UNIVERSITY OF LIVERPOOL

DYSKERIN (*DKC1*), TELOMERASE SUBUNIT, IS EXPRESSED AND REGULATED IN HEALTHY ENDOMETRIUM: IMPLICATIONS FOR ENDOMETRIAL PATHOLOGIES

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

By
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August 2020

Declaration

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

Rafah A. Alnafakh

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Acknowledgement

First and foremost, praises and thanks to God, the Almighty, for placing me in the Women's and Children's Health Department with this great team and for his showers of blessings throughout my research work.

I would like to express my deep and sincere gratitude to my supervisor, Professor Dharani Hapangama, for giving me the opportunity to do research and providing invaluable guidance, advice and support throughout this research. Her dynamism, vision, sincerity and motivation have deeply inspired me to carry out the research and to present the research works as clearly as possible. It was a great privilege and honour to work and study under her supervision. I sincerely thank her from the bottom of my heart and will be truly indebted to her throughout my lifetime. My sincere thanks also go to my secondary supervisor, Professor Sarah Coupland, for her support, invaluable suggestions and guidance. I could not have imagined having a better supervisory team for my PhD study.

I am extremely grateful to Jo and Anthony for all the scientific advice and technical support they have given me throughout my PhD study years. I am also thankful to Lisa, Jane, Sarah and Helen, for their technical support.

I would also like to thank Meera for help in PCR experiment as part of her own project; Areeg and Nic for scientific discussion; Chris for his help in producing 3D structural image using PyMOL software and Sam for sharing her immunofluorescence experiment figures.

Special thanks go to Dr Gabriele Saretzki from the University of Newcastle for her help, particularly for her proofreading of parts of my thesis, her positive criticism was always helpful, and I also thank her for providing me with TRAP data.

Great thanks also to Dr Angela Midgley for helping me with flow cytometry. Thanks to Stuart Ruthven in the Department of Pathology at the Royal Liverpool Hospital for providing access to pathology block. Many thanks to Dr Decruze for providing the updated outcome data of endometrial cancer patients and to Mr Steven Lane for his statistical advice.

I am particularly grateful to Dr Pierre Martin-Hirsch and Helen Stringfellow (Lancaster) for sharing resources.

I would also like to express my thanks to Dr Dada Pisconti, Dr Lakis Liloglou, Dr Roger Barraclough, Professor Phil Rudland from the the University of Liverpool and Dr Stephane Gross from Aston University for their valuable scientific advice, especially in transient transfection, PCR and Immunoblotting, I sincerely appreciate their help.

I am particularly grateful for Lucy Dobson for updating the outcome data at the very last minute.

I also thank my fellow PhD research students in Room 1121 for the stimulating discussions and valuable information gained while we were working together.

I would like to thank the Higher Committee for Education Development in Iraq for selecting me for this scholarship and for providing the financial support and continuous follow up.

I acknowledge the people who mean a lot to me, my parents; their endless love, help and encouragement were a great support during the study period. I also would like to thanks my sister, Zainab, for her selfless love and support, my brothers, for their moral help.

My heartfelt regard also goes to my mother and sisters in law and Dr Mohand; their moral and material support is invaluable, and I highly appreciated it.

I owe thanks to a very special person, my husband, Ahmed, for his continued patience, support and understanding during my pursuit of PhD degree that made the completion of this study possible. You were always around during the happy and hard moments to push me and motivate me. I greatly value his contribution and deeply appreciate his belief in me.

Thanks also to my boys Mustafa and Ali for their patience throughout these years. You have been really wonderful boys.

List of published papers

Papers published in peer-reviewed journals

Maclean A, Kamal AM, Adishesh M, **Alnafakh R**, Tempest N, Hapangama DK. Human Uterine Biopsy: Research Value and Common Pitfalls. *Int J Reprod* Med. 2020.

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Alnafakh R, Saretzki G, Midgley A, Flynn, J, Kamal AM, Dobson L, Natarajan P, Stringfellow H, Martin-Hirsch P, DeCruze SB1, Hapangama DK1. Aberrant dyskerin expression is related to proliferation and poor survival in endometrial cancer.

Adishesh M, **Alnafakh RA**, 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 a significant reduction in Long non-coding RNA, *TERRA*.

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Alnafakh R, Drury J, Midgely A, Decruze SB, Saretzki G, Hapangama DK. Dyskerin plays a negative role in endometrial epithelial cell proliferation and is associated with poor survival in endometrial cancer. ESGO, Athens, 2019 (Poster Presentation).

Alnafakh R, Midgley A, Hapangama D.K. Dyskerin Overexpression in Endometrial Cancer Cell Line, Faculty Poster Day, University of Liverpool, 2019 (Poster Presentation).

Alnafakh R, Saretzki G, Adishesh M, Drury J, Hapangama DK. Telomerase Component, Dyskerin (DKC1), is expressed and hormonally regulated in healthy endometrium: implications for endometrial pathologies, ESHRE Conference, Barcelona, Spain 2018 (Pitched Poster; Oral Presentation).

Alnafakh R, Adishesh M, Saretzki G, Drury J, Hapangama DK. Dyskerin (DKC1), telomerase Complex Component, is expressed and hormonally regulated in health endometrium: Implications for Endometrial Pathologies. Faculty Poster Day, University of Liverpool, Liverpool, 2018 (Poster Presentation).

Alnafakh R, Adishesh M, Saretzki G, Drury J, Hapangama D K. Dyskerin (DKC1), Telomerase complex component, is expressed and hormonally regulated in healthy endometrium: implications for endometrial pathologies. NWCR Centre, University of Liverpool. Annual Scientific Symposium, Liverpool 2018 (Poster Presentation).

Alnafakh R, Human endometrium expresses Dyskerin (DKC1) that is regulated by ovarian hormones, and dyskerin plays a role in the endometrial function and in carcinogenesis. Annual Academic Meeting, RCOG, London, 2017 (Oral Presentation).

Alnafakh R, Human endometrium expresses Dyskerin (DKC1) that is regulated by ovarian hormones and plays a role in endometrial in carcinogenesis. NWCR Centre-University of Liverpool. Annual Scientific Symposium, Liverpool 2017 (Poster Presentation).

Alnafakh R, Kamal AM, Popat K, Drury J, Decruze SB, Stringfellow H, Martin-Hirsch P, Hapangama DK. Human endometrium expresses DKC1 that is regulated by ovarian steroid hormones. ITM Research Day, University of Liverpool, 2017 (Poster Presentation).

List of Abbreviations

Ab: Antibody

ACTB: beta-actin

ALT: Alternative lengthening of telomeres pathway

AR: Androgen Receptor

ATM: Ataxia-telangiectasia mutated

ATR: Ataxia telangiectasia and Rad3

AU: Arbitrary units

BCA: bicinchoninic acid

BMI: Body mass index

bp: base pairs

BSA: Bovine serum albumin

BSCA: Base Space Cohort Analyzer application

CB: Cajal body

cDNA: Complementary deoxyribonucleic acid

CFSE: Carboxyfluorescein Diacetate Succinimidyl Ester

CNNB1: β catenin gene

COCP: Oral contraceptive pills

CREC: Cambridge Adult Research Ethics Committee

CRUK: Cancer Research UK

CS: Carcinosarcoma

CS-FBS: Charcoal stripped foetal bovine serum

CSS: Cancer-specific survival

Ct: Cycle to Threshold

CTE: C-terminal extension

DAB: 3,3′-Diaminobenzidine

DAPI: 4′,6-diamidino-2-phenylindole

DC: Dyskeratosis Congenita

DDK: Anti DYKDDDDK Tag

DDR: DNA damage response

DFS: Disease-free survival

DHT: Dihydrotestosterone

DKLD: Dyskerin-like domain

DMEM/F12: Dulbecco's Modified Eagle Medium/F12

DMSO: Dimethylsulfoxide

E2: 17β estradiol

EBRT: External beam radiotherapy

EC: Endometrial Cancer

EDCs: Endocrine-disrupting chemicals

EGTA: Ethylene Glycol Tetraacetic Acid

EH: Endometrial hyperplasia

EHA: Endometrial hyperplasia with cytological atypia

EIC: Endometrial in situ carcinoma

EIN: Endometrial intraepithelial neoplasia

ER: Estrogen Receptor

EV: Empty vector

FBS: Foetal bovine serum

FIGO: International Federation of Gynaecology and Obstetrics classification

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GnRH: Gonadotrophic realising hormone

GWAS: Genome-wide association studies

H2O2: Hydrogen peroxide

H&E: haematoxylin and eosin

HDR: Homology-directed recombination

HGEC: High-grade endometrial cancer

HHS: Hoyeraal-Hreidarsson syndrome

HRP: Horseradish peroxidase

HRT: hormone-replacement therapy

hTERC: Human telomerase RNA Component

hTERT: Human telomerase reverse transcriptase

IHC: Immunohistochemistry

IRES: Internal ribosomal entry site

IUDs: Intrauterine devices

LACRs: long-acting reversible contraceptives

LESQS: Liverpool endometrial steroid quickscore

LGEC: Low-grade endometrial cancer

LMP: Last menstrual period

LN: Lymph node

LNG-IUS: levonorgestrel-releasing intrauterine system

LREC: Liverpool research ethics committee

LVSI: Lymphovascular space invasion

LWH: Liverpool Women's Hospital

MAPK: Mitogen-activated protein kinase

MFI: Median fluorescence Intensity

MPA: Medroxyprogesterone acetate

MSI: Microsatellite instability

MW: Molecular weight

NBF: Neutral buffered formalin

NLSs: Nuclear localisation signals

NSAIDs: Non-steroidal anti-inflammatory drugs

nt: nucleotides

NT: Non-transfected

NTE: N-terminal extension

OD: Optical density

O/N: Overnight

OR: Odd ratio

OS: Overall survival

P4: Progesterone

Panck: Pancytokeratin

PBMC: Peripheral blood mono-nucleocyte

PBS: Phosphate Buffered Saline

PCOS: Polycystic ovarian syndrome

PI: Proliferative index

PM: postmenopausal

POLE: Polymerase (DNA) Epsilon

POPs: Progestogen-only preparations

POT1: Protection of telomeres 1

PP: Proliferative phase

PPIA: Peptidylprolyl Isomerase A

PR: Progesterone Receptor

PreM: Premenopausal

PTEN: Phosphatase and tensin homolog

PUA: Pseudouridine synthase and archaeosine transglycosylase

PVDF: Polyvinylidene fluoride

RAP1: Repressor/activator protein 1

RNA: Ribonucleic acid

RNP: Ribonucleoprotein

RR: Relative risk

RT: Room temperature

RT-qPCR: Real-Time: quantitative Polymerase Chain Reaction

scaRNPs: small cajal body RNPs

SDS-PAGE: Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis

SEER: Epidemiology and End Results

snoRNPs: small nucleolar RNPs

SP: Secretory phase

STR: Short Tandem Repeat profiling

T: Transfected cells

TA: telomerase activity

TBE: Tris-borate-EDTA

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline-Tween20

TCGA: The Cancer Genome Atlas

TERRAs: Telomeric repeat-containing RNAs

TGS: Tris-Glycine-SDS

TGX: Tris-Glycine eXtended shelf life

TMM: Telomere maintenance mechanisms

TP53: Tumour Protein 53

TPE: Telomere position effect

TPE-OLD: TPE–over long distance

TRAP: Telomere Repeat Amplification Protocol

TRF1 and TRF2: telomeric repeat binding factor 1 and 2

VBT: Vaginal intracavity brachytherapy

WHO: World Health Organisation

X-DC: X-linked Dyskeratosis congenita

YWHAZ: Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta

Abstract

Dyskerin (*DKC1*), telomerase subunit, is expressed and hormonally regulated in healthy endometrium: implications for endometrial pathologies

The huge increase of endometrial cancer incidence, with an associated rise in related mortality, has highlighted the need to find reliable markers to identify the high-risk endometrial cancers that are likely to recur. This is expected to help in developing more specialised and effective treatment strategies. The main aim of the work carried out during this PhD project, presented in this thesis, was to investigate the role of dyskerin, in the human endometrium, including its contribution to endometrial carcinogenesis. Dyskerin is one of the three core subunits of telomerase holoenzyme. High telomerase is an essential feature of most endometrial cancers. Since the other two core components of telomerase have been studied previously in human endometrium and in endometrial cancer, I focussed on dyskerin, the nucleolar multifunctional protein which is yet to be studied in the endometrium and in endometrial cancer. The endometrium is a hormone-regulated tissue, and telomerase is regulated by ovarian steroid hormones; therefore, I extended my studies to examine hormonal regulation of dyskerin in the endometrium.

My experimental workflow included human endometrial tissue samples, donated from a total of 279 women, comprising a group of healthy fertile women and those suffering from endometrial pathologies such as endometriosis and endometrial cancer. My initial work, presented in Chapter 3 of this thesis, comprehensively examined the levels of dyskerin protein (with immunohistochemistry) and *DKC1* mRNA levels (RT-qPCR) in the healthy endometrium across the menstrual cycle and in the postmenopausal period, describing their presence in the human endometrium, for the first time. I have also demonstrated that the levels of *DKC1* mRNA and dyskerin protein immunostaining were significantly higher in proliferatively quiescent postmenopausal samples, compared with the highly proliferative premenopausal proliferative phase endometrium. Despite the well established high telomerase activity (TA) levels, the endometrium of women with endometriosis did not demonstrate a significant alteration in dyskerin protein or gene expression levels.

Chapter 4 of this thesis describes my study that investigated the role of dyskerin in endometrial carcinogenesis. By initially examining the publicly available genomic data from uterine cancer cohort of The Cancer Genome Atlas (TCGA) and then by examining the endometrial samples from our Liverpool cohort of women with endometrial cancer, I found that both dyskerin protein and *DKC1* mRNA levels were significantly altered in endometrial cancer compared with healthy postmenopausal endometrial samples. Furthermore, my data demonstrated a better clinical outcome in high dyskerin protein containing endometrial cancers in comparison with endometrial cancers with low dyskerin, suggesting a prognostic relevance of dyskerin in endometrial cancer. The functional consequence of dyskerin in cell proliferation was suggested by the observed negative correlation of endometrial epithelial dyskerin protein immuno-scores with epithelial proliferative index (Ki67 immunoscores).

To ascertain if the dynamic dyskerin levels observed in the human endometrium (presented in Chapter 3), was regulated by the ovarian hormones, in Chapter 5, I examined the effect of endogenous and exogenous hormones on endometrial dyskerin (*DKC1*) levels. In addition to the healthy endometrium of regularly menstruating women who are not on hormonal medications across the menstrual cycle, I also examined endometrial samples from women on hormonal medications (synthetic progestogens and GnRH). Dyskerin immunoscores in the endometrial epithelial cells were significantly higher in healthy postmenopausal endometrial samples (not exposed to hormonal therapy) and in samples from women treated with GnRH analogue, both characterised by a decreased endometrial epithelial proliferation associated with a hypo-estrogenic status. Dyskerin levels decrease when endogenous estrogen is high in the proliferative phase samples and in secretory phase with high estrogen and progesterone levels, as well as in levonorgestrel-releasing intrauterine system (LNG-IUS) samples, where exogenous progesterone is the predominant hormonal signal. Using a hormonal responsive, endometrial cancer cell line Ishikawa (ISK), as an *in vitro* mono-cellular 2D endometrial epithelial cell culture model, I have also demonstrated that ovarian steroid hormones regulated *DKC1* mRNA expression levels, where both estradiol (E2) and dihydrotestosterone (DHT) (in supraphysiological doses) upregulated *DKC1*. However, at a physiological concentration, DHT did not affect *DKC1* mRNA level. Conversely, progesterone counteracted the estrogenic effect and decreased *DKC1* levels.

Finally, to examine the functional effect of dyskerin on endometrial epithelial cells, and thus its relevance to endometrial carcinogenesis, I examined the effect of the overexpression of *DKC1* gene on endometrial epithelial cell proliferation rates *in vitro*. The experimental data

presented in Chapter 6, illustrates the confirmation of transient overexpression of ISK cells with *DKC1* (immunoblotting, immunofluorescence) and the resulting significant reduction in cell proliferation effect assessed by direct flow cytometry.

In conclusion, the *in vivo* and *in vitro* data produced during this PhD studentship has conclusively demonstrated that dyskerin is expressed in the human endometrium for the first time; that it is hormonally regulated and that dyskerin protein and mRNA levels were significantly lower in endometrial cancer compared with healthy postmenopausal endometrium. Furthermore, the *in vitro* functional studies have confirmed the hormonal regulation of endometrial epithelial *DKC1* gene expression and manipulation of *DKC1* gene demonstrated that dyskerin has a negative impact on endometrial epithelial cell proliferation. Collectively, this data proposes that dyskerin might be a new prognostic and therapeutic target for endometrial proliferative disorders such as endometrial cancer.

1

Chapter One

General Introduction

Endometrial cancer (EC) is the commonest gynaecological malignancy in the UK (CRUK), and a known feature of EC is a high TA (Alnafakh et al., 2019). Although dyskerin protein is one of the earliest known core components of the telomerase holo-enzyme, there has not been any previous attempts to study dyskerin in the human endometrium or in EC. The main aim of the studies presented in this PhD thesis was to investigate the role of dyskerin protein in the healthy human endometrium and in EC, in order to fill the existing gap in the current literature.

1.1 The uterus and the endometrium

The human uterus is a "pear"-shaped muscular organ, located in the pelvis. Average measurements for normal premenopausal adult uterus are length (top of the fundus to ectocervix) 5-8 cm and width (intercornual distance) 3-5 cm (Scurry et al., 1993). It is capable of a significant expansion to accommodate the growing foetus until delivery. The term "uterus" usually excludes the fallopian tubes and the cervix; both of which exist as a continuum of the uterine body. The human uterine wall consists of 3 layers: the inner endometrium, the myometrium, and the outer serosa (Maclean et al., 2020).

The human endometrium is a highly regenerative tissue, undergoing monthly cycles of shedding and regeneration during a female's reproductive life. Unlike most other mammals, humans and the upper order primates (e.g., the Chimpanzee) menstruate (Tempest et al., 2020). The human endometrium, therefore, undergoes over 400 cycles of menstrual shedding and re-growth in a single woman's lifetime (Alnafakh et al., 2019). It is composed of two functionally distinct layers: the transient superficial stratum functionalis is only present during the reproductive years, while the permanent deeper stratum basalis layer that lies below the functionalis and adjacent to and resting on the outer myometrium, which exists throughout a woman's life (Hapangama et al., 2015a), (Figure 1.1).

The stratum functionalis is thought to be shed with menstruation and to be subsequently fully regenerated within two weeks, measuring up to a thickness of 16 mm in the mid-secretory phase of the menstrual cycle. This impressive regeneration ability suggests that a stem cell population must reside within the endometrium. The location of stem/progenitor cells of the endometrium is postulated to be within the stratum basalis, which remains after the menstrual shedding of the stratum functionalis (Gargett et al., 2016, Tempest et al., 2018a, Tempest et al., 2018b).

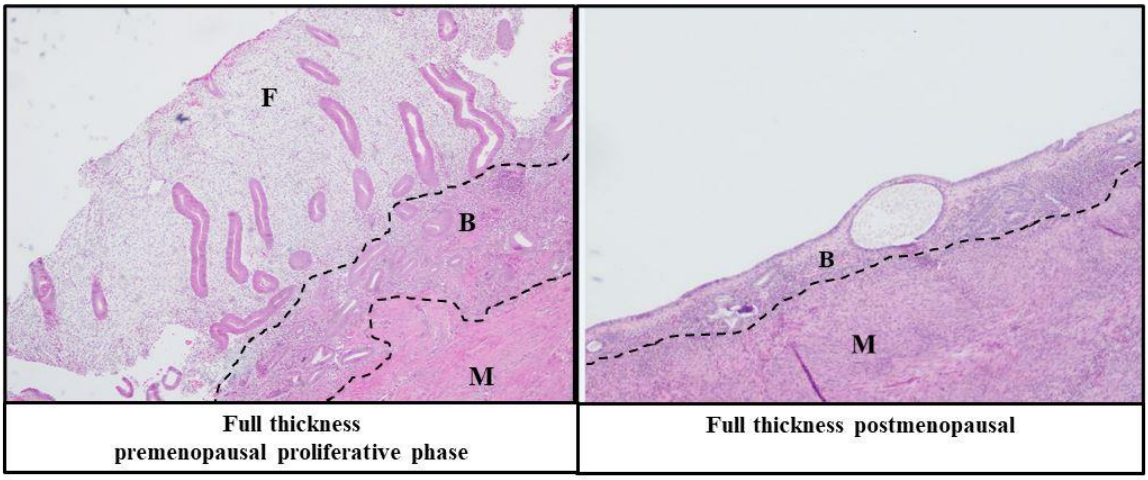

Figure 1.1 The layers of human endometrium. Magnification X40. **Abbreviations:** Functionalis (F), Basalis (B), Myometrium (M).

1.1.1 Pre and postnatal human endometrial gland development

Endometrial glands are present in all mammalian uteri and develop postnatally in sheep, pigs, rats and mice. In humans, however, this process is initiated in the foetus, continues after birth, and is only completed during puberty (Gray et al., 2001).

As seen in other mammals, the prenatal human uterus is also formed via the fusion of the paramesonephric ducts, which is initiated before the 8th week of gestation. Similar to rodents and ungulate (hoofed mammal) species, the simple columnar epithelium of the undifferentiated uterine body gives rise to several invaginations representing primordial glandular epithelium buds. By 20–22 weeks, the myometrium is well defined, but the glandular genesis is superficial (Song, 1964). At birth, uterine histoarchitecture resembles that of the adult but is less well developed. The neonatal endometrial luminal epithelium, comprised of either sparse low columnar or cuboidal glandular epithelial cells and stromal compartment, is limited to the adluminal stroma (Gray et al., 2001, Cooke et al., 2013a). From birth to puberty, there is a slow development of endometrial glands. By six years of age, the extension of the glands occurs from one-third to one-half of the endometrium (Cooke et al., 2013).

1.1.2 Adult human endometrial epithelium

At puberty, mature uterine histoarchitecture is observed, the endometrial glands extend to the inner circular myometrial layer (Cooke et al., 2013b). Human endometrium in adult composed of glands that are lined by columnar epithelial cells and surrounded by stromal cells (Maclean et al., 2020, Rinehart et al., 1988). Hormone-responsiveness is a feature of all endometrial cells (Hapangama et al., 2015a).

Recent 3D reconstruction of human endometrium has demonstrated unique histoarchitecture, particularly in the basalis layer; some non-branching, single, vertical functionalis glands originate from complex horizontal basalis glands that form a branching configuration. Tempest *et al.* reported that at least one stem-cell population exists in the basalis glands and can regenerate the whole complement of glandular lineages (Tempest et al., 2020).

Adenogenesis *in uteri* of adult women and primates is characterised by the adluminal development of endometrial glands from the basalis during the proliferative phase after menses, and possibly from the stroma (Okulicz et al., 1997, Huang et al., 2012). Recent evidence further suggests that the luminal epithelium may also contain a second stem niche, and therefore possibly may contribute to the postmenstrual glandular regeneration (Tempest et al., 2018b).

The epithelium is a defining cell type of the endometrium, and this is the main cell type particularly relevant to most endometrial proliferative pathologies, such as endometrial hyperplasia and cancer. This thesis mainly focuses on the malignant endometrial proliferative condition, EC and a holistic approach is taken to examine cancer tissue, in the context of a benign healthy endometrium; benign endometrial proliferative condition; endometriosis and the premalignant endometrial proliferative condition, endometrial hyperplasia.

1.2 Endometriosis

Endometriosis is a common, chronic, benign, estrogen-dependent inflammatory disease. It can cause chronic pelvic pain, dysmenorrhea, menorrhagia, dyspareunia and in some cases, infertility. Histologically, endometriosis is defined as the presence of endometrial glands and/or stroma-like tissue outside of the uterine cavity. Endometriotic (ectopic) lesions can be found mainly in the pelvic peritoneum, ovaries, and rectovaginal septum, and in rare cases, in the diaphragm, pleura, and pericardium (Sourial et al., 2014a, Giudice, 2010).

Endometriosis affects approximately 10% of women in the reproductive age, but the incidence increases to 35–50% of women with the symptoms of pelvic pain and infertility (Giudice, 2010, Carneiro et al., 2010). Retrograde menstruation, stem cells, genetic predisposition, dysfunctional immune response and aberrant peritoneal environment are all suggested as possible aetiological factors involved in the development and propagation of endometriotic lesions (Sourial et al., 2014a). The revised American Society for Reproductive Medicine (ASRM) staging system categorised endometriosis into minimal, mild, moderate, and severe (I-IV) stages, based on the number and character of peritoneal, ovarian and other organ lesions, and the presence, type, and extent of adhesions (Buttram, 1985, Rock, 1995). The uterine (eutopic) endometrium of women with endometriosis had been proposed to be different to that of the women without endometriosis (Hapangama et al., 2008b, Hapangama et al., 2009, Hapangama et al., 2012), and these changes have been postulated to contribute to the propagation of endometriosis (Sourial et al., 2014a, Hapangama et al., 2010).

1.2.1 Pathogenesis of endometriosis

The pathogenesis of endometriosis and the origin of the endometriotic lesion have been areas of extensive investigation for a long time; however, the aetiology behind endometriosis is still unknown. Several theories have been proposed, including retrograde menstruation, coelomic metaplasia, and the embryonic Mullerian rest theory (Sourial et al., 2014a).

Retrograde menstruation is the oldest theory to explain the aetiology of endometriosis (Sampson, 1927). This theory postulates that endometriotic implants occur as a result of retrograde flow of endometrial tissue through the fallopian tube into the pelvic cavity. Retrograde menstruation was demonstrated in 76-90% of women with patent fallopian tubes (Halme et al., 1984). Additionally, women with endometriosis have more retrograde menstrual fluid compared with those without endometriosis (Sasson and Taylor, 2008). Furthermore, this theory finds support from studies of congenital obstructed outflow tracts, where a high prevalence of endometriosis was reported in adolescent girls with congenital vaginal or cervical outlet obstruction (Sasson and Taylor, 2008). However, this theory failed to explain the reasons for the occurrence of endometriosis in pre-pubertal girls, newborns, or in males. Neonatal uterine bleeding occurs immediately after birth in most girls after the withdrawal of maternal ovarian hormones, similar to menstrual bleeding and retrograde flow of this uterine bleeding has been suggested as the reason of endometriosis in prepubertal girls (Sourial et al., 2014a).

Other theories include coelomic metaplasia, which proposes that endometriosis occurs as a result of the transformation of normal peritoneal tissue to ectopic endometrial tissue (Burney and Giudice, 2012). Endocrine-disrupting chemicals (EDCs) have been suggested as responsible agents for such transformation. The closely related 'induction hypothesis' proposed endogenous stimulating agents, such as a hormonal or immunologic factors that induce the differentiation of cells in the peritoneal lining to endometrial cells. Finally, the theory of embryonic Mullerian rests, or mullerianosis, purports cells residual from embryologic Mullerian duct migration maintain the capacity to develop into endometriotic lesions under estrogen effect at puberty or perhaps in response to estrogen mimetics. Further support for this theory is obtained from epidemiologic studies where the risk to develop endometriosis is doubled in women exposed to diethylstilbestrol *in utero* compared to non-exposed women (Burney and Giudice, 2012).

1.2.2 Risk factors or associations of endometriosis

1) Increased menstruation; commoner in nulliparous women (Moen and Schei, 1997).
2) Increased retrograde menstruation due to lower genital tract abnormalities such as vaginal atresia, cervical stenosis and imperforated hymen(Burney and Giudice, 2012, Veiga et al., 2018, Barbieri, 1998).
3) Ethnicity: Several studies have reported a nine-fold increase in risk in Asian women when compared to the European-American white females (Arumugam and Templeton, 1992, Sangi-Haghpeykar and Poindexter, 1995, Hasson, 1976, Dai et al., 2018).
4) Environmental factors: several reports showed that there is a link between endometriosis and some environmental factors; however, this association needs to be corroborated, such as elevated levels of phthalate esters, persistent organochlorine pollutants, perfluorochemicals and intrauterine exposure to cigarette smoke can cause endometriosis by inducing oxidative stress, altering hormonal homeostasis, or by changing the immune response (Buck Louis et al., 2012, Buck Louis et al., 2013, Louis et al., 2012, Missmer et al., 2004).
5) Other risk factors: for example, lower genital tract infections, have also been suggested as a possible cause of high risk to develop endometriosis (Lin et al., 2016, Dai et al., 2018).

1.2.3 Diagnosis

The gold standard for diagnosis of endometriosis is a combination of laparoscopy (or laparotomy) and histological confirmation of the presence of endometrium-like epithelium and/or stroma in endometriotic ectopic lesions. Such an invasive method of diagnosis is not desired for a mere diagnostic tool for a very common disease like endometriosis, due to the obvious risks and expenses involved. Many researchers have been (and presently are) in constant pursuit of non-invasive diagnostic methods. Many symptom-based questionnaires, biomarker-based studies and imaging methods have been examined by numerous authors to better diagnose endometriosis.

1.2.3.1 Laparoscopy and histopathology

The standard method for the diagnosis of endometriosis is the direct survey of the pelvic organs by an adequately trained gynaecologist to identify ectopic endometriotic lesions, routinely with a diagnostic laparoscopy (or incidental finding at laparotomy). Although some have proposed that the diagnosis should include laparoscopy and biopsy of the ectopic lesions for histological verification of the presence of endometrial glands and/or stromal cells, most national and international guidelines do not state the biopsy to be a mandatory requirement (Dunselman et al., 2014, Kuznetsov et al., 2017). This is due to many ectopic lesions not always containing the typical endometrium like epithelium and stroma, but fibrotic tissue only (Moen and Halvorsen, 1992).

At laparoscopy, endometriosis can be visualised as peritoneal implants, peritoneal windows, endometriomas, or deep infiltrating nodules, and all these lesions are commonly associated with adhesions.

Histopathological identification of endometriosis requires the presence of two or more of these histologic features: Endometrial epithelium, endometrial glands, endometrial stroma, and hemosiderin-laden macrophages (Hsu et al., 2010b).

Visualisation of endometriosis had been proposed to be inaccurate.; for example, Walter *et al.* (Walter et al., 2001) reported a significant discrepancy between the visual- and histological diagnosis of endometriosis, where 36% of the diagnoses were downstaged on the basis of

histological examination (Walter et al., 2001). A meta-analysis published in 2004 assumed a 20% prevalence of endometriosis stated that a positive finding on laparoscopy would be not correct in 50% of the cases (Wykes et al., 2004, Hsu et al., 2010a). However, the clinical consensus remains that in symptomatic women, visual detection of typical endometriotic lesions by a gynaecologist with sufficient training and skills would be sufficient to confirm the diagnosis of endometriosis (Kuznetsov et al., 2017).

Diagnosis of endometriosis by laparoscopy can be further substantiated by the combination of well-conducted, thorough gynaecological history, clinical examination as well as the pelvic imaging.

1.2.3.2 Imaging

Imaging has a limited role in the diagnosis of endometriosis as it does not have adequate resolution to detect adhesions or superficial peritoneal lesions. Recent developments in ultrasonographic techniques have significantly increased the detection of severe and deep infiltrating endometriosis when specialist sonographers survey the pelvis according to the International Deep Endometriosis Analysis group guidelines (Guerriero et al., 2016). Ultrasound is relatively cheap, widely available and less risky, but needs skilled sonographers. **Magnetic resonance imaging (MRI)** is also suitable to assess deep infiltrating endometriosis but is significantly more expensive and not sensitive enough to diagnose peritoneal and minimal disease (Foti et al., 2018). **Computerised tomography** (**CT) scan** of the pelvis lacks the ability to efficiently visualise pelvic organs; therefore, not useful in the diagnosis of endometriosis. However, there is a proposed role for CT scans with contrast to detect ureteral involvement and possible renal insufficiency (Hsu et al., 2010b).

1.2.3.3 Blood and other biological biomarker tests

To date, there is no single, clinically approved serum marker has been present to detect endometriosis with adequate sensitivity and specificity. Several studies have been searching for suitable markers to assess disease activity as well as to observe improvement. Serum cytokines, matrix metalloproteinases, adhesion molecules, and markers of angiogenesis or inflammation have been examined. Peritoneal markers have also been explored; however, the cyclic changes in hormonal influences and the variations in the amount of peritoneal fluid

makes this unfeasible and problematic for standardisation. Furthermore, obtaining peritoneal fluid is as invasive as a diagnostic laparoscopy; hence the usefulness of these markers is limited. The vast majority of studies have not correlated markers with disease activity or symptoms of endometriosis, partly because patient populations have not been well-characterized at the time the biosamples had been collected to assess these markers (Hsu et al., 2010a).

1.2.3.4 Endometrial nerve fibres

The presence of nerve fibres in the eutopic endometrium had been proposed as a diagnostic marker for endometriosis. Endometrial biopsy is being explored for the diagnosis of endometriosis. Several studies have shown an increased number of nerve fibres in eutopic endometrium of patients suffered from endometriosis compared with women without endometriosis (Al-Jefout et al., 2009, Bokor et al., 2009). These nerve fibres are described to be primarily small unmyelinated sensory C fibres in the functional endometrial layer, these fibres are recognised by their staining with anti-human protein gene product 9.5 (PGP9.5), anti-vasoactive intestinal peptide (VIP), and substance P, but not with neurofilament. Some evidence implies that endometriosis patients treated with hormones also have less number of nerve fibres in comparison with endometriosis patients who are not on hormonal treatment (Hsu et al., 2010a, Al-Jefout et al., 2009). However, the reproducibility and sensitivity of this method to be used in clinical practice are yet to be confirmed.

1.2.4 Treatment of endometriosis

Pharmacological and surgical treatments are available for endometriosis treatment. The aim of pharmacological agents is to alleviate the pain related to endometriosis, inhibit its further development and manage fertility. The empirical use of analgesics and hormones can abolish the painful symptoms without the need for laparoscopic surgery and is the recommended first-line treatment for endometriosis-associated symptoms other than subfertility. Nevertheless, before starting with this treatment, other causes of pelvic pain should be excluded if possible. The pharmacological treatment is primarily symptomatic and may stabilise or dry-out the lesions. Combined hormonal contraceptives, progestins and anti-progestins, GnRH agonists and antagonists, aromatase inhibitors, Danazol and non-steroidal anti-inflammatory drugs (NSAIDs) are the most common agents used in the management of endometriosis (Rafique and Decherney, 2017).

Pharmacological therapy is also often used as a complement to surgical treatment. It is used to reduce lesions before surgery and in the postoperative period. However, there is no evidence-based research to recommend systematic pre-operative hormonal therapy solely to prevent surgical complications or facilitate surgery. After surgery for endometriosis, combined hormonal contraceptives or 52mg LNG-IUS is recommended as a first-line treatment when pregnancy is not desired. Management of severe and deep infiltrating endometriosis is recommended to involve a multidisciplinary team, where physicians, gynaecological, colorectal, urology surgeons, and other professionals. The same approach is advised if the initial treatment failed, for the recurrent disease, or in the event of multiorgan involvement. The indications for the surgical treatment of endometriosis are pelvic pain not responding to conservative treatment, infertility, deep infiltrating endometriosis causing symptoms particularly due to invasion of the bowel, ureters or bladder, and large, symptomatic ovarian, endometrial cysts. The preferred method of surgical treatment is minimal access route, but with a deep infiltrating disease, there is a higher risk of complications as well as needing a laparotomy or open surgery. Surgical treatment of endometriosis might be associated with only local excision of lesions or sometimes require removal of all reproductive organs, i.e. hysterectomy with bilateral salphingo-oophorectomy; however, even this approach does not guarantee a cure for endometriosis and complete symptoms relief. Therefore, before embarking on a surgical treatment option, adequate counselling should be undertaken. Conservative treatment is the most frequent among reproductive-aged women where a complete excision of the lesions are desired with the preservation of genital organs and restoration of normal anatomy in the pelvis (Collinet et al., 2018, Krupa et al., 2019). It is important to consider that none of the aforementioned management approaches is curative and they are associated with a high rate of recurrence symptoms and possibly recurrent disease; for example, after conservative surgery, the reported recurrence rate is 21.5% after two years, 40% to 50% after five years (Guo, 2009). Therefore, further studies are needed to improve our understanding of natural history and the pathogenesis of endometriosis in order to develop new treatments. This will enable us to understand the mechanism of recurrence and propagation of the disease and to aid to identify biomarkers which will help in the diagnose and monitor endometriosis.

1.3 Endometrial hyperplasia (EH)

EH is a pathological condition characterised by hyperplastic changes in the endometrial glands and stroma. At a cellular level, cytological atypia of EH has been determined as the major

indicator for susceptibility to malignant transformation; therefore, EH is ranging from histologically subtle and spontaneously reversible proliferative lesions to premalignant changes that either immediately precedes, especially in postmenopausal women (25-30%) or co-occur with cancer (12.7% to 42.6%) (Litta et al., 2013, Montgomery et al., 2004, Mazur, 2005). For the ideal management of patients with EH, accurate diagnosis of premalignant endometrial lesions and exclusion of coexisting ECs are extremely important (Kurman et al.,2011).

The underlying cause of EH is usually a relative predominance of estrogen, combined with insufficient progesterone levels. This is commonly called unopposed estrogen (Emons et al., 2015). Estrogen has a mitotic effect on the human endometrial epithelium, and progesterone will counteract the estrogen-induced proliferation. Therefore, the high estrogen exposure without sufficient endogenous or exogenous progestogenic stimuli to reduce proliferation will have a net effect of excessive endometrial epithelial proliferation.

Typical premenopausal causes of progesterone insufficiency/ unopposed estrogen include corpus luteum insufficiency/anovulatory cycles, polycystic ovary syndrome and obesity with metabolic syndrome (aromatase conversion of ovarian androgens in adipose tissue). In postmenopausal women, in particular, inappropriate hormone replacement therapy (insufficient dosage of gestagens or just estrogens without any progestagens) or estrogen or androgen-producing tumour. In patients with Lynch syndrome (previously known as Hereditary nonpolyposis colorectal cancer (HNPCC)), tumorigenesis of hereditary endometrioid carcinoma also usually follows the natural progression pattern described in endometrioid cancers, with carcinogenesis originating from the initial development of hyperplasias (Emons et al., 2015).

Classification: The World Health Organisation (WHO) in 1994 categorised endometrial EH into four classes depending on the histological architecture and the presence or absence of cellular atypia:

1. Simple hyperplasia without atypia.
2. Complex hyperplasia without atypia.
3. Simple atypical hyperplasia.
4. Complex atypical hyperplasia (Emons et al., 2015).

However, this classification led to confusion among pathologists as they continued to debate if group 3 EHs existed or not. Furthermore, the use of the 1994 WHO classification caused confusion among clinicians because it did not adequately inform them of the subsequent premalignant potential, i.e., it did not help them to make clear treatment plans. Therefore, 20 years later, the WHO clarified the issue and published a new classification of EH in 2014, which distinguished only two classes of EH with a clear clinical implication:

1. Hyperplasia without atypia.
2. Atypical hyperplasia (EHA)/Endometrioid intraepithelial neoplasia (EIN), (Emons et al., 2015) (Table 1.1).

Table 1.1 The new classification of endometrial hyperplasia, table adapted from Emons *et al.* (Emons et al., 2015)

WHO classification (2014)	WHO classification (1994)	molecular genetic changes	The risk to develop EC, Risk Ratio (RR)
Hyperplasia without atypia	Simple hyperplasia without atypia, Complex hyperplasia without atypia,	No relevant genetic alterations	RR: 1.01–1.03
EHA/EIN	Simple EHA Complex EHA EIN	Show most of the mutations that associated with invasive endometrioid EC such as microsatellite instability, PAX2 inactivation, PTEN mutation and CTNNB1 (β-catenin) and KRAS mutation	RR: 14–45

Abbreviations: Atypical endometrial hyperplasia (EHA), Endometrioid intraepithelial neoplasia (EIN).

Hyperplasia without atypia is regarded as a benign and reversible condition (Mazur, 2005) that exhibit no relevant genetic changes and regress if treated with conservative measures such as progesterone therapy (administrated orally or locally, in an IUS). Prevention of future

progression to cancer with hysterectomy in these patients may only be considered in selected cases, such as those with a persistent disease not responsive to progestogenic treatment. Commonly this is seen in women with extreme obesity, and when weight loss is not achieved despite all attempts, hysterectomy may be considered (Trimble et al., 2012, Emons et al., 2015). However, the risk of surgery in those women still may outweigh the benefits of prevention of EC. Atypical EH is an entirely different condition, where up to 60% of women with a diagnosis of atypical EH may also have concomitant invasive endometrial carcinoma. This suggests that the histological diagnosis of atypical EH in an endometrial biopsy requires further investigations to confidently exclude EC and, therefore, they are often offered a hysterectomy. Even if extensive investigations exclude concomitant cancer, these women have a greater subsequent risk for developing invasive EC over the next few years.

Additionally, EHAs share many genetic mutations with invasive endometrioid EC. Therefore, a total hysterectomy, (not subtotal/supracervical hysterectomy) is the usual treatment of choice for atypical EH. However, in exceptional cases, for example, in younger patients wanting to have children – fertility-sparing therapy such as high-dose of progesterone, or specialist hysteroscopic resection techniques with appropriate subsequent follow up with regular histological assessment might serve as an alternative option to hysterectomy. Similar options may be considered in other patients with atypical EH who refuse hysterectomy for other indications or those with high anaesthesiologic and surgical risks (Litta et al., 2013, Trimble et al., 2012, Emons et al., 2015). EIN is a monoclonal endometrial preinvasive glandular proliferation that represents an immediate precursor of the endometrial type 1 EC. It is characterized by a proliferation of endometrial glands that exceeds the stroma with a maximum linear dimension >1mm (Horn et al., 2007).

Endometrial in situ carcinoma (EIC) is entirely different from EHA. It represents the precursor lesion of invasive serous carcinoma; it is a non-invasive glandular lesion characterised by epithelial cells with significant nuclear abnormalities that are similar to those seen in serous EC. Unlike EHA, EIC is not associated with high estrogen exposure, and it is seen in the atrophic endometrium of older postmenopausal women (Horn et al., 2007).

1.4 Endometrial cancer (EC)

EC is the fourth most common cancer in women in the UK, which makes it the commonest gynaecological cancer (CRUK). EC represents 5% of all new cancer cases diagnosed in women in the UK in 2017 (CRUK). Incidence rates for uterine cancer in the UK are highest in women aged 75 to 79 (2015-2017) (CRUK). Increasing obesity and longevity have both caused the incidence of EC to increase at an alarming rate. For example, in the UK, the incidence of EC increased by almost 55% between 2015 and 2017 (Figure 1.2A) (CRUK) compared with the rates of early 1990s. European estimates predict a 100% increase in the incidence of EC by 2025 not only in older postmenopausal women but also in younger women (Figure 1.2B) (Lindemann et al., 2010). Figures from the UK report that mortality associated with EC has risen by 23% over the last decade, which is concerning because this is observed in an era of improving survival rates for most other cancers. Additionally, the mortality rates are expected to increase by a further 19% by 2035 (CRUK).

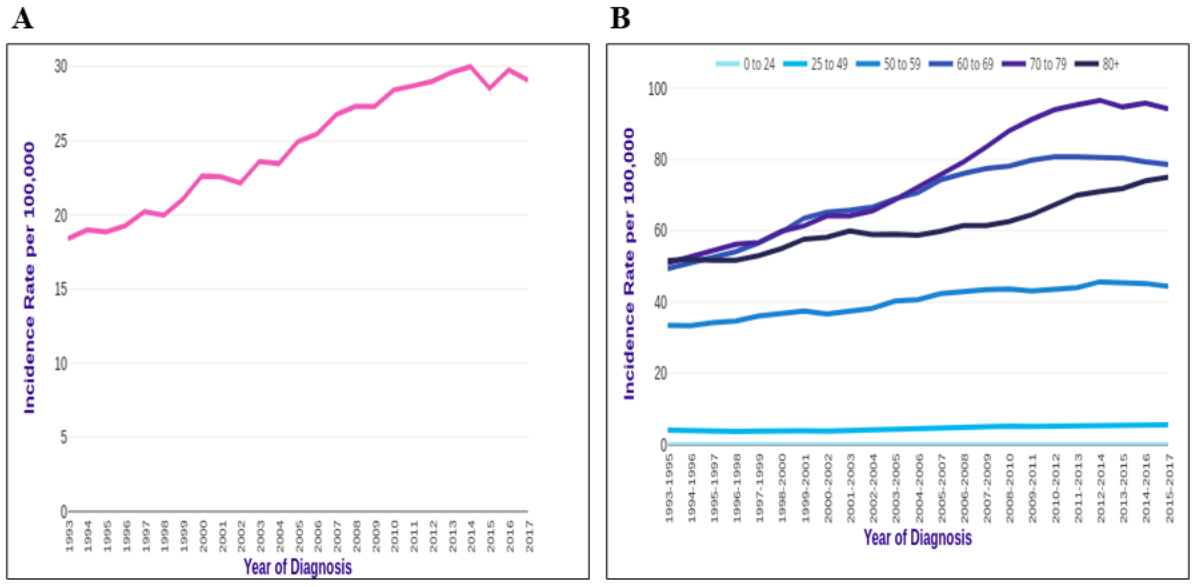

Figure 1.2 Endometrial cancer incidence in the UK. **A)** European Age-Standardised Incidence Rates, Females, UK, 1993-2017. **B)** European Age-Standardised Incidence Rates, By Age, Females, UK, 1993-2017 (CRUK).

The survival rates for high-grade EC are exceptionally poor, similar to ovarian cancer; and the traditional surgical treatment is associated with significant morbidity and mortality for many women even when presented with early disease due to frequently occurring co-morbidities and obesity in them (Orekoya et al., 2016). Urgent novel therapeutic options are therefore needed to prevent, treat as well as to avoid progression of EC.

Although EC is an important disease with a significant clinical and economic consequence, the molecular biology of endometrial carcinogenesis is not well-described or understood when compared with other female-specific malignancies, such as breast or ovarian cancer. The human endometrium is a unique organ with a massive regenerative potential (Tempest et al., 2018b), and it is the main target organ for ovarian steroid hormone action (Hapangama et al., 2015a). While being a hormonally responsive tissue, the endometrium responds rather differently to the same steroid hormones than other hormone-responsive organs, such as breast tissue (Kamal et al., 2016a, Hapangama et al., 2015a). This has made it difficult to translate the pioneering discoveries made in other cancers, such as breast cancer, to EC management and therapy.

1.4.1 Risk factors

Since a large part of this thesis is focused on EC, the important risk factors for EC that may also affect the endometrial expression of the proteins of interest are considered.

1.4.1.1 Factors associated with an increased risk to develop EC

1. **Estrogen therapy without progestogen protection**

Postmenopausal women treated with hormone-replacement therapy (HRT) containing estrogen alone are at increased risk of developing EC. An increasing incidence of EC was reported in the 1970s after estrogen therapy first became commonly used to treat menopausal symptoms. In addition, early data also showed that adding progestogen prevented the raised risk of EC with unopposed estrogen (Weiss et al., 1976). Therefore, the long-term unopposed effect of estrogen (without progestogen protection), is regarded as the most important aetiological factor for the development of EC. In a large study in 2005 where one million women were enrolled, it was revealed that the relative risk (RR) of estrogen therapy without progesterone protection in an intact uterus compared with those who never used HRT was 1.45 (Orekoya et al., 2016).

A meta-analysis of 30 observational studies showed That the RR of estrogen-only therapy was even higher; it was 2.3. The authors reported that estrogen monotherapy increased the risk for EC death (RR 2.7); whereas combined estrogen and progestin decreased the risk to develop EC (RR 0.4). The type of HRT, the duration of use and the cumulative dose of estrogen all modulate the risk of developing EC in the users (Kamal et al., 2016a).

2. Age

Ninety percent of EC cases occur in patients older than 50 years, with a median age of 63 years. In the UK, EC incidence rates are highest in females aged 75 to 79 (2015-2017) (CRUK). The risk of developing EC increases with age, Siegel *et al.* showing that a woman under the age of 49 years had a 0.3% of developing EC, whereas the risk increased to 1.3% in women over 69 years of age (Siegel et al., 2018).

3. Genetic predisposition

Approximately 90% of ECs are sporadic, whereas hereditary genetic mutations are responsible for nearly 10% of EC cases (Okuda et al., 2010), which typically occur 10 to 20 years earlier than sporadic ECs. An example of genetic predisposition of EC is Lynch syndrome (previously known as the hereditary nonpolyposis colorectal cancer), which is an autosomal dominant syndrome and is caused by a germline mutation in one of four DNA mismatch repair genes (MLH1, MSH2, MSH6, or PMS2). It is characterised by a significant increase in the lifetime risk of colorectal cancers and ECs; the risk of EC can be as high as 60% (Passarello et al., 2019). These patients are offered yearly screening with ultrasound scans and regular endometrial biopsies and prophylactic hysterectomy once the family is completed to reduce the risk of EC in them.

4. Obesity

In the UK, 34% of EC cases are associated with obesity (Brown et al., 2018). The risk of developing EC increased by 21% for each 0.1 increase in waist to hip ratio (Kyrgiou et al., 2017). Obese women have a two to fivefold higher EC risk compared with normal-weight women. Causative pathology is thought to be due to the obesity-associated increased levels of circulating estrogens (extra-ovarian aromatisation of adrenal androgens), likely accounting for their increased risk of EC.

5. Diabetes

Diabetes has also been significantly correlated with increased risk of EC. Results from the meta-analysis support a relationship between diabetes and increased risk of EC(Mueck et al., 2010). The analysis of the relationship between diabetes (mostly type 2) and EC, based on 16 studies, revealed that diabetes was statistically significantly associated with an increased risk of EC (Constantine et al., 2019a).

6. Reproductive factors

Certain reproductive factors might also affect the incidence of EC by influencing the relative estrogen/progesterone balance. For example, women with increased lifetime exposure to estrogens, such as those who underwent menarche at an early age, reached menopause at a later age, with lower parity, and who did not report the use of oral contraceptives, have been associated with a higher incidence of EC. However, according to a recent report, reproductive factors do not appear to be major contributors to the increase in the incidence of EC development in the Women's Health Initiative study (WHI) (Constantine et al., 2019a).

7. Polycystic ovarian syndrome (PCOS)

Premenopausal women (aged ≤54 years) with PCOS were at a significantly higher risk of developing EC, odds ratio (OR), 2.79 compared with the same age, non-PCOS control women (Barry et al., 2014). Chronic anovulation, obesity, and hyperinsulinaemia are all associated with PCOS and EC. The main causative mechanism that has been proposed to increase the risk of EC development in women with PCOS is increased exposure to estrogen either that is completely unopposed by progesterone with anovulation or in those who ovulate, estrogenic action in the endometrium is only weakly counteracted than in normal women without PCOS (Palomba et al., 2015).

8. Tamoxifen

Tamoxifen, a non-steroidal anti-estrogen, is used as an effective therapy for breast cancer treatment, but it increased the risk of EC. Tamoxifen is especially associated with non-endometrioid EC subtypes, and these cancers have poorer outcome among long term tamoxifen users. Tamoxifen has been categorised as a causative factor to develop EC by the International Agency for research on cancer. Breast cancer survivors aged 55-69 years have 3 times higher

risk to develop EC in those who used tamoxifen for approximately 5 years (Jones et al., 2012, Cohen, 2004).

9. Race and social factors

According to cancer research UK, EC is common in all women with white, Asian or black racial origins, and the incidence of EC in England is not associated with deprivation. However, it is reported that white women had the highest incidence of EC in comparison with other racial/ethnic groups in the USA. This pattern of EC incidence reported by Setiawan *et al.* (Setiawan et al., 2007) is consistent with the pattern observed in The Surveillance, Epidemiology, and End Results (SEER) Program for women in the same age and ethnic group. African Americans women had a 24% lower risk to develop EC compared with white women; however, the African American women were more likely to be diagnosed at advance stages of EC (Setiawan et al., 2007). Additionally, African Americans had higher grade and more aggressive cancers compared with white counterparts. Even within the same International Federation of Gynecology and Obstetrics (FIGO) stage at diagnosis, differences in grade and histological types between African Americans and Whites. Therefore, Setiawan *et al.* (Setiawan et al., 2007) suggested a biological basis for these observed differences. Dolly *et al.* have found that survival rates were significantly decreased with a longer interval of time from diagnosis to treatment and this delay was directly related to the insurance status of EC patients in the US (Dolly et al., 2016). Those without private insurance naturally suffered from significantly longer interval time from diagnosis to surgery compared with patients with private insurance. Race, BMI, and distance to treatment institution had no significant correlation with interval treatment time (Dolly et al., 2016). Therefore, it is likely that in a country where access to healthcare depends on social factors (economic status, racial background, education, etc.), the outcome of EC is dependable on the deprivation scores. In the UK, where the NHS is providing free, universally accessible, high-quality healthcare, these factors are expected to have less influence on the outcomes of EC.

10. Previous breast cancer

Breast cancer survivors have been proposed to have a higher risk of developing EC. The common causative factors such as the significant association between obesity with both breast

cancer and EC, common hereditary cancer syndromes and use of tamoxifen may explain this association (Constantine et al., 2019).

11. Endometrial polyps

Endometrial hyperplasia and cancer were identified in 5.42% of postmenopausal (PM) women with endometrial polyps compared with only 1.7% of women with endometrial polyps in reproductive age (Lee et al., 2010). Estradiol (E2) and tamoxifen (to a lesser extent) are suggested as the attributing factors to cause endometrial polyp in these women; however, there is no direct functional evidence to support malignant transformation in the benign polyps (Van Bogaert, 1988, Erdemoglu et al., 2008).

1.4.1.2 Factors associated with lowering the risk of EC

1. Contraceptives

The use of oral contraceptive pills (COCP) is a well-known treatment that decreases EC risk. The risk reduction increases with duration of use and will continue up to 30 years after the cessation of treatment. Kavanaugh and Jerman in 2018 analysed the National Survey of Family Growth database to examine levels of, correlates of and changes in the use of individual and grouped methods of contraception among US women aged 15–44 from 2008 to 2014. They observed a stable prevalence of oral contraceptive use in that period. However, altering the formulation of oral contraceptive pills resulting in lower progesterone relative to estrogen could potentially elevate the risk of EC. Nevertheless, the main trend in formulation changes of oral contraceptives has been a reduction in estrogen rather than in progesterone (Constantine et al., 2019b).

Setiawan and colleagues reported in 2013 that the use of COCP associated with lower risk to develop both types I and II EC apart from carcinosarcoma, where a non-significant negative correlation between the use of COCP and risk of cancer was observed (Felix et al., 2013). Premenopausal women, using COCP for ≥5 years before the first full-term pregnancy, have significantly reduced the risk of EC (Cook et al., 2014).

There are limited data on oral or injectable progestogen-only preparations (POPs) and the LNG-IUS, but similar protective effects are suggested(Mueck et al., 2010).

2. Intrauterine devices (IUDs)

IUDs were shown to protect against EC; this protective effect is higher with longer duration of treatment and sustained up to 5 years after cessation of use(Mueck et al., 2010). National Survey of Family Growth data has revealed that between 1982 and 2013, usage of long-acting reversible contraceptives (LACRs) has gained popularity (LARCs; IUDs and subdermal hormonal implants) in women aged 15–44 years. LARCs use was constant between 1998 and 2002 (1.5%); LARC use then doubled in 2006–2010 (3.8%) and then nearly doubled again for 2011–2013 (7.2%) (Branum and Jones, 2015). No study has yet reported whether newer IUDs types that release progesterone have any influence on EC risk; nevertheless, these IUDs are sometimes used to treat pre-cancers and early ECs (Constantine et al., 2019a); therefore their use is assumed to reduce risk of endometrial carcinogenesis.

3. Physical activity

Meta-analyses have shown that EC risk is decreased by 5% upon 1 hour/week increase in leisure-time physical activity, and 32% lower in women who spend the least time sitting compared with those who spend the most (Keum et al., 2014).

Additionally, a 20-40% reduction in EC risk can be observed in physically active women compared with those who are physically inactive. Although the prevalence of leisure-time physical inactivity has a gradual decrease over the past three decades in most U.S. states; yet, rates of physical inactivity persisted in being considerably high; therefore, there was no significant influence in the overall EC incidence after Women's Health Initiative (WHI) (Constantine et al., 2019a).

4. Smoking

The negative relationship between cigarette smoking and the risk of EC is well established. A 2014 study has shown that there was a significantly lower risk of developing EC among former and current smokers versus those who had no previous history of smoking (Felix et al., 2014).

This protective effect can be explained by the possible influence of cigarette smoking on estrogen metabolism, which occurs mainly in hepatic tissues and may be related to cigarette smoking (Felix et al., 2014). 16 alpha-hydroxyestrogen, opposed to 2-hydroxyestrogen, cause activation of cell proliferation and has been found to be associated with an elevated risk of endometrial and breast carcinomas Increased clearance of 16alpha-hydroxyestrone has been observed among smokers, that might clarify the inverse correlation between cigarette smoking and the risk of endometrial carcinoma (Felix et al., 2014). However, Brand *et al.*, in 2011, reported that Cigarette smoking is associated with an elevation of circulating levels of estrogens and androgens (Brand et al., 2011).

5. **High coffee intake**

Women with the highest intake of coffee have 21% less risk of EC compared with those with the lowest intake (CRUK).

1.4.2 Classification of EC

EC includes a biologically, clinically, pathological and genetically complex and heterogeneous group of tumour subtypes. Several classification systems have been suggested over the last decade in order to improve the outcome.

1.4.2.1 WHO

Based on the origin of the neoplastic cell and histological morphology, the WHO classified EC into 7 categories (Kurman et al.,2014).

1. Epithelial tumours, including endometrioid and non-endometrioid epithelial EC (Table 1.2).

2. Mesenchymal tumours, such as endometrial stromal and smooth muscle tumours.

3. Miscellaneous tumours, such as Mixed endometrial stromal and smooth muscle tumour

4. Mixed epithelial and mesenchymal tumours, such as carcinosarcoma.

5. Gestational trophoblastic diseases, such as Trophoblastic neoplasms.

6. Lymphoid and myeloid tumours, including Malignant lymphoma and Leukaemia.

7. Secondary tumours.

The main limitation of this classification is that carcinosarcoma is classified as a mixed epithelial and nonepithelial tumour in the uterine tumours' classification while it is an epithelial cancer in the WHO classification of ovarian tumours. This discrepancy reflects the confusion over the carcinosarcomas' histogenesis and classification in different anatomic sites. Recently, molecular genetic data support the notion that carcinosarcoma is a biphasic tumour, and its two components are clonally derived from a transformed epithelial cell. Therefore, many researchers now consider these neoplasms as poorly differentiated carcinomas that show sarcomatous metaplasia (Kurman et al., 2011). **Table 1.2 WHO histological classification of epithelial EC (Kurman et al., 2014).**

WHO histological classification of epithelial EC	
Endometrioid adenocarcinoma	**Variant with squamous carcinoma** **Villoglandular variant** **Secretory variant** **Ciliated cell variant**
Mucinous adenocarcinoma	
Serous adenocarcinoma	
Clear cell carcinoma	
Mixed adenocarcinoma	
Squamous cell carcinoma	
Transitional cell carcinoma	
Small cell carcinoma	
Undifferentiated carcinoma	
Others	**Rare types of epithelial EC such as adenoid cystic carcinoma**

1.4.2.2 Dualistic

A landmark oncology paper published in 1983 by Jan V. Bokhman, defined two types of EC (Bokhman, 1983) and this was the main classification of EC that prevailed since then until recently. Type I cancers represent approximately 80% of EC, and these tumours are thought to be estrogen-driven. They are of endometrioid type, mostly of low-grade histological differentiation and of early clinical stage and arise from a hyperplastic background. They are strongly associated with obesity, hyperlipidemia and other components of the metabolic syndrome. Women who suffer from this type are relatively younger, in either perimenopausal and PM period and have better five-year survival compared to type II EC sufferers. Type II cancers represent higher grade non-endometrioid tumours, which usually arise from a background of atrophic endometrium and have no association with ovarian hormones. They are inversely correlated with demographic features, such as obesity, and these tumours typically have an aggressive clinical course (Suarez et al., 2017). A major limitation of Bokhman's classification is the failure to identify some of the high-grade type I, endometrioid EC (Voss et al., 2012). Voss *et al.* in 2012 demonstrated that endometrioid EC grade 3, serous EC and clear cell EC had similar clinical, immunohistochemical parameters and survival outcomes. All type II tumours are regarded to be high-grade cancers due to their association with common molecular changes. In addition, there was no significant difference in the mean age of diagnosis or stage distribution at the time of initial treatment. It has been noticed that 68% of grade 3 endometrioid ECs were diagnosed at early stages. This is lower than the published data that 85–90% of all endometrioid carcinomas are diagnosed at early stages. Therefore, it is more suitable to include grade 3 endometrioid tumours in the type 2 group, class them all as high grade, and this could markedly improve management of these tumours (Voss et al., 2012, Barlin et al., 2013).

1.4.2.3 Molecular classification

An integrated genomic analysis by The Cancer Genome Atlas (TCGA) resulted in the molecular classification of endometrioid and serous carcinomas. This classification provides insights into disease biology and diagnostic classification that could have rapid therapeutic application. The TCGA project involved a combination of whole-genome sequencing, exome

sequencing, microsatellite instability (MSI) assays, and copy number analysis. This classification defines 4 classes of EC:

1. POLE ultramutated: These tumours have stable somatic copy number but highly mutant genes particularly POLE; a catalytic subunit of DNA polymerase epsilon included in nuclear DNA replication and repair; therefore, its mutation causes numerous genomic alterations (Mirza, 2020, McAlpine et al., 2018). The most important characteristic of this group is the very favourable prognosis, even in high-grade tumours (Meng et al., 2014, Billingsley et al., 2015, Church et al., 2015). This group includes 6.4% of the low-grade ECs (LGECs), 17.4% of high-grade endometrioid ECs and any of non-endometrioid ECs.

2. Hypermutated/microsatellite instability (MSI): MSI arises from defective post-replicative DNA mismatch repair system; therefore, these ECs have a high level of MSI and low MLH1 mRNA because of the methylation of MLH1 promoter in sporadic, and in hereditary Lynch syndrome it can be caused by mutations in any of the DNA mismatch repair genes (Morice et al., 2016). This group includes 28.6% of LGEC and 54.3% of high-grade ECs (HGECs) among TCGA dataset (Mirza, 2020). This subgroup consists mainly of the endometrioid tumours and has mutations tenfold more than copy number low, with less favourable outcomes.

3. Copy-number low/microsatellite stable: Resembles MSI group, tumours belong to this group mostly of endometrioid subtype. 60% of LGEC, 8.7% OF HGEC, 2.3% of serous EC and 25% of mixed histology ECs are gathered in this group. These tumours characterised by 100% mutation of chromosome 1 and 52% mutation in Beta-catenin (CNNB1) (Mirza, 2020).

4. Copy-number high: represents (serous-like): 5% of low-grade endometrioid EC, about 20% of high-grade endometrioid EC, most of serous EC (97.7%) and 75% of mixed histology EC are grouped in this subset of tumours (Le Gallo and Bell, 2014). It was noted that serous cancers are often displaying p53 mutations and high somatic copy number alterations (SCNAs). Tumours with high copy number (CN) have similar clinical behaviour and survival to that of serous ovarian cancer (Kandoth et al., 2013). Tumours in this group had significantly worse progression-free survival than endometrioid tumours groups (Mirza, 2020).

The first three subgroups encompass 97% of type I EC while the copy number high group comprises 94% serous EC. About 25% of high-grade endometrioid EC have molecular features

resembles those of serous EC, including frequent *TP53* mutations and extensive SCNA and have a poor outcome. The convincing similarities between this subset of endometrioid tumours and serous uterine carcinomas suggested that genomic-based classification might result in improvement in the management of these women (Talhouk et al., 2015).

1.4.2.4 Histological types of EC

Endometrioid ECs have a wide range of histological differentiation from a very well-differentiated carcinoma, which can be confused with atypical complex hyperplasia, to poorly differentiated tumours that are difficult to distinguish not only from undifferentiated carcinoma but also from various sarcomas. Endometrioid tumours are characterised by the presence of at least some glandular or villoglandular structures lined by stratified columnar cells. These tumours represent approximately 80% of EC cases (Kurman et al., 2011).

Grading

The FIGO Grading system is commonly used to grade endometrioid EC. It is determined by the microscopic appearance of cancer, which depends on the architectural pattern, nuclear features assessment or both of them. The architectural pattern is based on the amount of solid masses of cells within the tumour. The nuclear grading is determined by the variation in nuclear size and shape, distribution of the chromatin, and size of the nucleoli. Mitotic activity is an independent variable, but in general, it increases with the increase in nuclear grading. In endometrioid carcinomas that have squamous differentiation, exclusion of squamous epithelial masses is essential to determine the percentage of the solid growth (Kurman et al., 2011).

Endometrioid FIGO grades

Grade 1 tumours have ≤5% solid non-glandular, non-squamous growth and/or have oval nuclei, mildly enlarged, and have evenly dispersed chromatin.

Grade 2 tumours have from 6-50% of non-gland forming growth and/or nuclei have features intermediate between grades 1 and 3.

Grade 3 tumours have >50% non-glandular solid, non-squamous growth pattern and/or nuclei are markedly enlarged and pleomorphic, with irregular coarse chromatin, and prominent eosinophilic nucleoli (Kurman et al., 2011).

The presence of marked cytologic atypia increases the grade one level. For instance, cancer that is grade 2 by architecture but has nuclear grade 3 should be upgraded to grade 3 (Kurman et al., 2011).

Mucinous adenocarcinomas of the endometrium are closely related to endometrioid carcinomas; therefore, it is realistic to use FIGO grading for those carcinomas as well (Soslow et al., 2019).

Regarding other EC subtypes, by definition serous, clear cell, carcinosarcoma and undifferentiated carcinoma are not individually graded and considered in general as high grade (grade 3) tumours (Kurman et al., 2011, Soslow et al., 2019).

Serous ECs are high-grade ECs, accounting for less than 10% of all EC, characterised by a complex pattern of papillae with cellular budding. It is a highly aggressive cancer and not associated with estrogen stimulation, occasionally arising in endometrial polyps or from premalignant (EIN) lesions. Serous EC is usually developed in atrophic endometrium that mostly occurs in older aged patients. These tumours characterised by complex papillary and/or glandular structures, with diffuse severe nuclear pleomorphism (Gatius and Matias-Guiu, 2016, Kurman et al.,2014).

Budding and tufting are frequently seen in tumours of this subtype, classically resulting in luminal borders that appear ruffled, different from the linear, smooth contours of endometrioid carcinoma (Soslow et al., 2007). Serous carcinoma spreads commonly within the abdomen, similar to ovarian cancer. In the absence of myometrial invasion, early intra-abdominal metastases are often present at diagnosis (Kurman et al.,2011).

Clear cell ECs are typically characterised by glycogen-filled cells and ‘hobnail’ cells that protruded individually into lumens and papillary spaces. These tumours are arranged in solid, tubulocystic, papillary or a combination of these patterns. Clear cell ECs are less common than serous carcinoma (1-5%, of all ECs) and they are mainly seen in older women and usually, similar to serous ECs, diagnosed in advanced clinical stage. Nuclei are highly pleomorphic, often with bizarre and multinucleated forms. Psammoma bodies are present rarely in clear cell ECs. The stroma in clear cell ECs is distinctive has inflammation and is hyalinised and myxoid. Clear cell carcinomas usually have fewer mitoses than in serous carcinoma (Kurman et al., 2011; Soslow, 2013).

Carcinosarcoma ECs are metaplastic carcinomas or carcinomas with sarcomatous metaplastic foci rather than uterine sarcomas. Recent immunohistochemical and molecular genetic studies supported that both epithelial and mesenchymal-like elements shared the same clonal origin (McCluggage, 2002). Carcinosarcomas represents about 2-5% of all EC (Kurman et al., 2011) and are more common in women taking tamoxifen (Kloos et al., 2002). The development of these tumours is not secondary to estrogenic stimulation, but due to other mechanisms such as DNA adducts formation. The epithelial component typically has high-grade EC characteristics, often serous or clear cell carcinoma, although endometrioid patterns, including carcinoma with squamous differentiation, might be present. The sarcomatous component may be homologous or heterologous. The homologous sarcoma-like component often contains hypercellular sheets of small, hyperchromatic, round to spindle-shaped cells with a high mitotic index and they lack apparent differentiation. The epithelial component presents in most metastases, and usually, it determines the outcome of these tumours. Women with non-endometrioid epithelial component tumours have a poor prognosis compared with those with an endometrioid epithelial element (Kurman et al., 2011, Soslow, 2013).

Mixed tumours when type I tumour (endometrioid carcinoma, or its variants, or mucinous carcinoma) mixed with a type II EC (serous or clear cell) in which the minor type must comprise at least 5% of the total volume of the tumour. The percentage of the minor component should be stated in the pathology report. It is generally accepted that 5% or more of a type II tumour suggests a poor outcome, although the significance of lesser proportions is not well understood (Kurman et al., 2014).

The International Federation of Gynaecology and Obstetrics defined grades 1 and 2 endometrioid tumours as LGEC and FIGO grade 3 endometrioid, serous, and clear cell carcinomas considered as HGECs (Soslow et al., 2019).

Table in Appendix I summaried systems used to classify EC.

1.4.2.5 International Federation of Gynaecology and Obstetrics (FIGO) staging system

Tumour stage represents the degree of the spread of the disease at the time of diagnosis, which is a crucial requirement for treatment planning and outcome determining. To date, the FIGO staging system (Table 1.3) is generally used to stage EC (Pecorelli, 2009). The preoperative evaluation of EC including chest X-ray, clinical and gynaecological examination, a transvaginal ultrasound, blood counts and liver and renal function profiles. For examining the presence of extra-pelvic metastases, an abdominal CT scan is the best tool. Dynamic contrast-enhanced MRI is used to investigate cervical involvement (Colombo et al., 2013). Additionally, MRI has been shown to accurately evaluate the depth of myometrial invasion. The accuracy provided by transvaginal ultrasonography (TVU), when carried out by expert practitioners, is comparable to that of MRI according to prospective collaborative study (Savelli et al., 2008). Distant metastases can be detected accurately by [^{18}F]2-Fluoro-2- deoxy-D-glucose–positron emission tomography (FDG-PET)/ CT(Colombo et al., 2013).

For high risk of recurrence in apparently early-stage disease, several factors have been recognised according to ESMO Clinical Practice Guidelines in 2013: histological subtype, grade 3 tumour, myometrial invasion ≥50%, lymphovascular space invasion (LVSI), lymph node metastases and tumour diameter >2 cm. Therefore, stage I can be subcategorised into three risk classes: **i)** Low-risk group: endometrioid subtype, Stage IA (Grade 1 and Grade 2) **ii)** Intermediate risk: endometrioid EC, stage IA grade3 with Stage IB (grade1 and 2) **iii)** High risk: Stage IB grade 3 with endometrioid type and all stages with a non-endometrioid type (Pecorelli, 2009, Colombo et al., 2013).

Table 1.3 FIGO staging system 2009 (Pecorelli, 2009).

Stage	Description	
I Tumour confined to corpus uteri	**a**	No tumour invasion or tumour invades into less than inner half of the myometrium.
	b	Tumour invades into equal than or more than half of myometrium.
II		Tumour invades the cervical stroma but does not go outside uterus; endocervical gland involvement should be considered stage I.
III Local and/or regional spread of the tumour	**a**	Tumour invasion of the serosa of corpus uteri and/or adenxia.
	b	Tumour invades vagina and/or parametrium.
	c	**1** Pelvic lymph nodes involvement.
		2 Paraaortic lymph nodes.
IV	**a**	Tumour invades bladder and/or bowel mucosa
	b	Distant metastasis, including intra-abdominal and/or inguinal lymph nodes.

1.4.3 EC management

1.4.3.1 Surgical management

The treatment of choice for patients with EC is surgery. Total laparoscopic/abdominal hysterectomy with bilateral salpingo-oophorectomy without colpectomy is the standard approach to treat patients with early stages ECs with lymph node assessment. For women with unresectable tumours or with marked medical comorbidities, nonsurgical treatment might be presented, such as primary radiotherapy with or without chemotherapy or chemotherapy alone. Hormonal therapy might also be offered as an option in a selected group of premenopausal women (grade 1, stage IA non-invasive tumour) wanting to preserve

fertility; nevertheless, this is not considered typical of care, and these women should be carefully advised before pursuing conservative treatment (Colombo et al., 2013). The use of minimally invasive techniques has been widely accepted by many authors in the last two decades, where hysterectomy and bilateral salpingo-oophorectomy can also be performed using the laparoscopic approach (Colombo et al., 2013, Rabinovich, 2015). In stage III and IV ECs, complete macroscopic cytoreduction and comprehensive staging are recommended (Colombo et al., 2013).

1.4.3.2 Adjuvant treatment

The main aim of adjuvant therapy in newly diagnosed EC is to decrease the risk of relapse of the tumour. The recommendation for adjuvant therapy is guided by surgical stage, tumour histology, and adverse risk factors which include old age, presence of lymphovascular invasion, tumour grade, tumour size, surface cervical glandular involvement. Thus, in general, it is highly recommended to increase adjuvant therapy use as tumour grade, myometrial invasion, and cervical involvement worsen because the risk of recurrence raises in these situations. Type II ECs are never considered as a low or intermediate risk because of their high-risk histologies such as clear cell, serous, or carcinosarcoma, and are considered in the high-risk group regardless of the stage (Passarello et al., 2019).

1.4.3.2.1 Radiotherapy

The most common adjuvant treatment considered for EC is RT. It can be delivered by pelvic external beam radiotherapy (EBRT) or vaginal intracavity brachytherapy (VBT). Radiotherapy is also used as a definitive treatment for women who are medically unfit for surgery, for local recurrence or palliative treatment.

Three previous prospective randomised trial in 2000, 2004 and 2009 compared pelvic radiotherapy versus observation in early-stage EC patients in intermediate and high risk of recurrence ECs revealed that although postoperative radiotherapy reduced locoregional recurrence, it had no impact on overall survival. Additionally, postoperative radiotherapy increased treatment-related morbidity. Therefore, radiotherapy is not indicated in women below 60 with stage I EC and in patients with grade-2 tumours with superficial invasion (Keys et al., 2004, 2009, Creutzberg et al., 2000). A randomised clinical trial (PORTEC-2) in 2010

compared vaginal brachytherapy versus external beam radiation in intermediate-risk patients found that the two radiation therapies were similarly effective. However, women treated with vaginal brachytherapy had a better quality of life (Nout et al., 2010).

1.4.3.2.2 Chemotherapy

EC is considered as a chemotherapy-insensitive tumour and usually respond better to radiotherapy. However, postoperative Platinum-based chemotherapy is considered as a treatment modality for high-risk early-stage EC, advanced-stage tumour or recurrent disease (Moxley and McMeekin, 2010). There was no difference between adjuvant chemotherapy and external pelvic radiation in terms of progression-free survival or overall survival in a randomised controlled trial. Chemotherapy appeared to be superior to pelvic radiotherapy in older patients (aged >70 years) with >50% myometrial invasion, patients with grade 3 ECs, those with stage II or those with stage I disease and those who have positive peritoneal cytology (Maggi et al., 2006).

1.4.3.2.3 Combined radiotherapy–chemotherapy

Two randomised trials revealed that the use of a sequential combination of chemotherapy and radiotherapy reduced the risk of relapse and improved the progression-free survival in operated EC patients with no residual tumour and a high-risk profile in comparison with radiation therapy alone (Hogberg et al., 2010).

1.4.4 Prognosis

Generally, EC is associated with good prognosis. Diagnosis at an early stage is considered as a key factor attributed to this favourable outcome in most of EC cases. At the time of the diagnosis, stage, grade, depth of invasive disease, LVSI and histological subtype are the most important prognostic parameters. The 5-year survival is 83% in endometrioid ECs versus 62% in clear-cell and 53% in papillary carcinomas. A quarter of the cases have LVSI, and the five-year overall survival was 64% for those with LVSI, compared with 88% in those without LVSI.

Based on the 2009 FIGO staging system, survival for stage IA and IB was 89.6% and 77.6%. However, survival for stage IIIC1 was 57% compared with 49% for stage IIIC2 (Colombo et al., 2013, Lewin and Wright, 2011).

Although EC has a good prognosis, approximately 13% of all cancers recur (Fung-Kee-Fung et al., 2006), and the outcome of recurrent disease is poor; the median survival hardly exceeds 12 months. The absolute and proportional number of patients with recurrent EC have risen (Huijgens and Mertens, 2013, Odagiri et al., 2011). Therefore, it is essential to find markers to predict ECs that have a poor prognosis. Telomerase, a unique reverse transcriptase enzyme (Greider, 2010), is essential for cell proliferation. Telomerase is high in most human cancers, including EC. This is thought to be because TL maintenance by telomerase is used by a vast majority of cancer cells to facilitate unlimited proliferation (Buseman et al., 2012). Conversely, telomerase is low in most normal somatic cells. Thus, it is considered as a universal therapeutic target for the majority of human cancers. Despite this there is only one telomerase inhibitor available at the present time that is clinically applicable. Telomerase holoenzyme components hTERC and hTERT were studied previously in the human endometrium and in EC (Hapangama et al., 2017, Alnafakh et al., 2019). However, the third core-subunit, dyskerin has not been examined in human endometrium; thus, the work carried out for the purpose of this PhD thesis, examined the role of dyskerin in human endometrium and in EC to fill the gaps in the current knowledge.

1.5 Telomeres and Telomerase

Telomeres are specialised structures that are found at the ends of linear chromosomes, containing a tandemly repeated specific DNA sequence and associated protective proteins (Palm and de Lange, 2008). Telomeres have a protective function in preventing the loss of genomic DNA in proliferating cells (van Steensel et al., 1998, Karlseder et al., 1999, Sandell, Zakian, 1993). As telomeres shorten with each cell division, critically short telomeres initiate cellular senescence or an apoptotic pathway, leading to the cessation of cell division; therefore telomere shortening is a major tumour suppressor mechanism (Deng et al., 2008, Ohtani et al., 2009). In addition, oxidative stress is an important additional cause for telomere shortening (von Zglinicki et al., 1995, von Zglinicki, 2000). Telomerase is able to add repetitive telomeric sequences *de novo* onto telomeric ends (Blackburn et al., 1989) that are continually lost during

DNA replication due to oxidative stress and the “end replication problem” in mitotic cells. Thus, telomerase prevents shortening and maintains telomeres.

1.5.1 Telomeres

1.5.1.1 Structure

Human telomeres consist of a repetitive ‘TTAGGG’ hexanucleotide sequence bound by six-proteins forming the shelterin complex (de Lange, 2005) (Figure 1.3). In normal somatic cells, the average length of telomeres is around 5-15 kilobases, and they shorten *in vitro* by 30-200 base pairs (bp) during every cell division depending on the cell type and environmental conditions (Harley et al., 1990). Under increased oxidative stress, the telomere shortening rate per cell division can increase substantially, up-to 500 bp (von Zglinicki et al., 1995).

Most of the non-coding telomeric DNA is double-stranded whilst the terminal nucleotides (nt) form the single-stranded 3’ G-rich overhang, which serves as the primer for telomerase action (Cimino-Reale et al., 2001) and also protects telomeres from being recognised as DNA damage. This forms a D-loop (Displacement loop), facilitating repetitive DNA sequences to be added by telomerase (Greider, 1991). Another mechanism to protect telomeres from being recognised as DNA damage is the formation of a t-*loop*, which is a specific higher-order conformation. This large duplex loop-back structure is formed via invasion of the single-stranded telomeric 3′ overhang into the double-stranded telomeric repeat array (Griffith et al., 1999). The authors suggested that the t-loops are the basic mechanism by which the telomeric nucleoprotein complex sequesters chromosome ends from the DNA damage pathway, preventing inappropriate DNA repair and telomerase action (Griffith et al., 1999).

The shelterin complex (Figure 1.3) includes telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), which are homodimeric proteins that bind specifically to double-strand telomeric DNA (Fairall et al., 2001, Griffith et al., 1999). In contrast, Protection of telomeres 1(POT1) binds to the single-stranded region of the telomere (Lei et al., 2004) and forms a heterodimer with TPP1 (O'Connor et al., 2006). The Repressor/activator protein 1 (RAP1) is recruited through its relation with TRF2 (Palm and de Lange, 2008) and TRF1-interacting protein 2 (TIN2) is the central part of the shelterin complex (Nelson et al., 2018), and it interacts with TRF1, TRF2 (Ye et al., 2004), and POT1/TPP1 (Takai et al., 2011) to assure structural integrity of the complex.

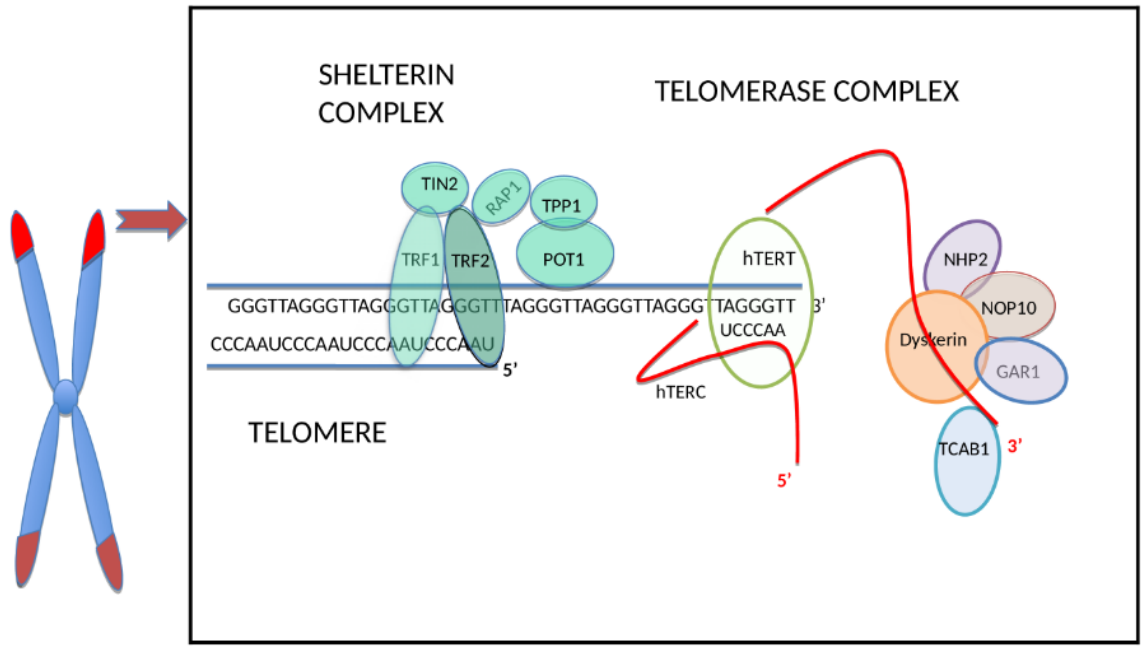

Figure 1.3 Schematic illustration of the telomere and main telomerase complex components, the human telomere and telomerase enzyme complex (only one half of the dimeric holoenzyme complex is shown for clarity), adapted from Hapangama *et al.*(Hapangama et al., 2017). From all sheltrin proteins only telomere repeat binding factors 1 (TRF1) and 2 (TRF2) (Fairall et al., 2001) bind directly to the double-stranded telomeric sequence, and protection of telomeres protein-1 (POT1) (Lei et al., 2004) binds to the single-stranded overhang; hence these are termed as telomere binding proteins, and they interact with remaining shelterin proteins TIN2 (binds to TRF1 and TRF2) (Nelson et al., 2018), (Ye et al., 2004), RAP1 (binds to TRF2) (Palm and de Lange, 2008) and TPP1 (binds to POT1) (Takai et al., 2011). The TERC H/ACA region located at the 3′ end binds to dyskerin and the other telomerase associated proteins: NOP10, NHP2, and GAR1 (Hapangama et al., 2017). The hTERC at the 3′ end binds also to telomerase Cajal body protein 1 (TCAB1) (Vogan et al., 2016).

Removal of individual shelterin proteins have been shown to stimulate a DNA damage response (DDR) pathway: TRF1 prevents the stimulation of both ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) pathways (Martinez et al., 2009); TRF2 and RAP1 inhibit the activation of the ATM pathway (Karlseder et al., 2004), (Li et al., 2015) and homology-directed recombination (HDR) (Rai et al., 2016) while TPP1 bound POT1

(POT1a/b in mouse) inhibit the ATR pathway (Kibe et al., 2016). TRF2 plays a vital role in facilitating this t-loop formation (Stansel et al., 2001). Super-resolution fluorescence light microscopy visualisation of the t-loop has shown that the strand invasion point can be located at almost any point along the duplex DNA, resulting in highly variable t-loops sizes (Doksani et al., 2013).

1.5.1.2 Functions of telomeres

The main function of telomeres is to protect chromosomal ends from degradation and end-to end-fusion (van Steensel et al., 1998) as well as to prevent the ends of chromosomes being recognised as DNA damage by the DNA damage response machinery of the cell (Griffith et al., 1999). However, when telomeres are critically short, they activate the apoptosis/senescence pathways, thereby preventing genetic material being lost by inhibiting inappropriate continuous DNA replication in the context of short telomeres. The telomere structure described above, prevents inappropriate DNA repair at these sites, for example, the loop conformation (D-loop) masks the single-stranded terminal DNA and enables its protection from the DNA damage response pathway (Griffith et al., 1999).

The shelterin complex supports the chromosome protective function of telomeres and stabilisation of telomere length (TL), and the complex interaction of shelterin proteins at the chromosomal ends have a key role in telomere maintenance via a negative feedback loop, which also has an inhibitory effect on the telomerase enzyme (Colgin et al., 2003).

In cells that have the replicative capability, telomere shortening can lead to chromosomal instability by promoting end-to-end fusions leading to multiple chromosomal aberrations, such as breakages, fusions, and translocations rendering the genome aneuploid and, therefore, promoting carcinogenesis. To maintain TL, the homeostasis mechanism that involves telomerase uses both TRF1 and TRF2 as negative regulators that stabilise and limit TL elongation (Smogorzewska et al., 2000, van Steensel and de Lange, 1997). Overexpression of both TRF1 and TRF2 was reported to cause telomere shortening (Smogorzewska et al., 2000), and this could be due to the binding of TRF1 and TRF2 along the length of the double-stranded telomeric repeat array which measures TL as demonstrated in yeast (Smogorzewska et al., 2000, van Steensel and de Lange, 1997). POT1 can either facilitate or inhibit telomerase accessing telomeres depending on its position relative to the DNA 3′-end (Bunch et al., 2005). Examining the high-resolution crystal structure of the human POT1-TTAGGGTTAG complex suggested that it would not be elongated by telomerase. When POT1 is bound at one telomeric

repeat before the 3′-end, leaving an 8-nucleotide 3′-tail, the resulting complex is elongated with increased activity and processivity (Lei et al., 2005). Replication protein A (RPA) is another ssDNA binding protein which has an important role in telomere replication by facilitating telomerase enzyme at the telomeres (Luciano et al., 2012, Rubtsova et al., 2009). It also recruits the ATR-ATRIP protein kinase complex to DNA damage sites and initiates the checkpoint signalling (Zou and Elledge, 2003, Haring et al., 2008). Collectively, the available evidence demonstrates that shelterin and other telomere-binding proteins are involved in the regulation of TL.

Gene regulation is another reported function of telomeres but with limited evidence available for it. Telomeric attrition extensively alters expression of some genes, and the difference in expression of genes proximal to telomeres may result from chromatin modifications, a conserved phenomenon termed as *telomere position effect* (TPE). TPE is a silencing mechanism spreading from the telomeres toward subtelomeric regions (Ottaviani et al., 2008). In humans, only a limited number of endogenous genes (e.g. ISG15) has been mentioned to be affected by TPE (Lou et al., 2009, Boussouar et al., 2013); however, microarray data suggest that the expression of many other genes close to telomeres may also be altered with the aid of a TL-dependent and DNA damage-independent mechanism, and this is known as *telomere position effect–over long distance* (TPE-OLD) (Robin et al., 2014). For example, the looping of chromosomes brought long telomeres closer to some genes which are over 10 Mb away from the telomere, but these same loci were completely separated from the telomeres when the telomeres were short (Robin et al., 2014). Further, microarray data support the notion that TL-dependent chromosome conformation can affect the transcription of non-subtelomeric genes (Robin et al., 2014). At the genome-wide level, the effect of this mechanism on gene expression has been proposed to occur earlier than replicative senescence, and that could potentially explain the increased incidence of age-related pathologies that are associated with old age without necessarily imposing a DNA damage signal from a critically-short telomere (Campisi, 1997, Robin et al., 2015).

TL is the main determinant of a cell's replicative life span. Dysfunctional telomeres that result from either progressive telomere shortening, internal DNA damage (Hewitt et al., 2012) or shelterin complex loss, provoke a strong DNA damage response and genomic instability (Martinez and Blasco, 2010). A plethora of experimental data has shown that tumorigenesis can be caused by genome instability resulting from telomere shortening (Maser and DePinho, 2002), (Deng et al., 2008). Nevertheless, in late generation telomerase knock-out mouse

models, telomere attrition was also a tumour suppressor mechanism through the induction of replicative senescence or apoptosis that repress tumourigenesis. Telomere shortening and telomere uncapping in metazoans stimulate ATM/ATR kinases to phosphorylate downstream kinases CHK1 and CHK2, which initiate p53-dependent replicative senescence and apoptosis pathways which inhibit tumour formation (Deng et al., 2008).

1.5.1.3 Regulation of TL and telomere maintenance mechanisms (TMM)

The most widely known classical telomere maintenance mechanism is dependent on telomerase reverse transcriptase activity, which will be detailed in section 1.5.2. However, there are two other TMM: (1) Telomerase-independent telomere maintaining pathway that has been described in cells that do not have measurable TA, termed alternative lengthening of telomeres (ALT) pathway (Bryan et al., 1997); (2) Telomeric repeat-containing RNAs (TERRAs) also have a role in TL regulation by mainly managing telomeric access of telomerase (Figure 1.4).

Cells can maintain their telomeres via a telomerase dependent or independent pathway (Bryan et al., 1997). New telomeric DNA is synthesised from a DNA template in ALT (Neumann et al., 2013) by homologous recombination (HR) (Dunham et al., 2000). The template could either be the telomere of another chromosome, another region of the same telomere by t-loop formation or sister telomere recombination. The first evidence for the presence of an ALT mechanism was described in several immortalised human cell lines that did not have TA but maintained TLs for hundreds of population doublings, and this mechanism occurs in ~15% of cancers including osteosarcomas, soft tissue sarcoma subtypes, and some glial brain tumours (Dilley and Greenberg, 2015, Min et al., 2017). In human cells, where ALT activity is elevated to a degree sufficient for TL maintenance, telomeres are characterised by their highly heterogeneous length, but the average length (>17 kb) is about double that of most cells where telomeres are elongated by telomerase (Bryan et al., 1995).

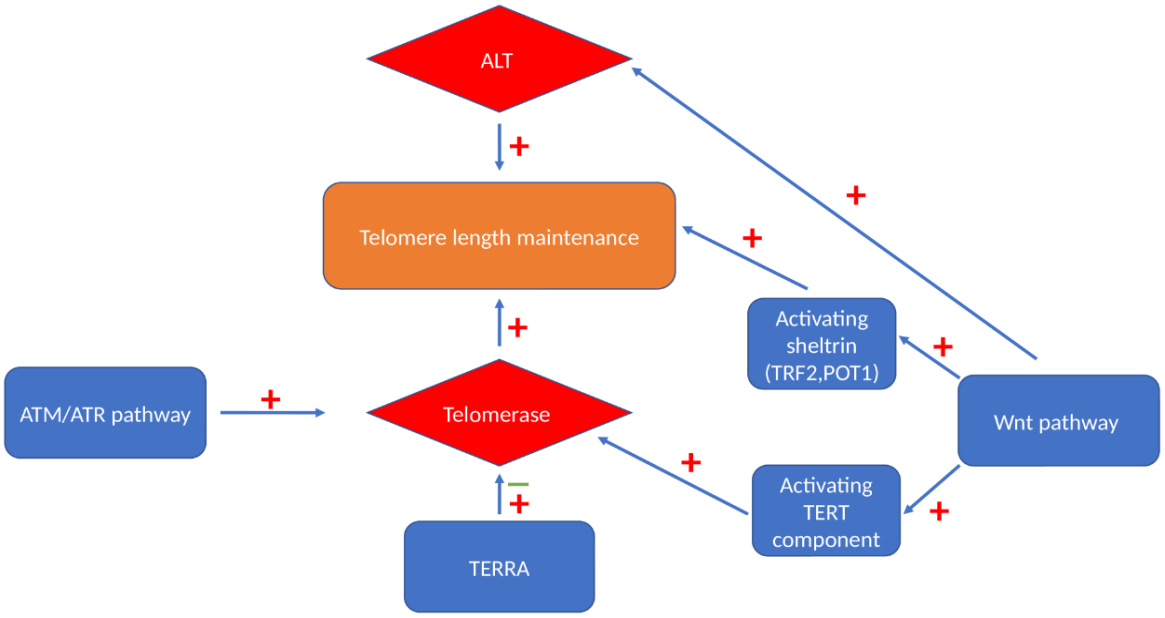

Figure 1.4 Telomere maintenance mechanisms. Cells can maintain their telomeres *via* either telomerase-dependent pathway or a telomerase-independent ALT pathway. Activated Wnt signalling pathway can maintain telomere length by activating both these maintenance mechanisms and by maintaining the level of TRF2 and POT1 sheltrin components that are essential for telomere protection (Diala et al., 2013). ATM and ATR also have a stimulatory effect on telomerase enzyme via triggering its recruitment and enhancing the assembly of this enzyme (Tong et al., 2015). TERRA binds independently to hTERC and hTERT telomerase subunits with an inhibitory effect on human telomerase enzyme (Redon et al., 2010), or it acts as a recruiter of telomerase enzyme rather than an inhibitor (Cusanelli et al., 2013).

Telomeres were initially thought to be transcriptionally silent, but they were found to be transcribed into telomeric repeat-containing, long non-coding RNAs, termed TERRAs (Azzalin et al., 2007). TERRAs have a role in telomere regulation and also regulate telomeric access of telomerase. A previous study stated that TERRA was found to bind to hTERC and hTERT components of telomerase independently, to function as an inhibitor of human telomerase enzyme (Redon et al., 2010). Another study has suggested that TERRA was induced in cells with short telomeres and acted as a scaffold for the spatial organisation of the telomerase components forming a TERRA-telomerase complex which helped in the

recruitment of telomerase to the telomere of its origin hence TERRA was proposed to be a recruiter of telomerase enzyme rather than an inhibitor (Cusanelli et al., 2013).

1.5.2 Telomerase

1.5.2.1 Structure of Telomerase

Telomerase, the only RNA dependent DNA polymerase in mammals, was first discovered in protozoans in 1985 (Greider and Blackburn, 1985), and subsequent studies demonstrated mammalian/human species in 1989 (Morin, 1989). The telomerase holoenzyme contains three core components: the RNA component harbouring the template region for telomere synthesis (hTR or hTERC), a catalytic protein with reverse transcriptase activity, hTERT (Cong et al., 2002) as well as dyskerin (Figure 1.3) which has been detailed in section 1.5.3. However, only the RNA component (TERC) and the catalytic subunit (TERT) are necessary and sufficient for *in vitro* TA (Weinrich et al., 1997).

1.5.2.1.1 Telomerase RNA component (hTERC or hTR)

The human telomerase RNA (TERC or hTR) consists of 451nt and is an essential constituent of the telomerase catalytic core complex. Although the length is variable among eukaryotes, the structure of TERC remains conserved. For example, the length ranges from ~150nt in ciliates, 400–600nt in vertebrates to ~1300nt in yeast (Chen and Greider, 2004). Additionally, in ciliates, polymerase III transcribes the telomerase RNA (Yu et al., 1990), whereas it is RNA polymerase II in yeast and vertebrates (Rubtsova et al., 2016).

Vertebrate TERC's secondary structure has four conserved elements: a pseudoknot domain (CR2/CR3), a CR4/CR5 (conserved region 4 and conserved region 5) domain, box H/ACA (CR6 /CR8) domain and a CR7 domain (Chen and Greider, 2004, Chen et al., 2000). The proximal template/pseudoknot domain and the distal CR4/5 domain represent the essential regions of TERC for TA (Tesmer et al., 1999).

As mentioned before, an active telomerase enzyme can be generated by combining the two RNA domains from the TERC subunit with the TERT protein on oligodeoxynucleotide substrates *in vitro* (Weinrich et al., 1997, Egan and Collins, 2012, Cristofari and Lingner, 2006, Mitchell and Collins, 2000). The human/vertebrate TERC has a third, conserved component, the H/ACA domain located at the 3′ end that has homologies to small nucleolar and small Cajal body (CB) ribonucleoproteins (snoRNPs and scaRNPs). The TERC H/ACA region binds to

telomerase associated proteins, such as dyskerin, NOP10, NHP2, and GAR1 (Hapangama et al., 2017), and this region is essential for telomerase biogenesis, and are important for RNA stability. Additionally, in the 3′ stem-loop of the H/ACA, there is another domain, the CB localisation box (CAB), for binding the telomerase CB protein 1 (TCAB1) (Vogan et al., 2016). Mutations in the H/ACA region decrease TERC accumulation, whereas mutations in the CAB cause TERC to accumulate in nucleoli instead of CBs (Podlevsky and Chen, 2012), (Venteicher et al., 2009). Although this mutant TERC has the capacity of forming catalytically active telomerase *in vivo*, it is highly impaired in telomere elongation because of the decreased association of telomerase with telomeres (Cristofari et al., 2007). This result emphasises that sub-nuclear localisation of telomerase as an important regulatory mechanism for the homeostasis of TL in human cells (Cristofari et al., 2007). TERC, therefore, not only provides the template, which identifies the telomere repeat sequence, but it also comprises motifs, which are crucial to reconstitute TA (Webb and Zakian, 2016). Furthermore, it plays a role in stability, maturation, accumulation, and functional assembly of the telomerase holo-enzyme.

1.5.2.1.2 Telomerase reverse transcriptase (hTERT)

TERT is the catalytic component of the telomerase enzyme, and as described above, together with TERC, it is essential for TAand thus for the maintenance of TL, chromosomal stability, and cellular immortality. The human TERT gene (hTERT) is located at chromosome 5p15 and encompasses more than 37 kb and contains 16 exons (Wick et al., 1999). The TERT protein consists of four conserved structural domains, the telomerase essential N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the central catalytic reverse transcription (RT) domain, and the C-terminal extension (CTE). Mutations in the RT conserved residues prevent telomerase enzymatic activity *in vitro* (Lingner et al., 1997). These mutated TERT proteins fail to maintain TLs *in vivo* (Alder et al., 2011), and many of these mutations have been identified in individuals with telomere-mediated disorders or telomeropathies (Opresko and Shay, 2017). As already stated above, TA can be reconstituted by hTERC and hTERT co-expression in yeast and mammalian extracts (Bachand and Autexier, 2001, Weinrich et al., 1997). TA is established in *Saccharomyces cerevisiae* via reconstitution of telomerase by hTERC and hTERT co-expression (Bachand and Autexier, 2001). Therefore, hTERC and hTERT are the minimal requirements for TA (Cong et al., 2002). However, biochemical TA, as measured by the telomere repeat amplification protocol (TRAP) assay, does not always mean that the enzyme has necessarily telomere elongation capacity *in vivo*. This was demonstrated when the hTERT protein was modified by attaching a hemagglutinin

(HA) epitope tag to the C terminus: while the catalytic activity of telomerase enzyme remained unaffected telomere maintenance function was lost *in vivo* due to loss of access to the telomere (Counter et al., 1998). Telomerase associated proteins are also essential for the full biological function of the enzyme but hTERT is the primary determinant of enzyme activity in most cells (Leao et al., 2018, Cristofari and Lingner, 2006).

1.5.2.2 Functions of telomerase

Telomerase is a specialised reverse transcriptase, which maintains and elongates telomeres at the 3'-single strand in the absence of a DNA template while using the inherent RNA (TERC) for the template function and is thus an RNA dependent DNA polymerase. In the subsequent S-phase of the cell cycle, the conventional DNA replication machinery can then replicate the complementary C-rich strand. Thus, telomerase ascertains chromosomal stability and cellular proliferation in proliferative somatic cells, tissue progenitor cells and in cancer cells (Yuan et al., 2014). When telomeres shorten beyond a critical threshold length, normal healthy cells in humans that are devoid of TA will assimilate a cellular senescence phenotype with an irreversible growth arrest and the classical morphological alterations (Kuilman et al., 2010). Somatic human cells lacking measurable telomerase yet expressing certain viral oncoproteins can overcome the senescence checkpoint and continue to proliferate, but they then accumulate chromosomal instability including aneuploidy, polyploidy and chromosomal fusions. On these grounds, high TA has been assigned a role in maintaining genome stability by preventing telomere shortening. Telomerase fulfils this important role via interaction with many key cellular pathways as detailed below.

ATM/ATR pathway: this pathway is related to (ATR) DNA damage response kinases which have essential roles in telomerase-mediated telomere maintenance (Tong et al., 2015). The conserved ATM and ATR family of serine-threonine kinase proteins mediates DNA damage and replication stress checkpoint responses (Harper and Elledge, 2007, Jeggo et al., 2016), therefore, play a crucial role in DNA repair, cell apoptosis, and cell senescence, and are closely associated with the development and progression of cancer in humans (Smith et al., 2010), (Stracker et al., 2013). ATM is required for the addition of new repeats onto telomeres by telomerase (Lee et al., 2015), and evaluation of bulk telomeres in both immortalised human and mouse cells showed that ATM inhibition suppressed elongation of telomeres while ATM stimulation through PARP1 led to an increase in TL (Lee et al., 2015).

Stalled replication forks increased telomerase localisation to telomeres in an ATR-dependent manner (Tong et al., 2015). Additionally, increased telomerase recruitment was observed upon phosphorylation of the shelterin component TRF1 at an ATM/ATR target site (S367) (Tong et al., 2015), and this led to TRF1 loss from telomeres and may, therefore, increase replication fork stalling (Sfeir et al., 2009). ATM and ATR depletion reduced assembly of the telomerase complex, and ATM was required for telomere elongation in cells expressing POT1ΔOB, an allele of POT1 that causes disruption in TL homeostasis (Tong et al., 2015). Hence from this data, it can be concluded that ATM and ATR are involved in triggering telomerase recruitment and facilitating its assembly (Tong et al., 2015).

WNT pathway: Wnt family proteins are essential for regulating cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (Zhang et al., 2012). A dysregulated Wnt/β-catenin signalling pathway is also associated with human tumourigenesis (Zhang et al., 2012). Due to the intricate relationship of telomeres and telomerase with similar cellular functions, their close interaction is not a surprise. An activated Wnt signalling pathway can reinforce the stability of telomeres by coupling and enhancing the two main telomere maintenance pathways: telomerase-dependent and ALT pathways. A Wnt-mediated telomere protective effect is particularly expected to have an important role during development, in adult stem cell function and oncogenesis (Diala et al., 2013).

The Wnt pathway may regulate telomere maintenance via its effect on several essential shelterin components, including TRF2 and POT1. Recently, in human somatic and cancer cells as well as in mouse intestinal tissue, activation of canonical Wnt/β-catenin pathway activated TRF2 and also increased telomere protection was demonstrated (Diala et al., 2013). In mice lacking telomerase, apoptosis of the Wnt-dependent intestinal crypt stem cell niche could be rescued by administration of Wnt agonists (Yang et al., 2017a). Additional evidence demonstrates that the Wnt pathway triggers APC- and β-catenin induced regulation of TRF2 and TCF4 which further regulate TRF1 and POT1 (Yang et al., 2017b, Sansom et al., 2004).

Further to the enhancement of shelterin protection, the Wnt/β-catenin signalling pathway also activates TERT (Hoffmeyer et al., 2012). Importantly, the use of Wnt pathway agonists can rescue telomere uncapping, suppress apoptosis and lead to elevated *Ascl2* transcripts as well

as Sox9 protein levels (Yang et al., 2017b) suggesting a therapeutic strategy for some conditions with aberrations in telomerase.

Non-canonical functions of TERT have been discovered later than TA, and they also play a role in tumorigenesis; for example, via TERT's role in regulating the Wnt signalling as a cofactor for the β-catenin pathway (Park et al., 2009). TERT has been shown to be inducible in ischaemic brain cells and to prevent apoptosis via a non-telomeric action via a shift of the cytosolic free Ca2+ into the mitochondria (Kang et al., 2004). Despite having normal TLs, lack of hTERT impairs the cellular capability to repair damaged DNA and fragmented chromatin (Masutomi et al., 2005). TERT also is demonstrated to have RNA dependent RNA polymerase function by interacting with the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*) and forming ribonucleoprotein complexes. These complexes produce double-stranded (ds) RNAs that serve as substrates for the generation of siRNA, which may regulate the expression of other genes related to stem cell biology (Maida et al., 2009). Further to the above, there are many other additional non-telomeric functions of TERT active in cancer, such as improved DNA repair, increased apoptosis resistance, changes in chromatin structure and altered gene expression (Saretzki, 2014).

Because hTERC and hTERT telomerase complex components have been studied previously in endometrium and dyskerin, the third telomerase complex subunit has never been studied in the endometrium; therefore, this thesis focuses mainly on dyskerin.

1.5.3 Dyskerin

Dyskerin, a nucleolar, 514-amino-acid long protein, is encoded by the *DKC1* gene at Xq28 (Marrone and Mason, 2003). Dyskerin is a part of a snoRNA ribonucleoprotein complex; it catalyses the formation of pseudouridine in ribosomal and certain small RNAs (Filipowicz and Pogacić, 2002). Dyskerin is also considered as part of the core telomerase complex (Cohen et al., 2007). It is necessary for the biogenesis of *hTERC*, which is transcribed from the *TERC* gene. The *hTERC* RNA contains a specific recognition domain for dyskerin.

1.5.3.1 Structure and function of dyskerin

1.5.3.1.1 Historical background

Dyskerin (*DKC1*) has gained a great biological significance since 1998 by the fact that its deficiency in human causes the X-linked dyskeratosis congenita (X-DC) disease. Hybridisation screening with 28 candidate cDNAs from unrelated patients revealed that some of them shared mutations in its coding region (Heiss et al., 1998, Angrisani et al., 2014). The classical triad of X-DC disease includes mucocutaneous features (skin pigmentation, nail dystrophy and mucosal leukoplakia) together with other symptoms such as bone marrow failure, stem cell abnormalities, premature ageing and mental retardation. DC patients are also susceptible to develop cancers, particularly skin cancers and leukaemias. After that, mutations in *DKC1* gene were proven to cause Hoyeraal-Hreidarsson syndrome (HHS) (Knight et al., 1999, Yaghmai et al., 2000). Patients with HHS suffer from cerebellar hypoplasia, immunodeficiency, progressive bone marrow failure, and intrauterine growth retardation. Recently, authors consider HHS as a severe form of X-linked DC disease (Glousker et al., 2015, Angrisani et al., 2014).

Initially, in yeast, *Cbf5* gene that is homologous to human *DKC1* gene was found to encode a centromere and microtubule-binding protein (Jiang et al., 1993), and it was the first member of this gene family to be recognised. Later on, in rats, a mammalian orthologue (*Nap57*) was discovered and hypothesised to be related to the nucleo-cytoplasmic transporting of pre-ribosomal structures (Meier and Blobel, 1994, Angrisani et al., 2014). After that, inactivation of yeast *Cbf5* was shown to be lethal due to defect in the processing of rRNAs precursors (Zebarjadian et al., 1999, Cadwell et al., 1997, Angrisani et al., 2014). Likewise, complete loss of dyskerin in mice is also lethal (Giordano et al., 1999). In Drosophila orthologue, *Nop60B/minifly* was identified (Phillips et al., 1998) and shown to be essential for proper maturation and pseudouridylation of rRNAs precursors (Giordano et al., 1999, Angrisani et al., 2014).

1.5.3.1.2 Function of dyskerin

Dyskerin has been proposed to be the third core component of the telomerase holoenzyme. It binds to TERC and participates in stabilising telomerase enzymatic complex (Rocchi et al.,

2014). Additionally, dyskerin acts as pseudouridine synthase. It is responsible for the conversion of uridine to pseudouridine in noncoding RNAs, a vital step in rRNA and ultimately ribosomal synthesis (Montanaro, 2010).

He and colleagues used the inducible Cre/loxP technology to produce deletions in the murine *DKC1* gene in early embryogenesis. They showed that *DKC1* gene-targeted disruption is lethal to mouse embryos. However, mice lacking telomerase (mTERC−/− and mTERT−/− mice) are viable and do not show a phenotype in early generations (He et al., 2002). The embryonic lethality of mice showed that dyskerin, unlike mTERC (Blasco et al., 1997) or mTERT (Liu et al., 2000) is necessary for mouse development. X-DC patients suffer from severe symptoms with a higher cancer susceptibility compared with autosomal dominant DC resulted from hTERC mutations. Whether this occurs because of defects in ribosome function or more severe reduction in TA is yet to be known (He et al., 2002).

Growing evidence suggested that the snoRNA/microRNA pathways can be interlaced, and that dyskerin-dependent RNA pseudouridylation is a flexible mechanism that can cause RNA function modulation variously such as via modulating splicing, changing mRNA coding properties or selectively regulating internal ribosomal entry sites (IRES) dependent translation(Angrisani et al., 2014). Angrisani *et al*. in their review in 2014 had proposed a speculative model that suggested that the dynamics of pre-assembly and nuclear import of H/ACA RNPs are essential regulatory steps that can be finely controlled in the cytoplasm in response to different stimuli such as developmental, differentiative and stress (Angrisani et al., 2014).

1.5.3.1.3 Dyskerin and cancer

Cancer susceptibility is increased by germline mutations of *DKC1*. Whereas overexpression of wild type dyskerin, not associated with mutation, is frequently observed in sporadic cancers. The exact role of dyskerin is not yet known. Alawi and Lin, in their study in 2011, found that acute loss of dyskerin by RNA interference resulted in H/ACA RNAs marked and specific reduction, *TERC* levels were also reduced in telomerase positive cells.

Depletion of dyskerin only transiently delayed maturation of rRNA but with no considerable effect on the levels of total 18S or 28S rRNA in human osteosarcoma cells (U2OS) cells (Alawi and Lin, 2011). Although rRNA processing defects typically trigger p53-dependent G1 arrest,

in contrast, dyskerin-depleted cells accumulated in G2/M by a p53-independent mechanism, and with the association of accumulated aberrant mitotic figures that were characterised by multipolar spindles (Alawi and Lin, 2011). In carcinogenesis, TA and rRNA processing rates are typically elevated, yet cumulative results of Alawi and Lin suggested that the role of dyskerin in carcinogenesis was not dependent on TA. They proposed that dyskerin may influence carcinogenesis through its role in rRNA processing, suggesting that both high or low dyskerin may play an important role in carcinogenesis (Alawi and Lin, 2011). Dyskerin expression levels are upregulated in several human cancer types, such as in breast cancers (Montanaro et al., 2006). In line with the known biological functions of the protein, breast cancers with low dyskerin levels have lower levels of pseudouridine and telomerase RNA than those with high dyskerin. Cancers with a high level of dyskerin usually showed worse histopathological features and outcome. Similar to these findings, a microarray study reported a significant increase in *DKC1* expression in a group of prostate cancers with a combination of molecular changes, such as chromosome 8 alterations and LINE-1 hypomethylation, characteristic for advanced cases (Sieron et al., 2009).

Critically short telomeres stimulated p53-dependent pathway causing cell cycle arrest, and that can cause cellular senescence, apoptosis, or rarely genomic instability and transformation. The same pathway is induced in response to DNA damage. *DKC1* mutations in DC disease are believed to act through such a mechanism, causing growth disadvantage in proliferative tissues and telomeric attrition. Gu *et al.* showed that pathogenic mutations in mouse *DKC1* caused growth defects and induced DNA damage response in the context of normal telomere length (Gu et al., 2008). Likewise, in sporadic chronic lymphocytic leukaemia, the expression of *DKC1* and other telomerase-associated factors were reduced (Poncet et al., 2008).

1.5.3.1.4 Structure of dyskerin relevance to its biological function

A homology model of human dyskerin was created by using a template crystal structure from saccharomyces cerevisiae which has a sequence identity of 73% with the human dyskerin. Rashid *et al*. have described the crystal structure of a specific complex of three archaeal H/ACA proteins, Cbf5, Nop10, and Gar1 of the *Pyrococcus furiosus* (Rashid et al., 2006). Cbf5 displays structural properties that are unique among known pseudouridine synthases and are consistent with its distinct function in RNA-guided pseudouridylation. The trimeric complex provides insights into H/ACA RNP assembly and the RNA-guided pseudouridylation

mechanism. The catalytic domain of Cbf5 is fused with a PUA domain, which is likely to be responsible for anchoring the H/ACA-specific RNA. The importance of this mechanism for life is highlighted by the high degree of conservation shown by aligning dyskerins from yeast to mammals. This feature was directly proved by swapping experiments, which showed that Drosophila or rat dyskerin could rescue yeast Cbf5 mutations (Phillips et al., 1998, Yang et al., 2000, Angrisani et al., 2014). At least three well-preserved functional domains were identified: the Dyskerin-like domain (DKLD) with an unknown function; the TruB_N pseudouridine synthase catalytic domain which comprises the active site, this domain is directly associated with pseudouridylation and the pseudouridine synthase and archaeosine transglycosylase (PUA) RNA binding domain which has a role in recognising RNAs of the H/ACA family. Additionally, two nuclear localisation signals (NLSs) identified as lysine-rich sequences) present in most metazoa, localised at the N- and C-termini, which thought to ensure the typical nucleolar localisation, (Figure 1.5) (Angrisani et al., 2014).

The main difference between eukaryal and archaeal dyskerins is in the N- and C-termini, where, the eukaryotic Cbf5 is longer than archaeal Cbf5 and these extensions (N-terminal extension (NTE), C-terminal extension (CTE) and long C-terminal tail) cannot be modelled from the archaeal Cbf5 structure(Marrone et al., 2005, Li et al., 2011d).

The eukaryal proteins harbour typical extensions and the length of the N-terminal extension is markedly higher in metazoans, in which a conserved sequence of approximately 30 aa (2–34 in humans), absent from the yeast protein. Most mutations in *DKC1* gene causing X-DC disease do not present at the catalytic domain, but, accumulated at the PUA RNA binding domain and the N-extension (Angrisani et al., 2014).

The C-terminal extension (390–514 in humans) is also absent in archaea. In eukaryotes, this C-terminal extension is highly variable in length and sequence. Its biological relevance might be attributed to the presence of a bipartite NLS and of several potentially modified residues (Angrisani et al., 2014). Additionally, work in yeast proposed that C-terminal truncated Cbf5p caused a delay at the G2/M phase, indicating that this domain might be associated with cell cycle control (Jiang et al., 1993, Angrisani et al., 2014). Furthermore, Vulliamy *et al.* presented a case of X-DC caused by a 2-kb deletion that removes the last exon of the gene (Vulliamy et al., 1999).

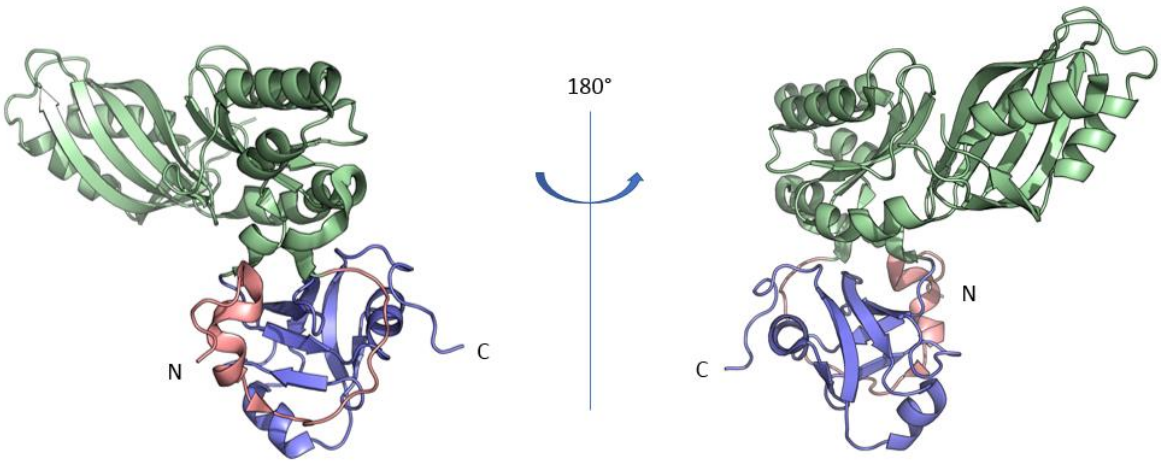

Figure 1.5 3D structure of cbf5 of saccharomyces cerevisiae (in front and back view, related by 180-degree rotation). (Li et al., 2011c, Angrisani et al., 2014), shows the following domains: TruB_N pseudouridine synthase catalytic domain (green), PUA RNA binding domain (purple), Dyskerin-like domain (DKLD) (red) and two nuclear localization signals (NLSs), localized respectively at the N- and C-termini. 3D structure was produced with PyMOL software (DeLano Scientific LLC) using the RCSB PDB database files.

Small deletions lacking only the last exon of the *DKC1* proved to be 100% lethal, and since the first generations of mice with no telomerase are viable, then the observed lethality is doubtful to be attributed to the effects of mutated dyskerin on TA (He et al., 2002). These reports confirm this particular region to be biologically relevant in mammals and in humans. Moreover, alternative truncated dyskerin isoforms that do not have variable tracts of the C-terminal domain have been recognised in Drosophila (Riccardo et al., 2007) as well as in several human tissues and cell lines (Angrisani et al., 2011, Turano et al., 2013). One of these alternative isoforms (isoform 3) includes the catalytic and the PUA domains but lacks the C-terminal NLS and showed a preference for cytoplasmic localisation. Angrisani *et al.* suggested that this variant of dyskerin might favour the transport of pre-ribosomal structures from the nucleus to the cytoplasm and might affect translation by contacting ribosomes or cytoplasmic mRNAs (Angrisani et al., 2011, Angrisani et al., 2014). Some clues about the possible roles of this isoform arise from its overexpression studies, that demonstrate important effects such as growth advantage, cell morphology changes and increase of cell-cell and cell-substratum

adhesion (Angrisani et al., 2011). Recently, another truncated dyskerin (lacking C-terminal) variant has been proposed to be able to boost energy metabolism and enhance respiration and eventually improving cellular ROS adaptive response and growth rate. These findings showed an unexpected involvement of *DKC1* in energy metabolism and confirming its role in the regulation and homeostasis of cellular metabolism (Angrisani et al., 2018).

1.5.4 H/ACA ribonucleoproteins (RNPs)

Dyskerin (pseudouridine synthase) is an integral part of H/ACA snoRNP complex and responsible for the catalytic activity, whereas other components are essential for the structural stabilisation and positioning of the guide and substrate RNAs for catalysis (Henras et al., 1998, Lafontaine et al., 1998, Jin et al., 2007). Therefore, understanding the function of dyskerin requires knowledge of H/ACA ribonucleoproteins. H/ACA snoRNPs and scaRNPs are small RNA–protein complexes that are crucial for the synthesis of larger RNPs such as ribosomes and telomerase (Yu et al., 1998, Dez et al., 2001, King et al., 2003, Kiss et al., 2010, Walbott et al., 2011). The mature H/ACA snoRNPs and scaRNPs are composed of a single or double hairpin guide RNA and four evolutionarily conserved proteins: Cbf5p, Nhp2p, Nop10p, and Gar1p in yeast; and dyskerin, NHP2, NOP10 and GAR1 in human (Meier, 2005, Ye, 2007), and a function-specifying, noncoding H/ACA RNA. The main functions of H/ACA RNPs are telomerase assembly, stabilisation, and posttranscriptional processing of nascent ribosomal RNA and spliceosomal RNA. Therefore, H/ACA RNPs are essential for ribosome biogenesis, pre-mRNA splicing, and maintenance of TL. 100-200 different species of H/ACA RNPs are present in every cell, most of them residing in nucleoli and CBs, each consisting of the same four core proteins and one function-specifying H/ACA RNA (Lin et al., 2015). Most of H/ACA particles have site-specific pseudouridine synthases function in an RNA-guided mechanism; the guide RNA provides base-pairing interactions that specify the target uridine (Li, 2008). Nevertheless, the H/ACA domain in telomerase is not required for pseudouridylation or TA as demonstrated in *in vitro* studies but is necessary for the in vivo accumulation and localisation of the mature telomerase RNP (Lukowiak et al., 2001, Mitchell et al., 1999a, Zhu et al., 2004). An ACA particles structure study further demonstrated a molecular base for the function of the RNP in pseudouridine synthesis (Walbott et al., 2011, Li and Ye, 2006, Duan et al., 2009, Liang et al., 2009).

1.5.5 Hormone regulation of telomerase in hormone-responsive tissues

Multiple studies provide evidence for telomerase to be under the regulation of steroid hormones in hormone-responsive tissues. This corroborates with the known direct regulation of cell fate and proliferation in such tissues by steroid hormones, for example, the ovarian hormone, estradiol, induces a mitotic response in endometrial epithelial cells (Hapangama et al., 2015b, Parkes et al., 2018). In different studies, telomerase is induced by estrogen, for example in macaque and human cell lines (Kyo et al., 1999, Valentijn et al., 2015). Androgens also upregulate telomerase in an ovarian cancer cell line (Nourbakhsh et al., 2010) but progesterone down-regulates telomerase in the endometrium (Valentijn et al., 2015). ATM silencing also down-regulated proteins such as ChK2, p53, and caspase 3, which were stimulated by the synthetic progestogen, medroxyprogesterone acetate (MPA) (Shan et al., 2015). This result suggested that MPA exerts its effects via the ATM-Chk2-p53-caspase-3 pathway protecting against carcinogenesis (Shan et al., 2015). The progestogenic effect on telomerase may also be mediated through this pathway. In LNCaP cells, the use of antiandrogen resulted in a decrease in the level of *hTERT* mRNA but had no effect on the level of dyskerin (Chung et al., 2007).

1.5.6 Role of telomeres and telomerase in premalignant and malignant proliferative disorders

Limitless proliferation is a cardinal feature of cancer cells; whist increased proliferation usually as a result of a stimulus is common to all premalignant changes, including hyperplasia. The excessive proliferation observed in these malignant/ premalignant conditions is maintained by avoiding senescence and crisis/apoptosis. Senescence/apoptosis exist as barriers for mitosis; thus, they are tumour suppressor mechanisms in normal cells, which are regulated intricately by telomeres and checkpoint activation. The unrestricted proliferation of cancer cells is thought, therefore, to be sustained by telomere maintenance mechanisms, which were detailed above. Since high TA is reported in over 85% of cancers, telomerase dependent telomere lengthening is believed to be the most common telomere maintenance mechanism relevant to carcinogenesis.

1.5.6.1 Evidence for altered TL in cancers

During ongoing proliferation in normal somatic cells without telomerase or other telomere-maintenance mechanisms, telomeres shorten until reaching a certain minimal length. Beyond this, when tumour suppressor checkpoints such as p53 are functioning, senescence or apoptosis can be induced. In contrast, when p53 or other important DNA damage checkpoints are not functioning, cells can enter a crisis state where ongoing proliferation promotes further telomere shortening and telomere dysfunction (Shay and Wright, 2005). This can cause various genomic instabilities such as end-to-end fusion of telomeres resulting in anaphase bridges in subsequent cell division cycles. Most of these cells usually die due to apoptosis and gross genomic instabilities. However, some rare cells acquire mutations in the TERT promoter that increase TA resulting in re-stabilisation of telomeres, Importantly, as long as telomeres are capped and protected, they can be rather short, and this situation is frequently found in epithelial cancer cells compared with adjacent healthy tissue. Several studies using telomere PNA-FISH have shown that breast, prostate, and pancreatic cancers are associated with telomere shortening (Meeker et al., 2004, Meeker et al., 2002, van Heek et al., 2002). Furthermore, around 40 to 97% of colorectal tumours have shorter telomeres compared with normal tissue, and telomere shortening is therefore considered to be one of the early events in tumourigenesis (Valls et al., 2011, Roger et al., 2013).

However, importantly, acquiring TA can stabilise even short telomeres in genetically unstable cells and provide sufficient capping for them to attain an unlimited proliferation potential. Thereby, telomerase re-activation conserves genomic mutations and instabilities and contributes further to tumourigenesis.

Significant TL shortening results in end-to-end fusion, thus increasing the potential for genome instability and carcinogenesis. There are few other generic associations that lead to telomere attrition, such as oxidative stress, lifestyle choices, environmental factors, smoking and obesity (Bojesen, 2013), and some of these also increase the risk of developing a variety of cancers. Telomere shortening can influence the progression of premalignant breast tissue to malignancy, and premalignant breast lesions had short telomeres leading to non-clonal chromosome aberrations (Raynaud et al., 2010).

Meta-analyses of available studies also revealed that shorter peripheral blood mono-nucleocyte (PBMC) telomeres are associated with a significant increase in the risk of developing cancer (OR=1.35, 95% CI=1.14-1.60) than longer telomeres (Ma et al., 2011, Wentzensen et al., 2011). Shorter PBMC telomeres could be related to oxidative stress endured by an organism, which is in agreement with the established mediatory role that oxidative stress plays between inflammation and cancer (Morgillo et al., 2018). When PBMC mean TLs were prospectively studied in the general population in Denmark; shorter TLs were also associated with decreased survival after cancer rather than the cancer risk itself (Weischer et al., 2013). Another systematic review has also reported a consistent inverse relationship between age and PBMC TL (Muezzinler et al., 2013).

Telomere dysfunction may also be a resultant of altered telomere-associated proteins that are also essential for regular end-capping function (Mizuguchi et al., 2017, Amir et al., 2018). For example, mutations in the C-terminal of POT1 can initiate genomic instability permissive for tumourigenesis (Chen et al., 2017). TRF1 flox/flox × K5-Cre transgenic mice, do not have TRF1 in stratified epithelia. These mice demised perinatally and showed skin hyperpigmentation and epithelial dysplasia and were associated with telomere initiated DNA damage, p53/p21 and p16 pathway activation and *in vivo* cell cycle arrest. Deficiency of p53 rescues mouse survival but causes an increase in the incidence of squamous cell carcinomas (Martinez et al., 2009). Alteration of the levels of TRF1, TRF2, TIN2 and POT1 has also been described in some human tumours (Blasco, 2005). A dysregulated expression of TRF1, RAP1 and TPP1 has been reported in patients with chronic lymphocytic leukaemia (Poncet et al., 2008). Likewise, TIN2, TRF1 and TRF2 mutations have been associated with some cases of DC and aplastic anaemia (Savage et al., 2006, Savage et al., 2008, Tsangaris et al., 2008, Walne et al., 2008), and both of these conditions increase the risk of developing some cancers. Defects in shelterin components naturally cause dysregulation of telomere homeostasis as explained above. This may operate as a tumour suppressor mechanism when it initiates the p53/pRb pathways, which in turn triggers senescence and prevents the tumorigenesis process. Alternatively, it can contribute to carcinogenesis with the fusion of dysfunctional telomeres or fusion between dysfunctional telomeres and double-strand breaks which trigger breakage-fusion-bridge cycles (Frias et al., 2012). In hepatocellular carcinomas, longer telomeres, increased hTERT expression and higher levels of TRF2 protein as “stemness markers” were associated with poorer prognosis and more chromosomal instability (Kim et al., 2013). Further studies have confirmed that different causal factors such as hepatitis B and C, and alcohol lead

to telomere dysfunction in hepatic cells hence initiating the carcinogenesis process (El Idrissi et al., 2013). A significant decrease in POT1 and RAP1 protein levels are described in familial papillary thyroid cancers (Cantara et al., 2012). TP53 disruption in haematological malignancies has been associated with the downregulation of expression in shelterin genes and severe telomere dysfunction and genomic instability (Guieze et al., 2016). Therefore, genetic mutations resulting in functional alterations in the essential components of the telomerase enzyme or shelterin components may repress TA and thus, shorter telomeres will be the consequence. The available evidence also suggests a concerted dysregulation in the expression of shelterin genes and protein levels with the commonly observed removal of cellular tumour suppressor mechanisms in premalignant conditions can lead to alteration in TLs that can trigger the tumourigenesis process.

1.5.6.2 Evidence for altered telomerase in cancers

Polymorphism in genes of the telomerase complex: such as *hTERT* and *hTERC* has been reported to affect individual susceptibility to cancers (Bayram et al., 2016, Polat et al., 2018). Variants in chromosome 5p15, the region that harbours the *hTERT* gene, have been identified by Genome-wide association studies (GWAS) to be associated with the risk of bladder, pancreas, brain, testicular, breast, prostate, skin and lung cancers (Rafnar et al., 2009, Kote-Jarai et al., 2011, Kote-Jarai et al., 2013, McKay et al., 2008, Petersen et al., 2010).

hTERT promoter mutations: Tumours with high *hTERT* promoter mutation frequencies have almost always originated in tissues with relatively low cell turnover rates. Contrastingly, tissues with rapid cell turnover seem to have different mechanisms to elongate telomeres and seem less likely to benefit from activating *hTERT* expression by mutations (Killela et al., 2013). Mutations that result in increased *hTERT* expression, TA or longer TLs have been identified in cancers of the central nervous system, thyroid, bladder, liver, tongue, adipose tissue and skin (Vinagre et al., 2013, Heidenreich et al., 2014, Killela et al., 2013). In thyroid cancers, when *hTERT* and *BRAF* mutations coexist, such tumours express high levels of *hTERT* (Liu and Xing, 2016).

Common inherited variants of telomere related genes, such as *TERC, TERT* and rare *POT1* mutations, have been found to be associated with a higher risk of developing gliomas. *TERT*

promoter and *ATRX* mutations were found to be the most recurrent somatic events, which led to a glioma-associated lengthening of telomeres (Walsh et al., 2015).

A high frequency of *hTERT* promoter mutations was also reported in follicular cell-derived thyroid carcinomas (Landa et al., 2013). An over-representation of *hTERT* promoter mutations had been detected in advanced thyroid cancers and these mutations were more prevalent in advanced disease (51%) compared with well-differentiated tumours (22%). Thus, *hTERT* promoter mutations have been suggested as biomarkers of tumour progression (Landa et al., 2013). *hTERT* promoter mutations usually cause an increased expression of the *hTERT* gene and paradoxically, these mutations were reported to occur together with short telomeres in tissues with low-rates of self-renewal and were also associated with poor patient survival in primary melanomas (Heidenreich et al., 2014). Tissue stem cells are reported to have active telomerase and daughter cells produced by these switch off telomerase upon differentiation, and subsequent reactivation of telomerase in these tissues have been proposed to be the reason for the observed short telomeres in thyroid cancers with high telomerase expression (Heidenreich et al., 2014). Rachakonda *et al.* showed that mutations of the *hTERT* promoter were also the most common somatic lesions in bladder cancer (Rachakonda et al., 2013). The authors also found that a common polymorphism rs2853669 in the *hTERT* promoter acts as a modulator of the mutations effect on survival and disease recurrence. The patients with the mutations had poor survival outcome in the absence but not in the presence of the variant allele of the polymorphism. The mutations without the presence of the variant allele were markedly correlated with tumour recurrence in patients with non-invasive and invasive T1 bladder tumours (Rachakonda et al., 2013). Polymorphisms in the *hTERT* gene were also associated with increased lung cancer risk in the Chinese Han population (Gao et al., 2014).

TA in cancers: The early observation that TA is absent in most human somatic tissues during differentiation but strongly upregulated in tumours, agrees with the hypothesis that telomerase playing an important role in the carcinogenesis process (Kim et al., 1994). In pancreatic ductal cell carcinoma, levels of TA were higher compared to other types of pancreatic cancer and benign pancreatic tissues (Hiyama et al., 2004). In gastric cancers, tumours with high TA had poorer prognosis and the authors concluded that detecting TA might be useful as a prognostic indicator of clinical outcome (Hiyama et al., 2004). TA was also detected in 90% of head and neck squamous cell cancers, in 100% hyperplastic squamous epithelium but not in normal mucosa (Mao et al., 1996). Colorectal cancers with high TA had a poorer prognosis in spite of

curative surgery in apparently disease-free patients; thus, the survival seems to have been associated with the level of TA (Tatsumoto et al., 2000). A systematic analysis of TA levels in many cancer types performed by Bacchetti and Shay in 1997 demonstrate high telomerase being a common observation in most of them (Shay and Bacchetti, 1997).

hTERC alterations in cancers: Recent work has proposed that hTERC maturation involves the poly(A)-specific ribonuclease (PARN) which is localized in the nucleolus and in the CB. The enzyme trims hTERC precursors by removing poly (A) tails and may be involved in the impairment of TA (Deng et al., 2018). Individuals with biallelic PARN mutations and PARN-deficient cells showed a reduction of expression of genes encoding several key telomerase components such as TERC, and DKC1. These cells also have critically short telomeres (Tummala et al., 2015). Improper hTERC processing and telomere dysfunction in premalignant diseases such as Pontocerebellar Hypoplasia 7 (PCH7) and DC had been proposed to have a mechanistic link (Deng et al., 2018). *hTERC* amplification was associated with the aggressive progression of cervical cancer, and the authors suggested that *hTERC* may serve as a surrogate marker for cancer progression and form a potential therapeutic target for cervical cancer (Zhu et al., 2018). However, it is important to appreciate that most cervical cancers initiated in a background of persistent papillomavirus infection in the transformed epithelial cells. hTERC over-expression has been reported in many other cancers, including prostate (Baena-Del Valle et al., 2018); breast (Heng et al., 2017); and oral squamous cell carcinoma (Dorji et al., 2015).

1.5.6.3 Dyskerin alterations in cancer

The low TA observed in DC patients during development and consequently shorter telomeres in many tissues resulting in a high susceptibility to develop a subset of cancers; therefore, wild type dyskerin protein has been suggested to act as a tumour suppressor. Conversely, wild-type dyskerin protein is upregulated in a number of human cancers such as in breast, prostate, colon and hepatocellular carcinomas (Montanaro et al., 2008, Sieron et al., 2009, Witkowska et al., 2010, Liu et al., 2012a) and in these cancers, high levels of dyskerin were associated with poor prognosis (Liu et al., 2012a). Acute loss of dyskerin function by RNA interference led to a marked reduction of steady-state levels of H/ACA RNAs, disruption of the morphology and repression of anchorage-independent growth of telomerase-positive and telomerase-negative human cell lines. The levels of dyskerin in cancer cells modulate TA through the regulation of TERC levels, independently of TERT expression (Montanaro et al., 2008). Dyskerin might

also contribute to tumour development through mechanisms where the presence of cellular TA is not essential, and which may be only partially dependent upon the protein's role in rRNA processing (Alawi and Lin, 2011).

1.5.7 Alteration of telomere biology in human endometrium

1.5.7.1 Telomerase in normal endometrium

TA as well as mean telomere TL change according to the ovarian cycle in whole healthy endometrial samples (Williams et al., 2001, Valentijn et al., 2015) suggesting an ovarian regulation and correlation with proliferative activity (Valentijn et al., 2015). Epithelial cells demonstrated significantly higher TA, but contrastingly, shorter telomeres compared with stromal cells across the cycle (Valentijn et al., 2015, Hapangama et al., 2017). In the endometrium, estrogen upregulates TA, while progesterone inhibits TA and *hTERT* (Valentijn et al., 2015). The telomere and telomerase biology of normal endometrium has recently been reviewed in detail (Hapangama et al., 2017).

1.5.7.2 The role of telomeres and telomerase in benign endometrial disorders

There are various benign gynaecological disorders, such as endometriosis (Sourial et al., 2014b), recurrent reproductive failure, subfertility with reported abnormal TA and TL aberrations (Hapangama et al., 2008b, Hapangama et al., 2008a). High TA, high hTERT mRNA and protein levels with longer mean endometrial TLs are characteristics of the eutopic secretory endometrium (Kim et al., 2007, Hapangama et al., 2008b, Hapangama et al., 2009), whereas epithelial cells of ectopic lesions also demonstrated longer mean TL (Valentijn et al., 2015).

These changes have been speculated to contribute to the pathogenesis of the disease that results in the clinical manifestation of subfertility and proliferation enhancement in ectopic lesions (Hapangama et al., 2008b).

The high TA, pro-proliferative and antiapoptotic phenotype (Hapangama et al., 2010, Hastings and Fazleabas, 2006, Hapangama et al., 2008b) in late-secretory endometrium of women with endometriosis might promote survival of cells that are shed into the peritoneal cavity during retrograde menstruation. The endometriotic deposits induce a local inflammatory response and

secrete various cytokines. Cytokines act on the eutopic endometrium to stimulate the pro-proliferative markers (Hapangama et al., 2010).

The advantage in the survival of these cells and their enhanced proliferative capacity resulted from high TA could facilitate implantation and maintenance of endometriotic deposits (Hapangama et al., 2010, Valentijn et al., 2015). This observation agrees with the high TA and hTERT mRNA/protein levels in active peritoneal ectopic endometriotic deposits. Additionally, ectopic epithelial cells display longer relative TL than eutopic epithelial cells from the same patient (Valentijn et al., 2015, Hapangama et al., 2008b). This corresponds well with the finding of progesterone resistance observed in the pathogenesis of endometriosis (Sourial et al., 2014a, Bulun et al., 2006). The development of ectopic endometriotic lesions may increase TA because endogenous progesterone fails to inhibit telomerase at the ectopic site. Additionally, in the baboon model of endometriosis, it has been shown that aberrant expression of telomerase is an important early event in endometriotic cells since high TA was required for the early establishment of ectopic lesions (Hapangama et al., 2010, Afshar et al., 2013). Furthermore, the establishment of ectopic deposits was associated with induction of high TA and TERT level in the eutopic endometrium (Hapangama et al., 2010).

The initial induction of endometriosis was associated with epidermal growth factor (EGF) signalling stimulation in the eutopic endometrium of the baboon model (Afshar et al., 2013). EGF signalling was associated with elevation of TA in normal ovarian surface epithelial cells (Bermudez et al., 2008). A similar scenario might be happening in the eutopic endometrium in the baboon model. Eutopic endometrial cells with high TA can subsequently initiate more ectopic lesions after retrograde menstruation contributing to a self-propagation cycle of the disease (Hapangama et al., 2010), reviewed in Hapangama *et al*. in 2017 (Hapangama et al., 2017).

Ovarian endometriotic epithelial cells were successfully immortalised by infection with various combinations of lentiviral vectors for expression of *cyclinD1*, *cdk4*, *dominant negative p53* and *hTERT*, whereas the introduction of hTERT alone, or together with cdk4, was insufficient to immortalise these cells (Bono et al., 2012). Therefore, telomerase alone may not be sufficient for the apparent survival advantage that characterised endometriotic cells (Hapangama et al., 2017).

The progesterone dominant window of implantation in healthy women has shown virtually no hTERT immunoreactivity (Hapangama et al., 2008b) and lowest TA (Williams et al., 2001), (Hapangama et al., 2008a). However, immunostaining for hTERT was significantly and differentially increased in various endometrial cellular compartments in women with recurrent reproductive failure (Hapangama et al., 2008a). These observations suggest that particular aberrations in cellular proliferation or causative dysregulation of telomerase to be important in endometrial pathologies. Furthermore, the normal TA seems to play a pivotal functional role in ensuring normal endometrial function (Hapangama et al., 2017).

1.5.7.3 Alteration of telomere biology in premalignant endometrial conditions and in EC

1.5.7.3.1 Alterations in TLs in EH

The involvement of telomere shortening in chromosomal instability has been associated with the initiation of carcinogenesis (Artandi and DePinho, 2010). There are only 2 studies that have examined TLs in endometrial hyperplasia. A study using a telomere-FISH (telo-FISH) assay to measure TLs, compared chromosomal arm loss or gain in premalignant endometrial lesions with normal endometrium, and reported TLs to be stable with the pathological transformation in EH and in EC (Maida et al., 2006). Albeit using small sample size, the authors conclude that unlike in cervical precancerous lesions, EH did not have widespread chromosomal alterations, implying that endometrial carcinogenesis involves mechanisms distinct from those of cervical carcinogenesis, which is almost always induced by a viral infection (Maida et al., 2006). However, close scrutiny of the data presented on different EHsubtypes suggested that atypical EH may be associated with higher TL heterogeneity. This may also be suggestive of the involvement of ALT mechanism in this premalignant condition, but larger studies are needed to confirm the ALT mechanism in the true premalignant EH subtype with atypia. Importantly, the analysis method utilised in the Maida study did not allow the inter-patient comparison of tissues samples (of different women) but was only suitable to compare adjacent cells of a single tissue sample. Therefore, the study presented insufficient data to conclude if there was a definite change in the TL in precancerous endometrial hyperplasia when compared with either normal or cancerous endometrium.

By using a three-dimensional (3D) imaging technique, a specific 3D arrangement of telomeres was revealed in tumour cell nuclei (Chuang et al., 2004). Unlike the non-overlapping nature of telomeres in normal nuclei, telomeres of cancer nuclei have the tendency to form aggregates (Chuang et al., 2004). Different numbers and sizes of such telomere aggregates can be found in tumour nuclei (Chuang et al., 2004). Telomere aggregate formation does not depend on TL and TA (Louis et al., 2005).

The existence of telomere aggregates in precancerous lesions, such as in human cervical intraepithelial neoplasia, supports the notion that changes in the organization of the 3D nucleus may facilitate tumorigenesis (Amiel et al., 2010). The "telomere-driven genome-instability" can happen as a result of the close contiguity of telomeres forming aggregates of different numbers and sizes that increase the risk of end-to-end telomeric fusions followed by cycles of breakage-bridge-fusion (Louis et al., 2005). A significantly increased number of telomere aggregates was observed in atypical hyperplastic cells in a mouse model which is also a specific feature of cancer cells. Moreover, the *PTEN* heterozygous mouse model further demonstrated that 3D telomere architectural changes occur before the complete loss of *PTEN* and prior to the development of histological characteristics of atypical hyperplasia and EC (Danescu et al., 2013). Therefore, the presence of telomere aggregates in hyperproliferative lesions with atypical nuclei may render them to be precancerous changes. Further studies, including larger sample size and both types of EH, are warranted to examine and conclude on changes in TL in precancerous endometrial hyperplasia lesions.

1.5.7.3.2 Telomerase in EH

High hTERT levels and elevated TA were reported in all types of endometrial hyperplasia, including simple, complex and complex with atypia subtypes (Saito et al., 1997a, Shroyer et al., 1997, Mazurek et al., 2001, Dong et al., 2004a, Dong et al., 2004b). This early observation prompted some investigators to propose that TA measured by the TRAP assay to be a suitable tool to screen the endometria of postmenopausal women with postmenopausal bleeding (Maida et al., 2002). The authors proposed that this method will determine endometrial premalignant and malignant conditions (Maida et al., 2002) from benign endometrium since TA was rarely detected in normal postmenopausal women, while the majority of endometrial hyperplasia and cancers contained high telomerase activity. However, there are other studies that reported a lack of measurable TA by TRAP assay in benign EH (Zheng et al., 1997). Further work also

found that it was possible to use hTERT immunohistochemical (IHC) analysis (Brustmann, 2005) as a marker for premalignant (atypical) EH. However, it is difficult to conclude on the diagnostic feasibility of TA or hTERT protein (IHC) in endometrial hyperplasia considering these studies, because of the inadequate sample sizes which were only n=12 atypical EH in Brustmann (2005) and n=18 simple and atypical EH in Maida *et al.* (2002) (Brustmann, 2005), (Maida et al., 2002). In addition, the studies did not clarify whether the existence of EH cells was confirmed in the analysed samples, particularly with TRAP assay and since EH can co-exist with either normal or cancerous endometrium, this may affect the results. Progesterone is one of the main current pharmacological therapies for treating EH (Kamal et al., 2016a), and telomerase being a (albeit indirect) downstream target of progesterone in the endometrium is of interest. This justifies future studies exploring the therapeutic utility of directly targeting telomerase in the treatment of endometrial hyperplasia.

1.5.7.3.3 Evidence for Telomere alterations in EC

A study in 1992 found that endometrial adenocarcinomas have reduced telomeric repeat sequences suggesting shorter telomeres compared with normal tissue (Smith and Yeh, 1992). A decade later, a second study demonstrated changes in TLs in 17/23 (73.9%) of ECs using a Southern blot technique (Wang et al., 2002b). Another study by Menon & Simha in 2003, using the same telomere restriction fragment (TRF) measurement, found that mean TRF lengths became shortened when normal endometrium underwent neoplastic changes (Menon and Simha, 2003). A study which used a telomere-oligonucleotide ligation assay demonstrated erosion of the telomere overhang length, rather than overall TL, and proposed that this might play a role in endometrial carcinogenesis and may be related to tumour aggressiveness (Hashimoto et al., 2005). All these studies utilised techniques that assess the average TL values of a tissue sample. However, when endometrial samples were harvested and frozen, they did not examine if the proportion of the endometrial sample examined for TL actually contained cancerous cells. Subsequently, 12 years ago, Maida *et al.* (2006) employed a telomere-FISH (telo-FISH) assay that assessed the relative TL in normal and pathological cells in intact tissue at the cellular level, and no significant difference was found between the TL of normal endometrium and EC (Maida et al., 2006). That study, however, did not specify the normal cell type that they used as the control (stromal/epithelium) and included only adenocarcinomas (Type I). A similar, but slightly modified version of telomere chromogenic in situ hybridization method was subsequently used by Akbay *et al.*, and the authors demonstrated a significant

telomere shortening in both type I and type II ECs in comparison with normal stromal cells (Akbay et al., 2008). They also reported that the adjacent normal stromal cells were compared with epithelial cancer cells to demonstrate telomere shortening only in type II cancers. The authors expanded the study to confirm their hypothesis in a rodent model. These animals were generated with shortened telomeres to show that telomere attrition contributes to the initiation of type II ECs and the progression of Type I ECs (Akbay et al., 2008). This is of interest, but caution should be taken when interpreting these results, as the endometrial stromal cells are known to possess longer telomeres when compared even with healthy epithelial cells (Valentijn et al., 2015), (Hapangama et al., 2017), and that has been hypothesised to be due to the difference in the cell proliferation rates, TA levels and different regulation of telomere maintenance in these two cell types (Hapangama et al., 2017). Therefore, the data may simply reflect cell type-specific difference in relative TLs but not demonstrating a true EC-associated change in TLs. Hashimoto *et al*. (2005) found that ECs show short 3' single-strand telomeric overhang length compared to normal endometrium (Hashimoto et al., 2005). They also found that poorly differentiated cancers or deeply invading ECs had a longer overhang length in comparison with well-differentiated cancers or superficial invading cancers and this may suggest that the 3' overhang may have a role in tumour progression (Hashimoto et al., 2005).

A recent paper that considered germline genetic variants in a GWAS as instrumental variables to appraise the causal relevance of TL for the risk of cancer demonstrated that their predicted increase in TLs was strongly associated with some specific cancers such as gliomas, low grade serous ovarian cancers, lung adenocarcinomas, neuroblastomas, bladder cancers, melanomas, testicular cancers, and also ECs (Haycock et al., 2017). However, this study did not measure the exact TL of the tissue of origin of cancers but assumed the particular genetic variance might promote longer TLs. With that assumption, the authors calculated a stronger association of presumed longer TLs and rarer cancers and cancers with a lower stem cell division rate (Haycock et al., 2017). However, this data should be considered with caution, since age associated tissue / cell specific TL change is a well-established fact, but that was not considered by the authors. Therefore, the postulated prediction in TL change may be relevant to the effect of genetic variants that were examined, in increasing cancer risk, but it does not provide direct or compelling evidence for a role for tissue TL change in endometrial carcinogenesis. When TLs were estimated for cancer cohorts in The Cancer Genome Atlas (TCGA) dataset; sarcomas, testicular germ cell tumours and low-grade gliomas were associated with longer telomeres whilst cervical, and ECs had shortest average TL (Barthel et al., 2017). This

observation has also been explained as a result of some tumours having high telomerase activity, thus shorter TLs that are stabilised (e.g., in testicular tumours (Albanell et al., 1999)), and others have long TLs accompanied by increased activity of the ALT mechanism (e.g., in low-grade gliomas and sarcomas). Longer TL in peripheral blood mononuclear cell (PBMC) has also been associated with a significantly increased risk of EC in a group of Caucasian Americans (Sun et al., 2015). Since ECs are known to have high telomerase activity, the ALT mechanism is less likely to be active in those cancers. Considering the above evidence, it is likely that ECs have relatively shorter TLs that are maintained by high TA compared with normal tissue. Further studies are warranted to examine subtype-specific TL aberrations and the relationship of TLs with the TA in the different types of ECs.

The protein and/or mRNA levels of the most conserved out of all shelterin proteins, POT1 (de Lange, 2005) was increased in many different cancers including gastric, thyroid, breast (Wan et al., 2011, Cantara et al., 2012, Kondo et al., 2004) and in ECs (Liu et al., 2012a). Higher levels of point mutations in the POT1 gene were observed in ECs, revealing that genetic variations in *POT1* may lead to carcinogenesis in the endometrium (Liu et al., 2012b). Simultaneous conditional inactivation of the shelterin protein POT1a with the tumour suppressor p53 in endometrial epithelial cells in a murine model, induced type II metastatic adenocarcinomas in 100% of the animals by 15 months (Akbay et al., 2013). This suggests that telomere dysfunction and loss of tumour suppressor genes can produce Type II ECs. This will obviously need to be accompanied by telomerase re-activation observed in most endometrial ECs supporting the cancer-associated increased cellular proliferation. The loss of POT1 proteins activates ATR (Flynn et al., 2012), and ATR activation requires Replication Protein A (RPA), which binds single-stranded (ss) DNA (Flynn et al., 2012); the POT1-TPP1 heterodimer protects telomere ends from being detected as DNA damage by excluding RPA from binding telomeric ssDNA. Therefore, the loss of POT1 described in EC may cause inappropriate telomere access of telomerase, resulting in compromised telomere capping and sustained telomere dysfunction, facilitating genetic instability. There are no published studies examining the expression or function of other shelterin proteins in EC to date.

1.5.7.3.4 Evidence for Telomerase role in EC

Kyo and colleagues, examining 13 ECs and 5 cell lines derived from ECs using a TRAP assay reported that 92% of cancer samples displayed detectable TA (Kyo et al., 1996). At that point

in time, the general consensus was that only specialised cells or cancer cells would have detectable TA. A year later, the same group increased their endometrial samples to 17, included 60 normal endometrial samples, and reported that being a somatic organ, the benign human endometrium, expresses dynamic levels of TA (measured by TRAP assay), with the highest levels observed in the late proliferative phase endometrium which was comparable to EC. They also indicated that endometrial telomerase levels are closely associated with proliferation and likely to be regulated by estrogen (Kyo et al., 1997) (Figure 1.6). During the same year, Saito *et al*, examined a larger and more diverse sample set and reported that activation of telomerase was found in many cancers such as gastric, prostate, bladder and skin (Saito et al., 1997b). They further confirmed the earlier work by Kyo *et al*., that 28/30 ECs had high TA and late proliferative phase to have the highest TA levels in the benign endometrial samples. Additionally, the authors found that endometrial hyperplasia demonstrated high TA similar to cancer, whereas no activity was detected in healthy postmenopausal endometria with or without bleeding problems, indicating TA to be a suitable diagnostic test for identifying post-menopausal endometrial pathology. The authors also noted that TA was increased by estrogen, which induced cell proliferation and was reduced in progesterone dominant conditions, indicative of an ovarian steroid hormonal regulation. The finding of high TA in ECs has been subsequently confirmed by many other groups (Tanaka et al., 1998, Ebina et al., 1999, Kyo et al., 1999, Lehner et al., 2002, Wang et al., 2002b, Maida et al., 2006, Gul et al., 2013, Valentijn et al., 2015). In addition to the high TA measured by the gold standard test, the TRAP assay, some authors studied expression levels of components of the telomerase holoenzyme using qPCR to detect gene expression levels. They concluded that hTERT levels correlated well with TRAP assay data (Kyo et al., 1999, Lehner et al., 2002), and both seem to be related to endometrial epithelial proliferation (Valentijn et al., 2015). In a relatively small study, Bonatz *et al*. have shown a significant correlation between higher TA and higher FIGO stage and grade, suggesting that TA is increased in advanced stages of EC (Bonatz et al., 2001). In their study, Wang *et al*. showed that 82% of their EC samples had TA, but they did not find any correlation between TL and TA in different gynaecologic cancers (cervical, ovarian and endometrial) (Wang et al., 2002b).

Detection of hTERT mRNA in PBMCs has been reported to be significantly higher in women with EC compared to patients with benign uterine diseases and healthy controls. Using a relatively moderate sample size (n=56 patients with EC, n=40 patients with benign uterine diseases and n=40 healthy control), the authors claimed that the exact levels of hTERT mRNA

would demarcate those with metastatic disease thus may be useful in stratifying patients for adjunctive therapy (Liang et al., 2016). This claim needs to be confirmed in a future study with an adequate sample size for statistical robustness.

Recently, in two progesterone responsive and progesterone-insensitive human EC cell lines (Shan et al., 2015), ATM protein was shown by reverse-phase protein array (RPPA) to participate in progesterone stimulation to suppress carcinogenesis in the endometrium (Shan et al., 2015). Additionally, a progressive loss of ATM levels from hyperplasia to the lowest levels was observed in type I EC lesions, and there was a negative relationship of the pathological grades and ATM levels (Shan et al., 2015).

Activating *hTERT* promoter mutations do not usually occur in a background of a loss of the tumour suppressor protein ARID1A (Wu et al., 2014). Recent data suggest that ARID1A negatively regulates *hTERT* transcription and telomerase activity; while induction of ARID1A represses transcription and histones via occupying SIN3A and H3K9me3 sites (Suryo Rahmanto et al., 2016). ARID1A is a member of the SWI/SNF chromatin remodelling complex, and it is frequently mutated in endometrial adenocarcinoma (Takeda et al., 2016); therefore, it is conceivable how *hTERT* might be upregulated in the EC with loss of *ARID1A*.

In EC cell lines, TA and expression of hTERT were both increased by estrogen in an estrogen receptor alpha (ERα) dependent and estrogen-responsive element (ERE) dependent effect in the *hTERT* promoter (Boggess et al., 2006). Additionally, a previous study showed that estrogen also induced TA via post-transcriptional Akt dependent phosphorylation of hTERT in human ovarian cancer cell lines (Kimura et al., 2004).

Lehner *et al.* compared hTERT mRNA levels and TA using TRAP assay in normal endometrium with EC, and they concluded that the levels and activity were significantly higher in cancer and low in normal endometrium during the secretory phase of the menstrual cycle as well as in atrophic endometrium (Lehner et al., 2002). Thus, they suggested that quantitative analysis of these parameters may be useful as markers for diagnosis of EC.

PTEN regulates TA, most likely through its known effects on the PI3-kinase/Akt pathway (Zhou et al., 2006). Reconstitution of PTEN in the PTEN-null Ishikawa EC cells resulted in inhibition of cell growth and suppression of Akt phosphorylation as well as a parallel decrease in TA and hTERT mRNA levels (Zhou et al., 2006). At present, there are no reports of different expression levels of other telomerase associated proteins. Interestingly DC, which is associated with an increase in the risk of developing some cancer types, has not been reported to be linked with an increased incidence in EC. There are no published studies examining the role of dyskerin in EC to date.

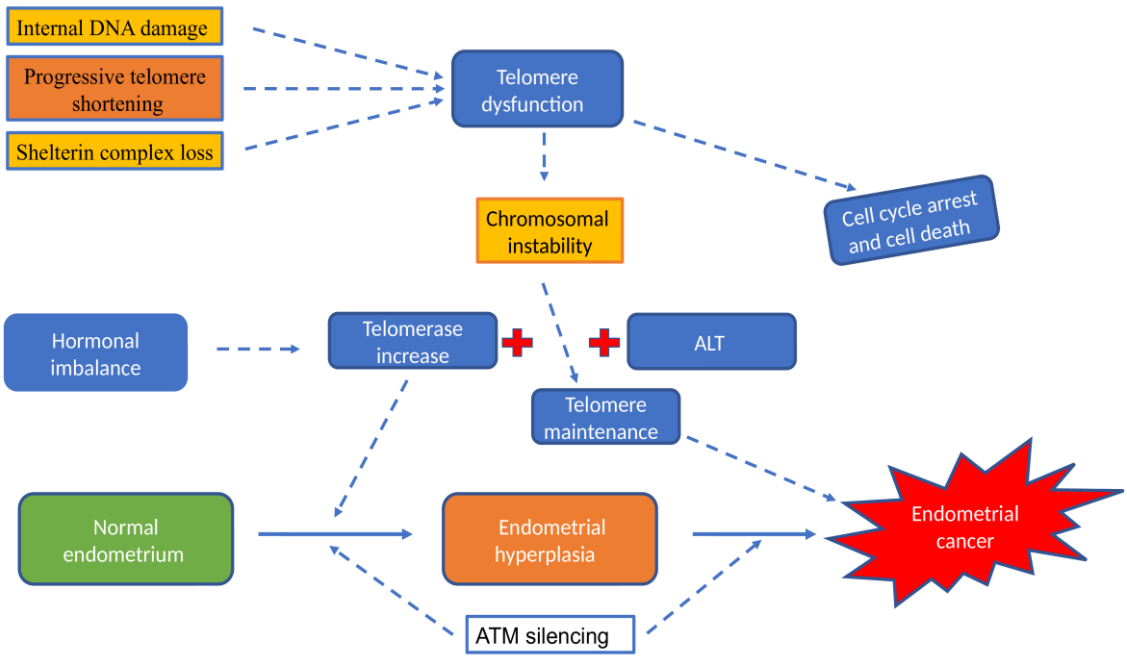

Figure 1.6 The role of telomere and telomerase activity in endometrial cancer. Hormonal imbalance (excess of estrogen or insufficient levels of progesterone) will increase telomerase and elevate telomerase activity was described in all types of endometrial hyperplasia and in endometrial cancer. Dysfunctional telomeres result in genomic instability, the first step in endometrial carcinogenesis. Telomerase dependent pathway is the most widely reported classical telomere length maintenance mechanism but ALT pathway; telomerase independent telomere maintenance was described in some cancer types that lack telomerase activity.

1.5.8 Anti-telomerase therapy

Telomerase was thought to be a suitable target for anti-cancer agents due to the high activity levels seen in most cancers. Available anti-telomerase strategies can be grouped into three main categories: (1) Telomerase inhibitors; (2) telomerase targeted immunotherapy; and (3) telomerase directed viral therapy. Imetelstat (GRN163L) is the only clinically applicable specific oligonucleotide telomerase inhibitor, which is a water-soluble, acid and nuclease resistant compound that forms stable RNA duplexes (Buseman et al., 2012). It prevents the 13-nucleotide region of TERC to form a complex with *hTERT*. Unfortunately, clinical data for Imetelstat has been disappointing with high toxicity (Salloum et al., 2016). The other anti-telomerase agents are also undergoing clinical trials, yet there is no conclusive data yet available for their clinical effectiveness in cancer. For those cancers harbouring activating TERT promoter mutations, directed immunotherapies have been proposed as part of personalised treatment.

Jagar and Walter reviewed the long-term effects of antitelomerase agents on highly proliferative cells that need telomerase for survival. These agents may induce negative effects and may interfere with telomere-independent physiological functions. In addition, only a few hTERT molecules are needed to overcome senescence in cancer cells, and telomerase inhibition requires dividing cells over a sufficient number of population doublings to elicit tumour suppressive senescence. These limitations may explain the moderate success rates in many clinical studies (Jäger and Walter, 2016).

Another review in 2017 discussed the recent approach of targeting telomeres by using guanine-rich oligonucleotides (GROs) homologous to the 3' telomere overhang sequence (T-oligos). T-oligos, a specific 11-base oligonucleotide (5'-dGTTAGGGTTAG-3') also known as T11. T-oligos has been shown to stimulate DNA damage responses (DDRs) like senescence, apoptosis, and cell cycle arrest in various cancer cell types with slight or no cytostatic effects in normal, non-transformed cells. The DDRs induced by T-oligos in cancer cells are similar to the effects seen after progressive telomere shortening in normal cells. The loss of telomeres is an important tumour suppressor mechanism that is frequently absent in transformed tumour cells and therefore, T-oligos have gained significant interest as a prospective anticancer therapeutics to fight cancer, though, little is understood about their mechanism of action (Ivancich et al., 2017).

Progestogens remain to be one of the main hormone-based chemotherapeutic agents that are used in early, advanced and recurrent EC with only modest benefit. The loss of response to progesterone or progressive disease despite progestogens has alluded to progesterone-induced downregulation of progesterone receptor (Kamal et al., 2016a), and the lack of progesterone receptor expression is a feature of advanced ECs (Kamal et al., 2016b). Since telomerase levels are high in most ECs and since telomerase seems to be a downstream target of progesterone in the endometrium, direct telomerase inhibition may have an added benefit in some women with EC. Those with the recurrent disease despite progesterone treatment or having PR negative advanced ECs may particularly respond to telomerase inhibition. However, the available limited *in vitro* data may suggest that Imetelstat may reduce TA but may not cause cell death (Valentijn et al., 2015). Since the *in vitro* data has been generated in a mono-cellular 2D culture system comprising of only epithelial cells, thus it may not accurately reflect the *in vivo* response to the medication (Parkes et al., 2018). Further studies using either physiologically more relevant 3D culture systems containing epithelial and stromal cells or animal models are warranted to explore this avenue further before embarking on clinical studies.

1.6 Hypotheses of this thesis

There is a significant amount of available data on human endometrial TERC and hTERT. However, the presence and alterations of dyskerin in healthy and pathological endometrium are yet unknown. Therefore, the work contained within this thesis is intended to fill the identified gaps that exist in the current literature regarding the role of dyskerin in human endometrium and in EC. My aim was to test five main hypotheses.

Hypothesis 1: Dyskerin is expressed in healthy human endometrium (pre and postmenopausal). To test this hypothesis, I have examined healthy endometrial samples from pre and postmenopausal women, and I have:

1) Quantified the dyskerin protein levels
    - by assessing immunoscores for dyskerin Immunohistochemical staining in both endometrial epithelial and stromal compartments using a semi-quantitative modified quickscore system.
2) Quantified *DKC1* gene expression with: Real-Time quantitative Polymerase Chain Reaction (RT-qPCR).

3) Assessed the associated TA levels using TRAP assay and *hTERC* mRNA levels measured by RT-qPCR.

Hypothesis 2: **Endometrial dyskerin levels are altered in benign proliferative condition, endometriosis**. To test this hypothesis, I have:

1) Quantified and compared dyskerin immunoexpression in eutopic secretory endometrium and ectopic endometriotic lesions from women with laparoscopically confirmed endometriosis for comparison with secretory endometrial samples from healthy, fertile women without endometriosis (surgically confirmed).
2) Compared *DKC1* mRNA levels measured by qPCR in secretory endometria collected from women with endometriosis with that of healthy fertile women.
3) Compared TA measured by TRAP assay and *hTERC* by qPCR in secretory endometria collected from women with endometriosis with that of healthy fertile women.

Hypothesis 3: Aberrant dyskerin levels are associated with endometrial carcinogenesis. To test this hypothesis, I have examined dyskerin protein and *DKC1* mRNA levels in endometrial samples collected from healthy women, women with precancerous EH and women with EC.

1) In Publicly available TCGA dataset EC cohort, I have:
   Evaluated *DKC1* RNA expression in ECs and examined the correlation with patient outcome.
2) Liverpool EC cohort, I have:
   a. Assessed *DKC1* mRNA levels in the context of TA and *hTERC* mRNA levels
   b. Quantified dyskerin protein levels with immunoblotting.
   c. Examined the levels of dyskerin protein semi-quantitatively at a cellular level using a modified quickscore.
3) Examined the correlation of dyskerin immunoscores with patient outcomes and the clinic-pathological features of EC samples.

Hypothesis 4: Dyskerin is regulated by ovarian steroid hormones. To test this hypothesis

I have:

1) Evaluated level of dyskerin according to endogenous hormone levels in healthy pre and postmenopausal endometrial samples.
2) Evaluated level of dyskerin in hormone (exogenous) treated endometrial samples (LNG-IUS) and GnRH).
3) Correlation of steroid receptors expression immunoscores with dyskerin immunoscores in healthy and EC samples.
4) Studied the *in vitro* effect of steroid hormones on *DKC1* mRNA expression levels in cultured ISK cells.

Hypothesis 5: Dyskerin has an important role in endometrial epithelial cell proliferation. To test this hypothesis, I have:

1) Correlated dyskerin immunoscores with the proliferative marker Ki67 in endometrial epithelial cells.
2) Transiently overexpressed *DKC1* in ISK cells using DDK tagged *DKC1* plasmid and Lipofectamine 2000.

3) Confirmed the successful *DKC1* transfection of the ISK cell system with immunoblotting.
4) Assessed the subcellular location of endogenous and exogenous dyskerin with indirect immunofluorescence, using anti-dyskerin and anti-DDK antibodies demonstrating DDK to colocalised with exogenous dyskerin.
5) Assessed the effect of *DKC1* overexpression on cell proliferation rates by staining ISK cells with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) and using direct flow cytometry technique and direct anti-DDK antibody, compared the cell proliferation levels in *DKC1* transfected cells with that of empty vector transfected and non-transfected cells.

These hypotheses are addressed in Chapters 3, 4, 5 and 6. Chapter 2 is General Materials and Methods, and Chapter 7 is the Final Discussion.

2

Chapter Two

General Materials and Methods

2.1 Ethics

The human tissue collection for studies included in this PhD thesis was approved by the following adult ethical committees: Liverpool Adult Research Ethics Committee (LREC 09/H1005/55, 11/H1005/4, 19/SC/0449); Cambridge Adult Research Ethics Committee (CREC 10/H0308/75). Tissue was collected from patients who attended Liverpool Women's Hospital (LWH) and Lancashire Teaching Hospital Trusts from 2009-2017. All specimens examined in this PhD thesis were collected after obtaining informed consent from the patients who donated to the tissue.

2.2 Study groups

The studies described in this thesis include the examination of endometrium from women: (1) healthy pre and postmenopausal without any known endometrial disease, who were not on any hormones, and those on hormonal treatments; (2) those with a surgical diagnosis of endometriosis; (3) those with EH and (4) those with EC (Table 2.1).

2.2.1 Healthy women

Normal endometrial tissue specimens were collected from women undergoing hysterectomy for benign gynaecological pathologies such as prolapse, heavy bleeding without known endometrial pathology (full-thickness samples) and those undergoing female sterilisation (pipelle biopsy) without any known endometrial diseases**.** The absence of endometriosis was confirmed in these women at the time of their surgery by the operating surgeons. Table 2.2 details the exclusion/inclusion criteria. This group includes the following:

Postmenopausal control (PM): Since EC is a disease mainly affecting PM women, 35 age-matched normal endometrial tissue samples were included as a control group. Since some authors indicate that the **proliferative phase control (PP) is** more suitable as a healthy comparator because EC is a proliferative disease, so, I had a second control group of 27 normal healthy premenopausal endometrial proliferative phase samples. **Healthy secretory phase (SP):** To examine menstrual cycle-dependent changes in the premenopausal endometrium and

to assess differences between benign endometrial pathology, endometriosis, 23 healthy secretory phase endometrial samples were also included in the healthy endometrial samples group.

Table 2.1 Study groups

Study group	
Proliferative	27
Secretory	23
Postmenopausal	35
EH	15
EC	109
LGEC	53
HGEC	56
Endometriosis	34
Hormonally treated	36
LNG-IUS	18
GnRH	18

Abbreviations: Endometrial hyperplasia (EH); Endometrial cancer (EC); low-grade cancers (LGEC); high-grade cancers (HGEC).

2.2.2 Women with endometriosis

Additionally, I examined specific changes in the uterine (eutopic) endometrium and ectopic endometriotic lesions: 19 eutopic endometrial samples and 15 ectopic endometriotic lesions were collected from women with symptomatic peritoneal endometriosis, who were not on hormonal treatment, and were undergoing surgical excision of endometriosis. The exclusion/inclusion criteria are detailed in Table 2.2.

Table 2.2 exclusion/inclusion criteria.

Study group	Inclusion criteria	Exclusion criteria
Healthy postmenopausal	Women should have amenorrhea for at least twelve consecutive months Hysterectomy for non-endometrial reasons	Premature menopause <40 years Exogenous hormone therapy in the last three months Endometrium with proliferative activity Any endometrial pathology
Healthy premenopausal	Women have a regular menstrual cycle Hysterectomy for non-endometrial causes	Exogenous hormone therapy in the last three months Irregular menstrual cycle Any endometrial pathology
Endometriosis	Women at reproductive age Women with a surgical diagnosis of active ectopic endometriosis at the time of sample collection	Women on hormonal therapy within the last three months Pregnant or breastfeeding women

2.2.3 Women on hormonal treatment

To study the hormonal regulation of dyskerin, samples were obtained from patients treated with two commonly used options to manage endometriosis, and these include 18 samples from women using slowly released synthetic progestin, LNG-IUS. Additionally, 18 samples from women taking GnRH analogue were also incorporated in the study group.

2.2.4 Endometrial hyperplasia group

A total of 15 hyperplastic endometrial biopsies with cytological atypia were collected from patients undergoing hysterectomy at LWH. Out of these, three women had prior histological evidence of hyperplasia in an endometrial biopsy with ongoing symptoms of irregular or heavy menstrual bleeding; other 12 samples were from paraffin blocks of hyperplastic changes adjacent to EC that were retrieved from the Histopathology Department archive at the Royal Liverpool University Hospital. None of the patients had received hormonal therapy in the three months prior to surgery.

2.2.5 EC groups

2.2.5.1 Local EC cohort

Data were obtained from a total of 109 histologically confirmed EC samples (Table 2.1), from patients who underwent staging operations at LWH. Out of the 109 samples, 60 were pipelle biopsies collected at the time of their hysterectomy as part of their primary surgical treatment for EC. The remaining samples were paraffin blocks retrieved from the Histopathology Department archive at the Royal Liverpool University Hospital, or Lancaster Teaching Hospital during the period between 2009 and 2017. The histological type and grade of EC specimens were assigned by experienced gynaecological pathologists according to the FIGO system. Metastases of 10 ECs were obtained during surgery at LWH, and their pathology paraffin blocks were also retrieved from the Histopathology Department archive at the Royal Liverpool University Hospital. In addition, 20 EC metastases were retrieved from the Pathology Department of Lancashire Teaching Hospitals archive. The sites of the metastases were as follows: lymph nodes (n=11), omentum (n=7), parametrium (n=5), soft tissue (n=4), fallopian tube (n=1), cervix (n=1), and urinary bladder (n=1). Demographic and clinicopathological details of patients were obtained prior to surgery or retrieved by reviewing the hospital notes and clinical databases (Somerset cancer registry, Meditech and EDMS). Body mass index (BMI) was available for (84) patients. None of the patients included had received hormonal treatment, chemotherapy or pelvic radiation prior to surgery when the endometrial samples were harvested. The clinicopathological features of the EC group were detailed in Table 2.3.

Table 2.3 Clinicopathological parameters of endometrial cancer cohort.

Parameter	N
Histological type	
Grade 1	34
Grade 2	19
Grade 3	12
Serous	12
clear	10
Carcinosarcoma	19
Dedifferentiated	1
Mixed cell adenocarcinoma	2
Tumour grade	
LGEC	53
HGEC	56
FIGO stage	
I	49
II	13
III	35
IV	8
Myometrial invasion	
<50%	56
≥50%	53
LVSI	
Negative	60
Positive	48
Cervical stromal invasion	
Negative	73
Positive	34
Extrauterine invasion	
Negative	69
Positive	40
Lymph node involvement	
Negative	10
Positive	15
Not resected	73

Abbreviations: Low-grade cancers (LGEC); High-grade cancers (HGEC); Lymphovascular invasion (LVSI)

2.2.5.2 TCGA EC cohort

The publicly-available "The Cancer Genome Atlas" (TCGA) cohort of uterine cancers included data for RNA expression levels (n=471); copy number variation (n=464); and somatic mutation (n=235) for *DKC1* that was interrogated using Illumina's Base Space Cohort Analyzer application (BSCA) (Robinson et al., 2010) (Software; https://www.illumina.com/informatics/research/biological-data-interpretation/nextbio.html; Illumina, San Diego, CA, USA). The normal endometrial control tissues were obtained from 35 EC patients 2-3 cm away from the cancer margin (Huang et al., 2016b). The TCGA dataset also contained patient follow up data for endometrioid (n=367) and serous ECs (n=109).

2.3 Collection of human endometrial samples

Trained members of the research team or the operating surgeon in the theatre collected endometrial biopsies. For healthy and hormone treated women who were not undergoing a hysterectomy, pipelle endometrial biopsies were collected under anaesthetic before their planned operation began, whereas women undergoing a hysterectomy, full-thickness biopsies were obtained by cutting a thin slice of endometrium attached to underlying myometrium straight after opening the anterior uterine aspect in the coronal plane.

In order to avoid interference with pathological diagnosis and staging, samples from EC were collected by introducing a pipelle (Laboratoire C.C.D., Paris, France) into the endometrial cavity. After gentle scraping, the inner piston of the pipelle was withdrawn to allow cancerous tissue to be sucked into the pipelle under vacuum effect.

Each sample was split into three to four containers: **(i)** 15 mL 10% neutral buffered formalin (10% NBF) (Sigma, Dorset, UK); **(ii)** 0.5 mL RNAlater (Sigma, Dorset, UK) for RNA extraction and PCR analysis; **(iii)** Immediately snap-frozen for immunoblotting and TRAP analysis; and **(iv)** 15 mL Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F-12) culture medium (Sigma, Dorset, UK) for subsequent stromal isolation.

2.4 Tissue preparation

2.4.1 Formalin-Fixed Paraffin-Embedded (FFPE) samples preparations

2.4.1.1 Tissue processing

All samples were preserved in 10% NBF directly after collection to avoid autolysis. After 1-5 days in NBF, the samples were ready to be processed in an automated processing machine (Shandon Citadel 1000, UK).

In the processor, the tissue specimens were dehydrated by transferring them automatically through increasing concentrations of ethanol prior to immersing the cassettes in xylene. Finally, the samples were placed within the paraffin.

2.4.1.2 Tissue embedding

Following automated processing, the tissue cassettes were manually put into block moulds using an embedding platform (Shandon HistoCentre3, Cheshire, UK). Pre-warmed tissue biopsies were picked out of the cassettes, aligned properly in a pre-warmed mould before molten paraffin was poured over them. The blocks then were labelled and left on the cold plate to cool. Finally, they were kept in a freezer overnight (O/N) or for at least 30 min before sectioning.

2.4.1.3 Sectioning

A Microm rotary microtome (Microm HM335, Thame, UK) was used to section the cooled blocks. 3-4µm sections were cut and collected on 3-Aminopropyltriethoxysilane (APES) coated glass slides, which were left O/N to dry at room temperature (RT).

2.4.2 Cycle staging

The premenopausal healthy endometrial tissue specimens were assigned according to the patients last menstrual period (LMP) and histological criteria (Dallenbach-Hellweg et al., 2010, Noyes et al., 1950) by examining the haematoxylin and eosin (H&E) stained endometrial

sections by 2 experienced pathologists in the group. These were further confirmed with the official pathology reports for the same samples issued by the NHS pathology service.

2.4.3 Endometrial cancer confirmation and classification

The histological type and grade of EC specimens were assigned by experienced gynaecological pathologists, according to FIGO system (Pecorelli, 2009). Before inclusion in the study, all collected samples were reviewed for adequacy by identification of representative malignant tissue in Haematoxylin and Eosin (H&E) stained sections. For descriptive and analytical purposes, EC has been grouped in this study into:

- Low-grade tumours (LGEC): This group includes grade 1 and grade 2 endometrioid EC, which generally have a better prognosis.
- High-grade tumours (HGEC): This group includes grade 3 endometrioid, serous, clear cell carcinomas and carcinosarcomas. The high-grade ECs represent more aggressive cancers (Voss et al., 2012).

2.5 Protein expression and quantification

2.5.1 Immunohistochemistry (IHC)

2.5.1.1 IHC protocol

2.5.1.1.1 Slide preparation

Slides were baked using Section Dryer Model E28.5 at 60ºC for 1 hour or 37ºC O/N to remove residual moisture prior to further processing.

2.5.1.1.2 Dewaxing

Slides were deparaffinised in xylene and rehydrated in decreasing concentrations of alcohol. Finally, they were placed in water prior to antigen retrieval.

2.5.1.1.3 Antigen retrieval and immunohistochemical staining

During the process of formalin fixation, cross-links form between different proteins which result in modification of the antigens epitopes within the sample (Shi et al., 1991). This can produce false-negative results and so 'antigen retrieval' is performed to free the antigen's epitopes and improve immunostaining. Antigen retrieval was performed by placing the sections in a heated pressurised chamber for 1-3 minutes with an appropriate buffer optimised according to antibody (Ab) affinity, the exact antigen retrieval period and the type of buffer used are shown in tables in the related chapters (Tables 3.2, 4.2, 5.2 and 6.1). The sections were then incubated in 0.3% Hydrogen peroxide (H_2O_2) in Tris-buffered saline (TBS) (Sigma, Aldrich, Dorset, UK) for 10 min to block endogenous peroxidase.

Sections were then incubated in a humidified chamber, with the specified Ab directly after blocking unspecific binding with horse serum block for 20 minutes. One drop of Vector ImmPRESS labelled polymer-Horseradish peroxidase (HRP) matching the primary antibody (Vector Laboratories, Peterborough, UK) was then added to each sample and incubated for 30 minutes followed by 3,3′-Diaminobenzidine (DAB) substrate for 10 minuntes then immersion in distilled water for 5 min. The principle of polymer labelled immunohistochemistry is shown in Figure 2.1. Tables 3.2, 4.2, 5.2 and 6.1 show primary antibody sources, concentrations and incubation conditions.

Each step in the staining protocol was carried out at RT unless stated otherwise. At the end of each step, sections were rinsed in TBS in order to hold the reaction and eliminate any unbounded material that would interfere with subsequent steps.

Finally, the sections were counterstained with Gill's haematoxylin (Thermo Scientific, Runcorn, UK) and mounted in synthetic resin after being dehydrated through graded alcohol.

Negative control was included in each run to assess the specificity of the primary Ab. For the negative control, the primary Ab was substituted with a matched type and concentration of isotype immunoglobulin. Internal positive control was also included in each run to detect any staining variation between runs.

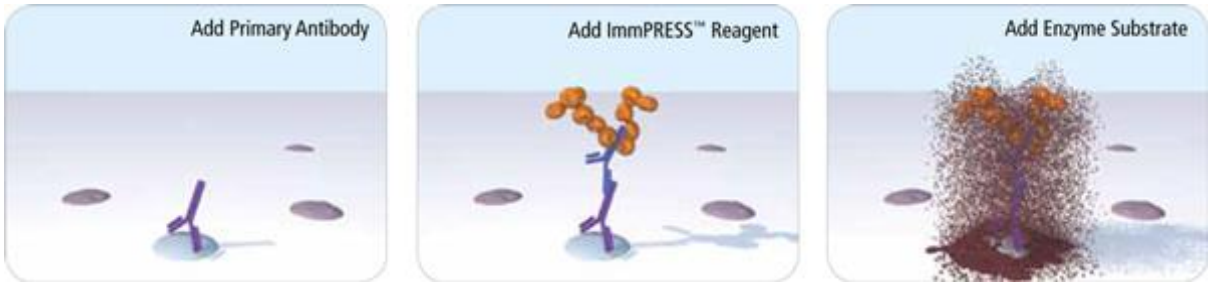

Figure 2.1 The principle of polymer labelled immunohistochemistry (https://vectorlabs.com/immpress-hrp-horse-anti-mouse-igg-peroxidase-polymer-detection-kit.html).

2.5.1.2 IHC analysis

Stained sections were analysed and scored using a light microscope (Nikon, Tokyo, Japan). Quickscore was performed by scanning the whole section and assessing the percentage of stained proportions in each of four intensities (0= no staining, 1= weak staining, 2= moderate staining, and 3= strong staining). The proportion bands were determined for each target protein; this will be detailed in related chapters. The total score out of 12 was calculated by multiplying the intensity score 0, 1, 2, 3 by the proportion score.

The Ki67 proliferative index (PI) was assessed as the percentage of positive cells of any intensity related to total cell number.

To identify the carcinomatous epithelial cells in carcinosarcoma (CS), a serial section for each CS sample was immunostained with pan-cytokeratin (Panck). Only Panck positive cells were scored for the target protein.

Consistency and reproducibility of immunoscores were assessed by revisiting randomly selected and blinded sections by myself (intraobserver) and with Dr Meera Adishesh (interobserver). The high agreement was achieved in both the Inter and intraobserver scoring. Conflicting scores were reviewed, and the agreed score was finally applied.

2.5.2 Immunoblotting

2.5.2.1 Protein extraction

Protein extraction from endometrial tissue

Tissues from human endometrial samples were weighed and then diced into very small pieces using a clean razor blade. 3ml of cold Radioimmunoprecipitation assay (RIPA) buffer was added per gram of tissue and supplemented with protease inhibitors (Sigma-Aldrich, Dorset, UK, 1:100) and phosphatase inhibitors (Sigma-Aldrich, Dorset, UK, one tablet in 10 mL).

Homogenisation of the tissue was achieved using IKA Labortechnik Ultra Turrax T25 Basic S2 homogeniser (Oxford, UK). All the steps were performed on ice; the samples next were transferred into Eppendorf tubes and incubated on ice for 30 minutes. The samples, then were spun at 12,000g for 30 minutes at 4^{o}C and the supernatant was transferred to fresh tubes, the pellets were discarded. The centrifugation step was repeated. The supernatant was obtained and stored in -20^{o}C.

Protein extraction from Cultured cells

Cultured cells were trypsinised and collected into a pellet and re-suspended in phosphate buffer saline (PBS), centrifuged at 500g for 5 minutes at 4^{o}C and the supernatant aspirated. This step was repeated twice to remove any traces of media before freezing the pellets at -70^{o}C. Pellets were thawed on ice, and 10µl of PBS was added to disperse the pelleted cells. RIPA buffer (Sigma-Aldrich, Dorset, UK) was used as a lysis buffer to extract protein from cultured ISK cells. Protease inhibitors (Sigma-Aldrich, Dorset, UK, 1:100 concentration in RIPA buffer) and phosphatase inhibitors (Sigma-Aldrich, Dorset, UK, one tablet in 10 mL of RIPA buffer) were used to block proteases and phosphatases, respectively. Protein lysates were centrifuged for 30-40 minutes at 12,000g at 4°C, and the supernatant was transferred to a new Eppendorf tube. The samples were then stored at -20°C.

2.5.2.2 Protein quantification

To determine the amount of protein to be loaded, a bicinchoninic acid (BCA) protein quantification assay (Thermofisher Scientific, UK) was performed according to the

manufacturer instructions. Briefly, 25µL of each of standard or unknown sample replicate was Pipetted into a microplate well (working range = 20–2000 µg/mL). Then 200µL of the Working Reagent was added to each well. After that, the plate was mixed thoroughly on a plate shaker for 30 seconds, covered and incubated at 37°C for 30 minutes. The plate was then cooled to RT, and the absorbance was measured at or near 562 nm using FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK).

2.5.2.3 Sample preparation

Samples were prepared by mixing equal volumes of cell or tissue lysate with 2x Laemmli loading buffer (Thermo Scientific, Loughborough, UK). The 2x Laemmli Solution contains 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approximately 6.8. The 2-mercaptoethanol reduces the intra- and inter-molecular disulfide bonds. The SDS detergent leads to denaturation of the proteins and subunits and gives each an overall negative charge, therefore allowing separation of proteins based on size. The bromophenol blue works as a dye front that runs ahead of the proteins and also serves to make it easier to see the sample during loading. The glycerol increases the density of the sample so that it will layer at the bottom of a gel's sample well. Additional 1x Laemmli loading buffer was added to equalise the volume of all samples. Samples were heated in a dry water bath for 3 minutes at 100^{o}C or 5 minutes at 95^{o}C to denature proteins, centrifuged briefly to collect the sample at the bottom of the tube and allowed to cool to RT.

2.5.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

10-15µg of the prepared lysates along with molecular weight marker were loaded into Precast gel Tris-Glycine eXtended shelf life (TGX) 12% (Bio-Rad, Hertfordshire, UK). Samples were electrophoresed in Tris-Glycine-SDS (TGS) running buffer (27mM Tris base, 192 mM glycine, 0.1% SDS in dH_2O) at 200V for 45 minutes. The protein was then transferred to an Immun-Blot Polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hertfordshire, UK) in transfer buffer (27mM Tris base, 192mM glycine, 20% (v/v) methanol pH 7.6) at 100V for 120 minutes or by using Trans-Blot Turbo Transfer System (BIO-RAD, Hertfordshire, UK) for 7 minutes. PVDF membrane was then blocked with 5% skimmed milk in Tris-buffered saline-Tween20

(TBS-T) for 2 hours. TBS is prepared by adding 18.15g of Tris base, 26.28g of Sodium chloride and 2.4L dH20, pH 7.6, the final volume of 3L was reached by adding dH20. TBS-T then was prepared by adding 1ml of Tween20 to 1L of TBS. After blocking, the membrane was incubated with primary Ab O/N at 4^{o}C. Next, the primary Ab was removed, and the blot was washed with TBS-T. After washing, the blot was incubated with the species-specific secondary antibody at RT for 1 hour with gentle agitation. After washing for 20 minutes in TBS-T, the membrane was incubated with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Loughborough, UK) for 5 minutes prior to detecting signals at a range of exposures using CL-Xposure Film (Thermo Scientific, Loughborough, UK).

2.6 Cell culture and hormone treatment

All cell culturing procedures were achieved under sterile conditions in BioMat Class II hoods (CAS, Manchester, UK). Cell culture reagents and steroid hormones were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise specified.

2.6.1 Ishikawa cell line culturing

The cell line Ishikawa (ISK) was established from a well-differentiated grade 1 human endometrial adenocarcinoma (Nishida et al., 1985). These cells have been shown to express all steroid receptors (Parkes et al., 2018). ISK cells were obtained from Public Health England (Salisbury, UK), and their gene profile was confirmed by Short Tandem Repeat profiling (STR).

These cells were maintained and grown in a 10cm culture dish containing DMEM/F12, 10% foetal bovine serum (10% FBS) supplemented with L-glutamine (2mM final concentration) and penicillin/streptomycin 1% (v/v). Cells were incubated at 37˚C under 5% CO2 in a 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 11th passages were used for the experiments.

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

2.6.2 Steroid hormone treatment

Hormone modulation was carried out *in vitro* by treating established ISK cells in monoculture with 17β estradiol (E2), 5α dihydrotestosterone (DHT) and progesterone (P4) (Merck, Nottingham, UK) individually or in combination. The steroid hormones were added from 1000-fold concentrated stocks made in absolute methanol to the desired concentration of E2 and P4. The exposure time for each hormone was approximately 12, 24, 48 or 72 hours as described in detail in chapter 5.

2.7 Messenger RNA (mRNA) expression and quantification

2.7.1 RNA isolation

RNA isolation is the most critical step that determines the reliability of the downstream outcome.

2.7.1.1 Extraction

The following methods were used to extract total RNA:

2.7.1.1.1 TRIzol/chloroform extraction (for RNA-later endometrial tissue samples)

Total RNA was extracted from frozen EC tissue specimens using TRIzol and PureLink™ RNA Mini Kit (Invitrogen Ltd., Paisley, UK) following the manufacturer's protocol. Briefly, RNA-later samples were homogenised in 1mL of TRIzol reagent using IKA Labortechnik Ultra Turrax homogeniser, (Oxford, UK). The resulting lysate was incubated for 5 minutes at RT to allow complete dissociation of nucleoprotein complexes. Chloroform (0.2mL) (Sigma, Dorset, UK) then was added and mixed by shaking the tube vigorously by hand for 15 seconds, followed by incubation for 2 minutes at RT. Samples were then centrifugated at 12,000g for 15 minutes at 4°C. The upper aqueous phase, which contains the RNA, was transferred to a new RNAse-free tube. Then, an equal volume of 70% (v/v) ethanol was added and vortexed.

2.7.1.1.2 Lysis buffer extraction (for adherent cultured cells)

Total RNA from cultured cells was extracted utilising lysis buffer of PureLink™ RNA Mini Kit (Invitrogen Ltd., Paisley, UK). 10µL of 2–mercaptoethanol was added to each 1mL of lysis buffer according to the manufacturers' protocol. 500µl of the prepared lysis buffer was added into each well. The content of each well was transferred into a 1.5 ml tube. Subsequently, the cell lysates were Vortexed for 2 minutes at RT. 500µL of 70% ethanol (v/v) was added to each tube. Then each tube was vortexed to mix thoroughly until no precipitate is visible.

2.7.1.2 Purification

Up to 700µL of the RNA/ethanol solution was then loaded onto a PureLink RNA Mini Kit spin cartridge and centrifuged at 12,000g for 15 seconds at RT. The spin cartridge includes a silica-based membrane to which the RNA binds during purification. The flow-through was discarded, and the above step was repeated until the whole sample was processed.

The washing step was carried out by adding 700µL of wash buffer I and centrifuging at 12,000g for 15 seconds at RT. Next, two washes using wash buffer II (500 µL) were performed and followed by centrifuge spin after each wash. Another centrifugation of spin cartridge at 12,000g for 1 minute at RT was performed to dry the membrane with the attached RNA. The spin cartridge was then transferred to a new recovery tube where 30µL of ribonuclease (RNase)-free water was added to the centre of the spin cartridge and incubated for 1 minute at RT, followed by centrifuge at 14,000g for 2 minutes at RT. The purified RNA samples were stored in a -80°C freezer.

2.7.2 DNase treatment

DNase treatment was performed to remove any contaminating genomic DNA before transcriptase reaction, 8µL of purified RNA was treated with 1µL (2 units) of DNase I enzyme (NEB, Hertfordshire, UK) with 1µL of (10X) DNase I reaction buffer and incubated for 10 minutes at 37˚C. Then, 1µL of Stop Buffer (Ethylene Glycol Tetraacetic Acid (EGTA) (Promega, Hampshire, UK) was added and incubated for 10 minutes at 75˚C to terminate the reaction.

2.7.3 RNA Quantification

RNA concentrations and purity were determined spectrophotometrically (260/280 ratio) using a NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). A260/A280 ratio of 1.8+ was considered as suitable for downstream applications.

2.7.4 First-strand DNA synthesis

To eliminate any secondary structure that may delay cDNA synthesis 0.3 -1µg of purified total RNA was first denatured at 70°C for 5 minutes in the presence of 2µL random primer mix and RNAase free water to make up to 8µL then rapidly cooled on ice to halt the reaction. Next, 10µL of 2X reaction mix and 2µL AMV reverse transcriptase RT (NEB, Hertfordshire, UK) was added to make a total volume of 20µL. The mixture then was incubated at 42°C for 60 minutes for maximum yield and length, then kept at 80°C for 10 minutes to stop the reaction. Lastly, 30µL of nuclease-free water was added to make a final volume of 50µL. The synthesised cDNA was stored at -20°C.

2.7.5 Real-time quantitative polymerase chain reaction (RT qPCR)

Real-time PCR is a highly sensitive technique enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real-time. The fluorescent dye SYBR Green binds all double-stranded DNA molecules non-specifically, emitting a fluorescent signal. The signal intensity amplifies with an increasing cycle number as a result of PCR products accumulation.

The PCR reaction constituted 5µL of iTaq Universal SYBR Green Supermix (Bio-Rad, Hertfordshire, UK), 0.25µL forward (F) and reverse (R) primers at 250 nM, 1µL of cDNA and nuclease-free water up to a final volume of 10µL. The reaction was set following the conditions optimised for each target at 37-40 cycles. All samples and negative controls were amplified in triplicate, and the obtained mean value was then used for further analysis.

The efficiency and reproducibility of qPCR experiments were evaluated through the generation of a standard curve for each target and reference gene (Figure 2.2). Primer sequences used for qPCR amplification and efficiency percentage shown in Table 2.4.

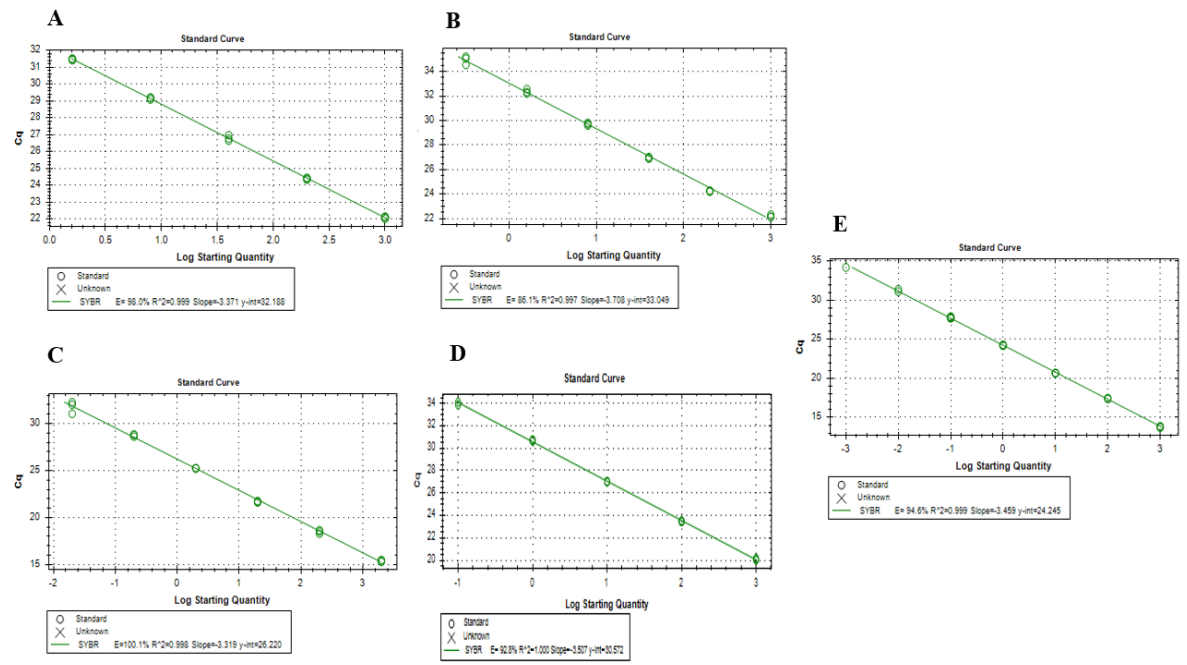

Figure 2.2 RT-qPCR primers efficiency and specificity. Standard curve showing assay efficiency, precision, and slope for **A)** DKC1, **B)** hTERC, **C)** PPIA, **D)** YHWAZ and **E)** ACTB.

A dilution series of cDNA spanned the concentration range expected for the experimental samples amplified. The results were then plotted with input nucleic acid quantity on the x-axis and cycle threshold (Ct) on the y-axis. The slope of the curve was used to determine the reaction efficiency, which was within the accepted range (90- 110%) for all primers used. The acceptable R^2 value of the standard curve was set at ≥0.98.

The *hTERC* mRNA data presented in this thesis for the same sample set were generated by Dr Meera Adishesh in our lab, and the results were included to present the endometrial *DKC1* levels in the context of both TA and *hTERC* levels.

Table 2.4 Primer sequences used for RT-qPCR amplification.

Primer	Sequence	Amplicon	Annealing temp (°C)	Efficiency (%)
DKC1 **(Turano et al., 2008)**	F:5'-CTCGGAAGTGGGGTTTAGGT-3 R:5'-ACCACTTCAGCAACCACCTC-3	166	62	98%
PPIA **(Jacob et al., 2013)**	F:5'- AGACAAGGTCCCAAAGAC-3 R:5'- ACCACCCTGACACATAAA-3	118	60	100.10%
YWHAZ **(Marullo et al., 2010)**	F:5'-CGTTACTTGGCTGAGGTTGCC-3 R:5'GTATGCTTGTTGTGACTGATCGAC-3	69	60	92.80%
TERC **(Chai et al., 2011)**	F:5'- GCCTTCCACCGTTCATTCTA-3 R:5'- CCTGAAAGGCCTGAACCTC-3	220	60	86.1%
ACTB **(Wang et al., 2015)**	F:5'TGTACGCCAACACAGTGCTG-3 R:5'GCTGGAAGGTGGACAGCGA-3	183	60	94.6%

2.7.6 Gene expression analysis

The normalised expression ΔΔCt is the method used to perform gene expression studies. It represents the relative quantity of the gene of interest is normalised to the geometric mean of normalisers across samples (reference genes as described in the related chapters).

2.7.7 Gel electrophoresis

After performing PCR, the products were resolved on 2-3% agarose SYBR Safe gel. Agarose powder (2-3g) (Sigma, Dorset, UK) was dissolved in 100mL 1x Tris-borate-EDTA(TBE)

buffer (89mM Tris, 89mM boric acid, 2mM EDTA) (Thermo Fisher Scientific, Loughborough, UK) and heated in the microwave until completely clear. Having cooled to approximately 60°C, 10µL of SYBR Safe (Invitrogen, Paisley, UK) was added, mixed gently and poured into the electrophoresis tray. At least 20 minutes were required for the gel to be set. Before loading the samples, TBE buffer was used to cover the agarose gel by 1-2mm. A mixture of 10µL of PCR product and 2µL of loading dye (NEB, Hertfordshire, UK) was loaded per well. Appropriate molecular weight marker (NEB, Hertfordshire, UK) was run alongside. The gel was run at 100V for 45 minutes. Bands were visualised using ChemiDoc-ItTS2 Imaging Systems (UVP, California, United States).

2.8 Transient transfection

2.8.1 Cell Preparation and transient overexpression

Approximately 24 hours before transfection, ISK cells were seeded at a density of $5x10^5$ cell/well in 6 well plate. Lipofectamine 2000 (Thermo Fisher Scientific, UK) and DNA plasmid were prepared immediately prior to transfection according to the manufacturers' instructions. Briefly, plasmid (DNA plasmid/empty vector) or Lipofectamine 2000 was diluted in Opti-MEM™ Reduced Serum Medium (Fisher Scientific UK). Each of the diluted plasmid or Lipofectamine was incubated for 5 minutes at RT. Then diluted DNA was mixed with diluted lipofectamine 2000, and the mixture was incubated for 20 minutes at RT. Before transfection, the culture medium of ISK cells was replaced with the same medium but without antibiotics. Then the mixture of lipofectamine and DNA was added dropwise to each well. The plate was gently rocked back-and-forth and side-to-side to achieve even distribution of the complexes, and the plate was incubated at 37° C. 4-6 hours after transfection, the medium with transfection reagents was removed and replaced with ordinary ISK cells culture medium and incubated for 24 to 48 hours at 37°C.

2.9 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Proliferation assay and flow cytometry

2.9.1 Cell labelling with CFSE

CFSE is an intracellular fluorescent stain with a fluorochrome wavelength in the range of the Fluorescein isothiocyanate (FITC) stain. It binds to intracellular molecules, especially lysine residues forming covalent bonds. It is retained in a cell for a very long time. CFSE has been used widely to measure cellular proliferation by flow cytometry. CFSE-labelled cell proliferation will cause spreading of the CFSE stain on to a greater number of cells (daughter cells) that appear on the histogram of the flow cytometry as a shift of the curve to the left with diminished peak height. 5mM CellTrace CFSE (Thermo Fisher Scientific, UK) stock solution was freshly prepared by dissolving the contents of one vial of CFSE (2µL) with 18µL of DMSO. The working solution was prepared by dilution in pre-warmed (37°C) PBS to the desired working concentration (1µM). ISK cells in 6 well plates were washed twice with PBS, and 2ml of the working solution was added per well for 20 min at 37°C in the dark. The CFSE solution was then removed, and cells were washed with culture medium containing at least 1% protein (FBS) twice, and then incubated for 24 hours at 37°C with fresh, pre-warmed complete culture medium (Figure 2.3).

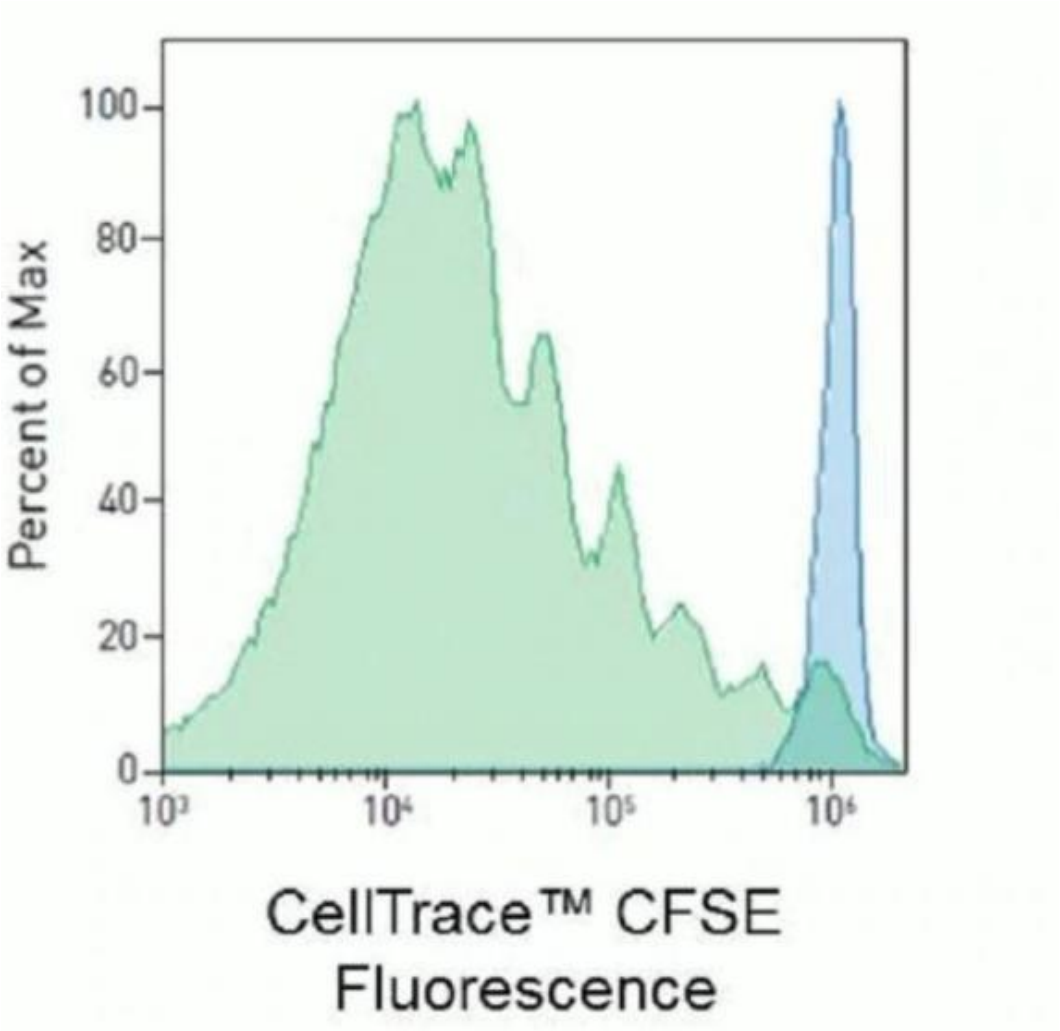

Figure 2.3 The principle of Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) assay. Cell proliferation was followed for seven generations using the CellTrace™ CFSE reagent. Human T lymphocytes were harvested and stained with CellTrace™ CFSE prior to stimulation with anti-human CD3 for five days. The discrete peaks in these histograms represent successive generations of live cells, green. The unstimulated parent generation is indicated in blue. The analysis was completed using an Attune® Acoustic Focusing Cytometer with 488 nm excitation and a 530/30 nm bandpass emission filter, (https://www.thermofisher.com/uk/en/home/references/protocols/cell-and-tissue-analysis/protocols/celltrace-cfse-cell-proliferation-protocol.html).

2.9.2 Flow cytometry

2.9.2.1 Cell preparation

ISK cells were trypsinised and pelleted. Then, the cells were washed by resuspending them with cold PBS, centrifuged at 500g for 5 minutes at RT, and the supernatant was discarded.

2.9.2.2 Fixation

The cells were resuspended in 10% NBF and fixed for 10 minutes at 37°C, followed by 5 minutes on ice. Next, they were centrifuged to a pellet at 500g for 5 minutes, and the supernatant was discarded. The cells were then resuspended in 1mL PBS, spun down to a pellet at 500g for 5 minutes and the supernatant discarded. Finally, the conical tubes were gently tapped to loosen the pellet.

2.9.2.3 Permeabilization

The cells were resuspended with a permeabilising solution (0.5% Tween 20 in PBS) at RT in the dark for 15 minutes to permeabilise the cell membrane; this step is important for intracellular antigens. Next, they were centrifuged to a pellet at 500g for 5 minutes. Then the supernatant was discarded. The cells were then resuspended in PBS and spun down to a pellet at 500g for 5 minutes, and the supernatant was discarded.

2.9.2.4 Immunostaining

The cells were resuspended with Blocking Buffer (3% BSA in PBS) and incubated for 30 minutes at 37°C. Then, fluorochrome-conjugated primary Ab and corresponding isotype control Ab were prepared separately in Blocking Buffer.

Next, the cells were resuspended with diluted primary antibody or isotype control antibody, approximately 100µL per tube) and incubated for 1 hour at 37°C in the dark. Following this, the cells were centrifuged to a pellet at 500g for 5 minutes, and the supernatant was discarded. Then, the cells were resuspended with washing buffer and spun down to pellet at 500g for 5 minutes, and then the supernatant was discarded, this step was repeated twice.

2.9.2.5 Second fixation

The fixation step was repeated with 10% NBF for 10 minutes at 37°C and 5 minutes on ice in order to minimise dissociation and prolong the life of the signal for up to 5 days. Subsequently, the cells were centrifuged at 500g for 5 minutes, and the supernatant was discarded, then resuspended with PBS and spun down to pellet, the supernatant was discarded. Each pellet was resuspended with 200µL of PBS and stored at 4°C in the dark before analysis. Flow cytometry was performed using a Guava Easycyte flow cytometer (Millipore, Germany). 10.000 cells per sample were analysed.

2.10 Immunofluorescence

Cells were cultured on polyLysine-coated coverslips in 6 well plates. The cells on the coverslips were washed with PBS for 5 minutes in RT and then fixed with 10% NBF for 10 minutes in the fume hood, then the cells were washed twice with PBS.

To permeabilise the cells, a permeabilising solution was used (0.2% Triton X-100 in PBS) for 5 minutes. The permeabilisation step was followed by washing with PBS twice, then blocking solution was added to the cells (1% BSA, 10% normal goat serum (NGS), PBST blocking buffer) to reduce unspecific binding of the Ab. The diluted primary Ab was added to wells (100-200µL) and incubated at RT for 1 hour. After primary Ab incubation, the cells were washed twice with PBS. Diluted secondary Ab was added to the corresponding wells (100-200µL) and incubated in the dark for 1 hour. 4′,6-diamidino-2-phenylindole (DAPI) containing mountant was used to stain the nuclei. Fluorescence was visualised with a Nikon Eclipse 50i microscope using NIS Elements F software.

2.11 Statistical analyses

Non-parametric tests (Kruskal–Wallis/ Mann–Whitney U-test) using Statistical Package for the Social Sciences (SPSS) version 25 was used to calculate the statistical differences among groups. Descriptive values were presented as median and range. The correlations between immuno-expression scores were examined with Spearman test and association between immuno-scores and the multiple clinicopathological parameters with Pearson Chi-square. Disease-free survival (DFS) was measured from the date of surgery to the date of EC recurrence or death from EC. Cancer-specific survival (CSS) was measured from the date of surgery to

the date of death from EC; the observations were censored at death for causes other than EC; recurrences were ignored. Overall survival (OS) was calculated from the date of surgery to the date of death from any cause; recurrences were not included in the analysis. All observations were censored at a loss to follow-up and at the last data update. For survival analysis, survival curves were obtained using the Kaplan-Meier method. Cumulative proportions of survivors in the high and low level of the protein of interest were compared using the Log-rank test; p value <0.05 was considered to be significant with adjustment by Bonferroni correction for multiple tests (using Pairwise comparisons of SPSS 25).

3

Chapter Three

Dyskerin Expression in Healthy Endometrium, and in the Benign Proliferative Condition, Endometriosis

3.1 Introduction

The human endometrium is a dynamic tissue that undergoes repetitive monthly cycles of cell proliferation, differentiation, shedding and regeneration. These characteristic endometrial changes are regulated by the ovarian steroid hormones (Kamal et al., 2016b, Hapangama et al., 2015a, Hapangama et al., 2017). The endometrium is comprised of two functionally distinct layers, the superficial functionalis, and the deeper basalis that lies on the underlying myometrium (Maclean et al., 2020). Every month, the endometrium grows from 1mm in thickness at the end of the menstrual shedding, to approximately 15 mm in the mid-luteal phase of cycle (Tempest et al., 2018b, Hapangama et al., 2017). The regeneration seen in the human endometrium after menstrual shedding, parturition or iatrogenic interventions (processes that remove or disrupt the whole functionalis layer) is full, without forming a scar (Tempest et al., 2018b). Therefore, unlike most other adult tissues, the endometrium has a huge and unique regenerative capacity. At menopause, when the ovarian steroid hormones ceased to be synthesised from the failing ovaries, the endometrium becomes proliferatively inactive without the hormonal cue. However, if appropriate exogenous ovarian steroid hormones are provided, a fully functional endometrium still can be generated from the remaining thin postmenopausal endometrium (Paulson et al., 2002).

As outlined in more detail in Chapter 1, telomeres are specialised heterochromatin nucleoprotein complexes at the chromosomal ends containing repeated nucleotide sequences ($(TTAGGG)_n$)(Blackburn and Gall, 1978). They protect the genome from nucleolytic degradation, unnecessary recombination, repair, and end-to-end chromosome fusion (Mandal, 2019) and they are maintained by a specialised enzyme, telomerase. Human telomerase enzyme is composed of 3 core subunits: (1) telomerase RNA component *hTERC*; (2) the catalytic subunit telomerase reverse transcriptase (hTERT), and (3) dyskerin protein (Cohen et al., 2007). TA showed dynamic changes in human endometrium correlating with the ovarian cycle and with glandular proliferation(Valentijn et al., 2015). Within the endometrium, stromal cells, regardless of the cycle phase, express very low levels or absent TA and *hTERC* in comparison with the epithelial cells (Yokoyama et al., 1998, Valentijn et al., 2015, Vidal et al., 2002, Hapangama et al., 2017). The proliferating endometrial epithelial cells express the highest TA (Valentijn et al., 2015), and Valentijn *et al.* suggested that the elevated TA in epithelial cells maintains the short telomeres, in order to protect from a critically short telomere length. Direct *in vitro* inhibition of TA using TERC inhibitor 'imetelstat' resulted in endometrial cell

proliferation inhibition and gland formation disturbance by healthy epithelial cells (Valentijn et al., 2015, Hapangama et al., 2017). The relatively quiescent (with regard to cell proliferation) postmenopausal endometrium expresses low levels of TA (Bryan et al., 1997, Tanaka et al., 1998).

As explained in Chapter 1, endometriosis is a common gynaecological disease, defined as the presence of endometrial glands and stroma like lesions outside the uterus (Sourial et al., 2014a). It is an estrogen-dependent disease that can cause significant morbidity. Unfortunately, since there are no curative treatments, women with endometriosis continue to suffer, and they have a decreased quality of life. None of the available treatments is universally acceptable or effective in managing the symptoms associated with this disease(Parasar et al., 2017). The pathogenesis of endometriosis is not fully known to develop new treatments for disease-associated pain and infertility.

The most accepted theory for the pathogenesis of endometriosis is retrograde menstruation, seeding of eutopic endometrium in the pelvis that initiates endometriotic deposits (Sampson, 1927). However, since this phenomenon of retrograde menstruation occurs in almost all women (Halme et al., 1984), but endometriosis is only detected in 15% of women; subsequently endometriosis researchers have modified this theory and postulated that the eutopic endometrium shed by women with endometriosis, have particular aberrations, enhancing their ectopic growth (Hapangama et al., 2019, Hapangama et al., 2010) and that the endometrium of women with endometriosis differs from that of healthy fertile women (Hapangama et al., 2012, Mathew et al., 2016). Additionally, many previous studies have also reported increased TA levels, higher hTERT gene expression and protein levels, associated with longer mean endometrial TL to be characteristics of the eutopic secretory endometrium of women with endometriosis when compared with the endometrium of healthy women (Kim et al., 2007, Hapangama et al., 2008b, Hapangama et al., 2009, Hapangama et al., 2010, Valentijn et al., 2013, Hapangama et al., 2017).

During retrograde menstruation, the elevated TA in late-secretory endometrium of women with endometriosis (Hapangama et al., 2008b) may cause the cells that are shed into the peritoneal cavity to survive and establish ectopic lesions (Hapangama et al., 2010, Valentijn et al., 2015). While TA, TL and the other 2 core components of the telomerase enzyme have been studied in the human endometrium; there have not yet been any reports published on the dyskerin levels in the endometrium to date.

In this chapter, the levels of dyskerin protein and *DKC1* mRNA were examined in normal endometrial tissue obtained from healthy women and in endometria collected from those with a surgical diagnosis of benign proliferative endometrial disease, endometriosis.

3.2 Study questions

1. Does healthy human endometrium express dyskerin (*DKC1*)?

I tested the hypothesis that healthy human endometrium expresses dyskerin (*DKC1*), in the context of TA and *hTERC*, another core-components of telomerase holo-enzyme. Both the dyskerin protein (IHC) and mRNA expression levels were examined.

2. Are there spatial and temporal differences in dyskerin protein levels in the human endometrium?

Subsequently, I examined the spatial and temporal IHC protein pattern of dyskerin protein as well as menstrual phase related *DKC1* mRNA (qPCR) levels in pipelle and full-thickness human endometrium collected from a cohort of pre and postmenopausal women without any endometrial pathology.

3. Are the endometrial dyskerin protein and *DKC1* mRNA levels altered in the benign proliferative disease, endometriosis?

Differences in dyskerin protein and *DKC1* mRNA levels were examined in eutopic and ectopic endometriotic endometrium and compared with healthy endometrium from women without endometriosis to identify the possible role of dyskerin in endometriosis.

3.3 Methods

3.3.1 Study cohort

As outlined in Chapter 2, full-thickness (endometrium and underlying myometrium) and pipelle biopsies (superficial functionalis endometrial layer) were collected from healthy (endometriosis surgically excluded) pre and postmenopausal women undergoing hysterectomy at the LWH. Eutopic endometrial samples from women with endometriosis during the secretory phase as well as the ectopic samples that were surgically excised as part of their surgical treatment, were also collected. All of the samples were collected during the period 2009 to 2017. Study groups included healthy premenopausal PP (n=27), SP (n=23) and normal PM samples (n=33). 34 samples were collected from women with endometriosis which included SP eutopic endometrium (n=19) and ectopic lesions (n=15) (not matched) (Table 3.1).

Additionally, Myometrium underlying the endometrium were also collected from (n=4) premenopausal women and Freshly harvested endometrial samples from 3 women were used for isolation of human endometrial epithelial and stromal fractions as previously described (Valentijn et al, 2015). Briefly, freshly harvested endometrial tissue were digested into epithelial and stromal fractions. EpCAM (epithelial cell adhesion molecule) microbeads (Miltenyi Biotec Ltd, Surrey, UK) was used to positively select the freshly isolated epithelial cells according to the manufacturer's protocol, while stromal fraction were EpCAM-depleted cells. The purity of the sorted cells was assessed by performing immunoblotting for expression of cytokeratin and vimentin. Additionally, cells maintained in culture were evaluated morphologically and by flow cytometry for the expression of glandular epithelial and stromal markers, CD9 and CD13 respectively.

The fractionated endometrium (epithelial and stromal cells) used in this chapter were isolated by Anthony Valentijn.

Table 3.1: Demographic features of study groups.

Study groups	No.	*Age (years)	*BMI (kg /m2)
Proliferative phase	27	40(30-57)	27(18-41)
Secretory phase	23	41(21-48)	26(19-40)
Postmenopausal	33	63(40-85)	26(20-40)
Endometriosis	34	24(24-48)	24(11-35)

*Data expressed as median (range).

3.3.2 IHC

As described in more detail in Chapter 2, standard IHC was undertaken on 3µm serial sections of FFPE endometrial tissue employing heat-induced antigen retrieval, and ImmPRESS polymerised peroxidase-based detection system (Vector Laboratories, Peterborough, UK) (Kamal et al., 2016b). The primary Ab sources, concentrations and incubation conditions are detailed in (Table 3.2). A matching isotype control (rabbit IgG) (0.5µg/mL) replaced the primary Ab as a negative control. A specific endometrial tissue sample with positive staining was included as an internal positive control with each staining experiment. Human tonsillar tissue was used as an external positive control.

3.3.2.1 IHC analysis/quantification

The nuclear dyskerin immunoreactivity in endometrial epithelial and stromal cells was quantified using a modified quickscore by scanning the whole section and estimating the percent of stained proportions in each of four intensities (0= no staining, 1= weak staining, 2= moderate staining and 3= strong staining). The total score out of 12 was calculated by multiplying the proportion of dyskerin positively stained cells (1%-25%=1, 26%-50%=2, 51%-75%=3 or >76=4) by the staining intensity score as previously described (Mathew et al., 2016).

The epithelial cell staining was scored in luminal epithelium, stratum functionalis and basalis of healthy premenopausal as well as stratum basalis of normal postmenopausal. Epithelial cells in eutopic endometrium and in ectopic endometriotic lesions were also scored. A subset of samples was scored by two independent observers. Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

Table 3.2. Primary antibodies and conditions for IHC.

Primary Ab	Type	Clone	Supplier	HIAR* (min)	Dilution	Incubation Time (hour)	Condition Temp (°C)
Dyskerin	Polyclonal		Santa Cruz biotechnology[1]	3	1:500	20	4

*HAIR by pressure cooking in citrate buffer pH 6. [1]Dallas, Texas, USA. **Abbreviations:** Antibody (Ab), Heat-induced antigen retrieval (HIAR).

3.3.3 TRAP assay for TA

TA was measured by our collaborator, Dr Gabriele Saretski from the University of Newcastle, UK, using TeloTAGGG™ Telomerase PCR ELISA kit (Roche Diagnostics Ltd, Burgess Hill, UK) according to the manufacturers' manual and as previously described (Valentijn et al., 2015), 1µg of lysate was used. PCR was performed for 30 cycles using the conditions in accordance with the manufacturers' manual. Optical density (OD) was measured at 450 nm in a Fluostar Omega Plate reader (BMG LABTECH, Aylesbury, UK) and presented as arbitrary units (AU).

3.3.4 RNA extraction and RT-qPCR

As detailed in Section 2.7, RNA from frozen human endometrial tissue preserved in RNAlater was extracted, quantified and reverse transcribed (Mathew et al.). cDNA was

amplified using iTaq universal SYBR Green supermix and CFX Connect Real-Time System (Bio-Rad, Hertfordshire, UK). The primer sequences and reaction conditions are listed in (Table 2.4). For each target and reference, a standard curve was produced, and efficiency was examined (Figure 2.2). The amplification products were run on an agarose gel (Figure 3.1) to verify the specificity of the primers and to exclude any off-targets. Normalised transcript level was calculated using the ΔΔCT method, relative to the reference genes, *DKC1* was normalised to peptidylprolyl Isomerase A *(PPIA*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), While *TERC* was normalised to *PPIA* and beta-actin (*ACTB*) (Romani et al., 2014, Sadek et al., 2012) using Bio-Rad CFX Manager software (Bio-Rad, Hertfordshire, UK).

3.3.5 Statistical analysis

As outlined in Section 2.11, statistical differences between groups were calculated by non-parametric tests (Kruskal–Wallis or Mann–Whitney U-test) using Statistical Package for the Social Sciences (SPSS) version 25 (IBM Corp, Armonk, NY, USA). Descriptive values were presented as median and range. Graphs were plotted using GraphPad Prism 5 (GraphPad Software, La Jolla California USA). The correlation between dyskerin immunoscores with hTERC RNA or TA levels was determined by Spearman test, p value <0.05 was considered to be significant.

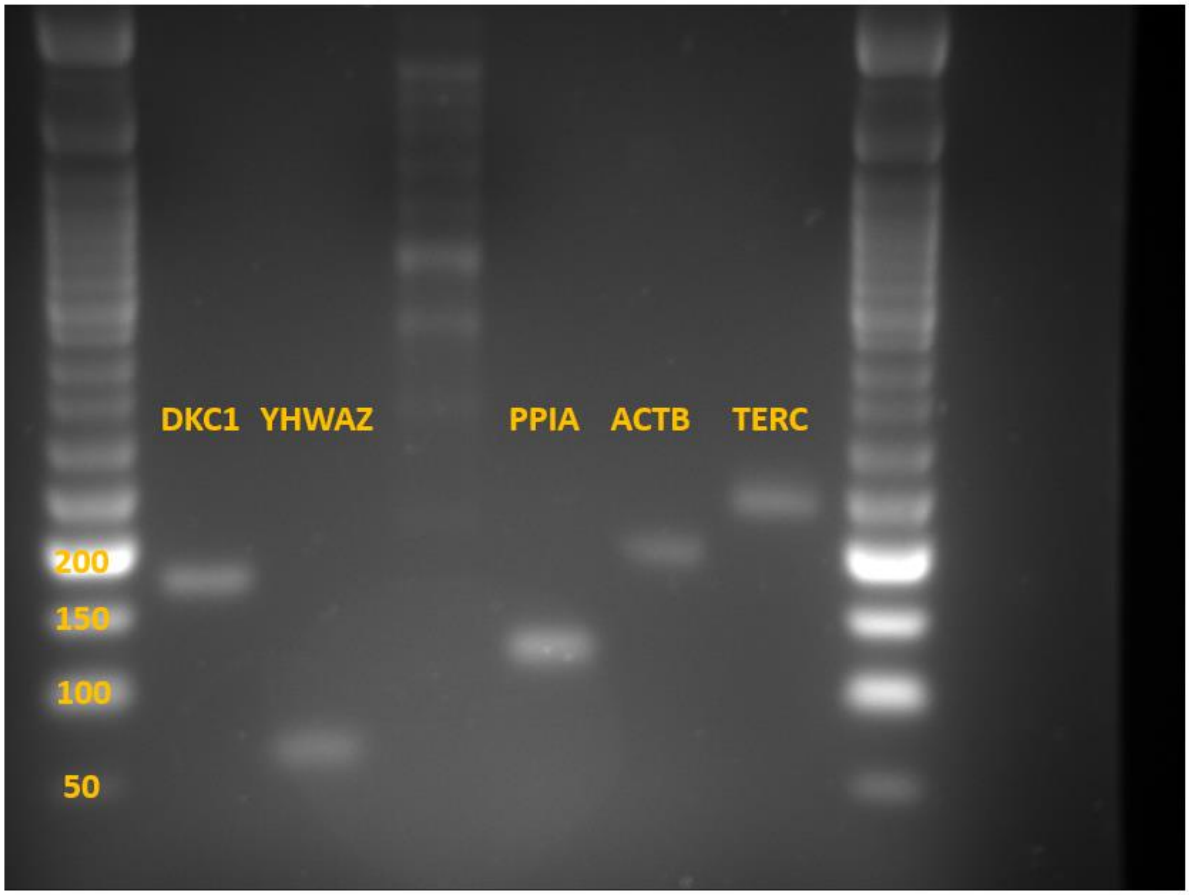

Figure 3.1 Image of agarose gel electrophoresis showing the specific band for *DKC1*, *YHWAZ*, *PPIA*, *ACTB* and *TERC*.

3.4 Results

3.4.1 Healthy endometrium express dyskerin protein and *DKC1* gene

3.4.1.1 TA in the normal human uterus

To define the cohort of healthy endometrial samples, I initially examined TA. The study was expanded to further delineate TA and *hTERC* RNA levels in different uterine wall layers, explore endometrial cell-specific levels and dynamic changes across the menstrual cycle phases of premenopausal and in postmenopausal endometrium.

3.4.1.1.1 Endometrium *versus* Myometrium

When I assessed the differential TA in the individual, distinct layers of the uterine wall, TA was low in both endometrial (secretory phase) and myometrial uterine layers; the difference in TA between these two layers was non-significant. (Figure 3.2A).

3.4.1.1.2 Epithelium *versus* Stroma

Agreeing with previously published evidence (Valentijn et al., 2015), the TA in freshly sorted primary epithelial cells appeared to be higher than in the matched endometrial stromal cells (Figure 3.2B).

3.4.1.1.3 Endometrial TA across the menstrual cycle

The observed differential TA levels were in keeping with previous publications with higher levels observed in the proliferative phase samples (Kyo et al., 1997). When I compared all the premenopausal endometrial samples together (both PP and SP) with the PM endometrial samples, premenopausal endometrium has higher TA in comparison with postmenopausal endometrial samples, but the difference was not significant (p=0.5) (Figure 3.2C). PP

endometrium showed a significant high TA compared with SP endometrium from healthy women (p=0.04). TA in PP samples was also higher compared with endometrial samples from healthy postmenopausal women, but the difference did not reach a statistical significant level (p=0.1) (Figure 3.2D).

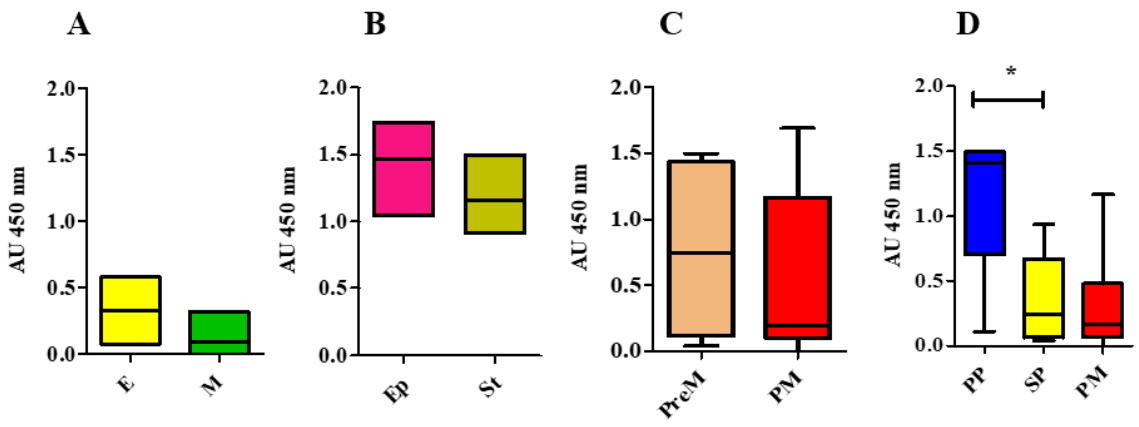

Figure 3.2 Telomerase activity (TA) in the normal human uterus

TA was measured using Telomerase Repeat Amplification Protocol (TRAP) assay in different uterine layers, endometrial cell specific levels and dynamic changes across the menstrual cycle phases of premenopausal and in postmenopausal endometrium. TA was measured in the following samples: **A)** Endometrium (E) (n=3) *versus* myometrium (M) (n=3) **B)** Isolated epithelial (Ep) (n=3) *versus* stromal endometrial cells (St) (n=3) **C)** Premenopausal (PreM) (n=12) *versus* postmenopausal (PM) (n=6) **D)** Proliferative (PP) (n=6), secretory (SP) (n=6) and PM endometrial samples (n=6)**.** *p<0.05, **p<0.01, ***p<0.001.

3.4.1.2 Telomerase holoenzyme components (*hTERC* and *DKC1*) levels in a normal uterus

Following this, I studied the mRNA levels of telomerase holoenzyme components *hTERC* and *DKC1*, using RT-qPCR in endometrial and myometrial layers of human uterus and in freshly isolated primary endometrial cells (epithelial and stromal), as well as assessing dyskerin protein

levels at the cellular level, using IHC in samples collected across the menstrual cycle in premenopausal and in postmenopausal endometrial samples.

3.4.1.2.1 *hTERC* RNA in a normal uterus

3.4.1.2.1.1 Endometrium *versus* myometrium

There was no significant difference (p=1) in *hTERC* levels in human uterine endometrial and myometrial samples (Figure 3.3A).

3.4.1.2.1.2 Epithelium *versus* stroma

In freshly sorted endometrial cells, I did not find a significant difference (p=0.7) in *hTERC* RNA in epithelial and stromal cells (Figure 3.3B).

3.4.1.2.1.3 *hTERC* level across the menstrual cycle

hTERC RNA level appeared to be higher in healthy postmenopausal endometria compared with normal premenopausal endometrial samples, which include both PP and SP samples (p=0.1) (Figure 3.3C).

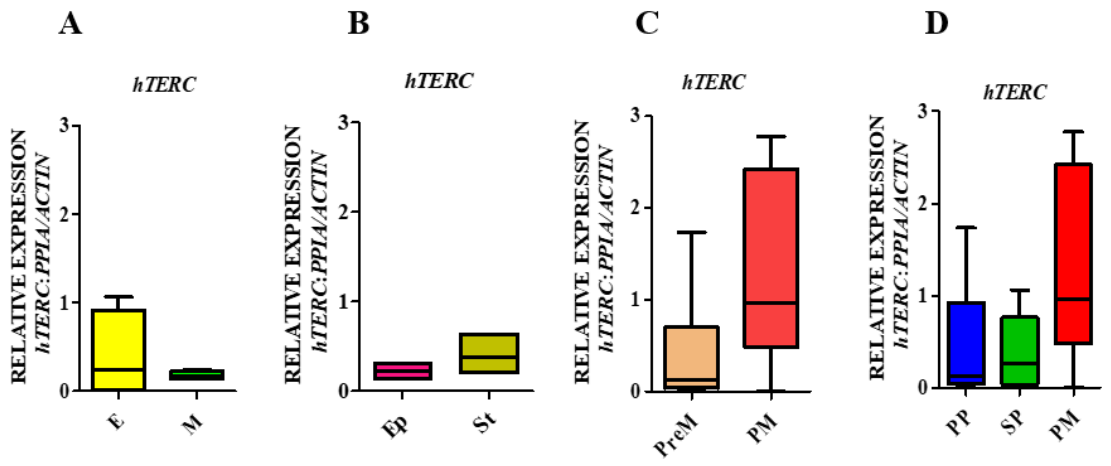

Figure 3.3 *hTERC* RNA levels in normal human uterus *hTERC* RNA levels were measured using RT-qPCR in the following samples**: A)** Endometrium (E) (n=4) *versus* myometrium (M) (n=4) **B)** Isolated epithelial (Ep) (n=3) *versus* stromal (St) (n=3) endometrial cells **C)** Premenopausal (PreM) (n=14) *versus* postmenopausal (PM) (n=5) **D)** Proliferative (PP) (n=6), secretory (SP)

(n=8) and PM (n=5) endometrial samples. *hTERC* was normalised to the geometric mean of peptidylprolyl Isomerase A (*PPIA)* and beta-actin (*ACTB*) reference genes. *p<0.05, **p<0.01, ***p<0.001.

When I compared the *hTERC* in the healthy PM and premenopausal PP and SP samples (separately), the PM endometrium continued to have high *hTERC RNA* level compared with PP and SP samples, but this difference did not reach a statistically significant level (p=0.2, p=0.1 respectively). There was no difference in *hTERC* RNA level between proliferative and secretory samples (p=0.8) (Figure 3.3D).

3.4.1.2.2 Dyskerin gene (DKC1) mRNA and protein levels in Normal uterus

3.4.1.2.2.1 *DKC1* mRNA in the normal uterine wall

3.4.1.2.2.1.1 Endometrium versus myometrium

When I sought to determine if there was a difference in *DKC1* mRNA levels between endometrial and myometrial tissue, *DKC1* levels appeared to be higher in myometrial samples (Figure 3.4A), but this difference was not statistically significant (p=0.1).

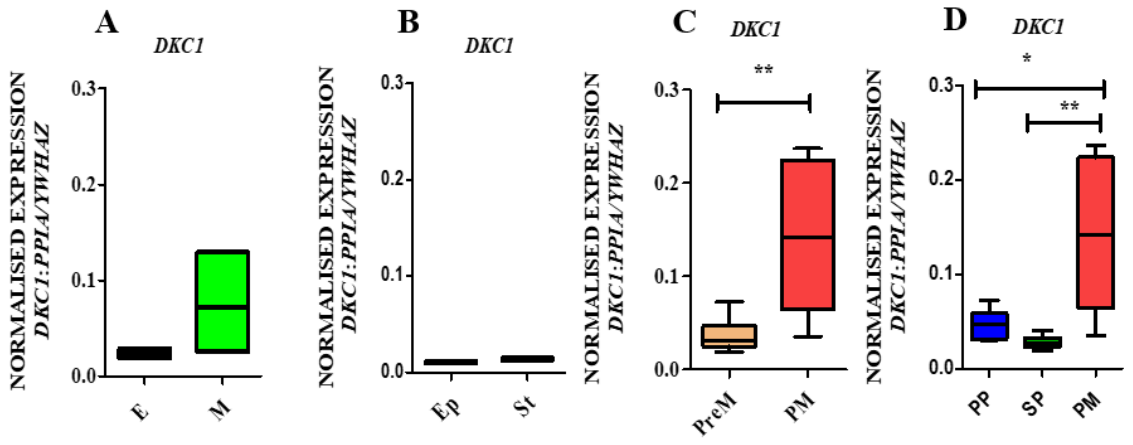

Figure 3.4 *DKC1* mRNA levels in normal human uterus *DKC1* mRNA was meassured in the following samples: **A)** Endometrium (E, n=4) *versus* myometrium (M) (n=4) **B)** Isolated epithelial (Ep) (n=3) *versus* stromal (St) (n=3) endometrial cells **C)** Premenopausal (PreM) (n=15) *versus* postmenopausal (PM) (n=6) **D)** Proliferative (PP) (n=7), secretory (SP) (n=8) and PM (n=6) endometrial samples, *DKC1* was normalised to the geometric mean of peptidylprolyl Isomerase A *(PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-

monooxygenase activation protein zeta (*YWHAZ*) reference genes. *p<0.05, **p<0.01, ***p<0.001

3.4.1.2.2.1.2 Epithelium versus stroma

When the levels of *DKC1* were compared in freshly sorted endometrial cells, there was no difference in *DKC1* mRNA levels between endometrial epithelial and stromal cells (p=0.1), (Figure 3.4B).

3.4.1.2.2.1.3 Across the menstrual cycle

The *DKC1* mRNA levels were significantly higher in normal PM samples in comparison with premenopausal tissue, which included endometrial samples from both proliferative and secretory phases (p=0.0021) (Figure 3.4C).

When the level of *DKC1* in the healthy postmenopausal group was compared with proliferative and secretory phase endometrial samples separately, I found that in postmenopausal *DKC1* levels were significantly higher compared with the endometrium from proliferative (P=0.01), and secretory phase endometrium (p=0.001). The lowest levels of *DKC1* mRNA were seen in secretory endometrium, but there was no significant difference between proliferative and secretory phase endometrial *DKC1* mRNA levels (Figure 3.4D).

In healthy human pre- and postmenopausal endometrial samples, there was a trend suggesting a positive correlation between *DKC1* and *hTERC* RNA levels (p=0.06, spearman r=0.44).

3.4.1.2.2.2 Dyskerin protein levels

3.4.1.2.2.2.1 Dyskerin protein across the menstrual cycle

IHC staining revealed the presence of dyskerin protein at a cellular level in both epithelial and stromal cells of the healthy pre- and postmenopausal endometrium, with immunostaining being primarily localised in the nucleus and/or nucleolus (Figure 3.5A). Dyskerin protein was also present in the uterine myometrial layer (Figure 3.5B).

Immunoreactivity for anti-dyskerin protein Ab was assessed using modified quickscore in the nuclei of epithelial and stromal endometrial cells in pre and postmenopausal endometrium (Figure 3.6A); dyskerin immunescores were significantly higher in healthy PM endometrial

epithelium compared with premenopausal endometrial samples including both PP and SP endometria (p=0.001), (Figure 3.6B).

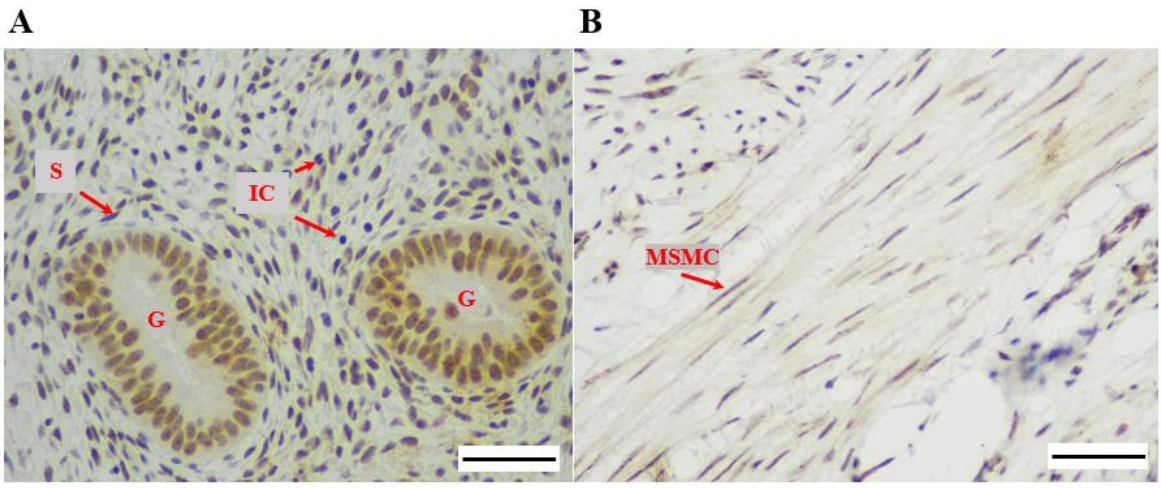

Figure 3.5 Microphotograph demonstrating dyskerin IHC staining at the cellular level in **A)** Human endometrium (proliferative phase). **B)** Myometrium. Magnification 400X. Scale bar 50µm. **Abbreviations:** Glands (G), Stromal cells (S), inflammatory cell (IC), Myometrial smooth muscle cell (MSMC).

When compared with PP and SP separately, dyskerin immunoreactivity in postmenopausal endometrial glands was significantly higher than the immunescores for the basalis glands of the PP (p=0.03) and SP endometrium (p=0.002) (Figure 3.6C). Compared with the epithelial cells, weaker dyskerin immunoreactivity was observed in healthy pre and postmenopausal stromal cells in functionalis or basalis layer (P<0.0001 each) (Figure 3.7A).

3.4.1.2.2.2.2 Dyskerin protein in luminal, functionalis and basalis endometrial epithelium

Dyskerin immunostaining was evaluated in the different epithelial sub-regions of premenopausal endometrium; dyskerin quickscores were similar in the epithelium of all layers of the endometrium (luminal, functionalis and basalis) (Figure 3.6D).

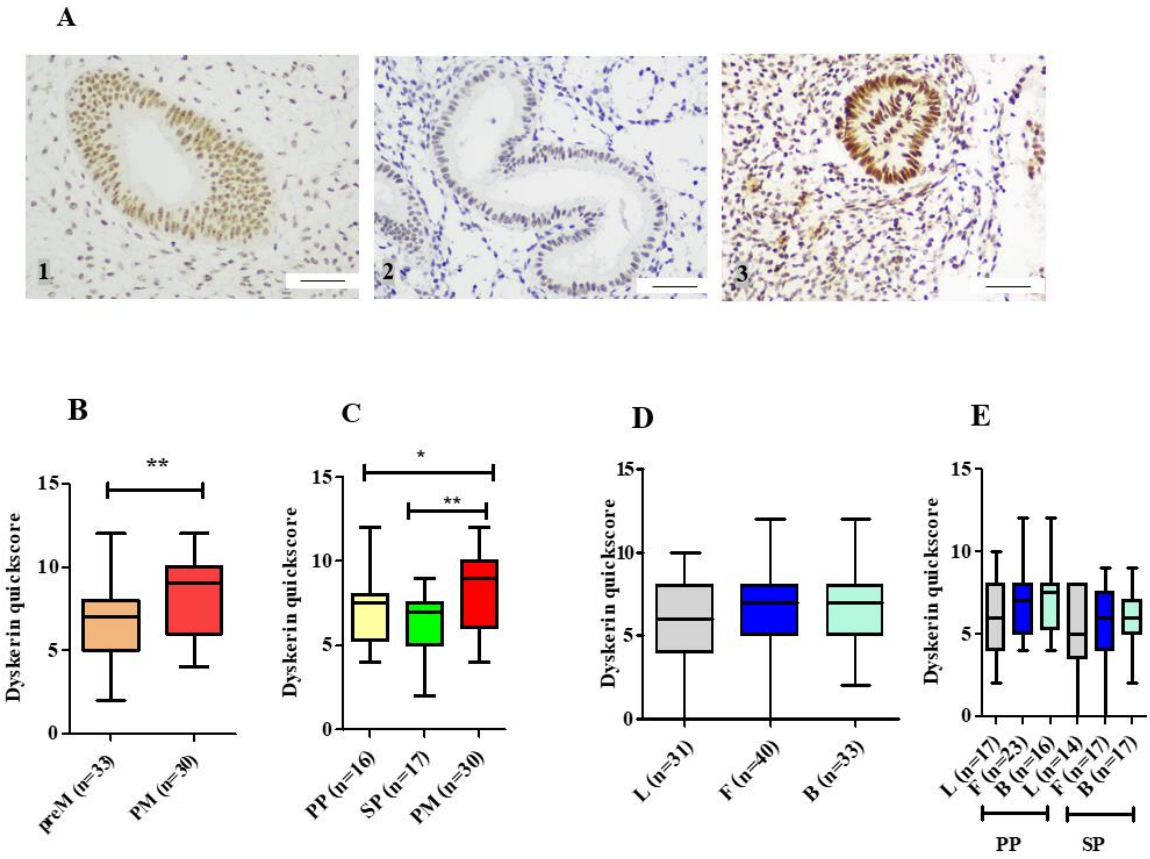

Figure 3.6 Dyskerin protein levels (quickscores) in human endometrial tissue samples A) Representative microphotographs illustrating dyskerin IHC staining in endometrial samples in (1) Normal proliferative phase (PP), (2) Secretory phase (SP) and (3) Healthy postmenopausal (PM) endometrium. Positive staining appears brown. Magnification 400X. Scale bar 50µm. B) Dyskerin protein levels in the endometrium epithelium (basalis) of healthy Premenopausal (preM) (n=33) and PM (n=30) and in C) PP (n=16), SP (n=17) and PM endometria (n=30). D) Dyskerin protein levels in epithelium of the three endometrial layers (luminal (L), functionalis (F) and basalis (B) of premenopausal endometrial samples. L (n=31), F (n=40) and B (n=33) and in E) PP, L (n=17), F (n=23), B (n=16) and in SP endometrial samples, L (n=14), F (n=17) and B (n=17). *p<0.05, **p<0.01, ***p<0.001.

In PP or SP, there was no significant difference in the 3 endometrial epithelial layers (Figure 3.6E). When the functionalis glands were compared between PP and SP, due to their known phase-specific differences, the dyskerin immunoscores were higher in the PP, but this difference did not reach statistical significant level (Figure 3.6E).

Dyskerin immunoreactivity was significantly lower in stromal cells compared with epithelial cells in both functionalis and in basalis of PP, SP or PM endometrial samples (Figure 3.7B).

There was no correlation between dyskerin immunoexpression with TA Spearman r=-0.1 p=0.7 or with *hTERC* RNA (Spearman r=0.5, p=0.1).

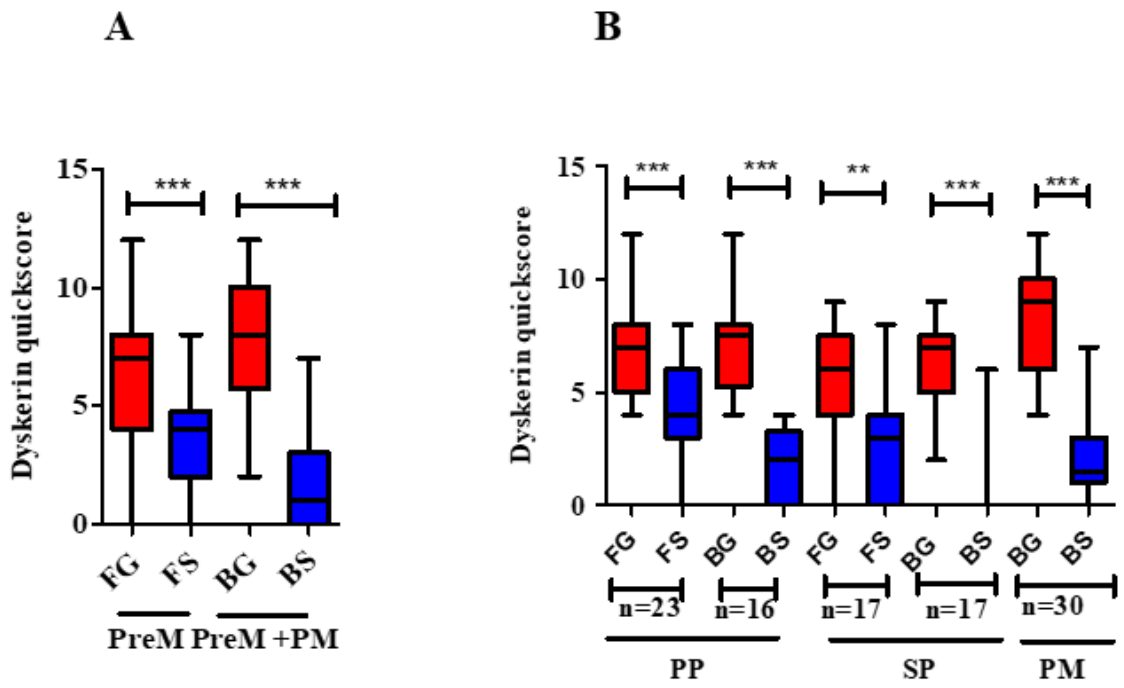

Figure 3.7 Dyskerin immunohistochemistry in pre and postmenopausal endometrial samples shows the difference of dyskerin immunostaining between epithelial and stromal compartments in: **A)** functionalis endometrial layer of PreM (n=40) and basalis of PreM (n=33) and PM (n=30) and in **B)** functionalis and basalis layers of PP, SP and PM. **Abbreviations:** Premenopausal (PreM), postmenopausal (PM), proliferative phase (PP), secretory phase (SP), functionalis gland (FG), Functionalis stroma (FS), basalis gland (BG), basalis stroma (BS). *p<0.05, **p<0.01, ***p<0.001.

3.4.2 Dyskerin *(DKC1)* levels in the endometrium of women with and without endometriosis

3.4.2.1 TA and *hTERC*

In eutopic secretory endometrium collected from patients with endometriosis, *hTERC* RNA levels were significantly higher (p=0.001) when compared with healthy secretory endometrial samples. TA, although non-significant, was higher in endometriotic secretory samples compared with healthy secretory endometrium (p=0.1) (Figures 3.8A & B).

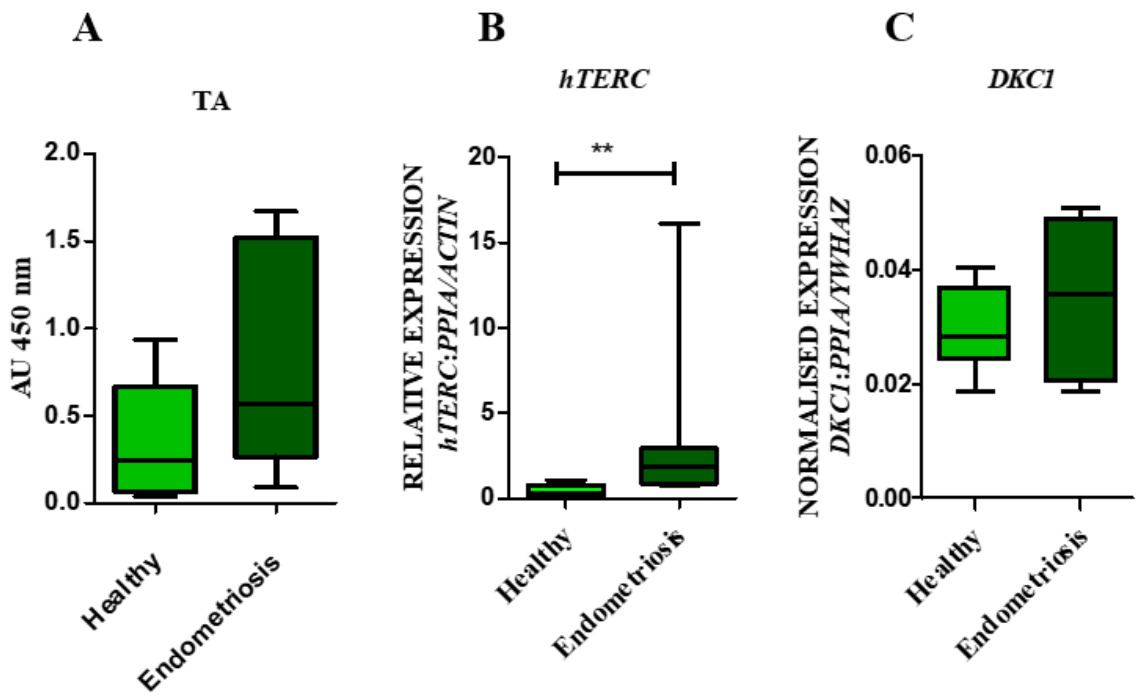

Figure 3.8 Eutopic secretory phase (SP) endometrium from women with confirmed endometriosis compared with SP from healthy women **A)** Telomerase activity (TA) in healthy SP (n=6) and in SP from women with endometriosis (n=10), **B)** Human telomerase RNA (*hTERC)* mRNA in healthy SP (n=8) and SP from women with endometriosis (n=10). The *hTERC* RNA was normalised to the geometric mean of peptidylprolyl Isomerase A (*PPIA)* and beta actin (*ACTB*) reference genes, **C)** *DKC1* mRNA in healthy SP (n=10) and eutopic SP from women with endometriosis (n=10). The *DKC1* mRNA was normalised to the geometric mean of *PPIA* and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) reference genes. *p<0.05, **p<0.01, ***p<0.001.

3.4.2.2 *DKC1* mRNA level

DKC1 mRNA level appeared to be higher in endometrial samples collected from women with endometriosis compared with secretory endometrial samples from healthy women (p=0.5) (Figure 3.8C).

3.4.2.3 Dyskerin protein

I first compared dyskerin immunoreactivity in the epithelium of the three layers of secretory eutopic endometrial (luminal, functionalis and basalis) samples collected from women with endometriosis with the corresponding layers of endometrial samples collected from healthy premenopausal women in the SP (Figure 3.9A). I found that dyskerin immunoscores appeared to be higher in the secretory luminal and functionalis epithelial regions of women with endometriosis compared with their correspondent layers in healthy endometrium (Figure 3.9B). Then I compared the endometriotic ectopic samples (Figure 3.9C) with eutopic SP luminal, functionalis and basalis epithelium from samples collected from women with endometriosis: a significant difference in dyskerin immunoscores between ectopic samples compared to any of the distinct regions of the eutopic endometrium was not found (p=0.5, p=0.8, p=0.4 respectively) (Figure 3.9C). In the same SP samples (healthy and endometriosis), I compared dyskerin immunostaining in functionalis epithelial and stromal cells. I found that dyskerin immunoscores was persistently and significantly higher in epithelium compared with stroma in both healthy samples and in samples from women with endometriosis (Figure 3.9D). There was no difference in dyskerin immunoscores in functionalis stroma in the presence or absence of endometriosis (Figure 3.9D).

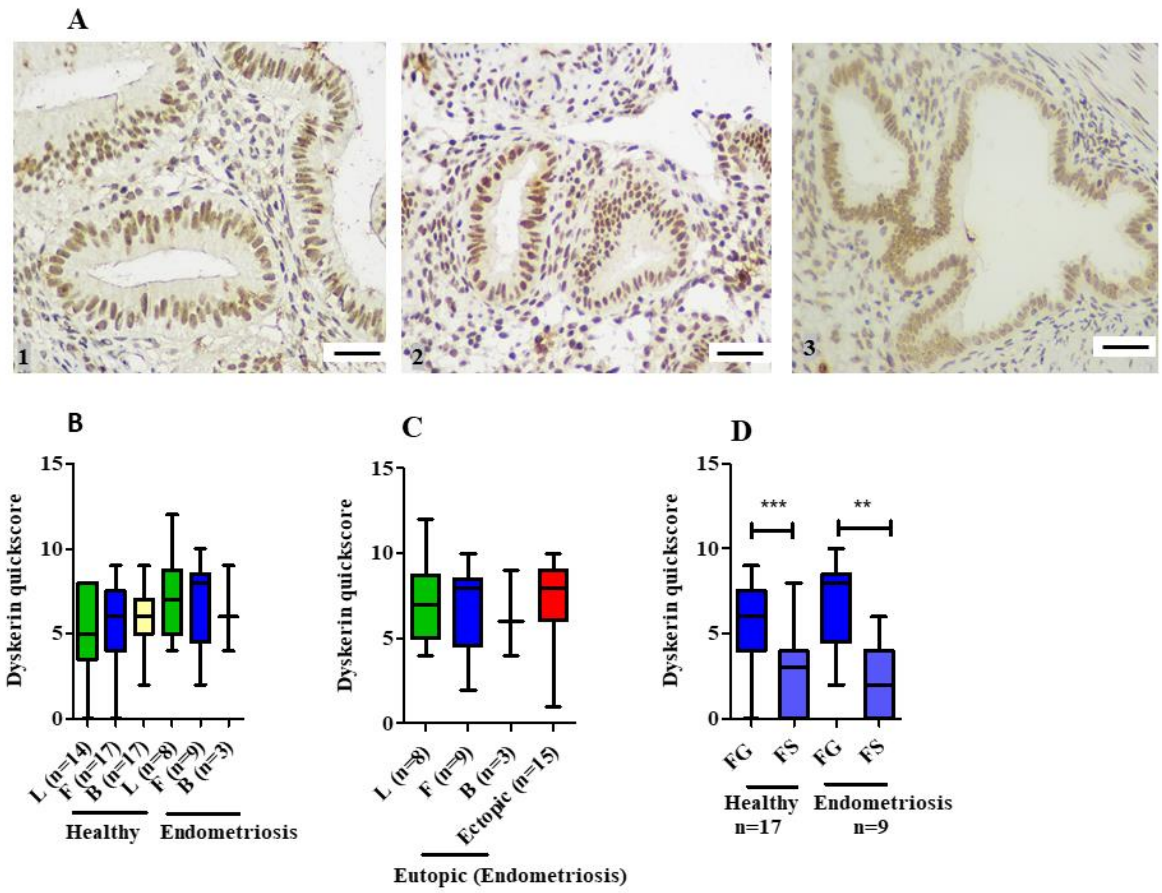

Figure 3.9 Dyskerin immunostaining in healthy secretory, secretory endometrium from endometriotic patients and ectopic endometriotic tissue **A)** Representative microphotographs illustrating dyskerin IHC staining in endometrial healthy secretory (**1**), eutopic secretory from endometriotic patient (**2**) as well as ectopic endometriotic tissue (**3**). Positive staining appears brown. Magnification 400X. Scale bar 50µm. **B)** Dyskerin immunoscores comparison in the three endometrial layers luminal (L), functionalis (F) and basalis (B) in healthy secretory phase and secretory eutopic endometrial samples from patients with endometriosis. **C)** Dyskerin immunoscores comparison among L, F and B endometrial layers from eutopic secretory phase (SP) endometriotic samples and ectopic endometriotic lesions. **D)** Dyskerin immunoscores in functionalis glands (FG) and stroma (FS) of SP samples from healthy women and from women with endometriosis *p<0.05, **p<0.01, ***p<0.001.

3.5 Discussion

This chapter presents novel data on dyskerin protein and *DKC1* mRNA levels in healthy pre- and postmenopausal human endometrium for the first time. Dyskerin (*DKC1*) mRNA and protein were also present in the human myometrial samples in both pre- and postmenopausal human samples. *DKC1* mRNA appeared to be higher in myometrium compared with endometrium.

In the present study, I demonstrated that both *hTERC* and *DKC1* mRNA levels in the normal uterus to be similar, in fractionated endometrial compartments (epithelial and stromal), with no difference observed between the mRNA levels related to the two genes in stromal and epithelial cells. There was no significant difference in the mRNA levels of *hTERC* or *DKC1* genes between proliferative and secretory phase endometrial samples. Furthermore, endometrial *hTERC* RNA levels were consistent with *DKC1* levels, reduced in healthy premenopausal endometrium and increased in postmenopausal samples. Therefore, it may suggest a common regulatory pathway for both genes. There is some prior evidence for such association, for example, the A353V mutation in *DKC1* gene, which is the most common mutation in X linked dyskeratosis congenita (X-DC) patients, was reported to cause a reduction in the levels of *hTERC* RNA and TA(Mochizuki et al., 2004) while another report revealed that loss of dyskerin to cause a degradation of *hTERC* and reduction in TA *in vivo*(Shukla et al., 2016). Further studies also suggest dyskerin to be involved in the stabilisation of *hTERC*, and impairment of dyskerin to result in a reduction of *hTERC* levels and TA, causing premature telomere attrition (Mitchell et al., 1999b). In breast cancer tissue, when *DKC1* mRNA levels were very low, the TA was significantly decreased, independently of the value of *hTERT*. Additionally, *in vitro* experiments showed that *DKC1* gene knock-down by RNA interference in breast cancer (MCF-7) cell line resulted in a significant reduction in *DKC1* mRNA and influenced the TA via the reducing *hTERC* levels and TA was only reduced when *hTERC* levels were markedly diminished (Montanaro et al., 2008). This might explain some of the findings of the present study, high TA levels observed in proliferative endometrium, when *DKC1* and *hTERC* levels were low. However, in the PM endometrium, where TA was low, *DKC1* was high; therefore, I propose that the association between *DKC1* and *hTERC* may or may not have a direct consequence on TA. In the healthy proliferative endometrium, which is known to have high proliferative activity, I observed a lower level of dyskerin protein and *DKC1* gene compared with postmenopausal endometrial samples. Ge et al. using an inducible deletion

approach to knock out *DKC1* in adult mice, found that only liver could survive with *DKC1* gene deletion for several months, while most tissues could not survive without dyskerin and became populated by cells that had escaped the deletion (Ge et al., 2010). In knockout animals, the turnover rate of hepatocytes in the liver was higher compared with the rate in wild-type animals. This might be due to the activation of apoptosis in a small fraction of cells and their replacement hepatocytes division that had presumably escaped the deletion (Ge et al., 2010). Whether low dyskerin would facilitate cell proliferation, is yet to be examined. My data also show that *DKC1* mRNA levels tended to be higher (although not reaching statistical significance) in the secretory phase eutopic endometrial tissue from women with endometriosis than that of healthy women with surgically excluded endometriosis. In addition, in the current study, dyskerin immunoscores appeared to be higher in the glands of luminal and functionalis layers of eutopic (secretory phase) endometrium of women with endometriosis compared with the corresponding layers obtained from secretory endometrial samples from healthy women. Researchers have found that the endometrium from patients with endometriosis is different from the endometrium of healthy women (Hapangama et al., 2019), which include high TA, longer mean TL, pro-proliferative and lower levels of expression of cellular senescence markers (Hapangama et al., 2010, Hastings and Fazleabas, 2006) in late-secretory endometrium of women with endometriosis (Hapangama et al., 2008b). Some of those endometrioses associated features will allow the endometrial cells to proliferate, implant and grow at ectopic sites and to escape the unfavourable condition in their new environment. In our study, there was no significant difference in dyskerin immunoreactivity in the epithelium of any of the 3 eutopic (SP) endometrial layers (luminal, functionalis or basalis) from women with surgically diagnosed endometriosis and ectopic endometriotic lesions. The highest dyskerin immunoscores were in functionalis layer, which is comparable to that in ectopic lesions. This may suggest similarity between the epithelial cells from functionalis endometrium to the establish ectopic deposits in the pelvis, in the context of dyskerin levels. The functional relevance of this observation is yet to be explored.

To characterise my sample set, in the context of telomerase, I initially examined the TA levels in our endometrial and myometrial samples. The data are in accordance with previously published studies (Hapangama et al., 2008b); I found that both healthy secretory endometrial and myometrial samples to have a low TA (Hapangama et al., 2008b).

A previous report using TRAP (stretch PCR) assay showed that TA is localised specifically in the endometrial epithelial cells (Tanaka et al., 1998); however, by using a more sensitive TRAP-ELISA assay I was able to demonstrate that TA was also present in endometrial stromal cells, which is consistent with other published studies using the same TRAP-ELISA assay (Valentijn et al., 2015). However, sorted epithelial cells appeared to have a higher TA compared with the corresponding stromal cells. A previous study also reported similar data, demonstrating higher TA in epithelial cells than the corresponding stromal cells, in the proliferative phase sample(Valentijn et al., 2015). Quantitative analysis of TA showed that it is regulated in a menstrual-phase-dependent manner and the levels altered markedly during the menstrual cycle (Tanaka et al., 1998). My results revealed the PP endometrium to have higher TA than that observed in SP and PM endometrial samples, agreeing with previous reports(Tanaka et al., 1998, Williams et al., 2001).

TA, in the current study, was higher in secretory phase endometrium from women with endometriosis compared with the same from healthy women, similar results also reported by a number of previous studies (Hapangama et al., 2008b, Mafra et al., 2014, Sofiyeva et al., 2017). I also found that *hTERC* RNA levels were significantly higher in SP endometrial samples from women with endometriosis compared with healthy secretory endometrium, that had not been previously studied (Hapangama et al., 2008b, Kim et al., 2007, Liu and Lang, 2011); thus, I proposed that the high *hTERC* RNA seen in the eutopic endometrium of women with the benign proliferative disease, endometriosis, is a reflection of increased TA, but dyskerin may not play a major role in the pathology of endometriosis.

The major limitations of this study include small sample size; and mRNA of ectopic lesions was not assessed. Additionally, the eutopic and ectopic endometriotic lesions used in this study were not from the same woman but matched for the cycle phase.

Future works are warranted mainly by increasing the sample size, evaluating *DKC1* and *hTERC* RNA levels in ectopic lesions, and by considering the use of a technique, such as a laser capture microdissection, which can be employed to extract a particular cell type from a frozen tissue section. This method will allow direct comparison and further in-depth to analyse endometrial epithelial and stromal cells.

In conclusion, I have demonstrated for the first time that *DKC1* gene is expressed and dyskerin protein is present in normal human endometrium across the menstrual cycle and that the levels

of the mRNA and protein were significantly higher in proliferatively quiescent postmenopausal samples, compared with the highly proliferative premenopausal endometrial samples from the proliferative phase. Additionally, I found that the level of *hTERC* RNA has a similar pattern with *DKC1* mRNA in healthy samples. Thus, I propose that dyskerin may either relevant to or influence endometrial glandular proliferation and decreased dyskerin protein levels may influence *hTERC* levels. These data warrant further investigation, particularly to examine if dyskerin is differentially expressed in benign and malignant proliferative diseases of the human endometrium.

Therefore, in chapter 4, I sought to examine alterations in dyskerin (*DKC1*) levels in the malignant proliferative condition of the human endometrium, EC samples from a local cohort of patients and from the uterine cancer cohort in TCGA dataset.

4

Chapter Four

Aberrant Dyskerin Expression is Associated with Poor Survival in Endometrial Cancer

4.1 Introduction

Telomeres are specialized nucleoprotein complexes consisting of tandem repeats of TTAGGG and associated specific shelterin proteins (de Lange, 2005). They prevent chromosomal ends from being identified as DNA damage and protect them from degradation and end to end fusion (Griffith et al., 1999, van Steensel et al., 1998). With each round of cell division, most human somatic cells lose about 50bps of telomeric DNA due to the inability of the conventional DNA replication mechanism to overcome the end replication problem (Levy et al., 1992). Therefore, in mitotic cells, telomeres can reach a critically short length resulting in the induction of cellular processes such as apoptosis and senescence (Campisi and d'Adda di Fagagna, 2007). Telomerase is a specialised reverse-transcriptase which maintains and elongates telomeres at the 3' ends of chromosomes (Blackburn et al., 1989). It is composed of the template-containing *TERC*, the catalytic component of the enzyme, hTERT; and the dyskerin protein (Venteicher et al., 2009). In most human somatic cells, TA is either undetectable or very low (Kim et al., 1994). However, human cells with high replicative demands such as lymphocytes (Liu et al., 1999) and epithelial cells (Yasumoto et al., 1996) as well as tissue stem cells have active or inducible telomerase (Hiyama and Hiyama, 2007). The human endometrium is a highly regenerative tissue with a dynamic TA corresponding to the epithelial proliferation status (Valentijn et al., 2015, Hapangama et al., 2017). Most cancer cells express constitutively high TA that provides cancer cells with an indefinite proliferative ability (Khattar et al., 2016).

EC is the commonest gynaecological malignancy in developed countries, with an increasing incidence associated with obesity and longevity (Hapangama et al., 2017). In an era of reducing cancer-related deaths reported for most other cancers, mortality due to EC is expected to increase (CRUK). Therefore, novel biomarkers to stratify high-risk patients for therapy, and novel therapeutic targets are urgently needed to reduce the rising EC associated mortality and morbidity.

High TA has been reported in over 90% of all ECs (Alnafakh et al., 2019). *hTERT* and *hTERC* expression levels and TA measured by TRAP assay have been previously reported in the healthy endometrium (Valentijn et al., 2015) and in ECs (Kyo et al., 1996, Ebina et al., 1999), yet dyskerin, which makes the foundation of the H/ACA lobe structure of the telomerase holoenzyme, has not been studied in normal or pathological endometrium. Dyskerin subunits bind to the base of each RNA hairpin and bind to the telomerase associated proteins NHP2, NOP10, GAR1 (Hapangama et al., 2017). The dyskerin protein is encoded by *DKC1* gene

located on the X chromosome (Heiss et al., 1998) and it stabilises *hTERC,* and enhances TA (Mitchell et al., 1999b, Montanaro et al., 2008). Dyskerin may also have an extra-telomeric function in ribosomal biogenesis (Montanaro et al., 2006, Ruggero et al., 2003).

Excessive function of dyskerin as well as its loss may be carcinogenic (Alawi and Lin, 2011, Alnafakh et al., 2019). High dyskerin levels have been reported in several human cancer types, for example, in breast and prostate cancers (Sieron et al., 2009, Montanaro et al., 2006). However, the reduction of dyskerin protein in primary human pituitary tumours resulted in an alteration of the translation of a group of mRNAs that contain an IRES and has been linked to carcinogenesis (Bellodi et al., 2010). Low dyskerin levels observed in DC (Parry et al., 2011) have been associated with an increased cancer-susceptibility before the age of 30 (Alter et al., 2009). In DC patients, a defective ribosomal function might influence the translation of a group of mRNAs encoding for tumour suppressors, and this may explain the apparent paradox of DC in which diminished cell proliferation is linked with an increase in the susceptibility to cancer (Montanaro, 2010). This observation is also in agreement with the only available animal model, where half of the hypomorphic *DKC1* mutant *(DKC1*^{m}) mice (with decreased DKC1 expression) developed various malignancies (Ruggero et al., 2003).

Since endometrial carcinogenesis is associated with dysregulation of TA, and for the reason that dyskerin and TA are intricately linked, I hypothesised that dyskerin expression levels might also be dysregulated in ECs. In this chapter, I have examined therefore *DKC1* RNA levels, somatic mutations and copy number alterations initially in the largest publicly available EC dataset TCGA; and then progressed to analyse dyskerin protein levels in endometrial samples derived from a local cohort of EC patients as well as healthy women, and those with premalignant hyperplastic endometrium.

4.2 Research questions

TCGA EC cohort:

1. Are the *DKC1* RNA levels altered in EC? And do they correlate with the RNA expression levels of other telomerase core components (*TERC* and *TERT*)?

2. Do *DKC1* RNA levels correlate with tumour grade or stage?

3. Is *DKC1* RNA levels correlate with the patient outcome?

Our Local EC cohort:

1. Are the *DKC1* RNA levels and protein levels altered in EC?

I tested the hypothesis that EC expresses dyskerin (*DKC1*), in the context of TA and *hTERC*. Both dyskerin protein (IHC and western blotting) and *DKC1* mRNA expression levels were examined.

2. How do dyskerin protein levels change in various stages of endometrial carcinogenesis; EH, different EC histological subtypes, surgical stage and metastatic lesions? And can dyskerin protein be utilised to stratify ECs into low-risk and high-risk groups for EC recurrance/ Poor outcome?

3. Do dyskerin protein levels correlate with clinicopathological parameters of the EC patients?

4. Is dyskerin a useful biomarker to predict cancer progression, cancer-specific or overall incidence of death, independently of other prognostic factors?

4.3 Material and Methods

4.3.1 Study groups:

4.3.1.1 TCGA Endometrial cancer cohort

The publicly-available TCGA cohort of ECs as outlined in Section 2.2.5.2, included data for RNA levels (n=471), copy number variation (n=464) and somatic mutation (n=235) for *DKC1* that was interrogated using Illumina's Base Space Cohort Analyzer application (BSCA)(Kupershmidt et al., 2010) (Software; https://www.illumina.com/informatics/research/biological-data-interpretation/nextbio.html; Illumina, San Diego, CA, USA) (Robinson et al., 2010).

4.3.1.2 Study cohort

Endometrial samples collected from women undergoing hysterectomy in the Liverpool Women's Hospital and Lancashire Teaching Hospitals Trusts from 2009 to 2017 were included in the study. Their demographic data are shown in Table 4.1. None of the patients received hormonal treatments, chemotherapy or pelvic radiation before surgery. Endometrial samples were categorised into healthy PP (n=21), healthy PM (n=35), EHA (n=15), and (n=109) EC samples. Further 30 metastatic EC lesions resected from distant sites, lymph nodes (n=11), omentum (n=7), parametrium (n=5), soft tissue (n=4), fallopian tube (n=1), cervix (n=1), and urinary bladder (n=1) from primary surgery were also included. Experienced gynaecological pathologists confirmed the histological type and grade of EC specimens according to FIGO classification (Pecorelli, 2009). Considering the clinically relevant outcome, EC samples were further categorised to LGEC, consisting of grade 1 and grade 2 endometrioid EC or HGEC, including grade 3 endometrioid, serous, clear-cell carcinomas, carcinosarcoma, Mixed cell adenocarcinoma and dedifferentiated ECs (Voss et al., 2012) as shown in Table 4.1. Healthy samples were assigned to PP and PM groups according to the last menstrual date and histological criteria (Noyes et al., 1975). Patient clinicopathological and demographic details were obtained by review of clinical notes in electronic hospital databases (Somerset cancer registry, Meditech and EDMS).

4.3.2 IHC

Standard IHC was performed on 3µm serial sections of FFPE endometrial tissue employing heat-induced antigen retrieval, and ImmPRESS Polymerised Reporter Enzyme Staining System (Vector Laboratories, Peterborough, UK) as outlined in 2.5.1.1. The primary antibody sources, concentrations and incubation conditions are detailed in Table 4.2.

4.3.2.1 IHC analysis/quantification

The nuclear dyskerin immunoreactivity in endometrial epithelial cells was assessed using a modified quickscore (Mathew et al., 2016). The final score out of 12 was calculated by multiplying the category of the percentage of positively dyskerin stained cells (1%-25%=1, 26%-50%=2, 51%-75%=3 or >76%=4) by the category of dyskerin staining intensity (0= no

staining, 1= weak staining, 2= moderate staining and 3= strong staining) (Mathew et al., 2016). The entire section was evaluated at 400x magnification. Epithelial cell staining was scored in the stratum basalis of healthy PP, normal PM, EH and in EC tissues by two independent observers. The level of dyskerin has not been previously reported in EC; therefore, several cut-off points were employed to identify the best value that could determine a group with the worst outcome and worst clinicopathological features. Quickscores 5, 6, 7 and 8 were examined for utility. Samples with scores lower than the tested cut-off figure were considered as low dyskerin, and those with scores equal to or more were considered as high dyskerin for the tested cut-off points. The score of 6 showed the best categorisation, thus considered as the final cut-off figure for this study.

Table 4.1: Demographic features of study groups

Study groups	No	%	*Age (years)	**BMI (kg /m2)
Healthy	56			
Proliferative phase	21		40(30-57)	27(18-41)
Postmenopausal	35		63(40-85)	26(20-40)
Endometrial hyperplasia	15		55(48-72)	36(24-57)
Endometrial cancer	109		68(37-96)	30(20-54)
LGEC	53	48.6	64(37-89)	32(21-54)
Endometrioid Grade 1	34	31.2	64(46-89)	33(21-53)
Endometrioid Grade2	19	17.4	60(37-78)	30(22-54)
HGEC	56	51.4	73(48-96)	30(20-43)
Endometrioid Grade 3	12	11	68(54-96)	28(24-43)
Serous	12	11	76(64-87)	29(23-39)
Clear cell	10	9.1	74(48-82)	30(27-39)
Carcinosarcoma	19	17.4	78(60-89)	26(20-37)
Dedifferentiated	1	0.9	79	32
Mixed cell adenocarcinoma	2	1.8	(63-66)	
Metastatic EC	34		68(27-96)	28(21-43)

Abbreviations: Body mass index (BMI); Low-grade endometrial cancer (LGEC); High-grade endometrial cancer (HGEC); *Data expressed as median (range); **BMI data were available for only 161 cases.

Table 4.2 Primary antibodies and conditions for IHC and immunoblotting

Primary Ab	Type	Clone	Supplier	HIAR* (min)	Dilution	Incubation Time (hour)	Condition Temp (°C)
IHC							
Dyskerin	P		Santa Cruz biotechnology[1]	3	1:500	20	4
Immuno-blotting							
Dyskerin	P		Santa Cruz biotechnology[1]		1:1000	20	4
Pancytokeratin	M	C-11+PCK-26+CY-90+KS-1A3+M20+A53-B/A2	Sigma-Aldrich[2]		1:8000	20	4
GAPDH	P		Sigma-Aldrich[2]		1:10000	20	4

* HIAR by pressure cooking in citrate buffer pH 6. [1]Dallas, Texas, USA; [2]Dorset, UK. **Abbreviations:** Antibody (Ab), Polyclonal (P), Monoclonal (M), Heat-induced antigen retrieval (HIAR), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH).

4.3.3 TRAP assay for TA

TA was measured by our collaborator, Dr Gabriele Saretski from the University of Newcastle, UK, as described in 3.3.3; using a *Telo*TAGGG™ PCR ELISA kit (Roche Diagnostics Ltd, Burgess Hill, UK) according to the manufacturers' manual and as previously described (Valentijn et al., 2015).

4.3.4 RNA extraction and RT- qPCR

Total RNA from tissue samples was extracted as detailed in section 2.7.1.1.1 and as previously described. cDNA was synthesised, treated with DNase and amplified by qPCR as described in 2.7.5. *DKC1* expression was normalised to the reference genes *YWHAZ* and *PPIA*. Relative

expression of *hTERC* was calculated against the reference genes *PPIA* and *ACTB*; the primers used are listed in Table 2.4. For each target and reference gene, a standard curve was constructed, and primer efficiency was determined (Figure 2.2). PCR products were resolved on an agarose gel to confirm the specificity of the primers and to exclude any off-target amplicons (Figure 3.1).

4.3.5 SDS-PAGE and immunoblotting

Protein from homogenised tissues was extracted in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors as described in 2.5.2. Lysates were analysed by SDS-PAGE under reducing conditions on precast 12% gels and transferred to an Immune-Blot PVDF. The primary antibody sources, concentrations and incubation conditions are detailed in Table 4.2. HRP-linked secondary antibodies were from (ThermoScientific, Loughborough, UK). Signal detection was performed using SuperSignal™ West Dura Extended Duration chemiluminescent Substrate and CL-Xposure film.

4.3.6 Statistical analysis

Statistical differences were calculated by non-parametric tests (Kruskal–Wallis or Mann–Whitney U-test) as appropriate using Statistical Package for the Social Sciences (SPSS) version 25 (IBM Corp, Armonk, NY, USA). Graphs were plotted using GraphPad Prism 5 (GraphPad Software, La Jolla California USA). Spearman rank correlation or Pearson correlation were used to analyse associations. All the observations were censored at the last date at which the patient was seen, and Kaplan-Meier survival curves were constructed. Further details are described in 2.11; p value <0.05 was considered to be significant.

4.4 Results

4.4.1 *In silico* interrogation of the TCGA endometrioid and serous EC dataset demonstrates dysregulation of *DKC1* to be associated with a poor survival

Analysis of the TCGA dataset demonstrated a more than 2-fold upregulation of *DKC1* RNA levels in 69/471 (14.65%) of the endometroid and serous ECs compared with a set of normal endometrial samples derived from a distance of 2-3 cm from the tumour margin. The normal samples were obtained from 35 EC patients (Huang et al., 2016a). High *DKC1* RNA level was significantly associated with poor prognosis (p=2E-5, cox-regression =0.91), as shown in Figure 4.1.

The mutation frequency of the *DKC1* gene in ECs was low (9/235, 3.69%) and consisted of mainly missense mutations that occurred in a background of no *TERC* gene mutations, (Figure 4.2). Patients with ECs harbouring mutant *DKC1* gene seemed to have a better clinical outcome compared with cancers that carry a wild-type *DKC1* gene (Figure 4.3). ECs also had a copy number variation (mostly loss) of the *DKC1* gene in 20/464 (4.31%). In the TCGA dataset, *DKC1* RNA level did not correlate with the tumour grade (r^2=0.19, p=9.61E-23) or clinical stage (r^2=0.02, p=7.27E-4), (Figures 4.4A & B). Similarly, there was no correlation observed between RNA levels of *DKC1* with steroid receptors genes, *TERT* (r^2=0.03, p=3.43E-4), (Figure 4.5A) and *TERC* expression (r^2=0.04, p=1.62E-4), (Figure 4.5B). High DKC1 RNA levels were observed in TP53 mutated ECs (p=1.23E-8) (Figure 4.6A), but contrastingly lower DKC1 RNA levels were observed in FGFR2 (p=7.90 E-3) (Figure 4.6B), PTEN (p=2.90E-6) (Figure 4.7A), PIK3R1 (p=0.02) (Figure 4.7B) and CTNNB1 (p=1.67E-3) mutated ECs. No significant difference in DKC1 expression was observed in TERC, TERT, POLE, PIK3CA, KRAS, and ARID1A mutated ECs compared with un-mutated EC samples.

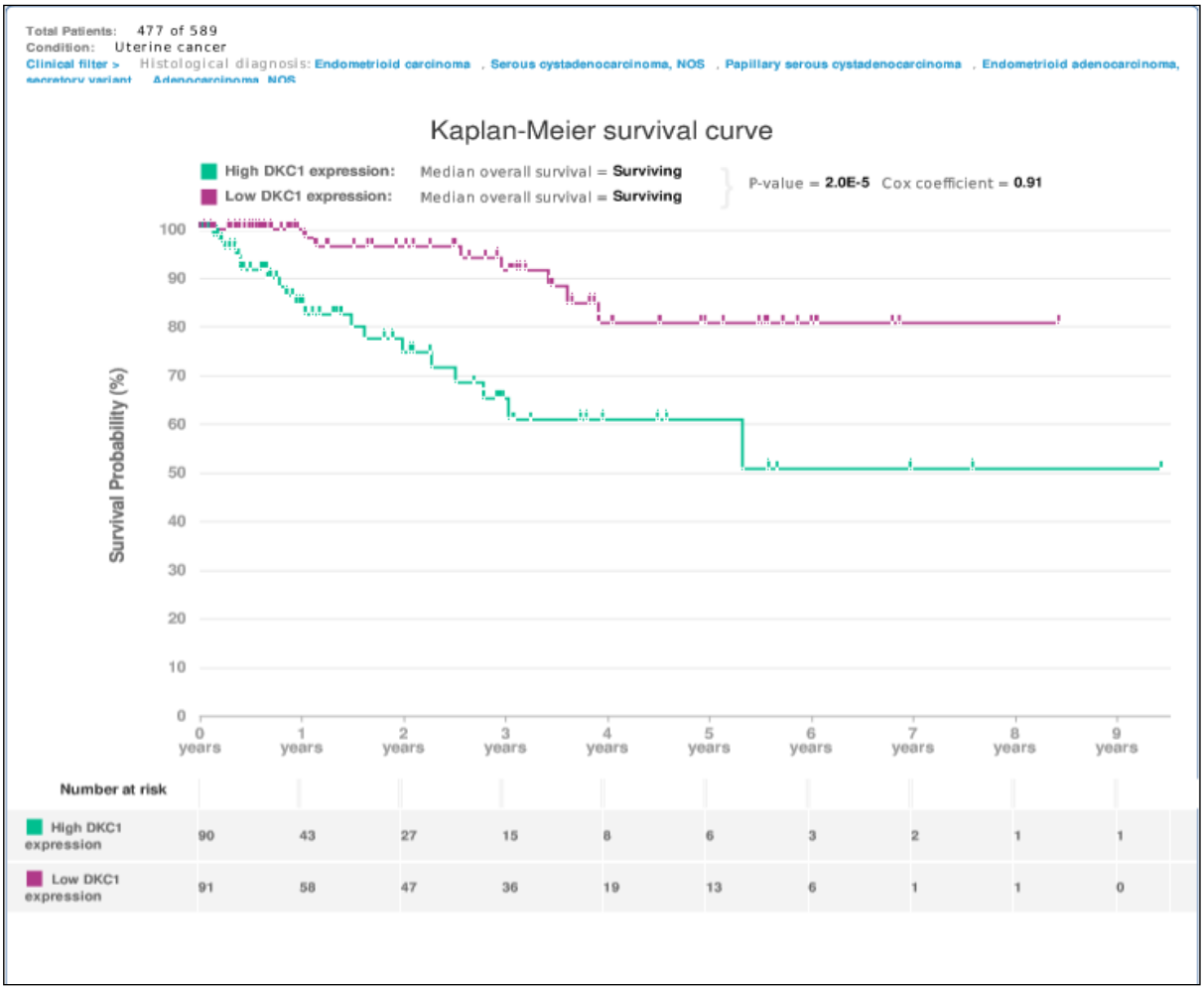

Figure 4.1 Kaplan-Meier survival curve for the association between *DKC1* RNA level and overall survival, (P=2E-5, cox-regression =0.91) in The Cancer Genome Atlas (TCGA) dataset (endometrioid and serous endometrial cancer) (n=477).

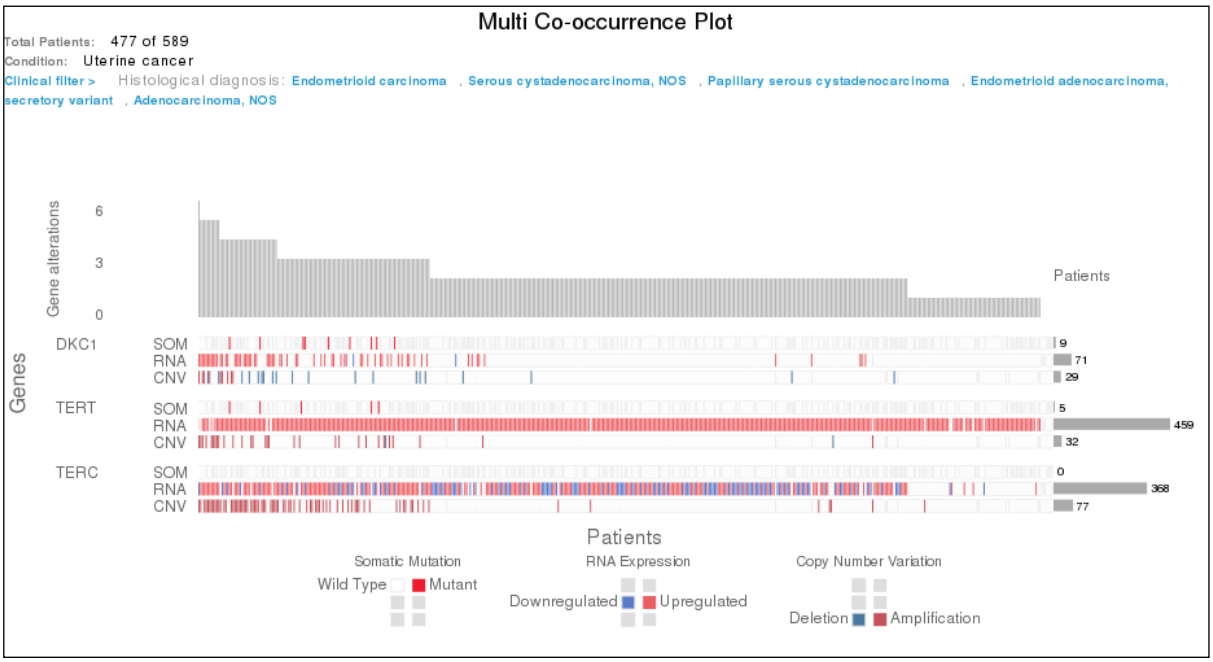

Figure 4.2 Multi co-occurrence plot of somatic mutation, RNA level and copy number variation of *DKC1*, *TERT* and *TERC* genes in The Cancer Genome Atlas (TCGA) dataset (endometrioid and serous endometrial cancer) (n=477)**.**

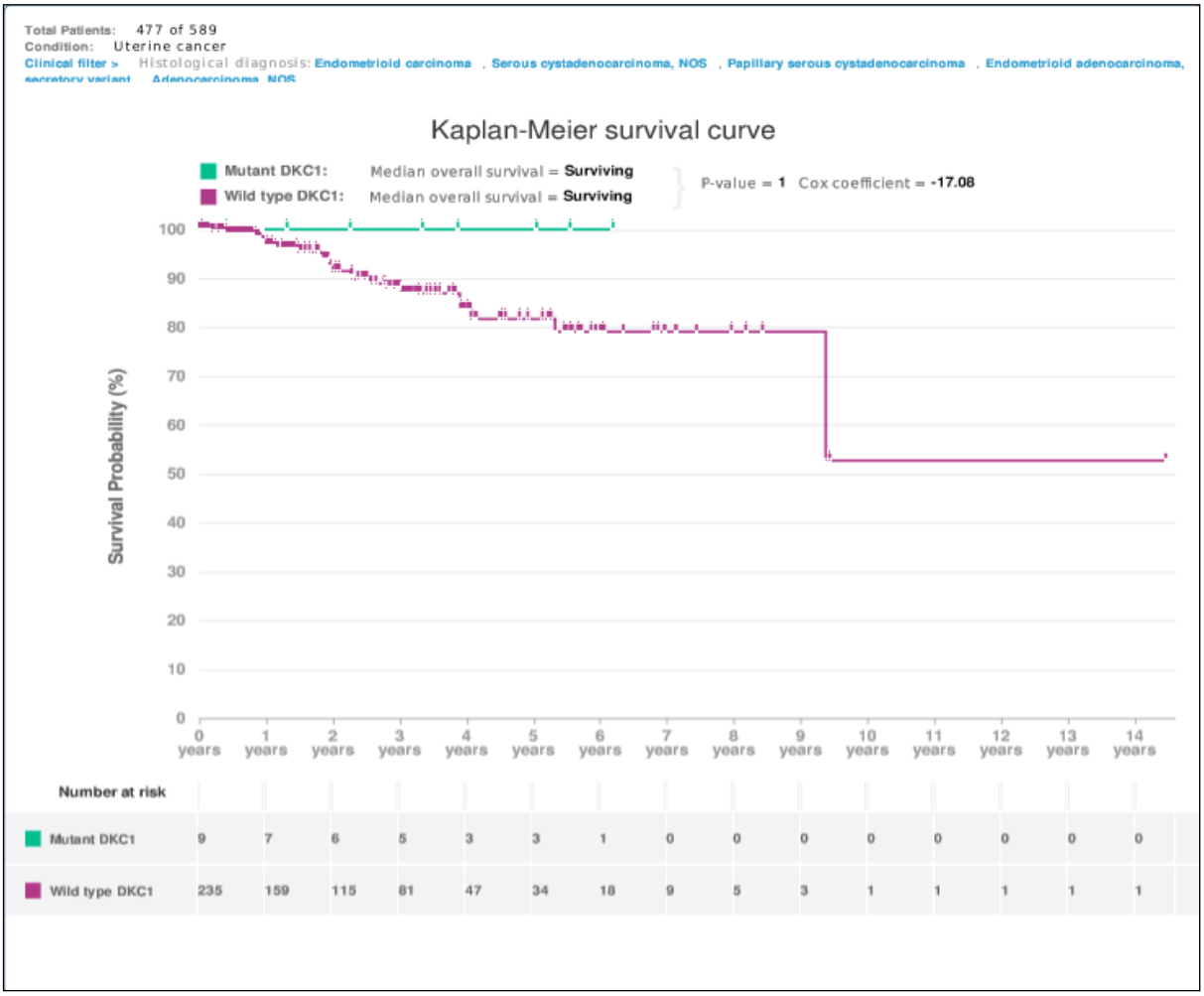

Figure 4.3 Kaplan-Meier survival curve shows the difference in overall survival between endometrial cancers (ECs) harbouring a mutant *DKC1* gene and ECs carrying the wild-type *DKC1* gene, in The Cancer Genome Atlas (TCGA) dataset (endometrioid and serous EC) (n=477), (p=1, cox-regression =-17.08).

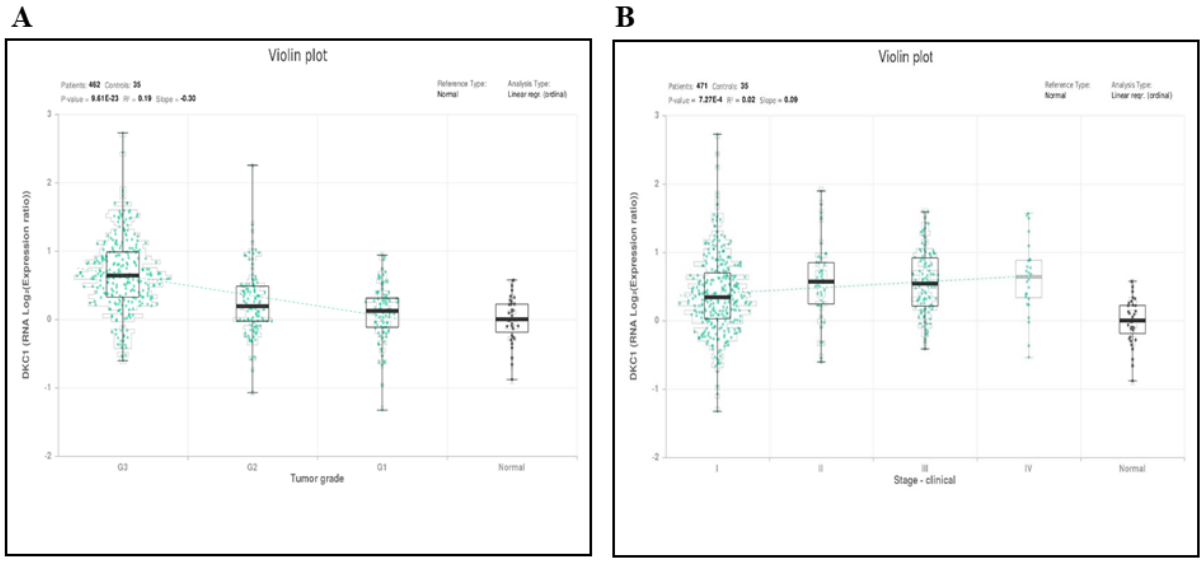

Figure 4.4 Violin plot demonstrating the correlation between *DKC1* RNA levels with **A)** Tumour grade (n=462), controls (n=35), (r^2=0.19, p=9.61E-23), analysis type linear regression, reference: normal tissue and **B)** Stage (n=471), controls (n=35), (r^2=0.02, p=7.27E-4), analysis type linear regression.

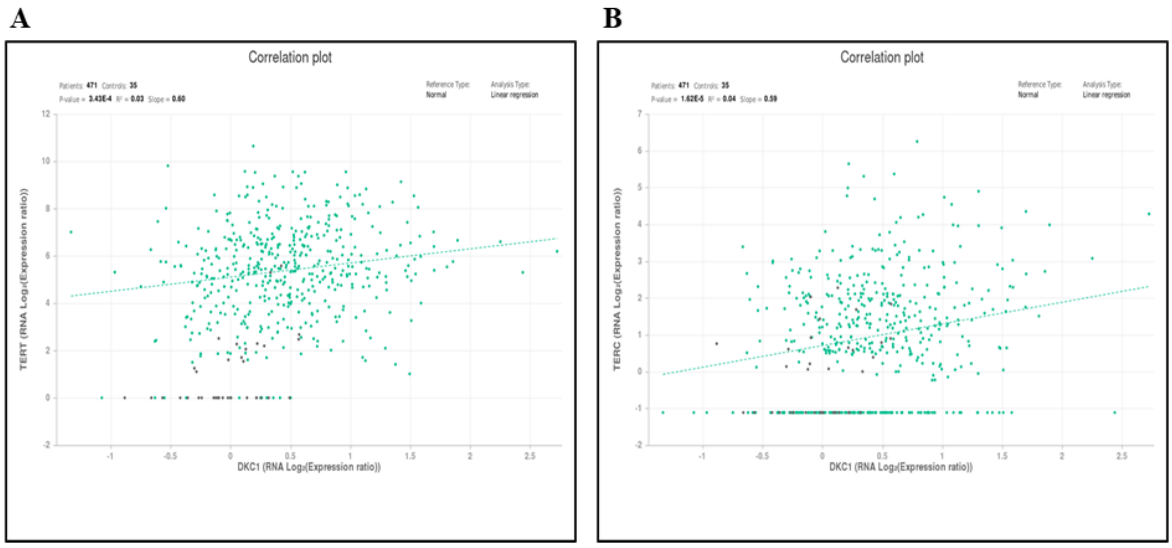

Figure 4.5 In The Cancer Genome Atlas (TCGA) dataset (endometrioid and serous endometrial cancer), *DKC1* RNA levels correlation with **A)** *TERT* RNA levels, (r^2=0.03 p=3.34E-4) and with **B)** *TERC* RNA levels, (r^2=0.04 p=1.62E-5). Patients (n=471), controls (n=35), analysis type linear regression.

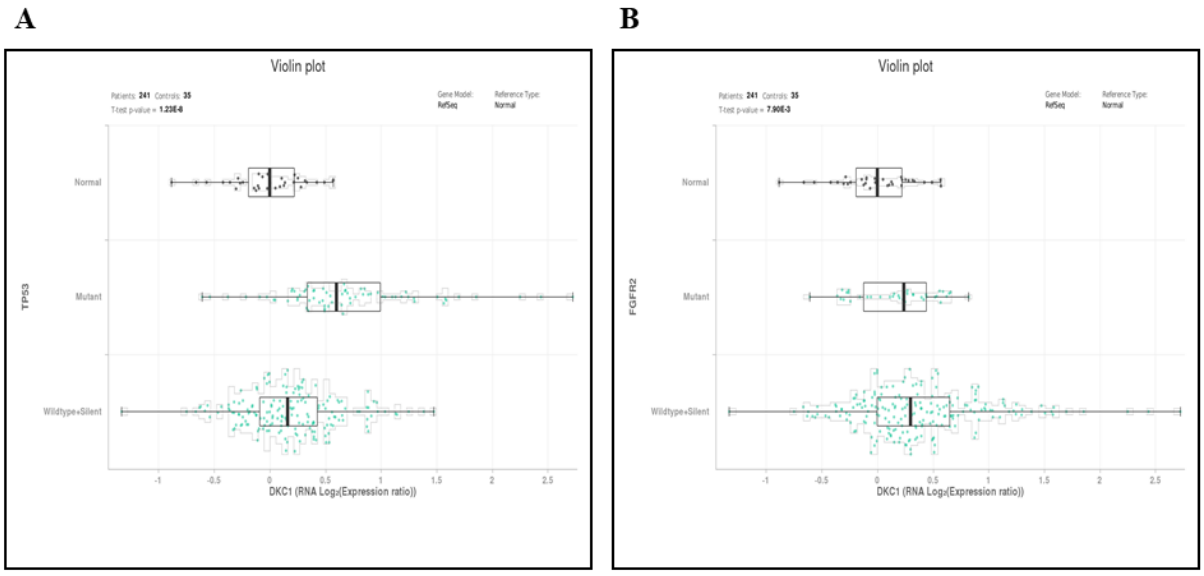

Figure 4.6 In the Cancer Genome Atlas (TCGA) dataset (endometrioid and serous endometrial cancer), violin plot shows the association between DKC1 RNA levels and mutation status: normal, mutant or wild-type + silent of **A)** *TP53* gene, t-test (p=1.23E-8), **B)** *FGFR2* gene, t-test (p=7.90E-3). Patients (n=241) and normal reference (n=35).

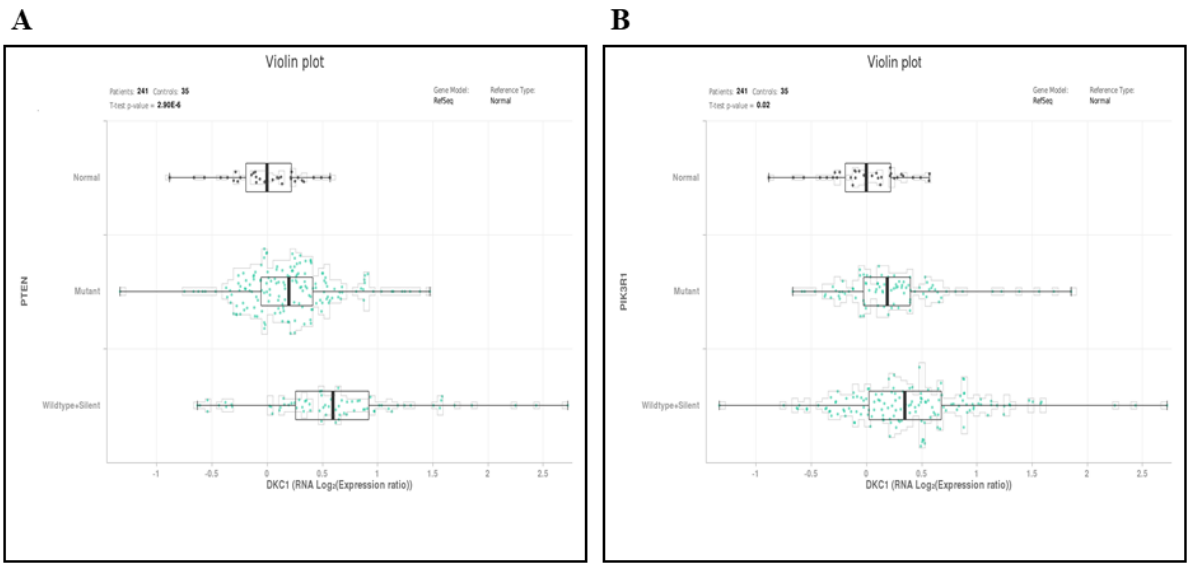

Figure 4.7 Violin plot shows the association between *DKC1* RNA levels and mutation status: normal, mutant or wild-type + silent of **A)** *PTEN* gene, t-test (p=2.90E-6), **B)** *PIK3R1* gene, t-test (p=0.02), in The Cancer Genome Atlas (TCGA) dataset (endometrioid and serous endometrial cancer), Patients (n=241) and normal reference (n=35).

4.4.2 Study cohort

4.4.2.1 Demographic data

Detailed demographics of the Liverpool/Lancaster patient cohort containing all subtypes of ECs are presented in (Table 4.1). All women in our EC cohort were PM. Women with high HGEC were significantly older than those with LGEC and healthy PM women ($p<0.001$, $p=0.002$ respectively). Women with serous ECs appear to be older when compared with healthy PM and endometrioid ECs ($p=0.07$, $p=0.09$ respectively). Significantly higher body mass index (BMI) was observed in EH compared with the healthy PM women ($p<0.001$) and in the EC group, there was an apparent, non-significant trend for LGEC group to have higher BMI compared with HGEC ($p=0.06$).

4.4.2.2 Dyskerin protein is significantly reduced in ECs when compared with healthy PM control endometrium.

In contrast to the TCGA data, in our patient samples, *DKC1* mRNA levels appeared to be downregulated in the ECs in comparison with normal endometrium from healthy PM women (p=0.06), (Figure 4.8A). No difference in *DKC1* mRNA level was observed between LGEC and HGEC.

When EC samples were compared with healthy PM endometrium, immunoblotting demonstrated significantly reduced dyskerin protein levels (normalised to the epithelial marker pancytokeratin, p=0.02), (Figures 4.8B & C) but significantly higher TA (p=0.03), (Figure 4.8D). There was no significant difference in h*TERC* RNA levels (P=0.35) between healthy PM and EC samples (Figure 4.8E).

IHC staining revealed the presence of dyskerin protein at a cellular level in both epithelial and stromal cells of the healthy PP and PM endometrium; immunostaining was primarily localised in the nucleus and/or nucleolus, with stronger staining in the epithelial compartment compared with the stromal cells (Figure 4.8F). In the hyperplasia and cancer groups, dyskerin staining was also localised in cell nucleoli and/or nuclei (Figures 4.8F & 4.9A).

Dyskerin immunoscores were significantly lower in PP compared with PM (p=0.03, Figure 4.8G). However, both dyskerin quickscores and *DKC1* mRNA levels did not correlate with TA (Spearman r=-0.12, p=0.16) and (Spearman r=0.04, p=0.77), respectively. Furthermore, no correlation was seen between *DKC1* and *hTERC* RNA levels in endometrial tissue samples. (Spearman r=0.11, p=0.5).

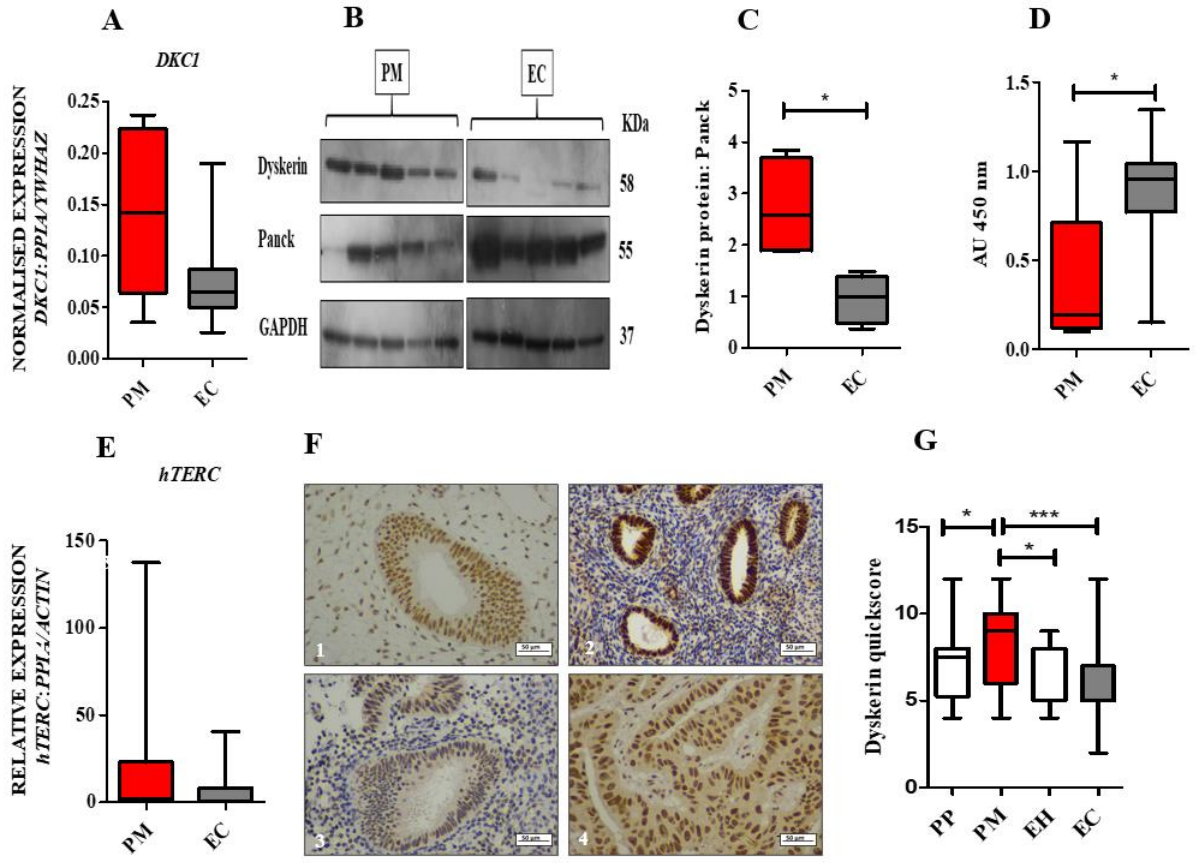

Figure 4.8 *DKC1* mRNA and dyskerin protein in human endometrium. **A)** *DKC1* mRNA is normalised to geometric means of Peptidylprolyl Isomerase A (*PPIA*) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (*YWHAZ*) and measured using qPCR in endometrial tissue samples: Healthy postmenopausal (PM) (n=6) and endometrial cancer (EC) (n=22). **B)** Immunoblotting was performed in healthy PM and EC, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used to ensure equal loading of protein. Dyskerin protein levels in epithelial cells of tissue samples were analysed by normalising to pancytokeratin (Panck). **C)**The amount of dyskerin protein was quantified using Image J in healthy PM (n=5), and EC (n=5), Dyskerin protein in epithelial cells of tissue samples was normalising to pancytokeratin (Panck). **D)** Telomerase activity (TA) in healthy PM (n=6) and EC (n=32) was measured using a Telomere Repeat Amplification Protocol (TRAP) assay. **E)** Human telomerase RNA component (*hTERC*) mRNA is normalised to geometric means of *PPIA* and beta-actin (*ACTB*) and measured by qPCR in endometrial tissue samples: healthy PM (n=7) and EC (n=24). **F)** Representative microphotographs illustrating dyskerin immunohistochemical staining at the cellular level in endometrial samples in (1) normal proliferative phase (PP), (2) healthy PM endometrium, (3) Endometrial hyperplasia (EH) and (4) EC. Positive staining appears brown. Magnification 400X. Scale bar 50µm. **G)** Dyskerin protein quickscores in the endometrium of healthy PP (n=16), PM (n=30), EH with atypia (n=15), EC (n=109). *p<0.05, **p<0.01, ***p<0.001.

4.4.2.3 Loss of dyskerin was a feature of pre-cancerous and cancerous endometrial epithelial cells

Dyskerin immunoscores in EH were significantly lower compared with normal PM endometrial epithelium (p=0.014, Figure 4.9G). ECs showed significantly reduced dyskerin immunoscores compared with healthy PM endometrial samples (p<0.001, Figure 4.9G). There were no significant differences in dyskerin immunostaining among different EC subtypes or between LGEC and HGEC (Figures 4.9B & C).

All ECs in this cohort showed lower dyskerin immunoscores compared with healthy PM endometrial tissue (Figure 4.9A), the difference was significant in endometrioid, carcinosarcoma and clear cell EC (p<0.0001, p<0.0001 and p=0.002, Figure 4.9B) and this reduction remained significant even when the low and high grades were considered separately (LGEC (p<0.001) and HGEC (p<0.001, Figure 4.9C).

The dyskerin immunoscores were significantly lower in advanced clinical stage ECs (FIGO stages III&IV) compared with early-stage (FIGO stages I&II) ECs (P=0.04, Figure 10C).

Metastatic lesions (Figure 4.10A), on the other hand, had significantly higher dyskerin immunoscores compared with matched primary tumours (Figure 4.10B, p=0.003).

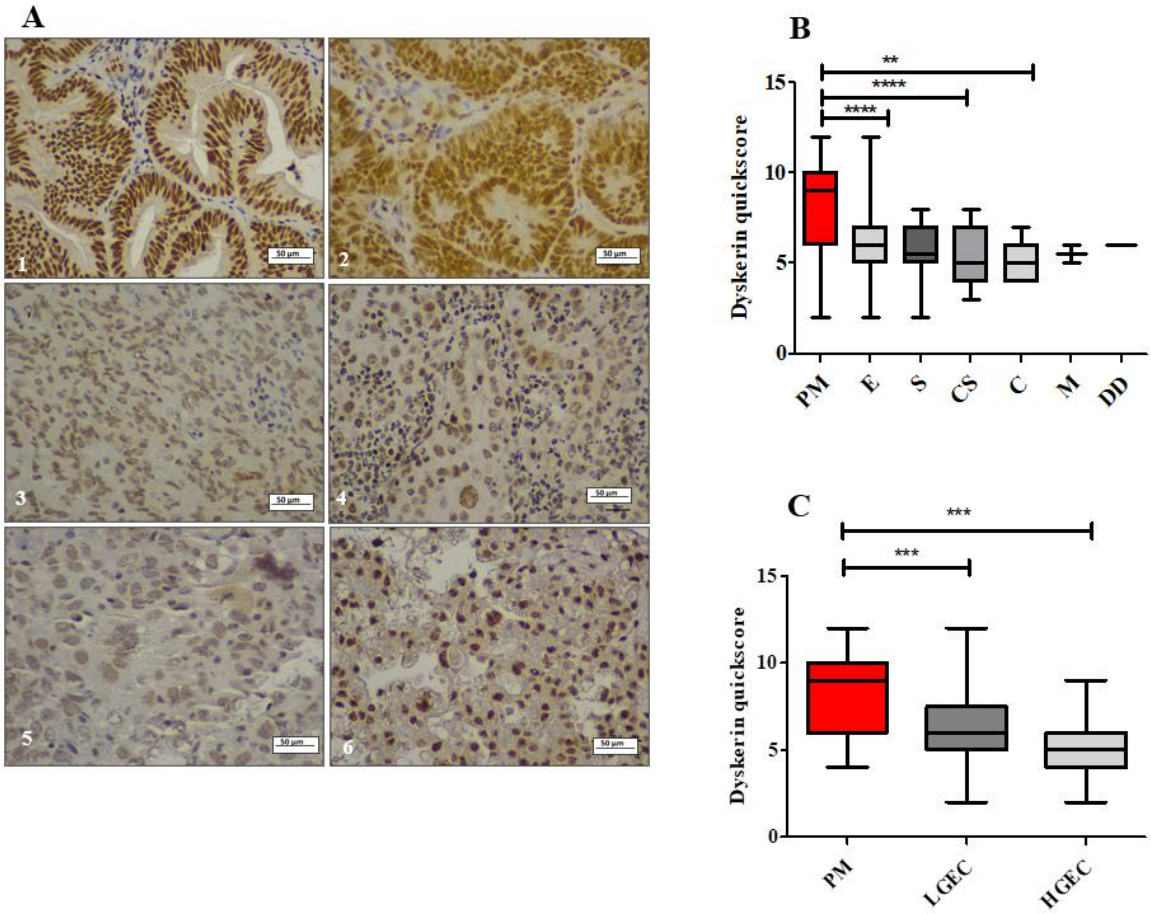

Figure 4.9 Immunostaining of dyskerin in endometrial cancer subtypes (n=109). **A)** Representative microphotographs of dyskerin in human EC. (1-3) grade 1-3 endometrioid carcinoma, (4) serous subtype, (5) Carcinosarcoma and (6) clear cell carcinomas. Positive staining appears brown Magnification 400X. Scale bar 50µm. **B)** Dyskerin immunoscores in healthy PM (n=30) and various EC subtypes including endometrioid (E) (n=65), Serous (S) (n=12), carcinosarcoma (CS) (n=19), clear cell carcinoma (C) (n=10), mixed cell adenocarcinoma (M) (n=2) and dedifferentiated EC (DD) (n=1). **C)** Dyskerin immuno-staining scores in human endometrial epithelium of healthy PM (n=30), LGEC (n=53) and HGEC (n=56). *p<0.05, **p<0.01, ***p <0.001, ****p<0.0001.

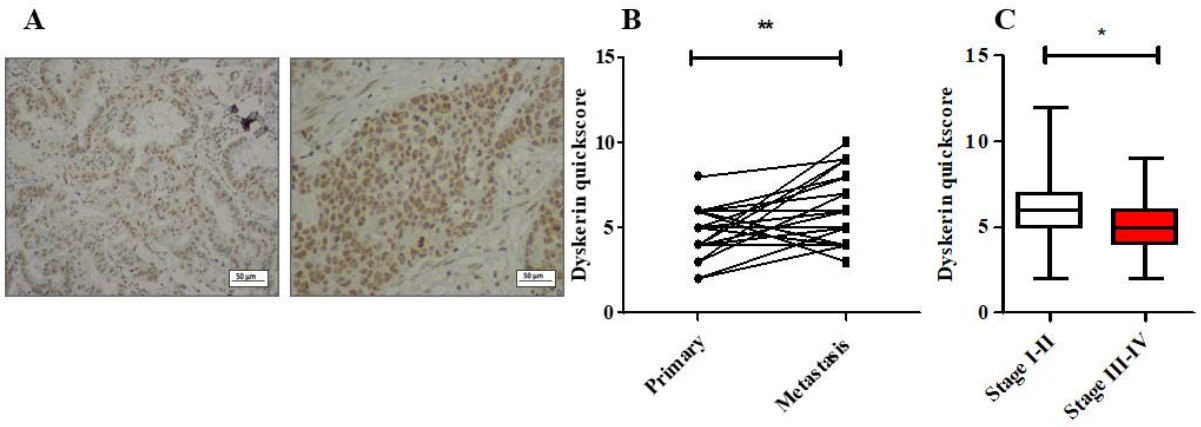

Figure 4.10 Dyskerin immunostaining in metastatic endometrial cancers (ECs) **A)** Representative microphotographs illustrating dyskerin immunohistochemical staining in primary EC (**1**) and matched metastatic lesion (**2**). Positive staining appears brown. Magnification 400X, Scale bar 50μm **B)** Difference in dyskerin immunoscores in primary EC samples versus matched metastatic lesions (n=30) each. **C)** The difference in dyskerin immunostaining between early-stage ECs (FIGO stage I-II) (n=63) and advanced stage ECs (FIGO stage III-IV) (n=43). *p<0.05, **p<0.01, ***p<0.001.

4.4.2.4 Survival analysis

According to national guidance, patients were followed up for at least 3 years after the primary surgery during the study period. By March 2020, follow-up data were available for 108 women in our cohort, and the median follow-up was 24 months (range 2-106 months) (Sundar et al., 2017). During this follow-up period, there were 11 recurrent tumours and 38 deaths (27 as a result of disease progression and 11 from other causes). Worse outcomes were seen in women with low dyskerin immunoscores. All outcomes analysed, including disease-free survival (DFS), cancer-specific survival (CSS) and overall survival (OS) suggested high dyskerin immunoscores to be favourable (P=0.08, P=0.07, P=0.06 respectively, Figure 4.11A 1, 2 & 3). When only endometrioid and serous ECs (n=77) were considered (similar to the available TCGA EC dataset), low dyskerin immunoscores were still suggestive of a worse clinical outcome (DFS, CSS and OS; P=0.1, P=0.3 and P=0.2 respectively, Figure 4.11B 1, 2 & 3).

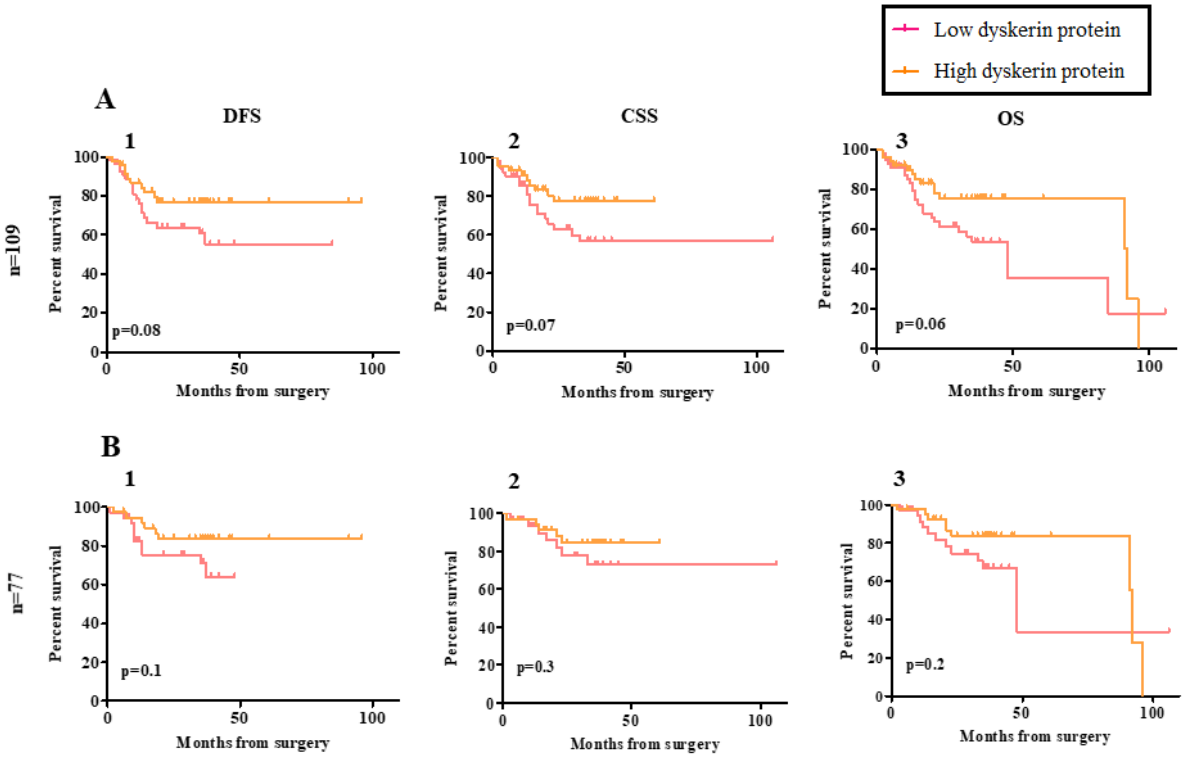

Figure 4.11 Kaplan Meier survival curves for the correlation between dyskerin immunoscores and patient outcome, **A)** Disease-free survival (DFS); (**1**) Cancer-specific survival (CSS) (**2**) and overall survival (OS) (**3**) in endometrial cancer (EC) samples (n=109); **B)** DFS (**1**); CSS (**2**); OS (**3**) in Endometrioid and serous ECs (n=77). Quickscore of 6 was chosen as the cut-off point. *p<0.05, **p<0.01, ***p<0.001.

When the clinicopathological features were considered, dyskerin immunoscores negatively correlated with cervical invasion (P=0.01, Table 4.3).

Table 4.3. The association between dyskerin protein immunoscores and clinicopathological parameters.

			Dyskerin				
Variables		**Total**	**<6**	**%**	**≥6**	**%**	**P-value**
Age	<65	105	23	53	20	47	0.49
	≥65		29	47	33	53	
BMI	<30	85	16	41	23	59	0.53
	≥30		22	48	24	52	
grade	LG	109	22	42	31	58	0.04
	HG		34	61	22	39	
FIGO stage	I-II	106	30	48	33	52	0.4
	III-IV		24	56	19	44	
Myometrial invasion	<50%	109	27	48	29	52	0.49
	≥50%		29	55	24	45	
LVSI	No	108	27	45	33	55	0.16
	Yes		28	58	20	42	
Cervical invasion	No	107	31	42	42	58	0.01
	Yes		23	68	11	32	
Extrauterine invasion	No	109	32	46	37	54	0.17
	Yes		24	60	16	40	
LN	No	25	8	80	2	20	1
	Yes		11	73	4	27	

Abbreviation: Lymphovascular space invasion (LVSI); Lymph node (LN).

4.5 Discussion

To my knowledge, this is the first study to examine the expression of the telomerase core-component, dyskerin in EC. My initial *in silico* interrogation of a published, large TCGA uterine cancer cohort, demonstrates the *DKC1* gene alteration profile in endometrioid and serous ECs. Subsequent wet laboratory-based study uses an independent local cohort of human ECs containing all histological subtypes with transcriptional and protein data and validates the initial findings of my *in silico* study. EC samples have significantly lower dyskerin protein levels when compared with healthy PM controls. Findings from the local cohort of EC patients are important for the following reasons: **i)** I examined the endometrial dyskerin protein levels with immunoblotting and at the cellular level with IHC for the first time, **ii)** the local patient cohort consisted of all EC subtypes, including carcinosarcomas, dedifferentiated, mixed cell adenocarcinoma and clear cell cancer types, precancerous endometrial hyperplasia samples and metastatic EC lesions as well as external control endometrium (both healthy PM and premenopausal PP samples) to increase the generalisability of the data. **iii)** Importantly, my data suggest a better clinical outcome in high dyskerin protein containing ECs in comparison with low dyskerin ECs. My results, therefore, fill the gaps in the current literature and in the TCGA dataset.

Several cancers, particularly adenocarcinomas with ductal and solid structure, e.g., hepatocellular carcinomas, are known to be associated with *DKC1* dysregulation (Vasuri et al., 2015). High incidence of cancers is seen with reduced *DKC1* gene expression in DC patients and in *DKC1* hypomorphic mice. Thus, at least in these examples, dyskerin may act as a tumour suppressor (Knight et al., 1998, Ruggero et al., 2003), since functional impairment increases the susceptibility to cancer. There are currently no reports of DC associated EC. However, EC is a disease mainly seen in PM women, and DC shortens the life span of patients (Dokal, 2011). Therefore, women with DC are likely to demise before they reach the age when the EC risk is high.

All ECs in this cohort were postmenopausal; therefore, I chose healthy postmenopausal samples as the best normal controls. The healthy quiescent PM endometrium with absent cellular proliferative activity having high dyskerin levels, also agree with a tumour suppressor function for dyskerin. The downregulation of dyskerin protein we have reported in ECs in comparison with the healthy PM endometrium seems to occur in a background of high TA (EC is known to have high TA and I confirmed a significantly higher TA in our EC samples

compared with healthy endometrium (Ebina et al., 1999, Pertynski T., 2002). TA positively correlates with endometrial epithelial proliferation (Valentijn et al., 2015). *DKC1* depletion has been reported to result in decreased *TERC* levels (Ibanez-Cabellos et al., 2018), yet neither the TCGA data nor our cohort data showed a significant correlation of *TERC* with *DKC1* gene expression in endometrial samples. There is ample evidence available to suggest that sufficient dyskerin levels are required for competent TA to overcome telomere attrition (Gu et al., 2008) and ECs are reported to be associated with short telomeres (Wang et al., 2002a). The observed dyskerin loss may also trigger DDR and produce a pro-oxidant environment in EC cells (Ibanez-Cabellos et al., 2018). Therefore, reduced levels of dyskerin protein in the context of the excessive cellular division may contribute to genomic instability known to be present in more advanced ECs. Dyskerin deficiency may also contribute to carcinogenesis by adversely influencing the translational machinery via affecting the balance in ribosomal proteins (Sbarrato et al., 2016) and by modifying the splicing of specific pre-mRNAs, or by altering the level of certain snoRNAs (Angrisani et al., 2014, Dos Santos et al., 2017). These mechanistic aspects need to be examined in future studies.

Although the importance of telomerase in endometrial epithelial proliferation and high TA in over 90% of ECs had been reported, the examination of the TCGA dataset only identified *DKC1* out of the three core telomerase components to have an alteration in gene expression in ECs with prognostic relevance. The protein data also suggests an associated difference in patient outcomes correlating to dyskerin immunoexpression scores. However, the exact function of dyskerin in the context of endometrial epithelial cell proliferation or TA is yet to be determined. Future work is warranted to examine these parameters in healthy endometrial regeneration and their role in pathological conditions.

Data from this cohort and the TCGA dataset jointly suggest a dysregulation of dyskerin in EC. However, my results differ from the TCGA data, where a reduction in dyskerin protein level in EC is seen compared with external control healthy PM endometrium. The TCGA data suggest that EC cells may have higher *DKC1* expression compared with tumour-adjacent normal endometrium. This discrepancy may be due to different “normal controls” used in the two studies and the fact that we examined protein levels rather than only the mRNA levels. It is important to appreciate that endometrioid/serous EC included in the TCGA data usually originates from a background of endometrial hyperplasia or EIN; thus, the normal tissue within 2cm from the tumour is likely to include hyperplastic or EIN lesion tissue rather than normal

endometrium. I have used external histologically confirmed healthy control tissue from an age-matched well-characterised population; therefore, I would propose that these data are generalizable.

Advanced primary ECs (stage III and IV) had significantly lower dyskerin protein level compared with early-stage ECs. This result may suggest a relevance for dyskerin protein levels in stratifying EC patients to high-risk groups after primary surgery for further therapy. The metastatic lesions also showed higher amounts of dyskerin protein, which is consistent with previous studies where metastatic lesions demonstrated less advanced/aggressive phenotype than the primary tumour (Kamal et al., 2016b).

In the context of telomerase biology, the exact functional role of dyskerin in normal endometrial function as well as in pathological conditions such as EC is yet to be determined. Since TA is known to play an intricate role in endometrial epithelial proliferation, further studies elucidating telomerase associated and other functions of dyskerin in the human endometrium and in EC are warranted. There is previous evidence suggested that two of telomerase complex subunits (hTERT and hTERC) are regulated by ovarian steroid hormones in human endometrium; therefore, in chapter 5, I examined the hormonal regulation of the remaining component of telomerase holoenzyme, dyskerin in human endometrium.

5

Chapter Five

Hormonal Regulation of Dyskerin

5.1 Introduction

The human endometrium is the primary target organ for ovarian steroid hormones (Hapangama et al., 2017). The reproductive lifespan of a woman begins with menarche and ends with menopause. During that period, an average woman has about 400 menstrual cycles, in which endometrial functionalis layer undergoes a well-defined cycle of proliferation, differentiation and menstrual shedding followed by regeneration (Kamal et al., 2016a, Hapangama et al., 2017). Ovarian steroid hormones, including estrogen, progesterone and androgen, regulate this endometrial cycle via their cognate receptors (Hapangama et al., 2015a, Kamal et al., 2016b). Progesterone is predominant in the luteal phase and the level of circulating estrogen peaks around the implantation window. At menopause, when the ovarian steroid hormones cease to be synthesised from the failing ovaries, the endometrium becomes proliferatively inactive without the hormonal cue. However, if appropriate exogenous ovarian steroid hormones are provided, a fully functional endometrium still can be generated from the remaining thin postmenopausal endometrium (Hapangama et al., 2017).

Emerging evidence suggests that sex steroid hormones have a dynamic role in the regulation of telomerase in target hormonally responsive tissues such as the endometrium. Estrogen is the trophic hormone that stimulates cellular growth and proliferation, while progesterone opposes the estrogenic induced proliferation and influences cellular differentiation (Hapangama, 2003). The third ovarian steroid hormone, androgen, is also proposed to influence the endometrial cycle, but unlike estrogen and progesterone, the exact androgenic regulatory role in the endometrium is not yet fully known.

Estrogen, directly and indirectly, increased TA and *hTERT* levels in the hormone-responsive breast cancer cell line (MCF-7) (Kyo et al., 1999). Estrogen response element (ERE), located in telomerase promoter(Kyo et al., 1999, Williams et al., 2001), can bind *in vitro* with human estrogen receptor α (ERα). Kyo *et al*. also found that estrogen upregulated c-Myc expression in MCF-7 cells and that E-boxes in the *hTERT* promoter that binds c-Myc/Max play additional roles in estrogen-induced *hTERT* transactivation (Kyo et al., 1999).

In the endometrium, estrogen is postulated to exert its effect via two main classical estrogen receptor (ER) isoforms, ERα and ERβ (Hapangama et al., 2015a). In hormone-responsive endometrial epithelial adenocarcinoma (ISK) cells, estrogen elevated TA and *hTERT* mRNA levels via a mitogen-activated protein kinase (MAPK) dependent pathway in an ERα dependent manner (Zhou et al., 2013). Endometrial TA dynamic changes are under ovarian

steroid hormones regulation are well-established and correlated with glandular proliferation (Williams et al., 2001, Hapangama et al., 2008b). The estrogen dominant proliferative phase of the cycle demonstrated high TA, but TA plummets in the progesterone dominant mid-secretory phase (Williams et al., 2001, Hapangama et al., 2008b). Similar dynamic changes in the mean endometrial TLs across the menstrual cycle are also confirmed by Valentijn *et al.* (Valentijn et al., 2015). Glandular epithelium of the endometrial functionalis possesses the shortest TL (Cervello et al., 2012) than the endometrial stromal cells in all phases of the menstrual cycle whilst having a higher TA.

Furthermore, the endometrial epithelial cells have the highest TA in the proliferative phase and have a negative correlation with TL (Valentijn et al., 2015). In postmenopausal endometrium, low levels of TA were observed (Tanaka et al., 1998, Brien et al., 1997). In ovariectomised female cynomolgus macaques (Macaca fascicularis), *TERC* levels in endometrial glands increased in response to long term treatment with clinically relevant doses of conjugated E2 (Vidal et al., 2002).

Compared to this well-described effect of estrogen, the effect of progesterone on TA and *hTERT* is less understood. Progesterone usually antagonises estrogen action in reproductive organs, including the effect on telomerase. In the endometrium, endogenous progesterone seems to oppose the estrogen action and reduce normal endometrial epithelial TA in normal SP endometrium compared with PP endometrial samples. Furthermore, administration of progesterone agonist Medroxyprogesterone acetate (MPA) to women inhibited TA, whilst PR antagonist, mifepristone administered in the early secretory phase, increased hTERT protein in the endometrium of normal women (Valentijn et al., 2015). However, *in vitro* evidence of progesterone on *hTERT* mRNA level seem to be diverse and time-dependent as well as being tissue-specific (Wang et al., 2000). In breast and EC cell lines expressing the progesterone receptor, progesterone led to a significant increase in the level of *hTERT* mRNA within 3 hours after exposure. This transient effect peaked at 12 hours and then decreased, whereas treatment with progesterone for more than 48 hours counteracted estrogen effects and repressed the estrogen-induced stimulation of *hTERT* level (Wang et al., 2000). In the primary endometrial explants and co-cultured primary epithelial/stromal cells in 2-dimensional culture, treatment with MPA or combined progesterone and estrogen reduced TA (Valentijn et al., 2015).

Androgen exerted an effect similar to estrogen on *hTERT* gene expression and TA (Calado et al., 2009); it increased *hTERT* mRNA and TA levels mainly via testosterone to estrogen aromatisation through ERα in normal peripheral blood lymphocytes and human bone marrow-

derived CD34(+) cells *in vitro* (Calado et al., 2009). However, it is difficult to determine if the observed effects of androgens were related to the direct impact on the androgen receptor (AR) or indirectly mediated *via* ER (Hapangama et al., 2017). In addition, oral treatment with Danazol (a synthetic steroid with weak androgenic properties) for 24 months caused a universal leucocyte telomere length increase in both male and female patients with diseases with genetic defects in telomere maintenance, such as bone marrow failure, liver cirrhosis and pulmonary fibrosis (Townsley et al., 2016). The *in vitro* exposure of normal peripheral blood lymphocytes and human bone marrow derived $CD34^+$ cells to androgens elevated the TA, together with higher *TERT* mRNA levels. Furthermore, Cells from patients with heterozygous telomerase mutations had low baseline TA, which was returned to normal levels after exposure to androgenic compounds (Calado et al., 2009).

Gonadotropin-releasing hormone (GnRH) is known to have an antiproliferative effect on the endometrium indirectly by inhibiting gonadotropin secretion and suppression of ovarian estradiol synthesis as well as directly through acting on GnRH receptor (GnRH-R) in the endometrium (Kamal et al., 2016a).

Taken together, there is ample evidence that telomerase enzyme activity and some of the core-components of telomerase are hormonally regulated in the endometrium. However, there are no published reports on the hormonal regulation of dyskerin (*DKC1*) in the human endometrium have to date; therefore, in this Chapter, I have explored for the first time regulation of dyskerin (*DKC1*) by ovarian steroid hormones *in vivo* and *in vitro*.

5.2 Research questions

1) Are endometrial dyskerin protein levels regulated by endogenous ovarian hormones?

   This was addressed by examining dyskerin protein and *DKC1* mRNA levels in different phases of the pre and postmenopausal healthy endometrial samples.

2) Do the levels of endometrial dyskerin correlate with the levels of ovarian steroid receptors?

   This was examined by correlating immunoscores of dyskerin with ovarian steroid hormones receptors immunoscores.

3) Do exogenous ovarian steroid hormones regulate dyskerin in human endometrium?

To answer this question, I evaluated the levels of dyskerin protein and *DKC1*mRNA in endometria of women on exogenous hormonal treatment.

4) What is the direct *in vitro* effect of steroid hormones on *DKC1* gene expression in endometrial epithelial cells?

The *in vitro* effect of estrogen, progesterone and androgen (DHT) on the *DKC1* mRNA levels was examined in an endometrial epithelial cell line model, (ISK cells).

5.3 Materials and Methods

5.3.1 Study groups

Endometrial samples were collected from 3 groups of patients.

Group (1): Healthy pre/postmenopausal women without any endometrial pathology. The premenopausal women had regular periods, and the postmenopausal women did not have any abnormal bleeding. All women in this latter group were not on any hormonal treatment for at least three months. Biopsies were collected in the PP (n=27) and SP (n=23) in premenopausal women and from 33 healthy PM women.

Group (2): Endometrial samples were also collected from women treated with exogenous hormones: The participants included in the study were taking, either Gonadotropin-releasing hormone (GnRH) agonists (n=18) or LNG-IUS (n=18) (Table 5.1).

Group (3): For the correlation of steroid receptor expression, endometrial samples from 109 women with endometrial carcinoma and 15 with EHA were also included.

Table 5.1. Demographic features of study groups

Study groups	No.	*Age (years)	*BMI (kg /m2)
Proliferative phase	27	40(30-57)	27(18-41)
Secretory phase	23	41(21-48)	26(19-40)
Postmenopausal	33	63(40-85)	26(20-40)
LNG-IUS	18	38(26-55)	28(19-38)
GnRH	18	41(20-52)	26(20-39)

*Data expressed as median (range)

5.3.2 IHC

Endometrial samples collected from women were processed, and 3µm FFPE tissue sections were immuno-stained using standard methods, as described in chapter 2. The primary antibody sources, their concentration and specific incubation conditions are detailed in (Table 5.2).

Table 5.2 Primary antibody sources and conditions for IHC.

Primary Ab	Type	Clone	Supplier	HIAR* (minutes)	Dilution	Incubation Time (hour)	Condition Temp (°C)
Dyskerin	P		Santa Cruz biotechnology[1]	3	1:500	20	4
ERα	M	6F11	NovoCastra[2]	2	1:50	2	18
ERβ	M	PPG5/10	Abcam[3]	2	1:50	20	4
PR	M	PgR 636	DAKO[4]	2	1:1000	1	18
AR	M	AR441	DAKO[4]	2	1:75	20	4

* HIAR by pressure cooking in citrate buffer pH 6. [1]Dallas, Texas, USA; [2]Newcastle, UK; [3]Cambridge, UK; [4]Ely, Cambridgeshire, UK. **Abbreviations:** Polyclonal (P); Monoclonal (M), Heat-induced antigen retrieval (HIAR).

5.3.2.1 Immunohistochemical analysis

The nuclear dyskerin immunoreactivity in endometrial epithelial cells was assessed using a modified quickscore by scanning the whole section and by estimating the percentage of the stained proportion of cells in each of the four intensities as previously described (the detailed description of this process is also presented in Chapters 3 & 4) (Mathew et al., 2016). The immunostaining for the four steroid receptors was evaluated semi-quantitatively using a four-tiered Liverpool endometrial steroid quickscore (LESQS), as previously described in Kamal *et al.* (Kamal et al., 2016b). The epithelial cell staining was scored in the basalis epithelium of endometrial samples, collected from healthy pre- and postmenopausal women as well as endometria from women treated with exogenous steroid hormones (LNG-IUS and GnRH). Since endometrial functionalis is only present in the PP and SP endometrium of premenopausal endometrium, the functionalis was examined separately, only in samples from premenopausal women included in Group (1).

5.3.3 Cell culture and hormonal treatment

The human endometrioid cancer cell line, ISK, was grown and maintained as previously described in 2.6.1 (Parkes et al., 2018). The cells were prepared for steroid treatment by preconditioning in DMEM/F12 (phenol red-free, Life Technologies, UK) supplemented with penicillin/streptomycin antibiotic and 2% CS-FBS for 48 hours prior to hormonal treatment. The cells were then treated with 17β estradiol (E2, 10^{-8}M), progesterone (P4, 10^{-6}M) (Merck, Nottingham, UK) and 5α dihydrotestosterone (DHT, 10^{-6}M and 10^{-8}M) individually or in combination for 12-72 hours. The medium containing progesterone was changed twice daily, to provide persistent concentration levels to simulate human physiology and to observe the expected progestogenic effects, as per advice from Prof. Sue Kimber (personal communication, University of Manchester, UK). The cells were subsequently pelleted for the TRAP assay and RNA extraction for qPCR. 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.

5.3.4 TRAP assay for TA

TA was measured using TeloTAGGG™ Telomerase PCR ELISA kit, as previously described (Valentijn et al., 2015) by our collaborator, Dr Gabriele Saretski from the University of Newcastle, UK. Endometrial cells and tissue were homogenised in TRAP-assay buffer for 30 minutes, centrifuged and protein quantification was determined using Bradford protein assay (Bio-Rad Laboratories). Amplification products after 30 cycles were measured as absorbance at 450nm in a Fluostar Omega Plate reader ((BMG LABTECH, Aylesbury, UK) and presented as arbitrary units (AU).

5.3.5 RT-qPCR

Messenger RNA was amplified, as described in section 2.7.5, using the primers listed in Table 2.4 and quantified relative to the geometric mean of reference genes YWHAZ and *PPIA* for *DKC1* or PPIA and ACTB for *hTERC*, as described previously (Mathew et al., 2016). A standard curve was generated for each target and reference gene. Primer efficiency was determined (Figure 2.2). The amplification products were run on an agarose gel to verify the specificity of the primers and rule out any off-target amplicons (Figure 3.1).

5.3.6 Statistical analysis

Descriptive data were presented as median and range. Statistical analyses were performed in IBM SPSS 25, as previously described in chapter 2; Graphs were plotted using GraphPad Prism 5.

5.4 Results

5.4.1 In vivo endogenous hormonal effects on dyskerin in the context of TA and *hTERC*

The physiological endogenous hormonal influence on dyskerin levels was examined in endometrial samples collected from healthy premenopausal women, who were having regular periods and not on hormonal medication as well as from healthy postmenopausal women. The premenopausal endometrium would contain functionalis layer in addition to basalis and luminal epithelial layers, whereas this was not present in the PM endometrium.

5.4.1.1 TA and *hTERC* RNA

The highest TA was observed in proliferative endometrial samples, while SP and PM samples showed low TA (Figure 5.1A). There was no difference in *hTERC* RNA level among healthy endometrial samples (p=0.2) (Figure 5.1B).

5.4.1.2 DKC1 mRNA level

DKC1 mRNA levels were significantly lower in PP and SP of the healthy endometrium compared with PM samples (p=0.01), (p=0.001) respectively. The endometrial mRNA levels were not significantly different between PP and SP of the cycle (Figure 5.1C).

5.4.1.3 Dyskerin immunoscores

In consistence with the qPCR results (mRNA levels), dyskerin protein immunoexpression in the basalis glands was significantly decreased in samples collected from healthy premenopausal women in both PP and SP compared with basalis glands of PM endometrial samples (p=0.03), (p=0.002), respectively (Figure 5.1D). When I compared the functionalis glands of PP and SP of the cycle, dyskerin immunoscores appeared to be higher in PP compared with SP (Figure 3.6E).

5.4.2 *In vivo* exogenous hormonal effects on dyskerin in the context of TA and *hTERC.*

The effects of exogenous hormonal changes were examined in endometrial samples collected from healthy premenopausal women, who were on GnRH analogues to simulate hypo-estrogenic status and those on synthetic progestogen, using a LNG-IUS. Both the hypo-estrogenic and high progestogenic status prevents the development of a functionalis layer; therefore, these samples only contained a thin endometrium that would contain epithelium similar to the basalis and luminal epithelial layers of the premenopausal endometrium. Therefore, the comparisons were made between the basalis of premenopausal endometria from women not on hormones for at least three months with the samples from these two hormone-treated groups.

5.4.2.1 TA and *hTERC* RNA

Samples collected from women using LNG-IUS and those on GnRH treatment showed low TA levels (Figure 5.1A). There was no significant difference in *hTERC* RNA level between endometrial samples from women on hormonal therapy (LNG-IUS and GnRH) (p=0.5) (Figure 5.1B).

5.4.2.2 *DKC1* mRNA

DKC1 mRNA level was significantly decreased in samples collected from women on GnRH analogues compared with either the healthy PM endometrial samples (p=0.0008) or the endometrial samples from women using local progestin (LNG-IUS) (p=0.0002) as shown in (Figure 5.1C). Additionally, the endometrial *DKC1* level in LNG-IUS group appeared to be lower than the level in healthy PM samples, but this difference was not statistically significant.

5.4.2.3 Dyskerin immunoscores

Dyskerin immunoreactivity was significantly lower in samples collected from women using LNG-IUS compared with those from women on GnRH analogue (p=0.01). Dyskerin protein level in endometria from women treated with GnRH and local progestin (LNG-IUS) appeared to be similar to its level in PM and SP samples, respectively (Figure 5.1D).

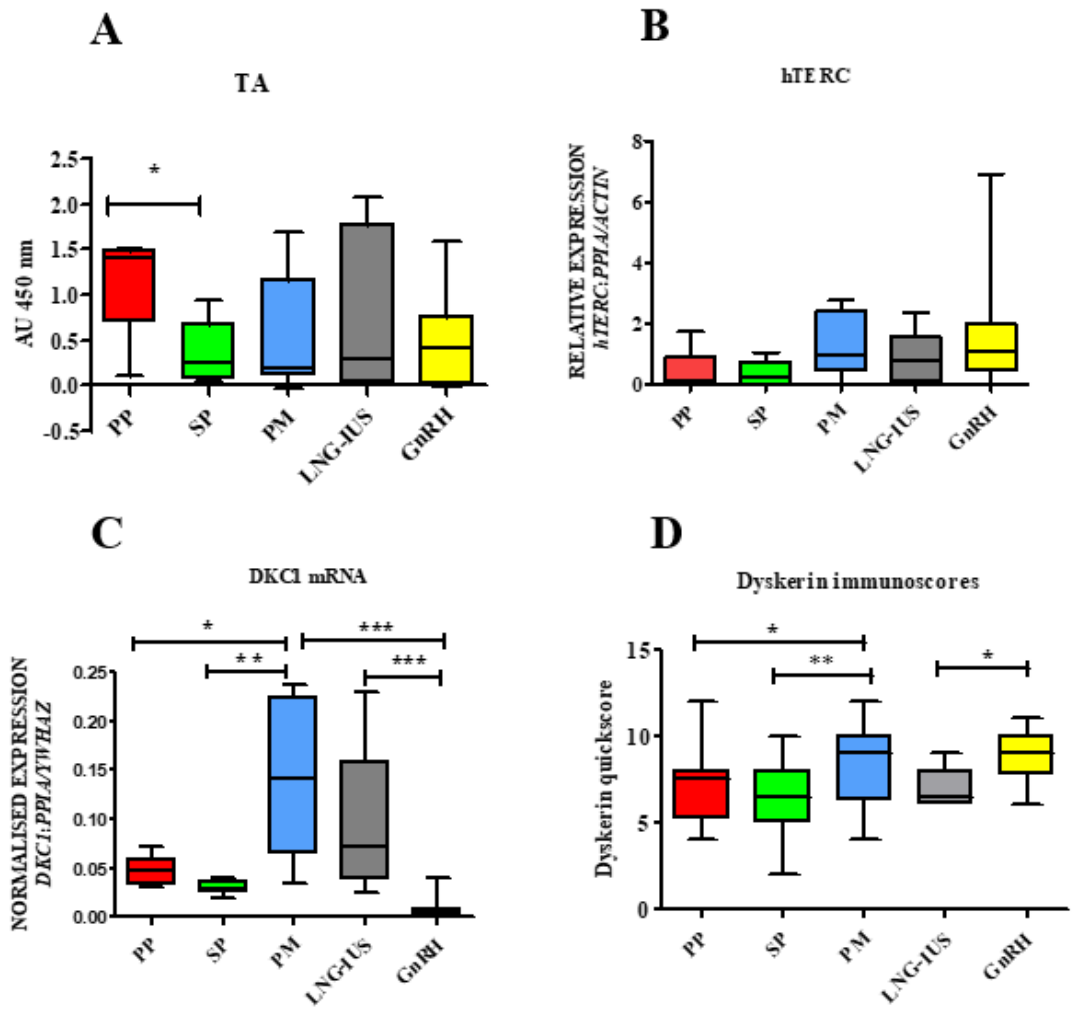

Figure 5.1 *In vivo* hormonal regulation of **A)** Telomerase activity (TA) in Healthy proliferative (PP) (n=6); secretory (SP) (n=6) and postmenopausal (PM) (n=7), in addition to samples collected from women treated with hormones: LNG-IUS (n=7) and GnRH (n=7); **B)** *hTERC* RNA in PP (n=6); SP (n=8); PM (n=5); LNG-IUS (n=11); GnRH (n=10); **C)** *DKC1* mRNA (PP (n=7); SP (n=10); PM (n=6); LNG-IUS (n=10); GnRH (n=9) and **D)** Dyskerin protein immunoscores in the following endometrial samples: PP (n=16); SP (n=17); PM (n=30); LNG-IUS (n=12); GnRH (n=10) *p<0.05, **p<0.01, ***p<0.001.

Data in Figures 5.1 A, B, C and D have been reproduced from Figures 3.2D, 3.3D, 3.4D and 3.6C, respectively to facilitate comparison with the LNG-IUS and GnRH samples.

5.4.3 Correlation of dyskerin protein with steroid receptors

To examine the correlation between dyskerin immunescores with endometrial epithelial steroid receptor scores, initially, only the normal endometrial samples of women not on hormonal treatments were considered. The dyskerin immunoscores in the basalis glands did not correlate with any of steroid receptors immunoscores (ERα Spearman r=-0.12, p=0.44); (ERβ Spearman r=0.23, p=0.14); (PR Spearman r=0.08, p=0.64); (AR Spearman r=-0.06, p=0.68).

In the functionalis glands of premenopausal endometrium (PP and SP), there was no significant correlation between dyskerin immunoscores with any of steroid receptors (ERα Spearman r=-0.35, p=0.05); (ERβ Spearman r=0.07, p=0.66); (PR Spearman r=0.36, p=0.05); (AR Spearman r=-0.24, p=0.17).

There was no significant correlation of dyskerin with steroid receptors in basalis glands of hormonally treated samples or in a group consists of both healthy endometrial samples from women not treated with hormones and samples from hormonally treated women, no significant correlation was observed between dyskerin with any of steroid receptors immunoscores.

In a group of samples includes premalignant and malignant samples, dyskerin is significantly correlated with ERβ (Spearman r=0.43, p<0.0001) and it showed a weak positive correlation with ERα (*r*=0.22, p=0.02); PR (*r*=0.23 p=0.01) and AR (*r*=0.24 p=0.01).

Finally, the correlation of dyskerin immunoscores with steroid receptors was examined in the extended sample set, that included the premalignant and malignant samples (EHA and EC); a significant positive correlation was found between dyskerin with ERβ (Spearman r=0.40, p<0.0001) and a positive trend was observed with PR (Spearman r=0.27, p<0.0008); whereas no correlation was observed with ERα or AR (Spearman r=0.14, p=0.08) and (Spearman r=0.13, p=0.1), respectively.

Figure 5.2 showed the immunostaining in endometrial samples from healthy women not on any hormonal therapy in the last three months and in samples from women treated with hormones.

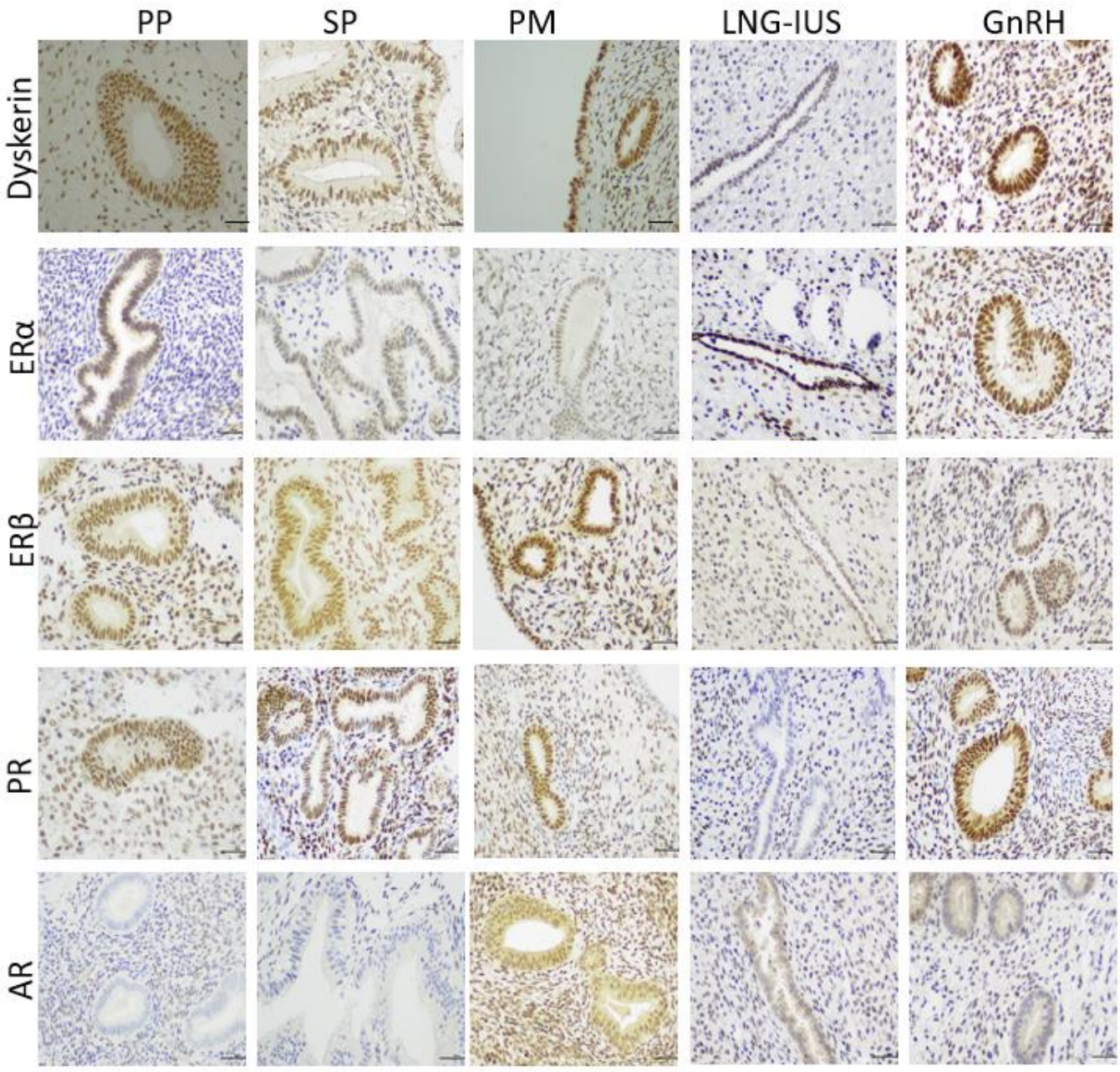

Figure 5.2 Representative microphotographs illustrate the IHC staining of dyskerin, ERα, ERβ, PR and AR in the following endometrial samples: Healthy proliferative (PP), secretory (SP) and postmenopausal (PM) as well as endometrial samples from women on hormone therapy (LNG-IUS and GnRH). Positive staining appears brown. Magnification 400X. Scale bar 50μm.

5.4.4 In vitro hormonal effect on dyskerin *(DKC1)* in the context of TA and *hTERC*

I examined the effect of E2 and P4 treatment (separately and combined) as well as DHT on *DKC1* mRNA level in an *in vitro* model of hormonal responsive EC (ISK) cell line in the context of TA and *hTERC* RNA levels.

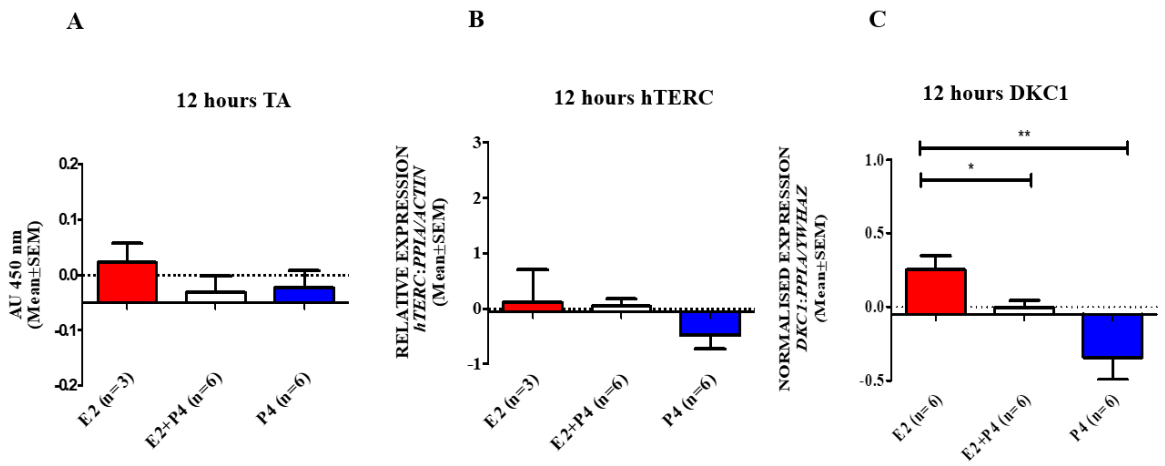

Figure 5.3 *In vitro* hormonal treatment of Ishikawa (ISK) cells, the difference in **A)** Telomerase activity (TA); **B)** Human telomerase RNA component (*hTERC*) mRNA and **C)** *DKC1* mRNA levels were observed in ISK cells treated for **12 hours** with 17β estradiol (E2), E2 and progesterone (E2+P4) and progesterone alone treatment (P4). TA was measured by telomeric repeat amplification protocol (TRAP) assay, *DKC1* and *hTERC* RNAs were measured by RT-qPCR and normalised to geometric means of *PPIA* and *YWHAZ* or *PPIA* and *ACTB*. Values are presented in logarithmic scale. *p<0.05, **p<0.01, ***p<0.001.

5.4.4.1 The effect of hormonal treatment on TA and *hTERC* RNA levels

Twelve hours after hormonal treatment, E2 slightly elevated TA in ISK cells. While P4, either alone or when combined with E2, reduced TA. P4 continued to downregulate TA below the vehicle level after 72 hours of treatment, and no difference in TA level was observed in cells treated with P4 alone or when combined with E2 at that time point (Figures 5.3A and 5.4A).

Low dose of DHT elevated TA level 12 hours following treatment but this effect disappeared after 24 hours. High dose of DHT did not affect TA level at all time points (12, 24, 48 hours) tested after treatment (Figure 5.5A, B & C).

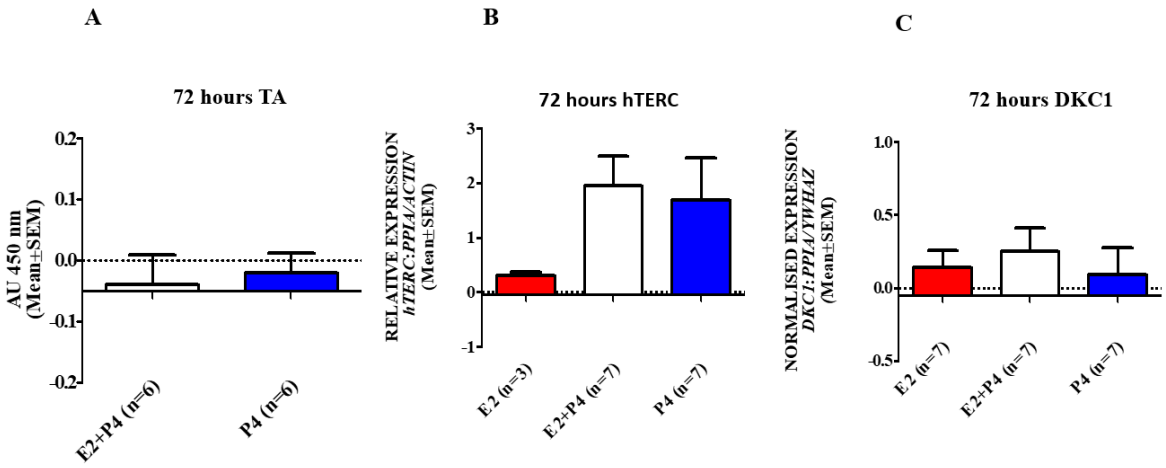

Figure 5.4 In vitro hormonal treatment of Ishikawa (ISK) cells, the difference in **A)** Telomerase activity (TA); **B)** Human telomerase RNA component (*hTERC*) mRNA and **C)** *DKC1* mRNA levels were observed in ISK cells treated for **72 hours** with 17β estradiol (E2), E2 and progesterone (E2+P4) and progesterone alone (P4). TA was measured by telomeric repeat amplification protocol (TRAP) assay, *DKC1* and *hTERC* RNAs were measured by RT-qPCR and normalised to geometric means of *PPIA* and *ACTB*. Values are presented in logarithmic scale. *p<0.05, **p<0.01, ***p<0.001

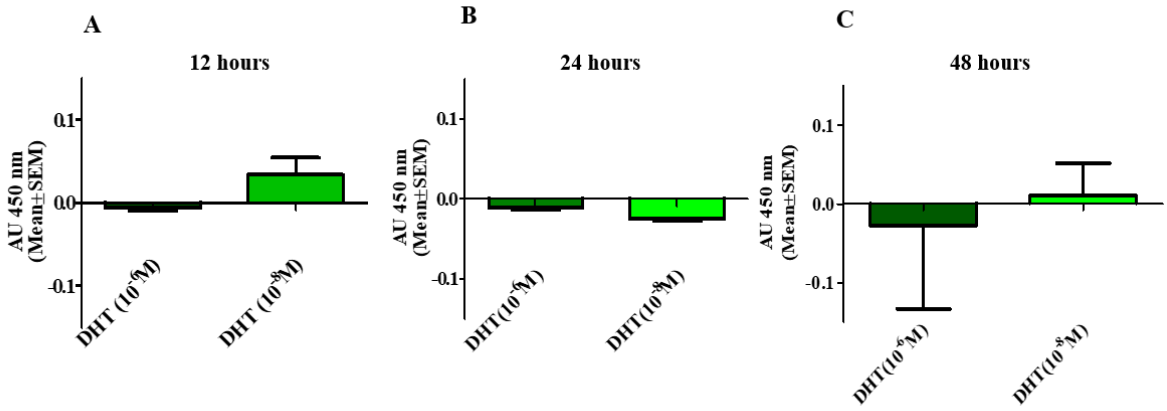

Figure 5.5 Telomerase activity (TA) in hormonally treated Ishikawa (ISK) cells. TA was measured by telomeric repeat amplification protocol (TRAP) assay. ISK cells (n=3) were treated with Dihydrotestosterone (DHT) (10^{-6}M) and DHT (10^{-8}M) for **A)** 12 hours, **B)** 24 hours and **C)** 48 hours. Values are presented in logarithmic scale. *p<0.05, **p<0.01, ***p<0.001.

No significant change on *hTERC* level was observed with E2 alone or with combined E2 and P4 at 12 hours of treatment. P4 alone decreased *hTERC* level below the vehicle (absolute methanol) level, but this effect did not reach the statistical significant level (Figure 5.3B). Seventy-two hours after treatment, P4 (separately or in combination with E2) upregulated *hTERC* level. Treatment with E2 alone slightly upregulated *hTERC* above the levels seen with control vehicle only treatment (Figure 5.4B).

Treatment with a high dose of DHT increased *hTERC* level at 48 hours following the treatment (Figure 5.6C), while the treatment at low (physiological) dose, decreased *hTERC* levels at all the time points (12, 24 & 48 hours) (Figure 5.6A, B & C) and the maximum effect was observed 12 hours following the treatment (Figures 5.6A).

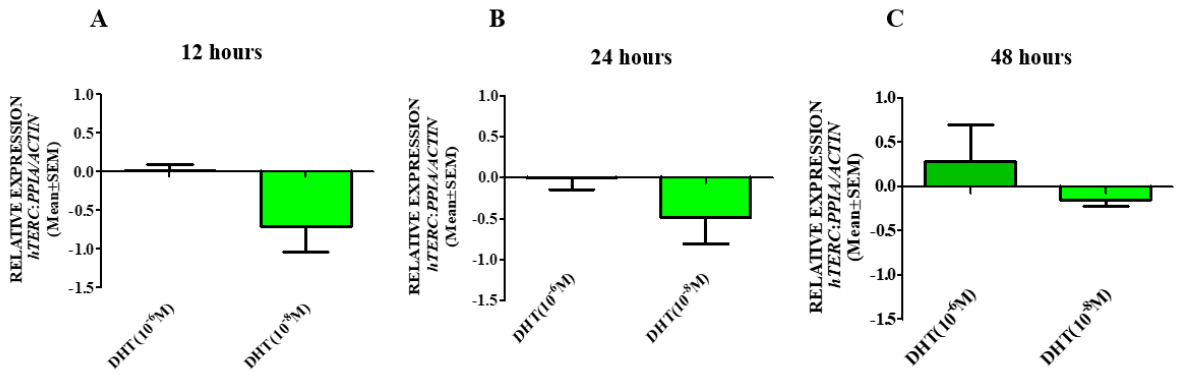

Figure 5.6 Human telomerase RNA component (*hTERC*) mRNA level was measured in hormonally treated Ishikawa (ISK) cells (n=3). *hTERC* RNA was normalised to geometric means of PPIA and ACTB. ISK cells were treated with Dihydrotestosterone (DHT) (10^{-6}M) and DHT (10^{-8}M) for **A)** 12 hours, **B)** 24 hours and **C)** 48 hours. Values are presented in logarithmic scale. *p<0.05, **p<0.01, ***p<0.001.

5.4.4.2 The effect of hormonal treatment on *DKC1* mRNA levels

When E2 was used alone, it significantly upregulated *DKC1* in comparison with its level when the cells treated with P4 alone (p=0.001) or combined E2 and P4 (p=0.04) (Figure 5.3C). P4 downregulated *DKC1* in ISK cells 12 hours after treatment. At 72 hours following treatment, the P4-induced downregulation of *DKC1* mRNA levels was not present, and both E2 and P4 treatment when used separately or in combination appeared to have resulted in elevated levels of *DKC1* mRNA slightly above the vehicle level (Figure 5.4C).

I also found that at 12 hours of treatment, high dose DHT (10^{-6} M) has a similar effect on *DKC1* mRNA levels to E2, and upregulated *DKC1* (Figure 5.7A), while the low DHT dose (10^{-8} M) had no significant effect on *DKC1* mRNA level (Figures 5.7A, B & C).

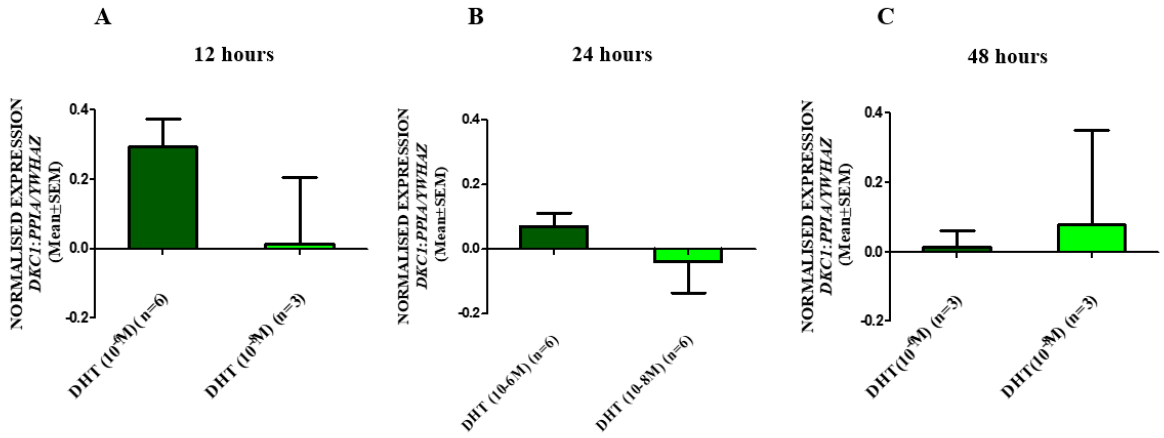

Figure 5.7 *DKC1* mRNA level in hormonally treated Ishikawa (ISK) cells. It was normalised to geometric means of PPIA and YWHAZ and measured by RT-qPCR. ISK cells were treated with Dihydrotestosterone (DHT) (10^{-6}M) or DHT (10^{-8}M) for **A)** 12 hours, **B)** 24 hours and **C)** 48 hours. Values are presented in logarithmic scale. *p<0.05, **p<0.01, ***p<0.001.

Figure 5.8 shows the effect of E2 and DHT on *DKC1* mRNA and TA levels in ISK cells 12, 24 and 48 hours after treatment.

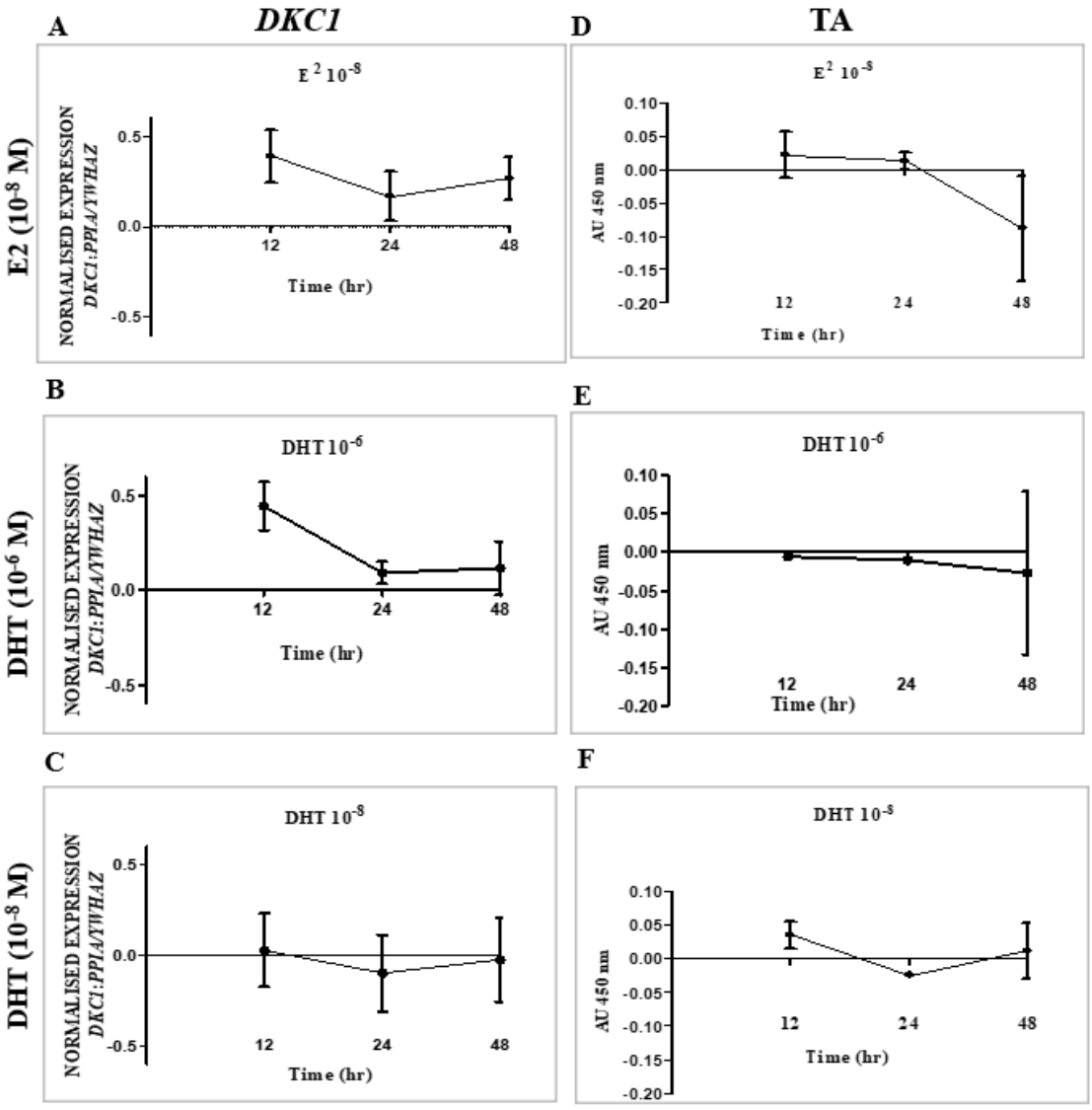

Figure 5.8 *DKC1* mRNA measured by RT-qPCR and telomerase activity (TA) measured by telomeric repeat amplification protocol (TRAP) in Ishikawa (ISK) cells treated with 17β estradiol (E2) (10^{-8}M), Dihydrotestosteron*e* DHT (10^{-6}M) and DHT (10^{-8} M) for 12,24 and 48 hours. **A)**, **B)** and **C)** DKC1 mRNA in ISK cells treated with E2, DHT (10^{-6}M) and DHT (10^{-8}M), respectively. **D)**, **E)** and **F)** TA in ISK cells treated with E2, DHT (10^{-6}M) and DHT (10^{-8} M), respectively. *DKC1* was normalised to geometric means of PPIA and YWHAZ. Values are presented in logarithmic scale (Mean±SEM). *p<0.05, **p<0.01, ***p<0.001.

5.5 Discussion

In the human endometrium, the dynamic alterations in TA in relation to the ovarian cycle have been well documented(Kyo et al., 1997, Tanaka et al., 1998, Williams et al., 2001). In the current study, I analysed the *in vivo* and *in vitro* effects of steroid hormones on endometrial dyskerin (*DKC1*) protein and mRNA levels. The *in vivo* study included healthy pre and postmenopausal endometrial samples of women not on hormones to examine the effect of endogenous ovarian hormones, and the effect of exogenous hormonal treatment was assessed by examining endometrial samples obtained from women using LNG-IUS (demonstrating a high local progestogenic effect on the endometrium) and also samples from women treated with GnRH analogue (depicting a hypo-estrogenic and antiproliferative effect on the endometrium). In the present study, I observed that both the protein and mRNA levels of dyskerin were affected by ovarian hormones *in vivo* and *in vitro*, suggesting a hormonal regulation.

Dyskerin immunoscores in the endometrial epithelial cells were significantly higher in healthy PM endometrial samples and samples from women treated with GnRH analogue, both characterised by a hyp-estrogenic status associated with a decreased endometrial epithelial proliferation. The data of the current study also showed that the dyskerin levels decrease when endogenous estrogen is high in the proliferative phase samples and in secretory phase with high estrogen and progesterone levels, as well as with LNG-IUS samples where exogenous progesterone is the predominant hormonal signal.

In agreement with the above IHC results, *DKC1* mRNA levels were also significantly higher in the healthy PM endometrium compared with healthy premenopausal endometrial samples. However, *DKC1* level was high in endometrial samples obtained from women using LNG-IUS (progestogen containing device), when compared with samples taken from women treated with GnRH analogues. The discrepancy between the IHC and PCR data could be due to many factors, and essentially the two techniques did not assess exactly the same cells. For example, IHC assessment only considered the endometrial epithelial immunoscores, whereas the mRNA levels obtained from the endometrial samples in PCR studies were the average levels of all cell types within the endometrium. In the PCR studies, the exact amount of endometrium included in the thin, atrophic endometrium from women treated with GnRH or those using LNG-IUS, obtained by scraping the uterine cavity can be highly heterogeneous. With IHC, the exact cell type and the presence of cells can be determined directly by visualisation; the PCR study is

subject to inclusion of blood, mucus etc. in variable amounts. I would envisage such issues to account for this observed discrepancy. Furthermore, some samples may also include strips of underlying myometrium, to add to this issue with contamination of samples used in gene studies.

Regarding TA, TRAP data were consistent with previous publications, and the highest levels of TA were observed in PP compared with other healthy endometrial samples and with hormonally treated samples. Previous reports also showed the proliferative phase endometrium to exhibit the highest TA, whereas the secretory and postmenopausal endometrium showed reduced TA levels (Tanaka et al., 1998, Hapangama et al., 2017, Valentijn et al., 2015, Williams et al., 2001). Both exogenous and endogenous progesterone had also previously shown to inhibit endometrial epithelial TA (Valentijn et al., 2015).

Interestingly, *hTERC* results did not show a significant difference among healthy women across the menstrual cycle phases or those on hormonal treatment endometrial samples.

TA and hTERT levels have been shown to be under hormonal regulation (Valentijn et al., 2015), and correspondingly, dyskerin immunoscores for endometrial samples demonstrated a significant correlation with ERβ expression scores. This may suggest dyskerin expression to be under estrogen regulation mainly via ERβ. ERβ is known to harness the estrogen-driven mitotic effect of ERα (Hapangama et al., 2015a); therefore, inducing endometrial dyskerin levels may also be a part of the ERβ associated proliferative inhibition. Further studies are required to examine the hormonal regulation of dyskerin in human endometrium.

In the *in vitro* study of hormonal treatment, I applied E2 at a physiologically relevant dose (10^{-8}M) to simulate the hormonal milieu of proliferative phase endometrium *in vivo*, and P4 and E2 together to replicate the secretory phase endometrial hormonal milieu. For our androgen studies, I used DHT in two concentration: **i)** high (10^{-6}M) supraphysiological dose to observe a direct exogenous androgenic effect; and at a **ii)** low (10^{-8}M) slightly higher than the physiological level to simulate the PM hormonal milieu, where androgen is the predominant hormone (Sivridis and Giatromanolaki, 2004).

My data show that E2 appeared to upregulate *DKC1* mRNA and TA, whereas *hTERC* RNA levels were minimally affected by E2. The addition of P4 counteracted E2 effect, and these findings are in agreement with the *in vivo* data. P4 treatment alone *in vitro* decreased *DKC1*, TA and *hTERC* levels. The *in vitro* P4 downregulation of *DKC1* and TA is comparable to the *in vivo* data (decreased *DKC1* and TA levels in the secretory phase with high endogenous

progesterone). However, *DKC1* mRNA levels were also low in the E2 dominant proliferative phase *in vivo*, whereas E2 treatment *in vitro* elevated the *DKC1* mRNA. This contradictory result could be attributed to the monocellular (epithelial cells only) culture model I used for the *in vitro* work. The hormonal effects, particularly some of the progestogenic effects on the endometrial epithelium is proposed to be via the adjacent stromal cells via inhibiting stromal production of several fibroblast growth factors that act as paracrine mediators of mitogenic estrogen effect on the epithelium (Li et al., 2011b); therefore, the *in vivo* observed effects may not be apparent in our laboratory model, which lacks stromal cells.

Similar to the results presented in this Chapter, Valentijn *et al.* found MPA to reduce PP TA, in endometrial explant cultures, after 24 hours treatment (Valentijn et al., 2015). They also reported that after five days of combined treatment with E2 and P4, TA was reduced by 50% compared with the treatment of E2 alone, in primary endometrial cell cultures comprised of both epithelial and stromal cells (Valentijn et al., 2015). Administration of 200 mg progesterone antagonist (mifepristone) in the early-secretory phase in healthy women, increased the endometrial epithelial immunoexpression for hTERT protein (Valentijn et al., 2015).

Similar to my results in ISK EC cells, Kyo *et al*. in cultured ER-positive MCF-7 breast cancer cells, TA was increased with 6 hours of E2 treatment (≥1 nM), and the effect persisted for least 48 hours(Kyo et al., 1999) when TA was examined using a quantitative telomerase assay. This was echoed by another study, which reported upregulation of TA and *hTERT* mRNA in ISK cells with E2 treatment through MAPK-dependant pathway in an ERα dependent manner (Zhou et al., 2006).

In the current study, I used two doses of DHT (0.01 and 1μM) to treat ISK cells, and demonstrated the high dose (1μM) of DHT to induce *DKC1* transcription similar to E2; and the highest effect was observed at 12 hours. This stimulatory effect of DHT on the *DKC1* gene could be mediated via either AR or ERα. DHT can directly bind to AR, but the intracellular metabolism of testosterone to estrogenic metabolites is well-described, especially in postmenopausal breast cancer patients (Sasano et al., 2008). At high doses, there may be higher levels of estrogenic metabolites of DHT available in ISK cells to bind to ERα to affect the typically estrogenic downstream effects. Further studies, using receptor-specific inhibitor are needed to ascertain this possibility.

DHT did not demonstrate a significant effect on TA at either supra-physiological doses (1μM) or at lower and more physiological doses (0.01μM). Similarly, no effect was observed with low dose DHT treatment on *DKC1* mRNA Contradictory to our TA results derived in the endometrium, Thalen *et al.* reported that DHT stimulated TA in an androgen-sensitive prostate cancer cell line (Thelen et al., 2004). This may be due to the cell-specific differences in the TA regulation since DHT is the main trophic stimuli for the prostate, whereas E2 is the main mitotic signal for the endometrium.

In summary, the results of this Chapter suggest that ovarian steroid hormones regulated *DKC1* in the endometrial epithelial cells, where estrogen and high dose DHT treatment stimulate *DKC1* transcription in ISK cells. Progesterone counteracted the estrogenic effect and decreased *DKC1* levels. Since this data suggest that dyskerin to be associated with endometrial epithelial proliferation, dyskerin may be a mediator of the well-known regulation of endometrial epithelial mitosis by the ovarian hormones. Therefore, future studies are warranted to further explore the hormonal regulation of *DKC1* to understand the role of dyskerin in mediating hormonal effects in human endometrium. In order to better comprehend the functional role of dyskerin, in Chapter 6, I have examined the effect of overexpression of *DKC1* gene on endometrial epithelial cell proliferation.

6

Chapter Six

Overexpression of *DKC1* Reduces Proliferation of Endometrial Cancer Cells

6.1 Introduction

Dyskerin is a pseudouridine synthase and a component of H/ACA small nucleolar ribonucleoprotein particles (Kirwan and Dokal, 2008). It is a fundamental protein that is highly conserved across species. Dyskerin is essential to many basic cellular events, including protein production, cell growth, and proliferation (Montanaro, 2010). Therefore, its functional loss is associated with premature ageing as well as subsequent multiple pathologies, e.g., as seen in the rare inherited X-DC (Di Maio et al., 2017). These include mucocutaneous abnormalities, bone marrow failure and increased susceptibility to cancer (Kirwan and Dokal, 2008). By the age of 50 years, nearly half of DC patients will have cancer. This suggests that loss of functional dyskerin increases the risk of carcinogenesis (Alter et al., 2009) and proposes a tumour suppressor role for dyskerin. The two best-described functions of dyskerin are: (i) The stabilisation of the telomerase RNA component, which in turn regulates TA; and (ii) The modification of rRNA via the conversion of specific uridine residues of ribosomal RNA to pseudouridine, thus allowing proper processing and function of ribosomes. In order to participate in these nuclear functions, dyskerin associates with the active telomerase holoenzyme (Cohen et al., 2007) and with specific scaRNPs. In addition, nuclear dyskerin is also associated with the hetero-pentameric complexes, H/ACA snoRNPs (Kiss et al., 2006) in which one molecule of an H/ACA small nucleolar RNA (snoRNA) is associated with dyskerin and three other highly conserved telomerase associated proteins: NOP10, NHP2 and GAR1 (Angrisani et al. 2014, Kiss et al., 2006). SnoRNAs have an RNA modification role and maturated snoRNAs; in particular, they produce short regulatory RNAs that are capable of modulation by alternative splicing (reviewed by Khanna and Stamm, 2010) (Khanna and Stamm, 2010). The discovery of this function drew specific attention to the association of dyskerin-H/ACA snoRNAs; in particular, some reports suggest that *DKC1* reduction may play a vital role in carcinogenesis through modulation of the splicing of specific mRNAs, or by altering the level of certain snoRNAs (reviewed by Angrisani *et al.* 2014) (Angrisani et al., 2014).

Correct localisation, for example with respect to telomerase and H/ACA RNP biology, is required for the appropriate function of dyskerin. Usually, dyskerin in the nucleus localises to both the nucleolus and Cajal bodies, which are important sites for trafficking, assembly and activity of telomerase. In addition, H/ACA RNP complex-guided pseudouridylation for rRNA and snRNA by dyskerin also occurs within these subnuclear organelles (MacNeil et al., 2019).

Some authors have suggested that dyskerin may have juxtaposing roles in tumorigenesis: suggesting gain as well as the loss of dyskerin function result in carcinogenesis (Alawi and Lin, 2011, Alnafakh et al., 2019). Overexpression of dyskerin has been proposed to be an essential feature in some cancer types, such as breast and prostate (Montanaro et al., 2006, Sieron et al., 2009). This is thought to provide the necessary increase in RNA and protein synthesis for cancer cells. *DKC1* mRNA levels in the same cancer types are also associated, albeit moderately, with *hTERC* levels, and this observation also supports the notion that for the essential maintenance of TA in the long-term growth of cancers *in vivo*, dyskerin may also be vital (Sieron et al., 2009, Montanaro et al., 2008). *In vitro* experiments showed that the impairment of *DKC1* function disturbs TA by decreasing *hTERC* levels, causing premature shortening of telomeres, which may lead to end-to-end chromosomal fusions, breakage and genomic rearrangements and subsequently results in carcinogenesis (Angrisani et al., 2014, Dos Santos et al., 2017). Furthermore, a defective ribosomal function in DC might influence the translation of a group of mRNAs encoding for tumour suppressors, and this may explain the apparent paradox of DC in which diminished cell proliferation is linked with an increase in the susceptibility to cancer (Montanaro, 2010).

The work described in this chapter tests the hypothesis that dyskerin has an important role in the proliferation of EC cells.

6.2 Research questions

1. Is there a correlation of dyskerin protein levels (immunostaining scores) with endometrial epithelial cell proliferation (measured by Ki67 proliferation index (PI)?
2. Can I develop an *in vitro* model of dyskerin overexpression in endometrial epithelial cells?
3. In the developed *in vitro* cell culture model, what is the subcellular location of exogenous dyskerin protein?
4. What is the functional effect (on cell proliferation) of dyskerin overexpression in the developed *in vitro* model?

To answer these research questions, the *DKC1* gene was transiently overexpressed in an *in vitro* model using an EC cell line ISK and the resulting changes in cell proliferation in the transfected cells, were analysed and compared with the empty vector transfected and non-transfected control cells by flow cytometry. The transfection success was confirmed with immunoblotting, to demonstrate the gain of additional dyskerin protein in the transfected cells.

The subcellular location of the overexpressed dyskerin was demonstrated with immunofluorescence, and exogenous dyskerin protein was aimed to be distinguished by co-localisation with the DDK tag protein (both the plasmid and empty vector utilised in this set of experiments were tagged with DDK tag protein), but unfortunately, the anti-DDK antibody was not demonstrating specific staining in our immunofluorescence experiments.

6.3 Materials and Methods

6.3.1 IHC staining and analysis

The samples included in the analyses were immunostained, as described in chapter 2. The primary antibody sources, concentration and incubation conditions are detailed in (Table 6.1) (Kamal et al., 2016b). The Ki-67 PI was evaluated by scanning endometrial sections under the microscope at 400X magnification as the percentage of positive cells, of any degree of nuclear staining intensity as previously described in Kamal *et al.* (Kamal et al., 2016b).

6.3.2 Cell culture

Cultured ISK cells were maintained in DMEM/F12 supplemented with 10% (v/v) FBS, L-glutamine, and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. All cell culture reagents were purchased from Sigma-Aldrich (Dorset, UK), and maintained as previously described (Parkes et al., 2018).

6.3.3 Transient transfection (overexpression)

Twenty-four hours prior to transfection, ISK cells were seeded in in a 6 well plate at a density of 0.5×10^6 cells/well. Immediately before the transfection, the medium was changed to DMEM/F12 without antibiotic (2ml per well). Cells were then transiently transfected by using a mixture of MYC-DDK tagged Dyskerin plasmid (OriGene, USA, 3µl) with Lipofectamine 2000 (Thermo Fisher Scientific, UK, 9µl); the plasmid or Lipofectamine 2000 was diluted in 250µl of Gibco Opti-MEM I (reduced serum medium) (Fischer Scientific, UK). Empty vector (Myc-DDK tagged pCMV6-Entry, OriGene, USA) at the same final concentration (as the main plasmid) and non-transfected cells were used as negative controls. 4-6 hours after transfection, the medium was replaced with fresh DMEM/F12 containing L-glutamine, FBS and antibiotic (Penicillin-Streptomycin). The cells were incubated at 37°C, 5% CO_2. The plasmids used were tagged with the synthetic DDK Tag protein to discern the transfected cells by using an anti-

DYKDDDDK (anti-DDK) Tag antibody. Figure 6.1 illustrates the three downstream experiments performed in this chapter.

Table 6.1 Primary antibodies and conditions for IHC, immunoblotting and immunofluorescence.

Primary Ab	Type	Clone	Supplier	HIAR* (min)	Dilution	Incubation Time (hour)	Condition Temp (°C)
IHC							
Dyskerin	P		Santa Cruz biotechnology[1]	3	1:500	20	4
Ki67	M	MM1	NovoCastra[2]	2	1:200	20	4
Immuno-blotting							
Dyskerin	P		Santa Cruz biotechnology[1]		1:1000	20	4
DDK Tag	M	5A8E5	Genscript[3]		1:1000	20	4
GAPDH	P		Sigma-Aldrich[4]		1:10000	20	4
Immuno-fluorescence							
Dyskerin	P		Santa Cruz biotechnology[1]		1:200	1	20
DDK Tag	M	5A8E5	Genscript[3]		1:1000	1	20

*HIAR by pressure cooking in citrate buffer pH 6. [1]Dallas, Texas, USA; [2]Newcastle, UK; [3]New Jersey, USA; [4]Dorset, UK. **Abbreviations:** Antibody (Ab), Polyclonal (P); Monoclonal (M) DYKDDDDK (DDK) Heat-induced antigen retrieval (HIAR), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH).

6.3.4 SDS-PAGE and immunoblotting

Protein was extracted using RIPA buffer (Sigma-Aldrich, Dorset, UK) supplemented with protease inhibitor (Sigma-Aldrich, Dorset, UK) and phosphatase inhibitor (PhosSTOP, Roche Diagnostics Ltd, Burgess Hill, UK), as described in section 2.5.2. Lysates were analysed by SDS-PAGE under reducing conditions on precast 12% gels (Mini-PROTEAN TGX, Bio-Rad, Hertfordshire, UK) and transferred to an Immune-Blot PVDF (polyvinylidene difluoride) membrane using Trans-Blot Turbo Transfer System (BIO-RAD, Hertfordshire).

Blots were blocked in 5% milk and then probed with primary antibodies (diluted in 5% skimmed milk). Matched HRP-linked secondary antibodies were used (ThermoScientific, UK) (1:3000, diluted in 5% skimmed milk). Signal detection was performed using SuperSignal™ West Dura Extended Duration Chemiluminescent Substrate (Thermo Scientific, UK) and CL-Xposure film (Thermo Scientific, UK). To validate the results, the experiment was repeated three times. Primary antibody sources, incubation conditions and concentrations are detailed in Table 6.1.

6.3.5 CFSE labelling and flow cytometry

6.3.5.1 CFSE labelling

To study the proliferation rates, ISK cells were labelled with 1µM CellTrace CFSE to provide sufficient fluorescence and minimal toxicity on ISK cells, as described in detail in chapter 2 and according to manufacturers' instructions.

6.3.5.2 Direct flow cytometry

ISK cells were trypsinised, pelleted, fixed and permeabilised as described in chapter 2. Then blocking was performed in 3% BSA in PBS. Fluorochrome-conjugated primary anti-DYKDDDDK (DDK) Tag Antibody (iFluor 647), (Genscript, USA) (1:500) and the corresponding fluorochrome-conjugated isotype control antibody Alexa Fluor 647 (Biolegend, UK) were prepared separately in Blocking Buffer, and the cells were resuspended with fluorochrome-conjugated primary antibody or isotype control antibody and incubated for 1 hour at 37°C in the dark. Then, the cells were centrifuged to pellet, washed twice and spun again to pellet. The fixation step was repeated to minimise dissociation and prolong the life of the signal for up to 5 days. The cells were subsequently centrifuged, resuspended with PBS and

spun down again to pellet. Each pellet was resuspended with 200µl of PBS and stored in 4°C in the dark before flow cytometry performed using a Guava EasyCyte flow cytometer (Millipore, Germany). FlowJo v10 software (Becton Dickinson, USA) was used to perform analysis.

6.3.6 Immunofluorescence

To differentiate between endogenous and exogenous overexpressed dyskerin protein, immunofluorescent staining of dyskerin was performed, allowing examination of their respective location within the ISK cell. Anti-DYKDDDDK (anti-DDK) Tag Ab (mouse) and anti-dyskerin Ab (rabbit) were added to the fixed cells which were seeded onto coated coverslips in a 6 well plate. The corresponding secondary Alexa Fluor conjugated antibodies (Anti-mouse and anti-rabbit IgG (H+L) (Alexa Fluor 555 and 488 Conjugate, respectively) (Cell signalling, USA, 1:1000) were applied. The cells were mounted in DAPI containing medium (Vector Laboratories, UK, one drop per coverslip). Fluorescence was visualised with a Nikon Eclipse 50i microscope using NIS elements F software. Rabbit and mouse isotype control antibodies were used as negative controls. The primary antibody sources, concentration and incubation conditions were detailed in Table 6.1.

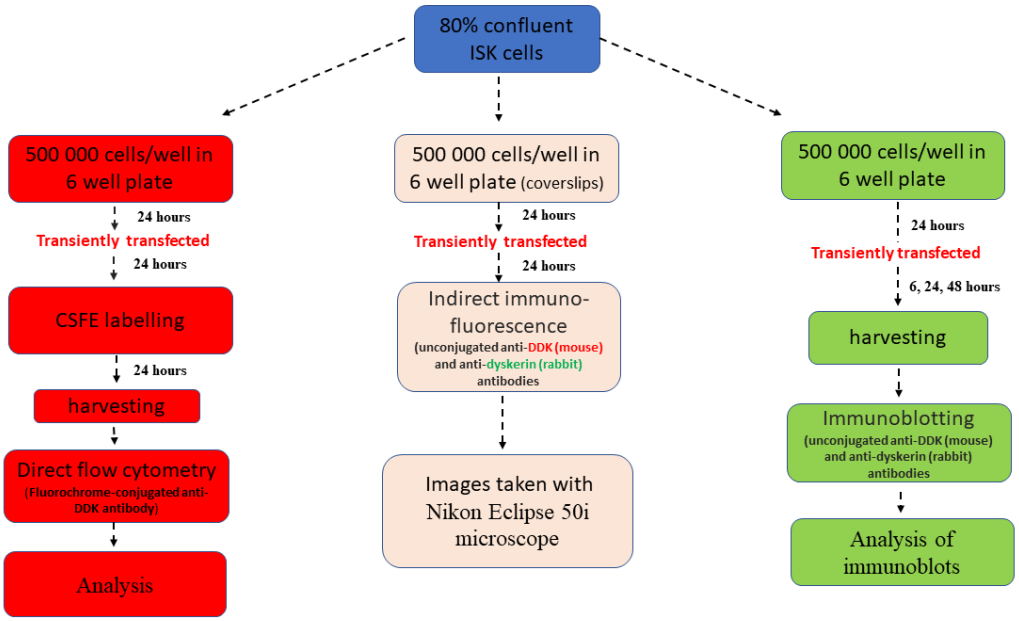

Figure 6.1 Flow chart summarising the three downstream experiments performed in this chapter: Direct flowcytometry, indirect immunofluorescence and immunoblotting. Cells were seeded (density of 500.000 cells/well) in 6 well plate 24 hours before transient transfection in each of the three experiments. **Abbreviations:** Carboxyfluorescein succinimidyl ester (CFSE); Ishikawa (ISK).

6.4 Results

6.4.1 Dyskerin protein levels correlate negatively with Ki67 PI

Ki67 PI in the healthy and EC endometrial samples included in this study demonstrated the well-established pattern (Figure 6.2A). That is, healthy PM epithelium had low Ki67 PI, whereas the EC samples generally demonstrated a significantly high Ki67 compared with PM ($p<0.0001$) or SP ($p<0.0001$). In healthy endometrial samples, Ki67 immunoscores levels were higher in PP compared with SP ($p=0.001$) and PM ($p<0.0001$) (Figure 6.2B). Considering all human endometrial samples included in this study, nuclear dyskerin immunoscores in endometrial epithelial cells demonstrated a negative correlation with the Ki67 proliferative index ($r=-0.33$, $p<0.0001$). Figure 6.2A showed the immunohistochemical staining of Ki67 in the endometrial samples.

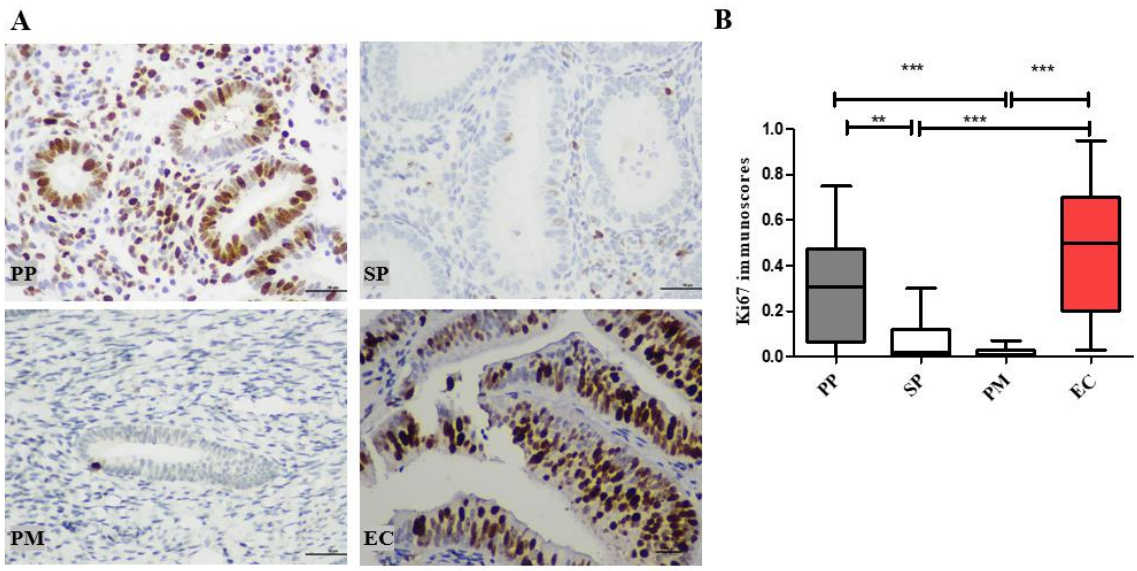

Figure 6.2 Ki67 proliferative index (PI) immunohitochemistry (IHC) staining in endometrial samples **A)** Representative microphotographs illustrate the IHC staining of Ki67 in the following endometrial samples: Healthy proliferative (PP), secretory phase (SP), postmenopausal (PM) and endometrial cancer (EC). Positive staining appears brown. Magnification 400X. Scale bar 50µm. **B)** Difference of Ki67 immunoscores in PP (n=22), SP (n=17), PM (n=28) and EC (n=107). *p<0.05, **p<0.01, ***p<0.001.

6.4.2 Overexpression of *DKC1* effect on EC proliferation *in vitro*

6.4.2.1 Dyskerin transfection efficiency

ISK cells were transiently transfected using Myc-DDK tagged dyskerin (*DKC1*) plasmid and empty vector and flow cytometric analysis was performed 48 hours after transfection to ascertain the transfection efficiency in each transfection experiments that were conducted, e.g. dyskerin (7-18%) (Figures 6.3A, B & Appendix III), and empty vector (11%) transfected cells; the false-positive level in this analysis, as identified in the non-transfected control was 1.76% (Figures 6.4A and B). Appendix III shows the negative controls used in this experiment.

6.4.2.2 *In vitro* transient transfection of ISK cells with the *DKC1* gene resulted in successful overexpression of dyskerin protein

A time-course experiment was conducted at 6, 24 and 48 hours after transfection, where endogenous and exogenous dyskerin levels were examined with immunoblotting.

A positive band corresponding to endogenous dyskerin was observed at the molecular weight (MW) of (58KDa) in negative controls (empty vector and non-transfected cells) and in transfected ISK cells at 6, 24 and 48 hours after transfection (Figures 6.5A and B). The tagged exogenous dyskerin protein was first observed at 24 hours and was still present at 48 hours (although decreased) in the *DKC1* transfected cells at the MW of 60KDa, (Figures 6.5A and C).

Confirmation of transfection was only seen with DDK tag protein at 24 and 48 hours; a positive signal at the MW of 60KDa demonstrates that the DDK tag peptide was present in transfected cells at 24 and 48 hours only.

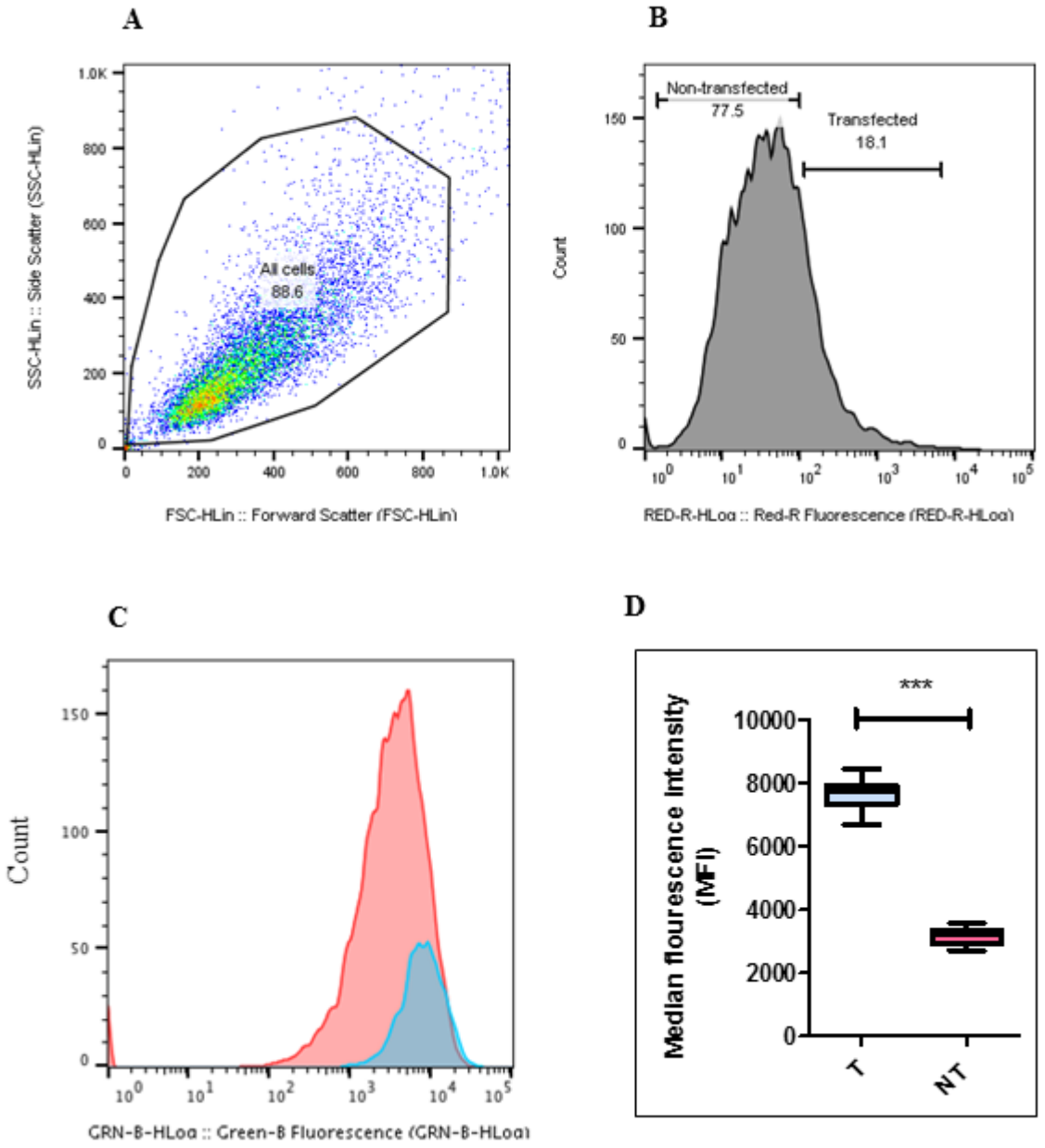

Figure 6.3 Direct flow cytometry following transient transfection of endometrial cancer cell line Ishikawa (ISK) with DDK tagged *DKC1* plasmid using Lipofectamine 2000 **A)** Dot-plot represents gated ISK cells exposed to DDK tagged DKC1 plasmid and transfection reagents. Fluorochrome-conjugated primary antibody was anti-DYKDDDDK (anti-DDK) Tag Antibody (Ab) (iFluor 647). Forward scatter (FSC); Side scatter (SSC). **B)** Two population of cells within the gate outlined in Figure 6.3A are represented in a histogram to evaluate the relative expression of anti-DDK Tag Ab in ISK cells, the cells stained with anti-DDK Tag Ab represent the transfected cells while the non-stained cells represent non-transfected cells.

C) Cell proliferation was analysed using the CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE). ISK were stained with CellTrace™ CFSE. Followed by direct flow cytometry staining with anti-Tag Ab (iFluor 647). Comparison was done between the two population of cells mentioned in Figure 6.3B: Transfected and non-transfected, blue and red curves respectively (both groups of cells were exposed to transfection material). Higher proliferation is suggested as the curve shifted to the left. Analysis was performed 48 hours after transfection and completed using a Guava easycyte with 488-nm excitation and a 530/30-nm bandpass emission filter for CellTrace™ CFSE. **D)** The difference in median fluorescence index (MFI) between transfected (T) and non-transfected (NT) cells was evaluated using paired t test, (n=8), *p<0.05, **p<0.01, ***p<0.001

6.4.2.3 Subcellular localisation of endogenous and exogenous dyskerin in transfected and non-transfected ISK cells

To examine the location of exogenous and endogenous dyskerin protein within ISK cell, double indirect immunofluorescence was performed, using anti-dyskerin and anti-DDK tag antibodies. The endogenous dyskerin was seen in all cells, including *DKC1* plasmid, empty vector transfected and in non-transfected cells and characterised by a punctuated pattern that was exclusively localised in the nuclei (Figure 6.6). The exogenous dyskerin was observed only in *DKC1* transfected cells and located both in the nucleus and in the cytoplasm, as illustrated in Figure 6.6. DDK staining was also attempted to confirm these findings, but unfortunately, the staining pattern of the anti-DDK antibody obtained did not work well with immunofluorescence (i.e. the antibody was non-specific) (Anti-DDK Antibody staining in *DKC1* transfected cells present in Appendix III). Therefore, it was not possible to ascertain useful information on the location of DDK-tagged *DKC1* gene.

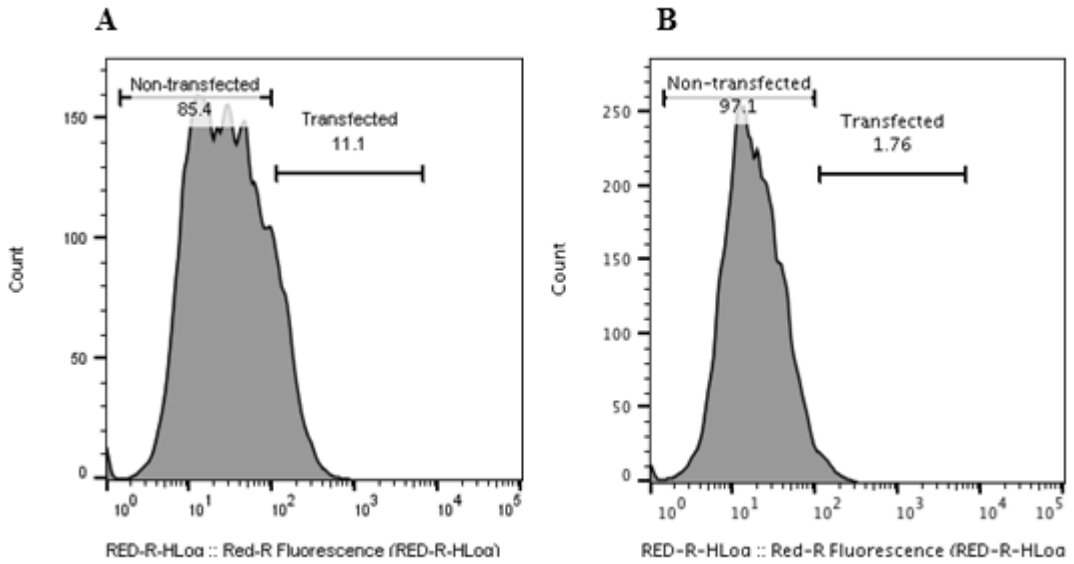

Figure 6.4 Direct flow cytometry of the negative controls using anti-DYKDDDDK (anti-DDK) tag antibody (Ab) (iFluor 647). A) Flow cytometry histogram to evaluate the relative expression of anti-DDK Tag Ab in empty vector transfected cells. Transfected cells represented by those cells that stained with the Ab, and the non-transfected cells are the cells that were negative to the aforementioned Ab. **B)** Flow cytometry histogram to evaluate transfection percentage in Ishikawa cells that were not subjected to the transfection reagents (negative control). The transfected cells represent a false positive value.

6.4.2.4 Transient overexpression of the dyskerin (*DKC1*) gene, resulting in reduced ISK cell proliferation *in vitro*

The rate of cellular proliferation assessed by flow cytometry in two populations of cells: The transfected cells group (stained with anti-DDK antibody) compared with non-transfected cells (subjected to transfection material) (not stained with anti-DDK antibody) 48 hours after transfection (Figure 6.3B). The proliferation was lower in transfected cells, as shown in Figure 6.3C (the proliferation is higher when the curve is shifted to the left). Additionally, the median fluorescence intensity (MFI) of CFSE staining was evaluated in these two groups of cells and it was significantly higher in transfected cells (T) compared with non-transfected (NT) cells ($P<0.0001$); the MFI decreases in the case of high cellular proliferation (CFSE fluorescence

intensity reduces when the rate of cell division is high as it passes from parent to daughter cells). (Figure 6.3 D). When the cellular proliferation rate was compared between dyskerin transfected, and empty vector transfected cells, cells transfected with dyskerin demonstrated a lower rate of cellular proliferation (Figure 6.7A).

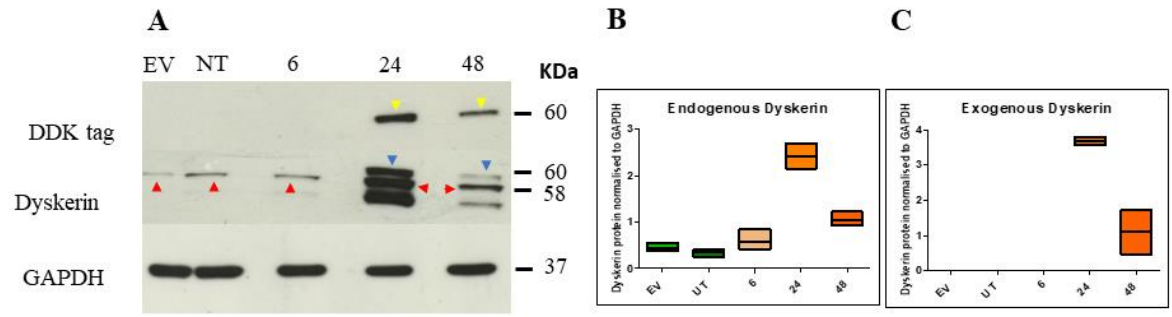

Figure 6.5 Immunoblotting following transient transection with DDK tagged *DKC1* plasmid **A)** Immunoblotting was performed using three primary antibodies: Anti DYKDDDDK Tag (anti-DDK), anti-dyskerin and anti- Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (loading control). Empty vector (EV) transfected, and non-transfected (NT) are the negative controls used in this experiment. Ishikawa (ISK) Cells were harvested 6, 24 and 48 hours following the transfection. Endogenous and exogenous dyskerin bands were present at the molecular weight (MW) of 58 and 60KDa (red and blue arrows) respectively. DDK bands (yellow arrow) were observed at 60KDa and GAPDH bands at 37KDa. **B)** Endogenous dyskerin protein was quantified using Image J. Dyskerin protein was normalised to GAPDH to ensure an equal amount of protein to be loaded in each well and Kruskal- Wallis test was used to evaluate the difference in dyskerin protein among all the 3-time points of transfected cells as well as the negative controls **C)** Exogenous dyskerin protein was quantified using image J. Dyskerin protein was normalised to GAPDH to ensure equal protein loading in all wells. Kruskal- Wallis test was used to evaluate the difference in dyskerin protein between transfected cells and negative controls.

The proliferation rates of ISK cells in the following experimental conditions were compared (1) Non-transfected cells, not exposed to any transfection materials, (2) Non-transfected cells, exposed to empty vector plasmid and transfection materials, (3) Non-transfected cells exposed to dyskerin plasmid and transfection materials. The lowest proliferation rates among the cells exposed to the above three conditions were observed in the cells that were exposed to dyskerin plasmid transfection (3) (Figure 6.7B).

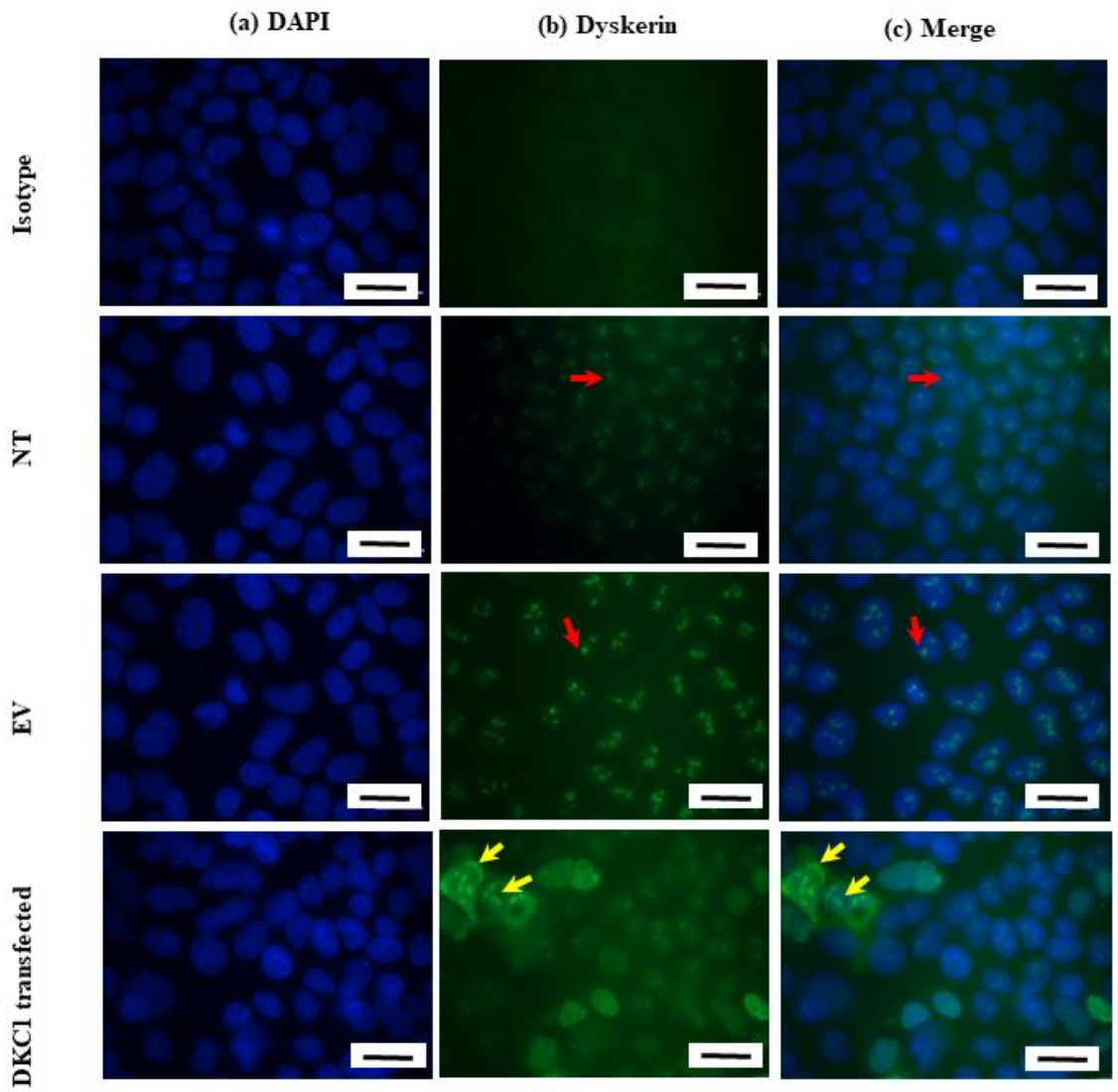

Figure 6.6 Immunofluorescence staining of ISK cells: Anti-dyskerin antibody (Ab) (primary Ab) and Alexa Fluor conjugated 488 (matched secondary Ab). Panel (a) 4′,6-diamidino-2-phenylindole (DAPI); (b) Anti-dyskerin antibody and (c) merge of (a) and (b). Isotype antibody staining, non-transfected negative control (NT), empty vector transfected cells (EV) and ISK cells transfected with *DKC1* plasmid. Blue colour represents staining of cell nuclei with (DAPI), green colour represents dyskerin (*DKC1*). Endogenous dyskerin (red arrows), exogenous dyskerin (yellow arrows). Magnification 100X. Scale bar 100μm.

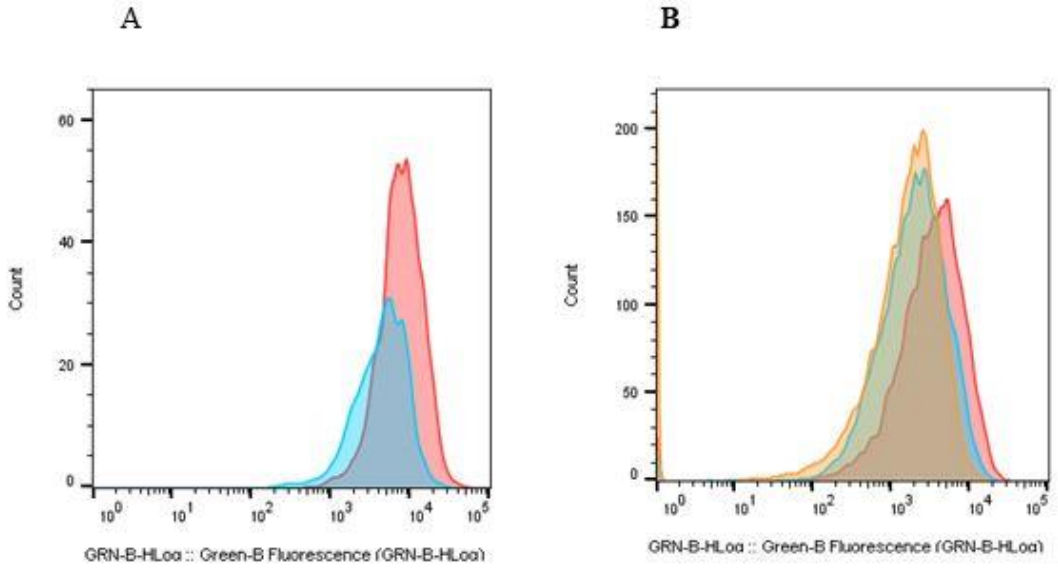

Figure 6.7 Cell proliferation was analysed using the CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE) in Ishikawa (ISK) cells: **A)** comparison of proliferation between cells transfected with *DKC1* plasmid (red curve) and cells transfected with empty vector (blue). The high proliferation was suggested when the curve is shifted to the left **B)** Cell proliferation of non-transfected cell of three different conditions: (1) ISK cells that did not expose to any transfection reagents (yellow curve), (2) Non-transfected cells but subjected to empty vector plasmid and transfection materials (blue curve) and (3) Non-transfected ISK cell exposed to *DKC1* plasmid and transfection materials (red curve).

6.5 Discussion

Involvement of the dyskerin protein in various essential cellular processes including telomere maintenance, splicing efficiency, ribosome biogenesis, snoRNAs stabilisation and stress response has been previously described (Angrisani et al., 2018). In this chapter, I demonstrated the involvement of dyskerin protein in the regulation of cellular proliferation in ISK EC cell line for the first time. Overexpressing dyskerin had a negative effect on cell proliferation suggested by the demonstration of low rates of cell proliferation in ISK cells transfected with a dyskerin plasmid (thus have high dyskerin protein levels) compared with non-transfected cells. These results are consistent with the *in vivo* data presented in chapter 4, which demonstrate that dyskerin levels are significantly lower in highly proliferative EC samples compared with normal PM endometrial samples, where it correlated negatively with Ki67-PI of the endometrial epithelial cells.

I have demonstrated that by using a Lipofectamine-based transfection method, dyskerin protein levels increase in the ISK cell line. Initially, immunoblotting was used to demonstrate that the endogenous dyskerin in human EC tissue has an MW of 57-58KDa and this observation agreed with published data (Parry et al., 2011, Norfilza Mohd Mokhtar et al., 2014). A band corresponding to the same MW was observed in all our cultured ISK cells (both transfected and negative control) representing the endogenous protein. The dyskerin plasmid used was tagged with DDK tag protein; thus, the MW of the exogenous dyskerin was expected to be higher than that of endogenous dyskerin. In agreement with that, a band representing exogenous dyskerin was observed at the MW of 60kDa in transfected cells, at 24 and 48 hours after transfection. Repeated experiments and corresponding immunoblots confirmed that this band corresponds to the exogenous dyskerin as a result of the transfection process, as it was not observed in the negative controls in any of the immunoblots.

Furthermore, this band was also absent at the 6 hours' time point after transfection in transfected cells, providing information on the expected time to detect functional changes relevant to increased exogenous dyskerin. The proliferation assays were conducted at 48 hours after transfection, for two reasons: (1) the immunoblotting confirmed the increased levels of the exogenous overexpressed dyskerin to persist at 48 hours (albeit lower than at 24 hours); (2) the earliest time the proliferation experiments can be performed after achieving adequate dyskerin overexpression, using our flow cytometric assay was at 48 hours. The immunoblot at 48 hours, therefore, confirmed the suitability of our experimental plan. There were additional

bands located around the MW 50KDa (MW lower than that of endogenous dyskerin), and I propose that these bands may represent a truncated variant of dyskerin. Several reports describe the truncated dyskerin variant, which lacks either C or N-terminal (nuclear localisation signal) and shows a prevalence in a cytoplasmic localisation (MacNeil et al., 2019, Angrisani et al., 2018, Angrisani et al., 2011).

The endogenous dyskerin in transfected cells at 24 and 48 hours after transfection also showed higher intensity compared with that at 6 hours and with the negative controls. This agrees with other reports of transfection associated changes to the endogenous gene; for example, Fiszer-Kierzkowska *et al.* found a significant induction of endogenous *Hspa1b* gene in cells treated with Lipofectamine 2000 by using DNA microarrays. Numerous other stress genes were also activated, together with several genes included in cellular metabolism, cell cycle control and pro-apoptotic pathways. We assume a similar effect may have contributed to explain our observation (Fiszer-Kierzkowska et al., 2011).

The immunofluorescence results of this study revealed that in the transfected Ishikawa cells, dyskerin was located in both the nucleus and cytoplasm, whereas in cells transfected with empty vector and in the non-transfected cells, dyskerin was located only in the nucleus. A previous study reported multiple minor dyskerin spliced variants, but their functions remain to be clarified (Angrisani et al., 2018). These authors suggested that the reported low abundance splice isoforms could contribute to a wide range of functions, including specialised functions or by playing significant roles in cell status alteration (Angrisani et al., 2018). For example, Angrisani *et al.* reported the biological roles of a truncated dyskerin variant that lacks the C-terminal nuclear localisation signal, to include non-canonical functions, such as homeostasis of cell metabolism (Angrisani et al., 2018). Future studies are required to ascertain if the overexpressed dyskerin in the altered sub-cellular location (cytoplasm) has a similar functional relevance in EC cells.

Critically short telomeres activate a p53-dependent pathway that leads to cell cycle arrest induction, which can cause senescence or apoptosis, both resulting in cell cycle arrest and decreased proliferation. Defects in *DKC1* in DC are thought to act via this mechanism, causing growth defects in proliferative tissues via telomere attrition (Gu et al., 2008). Excessive cellular proliferation is a cardinal feature of carcinogenesis, and my data indicate lower dyskerin levels were associated with the observed high cellular proliferation in the EC cells (Chapter 4). Therefore, I sought to examine the consequence of overexpressing the *DKC1* gene in an EC cell line that simulated low-grade ECs. There were no previously reported *in vitro* studies

examining *DKC1* overexpression; instead, the only *in vitro DKC1* gene manipulation studies available reported the effects of knocking down *DKC1* (Alawi and Lin 2011). As previously mentioned, complete dyskerin knock out is lethal, and all cells despite with or without detectable TA, express dyskerin gene/protein. Many studies on different cancer types, including those tissues/organs where carcinogenesis is associated with increased TA compared with healthy tissues (e.g., prostate, lung) (Sieron et al., 2009, Montanaro et al., 2006), reported overexpression of *DKC1* gene and dyskerin protein to be associated poor prognosis. For example, in contrast to our results on EC, reports on prostate, hepatocellular, lung and colorectal cancers showed that high *DKC1* is commonly associated with extensive tumour growth pattern (Sieron et al., 2009, Liu et al., 2012a, Turano et al., 2008, Penzo et al., 2015). Knocking down dyskerin in prostate carcinoma cell lines demonstrated that dyskerin is crucial in maintaining protein biosynthesis (Sieron et al., 2009). However, either up or downregulation of dyskerin function can contribute to carcinogenesis (Alawi and Lin, 2011). In cancers, such as prostate cancer (Sieron et al., 2009) and in neuroblastomas (O'Brien et al., 2016), high dyskerin is the norm; this was the anticipated reason for most other researchers assessing the therapeutic possibility of reduced expression of *DKC1* gene in those cancers, thus with siRNA-based gene interference to knock it down (Alawi and Lin, 2011, Sieron et al., 2009). I believe that my focus, EC, in this respect to be different, since the reduction of dyskerin/*DKC1* protein and gene expression was the observed change in dyskerin levels in the EC samples. Therefore, the appropriate avenue for my work was to overexpress dyskerin, to assess the consequential effect in cell proliferation in EC cell line ISK. My data are novel, describing the effect of increased levels of dyskerin on reduction in cell proliferation rates. The exact mechanism by which dyskerin exerts this observed antiproliferative effect on EC cells, however, remains to be explored in future studies.

I included multiple controls experiments to confirm the robustness of the data obtained from cells transfected with *DKC1* plasmid. Cells transfected with *DKC1* plasmid consistently showed lower proliferation rates compared with empty vector transfected cells or non-transfected cell. The difference in the rate of proliferation in the two negative controls suggests that the mere exposure to the transfection conditions may slightly affect cell proliferation.

Reduction of dyskerin is not unique to EC but has also been reported in some other cancers. For example, there was a significant reduction in *DKC1* expression compared with the controls in chronic lymphocytic lymphoma (CLL) (Dos Santos et al., 2017). Others have also suggested

that dysregulation of dyskerin affected the translational apparatus in CLL and that the patients with a decreased level of dyskerin have a significantly poor outcome (Sbarrato et al., 2016).

In the current study, the relatively low dyskerin levels in negative controls (empty vector and non-transfected cells) was associated with a higher rate of cell proliferation. This observation may be comparable with the loss of functional dyskerin in *DKC1* hypomorphic mice and X-DC patients, where cell proliferation is also impaired. Such impairment in *DKC1* function affected the translation of mRNAs containing internal ribosome entry site (IRES) sequences, including those encoding the tumour suppressor p27 and the anti-apoptotic factors Bcl-xL and XIAP (X-linked Inhibitor of Apoptosis Protein) (Yoon et al., 2006, Bellodi et al., 2010). The consequence of impairment of p27 IRES-mediated translation was a significant increase in the occurrence of spontaneous pituitary tumours in p27 heterozygous mice. Therefore, it can be concluded that there are functions of dyskerin that are essential for evading carcinogenesis independently of TA, and that dyskerin has a critical role in tumour suppression, at least in part through translational control of p27 (Bellodi et al., 2010).

A high incidence of cancer (particularly breast, lung carcinomas and B-cell lymphomas) was observed in the *DKC1* hypomorphic mutant mouse in which dyskerin expression was decreased. The decreased dyskerin level causes a reduction in both TA and rRNA pseudouridylation. The elevated susceptibility to cancers in this mouse model was detected to start as early as the first two generations when telomeres are still very long, while rRNA pseudouridylation was markedly reduced (Ruggero et al., 2003). Therefore, similarly, the observed cell proliferation may be related to the reduction in rRNA pseudouridylation.

Furthermore, a novel dyskerin-dependent mechanism has also been described to deactivate p53 via altering the translation of p53 mRNA. Low dyskerin levels triggered a defect in the p53 activity (independently of its role in telomerase activity) and caused a strong impairment in p53-IRES-mediated translation initiation in a subset of primary breast cancer specimens suggested that this dyskerin-dependent mechanism of p53 inactivation may be operating in a series of human breast carcinomas (Montanaro et al., 2010). In agreement with the above, embryonic fibroblasts from *DKC1* hypomorphic mice were also more resistant to p53-mediated oncogene-induced senescence and were easily transformed by oncogenic Ras expression (Montanaro, 2010).

Taking these observations together, I conclude that increased dyskerin protein levels in ISK cells might inhibit cell proliferation. Therefore, the observed loss of dyskerin in EC tissue may

contribute to the increase in cell proliferation and the progression of these ECs. Further studies are needed to explore the exact mechanism by which dyskerin exerts the antiproliferative effect and how this pathway can be manipulated to develop novel therapeutic options for this devastating disease.

7

Chapter Seven

Final Discussion

7.1 Overview of research findings

Endometrial cancer (EC) is the 8th most common cause of cancer death in females in the UK currently (CRUK and was responsible for 3% of all cancer mortality in women in 2017. Furthermore, in the UK, this continued rise in EC mortality rates seen over the last 10 years is projected to increase by at least 19%, to 9 deaths per 100,000 females by 2035 (CRUK). EC generally is associated with a good patient outcome and prognosis; however, recurrence is recorded in 13% of cases (Fung-Kee-Fung et al., 2006). The prognosis of recurrent disease is poorer than the primary tumour, and the median survival for women with recurrent EC is less than 10 months (Huijgens and Mertens, 2013). Additionally, the number of patients in the UK with recurrent or advanced-stage EC is also increasing (Odagiri et al., 2011). Therefore, it is necessary to find markers that identify ECs that are high-risk with respect to a poor prognosis and to identify tumours that are likely to recur at the initial stages of diagnosis. This will allow for the stratification of patients for further adjuvant therapy and for the development of novel therapeutics.

The role of telomeres and telomerase in the initiation and progression of human cancers has been well established (Heaphy and Meeker, 2011). The main function of telomeres is to protect the ends of chromosomes, whereas the specialised enzyme, telomerase, maintains TL, thus, allows unlimited cellular proliferation. Significant differences in TL and TA have been reported between malignant and healthy tissues. Therefore, many studies have evaluated the utility of TA or telomerase-associated proteins in cancer diagnosis or as a prognostic marker (Heaphy and Meeker, 2011). There are numerous reports of assessing TA and the 2 of the core components of telomerase, *hTERT* and *hTERC* in benign and cancerous endometrium (Valentijn et al., 2015, Hapangama et al., 2008b, Ebina et al., 1999, Tanaka et al., 1998, Maida et al., 2002, Lehner et al., 2002). High TA is present in 80-90% of human epithelial cancers, including EC (Alnafakh et al., 2019, Lehner et al., 2002). In the studies undertaken during this PhD studentship, I aimed to examine the remaining core telomerase complex component, dyskerin(Cohen et al., 2007), which is the only main subunit of telomerase holo-enzyme that is not yet studied in human endometrium or in EC.

This thesis, therefore, provides a comprehensive description of the dyskerin (*DKC1*) levels in healthy human endometrium and in EC samples; it evaluates its effectiveness as a marker that can stratify ECs into low-risk and high-risk groups, and assesses the possibility of utilising dyskerin protein as a prognostic biomarker for EC. Finally, the *in vitro* studies investigate the

functional role of dyskerin in endometrial epithelial cells and describe its effect on cell proliferation in EC cells.

7.1.1 Dyskerin protein (*DKC1*) is present in normal human endometrium

The experiments carried out initially describe that both *DKC1* mRNA and dyskerin protein were present in healthy endometrium (Chapter 3), and dyskerin protein was observed in both epithelial and stromal cells; this, to our knowledge, is the first report of detecting dyskerin in the human endometrium. The levels of both dyskerin protein and mRNA were significantly higher in proliferatively quiescent PM endometrial epithelial cells (Chapter 3) compared with proliferative premenopausal endometrium, suggesting an inverse relationship with epithelial cellular proliferation.

Previous studies examining telomerase have reported other telomerase component, *hTERT* (protein and mRNA) and TA levels were highest in proliferative endometrium, lowest in the SP and in PM atrophic endometrium (Kyo et al., 1997, Lehner et al., 2002, Valentijn et al., 2015, Hapangama et al., 2008b). When examining endometrial cellular compartments, stromal cells, regardless of the cycle phase, demonstrate absent or significantly lower TA and *hTERC* level compared with the epithelial cells (Tanaka et al., 1998, Valentijn et al., 2015, Vidal et al., 2002, Yokoyama et al., 1998). The high TA in healthy proliferating endometrial epithelial cells correlated negatively with TL (Valentijn et al., 2015). This suggested that the high TA in epithelial cells is to maintain the short telomeres and to avoid critical telomeric attrition (Valentijn et al., 2015).

In this context, dyskerin protein and *DKC1* mRNA levels seem to to have a contrasting expression pattern in the human endometrium compared with TA and hTERT. Therefore, the subsequent experimental pathway taken was to examine the role of dyskerin in pathological endometrial diseases where telomerase is known to be altered, to investigate the hormonal regulation of dyskerin in the endometrium and finally to examine the role of dyskerin in endometrial epithelial proliferation.

7.1.2 Dyskerin (*DKC1*) levels in benign proliferative endometrial disorder (Endometriosis)

There are many theories put forward to explain the development of endometriotic deposits, and one of the most accepted theories is the retrograde menstruation proposed by Sampson (Transplantation theory). He proposed that the disease is established when the shed endometrial cells at the time of menstruation from the uterus, is retrogradely transported and transplanted in the pelvic cavity (Sampson, 1927).

In the studies described in Chapter 3, I found dyskerin to be present in eutopic (SP) endometrium and in ectopic endometriotic lesions in women with surgically diagnosed endometriosis. I also showed that Dyskerin immunoscores (in luminal and functionalis endometrial glands) and *DKC1* mRNA levels appeared to be higher in SP of endometrial samples obtained from women with confirmed endometriosis compared with SP samples from healthy women; however, this difference did not reach statistical significant level (Chapter 3). Previous studies have shown TA and hTERT to be increased in the SP endometrium of women with endometriosis (Hapangama et al., 2008b, Kim et al., 2007) and our data also showed that *hTERC* levels to be significantly increased in women with endometriosis. Although the difference did not reach statistical significance, my data also suggest a higher TA value in these samples. It is, therefore, possible that the sample size I have included is too small to detect a significant difference. However, the levels of dyskerin present in these samples might facilitate the function of high *hTERC* and TA as stabilisation of *hTERC* is one of the main functions of dyskerin; it can be envisaged that this might enhance the survival probability of endometrial cells shed into the pelvic cavity during retrograde menstruation and cause endometriotic lesions to develop (Hapangama et al., 2008b). This hypothesis agrees with the several previous studies that have already reported that the late secretory endometrium of women with endometriosis to be characterised by high TA, pro-proliferative and antiapoptotic features (Hapangama et al., 2008b, Hapangama et al., 2010, Hastings and Fazleabas, 2006). Likewise the ectopic endometriotic lesions I have examined (Chapter 3), also demonstrated high dyskerin immunoscores in agreement with the proposed concept that the ectopic lesions have a greater ability to survive in the ectopic environment of the pelvis (Liu and Lang, 2011).

7.1.3 Hormonal regulation of dyskerin

Hormonal regulation of telomerase and ovarian steroid regulation of endometrial TA is well established. Dyskerin levels (mRNA and epithelial immunoscores) were higher in the hypo-estrogenic PM endometrium, compared with the premenopausal endometrium, where the ovarian estrogen and/or progesterone levels are high (Chapter 5). The endometrial epithelium of women, who were on GnRH analogue therapy simulating the hypo-estrogenic state, similarly demonstrated high dyskerin immunoscores. Conversely, the progestogen treated endometrial epithelial cells (LNG-IUS) demonstrated lower dyskerin levels than GnRH treated samples. In my *in vitro* experiments (Chapter 5), using an EC cell line (ISK), a short term (12 hours) treatment with E2 significantly upregulated *DKC1* mRNA levels, and progesterone counteracted that estrogen effect. In the same 2-dimensional cell culture model, which contained only epithelial cells, the potent androgen, DHT, in high supra-physiological doses increased *DKC1* levels; this was similar to the effect seen with E2. At lower, more physiologically relevant dose, DHT did not demonstrate an effect on *DKC1* levels. The *in vitro* increase in *DKC1* mRNA levels I have observed at 12 hours with E2 was accompanied by an increase in TA. These seemingly contradictory results may be due to the fact that our *in vitro* cell culture model system only contain epithelial cells. The inability of such mono-cellular cell culture systems to reproduce the *in vivo* hormonal effect observed in the human endometrium is well known. The *in vivo* effects of ovarian hormones on endometrial glandular epithelium can be via stromal cells, and the primary example of this is the anti-proliferative effect of progesterone on the endometrial epithelium. Progesterone induces basic helix-loop-helix transcription factor Hand2 in the endometrial stroma that inhibits the production of a number of fibroblast growth factors, which mediate the mitogenic effects of estrogen on the epithelium (Li et al., 2011b, Kurita et al., 1998, Marquardt et al., 2019, Li et al., 2011a). At least in this mono-cellular system, estrogen increases dyskerin in epithelial cells, in conjunction with an increase in TA levels. E2 treatment in the mono-culture system is known to induce cell proliferation and associated increased in TA may require a modest increase in *DKC1*. This highlights the importance of selecting the best model system to examine particular hormonal effects on the endometrial cells. I would propose that a co-culture system containing both stromal and epithelial cells would be more suitable to mimic the *in vivo* system.

7.1.4 Dyskerin (*DKC1*) levels in endometrial cancer and its prognostic value

High levels of dyskerin have been reported to be associated with poor patient outcomes in a number of human tumours of different origins. These include breast, prostate, head and neck, colon and hepatocellular carcinomas (Liu et al., 2012). In the current study, I found for the first time that dyskerin protein and mRNA levels are present in all EC subtypes; however, unlike in other cancer types, dyskerin levels were significantly lower in EC compared with healthy PM endometrium (Chapter 4). This is the opposite of what I expected from the well-known high TA levels reported in EC (Lehner et al., 2002). I also observed that high dyskerin in ECs was associated with better survival outcome and dyskerin protein was higher in early stages compared with ECs of advanced stages; primary ECs harboured lower dyskerin protein levels compared with matched metastatic lesions, and this agreed with a previous report which found that the metastatic EC lesions acquired a regressed phenotype (Kamal et al., 2016b) (Chapter 4). Importantly, my data suggest a better clinical outcome in high dyskerin protein containing ECs in comparison with low dyskerin ECs. On the other hand, analysis of TCGA dataset revealed that ECs with low *DKC1* RNA levels to be associated with a better prognosis, but this can be attributed to the fact that I have studied the association between the protein level and survival outcome of ECs, while in TCGA dataset, the association between *DKC1* RNA and survival outcome was examined. Additionally, in our study, the normal control was external healthy PM and premenopausal proliferative endometrial samples, while in TCGA, the normal reference were adjacent healthy tissues, 2-3cm from the tumour margin (Huang et al., 2016a) (Chapter 4). Furthermore, apart from *DKC1*, none of the other two core telomerase components neither *hTERT* nor *hTERC* was significantly associated with EC patient survival in the TCGA data.

Furthermore, the association I have consistently seen in the endometrium, low epithelial proliferation with higher dyskerin levels, and lower TA with paradoxically higher dyskerin all agree with low dyskerin levels in EC samples to provide a more aggressive phenotype and to adversely affect patient survival. However, the mechanism involved with this observed association is yet to be clarified.

7.1.5 The functional role of dyskerin and its effect on cell proliferation

Several reports proposed that excessive dyskerin to be associated with sporadic cancer development such as breast-, hepatocellular- and prostate cancer (Montanaro et al., 2006, Liu et al., 2012a, Sieron et al., 2009). However, a considerable body of evidence suggests that a reduction of dyskerin function is also linked with neoplasia. For instance, an X-DC disorder, resulting from mutations in *DKC1* gene, DC patients suffer from defects in highly regenerative tissues, such as skin and bone marrow, chromosome instability and a susceptibility to develop several types of cancers (Mitchell et al., 1999b). Another evidence is hypomorphic *DKC1* mutant *(DKC1m)* mice, the only available X-DC animal model; 50 % of these mice developed a variety of cancers (Ruggero et al., 2003). *DKC1*^{m} cells obtained from G1 and G2 mice have dysfunctional ribosomal RNA pseudouridylation before the onset of DC features. Telomeric shortening in *DKC1*^{m} mice became evident only in later generations (Ruggero et al., 2003). Additionally, chronic lymphocytic leukaemia (CLL) is also associated with loss of dyskerin function. Dyskerin has two main functions: stabilisation of *hTERC* and TA and rRNA processing and protein synthesis. Both TA and the rate of rRNA processing are typically increased during neoplasia. However, the pathways through which dyskerin dysregulation is associated with carcinogenesis are yet to be revealed.

I examined the effect of overexpression of *DKC1* gene on proliferation in EC cells (ISK) and found that transient overexpression of *DKC1* gene resulted in decreasing cell proliferation in comparison with negative controls. With that observation, I also propose that dyskerin might act as a tumour suppressor in EC; therefore, a decrease in its level results in an increase in proliferation (Chapter 6). In a mouse model for X-DC, impaired *DKC1* function affected the translation of specific mRNAs harbouring IRES elements, including the tumour suppressor, p27. Furthermore, in a human pituitary adenoma, Bellodi and colleagues showed that a novel mutation of *DKC1* (DKC1(S485G)), where a specific amino acid substitution significantly alters *DKC1* stability and pseudouridylation activity. This mutation correlated with downregulation of p27 protein levels. *DKC1*(S485G) mutation did not affect telomerase RNA levels. Thus, genetic mutations in *DKC1* could play a role in tumorigenesis associated with somatic cancers and establish a crucial function of *DKC1* in tumour suppression, at least in part, via translational control of p27 (Bellodi et al., 2010).

I assumed that dyskerin has an adverse effect on cell proliferation because endometrial epithelial dyskerin protein immunoscores correlates negatively with Ki67 proliferative index (Chapter 6). In keeping with that, overexpression of dyskerin decreased proliferation rates in an EC cell line (ISK) (Chapter 6).

7.2 Strength, Limitations and challenges of the study

The immunoreactivity of the proteins of interest in my study was analysed semi-quantitatively. Although immunoscoring was limited by observer subjectivity, I controlled this possible bias by independent scoring of a set of randomly selected samples by at least two observers (interobserver reproducibility); and I also rescored the slides again (intraobserver reproducibility). To study dyskerin level in ECs, I have included a similar number of LGEC and HGEC in the local cohort. This caused my sample to be deviated from the real incidence of non-endometrioid EC; but this offers me a better assessment of HGECs, which are usually associated with poor prognosis. My normal control for EC samples includes age-matched healthy PM endometrium and samples from healthy proliferative women. These were chosen because primarily EC is a disease of postmenopausal age and also is a proliferative disease. Contrastingly, in TCGA dataset, the control was presumed normal tissues obtained from EC patients, located 2-3cm from the margin of the tumour. Since most endometrioid or serous ECs originated from a background of endometrial hyperplasia with cytological atypia (EHA) or endometrial intraepithelial neoplasia (EIN), their approach increases the possibility of including hyperplastic or EIN lesions in the normal samples. Furthermore, RNA sequencing as a method would not allow to visualise cancerous cells in TCGA data, whereas my examination of tissue samples with IHC scoring allowed to visually identify cancerous cells directly and analyse their immunostaining.

The biggest challenge in this PhD studentship was the difficulties encountered in transfecting endometrial epithelial cells. Initially, I attempted to transfect ISK cells using two different plasmids. The first one had an unacceptably high cytotoxic effect on ISK cells; as such, most of the cultured cells died after a few hours following the transfection. This particular plasmid has the same sequence as the NCBI reference (NM_001363.5, Homo sapiens dyskerin pseudouridine synthase 1 (*DKC1*), transcript variant 1, mRNA). I then progressed to use a

second plasmid and subsequently in further experiments found that the second plasmid to be less cytotoxic to ISK cells. However, its sequence contains a single nucleotide difference from NCBI reference sequence containing G853T SNP (resulting in a valine to phenylalanine substitution at position 285 in the resulting translated dyskerin protein, Appendix III). This difference is a naturally occurring variation (e.g., polymorphisms).

To ascertain if this observation is only related to the ISK cells, another commercially available established EC cell line (HEC-1-A) was transiently transfected in our lab by Dr Sam Williams using the same methods and plasmids. With immunofluorescence, a similar pattern of immunofluorescence staining was observed in HEC-1-A cells as in the ISK cells with anti-dyskerin antibody demonstrating both endogenous and exogenous dyskerin (Appendix III), but because of the time limit I was not able to confirm the alteration of dyskerin protein level with immunoblotting, and I could not examine if HEC-1-A also demonstrate an anti-proliferative effect upon overexpressing *DKC1* by flow cytometry.

7.3 Future work

Further work immediately can be considered from the data I have generated to include the following avenues. My work did not include an assessment of dyskerin overexpression on TA or TLs in the ISK cells; this will require stable transfection of the cells, particularly to see an effect on TLs. The dyskerin overexpression data on ISK cells would also be further confirmed by repeating all experiments on a different cell line such as HEC-1-A, including immunoblotting and flow cytometry to confirm if overexpression of *DKC1* results in increasing dyskerin protein and if the *DKC1* overexpression has a similar effect on cell proliferation in HEC-1-A, thus endometrial epithelial cells in general. Such experiments can ideally be reproduced in primary healthy normal epithelial cells, if possible, to confirm the effect on healthy endometrium. Secondary methods to validate the effect on cell proliferation (such as PI Ki67 staining and quantification and/or MTT assay) would also confirm these observations.

The function of dyskerin includes two main pathways: stabilisation *hTERC* and enhancement of TA and rRNA modification /processing and protein production, including tumour suppressors. Therefore, it is important to study the effect of *DKC1* overexpression on these proteins such as P27 and P53 to elucidate the exact molecular pathway, through which dyskerin exerts its anti-proliferative activity.

7.4 Conclusions, impact and future directions

In summary, I confirmed for the first time that dyskerin (*DKC1*) is present in healthy human endometrium. Similar to the other two components of the telomerase complex (hTERT and hTERC), in the human endometrium, dyskerin also appears to be regulated by ovarian steroid hormones. Endometrial carcinogenesis was associated with a reduction in epithelial dyskerin protein levels, and my data suggest a poor clinical outcome in low dyskerin protein containing ECs in comparison with high dyskerin ECs. *DKC1* was transiently overexpressed in EC cells, and resultant high dyskerin protein levels demonstrated a negative impact on cell proliferation. Therefore, I propose that dyskerin might act as a tumour suppressor protein in the endometrial epithelium by suppressing cellular proliferation. Dyskerin could be used to stratify ECs into low and high-risk tumours, for further adjuvant therapy and for considering personalised clinical follow-up, since it may have a significant prognostic value to predict the patient outcome. Further studies are warranted to unravel the functional role of dyskerin in human endometrium and in EC, and to explore the molecular pathway through which dyskerin exerts the observed anti-proliferative effects on the endometrial epithelial cells.

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9 Appendices

9.1 Appendix I: Classification of EC

Summary of four systems used to classify ECs (Mirza, 2020; McAlpine et al., 2018; Morice et al., 2016).

	Based on	Classification	Histological type	Grade	Hormone receptor expression	Genomic instability	Molicular changes	prognosis	Associated clinical features
WHO	Histological features	Epithelial	Endometrioid grade 1 & 2 Endometrioid grade 3 Serous Clear Mixed cell Undifferentiated	LGEC HGEC HGEC HGEC HGEC HGEC	Positive Negative Negative Nagative Nagative Negative	Diploid Aneuploid Aneuploid Aneuploid Aneuploid Aneuploid		Good Poor Poor Poor Poor Poor	Metabolic syndrome None None None None
		Mixed (Epithelial/Mesenchymal) Mesenchymal Miscellaneous Gestational trophoblastic Lymphoid and myeloid Secondary	Carcinosarcoma	HGEC	Nagative	Aneuploid		Poor	None
Dualistic	Clinical and epidemiological features	Type I	Endometrioid	Grade 1-3	Positive	Diploid, frequent microsatellite instability (40%)	PTEN mutation (75–85%), PIK3CA mutation (50-60%), PIK3R1 mutation (40–50%), ARID1A mutation (35–40%), KRAS mutation (20–30%), CTNNB1 mutation (25%), FGFR2 mutation (12%)	Good (Overall survival 85% at 5 years)	
		Type II	Non endometrioid	HGEC	Negative	Aneuploid	**Serous ECs**: TP53 mutation (>90%), KRAS mutation (5%), PIK3CA amplifcation (45%),PIK3CA mutation (35%), PTEN mutation (11%), PIK3R1 mutation (12%), ERBB2 amplification (25–30%), FGFR2 mutation (5%) and frequent FGFR1, FGFR3 amplification and CTNNB1 mutation (3%), HER2 amplification (12-15%). **Carcinosarcomas:** TP53 mutation (60%), PTEN mutation (19%), PIK3CA mutation (35%), PIK3CA amplifi cation (14%), KRAS mutation (17%). ERBB2 amplification (13–20%), ERBB3 amplification or mutation (13%), FGFR3 amplifi cation (20%). **Clear cell carcinoma:** TP53 mutation (35%), PTEN loss (80%), PIK3CA mutation (18%), ERBB2 mutation (12%), ERBB2 amplification (16%), ARID1A (25%).	Poor (Overall survival 55% at 5 years)	
Dualistic with modification	G 3 endometrioid has been incorporated within type II EC	Type I	Endometrioid (Grade 1 and 2)	LGEC	Positive	Diploid		Good	
		Type II	Endometrioid Grade3 & Non endometrioid	HGEC	Negative	Aneuploid		Poor	
Molecular	TCGA	POLE ultramutated	Endometrioid	Grade 1-3 (60% HG)			Highly mutant genes (POLE mutation (100%), DMD (100%), CSMD1 (100%), FAT4 (100%), PTEN (94%), PIK3R1 (65%), PIXW7 (82%), PIK3CA (71%), KRAS 53%, TP53 (35%), MSI variable, very Low somatic copy number.	Very good prognosis even in HG	Lower BMI, Early stage (IA/IB), Early onset
		Microsatellite instability (MSI)	Endometrioid	Grade 1-3			Intermediate mutation load (PTEN (88%), PIK3R1 (42%), PIK3CA (54%), RPL22(37%), ARID1A (37%), KRAS (35%), MSI high, low rate of somatic copy number alteration.	Intermediate prognosis	Higher BMI Lynch syndrome
		Copy-number low	Endometrioid	Grade 1 and 2			Low mutation load (PTEN (77%), PIK3CA (53%), CTNNB1(52%), ARID1A (42%), PIK3R1 (33%), KRAS (16%)), MSI stable, low rate of somatic copy number alteration.	very good, intermediate or poor prognosis	Higher BMI
		Copy-number high	Serous, Endometrioid, mixed histology	Grade 3			Low mutation load (frequent TP53 (92%), PIK3CA (47%), PPP2R1A (22%), FBXW7 (22%), PIK3R1 (13%), few PTEN mutations (10%)), few MSI (6%), genomic instable, with frequent somatic copy number alterations.	poor prognosis	Lower BMI, Advanced stage

9.2 Appendix II: Standard operation procedures:

STANDARD OPERATING PROCEDURE

Number	09	Version & Issue	2.3
Title	ANTIGEN RETRIEVAL		
Author	Jo Drury	Date	November 2010
Approved		Date	
Review Date	November 2013	Last Reviewed	November 2012

BACKGROUND

The mechanisms of formalin-fixation are thought to be due to the formation of cross-linking bonds between tissue proteins, stabilising them to withstand subsequent processing (Mason and O'Leary 1991). Whilst preserving tissue morphology, the formation of cross-linking bonds may, however modify the antigen's epitopes and/or its electrostatic charges, thus producing weak or false negative staining during immunohistochemical detection of certain proteins. Restoring the epitopes enables the antigen to react with the paratope of the antibody. Methods of proteolytic pre-treatment and heat retrieval of tissue antigenicity aim to restore the avidity of the immune reaction.

Proteinase K is a type of proteolytic agent which is isolated from the saprophytic fungus *Tritirachium album*. It possesses a high specific activity which remains stable over a wide range of temperatures and pH values with substantially increased activity at higher temperature. It particularly unmasks antigens of proteins found in the basement membranes including laminin and collagen IV.

There are 2 commonly used heat retrieval buffers, which vary in their pH. Heat causes cross-linked protein epitopes to 'unfold' (in manner similar to DNA denaturation), while buffer solutions aid in maintaining the conformation of the unfolded protein. The citrate based solution is designed to break the protein cross-links, therefore unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies. Tris-EDTA is very useful for low affinity antibodies or when tissue antigens are not intense. This buffer works well for many antibodies, but it often gives high background staining (maybe due to endogenous biotin revealed after this pretreatment), so primary antibody can often be highly diluted.

HEALTH AND SAFETY REQUIREMENTS

Care needs to be taken when performing heat based retrieval procedures because there is a risk of burns from the hotplate and pressure cooker. There is also a risk of chemical burns from sodium hydroxide and there is a mild irritant effect from citric acid. Suitable personal protection equipment (Nitrile gloves, safety glasses and a lab coat etc) should be worn. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. DO NOT COMMENCE WORK WITH THE PRESSURE COOKER UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND RECEIVED AUTHORISATION FROM LISA HEATHCOTE OR JO DRURY. THE SEAL ON THE PRESSURE COOKER SHOULD BE INSPECTED PRIOR TO EVERY USE AND REPLACED ANNUALLY.

EQUIPMENT INFORMATION:

- Proteinase K supplied by QIAGEN. QIAGEN HOUSE, Fleming Way, Crawley West Sussex, RH10 9NQ
- Diluent
- Humidity Chamber
- Sodium Hydroxide pellets and citric acid are supplied by VWR International Ltd, Poole, BH15 1TD England (Future orders will be obtained from Sigma or Thermo Fisher).
- Trizma base (T1503, Sigma- Aldrich Chemical Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com)
- EDTA (disodium, dehydrate) is supplied by VWR International Ltd, Pole, BH15 1TD. Future orders will be obtained from Sigma (E4884).
- Tefal Clipso Easy 6L pressure cooker and Russell Hobbs hotplate are obtained commercially from John Lewis department store. Annual Insurance check TBC. The seal/gasket needs to be replaced annually. Gasket (part number SA793145) is obtained directly from: http://www.homeandcook.co.uk refer to section accessories/pressure cookers. 2010 price £8.50 +£1.50 delivery
- Slide racks are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

- Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, pH 9.0):
  Tris Base 12.1 g
  EDTA 3.7 g
  Distilled water 2 l to make 5x stock solution
  Mix to dissolve, pH is usually at 9.0. Store this solution at room temperature for 3 months or at 4°C for longer storage.

METHOD A (Proteinase K):

1. Prepare a volume of proteinase K with a 1:25 concentration of the working stock that is sufficient for 50µl per section. Allow larger volume for larger sections.
2. Once all slides have been de-waxed place into distilled water
3. Prepare humidified chamber
4. Remove slides from the staining dish, wipe the backs of the slides and place onto the humidified chamber.
5. Distribute 50µl of proteinase K onto each section and spread over whole of tissue using a piece of parafilm. Take care whilst spreading to ensure sections do not get dried or damaged in any way.
6. All sections should be incubated with proteinase K for 5 minutes at room temperature.
7. After 5 minutes tap the solution off the slide and place slides in a glass staining dish filled with TBS.
8. See SOP 12 for immunohistochemistry protocol.

METHOD B (Heat based antigen retrieval):

1. Either:
   (a) Prepare a 10mM solution of citrate buffer, pH=6.0 by adding 3.15g citric acid to 1.5 litre of distilled water and adjust pH to 6.0 with 2M NaOH.
   Or:
   (b) Dilute stock 5x Tris-EDTA buffer, pH=9.0 by adding 300 ml to 1.2 l of distilled water.
2. Place buffer in the pressure cooker and place on the hotplate.
3. Turn on the hotplate to the maximum heat setting.
4. Loosely place the lid back on pressure cooker. Bring the buffer to a rolling boil.
5. Immerse the slide rack in the buffer using long forceps.
6. Engage the lid, turn the valve to pressure symbol and press the lid down until it clicks.
7. Set timer for 1 minute - when steam starts to vent, start timer. After 1 minute, turn off the hotplate and release the pressure by turning the vent to the “steam”

symbol, and cool as quickly as possible by transferring the pressure cooker to the sink and running under cold water.

8. Transfer the slides back to the glass staining jar containing distilled water.
9. Transfer to TBS ensuring that the tissue does not dry out.
10. See SOP 12 for immunohistochemistry protocol.

Other useful SOPs associated with 06 Procedure

Embedding samples using the Shandon Histocentre 3 machine (SOP 05).

Preparation of RM biopsy samples (SOP 03).

Preparation of APES coated slides (SOP 07).

Tissue processing using Shandon processing machine (04).

Immunohistochemistry (SOP12).

SOP History

Original SOP prepared by J Drury and L Heathcote in June 2007.

Updated version was prepared in July 2009 to include changes in both Liverpool Women's Hospital and University of Liverpool logos.

Version 2.1 was prepared by K Palial to include Proteinase K antigen retrieval

Version 2.2 was prepared by J Drury to include Tris-EDTA antigen retrieval and generally update information.

Version 2.3 was prepared to update procedures following relocation of laboratories.

Appendices - Associated Documents

	Document	Location
1	Risk assessment for antigen retrieval	Risk assessment folder in the deputy departmental safety advisors office
2	Physical Hazards Code of Practice	Electronic copies are available on the University of Liverpool health and safety intranet: https://www.liv.ac.uk/intranet/safety/codes_of_practice/physical_hazards.pdf
3		

Review Date

November 2013

STANDARD OPERATING PROCEDURE

Number	12	Version & Issue	2.3
Title	Immunohistochemistry: ImmPRESS (Vector)		
Author	Jo Drury	Date	20.09.11
Approved	Dr D Hapangama	Date	
Review Date	May 2012	Last Reviewed	27.04.11

Background

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues. In order to perform the standard staining procedure, first the tissue section has to be prepared (SOP 04, 05 and 06), deparaffinized and then rehydrated (SOP 08). Antigen retrieval (SOP 09) is then performed to break any protein cross-links, therefore unmasking the antigens and epitopes in the tissue sections. The Vector immPRESS ™ reagent kit, HRP is a two-step Immunohistochemical staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequentially non-specific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper counts. Any endogenous peroxidase activity is quenched by incubating the specimen with Peroxidase block. The specimen is incubated with an appropriately characterised and diluted mouse primary antibody, followed by incubation with the labelled polymer. Staining is completed by incubation with 3'3-Diaminobenzindine (DAB)+ substrate - chromogen which results in a brown–coloured precipitate.

Definition

This SOP will explain how to stain tissue using an unlabelled antibody. Please read entire procedure before staining sections. Perform all antibody and staining incubations in a humid chamber and do not allow sections to dry out. Isotype and system controls should also be run. Carefully time all tests. Do not touch test specimens on slides during the staining procedure.

Health and Safety Precautions
The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, such as hydrogen peroxide (H_2O_2), xylene, ethanol, and DAB (3'3-diaminobenzindine). Gloves must always be worn when working with any of these chemicals.

EQUIPMENT INFORMATION

Staining dishes

Humidified chamber

Cover slips

General points:
Ensure that solutions cover the whole specimen – if necessary use a piece of Parafilm to spread.

Antibody information:
All current antibody datasheets are held in a labelled lever arch file in room 1128.
Additional information on appropriate antibody concentration, antigen retrieval conditions and incubation time/temperature is held in an Excel spreadsheet (C:\Documents and Settings\jadrury.livad\My Documents\Dharani\Dharani abs.xlsx).

Reagent information:
TBS: 6 g/l Trizma base (T1503, Sigma-Aldrich) + 8.7 g/l NaCl (S7653, Sigma-Aldrich). Adjust pH to 7.6 with HCl.
BSA: A3803 (Sigma Aldrich)
H_2O_2: H1009 (Sigma-Aldrich)
ImmPRESS anti-mouse IgG kit (50 ml) (MP-7402, Vector Laboratories)
ImmPRESS anti-rabbit IgG kit (50 ml) (MP-7401, Vector Laboratories)
ImmPRESS anti-goat IgG kit (50 ml) (MP-7405, Vector Laboratories)
ImmPACT DAB (120 ml) (SK-4105, Vector Laboratories)
Shandon Gill 2 Haematoxylin (Thermo Scientific)
1% acid alcohol: 1 ml HCl/100 ml 70% ethanol
Shandon Consul-Mount (Thermo Scientific)

Supplier information:
Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com.
Thermo Scientific, Bishop Meadow Road,Loughborough, LE11 5RG. Tel: 01509 231166 Fax: 01509 231893.
Vector Laboratories Ltd. 3, Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS. Tel: 01733 237999. Fax: 01733 237119. Web: www.vectorlabs.com.

METHOD (Paraffin sections):

1. Place slides in a staining dish containing TBS and incubate 5 minutes at room temperature.
2. Prepare 0.3% H_2O_2/TBS (2.5 ml 30% H_2O_2 + 247.5 ml TBS)
3. Incubate slides in 0.3% H_2O_2/TBS bath 10 minutes at room temperature.
4. Prepare humidified chamber by placing folded paper towels in the centre gulley's and soaking with distilled water.
5. Decant H_2O_2/TBS and tap off any remaining solution. Carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
6. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
7. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
8. Mark area to be stained with DAKO hydrophobic marker pen ensuring that the tissue is surrounded with sufficient space to allow spreading of antibodies.
9. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
10. Prepare antibody diluent (TBS/0.5 % BSA eg. 250 µl 10% BSA + 4750 µl TBS)
11. Prepare appropriate dilutions of each antibody, allowing 50 µl per section.
12. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
13. Place slides in humidified chamber.
14. If necessary, add one drop horse serum block to each section, spread with parafilm to ensure that the whole section is covered, and incubate 20 min at room temperature.
15. Tap off the serum block and proceed to step 17.
16. Steps 14 and 15 can be omitted if staining is clean without blocking.
17. Apply 50µl of the appropriate antibody to each section, spread with parafilm to ensure that the entire section is covered and incubate 30-120 min at room temperature or overnight at 4°C. See C:\Documents and Settings\jadrury.livad\My Documents\Dharani\Dharani abs.xlsx for specific conditions for each antibody.

18. Tap off the antibody solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
19. Incubate 5 minutes at room temperature.
20. Decant TBS and refill.
21. Incubate 5 minutes at room temperature.
22. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
23. Return slides to the humidified chamber and apply 1 drop labelled polymer-HRP. Ensure that the polymer matches the primary antibody.
24. Spread with parafilm to ensure that the entire section is covered and incubate 30 min room temperature.
25. Tap off the polymer solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
26. Incubate 5 minutes at room temperature.
27. Decant TBS and refill.
28. Incubate 5 minutes at room temperature.
29. Prepare substrate/chromagen solution: 30µl/1ml substrate (require 50 µl per section).
30. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
31. Return slides to the humidified chamber and apply 50 µl substrate/chromagen solution.
32. Spread with parafilm to ensure that the entire section is covered and incubate 10 min room temperature.
33. Place slides in staining rack and immerse immediately in tap water to stop the reaction.
34. Turn on fume hood in lab 4.
35. Counterstain using filtered Gill 2 haematoxylin in lab 4. Immerse for 1 min 30s.
36. Immerse in tap water and rinse until water is clear.
37. Dip briefly in acid alcohol, and immediately back into tap water (5 min).
38. Incubate 1 min in 70% ethanol.
39. Blot off excess and incubate 1 min in 90% ethanol.
40. Blot off excess and incubate 3 min in 100% ethanol.

41. Repeat step 40.
42. Blot off excess and incubate 5 min in xylene.
43. Blot off excess and incubate 10 min in xylene.
44. Remove a few slides at a time and apply sufficient mountant to cover the section.
45. Choose an appropriately sized coverslip and apply to the slide.
46. Remove air bubbles by using a cocktail stick or yellow pipette tip to gently press on the coverslip and "chase" bubbles to the edge.
47. Leave to dry in fume hood.

SOP History

Original SOP prepared by L Heathcote 21st June 2007.

Version 2.1 prepared by J Drury 16th July 2008.

Version 2.2 prepared by J Drury 20th September 2010 to update detection system from DAKO EnVision to Vector ImmPRESS.

Version 2.3 prepared by J Drury 27th April 2011 to generalise the SOP.

Other useful SOPs associated with 12 Procedure

Disinfection SOP 01, Transport of biological samples SOP 02 and Tissue Processing SOP 04, Embedding Procedure SOP 05, Cutting sections 06, Preparation of sections for staining SOP 08, Antigen retrieval SOP 09, Autoclave procedure SOP 28.

Appendices -Associated Documents

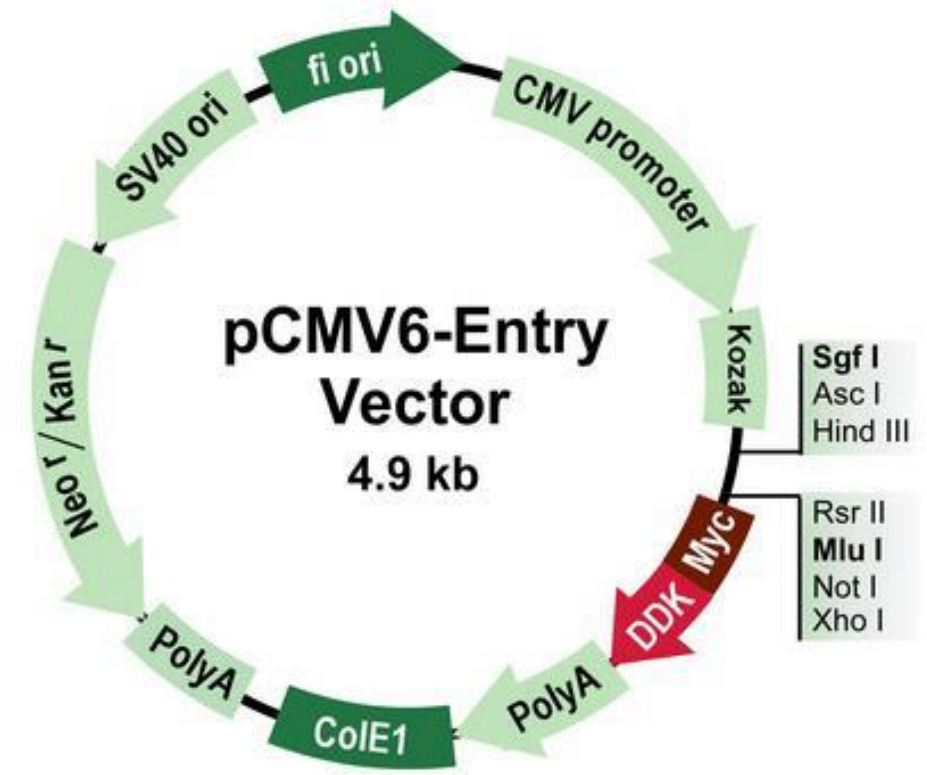
This will have a list of associated template letters and guidelines relevant to the SOP.

	Document	Location
1	Risk and COSHH assessments	Health and Safety folders in the departmental safety advisors office
2		
3		

Review Date: May 2012

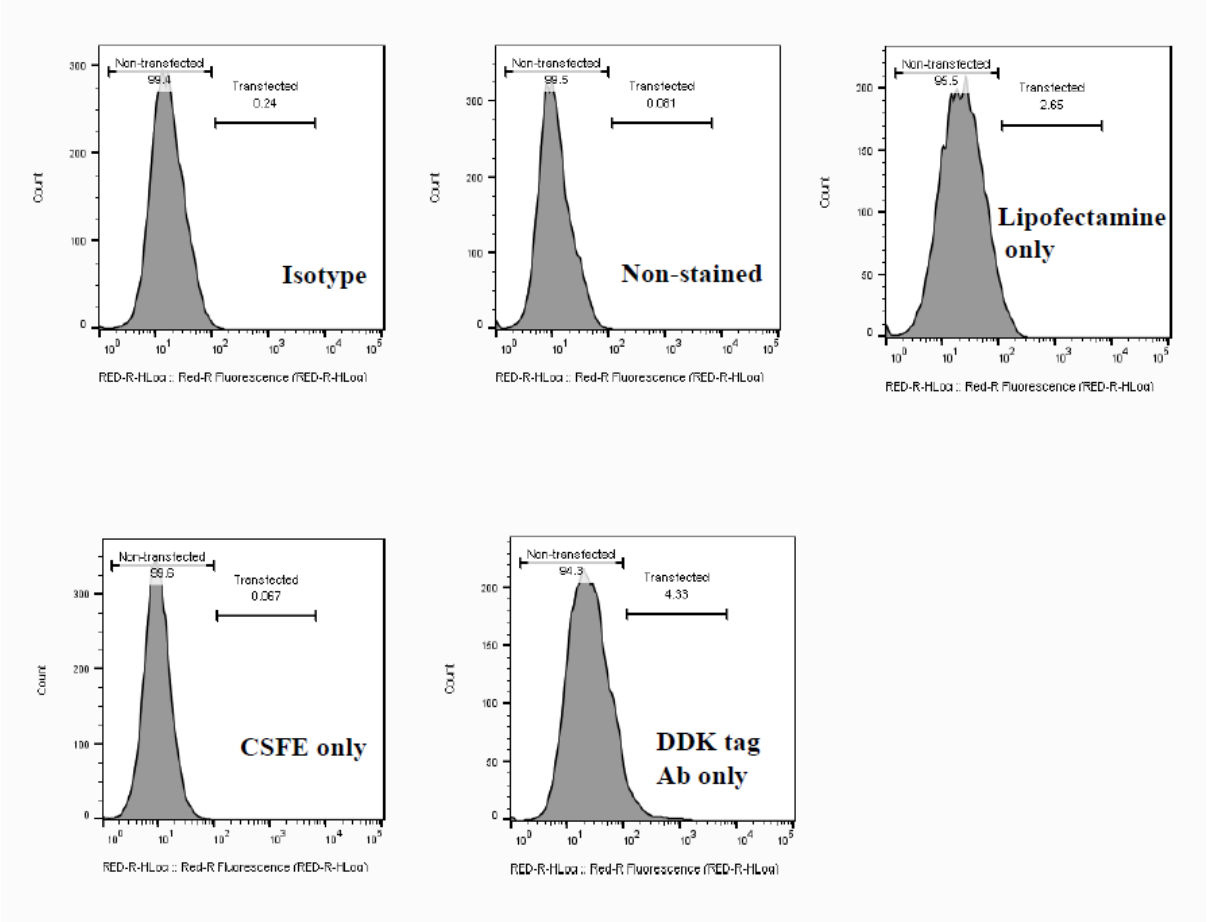
9.3 Appendix III: Transient transfection

pCMV6-Entry, mammalian vector with C-terminal Myc- DDK Tag (Origene,USA)

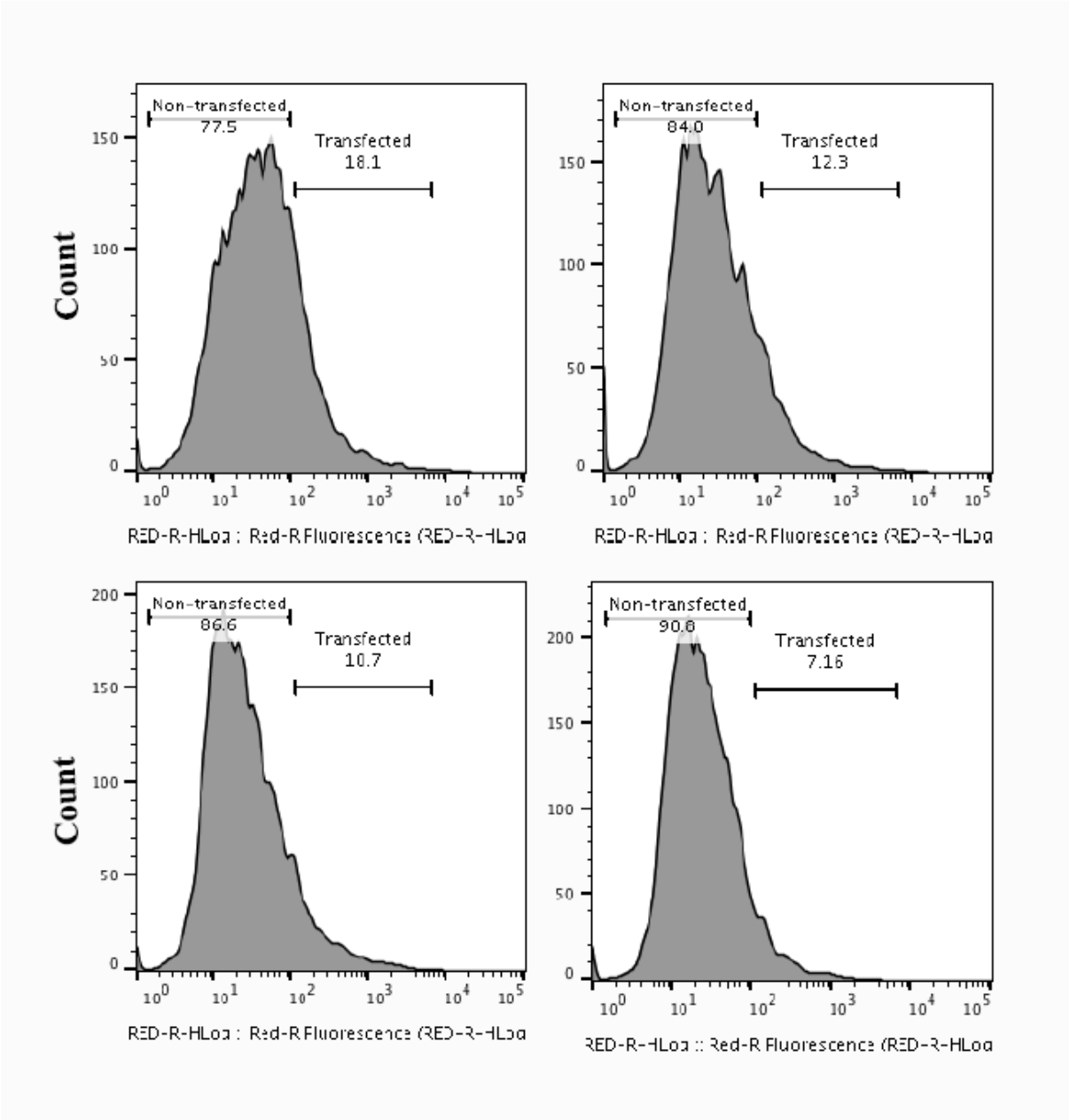


Protein sequence alignment of exogenous dyskerin showing that exogenous dyskerin protein differs from NCBI reference with one amino acid (valine to phenylalanine substitution at position 285) (red star) after transient transfection with DDK tagged DKC1 plasmid (Origene, USA). The alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

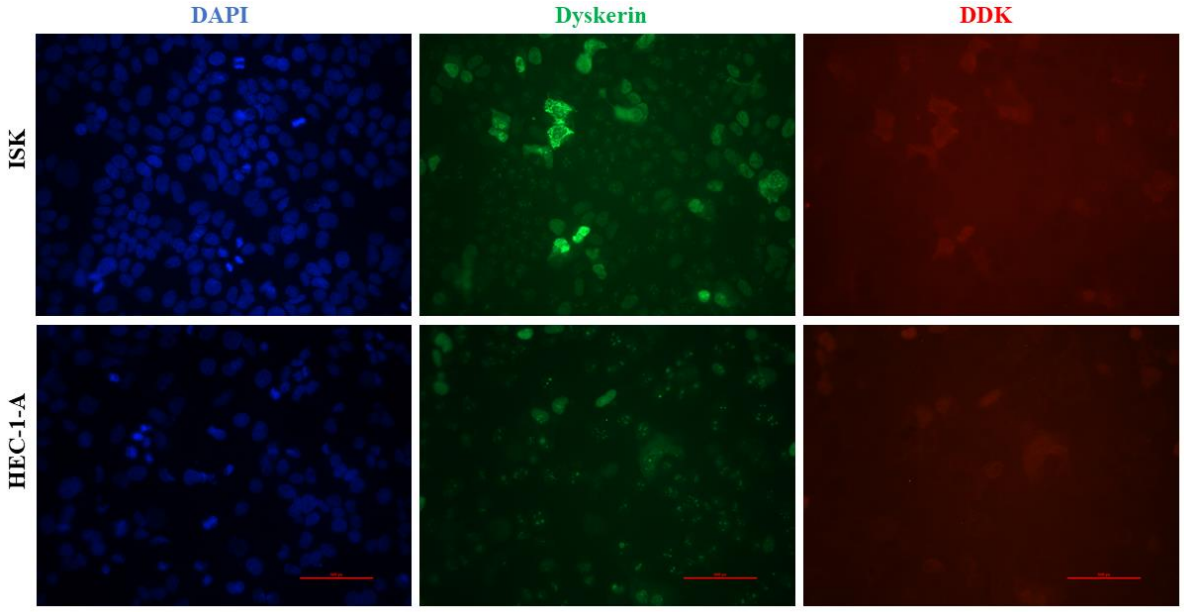
Negative controls used in the flow cytometry experiment



Transfection efficiencies in *DKC1* transfected ISK cells (flow cytometry)



Double immunofluorescence (Anti-DDK and anti-dyskerin antibodies) in endometrial cell lines (ISK and HEC-1-A). Both cell lines were transiently transfected using MYC-DDK tagged Dyskerin plasmid (OriGene, USA). Magnification 400X.



9.4 Appendix IV

frontiers in Oncology

REVIEW
published: 17 May 2019
doi: 10.3389/fonc.2019.00344

Telomerase and Telomeres in Endometrial Cancer

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Telomeres at the termini of human chromosomes are shortened with each round of cell division due to the "end replication problem" as well as oxidative stress. During carcinogenesis, cells acquire or retain mechanisms to maintain telomeres to avoid initiation of cellular senescence or apoptosis and halting cell division by critically short telomeres. The unique reverse transcriptase enzyme complex, telomerase, catalyzes the maintenance of telomeres but most human somatic cells do not have sufficient telomerase activity to prevent telomere shortening. Tissues with high and prolonged replicative potential demonstrate adequate cellular telomerase activity to prevent telomere erosion, and high telomerase activity appears to be a critical feature of most (80–90%) epithelial cancers, including endometrial cancer. Endometrial cancers regress in response to progesterone which is frequently used to treat advanced endometrial cancer. Endometrial telomerase is inhibited by progestogens and deciphering telomere and telomerase biology in endometrial cancer is therefore important, as targeting telomerase (a downstream target of progestogens) in endometrial cancer may provide novel and more effective therapeutic avenues. This review aims to examine the available evidence for the role and importance of telomere and telomerase biology in endometrial cancer.

Keywords: endometrial cancer, telomere, telomerase, endometrium, TERRA, TRAP, hTERT, hTERC

OPEN ACCESS

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Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *08 January 2019*
Accepted: *15 April 2019*
Published: *17 May 2019*

Citation:
Alnafakh RAA, Adishesh M, Button L, Saretzki G and Hapangama DK (2019) Telomerase and Telomeres in Endometrial Cancer. Front. Oncol. 9:344. doi: 10.3389/fonc.2019.00344

INTRODUCTION

Telomeres are specialized structures that are found at the ends of linear chromosomes, containing a tandemly repeated specific DNA sequence and associated protective proteins. The protective function of telomeres in preventing the loss of genomic DNA in proliferating cells is well-established (1–3). As telomeres shorten with each cell division, critically short telomeres initiate cellular senescence or an apoptotic pathway, leading to cessation of cell division, therefore telomere shortening is a major tumor suppressor mechanism (4, 5). In addition, oxidative stress is an important additional cause for telomere shortening (6, 7). Telomerase is a unique reverse transcriptase enzyme (8) that is able to add repetitive telomeric sequences *de novo* onto telomeric ends (9) that are continually lost during DNA replication due to oxidative stress and the "end replication problem" in mitotic cells. Thus, telomerase prevents shortening and maintains telomeres. However, most human somatic cells do not have significant levels of telomerase activity whereas cells, such as embryonic stem cells and most cancer cells exhibit high telomerase activity while adult tissue stem cells are potentially able to up-regulate telomerase upon activation (10–12).

Human endometrium is a unique somatic organ that contains a relatively high yet dynamic pattern of telomerase activity that changes according to the menstrual cycle, correlating with endometrial cellular proliferation (13, 14). Further evidence from benign endometrium also suggests that telomerase activity is a fundamental requirement for endometrial cell proliferation and survival (15). The involvement of telomerase in most cancer-related cellular abnormalities in cell fate regulatory pathways prompted many studies into telomerase and telomeres in a variety of cancers including endometrial cancer (16–18).

Endometrial cancer is the fourth common cancer in women in the UK and is the commonest gynecological cancer (CRUK). Increasing obesity and longevity have both caused the incidence of EC to increase at an alarming rate. For example, in the United Kingdom, the incidence of EC increased by more than 40% since 1993. European estimates predict a 100% increase in the incidence by 2025 not only in older post-menopausal women but also in younger women (19). Figures from the UK report that mortality associated with EC has risen by 21% over the last decade in an era of improving survival rates for most other cancers, highlighting the inequality and lack of translation of advances in cancer research to EC (CRUK) (20). The survival rates for high-grade EC are exceptionally poor, similar to ovarian cancer; and the traditional surgical treatment is associated with significant morbidity and mortality for many women even when presented with early disease due to frequently occurring co-morbidities and obesity (21). Urgent novel therapeutic options are therefore needed to prevent, treat as well as to avoid progression of EC.

Although EC is an important disease with a significant clinical and economic consequence, the molecular biology of endometrial carcinogenesis is not well-described or understood when compared with other female-specific malignancies, such as breast or ovarian cancer. Human endometrium is a unique organ with a massive regenerative potential (22) and is the main target organ for ovarian steroid hormone action (23). While being a hormonally responsive tissue, endometrium responds rather differently to the same steroid hormones than other hormone responsive organs, such as breast tissue (23, 24). This has made it difficult to translate the pioneering discoveries made in other cancers to EC management and therapy. Unlike most other somatic tissue, benign endometrial tissue demonstrate high telomerase activity, and telomerase has a pivotal functional role in healthy endometrial cell proliferation (14, 15). High telomerase activity is observed in most epithelial cancers, and the carcinogenesis process in those tissues involved ectopic expression of telomerase components and genetic alterations, such as activation mutations in promotors of the vital genes. In the endometrium however, the high telomerase activity is a feature even without being associated with driver mutations. It is therefore intriguing to explore the distinctive endometrial telomerase biology relevant to EC and we hypothesize EC to have a unique telomerase biology that is different to the other cancers. Furthermore, EC is a hormone driven disease and advanced and recurrent ECs are treated with progesterone which regress these tumors albeit without extending survival (24). It is therefore of particular interest to examine telomerase as a downstream progesterone target in the endometrium (15) which can be manipulated for therapeutic utility in progesterone resistant ECs. This review therefore focuses on the significance and role of telomerase and telomere biology in EC, highlighting recent findings proposing some aspects of telomerase biology as potential therapeutic targets for EC (25).

METHOD

We performed systematic PubMed (Medline) and Ovid searches using a combination of relevant controlled vocabulary terms and free-text terms related to telomeres and telomerase. The key words used included: telomerase, telomeres, telomere length, telomerase reverse transcriptase (TERT), telomeric RNA component (TERC), shelterin proteins, telomerase associated proteins, with endometrium, endometriosis, endometrial hyperplasia, endometrial cancer (EC), endometrial carcinomas, uterine cancer, cancers. All studies investigating telomerase or telomere biology in endometrium in women or animals or respective cell lines, either primary cells or tissue explants in culture, and published from database inception until December 2018, were included in this review.

TELOMERES

Structure

Human telomeres consist of a repetitive TTAGGG hexanucleotide sequence bound by six-proteins forming the shelterin complex [(26) **Figure 1**]. In normal somatic cells the average length of telomeres is around 5–15 kilobases and they shorten *in vitro* by 30–200 base pairs (bp) during every cell division depending on the cell type and environmental conditions (34). Under increased oxidative stress telomere shortening rate per cell division can increase substantially, up-to 500 bp (6).

Most of the non-coding telomeric DNA is double-stranded whilst the terminal nucleotides (nt) form the single stranded 3′ G-rich overhang, which serves as the primer for telomerase action (35) and also protect telomeres from being recognized as DNA damage. This forms a D-loop (Displacement loop) facilitating repetitive DNA sequences to be added by telomerase (36).

Another mechanism to protect telomeres from being recognized as DNA damage is the formation a t-*loop*, which is a specific higher order conformation. This large duplex loop-back structure is formed via invasion of the single-stranded telomeric 3′ overhang into the double stranded telomeric repeat array (37). The authors suggested that the t-loops are the basic mechanism by which the telomeric nucleoprotein complex sequesters chromosome ends from the DNA damage pathway, preventing inappropriate DNA repair and telomerase action (37).

The shelterin complex (**Figure 1**) includes telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), which are homodimeric proteins that bind specifically to double-strand telomeric DNA (27, 37). In contrast, Protection of telomeres 1(POT1) binds to the single-stranded region of the telomere (28) and forms a heterodimer with TPP1 (38). The Repressor/activator protein 1 (RAP1) is recruited through its relation with TRF2 (31) and TRF1-interacting protein 2 (TIN2) is the central part of

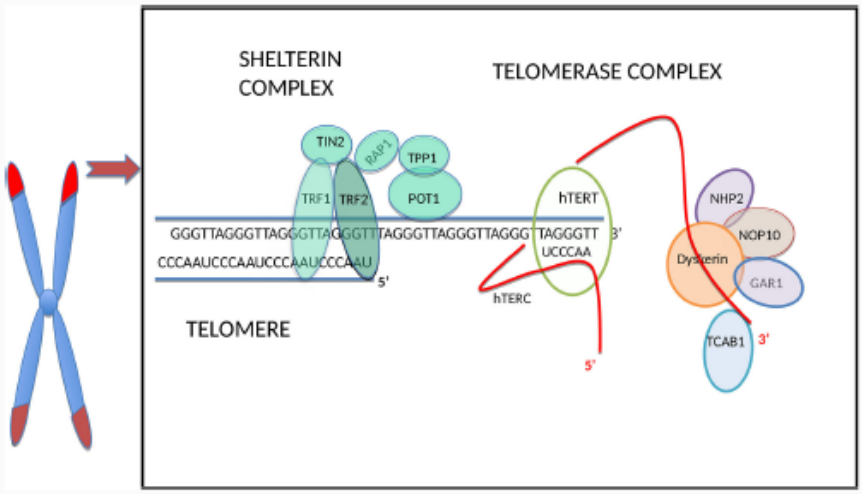

FIGURE 1 | Schematic illustration of the telomere and main telomerase complex components. The human telomere and telomerase enzyme complex (only one half of the dimeric holoenzyme complex is shown for clarity), adapted from Hapangama et al. (14). From all sheltrin proteins only telomere repeat binding factors 1 (TRF1) and 2 (TRF2) (27) bind directly to the double-stranded telomeric sequence, and protection of telomeres protein-1 (POT1) (28) binds to the single-stranded overhang; hence these are termed as telomere binding proteins and they interact with remaining shelterin proteins TIN2 (binds to TRF1 and TRF2) (29, 30), RAP1 (binds to TRF2) (31) and TPP1 (binds to POT1) (32). The TERC H/ACA region located at the 3′ end binds to dyskerin and the other telomerase associated proteins: NOP10, NHP2, and GAR1 (14). The hTERC at the 3′ end binds also to telomerase Cajal body protein 1 (TCAB1) (33).

the shelterin complex (29) and it interacts with TRF1, TRF2 (30), and POT1/TPP1 (32) to assure structural integrity of the complex. Removal of individual shelterin proteins has been shown to stimulate a DNA damage response (DDR) pathway: TRF1 prevents the stimulation of both ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) pathways (39); TRF2 and RAP1 inhibit the activation of the ATM pathway (40, 41) and homology-directed recombination (HDR) (42) while TPP1 bound POT1 (POT1a/b in mouse) inhibit the ATR pathway (43). TRF2 plays a vital role in facilitating this t-loop formation (44). Super-resolution fluorescence light microscopy visualization of the t-loop has shown that the strand invasion point can be located at almost any point along the duplex DNA, resulting in highly variable t-loops sizes (45).

Functions of Telomeres

The main function of telomeres is to protect chromosomal ends from degradation and end-to end-fusion (1) as well as to prevent the ends of chromosomes being recognized as DNA damage by the DNA damage response machinery of the cell (37). However, when telomeres are critically short, they activate the apoptosis/senescence pathways, thereby preventing genetic material being lost by inhibiting inappropriate continuous DNA replication in the context of short telomeres. The telomere structure described above, prevents inappropriate DNA repair at these sites, for example the loop conformation (D-loop) masks the single stranded terminal DNA and enables its protection from the DNA damage response pathway (37).

The shelterin complex supports the chromosome protective function of telomeres and stabilization of telomere lengths, and the complex interaction of shelterin proteins at the chromosomal ends have a key role in telomere maintenance via a negative feedback loop which also has an inhibitory effect on the telomerase enzyme (46).

In cells which have replicative capability, telomere shortening can lead to chromosomal instability by promoting end-to-end fusions leading to multiple chromosomal aberrations, such as breakages, fusions, and translocations rendering the genome aneuploid and therefore promoting carcinogenesis. To maintain telomere length, the homeostasis mechanism that involves telomerase, uses both TRF1 and TRF2 as negative regulators that stabilize and limit telomere length elongation (47, 48). Overexpression of both TRF1 and TRF2 was reported to cause telomere shortening (47) and this could be due to the binding of TRF1 and TRF2 along the length of the double stranded telomeric repeat array which measures telomere length as demonstrated in yeast (47, 48). POT1 can either facilitate or inhibit telomerase accessing telomeres depending on its position relative to the DNA 3′-end (49). Examining the high-resolution crystal structure of the human POT1-TTAGGGTTAG complex suggested that it would not be elongated by telomerase. When POT1 is bound at one telomeric repeat before the 3′-end, leaving an 8-nucleotide 3′-tail, the resulting complex is elongated with increased activity

and processivity (50). Replication protein A (RPA) is another ssDNA binding protein which has an important role in telomere replication by facilitating telomerase enzyme at the telomeres (51, 52). It also recruits the ATR-ATRIP protein kinase complex to DNA damage sites and initiates the checkpoint signaling (53, 54). Collectively, the available evidence demonstrates that shelterin and other telomere-binding proteins are involved in the regulation of telomere length.

Gene regulation is another reported function of telomeres but with limited evidence available for it. Telomeric attrition extensively alters expression of some genes, and the difference in expression of genes proximal to telomeres may result from chromatin modifications, a conserved phenomenon termed as *telomere position effect* (TPE). TPE is a silencing mechanism spreading from the telomeres toward subtelomeric regions (55). In humans, only a limited number of endogenous genes (e.g., ISG15) has been mentioned to be affected by TPE (56, 57), however, microarray data suggests that the expression of many other genes close to telomeres to be also altered with the aid of a telomere length-dependent and DNA damage-independent mechanism, and this is known as *telomere position effect–over long distance* (TPE-OLD) (58). For example, the looping of chromosomes brought long telomeres closer to some genes which are over 10 Mb away from the telomere, but these same loci were completely separated from the telomeres when the telomeres were short (58). Further microarray data supports the notion that telomere length-dependent chromosome conformation can affect the transcription of non-subtelomeric genes (58). At the genome-wide level, the effect of this mechanism on gene expression has been proposed to occur earlier than replicative senescence and that could potentially explain the increased incidence of age-related pathologies that are associated with old age without necessarily imposing a DNA damage signal from a critically-short telomere (59, 60).

Telomere length is the main determinant of a cell's replicative life span. Dysfunctional telomeres which result from either progressive telomere shortening, internal DNA damage (61) or shelterin complex loss, provoke a strong DNA damage response and genomic instability (62). A plethora of experimental data has shown that tumorigenesis can be caused by genome instability resulting from telomere shortening (4, 63). Nevertheless, in late generation telomerase knock-out mouse models, telomere attrition was also a tumor suppressor mechanism through the induction of replicative senescence or apoptosis that repress tumorigenesis. Telomere shortening and telomere uncapping in metazoans stimulate ATM/ATR kinases to phosphorylate downstream kinases CHK1 and CHK2, which initiate p53-dependent replicative senescence and apoptosis pathways which inhibit tumor formation (4).

TERRAs (Telomeric Repeat Containing RNAs)

Telomeres were initially thought to be transcriptionally silent, but recently they have been found to be transcribed into telomeric repeat containing, long non-coding RNAs, termed TERRAs (64). TERRAs have a role on telomere regulation and also regulate telomeric access of telomerase as described below in more detail.

REGULATION OF TELOMERE LENGTH AND TELOMERE MAINTENANCE MECHANISMS (FIGURE 2)

The most widely known classical telomere maintenance mechanism is dependent on telomerase reverse transcriptase activity. However, another telomerase-independent telomere maintaining pathway has been described in cells that do not have measurable telomerase activity, termed alternative lengthening of telomeres (ALT) pathway (69). TERRAs also have a role in telomere length regulation by mainly managing telomeric access of telomerase.

Telomerase

Structure of Telomerase (Figure 1)

Telomerase, the only RNA dependent DNA polymerase in mammals, was first discovered in protozoans in 1985 (70), and subsequent studies demonstrated mammalian/human species in 1989 (71). The telomerase holoenzyme contains three core components: the RNA component harboring the template region for telomere synthesis (hTR or hTERC), a catalytic protein with reverse transcriptase activity, hTERT (72) as well as dyskerin (**Figure 1**). However, only the RNA component (TERC) and the catalytic subunit (TERT) are necessary and sufficient for *in vitro* telomerase activity (73). **Table 1** lists some of the well-known telomerase associated proteins.

Telomerase RNA component (hTERC or hTR)

The human telomerase RNA (TERC or hTR) consists of 451 nt and is an essential constituent of the telomerase catalytic core complex. Although the length is variable among eukaryotes, the structure of TERC remains conserved. For example, the length ranges from ~150 nt in ciliates, 400–600 nt in vertebrates to ~1,300 nt in yeast (114). Additionally, in ciliates, polymerase III transcribes the telomerase RNA (115), whereas it is RNA polymerase II in yeast and vertebrates (116).

Vertebrate TERC's secondary structure has four conserved elements: a pseudoknot domain (CR2/CR3), a CR4/CR5 (conserved region 4 and conserved region 5) domain, box H/ACA (CR6/CR8) domain and a CR7 domain (114, 117). The proximal template/pseudoknot domain and the distal CR4/5 domain represent the essential regions of TERC for telomerase activity (118).

As mentioned before, an active telomerase enzyme can be generated by combining the two RNA domains from the TERC subunit with the TERT protein on oligodeoxynucleotide substrates *in vitro* (73, 119–121). The human/vertebrate TERC has a third, conserved component, the H/ACA domain located at the 3′ end that has homologies to small nucleolar (sno) and small Cajal body-specific (sca) RNAs. The TERC H/ACA region binds to telomerase associated proteins, such as dyskerin, NOP10, NHP2, and GAR1 (14), and this region is essential

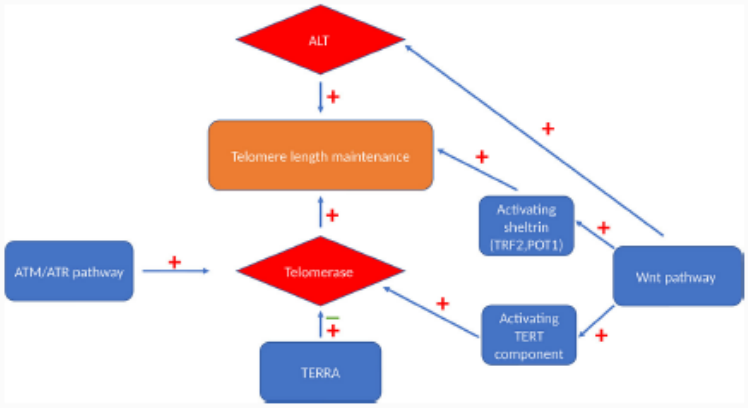

FIGURE 2 | Telomere maintenance mechanisms. Cells can maintain their telomeres via either telomerase-dependent pathway or a telomerase-independent ALT pathway. Activated Wnt signaling pathway can maintain telomere length by activating both these maintenance mechanism and by maintaining the level of TRF2 and POT1 sheltrin components that are essential for telomere protection (65). ATM and ATR also have stimulatory effect on telomerase enzyme via triggering its recruitment and enhancing the assembly of this enzyme (66). TERRA binds independently to hTERC and hTERT telomerase subunits with an inhibitory effect on human telomerase enzyme (67) or it acts as a recruiter of telomerase enzyme rather than an inhibitor (68).

for telomerase biogenesis, and are important for RNA stability. Additionally, in the 3′ stem-loop of the H/ACA, there is another domain, the Cajal body localization box (CAB), for binding the telomerase Cajal body protein 1 (TCAB1) (33). Mutations in the H/ACA region decrease TERC accumulation, whereas mutations in the CAB cause TERC to accumulate in nucleoli instead of Cajal bodies (122, 123). Although this mutant TERC has the capacity of forming catalytically active telomerase *in vivo*, it is highly impaired in telomere elongation because of the decreased association of telomerase with telomeres (124). This result emphasizes that sub-nuclear localization of telomerase as an important regulatory mechanism for the homeostasis of telomere length in human cells (124). TERC therefore, not only provides the template, which identifies the telomere repeat sequence, but it also comprises motifs, which are crucial to reconstitute telomerase activity (125). Furthermore, it plays a role in stability, maturation, accumulation, and functional assembly of the telomerase holo-enzyme.

hTERT

TERT is the catalytic component of the telomerase enzyme and as described above, together with TERC, it is essential for telomerase activity and thus for the maintenance of telomere length, chromosomal stability, and cellular immortality. The human TERT gene (hTERT) is located at chromosome 5p15, and encompasses more than 37 kb and contains 16 exons (126). The TERT protein consists of four conserved structural domains, the telomerase essential N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the central catalytic reverse transcription (RT) domain, and the C-terminal extension (CTE). Mutations in the RT conserved residues prevent telomerase enzymatic activity *in vitro* (127). These mutated TERT proteins fail to maintain telomere lengths *in vivo* (128), and many of these mutations have been identified in individuals with telomere-mediated disorders or telomeropathies (129). As already stated above, telomerase activity can be reconstituted by hTERC and hTERT co-expression in yeast and mammalian extracts (73, 130). Telomerase activity is established in *Saccharomyces cerevisiae* via reconstitution of telomerase by hTERC and hTERT co-expression (130). Therefore, hTERC and hTERT are the minimal requirement for telomerase activity (72). However, biochemical telomerase activity as measured by the telomere repeat amplification protocol (TRAP) assay does not always mean that the enzyme has necessarily telomere elongation capacity *in vivo*. This was demonstrated when the hTERT protein was modified by attaching a hemagglutinin (HA) epitope tag to the C terminus: while the catalytic activity of telomerase enzyme remained unaffected telomere maintenance function was lost *in vivo* due to loss of access to the telomere (131). Telomerase associated proteins are also essential for the full biological function of the enzyme but hTERT is the primary determinant of enzyme activity in most cells (120, 132).

Dyskerin

Dyskerin is a highly conserved, nucleolar, 514-amino-acid long protein, also known as NAP57 in rat (133) or Cbf5 in yeast (134) and has been proposed to be the third core component of the telomerase holoenzyme. Dyskerin is an essential member of the telomerase complex (but not required for biochemical telomerase activity as stated above); it binds to the telomerase RNA component (TERC) and participates in stabilizing the telomerase enzymatic complex (135). It is a pseudouridine synthase, encoded by the *DKC1* locus at Xq28 (136), which is

TABLE 1 | Telomerase associated proteins [adapted from Hapangama et al. (14)].

Protein	Function in Cancer
hTERT ASSOCIATED PROTEINS	
Hsp 90, P23	Hsp90 is an essential modulator for the proper folding and stabilization of several client proteins and it is a major contributor to carcinogenesis. Hsp90 and P23 act together to regulate telomerase DNA binding. Since heat shock protein 90 (Hsp90) client proteins have major cancer biological hallmarks, targeting Hsp90 provides the prospect for simultaneous disturbance of multiple oncogenic pathways. In triple-negative breast cancer, inhibition of Hsp90 has shown to be a promising therapeutic avenue (74–76)
Protein 14-3-3	These proteins are involved in regulating multiple cellular functions via their interaction with phosphorylated partners. An elevated level of 14-3-3 proteins facilitates tumor progression in a variety of malignancies. The observations of Seimiya et al. identified the 14-3-3 signaling proteins as human TERT (hTERT)-binding partners and suggested that 14-3-3 improves nuclear localization of TERT. A dominant-negative 14-3-3 redistributed hTERT into the cytoplasm, which was normally localized in the nucleus (77)
DHX36 (DEAH-Box Helicase 36)	It mediates AU-rich element mRNA degradation and as a resolvase for G-quadruplex DNA *in vitro* (78, 79). It involves in TERT stabilization and Correction of the positioning of the template domain of hTERT (80), it also Regulates p53 Pre-mRNA 3′-End Processing Following UV-Induced DNA Damage (81) and Prevents migration of colon cancer cells (82)
Pontin and reptin	Pontin and Reptin are conserved proteins belong to AAA + ATPases family, they have a role in various cellular processes that are critical for oncogenesis, such as transcriptional regulation, chromatin remodeling, DNA damage signaling and repair, assembly of macromolecular complexes, regulation of cell cycle/mitotic progression, and cellular motility, all of which contribute to their central roles in activating cell proliferation and survival (83–85). They also act together in telomerase assembly. Pontin and/or Reptin implicated in cancers of the esophagus, stomach, colon, and pancreas (86–90) Their exact functions are still entirely unclear as they interact with many molecular complexes with vastly various downstream effectors, with overexpression relating to factors, such as response to treatment, prognosis and outcome, reviewed in (91) Pontin and reptin have a well-established role in hepatocellular carcinoma (HCC), both were overexpressed in HCC tissues and associated with poor outcome (92, 93) Pontin and/or Reptin expression in both non-small cell lung cancer (NLSCLC) and small cell lung cancer (SCLC) with potential use as biomarkers in lung cancer (94–96). Pontin identified in screens of biomarker/autoantigen panels in breast cancer (99, 100) and both proteins are essential in cancers of white blood cells, resulting in lymphomas and leukemia (101)
hTERC ASSOCIATED PROTEINS	
Dyskerin	Dyskerin is one of H/ACA ribonucleoproteins (RNPs) which also include (NOP10, NHP2, and GAR1) (102), it is suggested in rRNA modification and processing, impaired dyskerin function in X-DC patients and DKC1 hypomorphic mutant model causes a decrease in the protein production which results in a reduction in tumor suppressor proteins (P53 and P27) reviewed in Montanaro (103). Dyskerin binds to the telomerase RNA component (TERC); thus dyskerin allows TERC stabilization and enhances telomerase activity. As a consequence, impaired dyskerin reviewed in Montanaro (103) Dyskerin protects from genetic instability. Loss and gain of dyskerin function may play critical roles in tumorigenesis (104)
NOP10	NOP10 as an H/ACA RNP contributes to telomerase enzyme assembly and stabilization, post-transcriptional processing of nascent ribosomal RNA and pre-mRNA splicing. Therefore, it is essential for ribosome biogenesis, pre-mRNA splicing, and telomere maintenance (105, 106) NOP10 mRNA level was reported to be decreased in patients with chronic lymphocytic leukemia (CLL) relative to controls (105)
NHP2	NHP2 has the same function as other H/ACA RNPs, increased NHP2 protein in gastric and colorectal cancer relative to healthy controls (107) Significant upregulation of the NHP2 protein encoding gene in colonic cancer, specifically those with high clinical stage (108)
GAR1	GAR1 is one of the four H/ACA RNPs. It also involved in telomerase assembly and stabilization, post-transcriptional processing of nascent ribosomal RNA and pre-mRNA splicing. All these RNPs are concentrated in nucleoli and Cajal bodies of mammalian cells, reflecting the location of H/ACA RNPs. GAR1 binds only to Dyskerin and it is crucial for the nucleolar localization and function of the RNP complex. In CLL patients, a significant decrease of GAR1 mRNA level in patients with CLL compared to controls (105)
TEP1 (telomerase protein component 1)	TEP1 is overexpressed in tumor cells compared to normal cells and it contributes to carcinogenesis and progression of renal cell carcinoma, bladder and prostate cancer (109). Additionally, Findings of Kohno study suggest TEP1 plays a role as a tumor suppressor gene in the genesis and progression of human lung cancer (110)
TCAB1 (telomerase and Cajal body protein 1, encoded by WRAP53)	TCAB1 is a subunit of active telomerase and is essential for the telomerase holoenzyme to be accumulated in Cajal bodies and to elongate telomeres (111), so it is involved in Cajal body maintenance, telomere maintenance and ribonucleoprotein biogenesis. Overexpression of TCAB1 seen in head and neck carcinoma clinical specimens as well as in carcinoma cell lines while depletion of TCAB1 decreased cellular proliferation and invasion potential both *in vitro* and *in vivo* (112)
A1/UP1	Findings of Nagata et al. suggested that UP1, a proteolytic product of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), can unfold the quadruplex structure of telomeric DNA into a single-stranded structure. Therefore, UP1 may enhance the telomerase activity via unfolding of the quadruplex structure of telomeric DNA and resultant provision of the accessible overhang. The authors assumed that both unfolding and recruitment by hnRNP A1/UP1 contribute to improve telomerase activity and maintain proper telomere length. Thus, hnRNP A1/UP1 may be promising targets to control telomerase activity which is associated with several cancers (113)

Hsp90, heat shock protein 90; CLL, chronic lymphocytic leukemia.

responsible for the conversion of uridine to pseudouridine in non-coding RNAs, a vital step in rRNA and ultimately ribosomal synthesis (103).

Complete dyskerin depletion is lethal in mice, Drosophila (they do not have telomerase activity therefore a non-telomerase related function) and yeast (137–139). In humans, germline mutation in the *DKC1* gene is the causative factor for X-linked dyskeratosis congenita (140).

Functions of Telomerase

Telomerase is a specialized reverse transcriptase, which maintains and elongates telomeres at the 3′-single strand in the absence of a DNA template while using the inherent RNA (TERC) for the template function and is thus a RNA dependent DNA polymerase. In the subsequent S-phase of the cell cycle, the conventional DNA replication machinery can then replicate the complementary C-rich strand. Thus, telomerase ascertains chromosomal stability and cellular proliferation in proliferative somatic cells, tissue progenitor cells and in cancer cells (141). When telomeres shorten beyond a critical threshold length, normal healthy cells in humans which are devoid of telomerase activity, will assimilate a cellular senescence phenotype with an irreversible growth arrest and the classical morphological alterations (142). Somatic human cells lacking measurable telomerase yet expressing certain viral oncoproteins can overcome the senescence checkpoint and continue to proliferate, but they then accumulate chromosomal instability including aneuploidy, polyploidy and chromosomal fusions. On these grounds, high telomerase activity has been assigned a role in maintaining genome stability by preventing telomere shortening. Telomerase fulfills this important role via interaction with many key cellular pathways as detailed below.

ATM/ATR pathway

Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad3 related (ATR) DNA damage response kinases have essential roles in telomerase-mediated telomere maintenance (66). The conserved ATM and ATR family of serine-threonine kinase proteins mediates DNA damage and replication stress checkpoint responses (143, 144), therefore, play a crucial role in DNA repair, cell apoptosis, and cell senescence, and are closely associated with the development and progression of cancer in humans (145, 146). ATM is required for the addition of new repeats onto telomeres by telomerase (147) and evaluation of bulk telomeres in both immortalized human and mouse cells showed that ATM inhibition suppressed elongation of telomeres while ATM stimulation through PARP1 led to an increase in telomere length (147).

Stalled replication forks increased telomerase localization to telomeres in an ATR-dependent manner (66). Additionally, increased telomerase recruitment was observed upon phosphorylation of the shelterin component TRF1 at an ATM/ATR target site (S367) (66) and this led to TRF1 loss from telomeres and may therefore increase replication fork stalling (148). ATM and ATR depletion reduced assembly of the telomerase complex, and ATM was required for telomere elongation in cells expressing POT1ΔOB, an allele of POT1 that causes disruption in telomere length homeostasis (66). Hence from this data it can be concluded that ATM and ATR are involved in triggering telomerase recruitment and facilitating its assembly (66).

WNT pathway

Wnt family proteins are essential for regulating cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (149). A dysregulated Wnt/β-catenin signaling pathway is also associated with human tumourigenesis (149). Due to the intricate relationship of telomeres and telomerase with similar cellular functions, their close interaction is not a surprise. An activated Wnt signaling pathway can reinforce the stability of telomeres by coupling and enhancing the two main telomere maintenance pathways: telomerase-dependent and ALT pathways. A Wnt-mediated telomere protective effect is particularly expected to have an important role during development, in adult stem cell function and oncogenesis (65).

The Wnt pathway may regulate telomere maintenance via its effect on several essential shelterin components, including TRF2 and POT1. Recently, in human somatic and cancer cells as well as in mouse intestinal tissue, activation of canonical Wnt/β-catenin pathway activated TRF2 and also increased telomere protection were demonstrated (65). In mice lacking telomerase, apoptosis of the Wnt-dependent intestinal crypt stem cell niche could be rescued by administration of Wnt agonists (150). Additional evidence demonstrates that the Wnt pathway triggers APC- and β-catenin induced regulation of TRF2 and TCF4 which further regulate TRF1 and POT1 (150, 151).

Further to the enhancement of shelterin protection, the Wnt/β-catenin signaling pathway also activates TERT (152). Importantly, the use of Wnt pathway agonists can rescue telomere uncapping, suppress apoptosis and lead to elevated *Ascl2* transcripts as well as Sox9 protein levels (150) suggesting a therapeutic strategy for some conditions with aberrations in telomerase.

Non-canonical functions of TERT

Non-canonical functions of TERT have been discovered later than telomerase activity, and they also play a role in tumorigenesis, for example via TERT's role in regulating the Wnt signaling as a cofactor for the β-catenin pathway (153). TERT has been shown to be inducible in ischemic brain cells and to prevent apoptosis via a non-telomeric action via shift of the cytosolic free Ca^{2+} into the mitochondria (154). Despite having normal telomere lengths, lack of hTERT impairs the cellular capability to repair damaged DNA and fragmented chromatin (155). TERT also is demonstrated to have RNA dependent RNA polymerase function by interacting with the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*) and forming ribonucleoprotein complexes. These complexes produce double-stranded (ds) RNAs that serve as substrates for the generation of siRNA which may regulate the expression of other genes related to stem cell biology (156). Further to the above, there are many other additional non-telomeric functions of TERT active in cancer, such as improved DNA repair, increased

apoptosis resistance, changes in chromatin structure and altered gene expression (157).

Hormone Regulation of Telomerase in Hormone Responsive Tissues

There is evidence from multiple studies that telomerase is under the regulation of steroid hormones in hormone responsive tissues. This corroborates with the known direct regulation of cell fate and proliferation in such tissues by steroid hormones, for example the ovarian hormone, estradiol, induces a mitotic response in endometrial epithelial cells (23, 158). In different studies, telomerase is induced by estrogen in various macaque and human cell lines (15, 159, 160). Androgens also upregulate telomerase in an ovarian cancer cell line (161) but progesterones down regulate telomerase in the endometrium (15). ATM silencing also down regulated proteins, such as ChK2, p53, and caspase 3, which were stimulated by the synthetic progestogen, medroxyprogesterone acetate (MPA) (162). This result suggested that MPA exerts its effects via the ATM-Chk2-p53-caspase-3 pathway protecting against carcinogenesis (162). The progestagenic effect on telomerase may also be mediated through this pathway. Hormonal regulation of telomerase in the healthy endometrium was recently reviewed in detail (14).

Telomerase-Related Telomere Regulation by TERRAs

Telomerase regulation by TERRAs has initially been examined in yeast although recent work also suggests a similar regulation in human cells. In yeast cells, TERRAs were found to sequester and direct telomerase to the specific telomeres which were the shortest (68). In addition, TERRA was found to bind to hTERC and hTERT components of telomerase independently, to function as an inhibitor of human telomerase enzyme (67). In telomerase negative cells with shortened telomeres, increase in TERRA levels trigger homology directed repair (HDR) whereas in telomerase positive cells, it results in recruitment of telomerase to the short telomeres (163). Absence of both telomerase and HDR accelerates the cell senescence pathway (164). Due to loss of Rat1 function, in yeast free TERRA accumulates at critically short telomeres which helps in recruiting the telomerase enzyme to that telomere and elongation of that telomere (165).

TERRA was found to be induced in cells with short telomeres and acted as a scaffold for spatial organization of the telomerase components forming a TERRA-telomerase complex which helped in recruitment of telomerase to the telomere of its origin hence TERRA was proposed to be a recruiter of telomerase enzyme rather than an inhibitor (68). Contrary to some *in vitro* studies, in human cancer cells, telomerase-led telomere elongation was not affected by the transcription of the telomere. In these cells, it was suggested that shortening of telomeres may not have been due to telomerase inhibition, but due to impaired replication due to integrity of the chromosomes affected by high levels of TERRAs (166). In general, the interaction of TERRAs and telomerase is complex and might depend on cell type and conditions, such as cell cycle phase, or telomere length.

Telomere Maintenance by Alternative Lengthening of Telomeres (ALT)

Cells can maintain their telomeres *via* a telomerase dependent pathway or a telomerase independent ALT pathway (69). New telomeric DNA is synthesized from a DNA template in ALT (167) by homologous recombination (HR) (168). The template could either be the telomere of another chromosome, another region of the same telomere by t-loop formation or sister telomere recombination.

The first evidence for the presence of an ALT mechanism was described in several immortalized human cell lines that did not have telomerase activity but maintained telomere lengths for hundreds of population doublings, and this mechanism occurs in ~15% of cancers including osteosarcomas, soft tissue sarcoma subtypes, and some glial brain tumors (169, 170).

In human cells, where ALT activity is elevated to a degree sufficient for telomere length maintenance, telomeres are characterized by their highly heterogeneous length, but the average length (>17 kb) is about double that of most cells where telomeres are elongated by telomerase (171).

Mutations in the ATRX/DAXX chromatin remodeling complex have been observed in cancers and cell lines that use the ALT mechanism, suggesting that ATRX may suppress the ALT pathway (172). In mortal cells or immortal telomerase-positive cells, knockout or knockdown of ATRX does not stimulate ALT (172). However, ATRX loss in SV40-transformed fibroblasts together with one or more unidentified genetic or epigenetic alterations was attributed to either a marked increase in the proportion of cells with an activated ALT (instead of telomerase) or significant decrease in the time taken for ALT activation (172). Loss of ATRX protein and mutations in the ATRX gene are also characteristic features of ALT-immortalized cell lines (172). In addition, ALT is associated with marked genome rearrangements, extensive micronucleation, a defective G2/M checkpoint and alteration in double-strand break (DSB) repair (173).

ROLE OF TELOMERES AND TELOMERASE IN PRE-MALIGNANT AND MALIGNANT PROLIFERATIVE DISORDERS

Alteration of Telomere Biology in Premalignant Conditions and in Cancers

Limitless proliferation is a cardinal feature of cancer cells, whist increased proliferation is common to all premalignant changes including hyperplasia. The excessive proliferation observed in these malignant/premalignant conditions is maintained by avoiding senescence and crisis/apoptosis. Senescence/apoptosis exist as barriers for mitosis, thus they are tumor suppressor mechanisms in normal cells, which are regulated intricately by telomeres and checkpoint activation (**Figure 3**). The unrestricted proliferation of cancer cells is therefore thought to be sustained by telomere maintenance mechanisms which were detailed above. Since high telomerase activity is reported in over 85% of cancers, telomerase dependent telomere lengthening is believed

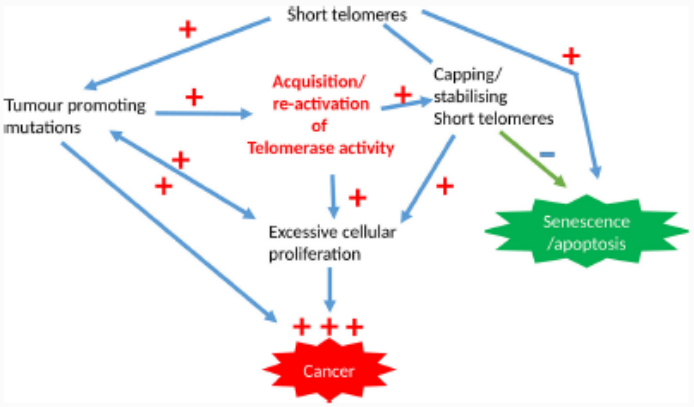

FIGURE 3 | The involvement of telomeres and telomerase activity in epithelial cancers. The initial acquisition of tumor promoting mutations is promoted by short dysfunctional telomeres which are subsequently stabilized by high telomerase activity levels that is characteristic for most cancer cells, with the overall result being pre-requisite for unregulated proliferation capacity.

to be the most common telomere maintenance mechanism relevant to carcinogenesis.

Evidence for Altered Telomere Lengths in Cancers

During ongoing proliferation in normal somatic cells without telomerase or other telomere-maintenance mechanisms, telomeres shorten until reaching a certain minimal length. Beyond this, when tumor suppressor checkpoints, such as p53 are functioning, senescence or apoptosis can be induced. In contrast, when p53 or other important DNA damage checkpoints are not functioning, cells can enter a crisis state where ongoing proliferation promotes further telomere shortening and telomere dysfunction (174). This can cause various genomic instabilities, such as end-to-end fusion of telomeres resulting in anaphase bridges in subsequent cell division cycles. Most of these cells usually die due to apoptosis and gross genomic instabilities. However, some rare cells acquire mutations in the TERT promoter that increase telomerase activity resulting in re-stabilization of telomeres, Importantly, as long as telomeres are capped and protected, they can be rather short and this situation is frequently found in epithelial cancer cells compared with adjacent healthy tissue. Several studies using telomere PNA-FISH have shown that breast, prostate, and pancreatic cancers are associated with telomere shortening (175–177). Furthermore, around 40 to 97% of colorectal tumors have shorter telomeres compared with normal tissue, and telomere shortening is therefore considered to be one of the early events in tumourigenesis (178, 179).

However, importantly, acquiring telomerase activity can stabilize even short telomeres in genetically unstable cells and provide sufficient capping for them to attain an unlimited proliferation potential. Thereby, telomerase re-activation conserves genomic mutations and instabilities and contributes further to tumourigenesis (**Figure 2**).

Significant telomere length shortening results in end-to-end fusion, thus increasing the potential for genome instability and carcinogenesis. There are few other generic associations which lead to telomere attrition, such as oxidative stress, lifestyle choices, environmental factors, smoking and obesity (180) and some of these also increase the risk of developing a variety of cancers. Telomere shortening can influence the progression of premalignant breast tissue to malignancy and premalignant breast lesions had short telomeres leading to non-clonal chromosome aberrations (181).

Meta-analyses of available studies also revealed that shorter peripheral blood mono-nucleocyte (PBMC) telomeres are associated with a significant increase in the risk of developing cancer (OR = 1.35, 95% CI = 1.14–1.60) than longer telomeres (182, 183). Shorter PBMC telomeres could be related to oxidative stress endured by an organism, which is in agreement with the established mediatory role that oxidative stress plays between inflammation and cancer (184). When PBMC mean telomere lengths were prospectively studied in the general population in Denmark, shorter telomere lengths were also associated with decreased survival after cancer rather than the cancer risk itself (185). Another systematic review has also reported a consistent inverse relationship between age and PBMC telomere length (186).

Telomere dysfunction may also be a resultant of altered telomere-associated proteins that are also essential for regular end-capping function (187, 188). For example, mutations in the C-terminal of POT1 can initiate genomic instability permissive for tumourigenesis (189). TRF1 flox/flox × K5-Cre transgenic mice, do not have TRF1 in stratified epithelia. These mice demised perinatally and showed skin hyperpigmentation and epithelial dysplasia and were associated with telomere initiated DNA damage, p53/p21 and p16 pathway activation and *in vivo* cell cycle arrest. Deficiency of p53 rescues mouse survival but

causes increase in the incidence of squamous cell carcinomas (39). Alteration of the levels of TRF1, TRF2, TIN2, and POT1 has also been described in some human tumors (190). A dysregulated expression of TRF1, RAP1, and TPP1 has been reported in patients with chronic lymphocytic leukemia (191). Likewise, TIN2, TRF1, and TRF2 mutations have been associated with some cases of Dyskeratosis congenita and aplastic anemia (192–195) and both these conditions increase the risk of developing some cancers. Defects in shelterin components naturally cause dysregulation of telomere homeostasis as explained above. This may operate as a tumor suppressor mechanism when it initiates the p53/pRb pathways which in turn triggers senescence and prevents the tumorigenesis process. Alternatively, it can contribute to carcinogenesis with the fusion of dysfunctional telomeres or fusion between dysfunctional telomeres and double strand breaks which trigger breakage-fusion-bridge cycles (196). In hepatocellular carcinomas, longer telomeres, increased hTERT expression and higher levels of TRF2 protein as "stemness markers" were associated with poorer prognosis and more chromosomal instability (197). Further studies have confirmed that different causal factors, such as hepatitis B and C, and alcohol lead to telomere dysfunction in hepatic cells hence initiating the carcinogenesis process (198). A significant decrease in POT1 and RAP1 protein levels are described in familial papillary thyroid cancers (199). TP53 disruption in hematological malignancies has been associated with the downregulation of expression in shelterin genes and severe telomere dysfunction and genomic instability (200). Therefore, genetic mutations resulting in functional alterations in the essential components of the telomerase enzyme or shelterin components may repress telomerase activity and thus shorter telomeres will be the consequence. The available evidence also suggests a concerted dysregulation in the expression of shelterin genes and protein levels with the commonly observed removal of cellular tumor suppressor mechanisms in premalignant conditions can lead to alteration in telomere lengths that can trigger the tumourigenesis process.

Evidence for Altered Telomerase in Cancers

Polymorphism in genes of the telomerase complex

Such as *hTERT* and *hTERC* has been reported to affect individual susceptibility to cancers (201, 202). Variants in chromosome 5p15, the region that harbors the *hTERT* gene, have been identified by Genome-wide association studies (GWAS) to be associated with the risk of bladder, pancreas, brain, testicular, breast, prostate, skin, and lung cancers (203–207).

hTERT promotor mutations

Tumors with high *hTERT* promoter mutation frequencies have almost always originated in tissues with relatively low cell turnover rates. Contrastingly, tissues with rapid cell turnover seem to have different mechanisms to elongate telomeres and seem less likely to benefit from activating *hTERT* expression by mutations (208). Mutations that result in increased hTERT expression, telomerase activity or longer telomere lengths have been identified in cancers of the central nervous system, thyroid, bladder, liver, tongue, adipose tissue and skin (208–210). In thyroid cancers, when *hTERT* and *BRAF* mutations coexist, such tumors express high levels of *hTERT* (211).

Common inherited variants of telomere related genes, such as *TERC*, *TERT*, and rare *POT1* mutations have been found to be associated with higher risk of developing gliomas. *TERT* promoter and *ATRX* mutations were found to be the most recurrent somatic events which led to glioma associated lengthening of telomeres (212).

A high frequency of *hTERT* promoter mutations was also reported in follicular cell-derived thyroid carcinomas (213). An over-representation of *hTERT* promoter mutations had been detected in advanced thyroid cancers and these mutations were more prevalent in advanced disease (51%) compared with well-differentiated tumors (22%). Thus, *hTERT* promoter mutations have been suggested as biomarkers of tumor progression (213). *hTERT* promoter mutations usually cause an increased expression of the *hTERT* gene and paradoxically, these mutations were reported to occur together with short telomeres in tissues with low-rates of self-renewal and were also associated with poor patient survival in primary melanomas (210). Tissue stem cells are reported to have active telomerase and daughter cells produced by these switch off telomerase upon differentiation, and subsequent reactivation of telomerase in these tissues have been proposed to be the reason for the observed short telomeres in thyroid cancers with high telomerase expression (210). Rachakonda et al. showed that mutations of the *hTERT* promoter were also the most common somatic lesions in bladder cancer (214). The authors also found that a common polymorphism rs2853669 in the *hTERT* promoter acts as modulator of the mutations effect on survival and disease recurrence. The patients with the mutations had poor survival outcome in the absence but not in the presence of the variant allele of the polymorphism. The mutations without the presence of the variant allele were markedly correlated with tumor recurrence in patients with non-invasive and invasive T1 bladder tumors (214). Polymorphisms in the *hTERT* gene were also associated with an increased lung cancer risk in the Chinese Han population (215).

Telomerase activity in cancers

The early observation that telomerase activity is absent in most human somatic tissues during differentiation but strongly upregulated in tumors, agrees with the hypothesis that telomerase playing an important role in the carcinogenesis process (216). In pancreatic ductal cell carcinoma, levels of telomerase activity were higher compared to other types of pancreatic cancer and benign pancreatic tissues (217). In gastric cancers, tumors with high telomerase activity had poorer prognosis and the authors concluded that detecting telomerase activity might be useful as a prognostic indicator of clinical outcome (217). Telomerase activity was also detected in 90% of head and neck squamous cell cancers, in 100% hyperplastic squamous epithelium but not in normal mucosa (218). Colorectal cancers with high telomerase activity had poorer prognosis in spite of curative surgery in apparently disease free patients, thus the survival seems to have been associated with the level of telomerase activity (219). A systematic analysis of telomerase activity levels in many cancer

types performed by Bacchetti and Shay in 1997 demonstrate high telomerase being a common observation in most of them (220).

hTERC alterations in cancer: Recent work has proposed that hTERC maturation involves the poly(A)-specific ribonuclease (PARN) which is localized in the nucleolus and in the Cajal body (CB). The enzyme trims hTERC precursors by removing poly (A) tails and may be involved in impairment of telomerase activity (221). Individuals with biallelic PARN mutations and PARN-deficient cells showed a reduction of expression of genes encoding several key telomerase components, such as TERC, and DKC1. These cells also have critically short telomeres (222). Improper hTERC processing and telomere dysfunction in premalignant diseases, such as Pontocerebellar Hypoplasia 7 (PCH7) and dyskeratosis congenita had been proposed to have a mechanistic link (221). *hTERC* amplification was associated with the aggressive progression of cervical cancer, and authors suggested that hTERC may serve as a surrogate marker for cancer progression and form a potential therapeutic target for cervical cancer (223). However, it is important to appreciate that most cervical cancers initiated in a background of persistent papilloma virus infection in the transformed epithelial cells. hTERC over-expression has been reported in many other cancers including prostate (224); breast (225); and oral squamous cell carcinoma (226).

Dyskerin alterations in cancer: Dyskeratosis congenita is a rare multisystemic syndrome characterized by low telomerase activity already during development and consequently, shorter telomeres in many tissues resulting in a high susceptibility to develop a subset of cancers, therefore, wild type dyskerin protein has been suggested to act as a tumor suppressor. Conversely, wild-type dyskerin protein is upregulated in a number of human cancers, such as in breast, prostate, colon and hepatocellular carcinomas (108, 227–229) and in these cancers, high levels of dyskerin were associated with an aggressive histopathological feature and poor prognosis (229). Acute loss of dyskerin function by RNA interference led to marked reduction of steady-state levels of H/ACA RNAs, disruption of the morphology and repression of anchorage-independent growth of telomerase-positive and telomerase-negative human cell lines. The levels of dyskerin in cancer cells modulate telomerase activity through the regulation of TERC levels, independently of TERT expression (227). The function of telomerase associated proteins in cancer is summarized in **Table 1**. Dyskerin might also contribute to tumor development through mechanisms where the presence of cellular telomerase activity is not essential, and which may be only partially dependent upon the protein's role in rRNA processing (104).

ENDOMETRIUM

The endometrium is the inner mucosal lining of the uterus that contains several cell types including tissue specific epithelial and stromal cells, as well as leucocytes and blood vessels (22, 230–233). It is the primary target organ for ovarian steroid hormone action (24) and healthy human endometrium is characterized by its regenerative and remodeling capacity that undergoes repetitive monthly cycles of proliferation, secretory changes, break-down and regeneration. These cycles of changes occur ~400 times in a female's reproductive life (22, 230) and are regulated by ovarian steroid hormones (23). Telomerase activity as well as mean telomere length change according to ovarian cycle in whole healthy endometrial samples (15, 234) suggesting an ovarian regulation and correlation with proliferative activity (15). Epithelial cells demonstrated significantly higher telomerase activity, but contrastingly, shorter telomeres compared with stromal cells across the cycle (14, 15) (**Figure 4**). In the endometrium, Estrogen upregulates telomerase activity. Whilst progesterone inhibits telomerase activity and *hTERT* expression (15). The telomere and telomerase biology of normal endometrium has recently been reviewed in detail (14).

The Role of Telomeres and Telomerase in Benign Endometrial Disorders (Table 2)

The role of telomeres and telomerase in benign endometrial disorders was recently reviewed in detail in Hapangama et al (14). There are various benign gynecological disorders, such as endometriosis (243), recurrent reproductive failure, subfertility with reported abnormal telomerase activity and telomere length aberrations (13, 235). High telomerase activity, high hTERT mRNA and protein levels with longer mean endometrial telomere lengths are characteristics of the eutopic secretory endometrium (13, 235, 242, 244), whereas epithelial cells of ectopic lesions also demonstrated longer mean telomere length (15).

The progesterone dominant window of implantation in healthy women has shown virtually no hTERT immunoreactivity (235) and lowest telomerase activity (13, 234). However, immunostaining for hTERT was significantly and differentially increased in various endometrial cellular compartments in women with recurrent reproductive failure (235). These observations suggest that particular aberrations in cellular proliferation or causative dysregulation of telomerase to be important in endometrial pathologies. Furthermore, normal telomerase activity seems to play a pivotal functional role in ensuring normal endometrial function.

Alteration of Telomere Biology in Endometrial Premalignant Conditions and in Endometrial Cancer

Endometrial Hyperplasia

Endometrial epithelial hyper-proliferation with increased glandular to stromal cell ratio is defined as endometrial hyperplasia. Pathogenesis of endometrial hyperplasia is virtually always associated with relative predominance of the mitotic estrogen signal, due to direct excess of Estrogen or due to insufficient levels of progesterone (24). Anovulatory cycles in premenopausal women, extra-ovarian aromatization of adrenal androgens in to estrogenic compounds in obese women and iatrogenic interventions, such as Tamoxifen and Estrogen only hormonal replacement therapy are common examples

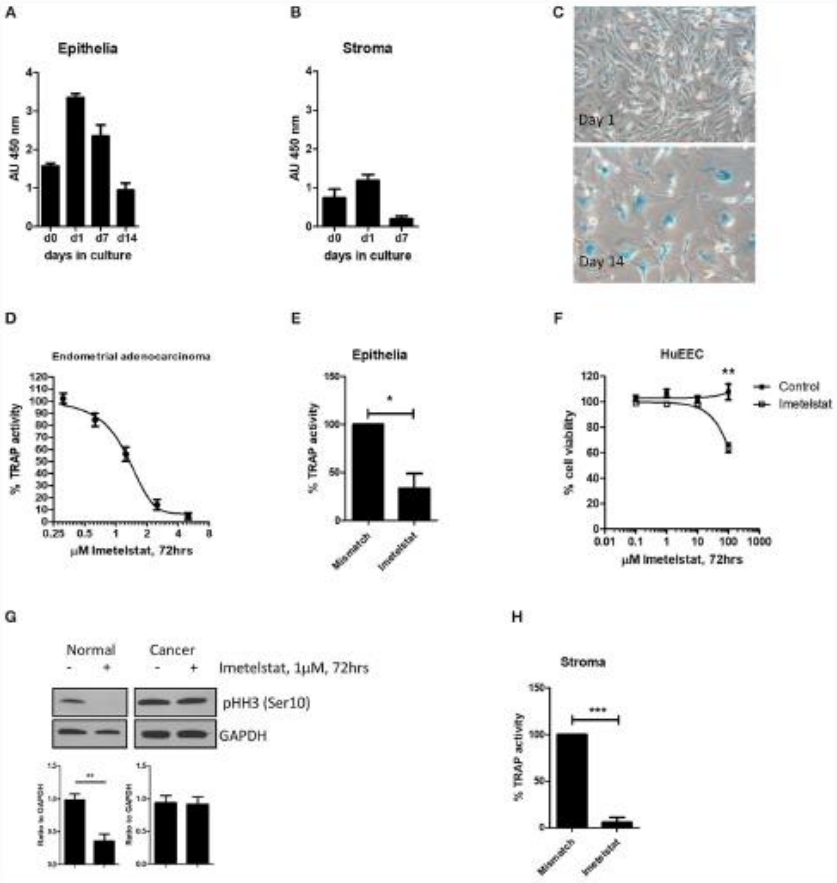

FIGURE 4 | From Valentijn et al. (15). Telomerase Inhibitor, Imetelstat affects Telomerase Activity and cell proliferation, but not viability of endometrial epithelial cells. **(A)** Epithelial cells and **(B)** stromal cells were maintained in monolayer culture for the indicated times prior to harvesting for TRAP assay. For each time point, $n \geq 4$; Patient group 2. **(C)** Epithelial cells maintained in long-term culture had a phenotype consistent with senescence. Note the enlarged cells and positive blue stain for β-galactosidase in the micrographs (representative of $n = 5$). **(D)** Epithelial cells were isolated from an adenocarcinoma of the human endometrium and maintained in culture as a cell line. The cells were treated with the concentrations of Imetelstat indicated for 72 h prior to TRAP. TRAP activity is expressed as a percentage relative to the activity of the mismatch control (mean ± SEM for $n = 3$ separate experiments). **(E)** Epithelial cells were maintained in culture for up to 3 days and then treated with 1 µM Imetelstat or mismatch control oligonucleotide for a further 72 h prior to TRAP assay. TRAP activity is expressed as a percentage of the mismatch control ($n = 4$). *T*-test, $^*p = 0.02$. **(F)** EEC ($n = 5$) were directly seeded into 96-well dishes, allowed to attach and treated the next day with Imetelstat or the mismatch control at the concentrations indicated for 72 h. Cell viability was assessed by MTT assay. Note significant loss in cell viability at 100 µM (Mann Whitney test, $p = 0.002$). **(G)** Cultures of normal epithelial cells and an adenocarcinoma of the endometrium treated with Imetelstat or mismatch control as before, and immunoblotted for phospho-H3 [phosphohistone H3 (Ser10)]. Histone H3 is only phosphorylated on Ser 10 during mitosis. Shown is a representative blot (top) of normal epithelial cells ($n = 5$) and the adenocarcinoma (representative of two separate experiments) and densitometric analysis (bottom). *T*-test, $^{**}p = 0.009$. **(H)** Stromal cells were grown for 24 h and then treated with 1 µM Imetelstat or mismatch control oligonucleotide for 72 h prior to TRAP assay. Telomerase activity is expressed as a percentage of the mismatch control. *T*-test, $^{***}p = 0.0004$. This previously published figure in human reproduction (15) is reused with permission.

of conditions related to endometrial hyperplasia. Importantly, the premalignant endometrial hyperplasia, which includes the category of atypical hyperplasia/endometrial intraepithelial neoplasia according to the 2014 World Health Organization (WHO) classification is the typical precursor of endometrioid endometrial cancers (245).

Alterations in telomere lengths in endometrial hyperplasia

The involvement of telomere shortening in chromosomal instability has been associated with the initiation of carcinogenesis (246). There are only 2 studies that have examined telomere lengths in endometrial hyperplasia. A study using a telomere-FISH (telo-FISH) assay to measure telomere lengths, compared chromosomal arm loss or gain in premalignant endometrial lesions with normal endometrium, and reported telomere lengths to be stable with the pathological transformation in endometrial hyperplasia and in endometrial carcinoma (247). Albeit using a small sample size, the authors conclude that unlike in cervical precancerous lesions, endometrial hyperplasia did not have widespread chromosomal alterations, implying that endometrial carcinogenesis involves mechanisms distinct from those of cervical carcinogenesis, which is almost always induced by viral infection (247). However, close scrutiny of the data presented on different endometrial hyperplasia subtypes suggested that atypical endometrial hyperplasia may be associated with higher telomere length heterogeneity. This may be also suggestive of the involvement of ALT mechanism in this premalignant condition, but larger studies are needed to confirm the ALT mechanism in the true pre-malignant endometrial hyperplasia subtype with atypia. Importantly, the analysis method utilized in the Maida study did not allow inter-patient comparison of tissues samples (of different women) but was only suitable to compare adjacent cells of a single tissue sample. Therefore, the study presented insufficient data to conclude if there was a definite change in the telomere length in precancerous endometrial hyperplasia when compared with either normal or cancerous endometrium.

By using a three-dimensional (3D) imaging technique, a specific 3D arrangement of telomeres was revealed in tumor cell nuclei (248). Unlike the non-overlapping nature of telomeres in normal nuclei, telomeres of cancer nuclei have the tendency to form aggregates (248). Different numbers and sizes of such telomere aggregates can be found in tumor nuclei (248). Telomere aggregate formation does not depend on telomere length and telomerase activity (249).

The existence of telomere aggregates in precancerous lesions, such as in human cervical intraepithelial neoplasia supports the notion that changes in the organization of the 3D nucleus may facilitate tumorigenesis (250). The "telomere-driven genome-instability" can happen as a result of the close contiguity of telomeres forming aggregates of different numbers and sizes that increase the risk of end-to-end telomeric fusions followed by cycles of breakage-bridge-fusion (249). A significantly increased number of telomere aggregates was observed in atypical hyperplastic cells in a mouse models which is also a specific feature of cancer cells. Moreover, the *PTEN* heterozygous mouse model further demonstrated that 3D telomere architectural changes occur before the complete loss of *PTEN* and prior to the development of histological characteristics of atypical hyperplasia and endometrial carcinoma (251). Therefore, the presence of telomere aggregates in hyperproliferative lesions with atypical nuclei may render them to be precancerous changes. Further studies including larger sample size and both types of endometrial hyperplasia are warranted to examine and conclude on changes in telomere length in precancerous endometrial hyperplasia lesions.

Telomerase in endometrial hyperplasia

High hTERT levels and elevated telomerase activity were reported in all types of endometrial hyperplasia, including simple, complex and complex with atypia subtypes (252–256). This early observation prompted some investigators to propose that telomerase activity measured by TRAP assay to be a suitable tool to screen the endometria of post-menopausal women with post-menopausal bleeding (257). The authors proposed that this method will determine endometrial premalignant and malignant conditions (257) from benign endometrium, since telomerase activity was rarely detected in normal post-menopausal women, while the majority of endometrial hyperplasia and cancers contained high telomerase activity. However, there are other studies that reported a lack of measurable telomerase activity by TRAP assay in benign endometrial hyperplasia (258). Further work also found that it was possible to use hTERT immunohistochemical (IHC) analysis (259) as a marker for premalignant (atypical) endometrial hyperplasia. However, it is difficult to conclude on the diagnostic feasibility of telomerase activity or hTERT protein (IHC) in endometrial hyperplasia considering these studies, because of the inadequate sample sizes which were only $n = 12$ atypical endometrial hyperplasia in Brustmann (259) and $n = 18$ simple and atypical endometrial hyperplasia in Maida et al. (257) and Brustmann (259). In addition, the studies did not clarify whether the existence of endometrial hyperplasia cells were confirmed in the analyzed samples, particularly with TRAP assay and since endometrial hyperplasia can co-exist with either normal or cancerous endometrium, this may affect the results. Progesterone is one of the main current pharmacological therapies for treating endometrial hyperplasia (24) and telomerase being a (albeit indirect) downstream target of progesterone in the endometrium is of interest. This justifies future studies exploring the therapeutic utility of directly targeting telomerase in the treatment of endometrial hyperplasia.

Endometrial Cancer

Traditionally, EC had been divided into two major groups: estrogen-dependent, type-I (endometrioid type) and estrogen-independent, type-II (non-endometrioid), with the former accounting for 80% of ECs. Five-years survival rates are exceptionally poor for advanced type-I and type-II (high grade) EC at 23% which is a far worse rate than for most other common cancers, such as breast cancer (CRUK). However, the recent trend had been to apply for an alternative classification system that more accurately defines ECs into prognostically distinct molecular subtypes that reflect the underlying molecular alterations with well-described underlying genomic aberrations (260). EC is a disease of post-menopausal women, however, obesity associated unopposed estrogen action is an established cause for the trend toward increasing incidence of this cancer even in younger women (23, 24, 261). ECs are hormone responsive tumors and even high grade ECs retain some

TABLE 2 | Published literature on telomerase biology in benign endometrial disorders, telomerase, and telomere length.

TA/hTERT/TL	Title	References	No. of samples	Methods	Conclusions
hTERT/TL	Endometrial telomerase shows specific expression patterns in different types of reproductive failure	(235)	Control group ($n = 15$), idiopathic recurrent loss of empty gestational sacs ($n = 10$), miscarriage following identification of fetal cardiac activity ($n = 10$) and recurrent implantation failure ($n = 10$)	IHC (telomerase protein level) real-time PCR (TL)	In recurrent reproductive failure samples, the immunostaining for telomerase was significantly high in various endometrial cellular compartments and this indicates that there are specific alterations occur in the regulation of endometrial cell fate are associated with recurrent reproductive failure various types
hTERT/TL/TA	Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length	(13)	Group 1: healthy fertile ($n = 27$), group 2 symptomatic endometriosis ($n = 29$)	IHC (Telomerase and ERβ) qPCR (Mean TL), TRAP (TA)	Either weak or absent telomerase immunoreactivity was observed in the endometria of fertile healthy women throughout the luteal phase. Increased telomerase protein level (IHC) during the implantation window and the premenstrual endometria of women with endometriosis. The mean TL were significantly longer in endometria of women with endometriosis during the implantation window This study suggested that aberrant expression of telomerase in endometrium alters the cell fate and enhances the cellular proliferation and that leads to the occurrence of endometriosis
hTERT	The expression levels of stem cell markers import in13, c-kit, CD146, and telomerase are decreased in endometrial polyps	(236)	Control (proliferative phase $n = 20$ and secretory $n = 20$), Endometrial polyp (proliferative phase $n = 20$ and secretory $n = 20$)	IHC (Telomerase protein)	In endometrial polyp tissue, the level of telomerase was decreased in comparison with normal endometrial tissue
hTERT	Enhanced differentiation and clonogenicity of human endometrial polyp stem cells	(237)	Endometrial polyp ($n = 6$)	Quantitative RT-PCR (TERT)	No telomerase reverse transcriptase (TERT) expression was noted in endometrial polyp tissue
hTERT	Aberrant Telomerase Expression in the Endometrium of Infertile Women with Deep Endometriosis	(238)	Control group: Fertile women without endometriosis ($n = 44$) and infertile women with endometriosis ($n = 25$) from which endometrium and endometriotic peritoneal lesions of the same patient were taken in the late luteal phase of the cycle	qRT-PCR (hTERT and GAPDH mRNA) based on TaqMan methodology	Telomerase (hTERT mRNA) level is associated with the development and progression of endometriosis
hTERT	The Status of Telomerase Enzyme Activity in Benign and Malignant Gynaecologic Pathologies	(239)	Benign endometrial tissue ($n = 7$): six endometrial polyps and one irregular proliferative-phase endometrium; endometriosic ectopic samples ($n = 13$) and endometrial cancer ($n = 6$)	Real-time reverse transcriptase polymerase chain reaction RT-PCR (hTERT mRNA)	hTERT was positive only in the irregular proliferative phase endometrium (14.2%) and hTERT was also positive in one of 13 endometriosis ectopic specimens (7.7%)
TA, hTERT, TL	Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis	(15)	Group 1 ($n = 85$) endometrial and matched blood, group 2 ($n = 74$) healthy endometrial biopsies (not on hormonal treatment) group 3 ($n = 5$) endometrial biopsies on medroxyprogesterone acetate (MPA) for contraception group 4 ($n = 10$) matched endometriotic ectopic and eutopic, group 5 ($n = 22$) healthy women in mid-secretory phase before ($n = 8$), and after administering 200 mg mifepristone ($n = 14$)	TRAP (TA), qPCR and Q-FISH (TL), immunoblotting (histone H3) (cell proliferation), 3D-culture (assess the ability of EECs to form spheroids, IHC (TERT and Ki67)	High TA and short TLs were observed in proliferating EECs *in vivo* and *in vitro*. In mid-secretory phase endometrial tissue where progesterone is dominant, TL was significantly shorter in comparison with the proliferative phase. Progestagen treatment repressed EEC TA *in vivo* and reduced endometrial TA in explants and *in vitro* cultures compared with non-treated cells

(Continued)

TABLE 2 | Continued

TA/hTERT/TL	Title	References	No. of samples	Methods	Conclusions
hTERT	Endometrial expression of telomerase, progesterone, and estrogen receptors during the implantation window in patients with recurrent implantation failure	(240)	Endometrial biopsies fertile (n = 30) and RIF (n = 30)	qRT-PCR (TERT, ER alpha and PR), western blotting and IHC (TERT and ER alpha)	Expression of endometrial telomerase was substantially increased as ER alpha decreased in women with RIF during the implantation window.
TA	Does telomerase activity have an effect on infertility in patients with endometriosis?	(241)	Healthy control (n = 16), endometriosis infertile (n = 14) and fertile (n = 17)	PCR (TA)	In peripheric blood analysis, telomerase activity is useless as a biomarker. Telomerase activity is absent in cystic wall and that suggesting a high differentiation of endometriosis tissue and that might be considered as a cause of low malignancy risk. Whereas, telomerase activity is high in the eutopic endometrium of the infertile group which may be the possible reason of endometriosis-related infertility.
hTERT, TA	Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis	(242)	Healthy control (n = 30), endometriosis (n = 30)	qRT-PCR (hTERT), TRAP (TA)	In the endometrium of endometriosis patients, the hTERT mRNA is overexpressed and telomerase activity is increased suggesting that the replication potential of endometrial cells might be crucial in the pathogenesis of endometriosis

TA, telomerase activity; TL, telomere length; IHC, immunohistochemistry; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; Q-FISH, quantitative fluorescent in situ hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EECs, endometrial epithelial cells; RIF, recurrent implantation failure.

hormone responsiveness as depicted by the expression of steroid hormone receptors (261).

Evidence for telomere alterations in endometrial cancer (Figure 5) (Table 3)

A study in 1992 found that endometrial adenocarcinomas have reduced telomeric repeat sequences suggesting shorter telomeres compared with normal tissue (262). A decade later a second study demonstrated changes in telomere lengths in 17/23 (73.9%) of endometrial cancers using a Southern blot technique (269). Another study by Menon and Simha (273), using the same telomere restriction fragment (TRF) measurement, found that mean TRF lengths became shortened when normal endometrium underwent neoplastic changes (273). A study which used a telomere-oligonucleotide ligation assay demonstrated erosion of the telomere overhang length, rather than overall telomere length, and proposed that this might play a role in endometrial carcinogenesis and may be related to tumor aggressiveness (274). All these studies utilized techniques that assess the average telomere length values of a tissue sample. However, when endometrial samples were harvested and frozen, they did not examine if the proportion of the endometrial sample examined for telomere length actually contained cancerous cells. Subsequently, 12 years ago, Maida et al. (247) employed a telomere-FISH (telo-FISH) assay that assessed the relative telomere length in normal and pathological cells in intact tissue at the cellular level and no significant difference was found between the telomere length of normal endometrium and endometrial cancer (247). That study however did not specify the normal cell type that they used as the control (stromal/epithelium) and included only adenocarcinomas (Type I). A similar, but slightly modified version of telomere chromogenic *in situ* hybridization method was subsequently used by Akbay et al. and the authors demonstrated a significant telomere shortening in both type I and type II endometrial cancers in comparison with normal stromal cells (270). They also reported that the adjacent normal stromal cells were compared with epithelial cancer cells to demonstrate telomere shortening only in type II cancers. The authors expanded the study to confirm their hypothesis in a rodent model. These animals were generated with shortened telomeres to show that telomere attrition contributes to the initiation of type II endometrial cancers and progression of Type I endometrial cancers (270). This is of interest, but caution should be taken when interpreting these results, as the endometrial stromal cells are known to possess longer telomeres when compared even with healthy epithelial cells (14, 15) and that has been hypothesized to be due to the difference in the cell proliferation rates, telomerase activity levels and different regulation of telomere maintenance in these two cell types (14). Therefore, the data may simply reflect cell type specific difference in relative telomere lengths but not demonstrating a true endometrial cancer associated change in telomere lengths. Hashimoto et al. (274) found that endometrial cancers show short 3′ single-strand telomeric overhang length compared to normal endometrium (274). They also found that poorly differentiated cancers or deeply invading endometrial cancers had a longer overhang length in comparison with well-differentiated cancers

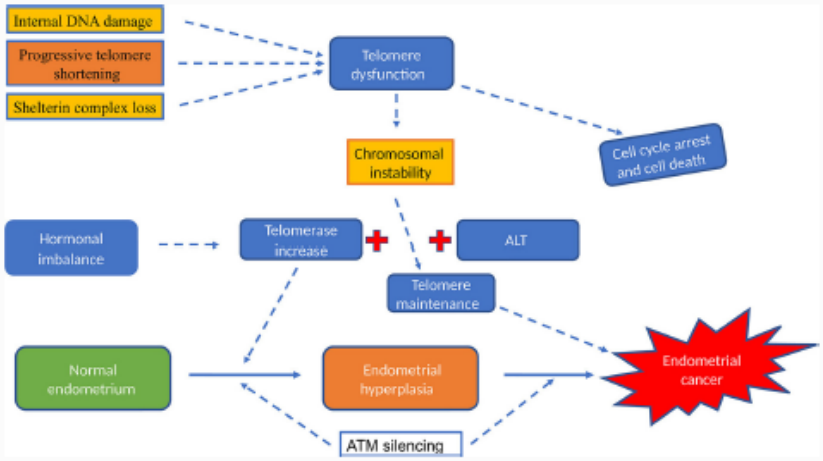

FIGURE 5 | The role of telomere and telomerase activity in endometrial cancer. Hormonal imbalance (excess of Estrogen or insufficient levels of progesterone) will increase telomerase and elevate telomerase activity was described in all types of endometrial hyperplasia and in endometrial cancer. Dysfunctional telomeres results in genomic instability, the first step in endometrial carcinogenesis. Telomerase dependent pathway is the most widely reported classical telomere length maintenance mechanism but ALT pathway; telomerase independent telomere maintenance was described in some cancer types that lack telomerase activity.

or superficial invading cancers and this may suggest that the 3′ overhang may have a role in tumor progression (274).

A recent paper that considered germline genetic variants in a genome wide association study (GWAS) as instrumental variables to appraise the causal relevance of telomere length for the risk of cancer, demonstrated that their predicted increase in telomere lengths was strongly associated with some specific cancers, such as gliomas, low grade serous ovarian cancers, lung adenocarcinomas, neuroblastomas, bladder cancers, melanomas, testicular cancers, and also endometrial cancers (275). However, this study did not measure the exact telomere length of the tissue of origin of cancers but assumed the particular genetic variance may promote longer telomere lengths. With that assumption, the authors calculated a stronger association of presumed longer telomere lengths and rarer cancers and cancers with a lower stem cell division rate (275). However, this data should be considered with caution, since age associated tissue/cell specific telomere length change is a well-established fact but that was not considered by the authors. Therefore, the postulated prediction in telomere length change may be relevant to the effect of genetic variants that were examined, in increasing cancer risk, but it does not provide direct or compelling evidence for a role for tissue telomere length change in endometrial carcinogenesis. When telomere lengths were estimated for cancer cohorts in The Cancer Genome Atlas (TCGA) dataset; sarcomas, testicular germ cell tumors and low grade gliomas were associated with longer telomeres whilst cervical and endometrial cancers had shortest average telomere length (276). This observation has also been explained as a result of some tumors having high telomerase activity, thus shorter telomere lengths that are stabilized [e.g., in testicular tumors (277)], and others have long telomere lengths accompanied by increased activity of the ALT mechanism (e.g., in low grade gliomas and sarcomas). Longer telomere length in PBMC has also been associated with a significantly increased risk of endometrial cancer in a group of Caucasian Americans (272). Since endometrial cancers are known to have high telomerase activity, the ALT mechanism is less likely to be active in those cancers. Considering the above evidence, it is likely that endometrial cancers have relatively shorter telomere lengths that are maintained by high telomerase activity compared with normal tissue. Further studies are warranted to examine subtype specific telomere length aberrations and the relationship of telomere lengths with the telomerase activity in the different types of endometrial cancers.

The protein and/or mRNA levels of the most conserved out of all shelterin proteins, POT1 (26) were increased in many different cancers including gastric, thyroid, breast (199, 278, 279) and in endometrial cancers (280). Higher levels of point mutations in the POT1 gene were observed in endometrial cancers, revealing that genetic variations in *POT1* may lead to carcinogenesis in the endometrium (280). Simultaneous conditional inactivation of the shelterin protein POT1a with the tumor suppressor p53 in endometrial epithelial cells in a murine model, induced type II metastatic adenocarcinomas in 100% of the animals by 15 months (281). This suggests that telomere dysfunction and loss of tumor suppressor genes can produce Type II endometrial cancers. This will obviously need to be accompanied by telomerase re-activation observed in most endometrial cancers supporting the cancer-associated increased cellular proliferation. The loss of POT1 proteins

activates ATR (282) and ATR activation requires Replication Protein A (RPA), which binds single stranded (ss) DNA (282); the POT1-TPP1 heterodimer protects telomere ends from being detected as DNA damage by excluding RPA from binding telomeric ssDNA. Therefore, the loss of POT1 described in endometrial cancer may cause inappropriate telomere access of telomerase resulting in compromised telomere capping and sustained telomere dysfunction facilitating genetic instability.

There are no published studies examining the expression or function of other shelterin proteins or TERRAs in EC to date.

Evidence for a role of telomerase in endometrial cancer (Figures 5, 6)

Kyo et al. examining 13 endometrial cancers and 5 cell lines derived from endometrial cancers using a Telomerase Repeated Amplification Protocol (TRAP) assay reported that 92% of cancer samples displayed detectable telomerase activity (263). At that point in time, the general consensus was that only specialized cells or cancer cells would have detectable telomerase activity. A year later, the same group increased their endometrial samples to 17, included 60 normal endometrial samples, and reported that being a somatic organ, the benign human endometrium, expresses dynamic levels of telomerase activity (measured by TRAP assay), with the highest levels observed in the late proliferative phase endometrium which was comparable to endometrial cancer. They also indicated that endometrial telomerase levels are closely associated with proliferation and likely to be regulated by estrogen (264). During the same year, Saito et al. examined a larger and more diverse endometrial cancer sample set and reported that activation of telomerase was found in most of these cancers, similar to the reports on gastric, prostate, bladder, and skin cancers (252, 283–286). Saito et al. further confirmed the earlier work by Kyo et al. that 28/30 endometrial cancers had high telomerase activity and late proliferative phase to have the highest telomerase activity levels in the benign endometrial samples. Additionally, the authors found that endometrial hyperplasia demonstrated high telomerase activity similar to cancer, whereas no activity was detected in healthy post-menopausal endometria with or without bleeding problems, indicating telomerase activity to be a suitable diagnostic test for identifying post-menopausal endometrial pathology (252). The authors also noted that telomerase activity was increased by estrogen which induced cell proliferation and was reduced in progesterone dominant conditions, indicative of an ovarian steroid hormonal regulation. The finding of high telomerase activity in endometrial cancers has been subsequently confirmed by many other groups (15, 25, 159, 239, 247, 267–269). In addition to the high telomerase activity measured by the gold standard test, the TRAP assay, some authors studied expression levels of components of the telomerase holoenzyme using qPCR to detect gene expression levels. They concluded that hTERT levels correlated well with TRAP assay data (159, 268) and both seem to be related to endometrial epithelial proliferation (15). In a relatively small study, Bonatz et al. (287) have shown a significant correlation between higher telomerase activity and higher International Federation of Gynecology and Obstetrics (FIGO) stage and grade, suggesting that telomerase activity is increased in advanced stages of endometrial cancer (287). In their study, Wang et al showed that 82% of their endometrial cancer samples had telomerase activity but they did not find any correlation between telomere lengths and telomerase activity in different gynaecologic cancers (cervical, ovarian and endometrial) (269).

Detection of hTERT mRNA in peripheral blood (PBMCs) has been reported to be significantly higher in women with EC compared to patients with benign uterine diseases and healthy controls. Using a relatively moderate sample size ($n = 56$ patients with endometrial cancer, $n = 40$ patients with benign uterine diseases and $n = 40$ healthy control) the authors claimed that the exact levels of hTERT mRNA will demarcate those with metastatic disease thus may be useful in stratifying patients for adjunctive therapy (288). This claim needs to be confirmed in a future study which includes an adequate sample size.

Recently, in two progesterone responsive and progesterone-insensitive human endometrial cancer cell lines (162), ATM protein was shown by reverse-phase protein array (RPPA) to participate in progesterone stimulation to suppress carcinogenesis in the endometrium (162). Additionally, a progressive loss of ATM levels from hyperplasia to the lowest levels was observed in type 1 endometrial cancer lesions and there was a negative relationship of the pathological grades and ATM levels (162).

Activating *hTERT* promotor mutations do not usually occur in a background of loss of the tumor suppressor protein ARID1A (289). Recent data suggest that ARID1A negatively regulates *hTERT* transcription and telomerase activity; while induction of ARID1A represses transcription and histones via occupying SIN3A and H3K9me3 sites (290). ARID1A is a member of the SWI/SNF chromatin remodeling complex, and it is frequently mutated in endometrial adenocarcinoma (291), therefore it is conceivable how hTERT might be upregulated in the endometrial cancer with loss of ARID1A.

In endometrial cancer cell lines, telomerase activity and expression of hTERT were both increased by estrogen in an estrogen receptor alpha (ERα) dependent and estrogen responsive element (ERE) dependent effect in the *hTERT* promoter (292). Additionally, a previous study showed that estrogen also induced telomerase activity via post-transcriptional Akt dependent phosphorylation of hTERT in human ovarian cancer cell lines (293).

Lehner et al. (268) compared hTERT mRNA levels and telomerase activity using TRAP assay in normal endometrium with endometrial cancer and they concluded that the levels and activity were significantly higher in cancer and low in normal endometrium during the secretory phase of the menstrual cycle as well as in atrophic endometrium (268). Thus, they suggested that quantitative analysis of these parameters may be useful as markers for diagnosis of endometrial cancer.

PTEN regulates telomerase activity, most likely through its known effects on the PI3-kinase/Akt pathway (294). Reconstitution of PTEN in the PTEN-null Ishikawa endometrial cancer cells resulted in inhibition of cell growth and suppression of Akt phosphorylation as well as a parallel decrease in telomerase activity and hTERT mRNA levels (294). At present, there are no reports of different expression levels of other telomerase associated proteins. Interestingly DC, which is associated with an

TABLE 3 | Published literature on telomerase biology in endometrial cancer: telomerase activity and telomere length.

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TL	Telomere reduction in endometrial adenocarcinoma.	(262)	Normal endometrium and EC samples ($n = 11$) and five endometrial carcinoma cell lines. Note: in this study the normal endometrial samples (control) were taken from areas adjacent to the cancer lesions	The relative number of telomeric repeat sequences in each sample was measured by hybridization of these deoxyribonucleic acids (DNA) to a probe specific for the human telomeric repeat. quantifion of the Hybridization signals were performed by autoradiography and a β-particle detection system	Telomeric repeat sequences were reduced in EC vs. normal endometium (in 10 out of 11 cases) and also reduced in endometrial carcinoma cell lines. the data of this study suggested that Telomeric reduction is a genetic characteristic of many endometrial cancers. Telomere reduction may play an essential role in the genesis and progression of endometrial carcinoma, or it may be a secondary effect of the tumorigenesis process
TA	Telomerase activity in gynecological tumors	(263)	EC cell lines ($n = 5$) EC ($n = 13$)	TRAP assay with dilution assay	5 of 5 EC cell lines displayed strong signals for TA 12 of 13 (92%) ECs positive for TA 4 of 13 ECs classified as high TA No significant correlation between high TA and clinical stage or pathological grade of EC
TA	Telomerase Activity in Human Endometrium	(264)	Normal ($n = 60$) EC ($n = 17$)	TRAP assay Immunohistochemistry	TA regulated during the menstrual cycle Highest TA in late proliferative phase No or faint TA in late secretory phase and during menstruation TA in EC comparable to that in late proliferative phase Low level of TA in post-menopausal samples telomerase enzyme is linked to cellular proliferation and it might be regulated by Estrogen
TA	Proliferation-associated regulation of telomerase activity in human endometrium and its potential implication in early cancer diagnosis	(252)	Normal ($n = 15$) PMB ($n = 6$) Hyperplastic PM ($n = 16$) Non-hyperplastic PM ($n = 9$) EC ($n = 30$)	TRAP assay (TA)	TA detected in 28 of 30 ECs TA demonstrated in all hyperplastic endometrial samples Early-proliferative phase showed no TA Late-proliferative phase showed the strongest TA Late-secretory, early pregnancy and post-menopausal samples showed no TA
TA	Telomerase expression in normal endometrium, endometrial hyperplasia, and endometrial adenocarcinoma.	(253)	Normal endometrium (pre and post-menopausal) ($n = 40$), EH ($n = 17$), EC ($n = 48$)	TRAP assay (TA)	Telomerase activity was detected in 40 of 48 cases of endometrial adenocarcinoma. In this study telomerase activity did not correlated with tumor grade, myometrial invasion, or cancer stage. However, there was a statistical significant association between telomerase activity in benign atrophic endometrium vs. any endometrial abnormality in women 52 years of age or older
TA	Telomerase Activity in Benign Endometrium and Endometrial Carcinoma.	(265)	EC ($n = 20$) Benign endometrium ($n = 14$)	TRAP -eze using PCR Quantitative DNA analysis using Feulgen method	Strong TA detected in 8 of 8 benign, premenopausal endometrial specimens (proliferative $n = 5$; secretory $n = 3$) Weak TA in 6 of 6 post-menopausal samples TA detected in 19 of 20 Ecs No correlation of positive TA with FIGO tumor grade, depth of myometrial invasion, or DNA content in the EC specimens

(Continued)

TABLE 3 | Continued

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TA	Telomerase activity in human gynecological malignancies.	(266)	EC ($n = 6$) Normal endometria ($n = 8$) Ovarian carcinomas ($n = 13$) Benign ovarian tumors ($n = 5$) Cervical carcinomas ($n = 6$) Normal cervices ($n = 5$)	TRAP assay using PCR	TA was detected in 6 of 6 EC TA was detected in 5 of the 8 normal endometrial samples
TA	Telomerase activity in gynecologic tumors.	(258)	EC ($n = 4$) Ovarian cancer ($n = 16$) Cervical cancer ($n = 16$) Benign (total $n = 8$; endometrial $n = 4$) Normal (total $n = 4$; endometrial $n = 1$)	TRAP assay using PCR	TA activity was detected in all ECs TA was not detected in any benign or normal samples
TA	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	(267)	Normal ($n = 52$) EC ($n = 19$)	TRAP assay (stretch PCR) *in situ* RNA hybridization of hTERC Cell culture and MTT assay	TA regulated in menstrual-phase-dependent manner Maximal TA in late proliferative phase Minimal TA in late secretory phase and post-menopausal TA in EC equivalent to that in late proliferative phase TA limited to epithelial glandular cells in proliferative phase Telomerase activation closely associated with cellular proliferative activity Estrogen may play a role in the regulation of TA
TA	Telomerase activity correlates with histo-pathological factors in uterine endometrial carcinoma.	(25)	EC ($n = 35$)	TRAP assay	TA detected in 31 of 35 ECs Of the 31 tumors showing positive TA: 15 tumors had high and 16 had low TA High TA in post-menopausal EC significantly correlated with the presence of pelvic lymph node metastasis and advanced surgical stage
TA	Human telomerase reverse transcriptase as a critical determinant of telomerase activity in normal and malignant endometrial tissues	(159)	Normal ($n = 32$) EC ($n = 23$) EC cell lines ($n = 5$)	TRAP assay	TA detected in 12 of 12 proliferative endometria TA detected in 4 of 13 secretory phase endometria TA detected in 3 of 7 atrophic endometria TA detected in 20 of 23 ECs Approximately 80% of ECs were concordant for positivity or negativity of hTERT expression and TA— suggesting hTERT is a critical factor directing TA in tumors
hTERT, TA	Quantitative analysis of telomerase hTERT mRNA and telomerase activity in endometrioid adenocarcinoma and in normal endometrium	(268)	Normal ($n = 20$) EC ($n = 28$)	RT-PCR of hTERT mRNA TRAP assay	In normal endometrium hTERT mRNA and TA levels were highest in the proliferative phase and relatively low in secretory and atrophic endometrium hTERT mRNA levels and TA levels significantly higher in EC than in normal endometrium
TA/TL	The relationship between telomere length and telomerase activity in gynecologic cancers	(269)	EC ($n = 23$) Ovarian ($n = 15$) Cervical ($n = 14$)	TRAP (EZE) ELISA kit (TA) Southern blot (TL)	TA detected in 18 of 22 ECs There was no detectable relationship between TL and stage of disease, pathologic diagnosis, or TA Rate and strength of telomerase activity increased progressively from clinical Stage I–III

(*Continued*)

TABLE 3 | Continued

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TA	Is the telomerase assay useful for screening of endometrial lesions?	(57)	Normal ($n = 82$) EC ($n = 15$) Hyperplasia ($n = 3$)	TRAP assay (TRAP-eze telomerase detection kit)	TA detected in 10 of 15 proliferative phase endometrial samples TA was detected in 5 of 20 secretory phase samples and 1 of 4 samples taken during menstruation TA was exhibited in 3 of 38 samples from the post-menopausal patients TA was detected in 12 of 15 EC pre-operative samples and 15 of 15 post-operative biopsies Lack of TA does not indicate an absence of endometrial lesions
TL	Differential Roles of Telomere Attrition in Type I and II Endometrial Carcinogenesis	(70)	EC ($n = 29$) Normal ($n = 29$)	Evaluated telomere lengths *in situ* using a novel chromogenic method (Telo-CISH) and Southern blot analysis	Telo-CISH demonstrates telomere shortening is a general feature of type I and II endometrial carcinogenesis Southern blot analysis confirmed significant telomere attrition in type I tumors relative to matched normal DNA
TL	Telomere length and genetic analyses in population-based studies of endometrial cancer risk.	(71)	EC ($n = 279$) Matched controls ($n = 791$)	Relative leukocyte TL measured using qPCR based telomere assay from blood sample	No relationship between leukocyte TL and EC
hTERT	The status of telomerase enzyme activity in benign and malignant gynaecologic pathologies.	(39)	EC ($n = 6$) Benign endometrium ($n = 7$) Ovarian ($n = 35$) Cervical ($n = 6$) Placental site trophoblastic tumor tissue ($n = 1$)	hTERT mRNA quantification using RT-PCR (presence of hTERT, not assessing TA)	6 of 6 ECs found to be hTERT positive Benign endometrial tissue samples: 6 endometrial polyps and 1 irregular proliferative-phase endometrium; hTERT positivity was found only in irregular proliferative phase endometrium
TL	Association of leukocyte telomere length in peripheral blood leukocytes with endometrial cancer risk in Caucasian Americans	(72)	EC ($n = 139$) Controls ($n = 139$)	Relative leukocyte TL measured using qPCR based telomere assay from blood sample	Normalized LTL was significantly longer in EC cases than in controls Individuals with long LTL had significantly increased risk of EC compared to those with short LTL

EC, endometrial cancer; EH, endometrial hyperplasia; TA, telomerase activity; TL, telomere length; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; hTERC, human telomerase RNA component.

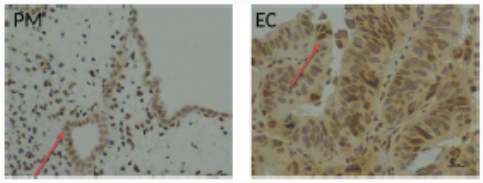

FIGURE 6 | Immunohistochemical staining with an anti-human telomerase antibody in healthy and endometrial tissue samples. Endometrial tissue sections demonstrating hTERT immunostaining in full thickness post-menopausal (PM) section and pipelle biopsy from a patient with endometrial cancer (EC) using a polyclonal rabbit anti-human telomerase antibody (ab27573, Abcam, Cambridge UK), detected with ImmPRESS anti-rabbit polymer and visualization with ImmPACT DAB (Vector Laboratories, Peterborough, UK). Positive nuclear hTERT brownish staining was observed in endometrial normal and cancer glands (red arrow). Magnification ×400, scale bar 10 μm.

increase in the risk of developing some cancer types, has not been reported to be linked with an increased incidence in EC. There are no published studies examining the role of dyskerin in EC to date.

ANTI-TELOMERASE THERAPY

Telomerase was thought to be a suitable target for anti-cancer agents due to the high activity levels seen in most cancers. Available anti-telomerase strategies can be grouped into three main categories: (1) Telomerase inhibitors, (2) telomerase targeted immunotherapy and (3) telomerase directed viral therapy. Imetelstat (GRN163L) is the only clinically applicable specific oligonucleotide telomerase inhibitor (**Figure 4**), which is a water soluble, acid and nuclease resistant compound that forms stable RNA duplexes (295). It prevents the 13-nucleotide region of TERC to form a complex with hTERT. Unfortunately, clinical data for Imetelstat has been disappointing with high toxicity (296). The other anti-telomerase agents are also undergoing clinical trials yet there are no conclusive data yet available for their clinical effectiveness in cancer. For those cancers harboring activating TERT promotor mutations, directed immunotherapies have been proposed as part of a personalized treatment (297). Anti-telomerase therapy and its relevance to cancer was reviewed in detail in several reviews recently (298, 299).

Progestogens remain to be one of the main hormone-based chemotherapeutic agents that are used in early, advanced and recurrent EC with only modest benefit (24). The loss of response to progesterone or progressive disease despite progestogens has been alluded to progesterone-induced down regulation of progesterone receptor (261) and the lack of progesterone receptor expression is a feature of advanced ECs (261). Since telomerase levels are high in most ECs and since telomerase seem to be a downstream target of progesterone in the endometrium, direct telomerase inhibition may have an added benefit in some women with EC. Those with recurrent disease despite progesterone treatment or having PR negative advanced ECs may particularly respond to telomerase inhibition. However, the available limited *in vitro* data may suggest that Imetelstat may reduce telomerase activity but may not cause cell death (**Figure 4**) (15). Since the *in vitro* data has been generated in a mono-cellular 2D culture system comprising of only epithelial cells, thus it may not accurately reflect the *in vivo* response to the medication (158). Further studies using either physiologically more relevant 3D culture systems containing epithelial and stromal cells or animal models are warranted to explore this avenue further before embarking on clinical studies.

CONCLUSION

Telomere and telomerase have an intricate relationship with cancer-related multiple cellular functional pathway aberrations. Collectively, the available evidence suggests that endometrial cancer tissues have relatively short telomeres that are maintained by high telomerase activity. Further studies should shed light into different endometrial cancer subtype-associated changes in telomere length, which might facilitate exploring alternative therapeutic strategies to prevent occurrence and progression or recurrence of this devastating disease. Future studies examining the involvement of various telomere and telomerase associated proteins as prognostic markers that potentially could be used in stratifying patients for adjuvant therapies in endometrial cancer are also warranted. In addition, a comprehensive understanding of the telomere and telomerase biology in endometrial cancer will facilitate assessment of targeting telomerase as a personalized therapeutic strategy in endometrial cancer.

AUTHOR CONTRIBUTIONS

DH conceived the manuscript. RA, MA, LB, and DH prepared the first draft. GS, RA, MA, and DH revised the manuscript critically for important intellectual content and RA, MA, and DH prepared the figures and references. All authors revised and read the manuscript and approved the submitted final version.

FUNDING

The authors would like to acknowledge the support from Wellbeing of Women project grant RG1487 (DH), Higher Committee for Education Development in Iraq (RA), Liverpool Women's Hospital cancer charity (MA).

ACKNOWLEDGMENTS

The authors are grateful for department of Women's and Children's Health of University of Liverpool in supporting this work (RA, MA, and DH).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.