *RNA Biol,* 13**,** 683-9.

WEIGELT, B. & BANERJEE, S. 2012. Molecular targets and targeted therapeutics in endometrial cancer. *Curr Opin Oncol,* 24**,** 554-63.

WEINRICH, S. L., PRUZAN, R., MA, L., OUELLETTE, M., TESMER, V. M., HOLT, S. E., BODNAR, A. G., LICHTSTEINER, S., KIM, N. W., TRAGER, J. B., TAYLOR, R. D., CARLOS, R., ANDREWS, W. H., WRIGHT, W. E., SHAY, J. W., HARLEY, C. B. & MORIN, G. B. 1997. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet,* 17**,** 498-502.

WELLCOME TRUST CASE CONTROL CONSORTIUM 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature,* 447**,** 661-78.

WERNER, H. M., TROVIK, J., MARCICKIEWICZ, J., TINGULSTAD, S., STAFF, A. C., ENGH, M. E., ODDENES, K., ROKNE, J. A., TJUGUM, J., LODE, M. S., AMANT, F. & SALVESEN, H. B. 2013. A discordant histological risk classification in preoperative and operative biopsy in endometrial cancer is reflected in metastatic risk and prognosis. *Eur J Cancer,* 49**,** 625-32.

WHITAKER, L. H., MURRAY, A. A., MATTHEWS, R., SHAW, G., WILLIAMS, A. R., SAUNDERS, P. T. & CRITCHLEY, H. O. D. 2017. Selective progesterone receptor modulator (SPRM) ulipristal acetate (UPA) and its effects on the human endometrium. *Human reproduction (Oxford, England)*.

WIDSCHWENDTER, A., MÜLLER, H. M., HUBALEK, M. M., WIEDEMAIR, A., FIEGL, H., GOEBEL, G., MUELLER-HOLZNER, E., MARTH, C. & WIDSCHWENDTER, M. 2004. Methylation status and expression of human telomerase reverse transcriptase in ovarian and cervical cancer. *Gynecol Oncol,* 93**,** 407-16.

WIEGAND, K. C., SHAH, S. P., AL-AGHA, O. M., ZHAO, Y., TSE, K., ZENG, T., SENZ, J., MCCONECHY, M. K., ANGLESIO, M. S., KALLOGER, S. E., YANG, W., HERAVI-MOUSSAVI, A., GIULIANY, R., CHOW, C., FEE, J., ZAYED, A., PRENTICE, L., MELNYK, N., TURASHVILI, G., DELANEY, A. D., MADORE, J., YIP, S., MCPHERSON, A. W., HA, G., BELL, L., FEREDAY, S., TAM, A., GALLETTA, L., TONIN, P. N., PROVENCHER, D., MILLER, D., JONES, S. J., MOORE, R. A., MORIN, G. B., OLOUMI, A., BOYD, N., APARICIO, S. A., SHIH IE, M., MES-MASSON, A. M., BOWTELL, D. D., HIRST, M., GILKS, B., MARRA, M. A. & HUNTSMAN, D. G. 2010. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med,* 363**,** 1532-43.

WILD, C. P., SCALBERT, A. & HERCEG, Z. 2013. Measuring the exposome: a powerful basis for evaluating environmental exposures and cancer risk. *Environ Mol Mutagen,* 54**,** 480-99.

WILLIAMS, C. D., BOGGESS, J. F., LAMARQUE, L. R., MEYER, W. R., MURRAY, M. J., FRITZ, M. A. & LESSEY, B. A. 2001. A prospective, randomized study of endometrial telomerase during the menstrual cycle. *J Clin Endocrinol Metab,* 86**,** 3912-7.

WINER, I., AHMED, Q. F., MERT, I., BANDYOPADHYAY, S., COTE, M., MUNKARAH, A. R., HUSSEIN, Y., AL-WAHAB, Z., ELSHAIKH, M. A., ALOSH, B., SCHULTZ, D. S., MAHDI, H., NUCCI, M. R., VAN DE VIJVER, K. K., MORRIS, R. T., OLIVA, E. & ALI-FEHMI, R. 2015. Significance of lymphovascular

space invasion in uterine serous carcinoma: what matters more; extent or presence? *Int J Gynecol Pathol,* 34**,** 47-56.

WOLF, C., JARUTAT, T., VEGA HARRING, S., HAUPT, K., BABITZKI, G., BADER, S., DAVID, K., JUHL, H. & ARBOGAST, S. 2014. Determination of phosphorylated proteins in tissue specimens requires high-quality samples collected under stringent conditions. *Histopathology,* 64**,** 431-44.

WYERS, F., ROUGEMAILLE, M., BADIS, G., ROUSSELLE, J. C., DUFOUR, M. E., BOULAY, J., RÉGNAULT, B., DEVAUX, F., NAMANE, A., SÉRAPHIN, B., LIBRI, D. & JACQUIER, A. 2005. Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell,* 121**,** 725-37.

WYNN, R. M. 1989. The Human Endometrium. *In:* WYNN, R. M. & JOLLIE, W. P. (eds.) *Biology of the Uterus.* Boston, MA: Springer US.

XI, L. & CECH, T. R. 2014. Inventory of telomerase components in human cells reveals multiple subpopulations of hTR and hTERT. *Nucleic acids research,* 42**,** 8565-8577.

XUE, Y., MARVIN, M. E., IVANOVA, I. G., LYDALL, D., LOUIS, E. J. & MARINGELE, L. 2016. Rif1 and Exo1 regulate the genomic instability following telomere losses. *Aging Cell,* 15**,** 553-562.

YANG, Q. 2008. Cellular senescence, telomere recombination and maintenance. *Cytogenet Genome Res,* 122**,** 211-8.

YANG, S., JIA, Y., LIU, X., WINTERS, C., WANG, X., ZHANG, Y., DEVOR, E. J., HOVEY, A. M., REYES, H. D., XIAO, X., XU, Y., DAI, D., MENG, X., THIEL, K. W., DOMANN, F. E. & LESLIE, K. K. 2014. Systematic dissection of the mechanisms underlying progesterone receptor downregulation in endometrial cancer. *Oncotarget,* 5**,** 9783-97.

YE, J. Z., DONIGIAN, J. R., VAN OVERBEEK, M., LOAYZA, D., LUO, Y., KRUTCHINSKY, A. N., CHAIT, B. T. & DE LANGE, T. 2004. TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J Biol Chem,* 279**,** 47264-71.

YEHEZKEL, S., SEGEV, Y., VIEGAS-PÉQUIGNOT, E., SKORECKI, K. & SELIG, S. 2008. Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. *Hum Mol Genet,* 17**,** 2776-89.

YILDIZ-AKTAS, I. Z., DABBS, D. J. & BHARGAVA, R. 2012. The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. *Mod Pathol,* 25**,** 1098-105.

YOKOI, S., YASUI, K., IIZASA, T., IMOTO, I., FUJISAWA, T. & INAZAWA 2003. TERC identified as a probable target within the 3q26 amplicon that is detected frequently in non-small cell lung cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research*.

YOO, H. H. & CHUNG, I. K. 2011. Requirement of DDX39 DEAD box RNA helicase for genome integrity and telomere protection. *Aging Cell,* 10**,** 557-71.

YOUNG, S. L. 2013. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reprod Biomed Online,* 27**,** 497-505.

YU, T. Y., KAO, Y. W. & LIN, J. J. 2014. Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase. *Proc Natl Acad Sci U S A,* 111**,** 3377-82.

YUILLE, M., VAN OMMEN, G. J., BRECHOT, C., CAMBON-THOMSEN, A., DAGHER, G., LANDEGREN, U., LITTON, J. E., PASTERK, M., PELTONEN, L., TAUSSIG, M., WICHMANN, H. E. & ZATLOUKAL, K. 2008. Biobanking for Europe. *Brief Bioinform,* 9**,** 14-24.

ZAINO, R. J., KURMAN, R. J., DIANA, K. L. & MORROW 1995. The utility of the revised International Federation of Gynecology and Obstetrics histologic grading of endometrial adenocarcinoma using a defined nuclear grading system. A Gynecologic Oncology Group study. *Cancer,* 75.

ZANDER, J., BRUEGEL, M., KLEINHEMPEL, A., BECKER, S., PETROS, S., KORTZ, L., DOROW, J., KRATZSCH, J., BABER, R., CEGLAREK, U., THIERY, J. & TEUPSER, D. 2014. Effect of biobanking conditions on short-term stability of biomarkers in human serum and plasma. *Clin Chem Lab Med,* 52**,** 629-39.

ZANG, H., SAHLIN, L., MASIRONI, B., ERIKSSON, E. & LINDEN HIRSCHBERG, A. 2007. Effects of testosterone treatment on endometrial proliferation in postmenopausal women. *J Clin Endocrinol Metab,* 92**,** 2169-75.

ZENG, S., LIU, L., SUN, Y., LU, G. & LIN, G. 2017. Role of telomeric repeat-containing RNA in telomeric chromatin remodeling during the early expansion of human embryonic stem cells. *FASEB J,* 31**,** 4783-4795.

ZHANG, J. M., YADAV, T., OUYANG, J., LAN, L. & ZOU, L. 2019. Alternative Lengthening of Telomeres through Two Distinct Break-Induced Replication Pathways. *Cell Rep,* 26**,** 955-968.e3.

ZHANG, Y., CHEN, L.-Y., HAN, X., XIE, W., KIM, H., YANG, D., LIU, D. & SONGYANG, Z. 2013. Phosphorylation of TPP1 regulates cell cycle-dependent telomerase recruitment. *Proceedings of the National Academy of Sciences,* 110**,** 5457-5462.

ZHONG, Z. H., JIANG, W. Q., CESARE, A. J., NEUMANN, A. A., WADHWA, R. & REDDEL, R. R. 2007. Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. *J Biol Chem,* 282**,** 29314-22.

ZHOU, J. M., ZHU, X. F., LU, Y. J., DENG, R., HUANG, Z. S., MEI, Y. P., WANG, Y., HUANG, W. L., LIU, Z. C., GU, L. Q. & ZENG, Y. X. 2006. Senescence and telomere shortening induced by novel potent G-quadruplex interactive agents, quindoline derivatives, in human cancer cell lines. *Oncogene,* 25**,** 503-511.

ZHOU, L., CHEN, B., HUA, X., ZHOU, P., GUO, L., PENG, Y. & QIU, K. 2013. Effect of newly identified hTERT-interacting proteins on telomerase activity. *Acta Biochim Biophys Sin (Shanghai),* 45**,** 674-82.

ZHU, X., KUMAR, R., MANDAL, M., SHARMA, N., SHARMA, H. W., DHINGRA, U., SOKOLOSKI, J. A., HSIAO, R. & NARAYANAN, R. 1996. Cell cycle-dependent modulation of telomerase activity in tumor cells. *Proc Natl Acad Sci U S A,* 93**,** 6091-5.

ZHU, Y., HAN, Y., TIAN, T., SU, P., JIN, G., CHEN, J. & CAO, Y. 2018. MiR-21-5p, miR-34a, and human telomerase RNA component as surrogate markers for cervical cancer progression. *Pathol Res Pract,* 214**,** 374-379.

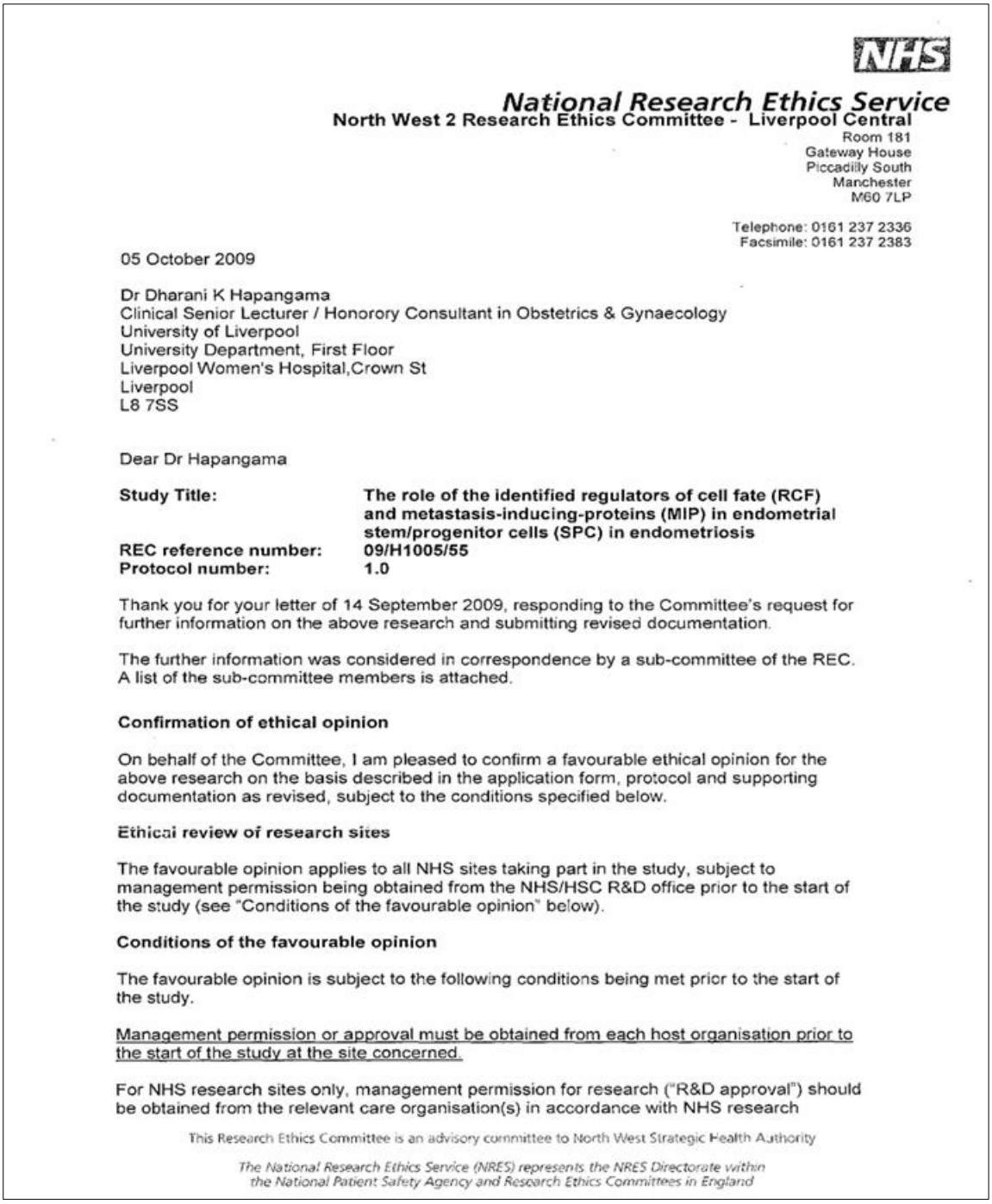
ZOU, L. & ELLEDGE, S. J. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science,* 300**,** 1542-8.

Appendices:

(i) Ethical approvals
(ii) Patient information leaflet
(iii) Consent form
(iv) Presentations and abstracts
(v) Publications
(vi) EC research tools
(vii) Published first author papers

(i) Ethical Approvals

LREC 09/H1005/55



NHS

National Research Ethics Service
North West 2 Research Ethics Committee - Liverpool Central

Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2336
Facsimile: 0161 237 2383

05 October 2009

Dr Dharani K Hapangama
Clinical Senior Lecturer / Honorory Consultant in Obstetrics & Gynaecology
University of Liverpool
University Department, First Floor
Liverpool Women's Hospital,Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study Title: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis**
REC reference number: **09/H1005/55**
Protocol number: **1.0**

Thank you for your letter of 14 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

This Research Ethics Committee is an advisory committee to North West Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		
REC application	2.2	
Protocol	1.0	02 July 2009
Investigator CV		
GP/Consultant Information Sheets		
Letter from Sponsor		02 July 2009
Referees or other scientific critique report		
Participant Information Sheet	2	09 September 2009
Participant Consent Form	2	09 September 2009
poster healthy + baby		
poster endometriosis		
Response to Request for Further Information		14 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

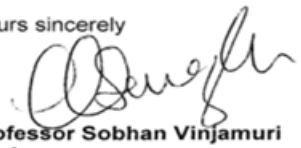
The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/55	Please quote this number on all correspondence

Yours sincerely



Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Mrs Gillian Vernon*

11/H1005/4

Liverpool Women's NHS
NHS Foundation Trust

Crown Street
Liverpool
L8 7SS

Tel: 0151 708 9988
www.lwh.nhs.uk

14th April 2011

Dr Dharani Hapangama
Clinical Senior Lecturer / Honorary Consultant in O&G
Liverpool Women's Hospital
University Department, First Floor
Crown Street
Liverpool L8 7SS

Direct dial: 0151 702 4346
Email: Gillian.vernon@lwh.nhs.uk

Dear Dharani

ID: LWH0877 – Study of the Role of Metastasis Inducing Proteins and cell fate regulators in the pathogenesis of Endometrial Cancer

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by the R&D Director. This approval relates to the documentation listed below:

- Ethics approval letter [11/H1005/4] dated 11th April 2011
- Protocol [version 1.0] 9th December 2010

The research is registered on the Trust's R&D database under the reference LWH0877, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) are the Trust and the University of Liverpool.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles and guidelines set out in ICH Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf.

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely

Vernon

Gillian Vernon
Research & Development Manager

Liverpool Women's

CREC 10/H0308/75

NHS

National Research Ethics Service

Cambridgeshire 2 Research Ethics Committee

Victoria House
Capital Park
Fulbourn
Cambridge
CB21 5XB

Telephone: 01223 597685
Facsimile: 01223 597645

18 October 2010

Dr Helen Stringfellow
Lancashire Teaching Hospitals NHS Trust
Pathology, Royal Preston Hospital
Sharoe Green Lane
PR2 9HT

Dear Dr Stringfellow

Title of the Research Tissue Bank: **Archival genito-urinary tissue, blood, urine and saliva collection**
REC reference: **10/H0308/75**
Designated Individual: **Dr Timothy P Dawson**

Thank you for your letter of 30 September 2010, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 15 October 2010. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
REC application	58126/139495/3/344	30 July 2010
Covering Letter	Dr Helen Stringfellow	30 July 2010

This Research Ethics Committee is an advisory committee to East of England Strategic Health Authority

Participant Information Sheet	2	30 September 2010
Response to Request for Further Information	Dr Helen Stringfellow	30 September 2010
Participant Consent Form	2	30 September 2010
Human Tissue Authority Licence		14 February 2008
Protocol for Management of the Tissue Bank	1	30 July 2010

Licence from the Human Tissue Authority
Thank you for providing a copy of the above licence.

Research governance

A copy of this letter is being sent to the R&D office responsible for Royal Preston Hospital. You are advised to check their requirements for approval of the research tissue bank.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by a research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Annual Reports. Please refer to the attached conditions of approval.
c) Amendments. Please refer to the attached conditions of approval.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk

10/H0308/75	**Please quote this number on all correspondence**

Yours sincerely

pp Nstorey

Dr Rowan Burnstein
Chair

E-mail: Nicky.Storey@ece.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

Standard approval conditions

Copy to: Dr Timothy Dawson
Lancashire Teaching Hospitals NHS Trust
Pathology, Royal Preston Hospital
Sharoe Green Lane
Preston
Lancashire
PR2 9HT

(ii) **Patient information leaflet**

UNIVERSITY OF LIVERPOOL

Liverpool Women's NHS
NHS Foundation Trust

Ethics Submission No:

PATIENT INFORMATION SHEET

"MIPs in Endometrial Cancer Study"

Role of metastasis-inducing-proteins in endometrial cancer

Version 1: For patients undergoing hysterectomy.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

Why are we doing the study?

Each year in the UK about 4500 women, commonly in their 50s and 60s, develop cancer of the lining of the womb (endometrium). The 'endometrium' builds up and is then shed each month as a 'period' before menopause. In women who unfortunately develop cancer, the cells in the endometrium multiply and behave abnormally. The survival rate of early stage endometrial cancer is good, however in extreme cases cancer cells can spread beyond the womb affecting the overall outcome of the disease. We are doing this study to better understand the changes that happen in endometrial cells. This will help us discover new targets to diagnose and design new treatment for endometrial cancer.

What is metastasis?

The spread of cancer cells beyond their origin, the womb, is called 'metastases'. This metastatic process is closely linked with the outcome of the disease, but is not fully understood. It is possible that there are special proteins that can encourage cancer cells to spread. These specific proteins are called 'metastasis-inducing-proteins' (MIPs), which we believe can cause cancer cells to invade healthy tissue. We would like to investigate the presence of these proteins in endometrial cancer cells and the role they play.

Why have I been chosen?

We are looking for a total of 160 women who are undergoing hysterectomy. We are specifically looking for 80 women who have endometrial cancer and another 80 healthy women undergoing surgery. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. The decision to not take part or withdraw at any time, will **NOT** affect the standard of care you receive.

What will happen to me if I take part?
Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed from you will be taken for the research.
Therefore, **NO** extra surgery or procedures will be performed for the study. A **blood sample** will also be taken from your veins.

What are the possible benefits of taking part?
We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometrial cancer.

What if something goes wrong?
We do not anticipate any harm to arise while taking part in this study, as we are not carrying out any additional procedures. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action. There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management
The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research, will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Surplus tissue
You can chose to give consent for any remaining tissue, after being used for this study, to be anonymised and stored in the Liverpool Women's Hospital which can be used for future ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact **Mr Jonathan Herod, Consultant Gynaecology Oncologist, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.**

(iii) Consent

Liverpool Women's NHS
NHS Foundation Trust

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: The role of metastasis-inducing-proteins (MIP) in endometrial cancer

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree for surplus tissue to be stored in the department of obstetrics & Gynaecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Version 1.1 6th April 2011

(iv) Presentation and abstracts

Oral – International

‘TERRA expression in human endometrium and in endometrial proliferative pathologies’, presented in ENITEC meeting , Liverpool 14 th June 2019.

‘Human endometrium demonstrates a dynamic TERRA expression and hormone regulation: implications in endometrial proliferative conditions’, ESHRE 2018 Annual Meeting in Barcelona, 1-4 July 2018.

‘Harmonisation of bio banking standards in Endometrial Cancer research’, ENITEC, Freiburg, Germany, Jun 2016

Oral – Regional

‘Effect of pre analytical variables on gene expression in endometrial samples for endometrial cancer research’, Live poster presentation BGCS May 2021

‘TERRAs: In health and in disease, the human endometrial perspective’, NOEGS, March 2018.

‘Developing Tools and Standard Operating Procedures for Data and Bio-banking Sample Collection in Endometrial Cancer Research’, NOEGS, Nov 2016.

Posters

Posters – International / National

Effect of preanalytical variables on endometrial gene expression- live poster presentation, BGCS 2021 annual scientific meeting, May 13-14, 2021

Long non-coding RNA, TERRA is downregulated in endometrial cancer, Annual academic meeting, RCOG, Feb 2018.

Long non-coding RNA, telomeric repeat containing RNAs are downregulated in endometrial cancer, ESGO, Vienna, Nov 2017.

Harmonisation of biobAnking STandards in Endometrial caNcer research (HASTEN), ESGO, Vienna, Nov 2017.

Harmonisation of biobAnking STandards in Endometrial caNcer research (HASTEN), Annual academic meeting, RCOG, Mar 2017.

Harmonisation of biobAnking STandards in Endometrial caNcer research (HASTEN), North of England meeting, Nov 2016.

(v) Publications

Research and Review Articles

- Maclean A, **Adishesh M**, Button L, Richards L, Alnafakh R, Newton E, Drury J, Hapangama DK. The effect of pre-analytical variables on downstream application and data analysis of human endometrial biopsies. Hum Reprod Open. 2022 Jun 13;2022(3):hoac026. doi: 10.1093/hropen/hoac026. PMID: 35775066; PMCID: PMC9240853.

- Alnafakh R, Choi F, Bradfield A, **Adishesh M**, Saretzki G, Hapangama DK. Endometriosis Is Associated with a Significant Increase in *hTERC* and Altered Telomere/Telomerase Associated Genes in the Eutopic Endometrium, an Ex-Vivo and In Silico Study. Biomedicines. 2020 Dec 9;8(12):588. doi: 10.3390/biomedicines8120588. PMID: 33317189; PMCID: PMC7764055.

- **Adishesh M**, Alnafakh R, Baird DM, Jones RE, Simon S, Button L, Kamal AM, Kirwan J, DeCruze SB, Drury J, Saretzki G, Hapangama DK. Human Endometrial Carcinogenesis Is Associated with Significant Reduction in Long Non-Coding RNA, TERRA. Int J Mol Sci. 2020 Nov 18;21(22):8686. doi: 10.3390/ijms21228686. PMID: 33217925; PMCID: PMC7698627

- Maclean A, Kamal A, **Adishesh M**, Alnafakh R, Tempest N, Hapangama DK. Human Uterine Biopsy: Research Value and Common Pitfalls. *Int J Reprod Med*. 2020;2020:9275360. Published 2020 Apr 28. doi:10.1155/2020/9275360

- Alnafakh RAA, **Adishesh M**, Button L, Saretzki G, Hapangama DK. Telomerase and Telomeres in Endometrial Cancer. *Front Oncol*. 2019;9:344. Published 2019 May 17. doi:10.3389/fonc.2019.00344

- **Adishesh, M**.; Hapangama, D.K. Enriching Personalized Endometrial Cancer Research with the Harmonization of Biobanking Standards. *Cancers* **2019**, *11*, 1734.

- **Adishesh M**, Fyson A, DeCruze SB, Kirwan J, ENITEC Consortium, Werner HMJ, Hapangama DK. Harmonisation of biobanking standards in endometrial cancer research. Br J Cancer. 29 June 2017. doi: 10.1038/bjc.2017.194

- Kamal A, Tempest N, Parkes C, Alnafakh R, Makrydima S, **Adishesh M** and Hapangama DK. Hormones and endometrial carcinogenesis. Horm Mol Biol Clin Invest 2016. DOI 10.1515/hmbci-2016-0005, Received January 12, 2016; accepted February 5, 2016

Book Chapters

Kamal A, Tempest N, Maclean A, **Adishesh M**, Bhullar J, Makrydima S and Dharani K. Hapangama (2020) 'Hormonal interactions in endometrial cancer' in Mirza, M. R. *Management of Endometrial Cancer*, 69-100 by Springer doi: 10.1007/978-3-319-64513-1

Under review

Kamal A, Tempest N, Makrydima S, **Adishesh M**, Bhullar J, McLean A, Hapangama DK. Hormone receptor expression in healthy endometrium and in endometrial cancer, chapter in Advances in Experimental Medicine and Biology (AEMB) series by Springer.

(vi) EC Research Tools

EC research tools as published in 'Adishesh, M., Fyson, A., DeCruze, S. *et al.* Harmonisation of biobanking standards in endometrial cancer research. *Br J Cancer* **117,** 485–493 (2017). https://doi.org/10.1038/bjc.2017.194'

1. ECPD
2. ECSD Minimal
3. ECSD Standard
4. ECBS
5. SOP-ECBS

1. ECPD

Study no.- _____ Date - _____

Endometrial Cancer Patient Data (ECPD) Collection Tool:

The doctors and nurses at the Hospital are collecting information from patients who are undergoing tests and treatment for endometrial cancer, along with samples for cancer research. We hope to use this information to improve treatments for patients in the future. We would be very grateful if you could complete this short questionnaire before you have your operation. The questionnaire will take 10 minutes to complete. If you require help to complete the questionnaire please ask a relative, friend or a nurse or doctor.

Thank you for your cooperation.

Age - _____ Ethnicity - _____

Weight - _____ Weight at 18 yrs of age (approximate) - _____Height- _____

Have your periods stopped? Yes/ No

Age periods stopped (menopause)? ____

What age did your periods start?_____

Were /are your periods- regular / irregular

How many days were/are in between your periods? ____

How many days do/did you bleed for? ____

Date of last menstrual period -

How many pregnancies have you had? ____

How many children do you have? ____

How old were you when you had your first pregnancy? _____

Did you have any problems becoming pregnant? Yes / No

Have you ever had IVF? Yes / No

Have you ever had any serious infectious disease? - Nil / HIV / Hepatitis B / Hepatitis C / Syphilis

Have you ever had pelvic inflammatory disease? - Yes / No

Is there a family history of cancer in any of your first degree relatives (eg. Mother/father/sister)? - None / Bowel / Breast / Ovarian / Thyroid / Other

_____

Please turn over

Study no.- _____ Date - _____

Have you had cancer in the past? – Yes / No

If Yes - Bowel / Breast / Ovarian / Thyroid / Other_____

Please circle any of these medical conditions that you have and inform us of any other medical conditions you have been diagnose with – Type I Diabetes / Type 2 Diabetes / Polycystic ovarian syndrome / Thyroid disorders / High Blood Pressure/ High cholesterol

_____

_____

Please circle any of these medications that you have ever been on and inform us of any other medications you are currently on – Oral Progesterone/ Mirena/Contraceptive pill / Tamoxifen / Metformin / HRT / Other

_____

_____

Do you smoke? Yes / No / Ex smoker / E-cigarette

If you have ever smoked how many did/do you smoke a day?_____ For how many yrs?_____

Do you drink any alcohol? – Yes / No/ In the past

If you have ever drunk alcohol, type of drink and approximately how much would you drink per week? _____ (e.g. 2 glasses of wine per week or 1 bottle of beer per week)

How often do you exercise (any form for 30 mins or above)? Daily / Once or twice a week / Once a month / Less than once a month / stopped / Never

If stopped, the reason for stopping:

_____

What investigations you have prior to diagnosis of endometrial cancer? Ultrasound / Hysteroscopy and biopsy (camera and biopsy) / Biopsy only / CT scan / MRI scan /Not known

Many Thanks for providing the above valuable and confidential information, this will be stored in our biobank database and will be used for cancer research purposes only.

2. ECSD Minimal

Endometrial Cancer Surgical Data (ECSD) Collection Tool – Minimal

Study ID no:

Age _____
Parity_____
BMI _____

Presenting symptom – PMB / None

Antecedental endometrial biopsy if definitive treatment:
Yes ☐ No ☐
If Yes – date of biopsy ___/__/__

Operation –
TLH/LAVH/TAH/VH
Diagnostic / Curative / Palliative
Findings: Uterus Normal ☐ Abnormal ☐
Tubes Normal ☐ Abnormal ☐
Ovaries Normal ☐ Cysts☐ Abnormal☐
Extrauterine extension –
Cervical involvement☐
Adnexae / Parametrium☐
Lymph Nodes☐
Locoregional metastasis (rectum/bladder) ☐
Bowel☐Liver☐Omentum☐

Histopathologic type –
Endometrioid / serous / clear cell / mucinous /
carcinosarcoma/undifferentiated/Mixed /Other_____
Grade – 1 / 2 / 3
LVSI present☐ LVSI absent ☐
FIGO stage – I / II / III / IV
Nodes sampled: No/ Yes, Pelvic ☐ Number of nodes - ___, Positive___ /
Negative___
Para aortic ☐ Number of nodes - ___, Positive___ / Negative___
Biomarkers performed: Yes ☐ / No ☐
P53 ☐ PTEN ☐ ER ☐ PR ☐ HER 2 ☐ P16 ☐ MLH1 ☐
MSH2 ☐ MSH6 ☐ PMS2 ☐ KRAS☐ PIK3Ca ☐ HE4 ☐ Stathmin
☐ L1CAM ☐
Status/ score:_____

Sample collection –
Timing of sample collection – At time of diagnosis ☐
Primary surgery☐
Relapse☐

Samples in:
NBF ☐ PBS☐ RNA later☐ Snap frozen☐

Sample type

☐ Uterine - Pipelle ☐ Currettings☐ surgical resection ☐
Dt __/__/__ Time __:__
☐ Extra uterine Dt __/__/__ Time __:__
☐ Urine Dt __/__/__ Time __:__
☐ Blood Dt __/__/__ Time __:__
☐ Endometrial fluid Dt __/__/__ Time __:__
☐ Peritoneal or ascitic fluid Dt __/__/__ Time __:_

Primary treatment – Surgery☐ Radiotherapy☐ Chemotherapy ☐

Outcome for patient: Date of last cancer follow up__/__/__
Date of Death __/__/__
Recurrence- Local / distant /unknown, Date __/__/__

3. ECSD Standard

Sample ID - _____ Date - _____

Endometrial Cancer Surgical Data (ECSD) Collection Tool - Standard:

Age _____ Parity_____
Performance status – 0 / 1 / 2 / 3
BMI _____

Presenting symptom – PMB / Other

Imaging –
US /MRI / PET CT
Findings: Tumour size _____ cm(MRI or USS)
Myometrial invasion <50%☐ >50% ☐
Extrauterine extension - Cervical involvement☐
Adnexae☐
Lymph Nodes☐
Locoregional metastasis (rectum/bladder) ☐
Distant metastasis☐
Other☐

Antecedent endometrial biopsy if definitive treatment:
Yes ☐ No ☐ date of biopsy ___/__/__

Operation –
TLH / LAVH / TAH /VH/Hysteroscopy
Anaesthetic time __:__ hrs
Diagnostic / Curative / Palliative
Findings Uterus - Normal ☐ Abnormal ☐
Tubes - Normal ☐ Abnormal ☐
Ovaries - Normal ☐ Cysts☐ Abnormal☐
Extrauterine extension - Cervical involvement☐
Adnexae / Parametrium☐
Lymph Nodes☐
Locoregional metastasis (rectum/bladder) ☐
Bowel☐Liver☐Omentum☐

Histopathologic type –
Endometrioid / serous / clear cell / mucinous /
carcinosarcoma/undifferentiated/Mixed /Other_____
Grade – 1 / 2 / 3
Lymphovascular invasion Present☐ Absent☐
FIGO stage – I / II / III / IV
Nodes sampled: No/ Yes, Pelvic☐ Number of nodes - _____ , Positive___ /
Negative ___

Sample ID - _____ Date - _____

Para aortic☐ Number of nodes - _____ , Positive___ / Negative ___

Biomarkers performed: Yes ☐ / No ☐
P53 ☐ PTEN ☐ ER ☐ PR ☐ HER 2 ☐ P16 ☐ MLH1 ☐ MSH2 ☐
MSH6 ☐ PMS2 ☐ KRAS☐ PIK3Ca ☐ HE4 ☐ Stathmin ☐ L1CAM ☐
Status/ score:_____

Sample collection –
Timing of sample collection – At time of diagnosis ☐
Primary surgery☐
Relapse☐

Samples in:
NBF ☐ PBS☐ RNA later☐ Snap frozen ☐

Sample type collected

A – Uterine - Date __/__/__ Time __:__
-Tissue / aspirate
-Hysteroscopic/ laparoscopic / open
-Medium used - saline /gas /glycine
-Location of sample collection - outpatient / general anaesthetic
-Pipelle/ curettage/ after hysterectomy
-Collected - Prior to uterine manipulator/ pre hysteroscopy /
Post hysteroscopy/ post hysterectomy

B – Extra uterine - Date __/__/__ Time __:__
-Tissue from metastatic lesion – source_____
-Laparoscopic / open
-Collected - Prior to uterine manipulator/ pre hysteroscopy /
Post hysteroscopy/ post hysterectomy

C – Urine –Collected – Date __/__/__ Time __:__
Collected pre theatre / intraoperative / post theatre
Mid stream sample / catheter sample

D - Blood–Collected – Date __/__/__ Time __:__
Collected pre theatre / intraoperative / post theatre

E – Peritoneal fluid – Date __/__/__ Time __:__
Collected at laparoscopy / open procedure

Sample ID - _____ Date - _____

F – Ascitic fluid – Date __/__/__ Time __:__
Collected at paracentesis / laparoscopy / open procedure

Primary treatment – Surgery☐ Radiotherapy☐ Chemotherapy ☐

Outcome for patient: Date of last cancer follow up__/__/__
Date of Death __/__/__
Recurrence- Local / distant /unknown, Date __/__/__

4. ECBS

Endometrial Cancer Biospecimen (ECBS) tool: To be filled by lab staff

Sample ID:_____

Processing and Storage details

Samples received: Date __/__/__ Time __:__hrs

Tissue - Endometrium / Extraendometrial		Blood		Endometrial fluid		Peritoneal /ascitic fluid		Urine		Saliva	
NBF		Whole		Plain		Plain		Plain		Plain	
Culture media		Serum		RNA stabilisation additives		RNA stabilisation additives		RNA stabilisation additives		RNA stabilisation additives	
RNA Later											
Frozen tissue											
Other (state)											

Details of tissue processing:

Endometrium

Tissue Processing	Number of blocks/pieces of tissue	Processing start time & Date	Date & time in storage	Storage Location
Frozen Tissue				
RNA later				
Paraffin embedded				

Extra uterine tissue

Tissue Processing	Number of blocks/pieces of tissue	Processing start time & Date	Date & time in storage	Storage Location
Frozen Tissue				
RNA later				
Paraffin embedded				

Please turn over

Details of fluid processing:
Endometrial fluid

Number of Aliquots			Total Volume	Processing start time	Date & time in storage	Storage location
Amount						
50 µl	250 µl	1 ml				

Peritoneal / Ascitic fluid

Number of Aliquots			Total Volume	Processing start time	Date & time in storage	Storage location
Amount						
50 µl	250 µl	1 ml				

Blood samples

Number of Aliquots				Total Volume	Processing start time	Date & time in storage	Storage location
Amount	50 µl	250 µl	1 ml				
serum							
whole							
plasma							

Saliva samples

Number of Aliquots			Total Volume	Processing start time	Date & time in storage	Storage location
Amount						
50 µl	250 µl	1 ml				

Urine samples

Number of Aliquots			Total Volume	Processing start time	Date & time in storage	Storage location
Amount						
50 µl	250 µl	1 ml				

Any variations or deviations from the SOP, problems, or issues:

_____

_____

_____

_____

5. SOP – ECBS

Standard Operating Procedures for collection, processing and storage of the tissue, blood, urine, saliva and fluid samples for endometrial cancer research (SOP-ECBS):

Processing and storage materials:

Biospecimen form
Log sheet to record the sample data
Labels suitable for long term storage or Bar codes

For frozen tissues:
Cryosafe aliquot vials
RNA stabilising solution
Freezers -80°C
Liquid nitrogen (LN_2) for snap freeze
Crushed ice (for transport prior to processing)

For fixed tissues:
Neutral buffered formalin 10 % (NBF), universal molecular fixative, paraffin wax, cassettes

For blood:
Blood collection tubes with EDTA and heparin for plasma, SST or no SST for serum
Crushed ice
Racks to hold the tubes in upright position
Temperature controlled centrifuge
Pipettes
Cryogenic vials with screw top gasket closure
Freezers -80°C or liquid nitrogen (LN_2)

For urine:
Sterile urine collection container with leak proof cap
Crushed ice
Pipettes
Dipstick
Temperature controlled centrifuge
Aliquot vials with screw top gasket closure
Freezers -80°C or liquid nitrogen (LN_2)

For saliva:
Sterile saliva container or manufacturer's provided container for DNA
Crushed ice
Pipettes
Qiagen's RNA Protect saliva kit

Temperature controlled centrifuge
Aliquot vials with screw top gasket closure
Freezers -80°C or liquid nitrogen (LN_2)

For peritoneal fluid:
20 ml suction device or laparoscopic needle and a 20 ml syringe
Normal saline solution
Crushed ice
Transfer pipette
Volume adjustable pipette
Temperature controlled centrifuge
Aliquot vials with screw top gasket closure
Freezers – 80°C or colder / liquid nitrogen (LN_2)

For endometrial fluid or aspirates:
Embryo transfer catheter and a 20 ml syringe or Cornier pipelle
Normal saline solution
Crushed ice, liquid nitrogen (LN_2) or dry ice
Transfer pipette
Volume adjustable pipette
1.5 mL Eppendorf tubes
Temperature controlled centrifuge
Freezers - 80°C / liquid nitrogen (LN_2)

1 Collection

- Prepare all the necessary materials needed for storing and for recording data
- Prepare all vials / tubes for surgical collection. Pre label each collection vial with unique identifier, participant ID, Date, time of collection and type of sample.
- Record on the log sheet, date and time of collection.

A Tissue:

a. If samples are to be snap frozen immediately in liquid nitrogen (in a specific safe area near theatre) or once obtained, tissue samples should be placed in suitable pre-chilled containers on wet ice so they can be transported to the lab for processing (This will minimise autolysis of RNA and any *ex vivo* changes in RNA/protein expression profiles). Alternatively an RNA

stabilisation solution can be used directly but this will be at the expense of being able to visualise tissue morphology by frozen sectioning. For hysterectomy specimens, the uterus should be transferred from theatre to the pathology department as quickly as possible to be opened and sampled by a pathologist. Again the sampled tissue should be placed into pre-chilled tubes and stored on wet ice prior to processing.

b. Record type of tissue (uterine/ metastatic/ peritoneal/ myometrium/recurrent lesions (specify location)) collected: method used (curettage, endometrial sampling device, hysterectomy, hysteroscopy specimen, using cold scalpel or diathermy or harmonic device).
c. Record whether endometrium collected 1) prior to anaesthesia, 2) after sedation but before anaesthesia, 3) after anaesthesia.
d. Deposition of sample: Insert tissues in prepared, labelled vials / tubes and snap freeze as soon as possible in liquid nitrogen in / near theatre in their final vials. If delays >15 min are expected (especially if hysterectomy specimens), immerse tiny fragments in an RNA stabiliser solution if required. If sample needed for frozen section rather than RNA then freeze in liquid nitrogen cooled isopentane.

B Blood

a. Collect blood using standard withdrawal procedures and in adequate setting by a suitably qualified personnel.
b. Collect samples after fasting for at least 10 hours and record the fasting time on the log sheet.
c. Order of sample collection: 1) EDTA plasma, 2) SST serum, 3) other tube types.
d. Tubes with anti-coagulants e.g. EDTA and heparin, need to be inverted 8 -10 times and placed in the rack in an upright position.
e. Keep the samples at room temperature if processed within 1 h. Place them on ice or refrigerator if delay for more than 1 h. The samples must be processed within 4 h of collection.

C Urine

a. Samples collection method: Clean catch first morning void. Patients should be provided with instructions on how to collect a clean catch urine sample and provided with a sterile specimen container and a leak proof cap.
b. Samples should be stored on wet ice immediately. If collected at home, samples should be kept refrigerated and delivered in an ice pack to the clinic (at 4°C).

c. Fasting status at time of collection: Fasted since: __: __ hrs. (except plain water).
d. Record whether First Morning Void or Spot Urine. Any urination during the night if first morning void.

D Saliva

a. Samples collection method: Record whether patient fasted and the duration of fast (at least 6 h).
b. Record recent exposure to toothpaste, gum, cigarettes, alcohol, meal, medications (in the last 24 h). Minimum criteria for collection: 1 h after brushing teeth, at least 1 h after eating a meal, at least 12 h after last alcohol consumption, at least 20 min after last acidic food consumption e.g. citrus fruits / high sugar food).
c. Patient should rinse mouth before collection.
d. Collect sample without spitting, by drooling into the container. Showing mouth-watering images such as lemons can increase saliva production. If for DNA, use manufacturer's protocol.
e. Obtain 2 ml of saliva apart from foam / bubbles.

E Peritoneal fluid

a. Samples collection method: after premedication or anaesthesia, using 20 mls suction device, laparoscopic needle and manual aspiration using syringe.
b. If small volume of peritoneal fluid / no fluid, then wash the pelvis with 20 ml sterile normal saline solution using laparoscopic needle under direct visual control.
c. Record date, time, volume and method of sample collection.
d. Transfer sample in screw top vial on wet ice within 30 min.

F Endometrial fluid

a. Samples collection method:
    1. Before premedication or anaesthesia
    2. Aspiration method using embryo-transfer catheter or cornier pipelle
    3. Lavage method – Infuse 4 ml of saline into uterine cavity slowly and slowly withdraw.
b. Record date, time, volume and method of sample collection.
c. Insert the tip of the catheter into a 1.5 ml Eppendorf tube
d. Transfer sample in screw top vial on crushed ice as soon as possible.

2 Sample Processing:

- Pre label aliquots with identifier number followed by unique aliquot ID number, date and time of sample creation. Record type, number and volume of aliquots prepared.
- Record time of starting sample processing on log sheet.
- Store aliquots in liquid nitrogen freezers or -80°c or colder freezers,
- Any SOP deviations, problems should be recorded.
- A detailed sample freezer log should be maintained

A Tissue

a. Visual inspection of tissue size should be made and tissue dissected to appropriately sized pieces. Collect in the following order: 1) snap freezing, 2) RNA stabilising solution followed by freezing, 3) universal molecular fixative or formalin fixation. Please note: If time to freezing is likely to be more than 5 min, then consider immersion into RNA stabiliser as first priority.
b. Fresh tissues: Fresh tissue can be stored in fluid (e.g. media or HEPES-buffered media containing antibiotics and fungicide for incubations carried out outside a gassed incubator) up to 24 h at 4°C. Transfer to waterbath (37°C) to keep cells alive for cell culture.
c. Frozen Tissues**:** Tissue samples should be stored in an aliquot vial with screw top gasket closure. Snap freeze in liquid nitrogen as soon as possible, record time, and transfer to freezers for long-term storage
d. RNA stabilizing solution: commercially available products like Allprotect Tissue Reagent, DNA/RNA shield, PAXgene tissue containers and RNAlater are all useful as tissue RNA stabilisers. Before immersion in RNA stabilizing solution, cut large tissue samples to size according to manufacturer's protocol. Tissue samples should be stored in an aliquot vial with screw top gasket closure containing RNA stabilizing solution within 15 min of collection, and stored at 4°C for 24 h prior to freezing.
e. Fixed tissues: Tissue samples should be mounted flat in a cassette into 20ml of 10% NBF, within 15 min (record time). The sample is transported at room temperature or on ice. The tissue should remain in NBF for no more than 24 h before transfer to graded alcohol solutions and paraffin embedding

B Blood

a. Allow SST tubes to clot for 30 min in upright position at room temperature and

b. Centrifuge samples for 10 min, at 2500 g at 4°C within 1 h of collection but store on ice if this time is exceeded.
c. Place the tubes upright on a rack and on ice during aliquotting.
d. Set number and pre label aliquot tubes, aliquot into small samples of 100 – 500µl to minimize the freeze thaw cycles.
e. Aspirate and transfer all the plasma or serum using a pipette carefully not disturbing the cell layer below, holding the tube at 45° angle, into a vial with screw top gasket closure. If there is contamination with cells then the vials can be re-centrifuged and transferred to new vials.
f. White blood cell (WBC) aliquots: Aspirate and transfer the buffy coat layer from the collection tube into a vial.
g. Red blood cell (RBC) aliquots: Gently mix and transfer the remaining erythrocytes into another vial.
h. Record volumes of plasma/serum, WBC, RBC in each aliquot.
i. Samples should be processed and stored within 1 h of collection

C Urine

a. All samples should be kept refrigerated and processed within 2h of collection.
b. Discard samples if they contain blood and record it.
c. Mix sample by swirling or repeated pipetting.
d. Dipstick analysis: retest if specific gravity <1.001 or >1.032, record all the findings
e. Store the unprocessed urine aliquots in LN_2 or in an -80°C or colder freezer.
f. Fill sterile tube with remaining urine and centrifuge at 1000-3000 g at 4°C for 5 min.
g. Place samples on wet ice and aspirate supernatant into aliquots. Label aliquots as above (in 5 and store processed urine as in 6).
h. Samples should be processed and stored within 2h

D Saliva

a. Samples should be stored on wet ice or refrigerator if more than one hour for processing.
b. Aliquot the necessary amount into vials for processing, remaining unprocessed saliva store in a screw top vial with gasket closure, label them and store in liquid nitrogen or - 80°C / lower freezers
c. Centrifuge saliva (to be processed) at 1000 g at 4°C for 2 min.
d. Aspirate supernatant into aliquots.
e. If for RNA extraction: Aliquot the centrifuged sample into a vial with RNA stabiliser (RNA Protect saliva kit by Qiagen). Label the aliquots consistent with initial sample label and store.
f. Saliva samples should be processed and stored within 4h

E Peritoneal/ Ascitic fluid

a. Record colour, clarity and volume of the sample
b. Centrifuge sample at 900 g at 4°C for 5 min.
c. Aspirate supernatant into aliquots with a screw top gasket closure and fill as close to the surface as possible. Transfer the pellet to an appropriate sized aliquot vial and stored.
d. Store fluid aliquots in liquid nitrogen freezers or -80°c or colder freezers

F Endometrial fluid / aspirate

a. If aspirate larger than 700ul, homogenise and split into two tubes to make two equal aliquots.
b. Add 1:1 ratio of PBS to the sample and shake manually by inverting the tube several times.
c. Centrifuge sample at 2500 g at 4°C for 20 min.
d. Aspirate supernatant into a new labelled eppendorf. Store the eppendorf tube with the pellet.
e. Store both fluid aliquots in liquid nitrogen freezers or -80°c or colder freezers

3. Storage and data recording

- Date and time of storage should be recorded on the log sheet. Also record the type, number of aliquots prepared on the biospecimen form. Store samples in LN_2 freezers (if available), as they have less temperature fluctuations than -80°C freezers.
- Record on the log sheet any variations or deviations from the SOP; also document if any problems, or issues (e.g. vial cracked during processing).
- Record the location of each sample into the freezer including freezer number, rack, box, and spot in the box along with all other sample attributes in a database.
- Keep a record of any freeze-thaw events that occurs with a sample for any reason.
- Track any change of location of a sample, including sending a sample out to an assay lab for processing.
- Track any new samples created from the original sample (i.e. a sub-sample) in the same manner as described above. Ensure that each sub-sample/aliquot is labelled with a unique ID.

4. Freezer check:

- Installing a centralized freezer/liquid nitrogen-monitoring system automatically records temperature fluctuations and makes call-outs to responsible persons in the event of a problem. If not, Check freezers twice weekly and keep a written-log of checks.

Have alarm systems setup on all freezers in addition to human twice-weekly checks.

- Aliquots of the same sample type and patient should be split between freezers where possible to ensure sample integrity in case of a freezer breakdown.
- Facilities should have an emergency -80°C freezer for the transfer of samples in the event of freezer breakdown.

5. Data recording checklist

1. Record protocols, specifying which steps are followed.
2. For each sample, record:
   a. Date and time of sample collection (Date: __/__/__ and __: __ hrs.).
   b. Start time of sample processing in the laboratory (Date: __/__/__ and __: __hrs.).
   c. Record type, number, volume of aliquots prepared
   d. Record fasting time or whether less than 1) 1 h after brushing teeth, 2) 1 h after eating a meal, 3) 12 h after last alcohol consumption, 4) 20 min after consuming acidic foods like citrus fruits/ high sugar foods – if applicable
   e. Date and time sample is stored into freezer (Date: __/__/__ and __: __hrs.).
   f. Any variations or deviations from the SOP, problems, or issues.
   g. Any freeze-thaw event that occurs with a sample for any reason.
3. Keep a log of twice weekly freezer checks.

(vii) Published first author papers

FULL PAPER

BJC

British Journal of Cancer (2017) 117, 485–493 | doi: 10.1038/bjc.2017.194 BJC OPEN

Keywords: endometrial cancer; standardisation; harmonisation; biospecimens; data collection tools

Harmonisation of biobanking standards in endometrial cancer research

M Adishesh[1,2], A Fyson[2], S B DeCruze[1], J Kirwan[1], ENITEC Consortium, H M J Werner[3,4] and D K Hapangama[*,1,2]

[1]*Liverpool Women's Hospital NHS Foundation Trust, Crown Street, Liverpool L8 7SS, UK;* [2]*Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool, Liverpool Women's Hospital, Crown Street, Liverpool L8 7SS, UK;* [3]*Department of Obstetrics and Gynaecology, Haukeland University Hospital, Jonas Liesvei 70, 50503 Bergen, Norway and* [4]*Department of Clinical Science, University of Bergen, Bergen 5021, Norway*

Background: Endometrial cancer is the most common gynaecological cancer and its incidence is predicted to escalate by 50–100% in 2025 with a parallel increase in associated mortality. Variations in the collection, processing and storage of biospecimens can affect the generalisability of the scientific data. We aimed to harmonise the collection of biospecimens, clinical data relevant to endometrial cancer and to develop standard operative procedures for the collection, processing and storage of endometrial cancer biospecimens.

Methods: We designed research tools, which were evaluated and revised through three consensus rounds – to obtain local/regional, national and European consensus. Modified final tools were disseminated to a panel ($n=40$) representing all stakeholders in endometrial cancer research for consensus generation.

Results: The final consensus demonstrated unanimous agreement with the minimal surgical and patient data collection tools. A high level of agreement was also observed for the other remaining standard tools.

Conclusions: We here present the final versions of the tools, which are freely available and easily accessible to all endometrial cancer researchers. We believe that these tools will facilitate rapid progress in endometrial cancer research, both in future collaborations and in large-scale multicentre studies.

Endometrial cancer (EC) is the most common cancer of the female genital tract in the developed world, and is the fourth most common cancer in women after breast, lung and colorectal cancer (Ferlay *et al*, 2015). In the United Kingdom in 2014, at least 6 women died of and 21 women were diagnosed with EC in the United Kingdom every day, with 9022 new cases and 2166 deaths reported that year (CRUK). The incidence rate of EC is increasing rapidly and is estimated to increase by 50–100% by 2025 (Lindemann *et al*, 2010). This increase in incidence is alarming, particularly due to the corresponding rise in mortality (CRUK). Increased efforts into finding new prevention, diagnostic, prognostic and therapeutic targets are therefore urgently required to reduce the high mortality and morbidity rates associated with EC. Traditionally, among others immunohistochemistry was used, based on formalin-fixed paraffin-embedded tissue, allowing only for the study of a limited number of proteins simultaneously. Further cell lines and animal studies have been applied in EC research; these however rarely give a perfect simulation of the *in vivo* human environment. Therefore, biobanks, collecting a wide range of different patient specimens, including for example fresh frozen tissue, urine, blood or saliva, have a vital role in providing valuable patient material for clinically relevant scientific discoveries and also aid to the rapid translation of basic scientific findings to clinical practice.

Through its nature, patient material stored in biobanks allows for studying multiple aspects of EC. This is of paramount importance with the emergence of novel technological platforms in genomics, proteomics, epigenomics and metabolomics that can be collectively and simultaneously applied to the same patient samples to gain the maximum amount of information. Such an all-

*Correspondence: Dr DK Hapangama; E-mail: dharani@liv.ac.uk

Received 25 January 2017; revised 1 June 2017; accepted 1 June 2017; published online 29 June 2017

encompassing approach is expected to reduce considerably the time taken for new basic scientific discoveries to reach patients as new treatments as well as allowing the samples donated by patients to be fully used.

The internal and external validity of the generated data depend on their quality, which is clearly dependent on the use of stringent standards in collecting the biospecimens and patient characteristics. Variations associated with collecting, processing, storing different biospecimens and the accompanying phenotypic and demographic data make it extremely difficult to extrapolate or to merge data from different studies (Tworoger and Hankinson, 2006; Ransohoff and Gourlay, 2010). This lack of quality standards and uniformity is recognised by the National Cancer Institute (NCI) as a roadblock in cancer research (NCI Best Practices for Biospecimen Resources, 2011). The irrevocable bias introduced by the irregularities and dissimilarities in specimens and data collection are well recognised by many and efforts are being made to overcome this by several international organisations and agencies (Morente *et al*, 2007; International Society for Biological and Environmental Repositories, 2008; Yuille *et al*, 2008; Vaught and Lockhart, 2012).

The NCI best practice guidance for biobanks (NCI Best Practices for Biospecimen Resources, 2011, 2016), which encourages optimisation of the resources available for cancer research, broadly mentions a limited list of preanalytic variables related to the donor or sample collection/processing. It has thereby been effective in raising the overall awareness and quality of research involving biospecimens.

Although this is an important start, many parameters and variables of interest, including choice of biospecimens and clinical data, are cancer-type-specific. Thus, universal biobanking standards are not necessarily applicable to every cancer type and should be adapted to each specific disease. The importance of cancer-specific harmonisation of biobanking standards is highlighted by the cancer genome atlas (Kandoth *et al*, 2013), which now contains over 532 EC samples with RNA sequencing, copy number variation, proteomic, mutation and microarray data. However, the extremely limited clinical data accompanying most of these samples and data sets severely affects the ability of researchers to draw clinically applicable information.

Therefore, EC-specific standardisation of the collection of biospecimens with distinctive and relevant accompanying clinical data sets is a fundamental unmet need in improving future EC research. This, we believe, will facilitate future large-scale internationally collaborative research into EC, which could lead to improved biomarker and target treatment discovery. Similar harmonisation projects have already been successfully implemented for other gynaecological conditions such as endometriosis – World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project and Ovarian Cancer Research Program (Wiegand *et al*, 2010; Heravi-Moussavi *et al*, 2012; Fassbender *et al*, 2014; Rahmioglu *et al*, 2014; Vitonis *et al*, 2014).

With this background, we initiated our study (Harmonisation of biobAnking STandards in Endometrial caNcer research – HASTEN) to achieve consensus among EC researchers; standardise the collection, processing and storage of all relevant biospecimens; and the accompanying clinical data for EC research through a joint effort with patients, surgeons/physicians/pathologists and the personnel of biobanks. We aimed to develop standards: standard operative procedure tools with a minimum and standard data set to be regularly updated and universally available for future researchers in EC.

MATERIALS AND METHODS

The method used to design the final tools in HASTEN is summarised in the flow diagram (Figure 1). We used a modified Delphi system to analyse and confirm the final consensus.

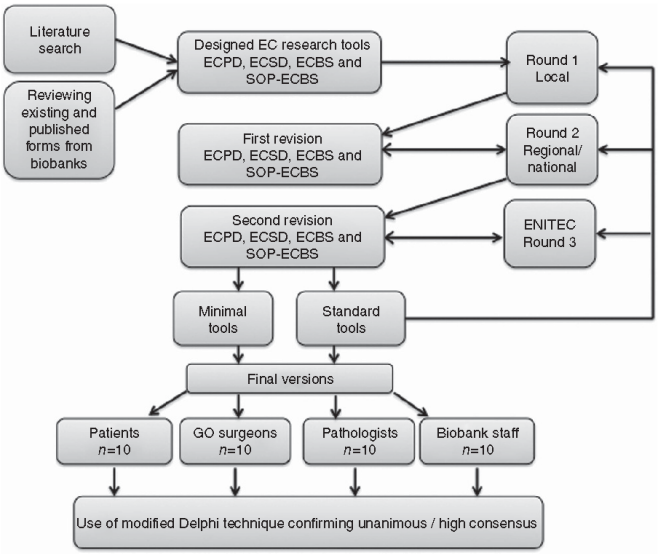


Figure 1. Flow chart illustrating our workflow in designing the EC research tools and the method of generating consensus (Endometrial cancer (EC), European Network of Individualised Treatment in Endometrial Cancer (ENITEC), Endometrial Cancer Patient Data Collection Tool (ECPD), Endometrial Cancer Surgical Data Collection Tool (ECSD), Endometrial Cancer Biospecimen Tool (ECBS), standard operating procedure for collection, processing and storage of tissue and fluid for EC research (SOP-ECBS)).

Generation of the initial tools

Literature search. We performed a systematic review of the literature using the keywords 'Endometrial Cancer', 'risk factors', 'age of presentation', 'parity', 'menopausal status', 'metformin', 'progestogens or Mirena', 'hormone replacement therapy', 'polycystic ovary syndrome', 'tamoxifen', 'bowel cancer', 'colorectal cancer', 'breast cancer', 'diabetes', 'hypertension', 'ethnicity', 'anthropometric assessment', 'smoking', 'standard operating procedure' and 'endometrium', 'blood or plasma or serum', 'saliva', 'urine', 'endometrial fluid', 'peritoneal fluid', 'biobank best practices' 'histopathology markers', 'outcomes', 'biomarkers', 'Laboratory processing procedures of tissue, blood and body fluids', 'biobanking standards', 'SOP's for collection of tissue, fluids, blood, saliva, urine' in Scopus, Discover and PubMed databases. The literature search was limited to studies published in the past 10 years. Out of 3464 papers identified in the initial search, 413 papers were selected for further detailed scrutiny based on the following inclusion criteria:

(1) Papers that investigated how the aforementioned factors affect an individual's risk of developing EC.
(2) Publications that proposed standard operating procedures (SOPs) or best practices for the collection, storage and processing of the different tissues or fluids.
(3) Papers in English language only.
(4) Papers available as full text via all available resources to the authors (e.g., online resources or library facilities at Liverpool Women's Hospital (LWH), University of Liverpool, British Medical Association or Royal College of Obstetricians and Gynaecologists.

We further conducted manual searches for the relevant manuscripts referenced in these selected papers and the relevant guidelines from the large biorepositories.

FURTHER DEVELOPMENT OF THE TOOLS

First local consultation. The local team at Liverpool, comprising of four members of surgical gynaecological oncology team, four Macmillan clinical cancer nurse specialists, two clinical academics with an interest in EC research, two pathologists, two biobank staff members and a medical student, developed the three forms (patient data collection tool, surgical data collection tool, biospecimen form) and a standard operative procedure. These forms and the SOP were based on: (a) the information gathered in the literature search; (b) by considering the forms that were already in use in LWH/University of Liverpool biobank to collect biospecimens and data in EC research studies. Liverpool Women's Hospital is a tertiary referral regional cancer centre for gynaecological cancers, and is part of the Cheshire and Merseyside strategic clinical networks, which serves a population of 2.4 million. The age-standardised incidence rate of EC in the Merseyside and Cheshire cancer network is 18.3 per 100 000 female members of the population (NCIN, 2013; Gynae Clinical Network Constitution, 2014–2015). (c) Standard operating procedures developed by the National Institutes of Health, Human Endometrial Tissue and DNA Bank for the collection, transport and storage of human endometrial tissue and blood samples of women undergoing endometrial biopsy or hysterectomy for non-malignant indications (Sheldon *et al*, 2011). (d) Sample handling and storage protocol published by the UK biobank to collect urine and blood samples (Elliott *et al*, 2008). UK biobank is a major national and international health resource, which was established by Wellcome trust, Medical Research Council, Department of Health, Scottish Government and The Northwest Regional Development Agency. The main aim of this was to improve prevention, diagnosis and treatment of many illnesses such as cancer, heart disease, stroke, diabetes, arthritis, osteoporosis and dementia.

The forms were revised and amended based on local consultation.

Second regional/national consultation. The modified versions of the three forms and the SOP mentioned above were disseminated among three regional and eight national research centres involved in EC research in the United Kingdom and forms were revised integrating their feedback and as a result, two different tools, a minimal and a standard tool were developed. This pragmatic and inclusive approach provides guidance for collecting either a minimal or the ideal 'standard' data set considering the available resources.

Third European consultation. The modified forms were then circulated to all researchers adhering to the European Network of Individualised Treatment in Endometrial Cancer (ENITEC) and were further revised according to feedback received. The revised tools were presented at the annual ENITEC face-to-face meeting in June 2016, where the minimal form was unanimously approved by all 47 attendees. Some further modifications were suggested for the standard tool, which was revised accordingly and the revised forms were repopulated to all participated in the consultations rounds 1–3 to obtain their final approval.

Consensus generation. A modified Delphi system was used to generate consensus regarding the final adapted tools. For this, the forms were disseminated to a group of selected panel members of representing all stakeholders included in all previous rounds, including patients, gynaecological oncologists, researchers, pathologists and biobank staff, randomly selected from the participants of the consultation ($n = 40$) to evaluate and score the tools using a scoring sheet recording their agreement.

Statistical analysis. The consensus was quantified using a modified Delphi technique and we have reported the median with an interquartile range and also percentages for each category of the Likert scale. A nine-point Likert scale was used, except for the patient data tool where the scale was reduced to five points to reduce complexity for patients.

RESULTS

Final tools

ECPD collection tool. A patient-friendly data collection tool (EC patient data (ECPD)) was devised to capture many important demographic variables that are directly relevant to EC research that can only be accurately recalled by the patient herself. For example, the available literature suggests that >20 kg of adult weight gain to be independently associated with increased risk of EC (Friedenreich *et al*, 2007) and this information is unlikely to be obtained easily other than directly from the patient. Many other risk factors for EC such as the age of presentation, the postmenopausal status, polycystic ovarian disease (Fearnley *et al*, 2010), nulliparity (Schonfeld *et al*, 2013), early age of menarche (Gong *et al*, 2015), family history of hereditary lynch syndrome-related cancers (Boilesen *et al*, 2008), past history of lynch syndrome-related cancers, medical conditions such diabetes (Friberg *et al*, 2007), previous use of tamoxifen (Bergman *et al*, 2000), hormone replacement therapy use (Beral *et al*, 2005) and exercise habits have been included in the tool. Some other factors with inconclusive links to EC at present such as smoking (Lindemann *et al*, 2008) were also included in anticipation of their confirmation in appropriate future studies. Table 1 and Figure 2 illustrate the outcome of the final round of consensus.

Table 1. Outcome of the final round of consensus for ECPD Tool using 5-point Likert scale

		Percentages of responses (%)				
Statements in the score sheet for patients	Score, median (IQR)	Strongly agree	Agree	Undecided	Disagree	Strongly disagree
The information asked in personal history is easy to fill	1 (1–2)	60	40	0	0	0
The questions in medical history section are easy to understand and fill	1 (1–2)	60	40	0	0	0
The questions in past history are easy to understand and fill	1 (1–2)	60	40	0	0	0
The questions in social history section are easy to understand and fill	1 (1–2)	60	30	10	0	0
Overall, the form is easy to understand and does not take much time to fill it	1.5 (1–2)	50	50	0	0	0

Abbreviations: ECPD = Endometrial Cancer Patient Data; IQR = interquartile range. IQR, five-point Likert scale: Strongly agree, Agree, Undecided, Disagree and Strongly disagree; $n = 10$.

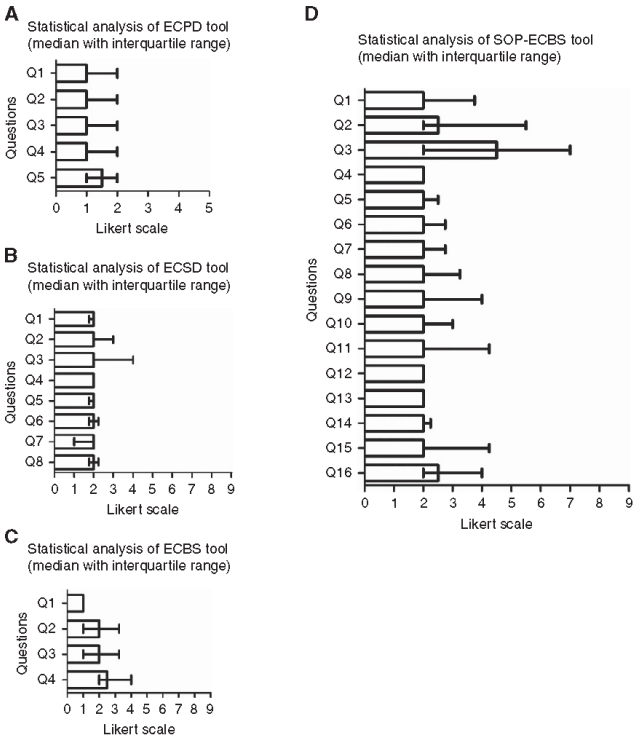


Figure 2. Statistical analysis of Endometrial Cancer Patient Data Collection Tool (ECPD), Endometrial Cancer Surgical Data Collection Tool (ECSD), Endometrial Cancer Biospecimen Tool (ECBS) and standard operating procedure for collection, processing and storage of tissue and fluid for EC research (SOP-ECBS) tools.

Score for each question in ECPD was obtained using the Likert scale, which assesses the acceptability and usability ($n = 10$). Among the panel members, only 2% were undecided on the clarity of the questions in social history section, and overall, 98% patients agreed that the tool was easy to use (Supplementary Figure S1).

ECSD collection tool. The EC surgical data (ECSD) tool included salient demographic, histological and pre/postoperative features. Demographic features such as body mass index (BMI) were included. Body mass index instead of waist-to-hip ratio or waist circumference was chosen because of its universal use and reproducibility. Although all anthropometric assessments (BMI, waist-to-hip ratio, waist and hip circumferences) are found to be strongly associated with increased risk of EC (Friedenreich *et al*, 2007), accurate data on waist-to-hip ratio or waist circumferences require additional effort using the same reference points by health-care team and thus accurate data collection is unlikely to be universally feasible. In a recent study (Painter *et al*, 2016), BMI was

found to be a causal factor and was associated with EC compared with waist-to-hip ratio. The preoperative imaging details are helpful to assess the spread locally and to rule out distant metastases. Discordance between endometrial biopsy and final histology results has been shown to be associated with poorer survival outcome (Werner *et al*, 2013); hence, preoperative biopsy results are important. Staging details including operative findings and final histopathologic details after surgery are important when correlating with outcomes. Immunohistochemical biomarkers can be used to distinguish ECs from ovarian or cervical or other malignancies, but importantly also as prognostic biomarkers that are associated with clinical outcome (Li *et al*, 2013; Kamal *et al*, 2016). Information when collected in a standard way together with biosamples will naturally increase the internal and external validity of the generated data. The patient data collection, including follow-up and accurate documentation of cause of demise, should be updated regularly until the completion of standard follow-up period (either 3 years (minimum) or 5 years, depending on local practice). The form is arranged into three sections:

(1) Surgical data: Completed at the time of sample collection.
(2) Histopathology details: Completed after final staging and treatment.
(3) Outcome data: Documented during follow-up and finally at the end of follow-up

The results of final consensus are as shown in Table 2 and Figure 2, wherein we have calculated the median with an interquartile range. There was a high level of agreement among the panellists for all sections, except that a number of the respondents considered sections on the history, antecedent biopsy details and sample collection details to be not relevant. Overall, 96.25% of panel members agreed on different aspects of the tool (Supplementary Figure S1).

EC Biospecimen tool. Variations in the collection methods and biobanking conditions (processing and storage) may alter the molecular composition, expression and stability of biomarker profile (Zander *et al*, 2014); thus, consistency and strict adherence to standard operating procedures is vital (Moore *et al*, 2011). Therefore, biobank staff with applied experience and knowledge on clinical biobanking participated in designing, revising and obtaining final consensus on the biospecimen form. Only few respondents felt that the tissue processing (both uterine and extrauterine) section of the form was difficult to understand, while all respondents agreed on the relevance and clarity of all other sections. Overall, there was a 94% level of agreement on the different aspects of this tool. The detailed results were as shown in Table 3, Figure 2 and Supplementary Figure S1.

Standard operating procedure for collection, processing and storage of tissue and fluid for EC research. Different tissue types (both uterine and extrauterine) and body fluid types are studied in EC research. The routine investigations of these biospecimens may involve extraction of protein, RNA and DNA to be evaluated using a variety of techniques such as proteomics, genomics and metabolomics. The final SOP was designed amalgamating a number of available separate, detailed methodological protocols (e.g., for centrifugation, filtration, addition of preservatives, as well as storage temperatures). Availability of such information from a biobank will allow the scientists to accurately interpret their data, for example, to examine the metabolic profile of samples such as blood, tissue, endometrial fluid or aspirate and detect disease-specific changes with confidence, especially in multicentre studies (Assfalg *et al*, 2008; Bernini *et al*, 2009). Studies examining hormones are of major relevance to the endometrium, and in addition to more traditional samples such as blood, some have studied noninvasive specimens including saliva and urine (Shirtcliff *et al*, 2001). Noninvasive tests are of a particular interest in clinical research and future work is expected to focus more on them.

The outcome details of the final round of consensus regarding the standard operating procedure for collection, processing and storage of tissue and fluid for EC research (SOP-ECBS) are as presented in Table 4 and Figure 2. There was a general agreement

Table 2. Outcome of the final round of consensus for ECSD Tool using nine-point Likert scale

		Percentages of responses (%)								
Questions in the score sheet for gynaeoncologists	Score, median (IQR)	Strongly agree	Agree	Moderately agree	Mildly agree	Undecided	Mildly disagree	Moderately disagree	Disagree	Strongly disagree
Is the general information about patient relevant?	2 (1.75–2)	20	80	0	0	0	0	0	0	0
Is the section on history relevant?	2 (2–3)	10	60	20	0	0	0	0	10	0
Are the Imaging details relevant and sufficient?	2 (2–4)	10	80	0	30	0	0	0	0	0
Are Antecedent biopsy details relevant?	2 (2–2)	10	80	0	0	0	0	0	0	10
Is the Operative findings section relevant?	2 (1.75–2)	20	80	0	0	0	0	0	0	0
Is the Histopathology type details section relevant and sufficient?	2 (1.75–2.25)	20	60	10	10	0	0	0	0	0
Is the Sample collection details section easy to complete?	2 (1–2)	40	50	0	0	0	0	0	0	10
Are Outcome details relevant?	2 (1.75–2.25)	20	60	10	10	0	0	0	0	0

Abbreviations: ECSD = Endometrial Cancer Surgical Data; IQR = interquartile range. IQR, nine-point Likert scale: Strongly agree, Agree, Moderately agree, Mildly agree, Undecided, Mildly disagree, Disagree and Strongly disagree; $n = 10$.

Table 3. Outcome of the final round of consensus for ECBS Tool using nine-point Likert scale

		Percentages of responses (%)								
Questions in the score sheet for biobank staff	Score Median (IQR)	Strongly agree	Agree	Moderately agree	Mildly agree	Undecided	Mildly disagree	Moderately disagree	Disagree	Strongly disagree
Sample ID–Is this relevant?	1 (1–1)	90	10	0	0	0	0	0	0	0
Methods of tissue processing (Endometrium)–Is this section easy to understand?	2 (1–3.25)	40	30	10	10	0	0	0	10	0
Methods of tissue processing (Extra uterine tissue)–Is this section easy to understand?	2 (1–3.25)	40	30	10	10	0	0	0	10	0
Methods of fluid processing (Endometrial/Peritoneal/Blood/Saliva/Urine)–Is this section easy to understand?	2.5 (2–4)	10	40	10	40	0	0	0	0	0

Abbreviations: ECBS = Endometrial Cancer Biospecimen; IQR = interquartile range. IQR, nine-point Likert scale: Strongly agree, Agree, Moderately agree, Mildly agree, Undecided, Mildly disagree, Disagree and Strongly disagree; $n = 10$

on the user-friendliness and relevance of the tool. Few panellists responded that tissue and blood collection details could be modified further for clarity. Overall, 83.75% of panellists agreed, 8.75% were undecided and 7.5% disagreed with different sections of this tool.

DISCUSSION

We have developed evidence-based standard data collection forms ECPD, ECSD (minimal), ECSD (Standard) and an SOP-ECBS with inclusive participation and approval of all stakeholders in EC cancer biobanking. The final tools were approved by a large multidisciplinary team of reviewers and after reaching consensus (see Supplementary Figure S1), they are published as Supplementary Information with this open access manuscript. They will therefore be freely available to all EC researchers internationally. These tools provide a means by which to reduce confounding factors in the collected data and facilitate larger multicentre studies.

Our choice of the exact information to collect was based on critical appraisal of the best available evidence. Where no published evidence was available, consultation of the experts' opinion and the SOPs of the larger biobanks were considered. The centrifugation speed in processing blood was one such example.

We have used a modified Delphi technique, with multiple alterations from the standard technique, including multiple rounds of feedback, which allows the same panel members to reassess or reconsider initial judgment, participant anonymity, controlled feedback and statistical analysis to interpret data between the rounds. Similar variations to original Delphi system, for example, restricting the ability of the experts to respond to the original question and alterations in the expert groups, as well as changing the end point, have been used previously (Thompson, 2009).

Repeated use of a homogenous panel was unjustifiable for our research aims for the following reasons. Our endeavour was to generate separate forms for diverse end points, for example, patient data collection, surgical data collection, tissue processing information and the standard operative procedures. These obviously required panel members of diverse backgrounds, with different fields of expertise and therefore our panellists were not a homogenous group.

The main deviation from the classic technique was the number of consultation rounds and the end point. Our first two rounds were descriptive to generate opinions and ideas from different expert panels. We included their feedback to generate the finalised forms and SOP. In the final round of the consensus, we distributed a score sheet to each of the panellists along with the forms to evaluate their agreement with the final tools. Our final panel included stakeholders representing those involved in all previous panels. The high percentage of agreement observed with the statistical analysis of data obtained from the third and final round precluded the need for any further consensus rounds.

As more detailed, standardised surgical data collection will allow a comprehensive assessment of the relationship between the surgical phenotypical data with the outcomes of treatments, we strongly advise the use of the standard rather than the minimal ECSD tool. However, if the collection or quality of the large set of data or specimens cannot be guaranteed, the minimal set should be employed. We plan to regularly update these tools in the future through information obtained by feedback and review of future literature, initially on a yearly basis and 5 yearly thereafter. Future considerations in the context of our initiatives include creating an internationally funded web-based central database system allowing voluntary deposition of the information on all biospecimens collected by EC researchers worldwide, which will be easily accessible to all. This approach, we believe, will reduce costs and time spent by individual units while increasing the credibility of the data generated and will offer a transparent, common platform for newer collaborations.

'Molecular Pathological Epidemiology' (MPE) integrates pathology and epidemiology to understand the interrelationships between exogenous and endogenous factors that affect carcinogenesis, progression and response to treatment. It is a constantly evolving field in cancer research (Ogino and Stampfer, 2010). Statistical methods have also been developed to consider both molecular pathology and epidemiology to ensure novel discoveries with high clinical impact. However, the generation of such high-impact MPE studies are impeded by similar challenges including

Table 4. Outcome of the final round of consensus for SOP-ECBS Tool using nine-point Likert scale

		Percentages of responses (%)								
Questions in the score sheet for pathologists	Score Median (IQR)	Strongly agree	Agree	Moderately agree	Mildly agree	Undecided	Mildly disagree	Moderately disagree	Disagree	Strongly disagree
Is section: Processing and storage materials, relevant and easy to understand?	2 (2–3.75)	10	50	20	0	0	20	0	0	0
Is section: Collection–Tissue, relevant and easy to understand?	2.5 (2–5.5)	10	40	10	10	10	0	20	0	0
Is section: Collection–Blood, relevant and easy to understand?	4.5 (2–7)	10	30	0	10	10	10	30	0	0
Is section: Collection – Urine, relevant and easy to understand?	2 (2–2)	10	80	10	0	0	0	0	0	0
Is section: Collection – Saliva, relevant and easy to understand?	2 (2–2.5)	10	70	0	10	10	0	0	0	0
Is section: Collection – Peritoneal fluid, relevant and easy to understand?	2 (2–2.75)	0	80	0	0	20	0	0	0	0
Is section: Collection – Endometrial fluid/uterine aspirates, relevant and easy to understand?	2 (2–2.75)	0	80	0	0	20	0	0	0	0
Is section: Sample processing – Tissue, relevant and easy to understand?	2 (2–3.25)	0	70	10	10	10	0	0	0	0
Is section: Sample processing – Blood, relevant and easy to understand?	2 (2–4)	0	60	10	20	10	0	0	0	0
Is section: Sample processing – Urine, relevant and easy to understand?	2 (2–3)	0	70	20	0	0	10	0	0	0
Is section: Sample processing – Saliva, relevant and easy to understand?	2 (2–4.25)	0	60	10	10	10	10	0	0	0
Is section: Sample processing – Peritoneal fluid, relevant and easy to understand?	2 (2–2)	10	80	0	0	10	0	0	0	0
Is section: Sample processing – Endometrial fluid/uterine aspirates, relevant and easy to understand?	2 (2–2)	10	80	0	0	10	0	0	0	0
Is section: Storage and data recording, relevant and easy to understand?	2 (2–2.25)	10	70	10	0	0	10	0	0	0
Is section: Freezer check, relevant and easy to understand?	2 (2–4.25)	0	60	10	10	10	10	0	0	0
Is section: Checklist, relevant and easy to understand?	2.5 (2–4)	0	50	10	30	10	0	0	0	0

Abbreviations: IQR = interquartile range; SOP-ECBS = standard operating procedure for collection, processing and storage of tissue and fluid for EC research. IQR, nine-point Likert scale: Strongly agree, Agree, Moderately agree, Mildly agree, Undecided, Mildly disagree, Disagree and Strongly disagree; $n = 10$.

selection and recall bias, measurement errors and misclassification comparable to the traditional molecular biological studies (Hughes *et al*, 2012; Campbell *et al*, 2017). Variability in tissue retrieval rate and sample sizes leads to random and non-random selection bias, resulting in large variation of an effect estimate with wide confidence intervals and publication bias (Ogino *et al*, 2011, 2016). The use of our tools by EC biobanks will provide means with which to streamline the collection of a large amount of standardised quality assured material from well-phenotyped patients. This will in turn facilitate adequately powered studies, giving high clinical impact while also facilitating high-quality research that is attainable within an acceptable timescale.

ACKNOWLEDGEMENTS

We acknowledge the support from Liverpool Women's NHS Foundation Trust (MA/DKH) and Institute of Translational Medicine at the University of Liverpool (DKH/MA). We are grateful to Liverpool Women's Hospital Gynaeoncology Oncology Department, ENITEC, Dr Steven Lane and all panel members for reviewing the forms and providing feedback. The support received from Dr Nicola Tempest, Jo Drury in preparation of this manuscript, and Druvi Edirisinghe for proof reading is also acknowledged.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Assfalg M, Bertini I, Colangiuli D, Luchinat C, Schäfer H, Schütz B, Spraul M (2008) Evidence of different metabolic phenotypes in humans. *Proc Natl Acad Sci USA* **105**(5): 1420–1424.

Beral V, Bull D, Reeves G, Collaborators MWS (2005) Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet* **365**(9470): 1543–1551.

Bergman L, Beelen ML, Gallee MP, Hollema H, Benraadt J, van Leeuwen FE (2000) Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. *Lancet* **356**(9233): 881–887.

Bernini P, Bertini I, Luchinat C, Nepi S, Saccenti E, Schäfer H, Schütz B, Spraul M, Tenori L (2009) Individual human phenotypes in metabolic space and time. *J Proteome Res* **8**(9): 4264–4271.

Boilesen AE, Bisgaard ML, Bernstein I (2008) Risk of gynecologic cancers in Danish hereditary non-polyposis colorectal cancer families. *Acta Obstet Gynecol Scand* **87**(11): 1129–1135.

Campbell PT, Rebbeck TR, Nishihara R, Beck AH, Begg CB, Bogdanov AA, Cao Y, Coleman HG, Freeman GJ, Heng YJ, Huttenhower C, Irizarry RA, Kip NS, Michor F, Nevo D, Peters U, Phipps AI, Poole EM, Qian ZR, Quackenbush J, Robins H, Rogan PK, Slattery ML, Smith-Warner SA, Song M, VanderWeele TJ, Xia D, Zabor EC, Zhang X, Wang M, Ogino S (2017) Proceedings of the third international molecular pathological epidemiology (MPE) meeting. *Cancer Causes Control* **28**(2): 167–176.

CRUK. *Uterine Cancer Statistics,* Vol. 2015. Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer.

Elliott P, Peakman TC, Biobank U (2008) The UK Biobank sample handling and storage protocol for the collection, processing and archiving of human blood and urine. *Int J Epidemiol* **37**(2): 234–244.

Fassbender A, Rahmioglu N, Vitonis AF, Vigano P, Giudice LC, D'Hooghe TM, Hummelshoj L, Adamson GD, Becker CM, Missmer SA, Zondervan KT, Group WEW (2014) World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. *Fertil Steril* **102**(5): 1244–1253.

Fearnley EJ, Marquart L, Spurdle AB, Weinstein P, Webb PM, Australian Ovarian Cancer Study Group and Australian National Endometrial Cancer Study Group (2010) Polycystic ovary syndrome increases the risk of endometrial cancer in women aged less than 50 years: an Australian case–control study. *Cancer Causes Control* **21**(12): 2303–2308.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **136**(5): E359–E386.

Friberg E, Orsini N, Mantzoros CS, Wolk A (2007) Diabetes mellitus and risk of endometrial cancer: a meta-analysis. *Diabetologia* **50**(7): 1365–1374.

Friedenreich C, Cust A, Lahmann PH, Steindorf K, Boutron-Ruault MC, Clavel-Chapelon F, Mesrine S, Linseisen J, Rohrmann S, Boeing H, Pischon T, Tjønneland A, Halkjaer J, Overvad K, Mendez M, Redondo ML, Garcia CM, Larrañaga N, Tormo MJ, Gurrea AB, Bingham S, Khaw KT, Allen N, Key T, Trichopoulou A, Vasilopoulou E, Trichopoulos D, Pala V, Palli D, Tumino R, Mattiello A, Vineis P, Bueno-de-Mesquita HB, Peeters PH, Berglund G, Manjer J, Lundin E, Lukanova A, Slimani N, Jenab M, Kaaks R, Riboli E (2007) Anthropometric factors and risk of endometrial cancer: the European prospective investigation into cancer and nutrition. *Cancer Causes Control* **18**(4): 399–413.

Gong TT, Wang YL, Ma XX (2015) Age at menarche and endometrial cancer risk: a dose-response meta-analysis of prospective studies. *Sci Rep* **5**: 14051.

Gynae Clinical Network Constitution (2014–2015) Available at: http://www.nwcscnsenate.nhs.uk/files/2314/1172/3867/Gynae_CNG_Constitution_2014.pdf.

Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, Yang W, Prentice L, Fejes AP, Chow C, Tone A, Kalloger SE, Hamel N, Roth A, Ha G, Wan AN, Maines-Bandiera S, Salamanca C, Pasini B, Clarke BA, Lee AF, Lee CH, Zhao C, Young RH, Aparicio SA, Sorensen PH, Woo MM, Boyd N, Jones SJ, Hirst M, Marra MA, Gilks B, Shah SP, Foulkes WD, Morin GB, Huntsman DG (2012) Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N Engl J Med* **366**(3): 234–242.

Hughes LA, Williamson EJ, van Engeland M, Jenkins MA, Giles GG, Hopper JL, Southey MC, Young JP, Buchanan DD, Walsh MD, van den Brandt PA, Alexandra Goldbohm R, Weijenberg MP, English DR (2012) Body size and risk for colorectal cancers showing BRAF mutations or microsatellite instability: a pooled analysis. *Int J Epidemiol* **41**(4): 1060–1072.

International Society for Biological and Environmental Repositories (2008) *Best Practices for Respositories: Collection, Storage, Retrieval and Distribution of Biological Materials for Research*. In Cell preservation technology, Vol 6, 2nd edition. Mary Ann Liebert, Inc.: New Rochelle, NY, USA.

Kamal A, Tempest N, Parkes C, Alnafakh R, Makrydima S, Adishesh M, Hapangama DK (2016) Hormones and endometrial carcinogenesis. *Horm Mol Biol Clin Invest* **25**(2): 129–148.

Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, Shen H, Robertson AG, Pashtan I, Shen R, Benz CC, Yau C, Laird PW, Ding L, Zhang W, Mills GB, Kucherlapati R, Mardis ER, Levine DA, Network CGAR (2013) Integrated genomic characterization of endometrial carcinoma. *Nature* **497**(7447): 67–73.

Li MZL, Qi W, Shen D, Li X, Wang J, Wei L (2013) Clinical implications and prognostic value of five biomarkers in endometrial carcinoma. *Chin-German J Clinl Oncol* **12**(12): 586–591.

Lindemann K, Eskild A, Vatten LJ, Bray F (2010) Endometrial cancer incidence trends in Norway during 1953–2007 and predictions for 2008–2027. *Int J Cancer* **127**(11): 2661–2668.

Lindemann K, Vatten LJ, Ellstrøm-Engh M, Eskild A (2008) Body mass, diabetes and smoking, and endometrial cancer risk: a follow-up study. *Br J Cancer* **98**(9): 1582–1585.

Moore HM, Kelly A, Jewell SD, McShane LM, Clark DP, Greenspan R, Hainaut P, Hayes DF, Kim P, Mansfield E, Potapova O, Riegman P, Rubinstein Y, Seijo E, Somiari S, Watson P, Weier HU, Zhu C, Vaught J (2011) Biospecimen reporting for improved study quality. *Biopreserv Biobank* **9**(1): 57–70.

Morente MM, de Alava E, Fernandez PL (2007) Tumour banking: the Spanish design. *Pathobiology* **74**(4): 245–250.

National Cancer Institute (2011) Best Practices For Biospecimen Resources. NIH: Bethesda, MD, USA. Available at: https://biospecimens.cancer.gov/practices/2011bp.asp.

National Cancer Institute (2016) Best Practices For Biospecimen Resources. NIH: Bethesda, MD, USA. Available at: https://biospecimens.cancer.gov/bestpractices/2016-NCIBestPractices.pdf.

NCIN (2013) *Outline of Uterine Cancer in the United Kingdom: Incidence, Mortality and Survival*. NCIN. PHE Knowledge and Intelligence Team (East Midlands): Sheffield, UK.

Ogino S, Chan AT, Fuchs CS, Giovannucci E (2011) Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* **60**(3): 397–411.

Ogino S, Nishihara R, VanderWeele TJ, Wang M, Nishi A, Lochhead P, Qian ZR, Zhang X, Wu K, Nan H, Yoshida K, Milner Jr DA, Chan AT, Field AE, Camargo Jr CA, Williams MA, Giovannucci EL (2016) Review article: the role of molecular pathological epidemiology in the study of neoplastic and non-neoplastic diseases in the era of precision medicine. *Epidemiology* **27**(4): 602–611.

Ogino S, Stampfer M (2010) Lifestyle factors and microsatellite instability in colorectal cancer: the evolving field of molecular pathological epidemiology. *J Natl Cancer Inst* **102**(6): 365–367.

Painter JN, O'Mara TA, Marquart L, Webb PM, Attia J, Medland SE, Cheng T, Dennis J, Holliday EG, McEvoy M, Scott RJ, Ahmed S, Healey CS, Shah M, Gorman M, Martin L, Hodgson SV, Beckmann MW, Ekici AB, Fasching PA, Hein A, Rübner M, Czene K, Darabi H, Hall P, Li J, Dörk T, Dürst M, Hillemanns P, Runnebaum IB, Amant F, Annibali D, Depreeuw J, Lambrechts D, Neven P, Cunningham JM, Dowdy SC, Goode EL, Fridley BL, Winham SJ, Njølstad TS, Salvesen HB, Trovik J, Werner HM, Ashton KA, Otton G, Proietto A, Mints M, Tham E, Bolla MK, Michailidou K, Wang Q, Tyrer JP, Hopper JL, Peto J, Swerdlow AJ, Burwinkel B, Brenner H, Meindl A, Brauch H, Lindblom A, Chang-Claude J, Couch FJ, Giles GG, Kristensen VN, Cox A, Pharoah PD, Tomlinson I, Dunning AM, Easton DF, Thompson DJ, Spurdle AB, Group A, RENDOCAS f, (NSECG) NSoECGG, (ANECS) ANECSG (2016) Genetic risk score Mendelian randomization shows that obesity measured as body mass index, but not waist:hip ratio, is causal for endometrial cancer. *Cancer Epidemiol Biomarkers Prev* **25**(11): 1503–1510.

Rahmioglu N, Fassbender A, Vitonis AF, Tworoger SS, Hummelshoj L, D'Hooghe TM, Adamson GD, Giudice LC, Becker CM, Zondervan KT, Missmer SA, Group WEW (2014) World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil Steril* **102**(5): 1233–1243.

Ransohoff DF, Gourlay ML (2010) Sources of bias in specimens for research about molecular markers for cancer. *J Clin Oncol* **28**(4): 698–704.

Schonfeld SJ, Hartge P, Pfeiffer RM, Freedman DM, Greenlee RT, Linet MS, Park Y, Schairer C, Visvanathan K, Lacey JV (2013) An aggregated analysis of hormonal factors and endometrial cancer risk by parity. *Cancer* **119**(7): 1393–1401.

Sheldon E, Vo KC, McIntire RA, Aghajanova L, Zelenko Z, Irwin JC, Giudice LC (2011) Biobanking human endometrial tissue and blood specimens: standard operating procedure and importance to reproductive biology research and diagnostic development. *Fertil Steril* **95**(6): 2120–2122.e1–e12.

Shirtcliff EA, Granger DA, Schwartz E, Curran MJ (2001) Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology* **26**(2): 165–173.

Thompson M (2009) Considering the implication of variations within Delphi research. *Fam Pract* **26**(5): 420–424.

Tworoger SS, Hankinson SE (2006) Use of biomarkers in epidemiologic studies: minimizing the influence of measurement error in the study design and analysis. *Cancer Causes Control* **17**(7): 889–899.

Vaught J, Lockhart NC (2012) The evolution of biobanking best practices. *Clin Chim Acta* **413**(19-20): 1569–1575.

Vitonis AF, Vincent K, Rahmioglu N, Fassbender A, Buck Louis GM, Hummelshoj L, Giudice LC, Stratton P, Adamson GD, Becker CM, Zondervan KT, Missmer SA, Group WEW (2014) World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: II. Clinical and covariate phenotype data collection in endometriosis research. *Fertil Steril* **102**(5): 1223–1232.

Werner HMJ, Trovik J, Marcickiewicz J, Tingulstad S, Staff AC, Engh ME, Oddenes K, Rokne JA, Tjugum J, Lode MS, Amant F, Salvesen HB (2013) A discordant histological risk classification in preoperative and operative biopsy in endometrial cancer is reflected in metastatic risk and prognosis. *Eur J Cancer* **49**: 625–632.

Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih IeM, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* **363**(16): 1532–1543.

Yuille M, van Ommen GJ, Bréchot C, Cambon-Thomsen A, Dagher G, Landegren U, Litton JE, Pasterk M, Peltonen L, Taussig M, Wichmann HE, Zatloukal K (2008) Biobanking for Europe. *Brief Bioinform* **9**(1): 14–24.

Zander J, Bruegel M, Kleinhempel A, Becker S, Petros S, Kortz L, Dorow J, Kratzsch J, Baber R, Ceglarek U, Thiery J, Teupser D (2014) Effect of biobanking conditions on short-term stability of biomarkers in human serum and plasma. *Clin Chem Lab Med* **52**(5): 629–639.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

cancers

MDPI

Review

Enriching Personalized Endometrial Cancer Research with the Harmonization of Biobanking Standards

Meera Adishesh [1,2] and Dharani K. Hapangama [1,2,*]

[1] Department of Women's and children's health, Institute of Translational medicine, University of Liverpool, Liverpool L8 7SS, UK; meeadish@liverpool.ac.uk
[2] Liverpool Women's Hospital, Liverpool L8 7SS, UK
* Correspondence: dharani@liv.ac.uk; Tel.: +44-151-795-9559

Received: 30 September 2019; Accepted: 2 November 2019; Published: 5 November 2019

Abstract: Endometrial cancer is the commonest gynecological cancer, with an incidence predicted to escalate by a further 50–100% before 2025, due to the rapid rise in risk factors such as obesity and increased life expectancy. Endometrial cancer associated mortality is also rising, depicting the need for translatable research to improve our understanding of the disease. Rapid translation of scientific discoveries will facilitate the development of new diagnostic, prognostic and therapeutic strategies. Biobanks play a vital role in providing biospecimens with accompanying clinical data for personalized translational research. Wide variation in collection, and pre-analytic variations in processing and storage of bio-specimens result in divergent and irreproducible data from multiple studies that are unsuitable for collation to formulate robust conclusions. Harmonization of biobanking standards is thus vital, in facilitating international multi-center collaborative studies with valuable outcomes to improve personalized treatments. This review will detail the pitfalls in the biobanking of biosamples from women with cancer in general, and describe the recent international harmonization project that developed standardized research tools to overcome these challenges and to enhance endometrial cancer research, which will facilitate future development of personalized novel diagnostic strategies and treatments.

Keywords: biobanking; biospecimens; harmonization; translational research; endometrial cancer

1. Introduction

Endometrial cancer (EC) is the 4th most common cancer in women, and it is therefore the commonest gynecological cancer. Around 9000 new cases of EC were diagnosed in the UK in 2013 [1]. Whilst the incidence of many other cancers is reducing, and in general, cancer-associated death rates are declining, the incidences of EC and EC-associated mortality rates are on the rise [2]. In the UK, there has been a 43% increase in age-standardized incidence of EC compared to the 1990s [1], accounting for about 3% of all female deaths (2012). Similarly, UK survival figures indicate the mortality rates from EC have gone up by 21% over the last decade, with a projected rise of 19% by 2035 [3]. The rise in EC rates is a global phenomenon, as shown by European and North American studies due to reasons detailed later in this review. For example, in Norway, the estimated rise in the incidence of EC is 50–100% by year 2025 [4]. Increased efforts into finding new preventative and diagnostic strategies and determining personalized prognostic and therapeutic targets are therefore urgently required in order to reduce the high mortality and morbidity rates associated with EC.

Our current understanding of the human endometrium is relatively poor, due to its species-specific functional and regulatory differences. For example, regular menstrual shedding, scar-less repair and regeneration, are hallmarks of the human endometrium, but these processes are not seen in most other

mammals [5,6]. This precludes the translation of knowledge on endometrial function derived from the other mammals, to the human endometrium.

Estrogen exerts its trophic/mitotic effects mainly via estrogen receptor alpha (ERα), whilst ligand-activated ERβ counteracts and regulates ERα action [5]. Although endometrium is a target organ for ovarian sex steroid hormones, compared with the other comprehensively researched, hormone responsive organs, there are further striking dissimilarities in the responses of endometrial cells to ovarian hormones in humans vs. other mammals. This is exemplified by the fact that estrogen plays an important trophic role in both breast and endometrial tissue, promoting carcinogenesis. However, tamoxifen, an estrogen receptor modulator, inhibits estrogenic action in breast tissue, and thus is an anti-cancer agent used in breast cancer treatment, while it is an inducer of endometrial growth and EC. This further highlights the urgent need for improving our current understanding of the normal endometrial function and EC, as well as the need for expansion of EC research using human samples.

2. Reasons for the Increase in the Incidence of EC

We aim to highlight the need for harmonizing biobanking in EC in this review. The reasons for the enduring increase in the incidence and the EC-associated mortality are multifactorial [7]. The influence of these factors in a particular biological sample is important and relevant to studies exploring either the pathogenesis or the therapeutic targets of EC. Therefore, whilst such information may not be relevant to other types of cancer, they should accompany EC-biospecimens. Some of these factors are listed below for clarity.

Obesity is a significant risk factor for developing EC, and is also responsible for an increase in perioperative morbidity [8]. EC is an *age*-related disease that is commonly present in postmenopausal women [1]. Since the endometrium is exquisitely sensitive to ovarian hormones [5], the *exposure to excessive exogenous or endogenous estrogen* in particular increases the risk of EC [7]. *Hormone replacement therapy (HRT)* that is commonly used to alleviate the menopausal symptoms by peri- and postmenopausal women is associated with an increased risk of EC [9,10]. *Tamoxifen*, a selective estrogen receptor modulator (SERM), is used to reduce the risk of a recurrence of breast cancer. On breast tissue, tamoxifen has anti-estrogenic effects, while moderate estrogenic effects are seen on the endometrium; therefore, in standard doses it causes endometrial proliferation leading to hyperplasia, polyp formation and invasive cancer [11–13].

Increased, unopposed endogenous estrogen activity in women with *Polycystic ovarian syndrome (PCOS)* increases the incidence of EC by three to four fold, with a lifetime risk of 9% in comparison with 3% in the general population [14]. Hyperandrogenism and peripheral aromatization of androgens, which occurs in adipose tissue and high BMI, are all important features of the PCOS-intensifying estrogenic effect on the endometrium. Late menarche reduces the risk of EC, whilst late menopause increases it [15,16]. In contrast to HRT, the use of hormonal contraception is protective to the endometrium [17]. The reduction of this risk is proportional to duration of use, for every 5 years of use is associated with an RR of 0.76, and this effect persists for about 30 years, and it may be amplified as time progresses [18].

Lynch syndrome is an inherited syndrome, which is associated with a high risk of colorectal, endometrial, ovarian and urinary tract cancers. Lifetime risk of EC in women with Lynch syndrome is about 60% [19]. EC in these patients when it occurs as sentinel cancers, occurs in younger and low BMI women when compared with sporadic tumors. Patients with medical conditions such as Diabetes, and Parkinson's disease also have increased predisposition to EC, this may be due to increased insulin resistance or other unknown factors [20].

Nulliparous women are at higher risk of EC than multiparous women (nulliparous vs parous: HR, 1.42; 95% CI, 1.26–1.60) [21]. Factors such as increased physical activity and decreased sedentary time are associated with decreased risk of EC [22]. Both former smokers and current smokers have a reduced incidence of EC compared to non-smokers, and this effect can be explained by hormonal modulation affecting hormone-producing organs, adrenals and ovaries [23].

Increasing life span, obesity and *a sedentary lifestyle* are global phenomena that will continue to influence the increasing incidence of EC. The presence of all these risks is important to consider when assessing patient samples in EC research.

They may also influence the clinical success of a study planned for biomarker identification, and thus should be considered initially when collecting bio-samples and also during their analysis. However, unfortunately, many studies have been conducted without these important considerations. This may be the explanation for the frequent observation that many promised biomarkers emerged from initial studies, failing to show sufficient clinical efficiency in larger clinical studies.

3. Importance of Patient Derived Samples/Biospecimens in Cancer Research and in Personalized Medicine

Personalized medicine and translational research aim to use clinical and molecular data from individual patients, to develop and validate therapies with greater specificity, thus reducing the number of side effects whist focusing on determining disease predisposition to develop preventative strategies. Human bio-specimens form a crucial link between molecular signatures of an individual's specific cancer and their response to clinical treatment. Therefore, the information generated from the bio-specimens provides the basis for subsequent treatment [24]. In recent years, the 'Omics' revolution has been driving the field of cancer research, providing alternative ways to study biology, heterogeneity and evolution of tumors [25]. Both the genetic background and environmental factors influence the crucial changes in cellular function that result in tumorigenesis, and they also converge to influence the individuals' risk of developing cancer. Therefore, improving our knowledge in these areas forms the basis of cancer prevention through targeted therapies [26]. For example, epigenetic research depends upon the analysis of biospecimens, and blood and tumor tissue are the commonest types of specimens that have been used. In addition, patient-derived samples, and patient-derived primary cell lines that retain the phenotype and functional characteristics of the parent tumor, are invaluable in a variety of research studies. Functional studies using them may provide more clinically relevant data, such as the response to the chemotherapeutic agents of a tumor, and they will be more representative of the tumor type/population. Therefore, the overall clinical relevance will be high. The internal and external validity of the generated data depends on the use of stringent standards in collecting the biospecimens and the accompanying patient characteristics pertinent to the specific cancer type. This helps researchers to draw direct clinically translatable conclusions, and enables them to tailor the therapeutic options for individualized treatment with the maximum effectiveness, whilst reducing side effects [27]. However, heterogeneity in the collection, processing and storage of the biospecimens can seriously hamper this seemingly straightforward process, leading to questionable molecular integrity of the biospecimens and irreproducible results that impede development of effective diagnostic and therapeutic strategies [24].

4. Translational, Personalized Research and Role of Biobanks

The main aim of translational research is to accelerate the process of the transition of scientific discoveries from the lab to the patients who will benefit from those findings. Having a sustainable supply of well-documented and high quality biospecimens is a crucial resource for translational research with a specific and personal relevance. Therefore, biobanks form a critical platform, where all such suitable biospecimens are stored for use in research [28]. Disease-specific biobanks have a huge impact on the discovery of bio-markers, therapeutic targets, and in general, for research on treatment of any diseases or specifically of cancers [29]. In this respect, Biobanks are the cornerstone for research, and they are a valuable educational resource, bringing together all the stakeholders in research, and they lead in the validation of standards used even in standard and routine clinical pathology. Biobanks also play a vital role in improving our understanding of epidemiology, pathogenesis and genetics, relevant to particular pathologies. For example, in EC, they provide the means to rapidly embrace

the arrival of the next generation of novel technologies in translational medicine, which encompass genomics, proteomics, epi-genomics and metabolomics.

The main diagnostic approach (e.g., diagnosing cancers and many other diseases) in clinical care has always been the expert pathological scrutiny of the resected tissue and other biological samples routinely collected from patients during their medical procedures. Traditionally, in cancer research, the pathological characteristics identified by histological means are further analyzed using additional techniques such as immunohistochemistry. Therefore, the commonest way the samples are processed and stored is still by preservation in fixative agents such as formalin, followed by paraffin embedding and subsequent storage as blocks. Although this method preserves the tissue architecture for long-time retention, it only allows the consequent use of a limited number of techniques. To examine the functional aspects of a molecule, or to assess the response of a tumor to a chemotherapeutic agent in the laboratory, samples preserved in that way, are not suitable. To rectify this issue, researchers have developed methods suitable for in vitro and in vivo studies that use patient-derived cell lines and freshly collected/freeze-thawed tissue. These can be incorporated into laboratory in vitro models and in-vivo animal models that are preferentially being applied in cancer research. Although these are not the perfect simulation of the in vivo human environment, there is substantial homology, thus they may reduce the need for testing novel therapeutic agents directly in humans and reduce the burden on patients. Therefore, Biobanks, collecting and storing a wide range of different patient specimens (including fresh tissue, fresh frozen tissue, processed tissue, urine, blood or saliva samples and many other specimens), play a vital role in providing valuable patient material for clinically relevant scientific discoveries. Consequently, they support the rapid translation of basic scientific findings to clinical practice for the benefit of cancer patients.

5. Quality of the Biospecimen as a Cause of Bias in Translational Research/Personalized Medicine

A major setback in cancer research at present is the difficulty in identifying clinically effective molecular targets for early detection and for predicting prognosis. Such markers will facilitate efficient stratification of patients for specific treatments, thus personalizing therapy [24]. The reliability of studies investigating this aspect is largely dependent upon the quality and consistency of the standards used for biospecimen handling. The potential variation in collecting, processing and storing different biospecimens, and the accompanying phenotypic and demographic data, [30,31] may lead to different studies providing divergent results that are extremely difficult to evaluate and merge. This lack of uniformity and inadequate adherence to quality standards in biospecimen handling is recognized by the national cancer institute (NCI) as a roadblock in cancer research, thus efforts are being made to overcome this by several international organizations and agencies [32–34].

5.1. Factors Directly Influencing the Usefulness of a Bio-Specimen

The collection methods, transportation, processing and storage conditions/methods will all determine the final quality of the biospecimen that is being analyzed. Different protocols used in each step of this pathway, from a sample being donated by the patient, to it being received by the researcher in the laboratory, may add a pre-analytic bias to the result obtained from it. Such biases can be introduced prior to the specimens reaching the laboratory, and they may or may not be recognized by the researcher [30]. Even if recognized, they may be difficult to adjust for in an analysis. Bias may also be introduced in the laboratory, producing results which may be related to artefacts of sample processing, but not due to disease specific pathology. Therefore, inequality of biospecimens remains to be the biggest flaw in the biomarker discovery. That can introduce bias early on in the studies, and 60–70% of all pre-analytical errors are due to the mishandling of samples during collection and processing [35]. Invalid proteomic and HER2 analyses [36] data from a clinical assay due to not adequately controlling the pre-analytical variables, is an example of a harmful outcome of using bio-specimens of poor or unknown quality.

The sample collection is a team effort, which typically involves patients, clinicians, researchers and biobank personnel. For a sample to truly represent a patient's tumor biology, and to make a valuable contribution to biomarker discovery, it needs to be properly collected and stored. This crucially requires the coordinated work and communication between all the essential members in the team, who follow explicit, best practice guidelines in every step in the biospecimen's journey, from the patient agreeing to donate it, to the specimen reaching the laboratory and going through the analysis. Therefore, it is important to bear in mind how a simple alteration, for example, tissue manipulation either before or after collection, can affect the end-result. Such aberrations could be erroneously reported as the changes in the expression levels of different genes and downstream targets, specific to a pathology. Therefore, it is an important responsibility of cancer researchers to remove such aberrations and to collect samples in optimal conditions.

5.2. *Biobanking Standards*

Controlling pre-analytical variability is challenging and complex. However, as the quality of data obtained from the sample is directly dependent upon the pre-analytic factors, it is important to consider using the most appropriate samples and the most robust biomolecule analytical method to obtain useful data. In this respect, biobanks have an important role to play in adhering to stringent and explicit standards when handling samples. In general, most biobanks have their own standardized way of sample handling and local standard operating procedures (SOPs) and protocols. Unfortunately, between biobanks, there are wide variations in biobanking practices, such as the type of samples collected, sample quality, demographic data collected, ethical approval process, available patient consent, processing techniques and storage workflows. This can create challenges for the researchers to obtain suitable and comparable samples for collaborative research projects.

The quality control of bio-banked samples can be regulated by (1) multidisciplinary scientific teams agreeing on the SOPs to adhere to at each stage of the biospecimen accruement, (2) standardizing these and communicating these with other scientific teams (3) by conducting specific-relevant research to identify new ways that will predict bio-sample integrity and quality [37–39].

5.3. *The Factors/Issues Affecting Analytical Results*

The results obtained from a biosample may be vulnerable to the quality of the biosample, in the context of the class of molecule analyzed, type of analytical method, the specificity, sensitivity and robustness of the method of analysis and the researcher controlling for the pre-analytical variables. Therefore, the researchers need to be fully aware of these issues pertinent to their samples and the employed methodology. Pre analytical variables such as biospecimen handling (e.g. snap freezing a sample immediately after collection as opposed to being transported in room temperature for several hours before freezing) may have an obvious effect on the integrity of the biospecimen and consequently on the downstream analyses [40]. Similarly, freeze thawing of samples after their acquisition in the laboratory can also affect the results, and should be considered by the researchers. Employing multiple techniques to confirm the data obtained from a biospecimen, examining multiple specimens from the same patient, and using a large number of samples from different sources to verify and to test the reproducibility of the results, are ways to reduce these pre/post-analytical biases in studies.

Genomics studies and transcriptomic analysis (e.g., microarrays/polymerase chain reaction (PCR)) depend on the sample stability and preservation of RNA integrity, hence even small temperature changes in collection, processing and storage can affect the scientific results. The SOPs used in different biobanks vary, depending on local resources, thus the quality of the samples can also differ widely. Implementation of quality management systems in biobanks and standardizing the best practice can lead to minimize the influence of these variations. Researchers can then obtain comparable samples for their research and conduct collaborative studies whilst facilitating the external validation of the promising results generated in smaller studies.

Presently, analysis of big-data at high throughput speed is revealed in the scientific world, and thus importantly, considering biospecimens, we should strive to focus on quality, rather than the quantity to prevent wastage of time and resources. SOPs should be developed with input from all stakeholders and implemented in biobanks to minimize the variability while improving quality. This will encourage consideration of all possible but rectifiable aspects in sample handling.

Making explicit records of the pre-analytic variables the specimens are subjected to is called the pre-analytical characterization. They should be part of the documentation held in biobanks, as they allow accurate grouping of similar samples during analysis.

6. Role of Harmonization of Biobanking and Existing Initiatives

As previously mentioned, many agencies have recognized the importance of the harmonization of the biobanking of human biological samples (Table 1). The welcome trust case control consortium and The Cancer Genome Atlas (TCGA) project have recognized issues with inconsistent sample quality. Different data from different sources [41] recommend consistency with biospecimens quality [42]. However, studies still report difficulties in obtaining sufficient high-quality bio-samples of diseased and control biological materials to come to definite conclusions [43,44].

The European Prospective Investigation into Cancer study coordinated from the International Agency for Research on Cancer, and the Telethon Network of Genetic Biobanks in Italy, have ventured into standardizing the SOPs, their consent, transfer policies and procedures [45,46]. The European strategy forum on research infrastructures recognized that major synergy, gain of statistical power and economy of scale is by interlinking, standardizing, harmonizing or just cross referencing with a large variety of well qualified, existing, up-to-date national resources [47]. This foresaw the development of the Biobanking and Biomolecular resources Research Infrastructure [48]. International biobanking platforms like 'The Marble Arch International Working Group on Biobanking for Biomedical Research' and the 'International society for Biological and Environmental Repositories' have also been working on standardization of biobanking at global level [49–51]. More than a decade ago, NCI launched an investigation to understand the state of resources and the quality of biospecimens used in cancer research, developing a detailed NCI-best practice guidance for biobanks [52]. This has established guiding principles for practice, promoting biospecimen and data quality maintenance, and also details the ethical and legal considerations. Although their adaptation is voluntary, they support the optimization of the resources available for cancer research on a global level.

Adapting and applying the current established best practice documents from some national institutes such as the 'National Institute of Health/NCI's Biorepositories and Biospecimen Research Branch Best Practices for Biospecimen Resources', the 'International Society of Biological and Environmental Repositories Best Practices for Repositories: Collection, Retrieval, and Distribution of Biological Materials for Research' and the 'World Health Organization International Agency for Research on Cancer Common Minimum Standards and Protocols for Biological Resource Centers Dedicated to Cancer Research' will assist to improve the harmonization process for biobanks, and by raising the overall awareness and quality of research involving bio specimens.

The huge efforts that have already been made as described above, have ensued many individual biobanks to be well-organized and to be accessible bio-sample repositories. However, this is not a uniform process. The prevailing bank-specific variations are still too large to source samples from all biobanks to a single study and to generate robust results. Hence, further harmonization is a necessity to utilize the available resources to their maximum potential.

Table 1. List of International and National Efforts of Harmonization of biobanking.

Year	Project	Role	References
2003	Public Population Project in Genomics (P3G)	Not for profit international consortium, promoted collaboration between researchers in genomics.	[53]
2005	Wellcome Trust Case Control Consortium	UK wide consortium, explored utility, design and analyses of Genome wide association studies.	[54]
2005	International Society for Biological and Environmental Repositories (ISBER)	Global forum which addressed the harmonisation of scientific, technical, legal, ethical issues of repositories.	[51]
2006	The Cancer Genome Atlas Project	Cancer genomics program, a joint project between National cancer institute and National human genome research institute.	[55]
2006	European Human Frozen Tumor Tissue Bank TUBAFROST	Virtual European human frozen tumor tissue bank, has access to high quality tissue collections, which are made available for the researchers.	[56]
2006	International Agency of Research on Cancer (IARC)	International project funded by WHO, international collaboration on cancer research for cancer prevention.	[57]
2006	First-Generation Guidelines for NCI-Supported Biorepositories	National Cancer Institute (NCI) drafted guidelines to standardize and enhance the quality of research material collected by the repositories.	[58]
2007	Biobanking and Biomolecular Resources Infrastructure (BBMRI)	European network, with biobanking focus on human biosamples.	[33]
2014	World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project (WERF EPhect)	International working group, which achieved global consensus in standardizing the data collection tools and protocols in endometriosis research.	[59]

7. Need for Specific Tools for Collection of Accurate Data

Biomarker-discovery studies have a wide variation and conflicting results, and these may be due to the lack of some essential data. For example, surgical phenotypic data, details of patient symptoms, together with other relevant information regarding sample handling that are specific to a particular condition/pathology, can influence the results. There is inconsistency in the type of data collected and the protocols used; hence, prior biobanked samples are not easy to be used in new, large, international, collaborative projects. This leads to the regular publication of a large number of studies with insufficient power that are simply ineffectual in making useful conclusions [59]. This huge waste in time and resources can be avoided with a harmonized biobanking practice that facilitates the easy organization of highly sought after, large scale, international, collaborative studies. Detailed surgical, clinical and epidemiological data pertinent to a specific cancer type can then be collected to accompany the biosamples from cancer patients, and thus will support many scientifically valid enquiries, producing a maximum return from the resources employed in sample collection. For standardization, it is important to have SOPs for the collection, processing and storage of the particular biospecimens and their accompanying clinical, surgical and other relevant data.

The surgical team is best placed to collect important clinical/surgical information, for example, intraoperative findings relevant to the clinical-staging of the cancer and complications. They will help to enlighten researchers both in basic science and in clinical research. This can be a time consuming process imposed on the surgical team, to collect and record the data. However, the engagement of the surgical team validates the clinical details, and they may also contribute their knowledge and understanding of the disease to link scientific discoveries with clinical outcomes. Information about clinical symptoms, previous relevant medical history of the patient, up to date comorbidities, medications, etc. can be

directly acquired from the patients who are the most accurate source of information in those aspects. This can be done by means of a self-completed questionnaire. As previously mentioned, it is important to recognize and minimize the variability by standardizing the collection, processing and storage of biological samples. Formulation of SOPs in advance, which are diligently adhered to by the biobank personnel is thus warranted.

8. Methods of Harmonization

Previous research has used several consensus generation methods, which mainly aim to achieve agreement of opinion on a particular topic, especially where published literature is inadequate. Consequently these methods have been generally used for problem solving or idea generation [60]. Three main consensus generation methods commonly adapted are: (i) The nominal group technique, (ii) consensus development conference and (iii) Delphi process. These methodologies are generally helpful in overcoming the disadvantages relevant to other less favored methods, such as committee meetings that can be dominated by one person or a group, usually with stakes or perception bias. In contrast, the focus of the more acceptable and thus favored consensus generation methods, is to assess the extent of agreement and to resolve disagreement. Therefore, the final result of these main methods is the inclusive and comprehensive agreement.

8.1. Nominal Group Technique

The nominal group technique is a structured face-to-face meeting where the panelists rate, discuss and rerate a number of questions. This has been mainly used in assessing the appropriateness of clinical interventions, education, training, and practice developments in the healthcare setting [61].

8.2. Consensus Development Conference

Consensus development conferences have been used for safety, effectiveness and appropriateness of medical care and technology. These are run informally in terms of criteria for generating consensus. The definition of consensus in these conferences is unanimous agreement with the consensus statement. The processes used can affect the value, validity and hence its reproducibility [62]. As in any consensus generation methodology, investment of time is necessary, but this is particularly true with conferences, as conferences need to be organized, and participants have to attend all of the meetings to ensure its reliability and validity in reaching a consensus. Hence, this method has the added complexity of being more expensive and time consuming. Debates and disagreements during the consensus generation conferences may deviate the attention and focus of the entire group [62]. Some of the well-known examples of the use of this method are those conducted by the National institute of Health (NIH) [63] and World endometriosis research foundation endometriosis phenome and biobanking harmonization project [59].

8.3. Delphi Process

The Delphi process is also a structured process, but here, the interaction amongst the panel members is through questionnaires, hence preserving anonymity. In this Delphi process, relevant individuals are invited to provide opinions, and they are also invited to participate in responding to different rounds of questionnaires. During each round, opinions are grouped together, and the questionnaire is redrafted until consensus is achieved on all topics included in the questionnaire. Although this appears to be a vigorous and sensible approach, there have also been various criticisms, such as the lack of evidence on the reliability of the Delphi process and its validity. Poor response rates may impede the process, and this is also another limitation of this method, and currently there are no guidelines on the number of consultation rounds that should be used as a standard, hence process can be variable [64].

Ironically, there is no general consensus or clear evidence as to which consensus generating method out of the above, is the best. Therefore, usually in a particular study, it is sufficient to clearly

justify the reasons for choosing the specific method, and to present the findings and their relevance in the context of the method [61].

9. Key Stakeholders in Cancer Research

Participation of all key stakeholders is paramount in the consensus generation exercise since they are the end users, which increases the acceptability through a sense of ownership and engagement. The benefit of involving patients is increasingly recognized [65] and includes procuring more accurate and reliable personal, past medical and past surgical data for research. Examples of information which can be obtained from patients include current weight, lifestyle data and family history. Although patients are likely to recall personal information on past medical, surgical and family histories more accurately, self-completed patient questionnaires used in this regard should be adequately prepared, with extensive patient/public involvement. This is because the accuracy of the information gathered is dependent upon the acceptability, user friendliness and clarity of the questions. A stringent methodology in developing the patient questionnaires and testing its reproducibility, suitability and acceptability to patients from different social, ethnic and cultural backgrounds, is therefore essential.

The healthcare professional involvement in basic science research is vital, since they are instrumental in translating scientific discoveries into clinical practice. They can convey the relevance of the research to patients well, and thus recruit participants appropriately for the studies. Clinicians with adequate knowledge/experience can ensure documenting and verifying accurate clinical-surgical information, such as details about a particular operation, surgical findings, cancer staging and patient follow up data relevant to cancer research [66]. Clinicians partake in the current standard clinical management pathway, and they can thus bridge the gap between basic and applied research [67].

Pathologists make the diagnostic confirmation of cancer, and they are key members of the biobanking team who procure the surplus clinical diagnostic material as biospecimens for research to be stored in biobanks. Pathologists thus are quality controllers of each sample, and contribute to developing robust SOPs for biospecimen collection, processing and storage [68].

Researchers and biobank personnel carry out fundamental work in specimen processing, storage and data management. Detailed information on the time of processing a sample, whether SOPs were accurately followed, if there were any deviations, and reasons for deviation from the protocol, are all valuable in harmonizing biospecimens, since that would allow comparison between groups and individuals. These are recorded by the biobank personnel for the samples collected, and they reduce bias and enable large scale collaborative studies [69].

10. Developing Tools for Harmonization of Biobanking Standards in Endometrial Cancer Research—HASTEN Study

Suitably collected patient material stored to high standards in Biobanks allows the study of multiple aspects of a single EC tumor, using novel technological platforms in genomics, proteomics, epigenomics and metabolomics, thus to simultaneously generate a large amount of information. Such an all-encompassing approach is expected to considerably reduce the time taken for new basic scientific discoveries to reach patients in the form of new treatments, as well as allowing the samples donated by patients to be fully utilized.

As described above, there are many generic biobanking standards and initiatives in place already. Although they are an important start, many parameters and variables of interest, including the choice of biospecimens and clinical data, are cancer-type specific. Thus, universal biobanking standards are not necessarily applicable to every cancer-type and should be adapted to each specific disease. The importance of a cancer-specific harmonization of biobanking standards is highlighted by the TCGA [70], which now contains over 532 EC samples with RNA sequencing, copy number variation, proteomic, mutation and microarray data. However, the extremely limited clinical data accompanying most of these samples and datasets severely affects the ability of researchers to draw clinically applicable information.

Therefore, EC specific standardization of the collection of biospecimens with distinctive and relevant accompanying clinical data sets, was a fundamental unmet need in improving future EC research. This, we believe, will facilitate future large-scale internationally collaborative research into EC, which could lead to improved biomarker and target treatment method discovery. Similar harmonization projects have already been successfully implemented for other gynecological conditions such as endometriosis, with the World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project (WERF EPHect), and the Ovarian Cancer Research Program (OVCARE) [71–75].

With this background, we initiated our study (Harmonization of biobAnking STandards in Endometrial caNcer research—HASTEN) in 2016, to achieve consensus amongst EC researchers. This was to standardize the collection, processing and storage of all relevant biospecimens, and the accompanying clinical data for EC research through a joint effort with all stakeholders of EC research.

Harmonization of EC research required the inclusion of all the above-mentioned variables, which increases the risk of EC in a woman. We also considered the common variations in the sample collection process; for example, the samples could be obtained during the diagnostic process (as a pipelle/curettings, Figure 1) or during the therapeutic procedure (from a hysterectomy sample). Variations in the handling of the samples were also considered, for example, frozen samples of presumed EC may or may not contain actual cancer cells, but only the background/adjacent hyperplasia or normal endometrium (Figure 2); thus diligent confirmation of the actual phenotype of the cellular content included in the specimen by histological scrutiny is required.

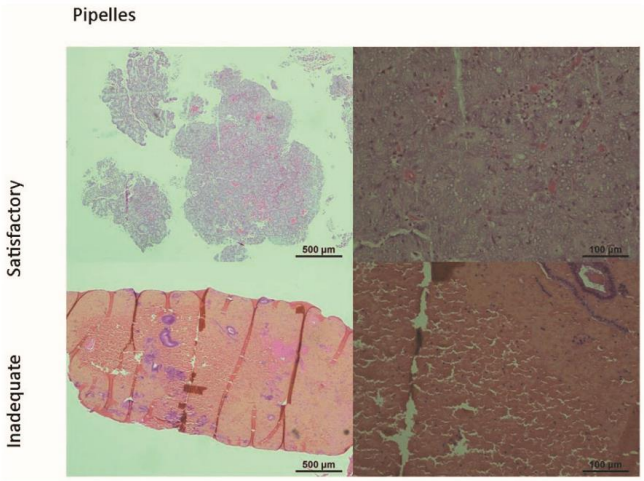


Figure 1. *Cont.*

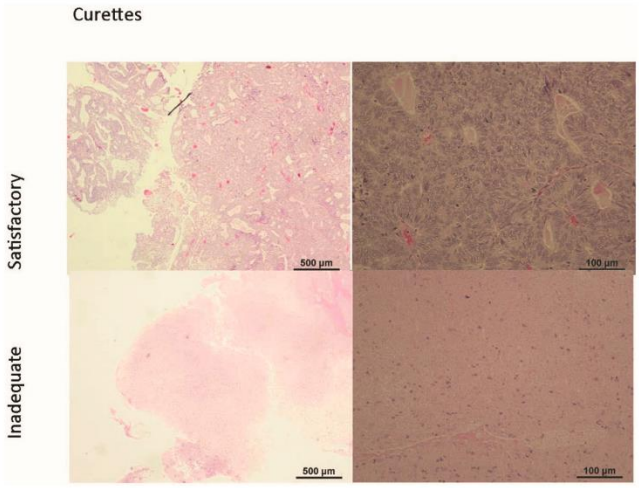


Figure 1. Micrographs of endometrial biopsies obtained during diagnostic procedures from patients with endometrial cancer. Pipelle (upper panel) and curettage (lower panel) samples may contain either satisfactory or inadequate amounts of cancer tissue as shown in this Haematoxylin- and Eosin-stained formalin fixed and paraffin-embedded tissue sections. This may be due to the skill of the clinician obtaining the sample, the endometrial thickness and the presence of mucus/blood, but they are inherent and unpredictable problems associated with these methods. Therefore, when a sample is collected by using these methods, and it is directly assigned for genomic and proteomic studies without confirming their cellular/tissue content, they may not produce credible data.

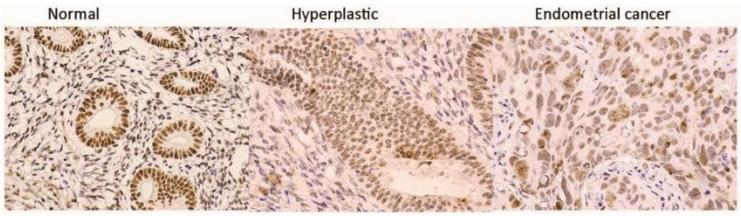


Figure 2. Micrographs of three separate endometrial samples obtained from the same patient, containing normal endometrial glands, hyperplastic glands and frank endometrial cancer tissue. The exact pathology included in the part of the sample studied with high throughput methods will directly influence the data generated. As shown here, the three separate parts of the endometrium biopsied from the same hysterectomy sample contained a histologically different pathological phenotype in the epithelial cells.

After an initial, thorough literature search and a critical appraisal of the available current evidence, four tools were consequently developed. Local, regional and European consensus on these tools was obtained through a comprehensive consultation process. When the final versions of the harmonization tools were developed, and final consensus was generated by a modified Delphi system. The modified Delphi system included sending the tools to panel members representing all the stakeholders in EC research, which included patients, gynecological oncologists, researchers, pathologists and biobank staff. The tools went through several rounds of revision according to the comments received, until unanimous consensus was reached. The final tools developed are freely available for any researcher via open access publication and the European Network for Individualized Treatment in EC (ENITEC) website. They include an EC patient data collection tool, an EC surgical data collection tool, an EC

biospecimen tool and a Standard operating procedure for the collection, processing and storage of tissue or fluid for EC research [76] (Figure 3).

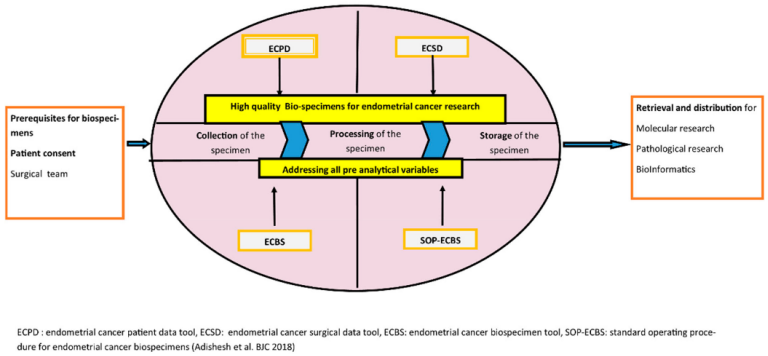


Figure 3. Journey of a biospecimen. Schematic representation of the utility of the tools developed with the HASTEN study (Harmonization of biobAnking STandards in Endometrial caNcer research). ECPD: Endometrial cancer patient data tool, ECSD: Endometrial cancer surgical data tool, ECBS: Endometrial cancer biospecimen tool, SOP-ECBS: Standard operating procedure for endometrial cancer biospecimens (Adishesh et al. BJC 2018).

10.1. Endometrial Cancer Patient Data Collection Tool (ECPD)

This user-friendly data collection tool captures important demographic variables that are relevant to EC research. These we believe can only be accurately recalled by the patients [76]. For example, the available literature suggests that >20 kg of adult weight gain to be independently associated with an increased risk of EC. However, this information is unlikely to be obtained easily from any other mean, but directly from the patient. Many other risk factors for EC, such as the age of presentation, the postmenopausal status, history of polycystic ovarian syndrome, nulliparity, early age of menarche, family history of hereditary lynch syndrome-related cancers, past history of lynch syndrome-related cancers, medical conditions such diabetes, previous use of tamoxifen and hormone replacement therapy and exercise habits, are similarly best recalled by the patient, and therefore are also included in the tool (Supplementary Document 1 in [76]).

10.2. Endometrial Cancer Surgical Data Collection Tool (ECSD)

The surgical data tool includes salient demographic, histological and pre/postoperative features [76] relevant to EC. It also includes information about preoperative imaging details and preoperative investigations such as endometrial biopsy results. Immunohistochemical biomarkers can be used to distinguish ECs from ovarian, cervical or other malignancies, but importantly they may also serve as prognostic biomarkers that are associated with clinical outcome. This tool is organized into different sections containing; surgical data: To be completed at the time of sample collection; histopathology details: To be completed after final staging and treatment and; outcome data: To be documented during follow-up and finally at the end of follow-up period (Supplementary Documents 3 and 4 in [76]).

10.3. Endometrial Cancer Biospecimen Tool (ECBS)

Variations in the collection methods at the time of diagnosis or treatment, and biobanking variables such as processing and storage, may alter the molecular composition, expression and stability of biomarker profiles, and thus, consistency and strict adherence to SOPs is vital. Therefore, information regarding the times of processing, storage and any deviations from this SOP needs to be documented clearly by the biobanking personnel (Supplementary Document 2 in [76]).

10.4. Standard Operating Procedure for Collection, Processing and Storage of Tissue or Fluid for Endometrial Cancer Research (SOP-ECBS)

The methods used for investigating different tissue and fluid biospecimens collected may involve the extraction of protein, RNA and DNA, using a variety of techniques, such as proteomics, genomics and metabolomics. A SOP amalgamating a number of separate, detailed methodological protocols (e.g., for centrifugation, filtration, addition of preservatives, as well as storage temperatures) is required, and therefore, was devised [76]. Studies examining specimens collected via non-invasive means, including saliva and urine, are of a particular interest in clinical research. Future work is expected to focus more on them, hence SOPs need to include all samples which could be collected for EC research and are considered in this tool (Supplementary Document 5 in [76]).

11. Conclusions

Incidences of EC, and EC associated surgical morbidity and mortality, are increasing at an alarming rate. The causative factors, such as obesity and longevity, with their associated co-morbidities, are only expected to increase in the future, adding further pressure on clinicians and researchers to find novel, personalized diagnostic, therapeutic, prognostic and preventative strategies.

For EC research and personalized EC treatment to be benefitted from the advances in 'Omics' technology, robust and extensive repositories of patient derived biological samples with accompanying detailed surgical, clinical and epidemiological data, is essential. Thus, harmonization of biobanking standards is a vital step toward high quality standardized, large-scale international collaborative projects to generate data that is translated into personalized clinical practice. The HASTEN project devised EC-specific tools and SOP through a comprehensive consensus generation process for the first time, and these will provide the necessary guidance and means for all EC researchers to standardize biobanking EC-related biospecimens. This, we envisage, will be a significant step in improving the quality of EC research in general, and will in the future result in enhancing clinical care through personalized management for the benefit of many women suffering from EC.

Author Contributions: Conceptualization, D.K.H.; methodology, D.K.H. and M.A.; writing—original draft preparation, M.A. and D.K.H.; writing—review and editing; visualization, and supervision, D.K.H.; project administration, M.A..; funding acquisition, D.K.H. and M.A.

Funding: This work was funded by Liverpool Women's Hospital Cancer Charity (M.A.), Liverpool Women's Hospital NHS trust (D.K.H. and M.A.); Department of Women's Health, University of Liverpool (D.K.H.), and Wellbeing of Women project grant numbers RG1487 and RG2137.

Acknowledgments: The authors are grateful to the patients, panellists, gynaecological oncology surgical team members at Liverpool Women's Hospital, members of Endometrial Research team, members of ENITEC, Dr Henrica Werner for their invaluable contribution for the HASTEN project.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. CRUK. Available online: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer (accessed on 29 September 2019).
2. Wartko, P.; Sherman, M.E.; Yang, H.P.; Felix, A.S.; Brinton, L.A.; Trabert, B. Recent changes in endometrial cancer trends among menopausal-age U.S. women. *Cancer Epidemiol.* **2013**, *37*, 374–377. [CrossRef]
3. Smittenaar, C.R.; Petersen, K.A.; Stewart, K.; Moitt, N. Cancer incidence and mortality projections in the UK until 2035. *Br. J. Cancer* **2016**, *115*, 1147–1155. [CrossRef] [PubMed]
4. Lindemann, K.; Eskild, A.; Vatten, L.J.; Bray, F. Endometrial cancer incidence trends in Norway during 1953–2007 and predictions for 2008–2027. *Int. J. Cancer* **2010**, *127*, 2661–2668. [CrossRef] [PubMed]
5. Hapangama, D.K.; Kamal, A.M.; Bulmer, J.N. Estrogen receptor β: The guardian of the endometrium. *Hum. Reprod. Update* **2015**, *21*, 174–193. [CrossRef] [PubMed]

6. Tempest, N.; Baker, A.M.; Wright, N.A.; Hapangama, D.K. Does human endometrial LGR5 gene expression suggest the existence of another hormonally regulated epithelial stem cell niche? *Hum. Reprod.* **2018**, *33*, 1052–1062. [CrossRef] [PubMed]
7. Kamal, A.M.; Bulmer, J.N.; DeCruze, S.B.; Stringfellow, H.F.; Martin-Hirsch, P.; Hapangama, D.K. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. *Br. J. Cancer* **2016**, *114*, 688–696. [CrossRef] [PubMed]
8. Calle, E.E.; Rodriguez, C.; Walker-Thurmond, K.; Thun, M.J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N. Engl. J. Med.* **2003**, *348*, 1625–1638. [CrossRef] [PubMed]
9. Morch, L.S.; Kjaer, S.K.; Keiding, N.; Lokkegaard, E.; Lidegaard, O. The influence of hormone therapies on type I and II endometrial cancer: A nationwide cohort study. *Int. J. Cancer* **2016**, *138*, 1506–1515. [CrossRef] [PubMed]
10. Sjogren, L.L.; Morch, L.S.; Lokkegaard, E. Hormone replacement therapy and the risk of endometrial cancer: A systematic review. *Maturitas* **2016**, *91*, 25–35. [CrossRef]
11. Sismondi, P.; Biglia, N.; Volpi, E.; Giai, M.; de Grandis, T. Tamoxifen and endometrial cancer. *Ann. N.Y. Acad. Sci.* **1994**, *734*, 310–321. [CrossRef]
12. Bissett, D.; Davis, J.A.; George, W.D. Gynaecological monitoring during tamoxifen therapy. *Lancet* **1994**, *344*, 1244. [CrossRef]
13. Fisher, B.; Costantino, J.P.; Redmond, C.K.; Fisher, E.R.; Wickerham, D.L.; Cronin, W.M. Endometrial cancer in tamoxifen-treated breast cancer patients: Findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J. Natl. Cancer Inst.* **1994**, *86*, 527–537. [CrossRef] [PubMed]
14. Haoula, Z.; Salman, M.; Atiomo, W. Evaluating the association between endometrial cancer and polycystic ovary syndrome. *Hum. Reprod.* **2012**, *27*, 1327–1331. [CrossRef]
15. Gong, T.T.; Wang, Y.L.; Ma, X.X. Age at menarche and endometrial cancer risk: A dose-response meta-analysis of prospective studies. *Sci. Rep.* **2015**, *5*, 14051. [CrossRef] [PubMed]
16. Ali, A.T. Reproductive factors and the risk of endometrial cancer. *Int. J. Gynecol. Cancer* **2014**, *24*, 384–393. [CrossRef]
17. Mueck, A.O.; Seeger, H.; Rabe, T. Hormonal contraception and risk of endometrial cancer: A systematic review. *Endocr. Relat. Cancer* **2010**, *17*, R263–R271. [CrossRef]
18. Cook, L.S.; Dong, Y.; Round, P.; Huang, X.; Magliocco, A.M.; Friedenreich, C.M. Hormone contraception before the first birth and endometrial cancer risk. *Cancer Epidemiol. Biomark. Prev.* **2014**, *23*, 356–361. [CrossRef]
19. Bats, A.S.; Rossi, L.; Le Frere-Belda, M.A.; Narjoz, C.; Cournou, C.; Gosset, M.; Ngo, C.; Delomenie, M.; Nos, C.; Blons, H.; et al. [Lynch syndrome and endometrial cancer]. *Bull. Cancer* **2017**, *104*, 1013–1021. [CrossRef]
20. Yin, X.H.; Jia, H.Y.; Xue, X.R.; Yang, S.Z.; Wang, Z.Q. Clinical analysis of endometrial cancer patients with obesity, diabetes, and hypertension. *Int. J. Clin. Exp. Med.* **2014**, *7*, 736–743.
21. Schonfeld, S.J.; Hartge, P.; Pfeiffer, R.M.; Freedman, D.M.; Greenlee, R.T.; Linet, M.S.; Park, Y.; Schairer, C.; Visvanathan, K.; Lacey, J.V., Jr. An aggregated analysis of hormonal factors and endometrial cancer risk by parity. *Cancer* **2013**, *119*, 1393–1401. [CrossRef]
22. Moore, S.C.; Gierach, G.L.; Schatzkin, A.; Matthews, C.E. Physical activity, sedentary behaviours, and the prevention of endometrial cancer. *Br. J. Cancer* **2010**, *103*, 933–938. [CrossRef] [PubMed]
23. Felix, A.S.; Yang, H.P.; Gierach, G.L.; Park, Y.; Brinton, L.A. Cigarette smoking and endometrial carcinoma risk: The role of effect modification and tumor heterogeneity. *Cancer Causes Control* **2014**, *25*, 479–489. [CrossRef] [PubMed]
24. Moore, H.M.; Compton, C.C.; Lim, M.D.; Vaught, J.; Christiansen, K.N.; Alper, J. 2009 Biospecimen research network symposium: Advancing cancer research through biospecimen science. *Cancer Res.* **2009**, *69*, 6770–6772. [CrossRef] [PubMed]
25. Gambara, G.; Gaebler, M.; Keilholz, U.; Regenbrecht, C.R.A.; Silvestri, A. From Chemotherapy to Combined Targeted Therapeutics: In Vitro and in Vivo Models to Decipher Intra-tumor Heterogeneity. *Front. Pharmacol.* **2018**, *9*, 77. [CrossRef] [PubMed]
26. Verma, M.; Rogers, S.; Divi, R.L.; Schully, S.D.; Nelson, S.; Joseph Su, L.; Ross, S.A.; Pilch, S.; Winn, D.M.; Khoury, M.J. Epigenetic research in cancer epidemiology: Trends, opportunities, and challenges. *Cancer Epidemiol. Biomark. Prev.* **2014**, *23*, 223–233. [CrossRef] [PubMed]

27. Mill, J.; Heijmans, B.T. From promises to practical strategies in epigenetic epidemiology. *Nat. Rev. Genet.* **2013**, *14*, 585–594. [CrossRef] [PubMed]
28. Hewitt, R.; Hainaut, P. Biobanking in a fast moving world: An international perspective. *J. Natl. Cancer Inst. Monogr.* **2011**, *2011*, 50–51. [CrossRef]
29. Knoppers, B.M.; Zawati, M.H.; Kirby, E.S. Sampling populations of humans across the world: ELSI issues. *Annu. Rev. Genom. Hum. Genet.* **2012**, *13*, 395–413. [CrossRef]
30. Ransohoff, D.F.; Gourlay, M.L. Sources of bias in specimens for research about molecular markers for cancer. *J. Clin. Oncol.* **2010**, *28*, 698–704. [CrossRef]
31. Tworoger, S.S.; Hankinson, S.E. Use of biomarkers in epidemiologic studies: Minimizing the influence of measurement error in the study design and analysis. *Cancer Causes Control* **2006**, *17*, 889–899. [CrossRef]
32. Vaught, J.; Lockhart, N.C. The evolution of biobanking best practices. *Clin. Chim. Acta* **2012**, *413*, 1569–1575. [CrossRef] [PubMed]
33. Yuille, M.; van Ommen, G.J.; Bréchot, C.; Cambon-Thomsen, A.; Dagher, G.; Landegren, U.; Litton, J.E.; Pasterk, M.; Peltonen, L.; Taussig, M.; et al. Biobanking for Europe. *Brief. Bioinform.* **2008**, *9*, 14–24. [CrossRef] [PubMed]
34. Morente, M.M.; de Alava, E.; Fernandez, P.L. Tumour banking: The Spanish design. *Pathobiology* **2007**, *74*, 245–250. [CrossRef] [PubMed]
35. Lippi, G.; Bowen, R.; Adcock, D.M. Re-engineering laboratory diagnostics for preventing preanalytical errors. *Clin. Biochem.* **2016**, *49*, 1313–1314. [CrossRef]
36. Portier, B.P.; Wang, Z.; Downs-Kelly, E.; Rowe, J.J.; Patil, D.; Lanigan, C.; Budd, G.T.; Hicks, D.G.; Rimm, D.L.; Tubbs, R.R. Delay to formalin fixation 'cold ischemia time': Effect on ERBB2 detection by in-situ hybridization and immunohistochemistry. *Mod. Pathol.* **2013**, *26*, 1–9. [CrossRef]
37. Moore, H.M. The NCI Biospecimen Research Network. *Biotech. Histochem.* **2012**, *87*, 18–23. [CrossRef]
38. Betsou, F.; Lehmann, S.; Ashton, G.; Barnes, M.; Benson, E.E.; Coppola, D.; DeSouza, Y.; Eliason, J.; Glazer, B.; Guadagni, F.; et al. Standard preanalytical coding for biospecimens: Defining the sample PREanalytical code. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 1004–1011. [CrossRef]
39. Betsou, F.; Beaudeux, J.L.; Berthelaix, A.; Borg, J.; Dupuy, A.; Lizard, G.; Peoc'h, K.; Quillard, M.; Lehmann, S. "Standard" protocol for evaluating the impact of preanalytical variables on peptidic and proteic analytes and standard coding of preanalytical procedures. *Ann. Biol. Clin.* **2009**, *67*, 641–649. [CrossRef]
40. Ellervik, C.; Vaught, J. Preanalytical variables affecting the integrity of human biospecimens in biobanking. *Clin. Chem.* **2015**, *61*, 914–934. [CrossRef]
41. Bellenguez, C.; Strange, A.; Freeman, C.; Donnelly, P.; Spencer, C.C. A robust clustering algorithm for identifying problematic samples in genome-wide association studies. *Bioinformatics* **2012**, *28*, 134–135. [CrossRef]
42. Solomon, D.A.; Kim, J.S.; Ressom, H.W.; Sibenaller, Z.; Ryken, T.; Jean, W.; Bigner, D.; Yan, H.; Waldman, T. Sample type bias in the analysis of cancer genomes. *Cancer Res.* **2009**, *69*, 5630–5633. [CrossRef] [PubMed]
43. Chantrill, L.A.; Nagrial, A.M.; Watson, C.; Johns, A.L.; Martyn-Smith, M.; Simpson, S.; Mead, S.; Jones, M.D.; Samra, J.S.; Gill, A.J.; et al. Precision Medicine for Advanced Pancreas Cancer: The Individualized Molecular Pancreatic Cancer Therapy (IMPaCT) Trial. *Clin. Cancer Res.* **2015**, *21*, 2029–2037. [CrossRef]
44. Rush, A.; Spring, K.; Byrne, J.A. A critical analysis of cancer biobank practices in relation to biospecimen quality. *Biophys. Rev.* **2015**, *7*, 369–378. [CrossRef] [PubMed]
45. Bricarelli, M.F.; Chiara, B.; Stefano, G.; Alessandra, R.; Corrado, A.; Maurizio, M.; Marina, M.; Giuseppe, M.; Luisa, P.; Barbara, G.; et al. Telethon Network of Genetic Biobanks: A key service for diagnosis and research on rare diseases. *Orphanet J. Rare Dis.* **2013**, *8*, 1–11. [CrossRef]
46. Riboli, E.; Hunt, K.J.; Slimani, N.; Ferrari, P.; Norat, T.; Fahey, M.; Charrondiere, U.R.; Hemon, B.; Casagrande, C.; Vignat, J.; et al. European Prospective Investigation into Cancer and Nutrition (EPIC): Study populations and data collection. *Public Health Nutr.* **2002**, *5*, 1113–1124. [CrossRef] [PubMed]
47. Dinges, M.; Bouttier, R.; Schiffbanker, H.; Holzinger, F.; van der Giessen, A.M.; Lehenkari, J.; Deschryvere, M.; Kuittinen, H.; Rammer, C. Analysis of the ERA State of Play in Member States and Associated Countries:Focus on Priority Areas. Available online: https://www.researchgate.net/publication/266399729_Analysis_of_the_state_of_play_of_the_European_Research_Era_in_Member_States_and_Associated_Countries_focus_on_priority_areas (accessed on 29 September 2019).

48. BBMRI. Available online: https://cordis.europa.eu/project/rcn/88365/reporting/en (accessed on 29 September 2019).
49. Riegman, P.H.; Morente, M.M.; Betsou, F.; de Blasio, P.; Geary, P. Biobanking for better healthcare. *Mol. Oncol.* **2008**, *2*, 213–222. [CrossRef] [PubMed]
50. Doucet, M.; Becker, K.F.; Björkman, J.; Bonnet, J.; Clément, B.; Daidone, M.G.; Duyckaerts, C.; Erb, G.; Haslacher, H.; Hofman, P.; et al. Quality Matters: 2016 Annual Conference of the National Infrastructures for Biobanking. *Biopreserv. Biobank.* **2017**, *15*, 270–276. [CrossRef]
51. Campbell, L.D.; Astrin, J.J.; DeSouza, Y.; Giri, J.; Patel, A.A.; Rawley-Payne, M.; Rush, A.; Sieffert, N. The 2018 Revision of the ISBER Best Practices: Summary of Changes and the Editorial Team's Development Process. *Biopreserv. Biobank.* **2018**, *16*, 3–6. [CrossRef]
52. NCI. NCI Best Practices for Biospecimen Resources. Available online: https://biospecimens.cancer.gov/bestpractices/2011-NCIBestPractices.pdf (accessed on 29 September 2019).
53. P.G. Consortium. Available online: http://www.p3gconsortium.org/resources/biobank-toolkit (accessed on 29 September 2019).
54. The Wellcome Trust Case Control Consortium; Burton, P.R.; Clayton, D.G.; Cardon, L.R.; Craddock, N.; Deloukas, P.; Duncanson, A.; Kwiatkowski, D.P.; McCarthy, M.I.; Ouwehand, W.H.; et al. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **2007**, *447*, 661. [CrossRef]
55. Waltz, E. Pricey cancer genome project struggles with sample shortage. *Nat. Med.* **2007**, *13*, 391. [CrossRef]
56. Morente, M.M.; Mager, R.; Alonso, S.; Pezzella, F.; Spatz, A.; Knox, K.; Kerr, D.; Dinjens, W.N.M.; Oosterhuis, J.W.; Lam, K.H.; et al. TuBaFrost 2: Standardising tissue collection and quality control procedures for a European virtual frozen tissue bank network. *Eur. J. Cancer* **2006**, *42*, 2684–2691. [CrossRef] [PubMed]
57. IARC. Available online: http://www.iarc.fr/News/RecommendationsBRC.pdf (accessed on 29 September 2019).
58. NCI. Available online: https://www.federalregister.gov/documents/2006/04/28/06-3997/first-generation-guidelines-for-nci-supported-biorepositories (accessed on 29 September 2019).
59. Becker, C.M.; Laufer, M.R.; Stratton, P.; Hummelshoj, L.; Missmer, S.A.; Zondervan, K.T.; Adamson, G.D. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: I. Surgical phenotype data collection in endometriosis research. *Fertil. Steril.* **2014**, *102*, 1213–1222. [CrossRef] [PubMed]
60. McMillan, S.S.; King, M.; Tully, M.P. How to use the nominal group and Delphi techniques. *Int. J. Clin. Pharm.* **2016**, *38*, 655–662. [CrossRef] [PubMed]
61. Jones, J.; Hunter, D. Consensus methods for medical and health services research. *BMJ* **1995**, *311*, 376–380. [CrossRef] [PubMed]
62. Halcomb, E.; Davidson, P.; Hardaker, L. Using the consensus development conference method in healthcare research. *Nurse Res.* **2008**, *16*, 56–71. [CrossRef] [PubMed]
63. NIH. National Institutes of Health Consensus Development Conference Statement on Celiac Disease, June 28–30, 2004. *Gastroenterology* **2005**, *128*, S1–S9. [CrossRef]
64. Keeney, S.; Hasson, F.; McKenna, H.P. A critical review of the Delphi technique as a research methodology for nursing. *Int. J. Nurs. Stud.* **2001**, *38*, 195–200. [CrossRef]
65. Chalmers, I.; Bracken, M.B.; Djulbegovic, B.; Garattini, S.; Grant, J.; Gulmezoglu, A.M.; Howells, D.W.; Ioannidis, J.P.; Oliver, S. How to increase value and reduce waste when research priorities are set. *Lancet* **2014**, *383*, 156–165. [CrossRef]
66. NCRI. NCRI Surgical Research Report. Available online: https://www.ncri.org.uk/wp-content/uploads/2013/07/2012-NCRI-surgical-research-report.pdf (accessed on 29 September 2019).
67. Kahn, K.; Ryan, G.; Beckett, M.; Taylor, S.; Berrebi, C.; Cho, M.; Quiter, E.; Fremont, A.; Pincus, H. Bridging the gap between basic science and clinical practice: A role for community clinicians. *Implement. Sci.* **2011**, *6*, 34. [CrossRef]
68. NCRI. Fostering the Role of Pathology in Research: Executive Summary. Available online: https://www.ncri.org.uk/wp-content/uploads/2013/08/2009-NCRI-Fostering-the-role-of-pathology-in-research.pdf (accessed on 4 November 2019).
69. Capocasa, M.; Anagnostou, P.; D'Abramo, F.; Matteucci, G.; Dominici, V.; Destro Bisol, G.; Rufo, F. Samples and data accessibility in research biobanks: An explorative survey. *PeerJ* **2016**, *4*, e1613. [CrossRef]

70. Kandoth, C.; Schultz, N.; Cherniack, A.D.; Akbani, R.; Liu, Y.; Shen, H.; Robertson, A.G.; Pashtan, I.; Shen, R.; Benz, C.C.; et al. Integrated genomic characterization of endometrial carcinoma. *Nature* **2013**, *497*, 67–73. [CrossRef] [PubMed]
71. Vitonis, A.F.; Vincent, K.; Rahmioglu, N.; Fassbender, A.; Buck Louis, G.M.; Hummelshoj, L.; Giudice, L.C.; Stratton, P.; Adamson, G.D.; Becker, C.M.; et al. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: II. Clinical and covariate phenotype data collection in endometriosis research. *Fertil. Steril.* **2014**, *102*, 1223–1232. [CrossRef] [PubMed]
72. Rahmioglu, N.; Fassbender, A.; Vitonis, A.F.; Tworoger, S.S.; Hummelshoj, L.; D'Hooghe, T.M.; Adamson, G.D.; Giudice, L.C.; Becker, C.M.; Zondervan, K.T.; et al. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil. Steril.* **2014**, *102*, 1233–1243. [CrossRef] [PubMed]
73. Fassbender, A.; Rahmioglu, N.; Vitonis, A.F.; Vigano, P.; Giudice, L.C.; D'Hooghe, T.M.; Hummelshoj, L.; Adamson, G.D.; Becker, C.M.; Missmer, S.A.; et al. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. *Fertil. Steril.* **2014**, *102*, 1244–1253. [CrossRef]
74. Wiegand, K.C.; Shah, S.P.; Al-Agha, O.M.; Zhao, Y.; Tse, K.; Zeng, T.; Senz, J.; McConechy, M.K.; Anglesio, M.S.; Kalloger, S.E.; et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N. Engl. J. Med.* **2010**, *363*, 1532–1543. [CrossRef]
75. Heravi-Moussavi, A.; Anglesio, M.S.; Cheng, S.W.; Senz, J.; Yang, W.; Prentice, L.; Fejes, A.P.; Chow, C.; Tone, A.; Kalloger, S.E.; et al. Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N. Engl. J. Med.* **2012**, *366*, 234–242. [CrossRef]
76. Adishesh, M.; Fyson, A.; DeCruze, S.B.; Kirwan, J.; Werner, H.M.J.; Hapangama, D.K. Harmonisation of biobanking standards in endometrial cancer research. *Br. J. Cancer* **2017**, *117*, 485–493. [CrossRef]

International Journal of
Molecular Sciences

MDPI

Article

Human Endometrial Carcinogenesis Is Associated with Significant Reduction in Long Non-Coding RNA, TERRA

Meera Adishesh [1,2], Rafah Alnafakh [2], Duncan M. Baird [3], Rhiannon E. Jones [3], Shannon Simon [2], Lucy Button [2], Areege M. Kamal [2,4], John Kirwan [1], S. Bridget DeCruze [1], Josephine Drury [2], Gabriele Saretzki [5] and Dharani K. Hapangama [1,2,*]

1 Liverpool Women's Hospital NHS Foundation Trust, Liverpool L8 7SS, UK; meeadish@liverpool.ac.uk (M.A.); John.Kirwan@lwh.nhs.uk (J.K.); shandyabdecruze@gmail.com (S.B.D.)
2 Department of Women's and Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool, Member of Liverpool Health Partners, Liverpool L8 7SS, UK; R.A.A.Alnafakh@liverpool.ac.uk (R.A.); shannonsimon96@gmail.com (S.S.); lucyfbutton@gmail.com (L.B.); areegekamal@gmail.com (A.M.K.); jadrury@liverpool.ac.uk (J.D.)
3 Division of Cancer and Genetics, Cardiff University, Cardiff CF14 4XN, UK; bairddm@cardiff.ac.uk (D.M.B.); jonesr47@cardiff.ac.uk (R.E.J.)
4 The National Centre for Early Detection of Cancer, Oncology Teaching Hospital, Baghdad Medical City, Baghdad, Iraq
5 Biosciences Institute, Newcastle University, Newcastle upon Tyne NE4 5PL, UK; gabriele.saretzki@newcastle.ac.uk
* Correspondence: dharani@liv.ac.uk; Tel.: +44-151-795-9553

Received: 3 November 2020; Accepted: 16 November 2020; Published: 18 November 2020

Abstract: Telomeres are transcribed as long non-coding RNAs called TERRAs (Telomeric repeat containing RNA) that participate in a variety of cellular regulatory functions. High telomerase activity (TA) is associated with endometrial cancer (EC). This study aimed to examine the levels of three TERRAs, transcribed at chromosomes 1q-2q-4q-10q-13q-22q, 16p and 20q in healthy (n = 23) and pathological (n = 24) human endometrium and to examine their association with cellular proliferation, TA and telomere lengths. EC samples demonstrated significantly reduced levels of TERRAs for Chromosome 16p (Ch-16p) ($p < 0.002$) and Chromosome 20q (Ch-20q) ($p = 0.0006$), when compared with the postmenopausal samples. No significant correlation was found between TERRA levels and TA but both Ch-16p and Ch-20q TERRA levels negatively correlated with the proliferative marker Ki67 ($r = -0.35$, $p = 0.03$ and $r = -0.42$, $p = 0.01$ respectively). Evaluation of single telomere length analysis (STELA) at XpYp telomeres demonstrated a significant shortening in EC samples when compared with healthy tissues ($p = 0.002$). We detected TERRAs in healthy human endometrium and observed altered individual TERRA-specific levels in malignant endometrium. The negative correlation of TERRAs with cellular proliferation along with their significant reduction in EC may suggest a role for TERRAs in carcinogenesis and thus future research should explore TERRAs as potential therapeutic targets in EC.

Keywords: TERRA; long non-coding RNA; telomerase; endometrium; telomeres; telomere length; endometrial cancer

1. Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in the western world with an increasing incidence due to the rise in obesity and longevity [1]. This increase in frequency of EC is

accompanied by a concomitant rise in cancer-associated mortality, a trend that is expected to continue for the next few decades [2]. This is a contrasting and concerning statistic, compared with many other cancers, where the incidence of new cancers remains stable and cancer-associated mortality is decreasing. Therefore, it poses an urgent need to develop novel preventative and treatment strategies, which require advancement in our current understanding of endometrial carcinogenesis. The mortality rates for high-grade EC are especially high, similar to ovarian cancer. Some women with EC are not suitable for the conventional surgical treatment, hysterectomy [3], and surgical treatment is associated with significant morbidity and mortality in those with co-occurring multi-morbidities and obesity. Biomarkers that would inform us of potential prognostic and therapeutic targets are thus invaluable in developing future strategies to reduce the cancer related morbidity and mortality of EC.

High activity levels of telomerase enzyme are a feature of most carcinomas, with telomere maintenance being its main function. Telomeres are specialized nucleoprotein complexes at the ends of chromosomes that prevent chromosomal ends from being recognized as DNA damage foci that can mount a DNA damage response (DDR) [4]. Telomeres contain a repetitive hexanucleotide sequence TTAGGG bound by six-shelterin proteins, namely telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), Protection of telomeres 1 (POT1), tripeptidyl peptidase 1 (TPP1), Repressor/activator protein 1 (RAP1) and TRF1- and TRF1-interacting protein 2 (TIN2), and ends with single-stranded G-rich overhang [5]. Telomeric DNA is lost with each round of cell division, due to the end replication problem, and, unless the lost DNA is replaced by the action of telomerase, telomeres will shorten in proliferating cells. Critically short telomeres can initiate a DDR and induce apoptotic or cellular senescence pathways with permanent cell cycle arrest [6]. However, specialized cells and cancer cells have sufficiently high telomerase activity (TA) for telomere maintenance and elongation, which prevents senescence and allows unrestrained proliferation [7].

Telomeres were considered to be transcriptionally silent until recently, however telomeric repeat-containing RNA (TERRA) molecules are transcribed by RNA polymerase II from CpG-island containing sub telomeric promotors [8]. TERRA molecules are heterogenous long non-coding RNAs (lncRNA) [9], and they have been identified in vertebrates and in several other species including yeasts and plants [10,11]. Transcription of TERRA has been proposed to assist in telomere replication and thus support chromosome stability [12]. The action of TERRA may be relevant to the telomere of origin or other telomeres in general [13]. Their expression is inversely correlated with the length of the corresponding telomeres [14]. TERRAs are involved in the recruitment of telomerase to telomeres [15] when they remain partly associated with the telomere of origin, establishing an RNA G-quadruplex structure that protects the telomeres [16]. The TERRA containing RNA-DNA hybrids at the telomeric end may also have a physiological role in stalling replication forks, hence allowing DNA repair [17]. Conversely, TERRAs are also proposed to inhibit telomerase function by binding to the *hTERC* and hTERT components of telomerase [18], as well as by forming RNA–DNA hybrids [10]. TERRA levels are cell specific [9] and fluctuate with the cell cycle [19]. Shelterin proteins TRF1 and TRF2 are known to interact with TERRA and TERRAs mediate cell-fate and cellular reprogramming via a TRF1-dependent pathway [20]. Therefore, TERRAs are thought to participate in many essential cellular and telomere/telomerase regulatory functions [14]. A study that examined the predicted features of 18 TERRA loci in human cell confirmed transcripts arising from only 20q and Xp loci to have these expected TERRA features, and subsequently demonstrated that only deletion of the 20q locus to cause a dramatic decrease in TERRA levels [13]. By deleting the 20q-TERRA locus, the authors demonstrated an increase in telomere damage foci and confirmed that TERRA transcripts to be essential for the maintenance of a functional telomere cap.

Human endometrium is a dynamic tissue, which undergoes a repetitive cycle of cell proliferation, differentiation, shedding and regeneration during the reproductive years of a woman's life. It is regulated by the cyclically secreted ovarian hormones on a monthly basis [21]. Although a somatic tissue, human endometrium expresses dynamic TA levels, and highly proliferative endometrial cells during the estrogen dominant, proliferative phase of the menstrual cycle have high TA levels, while the

cessation of the epithelial proliferation under the regulation of progesterone in the secretory phase of the cycle demonstrated lowest TA levels [4,22]. Endometrial epithelium demonstrates distinctively higher TA than the stromal cells, yet conversely shorter relative telomere lengths were reported in the epithelial cells when compared with the stromal cells from the same samples [22]. This observation had been explained as high TA maintaining the short epithelial telomeres beyond the critical length, thus allowing epithelial cells to avoid cell cycle arrest/cellular senescence [4,22]. Therefore, TA is implicated in endometrial epithelial cell proliferation; high TA is a reported feature in the normal proliferative phase endometrium, in benign endometrial proliferative conditions such as endometriosis, and in endometrial carcinogenesis [23,24].

As mentioned above, many authors have already reported the involvement of TA, telomeres and many telomere/telomerase associated proteins and genes in endometrial function including their aberrations in endometrial carcinogenesis [25–30]. However, the existence of TERRAs in healthy endometrium or their disease-specific alterations have not yet been elucidated. Considering the intricate relationship between telomere/telomerase biology and endometrial cellular proliferation [22] and carcinogenesis [31], we hypothesized that TERRA levels may also be altered in EC. We therefore sought to examine the expression levels of TERRAs in human endometrium, from both healthy women and those with EC. We utilized TERRA primers that measured collective TERRA levels from various chromosome ends (chromosomes 1q, 2q, 4q, 10q, 13q and 22q) and two further specific TERRAs that are transcribed from chromosomes 16p and 20q.

We also examined the relationship of TERRA levels with epithelial cell proliferative index and steroid hormone receptors immuno-scores assessed using immunohistochemistry, as they are established markers predicting prognosis in EC. We also examined the immunoreactivity of two shelterin proteins known to regulate TERRAs, TRF1 (positive regulator) and TRF2 (negative regulator), in the same EC samples to examine their possible correlation with the corresponding TERRA levels. Finally, we analyzed the differential telomere length distributions using high-resolution single telomere length analysis (STELA) at the chromosome XpYp telomeres (which is representative of the genome-wide telomere length) in the same human endometrial samples.

2. Results

2.1. TERRA Transcription Is Observed in Normal Human Endometrium and the Levels Are Significantly Reduced in Endometrial Cancer

Both pre- and postmenopausal endometrium expressed all TERRAs examined (chromosomes 1q-2q-4q-10q-13q-22q, 16p and 20q) (Figure 1A–C). Compared with the proliferative phase samples, the proliferatively quiescent postmenopausal samples had higher levels of Ch-16p and Ch-20q TERRA levels (Figure 1B,C).

Ch-16p ($p = 0.002$) and Ch-20q ($p = 0.0006$) TERRAs were significantly reduced in EC samples when compared with the healthy postmenopausal endometrium (Figure 1B,C). However, TA was high in proliferative phase and EC samples ($p = 0.19$, Figure 1D). In our sample set, the only significant difference in TA was found between the proliferative and secretory phase samples from pre-menopausal women ($p = 0.04$) as previously described [4,32].

When the histological grading (as a measure of cellular anaplasia) of EC samples or individual subtypes of type II ECs were considered, neither TERRA levels nor TA changed significantly with the tumor grade/subtype (Figure 2).

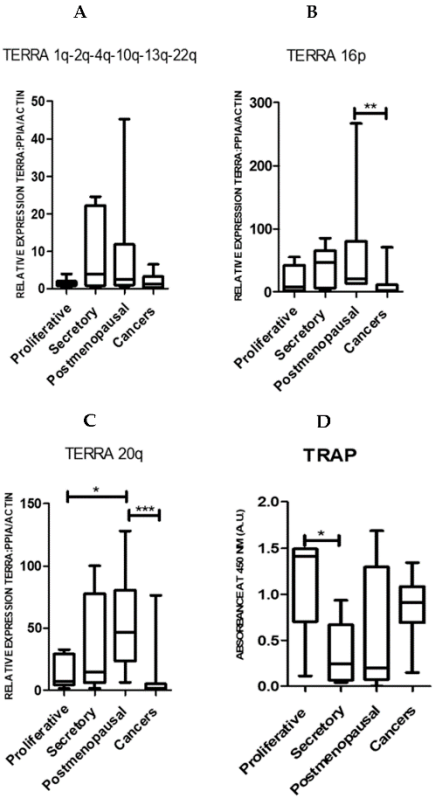


Figure 1. TERRA levels in human endometrium. TERRA levels by qPCR and Telomerase activity by Telomere Repeat Amplification Protocol (TRAP) assay in proliferative (n = 6), secretory (n = 8), postmenopausal (n = 7) human endometrium and endometrial cancers (n = 24) for: chromosome 1q-2q-4q-10q-13q-22q (**A**); chromosome 16p (**B**); and chromosome 20q (**C**). When compared to age-matched postmenopausal endometrium (n = 7), women with ECs (n = 24) showed no changes in TERRA levels for chromosomes 1q-2q-4q-10q-13q-22q (**A**) but a significant decrease in Ch-16p (** p = < 0.002), Mann–Whitney-U test (**B**) and Ch-20q (*** p = <0.001, * p < 0.05); Mann–Whitney-U test (**C**) but no changes in TA (**D**).

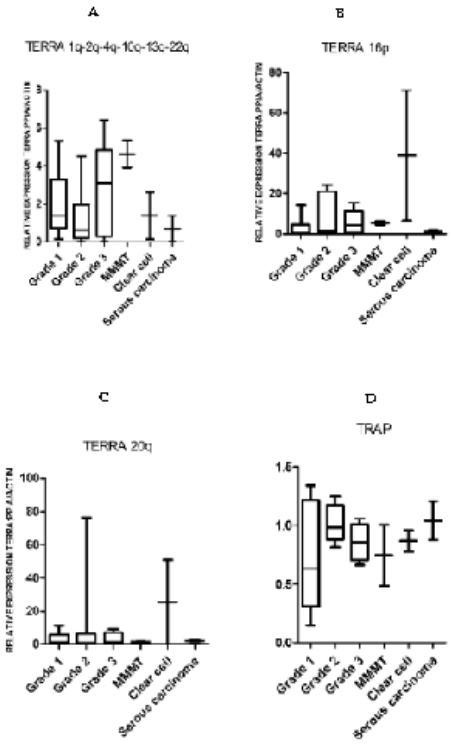


Figure 2. TERRA and TRAP levels in different grades and types of endometrial cancers. Grade 1 adenocarcinoma, $n = 6$; grade 2 adenocarcinoma, $n = 7$; grade 3 adenocarcinoma, $n = 5$; carcinosarcoma/malignant mixed Müllerian tumor (MMMT), $n = 2$; clear cell carcinoma, $n = 2$; serous carcinoma, $n = 2$. Relative normalized expression of chromosomes: 1q-2q-4q-10q-13q-22q (A); 16p (B); and 20q (C). TERRA levels were measured in all types of endometrial cancers. No difference in TERRA levels were found in different grades of endometrioid cancers or individual subtypes of type 2 cancers. All types of endometrial cancers had high telomerase activity as assessed by TRAP assay (D).

2.2. *Endometrial TERRA Levels Correlated with Each Other, the Proliferative Marker Ki67, Steroid Receptor PR and Shelterin Protein TRF1 but Did Not Correlate with TA or with TLs at the XpYp Chromosomes*

Levels of all three TERRAs demonstrated a positive correlation with each other in all endometrial samples (healthy and pathological) examined, suggesting a shared regulation (Table 1: Ch-16p and 20q, $r = 0.78$, $p < 0.0001$; Ch-16p and Ch-1q-2q-4q-10q-13q-22q, $r = 0.49$, $p = 0.001$; Ch-20q and Ch-1q-2q-4q-10q-13q-22q, $r = 0.48$, $p = 0.001$). However, TERRA levels did not correlate with TA. Ki-67 proliferative indices correlated negatively with Ch-16p and Ch-20q TERRAs (Table 1: $r = -0.35$, $p = 0.03$; $r = -0.42$, $p = 0.01$ respectively) suggesting a role related to cell proliferation. Ki67 scores did not correlate with Ch-1q-2q-4q-10q-13q-22q. Ch-16p TERRA also correlated negatively with the Liverpool endometrial steroid quick score (LESQS) for PR ($r = -0.40$, $p = 0.02$) but the LESQS for other hormone receptors did not show a significant association with TERRA levels (Table 1 and Figure 3). The publicly available "The Cancer Genome Atlas" (TCGA) cohort of uterine cancers dataset suggests high expression of TRF1 gene (*TERF1* RNA) in ECs to be associated with decreased survival (Figure S1). Immunostaining quick-scores for TRF1 protein levels correlated positively with Ch-20q TERRA levels ($r = 0.71$, $p = 0.001$, Table 1).

Table 1. Correlation of chromosomes 1q-2q-4q-10q-13q-22q (TERRA 1), TERRA 16 TERRA 20, LESQS scores for AR, PR, ERα and ERβ and immuno-scores for Ki67, TRF1, TRF2 and TA measured by TRAP assay in all endometrial samples. Values in **bold letters** show significant correlations

	TERRA1		TERRA16		TERRA20	
	r	*p*	r	*p*	r	*p*
TERRA1			**0.4885**	**0.0008**	**0.4841**	**0.0009**
TERRA16	**0.4885**	**0.0008**			**0.7792**	**<0.0001**
TERRA20	**0.4841**	**0.0009**	**0.7792**	**<0.0001**		
AR	−0.08156	0.6465	−0.2828	0.1051	−0.1727	0.3288
PR	−0.1068	0.5543	**−0.3997**	**0.0212**	−0.2094	0.2423
ERα	−0.0579	0.7411	0.1462	0.4019	0.198	0.2543
ERβ	0.01555	0.9293	0.1325	0.4479	0.08311	0.635
Ki67	−0.147	0.3854	**−0.3487**	**0.0319**	**−0.4156**	**0.0095**
TRF1	0.4547	0.0505	0.4414	0.0585	**0.7083**	**0.0007**
TRF2	0.08588	0.8432	0.4269	0.2499	0.4809	0.1938
TRAP	−0.1997	0.2292	−0.251	0.1285	−0.09328	0.5775

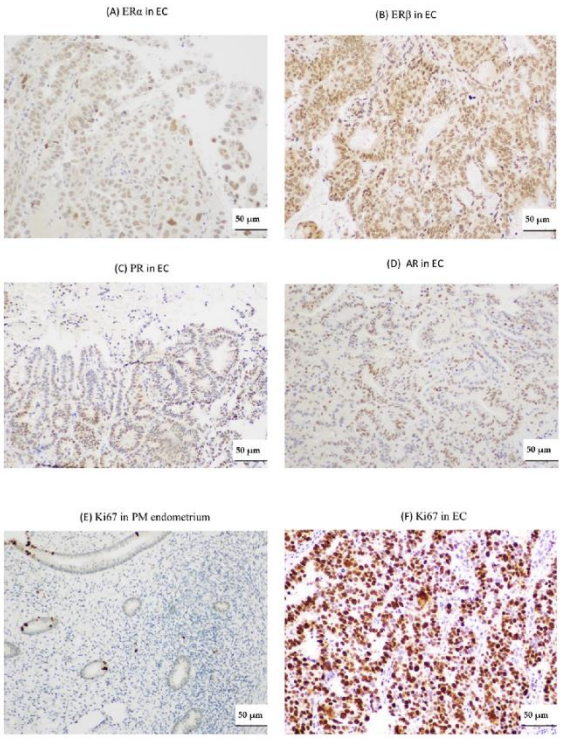


Figure 3. Representative micrographs of steroid receptors and Ki67. Micrograph showing immunostaining in endometrial cancer (EC) of: estrogen receptor α (ERα) (**A**); estrogen receptor β (ERβ) (**B**); progesterone receptor (PR) (**C**); and androgen receptor (AR) (**D**). Micrographs of Ki67 immunostaining: in postmenopausal (PM) endometrium (**E**); and in endometrial cancer (**F**). Scale bar 50 µm, 200× magnification.

Clear differences were observed between healthy postmenopausal and EC samples in terms of both telomere lengths and variance (SD) of the distribution at the XpYp chromosomes (which is representative of the genome-wide telomere length) (Figure 4A). Postmenopausal endometrial samples displayed considerable telomere length heterogeneity with telomeres at XpYp chromosomes ranging in one sample from 1.3 to 21.7 kb. Consistent with previous reports (reviewed in [5]), ECs displayed shorter telomeres when compared with healthy postmenopausal endometrium ($p = 0.002$, Figure 4B). We found a trend towards a decrease of telomere lengths in older women; however, this difference was not significant (Spearman correlation $r = -0.49$, $p = 0.09$, Figure 4C).

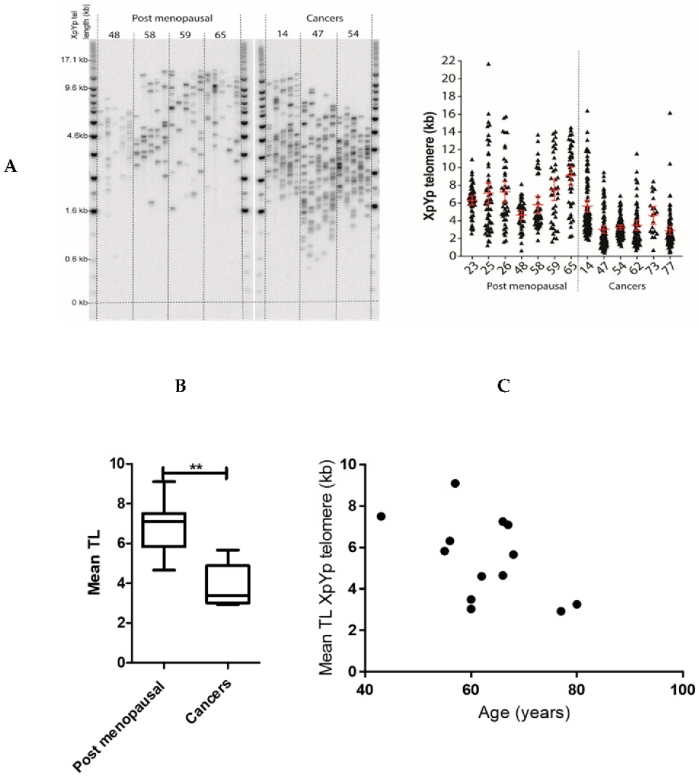


Figure 4. Single telomere length analysis at XpYp telomeres, telomere length distributions in healthy post-menopausal endometrial and endometrial cancer tissue. Representative STELA gel image, together with the distribution of telomere lengths represented as a scatter plot (**A**). Mean telomere lengths in healthy postmenopausal endometrium compared to endometrial cancers. Telomere lengths are significantly shorter in endometrial cancers compared to postmenopausal endometrium (** $p = 0.002$, Mann–Whitney-U test) (**B**). Telomere length correlated with age of the patients, demonstrating no significant association with age and mean telomere lengths (Spearman correlation, r = −0.49, $p = 0.09$) (**C**).

3. Discussion

We report here the long non-coding RNA, TERRA, levels in healthy human endometrium from pre- and postmenopausal women and their significant reduction in expression in EC. Although TERRAs have been previously described in human cancers, to our knowledge, the evaluation of TERRA levels in endometrial cancers and their comparison with healthy human endometrial tissues has not been

previously reported. The levels of different TERRAs are described in the context of TA, telomere lengths at chromosomes XpYp, immunoscores for ovarian hormone receptors, levels of shelterin proteins TRF1/2 and proliferative indices from the same patient-derived endometrial samples.

We employed a qPCR-based assessment of TERRA levels for its superior quantification quality and reproducibility. Human endometrium is a unique somatic tissue demonstrating dynamic TA, which regulates epithelial proliferation [4]. When we considered the highly proliferative epithelial cells in the proliferative phase endometrium from premenopausal women, which has been associated with high TA and relatively longer average telomere length [22], TERRA levels were remarkably low. The inverse correlation of TERRA levels with epithelial proliferative indices we observed further suggests an association with cellular quiescence for TERRAs in the human endometrium.

Each TERRA had a unique expression pattern, suggesting chromosome-specific regulation of TERRAs. Non-replicative cells with shorter telomeres undergo sub-telomeric rearrangements in yeasts during the quiescent phase of the cell cycle [33], and this further results in increased transcription of non-coding TERRA, agreeing with our data for Ch-20q TERRA in human tissue.

TRF1 and TRF2 are shelterin proteins which protect the chromosomal ends from fusion and initiation of a DDR and they help to regulate TA through a negative feedback mechanism [34]. TRF1 may provide a telomere stabilization function to the short telomeres that we and others have shown in ECs [5]. This may prevent arrest of the cell cycle, thus allowing EC cells to proliferate continuously. The publicly available "The Cancer Genome Atlas" (TCGA) cohort of uterine cancers dataset suggests high expression of TRF1 in ECs to be associated with decreased patient survival (Figure S1). Furthermore, TERRAs can bind to chromosomal locations other than telomeres [35], while the interaction between TERRA and TRF1/2 ensures that TERRA transcripts remain tethered to the telomeric domains [36]. We observed TRF1 protein levels to correlate positively with Ch-20q TERRA levels in EC samples. In contrast, the opposite was observed in benign mouse embryonic stem cells, where an increase in TERRA transcription was observed following abrogation of TRF1 and this may suggest a potential differential interaction between TRF1 and Ch-20q TERRA that may be specific to either human cancers or to EC [20].

We observed high TERRA levels in the proliferatively quiescent PM endometrium, which has low TA levels. In fission yeasts, upregulation of telomere-engaged TERRA can support telomerase-independent telomere maintenance [37]. The high TERRA levels that we report in the TA deficient healthy postmenopausal human endometrium may suggest similar function for human TERRAs, in sustaining telomeres in PM endometrium, preventing genetic instability. A recent in silico study, which examined the TCGA uterine cancers dataset, identified many critical genes associated with telomere maintenance that were previously unknown to contribute to endometrial carcinogenesis and prognosis [25]. However, the intricate relationship between these telomere and telomerase associated genes and proteins with TERRAs is yet to be fully elucidated. Considering the interesting and significant reduction in TERRA levels in EC samples we observed, further examination of this potential interaction is warranted in future studies.

A recent study analyzing endometrioid and serous cancer samples from the TCGA dataset reported EC to be one of the human cancers with the shortest mean telomere lengths among 31 different cancer types [38]. Our data examining individual telomere lengths using STELA at Chromosomes XpYp (which is representative of the genome-wide telomere length) [39] also demonstrate significantly shorter telomeres in EC. Although we were not able to assess the specific telomere lengths at the chromosomes corresponding to the TERRAs tested (Ch16 and 20), collectively, the above data suggest EC to have shorter telomeres. The complex mechanism of TERRA's role in regulation of telomere length has been previously reviewed and variable mechanisms involving different pathways have been proposed in different physiological conditions. For example, TERRA binding of TLS to G-quadruplex leading to accumulation of H4K20 trimethylation and TERRAs associating with LSD1 and MRE 11 in telomeric 3'G overhang removal leading to lack of protection to chromosomal ends from being recognized as sites of DNA damage, therefore resulting in telomere shortening, were reported [40–43]. However, our data are the first to evaluate the comparative TERRA levels in healthy and malignant human endometrial tissue,

thus we hypothesize that concurrent reduction in TERRA levels and telomere lengths observed in EC may be a global EC related phenomenon. At least, before telomerase re-activation, shorter telomeres may facilitate EC progression due to telomere dysfunction by initiating a "telomere crisis" that drives genomic instability and clonal evolution [44]. We therefore present this interesting observation of reduced TERRA levels in ECs, which co-exist with high TA and short telomeres, thus suggesting a complex, cancer-specific telomere biology in the endometrium. Furthermore, considering the proposed prevention of telomerase accessing telomeres by TERRA [40], the reduction of TERRA may provide a telomere maintenance function and thus proliferative advantage for the ECs. This aspect warrants further studies to ascertain the functional role of TERRA in the endometrium and in EC.

4. Materials and Methods

4.1. Endometrial Tissue Samples

Endometrial biopsies were obtained from 47 women undergoing gynecological surgery at Liverpool Women's Hospital. Ethical approval was obtained from the Liverpool Adult Local Research Ethics Committee (LREC; 09/H1005/55, 9 October 2009, NRES Committee North West—Liverpool Central, and 11/H1005/4 6 April 2011, NRES Committee North West—Liverpool Central) and informed written consent was obtained from all patients. Control endometrial samples were collected from women who were not on any hormonal treatment for at least 3 months, with no known endometrial pathology and with regular periods in proliferative phase ($n = 7$), secretory phase ($n = 9$) and postmenopausal ($n = 7$) women, as well as EC samples donated by 24 women with a prior diagnosis of EC (Endometrioid (grade 1 $n = 6$, grade 2 $n = 7$ and grade 3 $n = 5$) and 6 type 2 ECs (serous $n = 2$, carcinosarcoma/malignant mixed Müllerian tumor (MMMT) $n = 2$ and clear cell $n = 2$)) undergoing hysterectomy without receiving any pre-surgical treatment. Patient clinico-pathological and demographic details were retrieved from the clinical notes and electronic databases (Table 2).

Table 2. Demographic features of study groups.

Study Groups (*n*)	* Age (years)	* BMI (kg/m^2)
Proliferative phase (7)	43 (32–57)	27.8 (22–40.5)
Secretory phase (9)	41 (21–47)	22.6 (18.9–31.6)
Postmenopausal (7)	62 (52–85)	24.3 (20–39.6)
Total Endometrial cancer pts (24)	67 (37–80)	30 (23.9–54.4)
Endometrioid Grade 1 (6/24)	61 (46–73)	37.8 (28.3–46.1)
Endometrioid Grade 2 (7/24)	60 (37–77)	28.9 (25.8–54.4)
Endometrioid Grade 3 (5/24)	68 (60–80)	29.8 (23.9–42.7)
Malignant Mixed Mullerian Tumor (2/24)	72.5 (65–80)	28.6 (24.2–32.9)
Clear Cell Carcinoma (2/24)	71.5 (61–82)	28.4 (26.6–30.1)
Serous Carcinoma (2/24)	73 (68–78)	32.7 (NK **-32.7)

* Data expressed as median (range). ** BMI not known (NK) for 1 patient.

4.2. RNA Extraction and Real Time-qPCR

RNA was extracted from tissue samples using the Trizol Plus RNA extraction kit (Invitrogen, Life Technologies, Paisley, UK) and quantified using Nanodrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK) as previously described [32]. One microgram of RNA was reverse transcribed with random hexamers using AMV reverse transcriptase (New England Biolabs, Hertfordshire, UK). Two micrograms of cDNA were amplified in triplicate for 40 cycles using iTaq universal SYBR Green supermix and CFX Connect Real Time System (Bio-Rad, Hertfordshire, UK). The specific oligonucleotides used to amplify TERRA transcripts from different chromosome ends (Ch 1q-2q-4q-10q-13p-22p, Ch16p,

Ch 20q) and reaction conditions are listed in Table S1. No template and no reverse transcriptase controls were included on each plate and the melt curves were examined to ensure that only a single product was obtained. Relative transcript level was calculated using the ΔΔCT method, normalized to the reference genes, beta actin (*ACTB*) and peptidylprolyl Isomerase A (*PPIA*) using Bio Rad CFX Manager (Bio-Rad, Hertfordshire, UK).

4.3. Telomerase Repeat Amplification Protocol (TRAP) Assay

TA was measured using TeloTAGGG TRAP assay (Roche Diagnostics Ltd., Burgess Hill, UK) using 1 μg of lysate [22]. Amplification products after 30 cycles were measured as absorbance at 450 nm in a Fluostar Omega Plate reader (BMG LABTECH, Aylesbury, UK) and presented as arbitrary units (AU).

4.4. Single Telomere Length Analysis (STELA)

DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Manchester, UK). For telomere length analysis at the XpYp telomere, we used the single telomere length analysis (STELA) assay, as previously described [39,44], Genomic DNA was solubilized and diluted in 10 mmol/L Tric-HCl (pH 7.5) to 10 ng/μL. Ten nanograms of DNA were further diluted with 1 μmol/L Telorette2 linker and 1 mM Tris-HCl to 250 pg/μL in a volume of 40 μL. Multiple polymerase chain reactions were conducted to test the DNA sample and were cycled in a Tetrad2 thermocycler (BioRad, Hertfordshire, UK) (22 cycles of 94 °C for 15 s, 65 °C for 30 s, 68 °C for 8 min). DNA fragments were resolved by 0.5% TAE agarose gel electrophoresis and detected by southern hybridization. The hybridized fragments were detected using a phosphorimaging with a Typhoon FLA 9500 phosphoimager (GE healthcare, Chalfont St Giles, UK). The molecular weights of the DNA fragments were calculated using the Phoretix ID quantifier (Nonlinear Dynamics, Newcastle Upon Tyne, UK).

4.5. Immunohistochemistry

Formalin-fixed paraffin embedded 3 μm tissue sections were immuno-stained with anti-human TRF1, TRF2, steroid receptors and Ki67 antibodies after antigen retrieval at pH6 as previously described [45]. Antibody sources, concentrations and incubation conditions are detailed in Table S2. Matching isotype (0.5 μg/mL) replaced the primary antibody as a negative control. A specific endometrial tissue sample with positive staining was included as the internal positive control with each staining experiment.

Steroid receptor, TRF1 and TRF2, immunostaining was assessed semi-quantitatively using a four-tiered Liverpool endometrial steroid quick score (LESQS) for steroid receptors and a standard quick score for TRF1 and TRF2 as previously described [45]. The Ki67 proliferative index (PI) was evaluated as the percentage of immunopositive cells of any intensity. Epithelial and stromal cell staining was scored separately based on morphological criteria in postmenopausal and malignant endometrium and stratum basalis of healthy proliferative phase and secretory phase endometrium by two independent observers. Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

4.6. Analysis of TCGA Dataset

The publicly available TCGA cohort of uterine cancers included data for RNA levels for *TERF1* and was interrogated using Illumina's Base Space Cohort Analyzer application (BSCA) (https://www.illumina.com/informatics/research/biological-data-interpretation/nextbio.html; Illumina, San Diego, CA, USA) [46].

4.7. Statistical Analysis

Gene expression data were analyzed using GraphPad Prism software version 5 (San Diego, CA, USA) using non-parametric tests (Mann–Whitney U test or Spearman correlation as appropriate, not-assuming Gaussian distribution). The criterion for significance was $p \leq 0.05$.

5. Conclusions

TERRAs seem to be dynamically expressed in the healthy human endometrium, and significantly lower TERRA levels are found in ECs. Since our study showed TERRA levels to be inversely related to endometrial epithelial cellular proliferative indices, further studies are required to determine whether these levels can be successfully modified and tailored as a therapeutic target in endometrial proliferative conditions.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/22/8686/s1. Figure S1: The Cancer Genome Atlas (TCGA) RNA Sequencing Data (endometrioid and serous cancers) for shelterin protein TRF1, Table S1: Oligonucleotides used for qPCR amplification, Table S2: Primary antibodies and conditions for IHC.

Author Contributions: D.K.H. conceived the study and obtained ethical approval. D.K.H., M.A., S.B.D. and J.K. collected samples. M.A., R.E.J., D.M.B., J.D., A.M.K., G.S., R.A., L.B. and S.S. carried out experiments. M.A. and D.K.H. produced the first draft and all authors were involved in revising the paper. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge the support by Wellbeing of Women project grants RG1073 and RG1487 (DKH) and Liverpool Women's Hospital Cancer Charity Fund (MA), Liverpool Women's Hospital Foundation Trust (MA, JAD, JK, SBD and DKH), Institute of Translational Medicine (MA, DKH), and Higher Committee for Education Development in Iraq (RA and AMK). The Baird laboratory was supported by Cancer Research UK (C17199/A18246/A29202).

Acknowledgments: The authors would like to thank Annabelle Decottignies of Université Catholique de Louvain for advice on TERRA qPCR optimization. We are grateful to all patients who participated and kindly donated tissue towards research and all staff at Liverpool Women's Hospital and Women's health, Institute of Translational medicine, University of Liverpool who helped for this project.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Lindemann, K.; Eskild, A.; Vatten, L.J.; Bray, F. Endometrial cancer incidence trends in Norway during 1953-2007 and predictions for 2008–2027. *Int. J. Cancer* **2010**, *127*, 2661–2668. [CrossRef] [PubMed]
2. CRUK. Available online: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer/mortality (accessed on 3 February 2020).
3. Barcellini, A.; Roccio, M.; Laliscia, C.; Zanellini, F.; Pettinato, D.; Valvo, F.; Mirandola, A.; Orlandi, E.; Gadducci, A. Endometrial Cancer: When Upfront Surgery Is Not an Option. *Oncology* **2020**, 1–7. [CrossRef] [PubMed]
4. Hapangama, D.K.; Kamal, A.; Saretzki, G. Implications of telomeres and telomerase in endometrial pathology. *Hum. Reprod. Update* **2017**, *23*, 166–187. [CrossRef] [PubMed]
5. Alnafakh, R.A.A.; Adishesh, M.; Button, L.; Saretzki, G.; Hapangama, D.K. Telomerase and Telomeres in Endometrial Cancer. *Front. Oncol.* **2019**, *9*, 344. [CrossRef]
6. Smogorzewska, A.; de Lange, T. Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem.* **2004**, *73*, 177–208. [CrossRef]
7. Kim, N.W.; Piatyszek, M.A.; Prowse, K.R.; Harley, C.B.; West, M.D.; Ho, P.L.; Coviello, G.M.; Wright, W.E.; Weinrich, S.L.; Shay, J.W. Specific association of human telomerase activity with immortal cells and cancer. *Science* **1994**, *266*, 2011–2015. [CrossRef]
8. Nergadze, S.G.; Farnung, B.O.; Wischnewski, H.; Khoriauli, L.; Vitelli, V.; Chawla, R.; Giulotto, E.; Azzalin, C.M. CpG-island promoters drive transcription of human telomeres. *RNA* **2009**, *15*, 2186–2194. [CrossRef]
9. Azzalin, C.M.; Reichenbach, P.; Khoriauli, L.; Giulotto, E.; Lingner, J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science* **2007**, *318*, 798–801. [CrossRef]
10. Luke, B.; Panza, A.; Redon, S.; Iglesias, N.; Li, Z.; Lingner, J. The Rat1p 5′ to 3′ exonuclease degrades telomeric repeat-containing RNA and promotes telomere elongation in Saccharomyces cerevisiae. *Mol. Cell* **2008**, *32*, 465–477. [CrossRef]

11. Vrbsky, J.; Akimcheva, S.; Watson, J.M.; Turner, T.L.; Daxinger, L.; Vyskot, B.; Aufsatz, W.; Riha, K. siRNA-mediated methylation of Arabidopsis telomeres. *PLoS Genet.* **2010**, *6*, e1000986. [CrossRef]
12. Beishline, K.; Vladimirova, O.; Tutton, S.; Wang, Z.; Deng, Z.; Lieberman, P.M. CTCF driven TERRA transcription facilitates completion of telomere DNA replication. *Nat. Commun.* **2017**, *8*, 2114. [CrossRef] [PubMed]
13. Montero, J.J.; López de Silanes, I.; Graña, O.; Blasco, M.A. Telomeric RNAs are essential to maintain telomeres. *Nat. Commun.* **2016**, *7*, 12534. [CrossRef] [PubMed]
14. Arnoult, N.; Van Beneden, A.; Decottignies, A. Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1α. *Nat. Struct. Mol. Biol.* **2012**, *19*, 948–956. [CrossRef] [PubMed]
15. Smekalova, E.; Baumann, P. TERRA -a calling card for telomerase. *Mol. Cell* **2013**, *51*, 703–704. [CrossRef]
16. Hirashima, K.; Seimiya, H. Telomeric repeat-containing RNA/G-quadruplex-forming sequences cause genome-wide alteration of gene expression in human cancer cells in vivo. *Nucleic Acids Res.* **2015**, *43*, 2022–2032. [CrossRef]
17. Graf, M.; Bonetti, D.; Lockhart, A.; Serhal, K.; Kellner, V.; Maicher, A.; Jolivet, P.; Teixeira, M.T.; Luke, B. Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle. *Cell* **2017**, *170*, 72–85.e14. [CrossRef]
18. Redon, S.; Reichenbach, P.; Lingner, J. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res.* **2010**, *38*, 5797–5806. [CrossRef]
19. Porro, A.; Feuerhahn, S.; Reichenbach, P.; Lingner, J. Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways. *Mol. Cell. Biol.* **2010**, *30*, 4808–4817. [CrossRef]
20. Marion, R.M.; Montero, J.J.; Lopez de Silanes, I.; Grana-Castro, O.; Martinez, P.; Schoeftner, S.; Palacios-Fabrega, J.A.; Blasco, M.A. TERRA regulate the transcriptional landscape of pluripotent cells through TRF1-dependent recruitment of PRC2. *eLife* **2019**, *8*. [CrossRef]
21. Hapangama, D.K.; Bulmer, J.N. Pathophysiology of heavy menstrual bleeding. *Womens Health (Lond.)* **2016**, *12*, 3–13. [CrossRef]
22. Valentijn, A.J.; Saretzki, G.; Tempest, N.; Critchley, H.O.; Hapangama, D.K. Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis. *Hum. Reprod.* **2015**, *30*, 2816–2828. [CrossRef] [PubMed]
23. Menon, M.M.; Simha, M.R. Telomerase and telomere length in normal and malignant human endometrium as prognostic markers. *Indian J. Pathol. Microbiol.* **2003**, *46*, 394–398. [PubMed]
24. Saygan-Karamursel, B.; Dikmen, G.; Dogan, P.; Aksu, T.; Guven, S.; Ayhan, A. Quantitative telomerase activity in malignant, benign and normal gynecological tissues. *Eur. J. Gynaecol. Oncol.* **2005**, *26*, 83–86. [PubMed]
25. Bradfield, A.; Button, L.; Drury, J.; Green, D.C.; Hill, C.J.; Hapangama, D.K. Investigating the Role of Telomere and Telomerase Associated Genes and Proteins in Endometrial Cancer. *Methods Protoc.* **2020**, *3*. [CrossRef] [PubMed]
26. Williams, C.D.; Boggess, J.F.; LaMarque, L.R.; Meyer, W.R.; Murray, M.J.; Fritz, M.A.; Lessey, B.A. A prospective, randomized study of endometrial telomerase during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 3912–3917. [CrossRef]
27. Tanaka, M.; Kyo, S.; Takakura, M.; Kanaya, T.; Sagawa, T.; Yamashita, K.; Okada, Y.; Hiyama, E.; Inoue, M. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. *Am. J. Pathol.* **1998**, *153*, 1985–1991. [CrossRef]
28. Wang, S.-J.; Sakamoto, T.; Yasuda, S.-i.; Fukasawa, I.; Ota, Y.; Hayashi, M.; Okura, T.; Zheng, J.-H.; Inaba, N. The Relationship between Telomere Length and Telomerase Activity in Gynecologic Cancers. *Gynecol. Oncol.* **2002**, *84*, 81–84. [CrossRef]
29. Kyo, S.; Kanaya, T.; Ishikawa, H.; Ueno, H.; Inoue, M. Telomerase activity in gynecological tumors. *Clin. Cancer Res.* **1996**, *2*, 2023–2028.
30. Kyo, S.; Takakura, M.; Kohama, T.; Inoue, M. Telomerase activity in human endometrium. *Cancer Res.* **1997**, *57*, 610–614.
31. Shay, J.W. Role of Telomeres and Telomerase in Aging and Cancer. *Cancer Discov.* **2016**, *6*, 584–593. [CrossRef]

32. Mathew, D.; Drury, J.A.; Valentijn, A.J.; Vasieva, O.; Hapangama, D.K. In silico, in vitro and in vivo analysis identifies a potential role for steroid hormone regulation of FOXD3 in endometriosis-associated genes. *Hum. Reprod.* **2016**, *31*, 345–354. [CrossRef] [PubMed]
33. Maestroni, L.; Audry, J.; Matmati, S.; Arcangioli, B.; Geli, V.; Coulon, S. Eroded telomeres are rearranged in quiescent fission yeast cells through duplications of subtelomeric sequences. *Nat. Commun.* **2017**, *8*, 1684. [CrossRef] [PubMed]
34. van Steensel, B.; de Lange, T. Control of telomere length by the human telomeric protein TRF1. *Nature* **1997**, *385*, 740–743. [CrossRef] [PubMed]
35. Schoeftner, S.; Blasco, M.A. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat. Cell Biol.* **2008**, *10*, 228–236. [CrossRef] [PubMed]
36. Deng, Z.; Norseen, J.; Wiedmer, A.; Riethman, H.; Lieberman, P.M. TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. *Mol. Cell* **2009**, *35*, 403–413. [CrossRef]
37. Hu, Y.; Bennett, H.W.; Liu, N.; Moravec, M.; Williams, J.F.; Azzalin, C.M.; King, M.C. RNA–DNA Hybrids Support Recombination-Based Telomere Maintenance in Fission Yeast. *Genetics* **2019**, *213*, 431–447. [CrossRef]
38. Barthel, F.P.; Wei, W.; Tang, M.; Martinez-Ledesma, E.; Hu, X.; Amin, S.B.; Akdemir, K.C.; Seth, S.; Song, X.; Wang, Q.; et al. Systematic analysis of telomere length and somatic alterations in 31 cancer types. *Nat. Genet.* **2017**, *49*, 349–357. [CrossRef]
39. Baird, D.M.; Rowson, J.; Wynford-Thomas, D.; Kipling, D. Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat. Genet.* **2003**, *33*, 203–207. [CrossRef]
40. Wang, C.; Zhao, L.; Lu, S. Role of TERRA in the regulation of telomere length. *Int. J. Biol. Sci.* **2015**, *11*, 316–323. [CrossRef]
41. Cusanelli, E.; Romero, C.A.; Chartrand, P. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. *Mol. Cell* **2013**, *51*, 780–791. [CrossRef]
42. Takahama, K.; Takada, A.; Tada, S.; Shimizu, M.; Sayama, K.; Kurokawa, R.; Oyoshi, T. Regulation of telomere length by G-quadruplex telomere DNA- and TERRA-binding protein TLS/FUS. *Chem. Biol.* **2013**, *20*, 341–350. [CrossRef] [PubMed]
43. Porro, A.; Feuerhahn, S.; Lingner, J. TERRA-reinforced association of LSD1 with MRE11 promotes processing of uncapped telomeres. *Cell Rep.* **2014**, *6*, 765–776. [CrossRef] [PubMed]
44. Lin, T.T.; Letsolo, B.T.; Jones, R.E.; Rowson, J.; Pratt, G.; Hewamana, S.; Fegan, C.; Pepper, C.; Baird, D.M. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: Evidence for a telomere crisis. *Blood* **2010**, *116*, 1899–1907. [CrossRef]
45. Kamal, A.M.; Bulmer, J.N.; DeCruze, S.B.; Stringfellow, H.F.; Martin-Hirsch, P.; Hapangama, D.K. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. *Br. J. Cancer* **2016**, *114*, 688–696. [CrossRef] [PubMed]
46. Kupershmidt, I.; Su, Q.J.; Grewal, A.; Sundaresh, S.; Halperin, I.; Flynn, J.; Shekar, M.; Wang, H.; Park, J.; Cui, W.; et al. Ontology-based meta-analysis of global collections of high-throughput public data. *PLoS ONE* **2010**, *5*, e13066. [CrossRef]