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Probing the impact of the maternal environment on foetal development: Can high-resolution telomere analysis predict prenatal adversity?

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Acknowledgments

The first time I was in Cardiff was for a MRC scholarship. It was raining all the time and the result of the interview was not successful, reasons that made me think of Cardiff as a grey and ugly city. Six months later I came back thanks to Ros and Duncan. I can say now that Cardiff has become home for me. It is a long time since I complained about the weather, instead I always carry a mini umbrella and try to get the most from the sunny days. I am in love with this city. I would like to thank my supervisors Ros and Duncan for given me the opportunity to undertake this PhD. I have enjoyed a lot our meetings in BIOSI, where we talked about science among other things. You have been to me great mentors and most importantly awesome human beings. Throughout the last 4 years I have grown as a scientist, feeling your support and trust in every step of the process. I am very grateful for that.

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Abstract

Telomeres are nucleoprotein structures located at the end of eukaryotic chromosomes. They maintain genomic stability avoiding fusion and degradation events. Telomeres shorten as cells divide and the length of a telomere is related to its function. Studies in animal models and humans suggest that telomere shortening contributes to the development of cancer, several age-related diseases and premature ageing syndromes. In the context of human pregnancy, it has been hypothesised that *in utero* exposure to different forms of physiological stress can impact the telomere biology of the foetus accelerating dysfunction, ageing and disease risk. Given the potential importance of critically shortened telomeres, detecting and using them as a biomarker of pregnancy complications linked to placental dysfunction is of significant interest.

The primary aim of this thesis was to utilise single telomere length analysis (STELA), a high-resolution single-molecule technique, to examine telomere length distributions at individual chromosome ends in the human placenta. Telomere length profiles were obtained from placental samples from two different human cohort studies, including healthy pregnancies and pregnancies complicated by prenatal depression and gestational diabetes. Placental telomeres were also analysed in relation to maternal lifestyle factors.

STELA revealed no effect of sampling site, mode of delivery or foetal sex on telomere length when placenta from healthy pregnancies were examined. However, the first novel finding was that human placenta exhibit substantial telomere length heterogeneity, that may be related to the number of cell divisions taken to generate the term tissue. GDM did not impact the telomere length distributions in placenta from female infants, but placental telomeres from male infants exposed to GDM in medically untreated pregnancies were significantly shorter than placental telomeres from control male infants ($P = 0.02$). This was not observed in GDM pregnancies treated with metformin and/or insulin ($P = 0.003$). In contrast to GDM, prenatal depression symptoms were associated with telomere shortening in female placenta but not male placenta ($P = 0.026$).

In conclusion, this work suggests that prenatal adversities can have a sexually dimorphic impact on placental telomere length and distribution detectable using STELA. Telomere length therefore provides a useful biomarker of prenatal adversities.

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List of abbreviations

AA	Aplastic anaemia
ACTH	Adrenocorticotropic hormone
AF	Amniotic fluid
AFI	Amniotic fluid index
ALT	Alternative lengthening of telomeres
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ATM	Ataxia telangiectasia-mutated
ATR	Ataxia telangiectasia and Rad3 related
BLM	Bloom syndrome
BMI	Body mass index
BSA	Bovine serum albumin
CLL	Chronic lymphocytic leukaemia
CRH	Cortisol-released hormone
CRP	C-reactive protein
CTE	C-terminal extension domain
CVD	Cardiovascular diseases
DC	Dyskeratosis congenita
DKC1	Dyskeratin Pseudouridine Synthase 1
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
EMA	Ecological momentary assessment of emotional experiences
EPDS	Edinburg Postnatal Depression Scale
FANC	Fanconi anaemia
FFQ	Food frequency questionnaire
FISH	Fluorescence *in situ* hybridisation
GDM	Gestational diabetes mellitus
GLUTS	Glucose transporters
GTT	Glucose tolerance test
HDL	High-density lipoprotein
HDR	Homology-directed repair
HGPS	Hutchinson-Gilford progeria syndrome
HHS	Hoyerall-Hreidarsoon syndrome
HPA	Hypothalamic pituitary adrenal
ICM	Inner cell mass
IL-6	Interleukin 6
IPF	Idiopathic pulmonary fibrosis
IUGR	Intrauterine growth restriction
IVF	*In vitro* fertilisation
LGA	Large for gestational age
LSD	Least significant difference
MDD	Major depressive syndrome
NET	Noradrenaline transporter
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining

OB	Oligonucleotide/oligosaccharide
OGTT	Oral glucose tolerance test
P53	Tumor protein p53
PBMC	Peripheral blood mononuclear cells
PE	Preeclampsia
PNA	Fluorescent peptide nucleic acid
POT1	Protection of Telomere 1
q-FISH	Quantitative fluorescence *in situ* hybridisation
q-PCR	Quantitative polymerase chain reaction
RAP	Repeat addition processivity
RAP1	Repressor / Activator Protein 1
ROS	Reactive oxygen species
RPA	replication protein A
RT	Reverse transcriptase domain
RT-PCR	Real time-PCR
SASP	Senescence-associated secretory phenotype
SERT	Serotonin transporter
SB	Southern blot
SMSFAs	Small to medium chain saturated fatty acids
SSRI	Serotonin reuptake inhibitors
STAI	State-trait anxiety inventory
STELA	Single telomere length analysis
T-SCES	Telomere sister chromatid exchanges
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TCAB1	Telomerase cajal body protein 1
TEN	Telomerase essential N-terminal domain
TERC	Telomerase RNA component
TERRA	Telomeric repeat-containing RNAs
TERT	Telomerase reverse transcriptase
TIN2	TRF1- and TRF2-Interacting Nuclear Protein 2
TNF-α	Tumor necrosis factor alpha
TPE	Telomere position effect
TPP1	TINT1, PTOP or PIP1
TRAP	Telomeric repeat amplification protocol
TRBD	Telomerase RNA binding domain
TRF	Terminal restriction fragment
TRF1	Telomere Repeat binding Factor 1
TRF2	Telomere Repeat binding Factor 2
WIMD	Welsh Index of Multiple Deprivation
WRN	Werner syndrome
WT1	Wilm's tumor suppressor protein

Publications

- Garcia-Martin, I., Janssen, A. B., Jones, R. E., Grimstead, J. W., Penketh, R.J.A., Baird, D.M., & John, R.M. 2017. **Telomere length heterogeneity in placenta revealed with high-resolution telomere length analysis**. *Placenta*, *59*, 61–68.

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Chapter 1: Introduction

1.1.Telomeres

1.1.1. History of telomeres

The concept of telomeres was first described in 1938 by Herman Muller, whose studies using X-rays to induce chromosomal rearrangements in *Drosophila melanogaster* showed that the ends of the chromosomes were not affected. From these studies, he speculated about the protective nature of chromosomal ends and named them telomeres (from the Greek, *telo* = end, and *mere* = part) (Muller 1938). Also in 1938, Barbara McClintock observed that the ends of X-rays induced broken chromosomes in maize are likely to fuse with other broken chromosomes forming dicentric chromosomes. These structures where then subjected to cycles of anaphase bridging, breakage and fusion. This will continue after every cell division until the chromosome acquires a new functional telomere, most commonly by translocation of the end of another chromosome (McClintock 1938). McClintock inferred one of the essential functions of telomeres: the protection of chromosomes ends from fusing between each other and with induced double strand breaks.

In the 1970s Alexey Olovnikov and James Watson separately pointed out that complete synthesis of the ends of linear DNA present in eukaryotic chromosomes cannot be fully achieved (Olovnikov 1971, Watson 1972). Since DNA replication is bidirectional and DNA polymerase can only add nucleotides to a pre-existing primer, the gap at the very end cannot be filled and the new strand of DNA will be shorter than the template. This is the so called “DNA end-replication problem” and was used by Olovnikov to propose that every time a cell divides chromosomes becomes shorter until a critical telomere length is reached, thereby stopping cell replication and triggering cellular senescence (Olovnikov 1973).

In 1978, Elizabeth Blackburn working with *Tetrahymena* discovered that telomeres were composed of the hexameric repeat sequence TTGGGG (Blackburn and Gall 1978). Soon after that, Blackburn and Jack Szostak

conducted an experiment where *Tetrahymena* telomere repeat sequences were added to linear yeast plasmids, providing chromosomal stability to the ends and therefore allowing replication. These tandem repeat sequences seemed to be conserved throughout evolution and in eukaryotes a common underlying mechanism for the maintenance of telomeres was proposed (Szostak and Blackburn 1982).

1.1.2. Telomere structure

Telomeres are nucleoprotein structures located at the ends of Eukaryotic chromosomes. They maintain genomic stability avoiding fusion and degradation events. Tandem repeats of a TG-rich sequence on one strand and CA-rich on the complementary strand are found in Eukaryotic telomeres. In humans and other vertebrates, the repeating sequence is the hexameric DNA sequence TTAGGG (Moyzis *et al.* 1988). Telomere length in human adult cells ranges from 5 to 15 kb (Samassekou *et al.* 2010). Adjacent to the terminal $(TTAGGG)_n$ array are 100-300 kb of telomere associated repeats (TAR) (Brown *et al.* 1990).

Telomeric repeats have been highly conserved during evolution, probably as a result of the unique interaction between telomeric DNA sequence and telomere-specific binding proteins (Watson and Riha 2010). Different species show little variation in the sequence repeat, for example, TTGGGG in *Paramecium*, TAGGG in *Trypanosoma*, TTTAGGG in *Arabidopsis*, and TTAGGG in *Homo sapiens* (Strachan and Read 2004).

The terminus of the telomere is composed of a single-stranded G-rich strand, which is longer than the C-rich strand, forming a 3' G-overhang which is maintained throughout the replicative lifespan of the cell (Chai *et al.* 2005). In mammals the 3' overhang length is longer compared with most eukaryotes and ranges from 50-500nt (Palm and de Lange 2008), and in humans consists of 50-200 nucleotides of TTAGGG repeats (Makarov *et al.* 1997). A tertiary configuration called the T-loop has been suggested as the mechanism by which telomeres protect those fragile ends. T-loops are created when the G-overhang folds back and invades the duplex telomeric DNA, thereby hiding the termini of the chromosome and avoiding be considered as a double-stranded DNA break.

These T-loop structures has been found in protozoa, yeast, plants and human (Griffith *et al.*1999).

The "DNA end-replication problem" (**Figure 1.1**) is solved by the reverse transcriptase enzyme, telomerase. This enzyme was originally found in *tetrahymena* and it is capable of adding tandem TTGGGG onto the 3'ends for completing the replication of chromosomes ends (Greider and Blackburn, 1985).

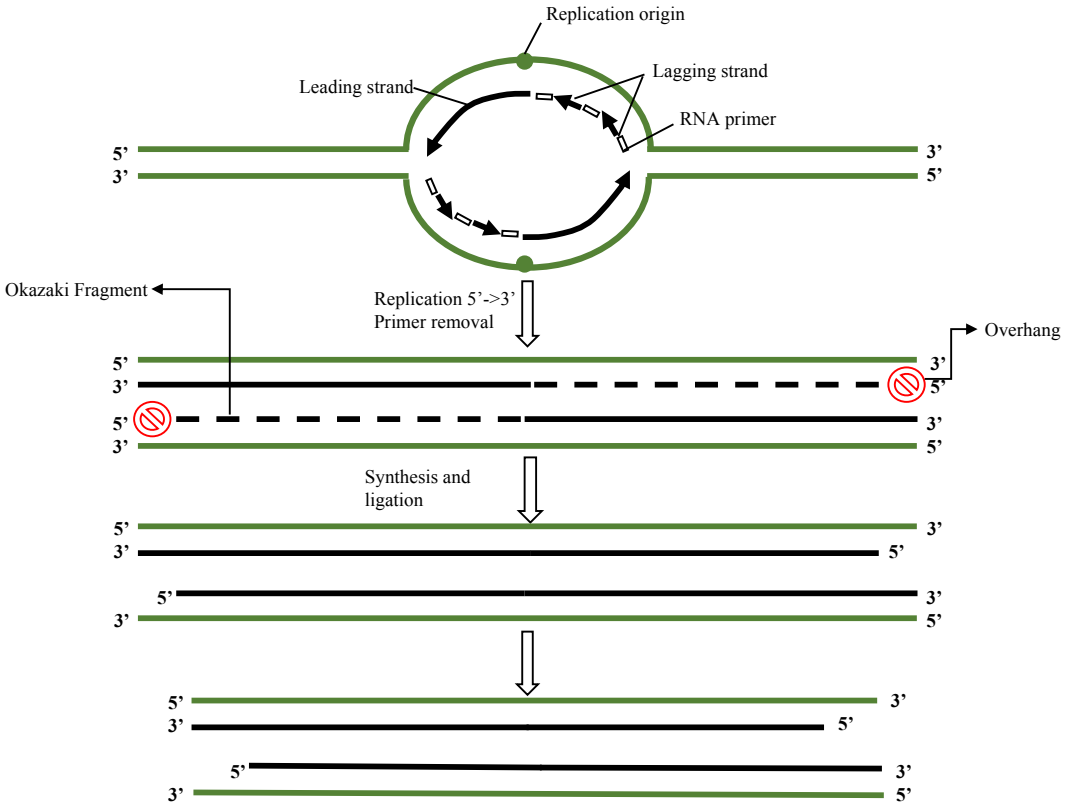


Figure 1.1. The End replication problem. DNA polymerase I synthesises DNA in the 5' to 3' direction resulting in a leading and a lagging strand. The lagging strand should be synthesised in a discontinuous manner because RNA primers are required to start the replication. Removal of RNA primers results in small fragments of DNA called Okazaki which are separated by gaps. Gaps are filled with nucleotides and ligated but the removal of the terminal primer leaves a gap that cannot be filled, a G-rich overhang.

1.1.3. Shelterin complex

The maintenance and function of telomeres depends on three factors: telomeric DNA sequence, the shelterin complex and the telomerase enzyme. Telomeric tandem repeats associate with a complex of six proteins (Telomere Repeat binding Factor 1 (TRF1), Telomere Repeat binding Factor 2 (TRF2), Protection of Telomere 1 (POT1), TRF1- and TRF2-Interacting Nuclear Protein 2 (TIN2), Repressor / Activator Protein 1 (RAP1) AND TPP1 (TINT1, PTOP or PIP1) called shelterin (**Figure 1.2**). Shelterin represses recognition and repair by the DNA damage response apparatus thereby allowing the cell to distinguish between natural chromosome ends and DNA double-strand breaks. The shelterin complex is required for the regulation of telomerase mediated telomere maintenance (Palm and de Lange 2008).

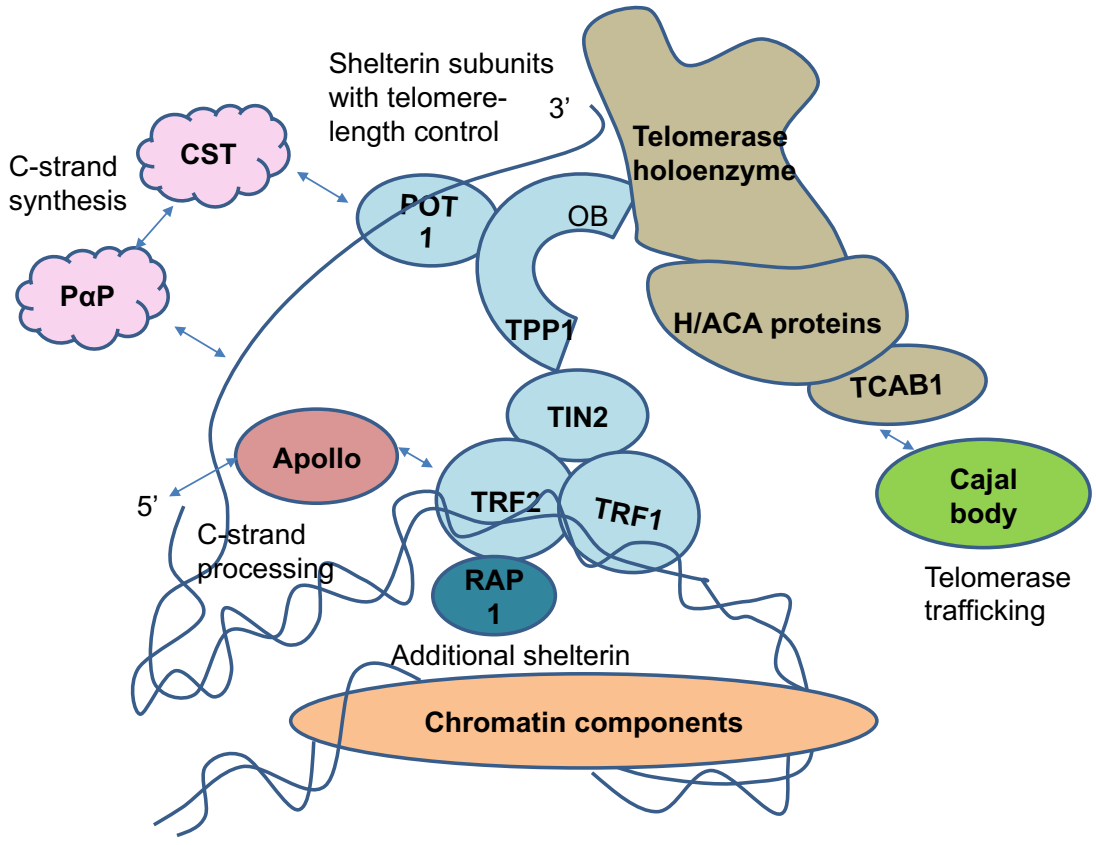


Figure 1.2. The human telomeric system. Interactions between single and double-stranded telomeric DNA, the shelterin complex and the telomerase enzyme. Adapted from (Hockemeyer and Collins 2015).

1.1.3.1. TRF1 and TRF2

TRF1 and TRF2 are orthologs of the fission yeast taz1 protein. They are extremely abundant and it is believed that telomere are coated with thousands of dimers (Palm and de Lange 2008). TRF1 and TRF2 share a common domain structure consisting of a flexible hinge domain which connects the TRF homology domain and a c-terminal SANT/Myb DNA-binding domain (Bianchi *et al.* 1997). However, these two proteins differ at their N-termini, while the N-terminus of TRF2 is basic (contains a Gly/Arg-rich domain), the N-terminus of TRF1 has acidic amino acids. The SANT/Myb domains of both proteins confer specificity for the half-site 5'YTAGGGTTR3' in double-stranded DNA (Bianchi *et al.* 1999).

These proteins bind DNA as homodimers or oligomers formed through interactions within the TRFH domain, although TRF1 and TRF2 do not interact directly (Broccoli *et al*. 1997). TRH domain plays an essential role also providing a site through which other proteins are recruited to telomeres (Chen *et al*. 2008). TRF1 and TRF2 have the ability to alter the higher order structure of a telomere: TRF1 can loop and pair stretches of telomeric DNA whereas TRF2 has been reported to form t-loop structures on a model telomere substrate (Stansel *et al.* 2001).

TRF1 negatively regulates telomere length: when overexpressed leads to telomere attrition, whereas a dominant-negative form of TRF1 that leads to the loss of this protein from telomeres have been reported to cause telomere elongation (Van Steensel and de Lange 1997). Moreover, TRF1 deletion in mouse is lethal (Iwano *et al.* 2004). It has a specific function during the S phase, where it has the ability of facilitate the replication of telomeres, preventing ATR (Ataxia Telangiectasia and Rad3 related) activation and the formation of fragile telomeres in metaphase (Sfeir *et al.* 2009).

Overexpression of TRF2 leads to telomere attrition suggesting a role in telomere length regulation (Smogorzewska *et al.* 2000). TRF2 is important for telomere capping and chromosome ends protection throughout the cell cycle (Sfeir and de Lange 2012). Deletion of TRF2 results in loss of the 3' overhang, since TRF2 is not promoting t-loop formation, it also leads to covalent fusion of telomeres and induction of ATM (Ataxia telangiectasia mutated) and Tumor Protein 53 (p53)

dependent-apotosis (van Steensel *et al.* 1998; Karlseder *et al.* 1999). TRF2 prevents Non-Homologous End-Joining (NHEJ) at telomeres by preventing ERCC1/XPF- mediated removal of the 3'overhang (Zhu *et al.* 2003).

1.1.3.2. RAP1

Rap1 is the most highly conserved shelterin component (Rai *et al.* 2016). It is the TRF2-interacting protein in the shelterin complex (Li *et al.* 2000) but mammalian Rap1 lacks the ability to bind directly to DNA. It forms a 1:1 heterodimer complex with TRF2 and its location and stability depends on the latter (Celli and de Lange 2005). RAP1 also relies on TRF2 protein for stable expression (Takai *et al.* 2010).

In humans, Rap1 seems to alter affinity and binding preference of TRF2 on DNA, these proteins together exert a greater effect remodelling telomeric DNA together than either constituent alone (Arat and Griffith 2012). Rap1 has been reported as a negative regulator of telomere length (Li and de Lange 2003). It also displays a telomere end-protective function, which is essential to maintain genomic stability.

It supresses DNA repair pathways: in humans Rap1 has been correlated with NHEJ inhibition at telomeres. However, mice studies reported that in presence of TRF2, but not RAP1, mouse telomeres are still protected from end-to-end chromosome fusions, suggesting that RAP1 is dispensable for protection from NHEJ-mediated repair of telomeres and instead appears to be involved in the suppression of homologous recombination (Chen *et al.* 2011). A role in transcription has also been suggested for Rap1 (Martinez *et al.* 2013).

1.1.3.3. TIN2

Located in the central position within the shelterin complex, TIN2 is able to bind to TRF1, TRF2 and TPP1, providing a bridge between the shelterin components that bind to double and single strand telomeric DNA (de Lange 2005). While TRF1-TIN2 interaction is mediated by the TRFH domain of TRF1 and the FxLxP motif in the C-terminus region of TIN2, a short section in the hinge region of TRF2 interacts with a region located in the N-terminus of TIN2. These interactions may occur, simultaneously positioning TIN2 as the link between TRF1 and TRF2 (Chen *et al.* 2008). Overexpression of wild type TIN2 has been reported to shorten telomeres a little. Expression of mutant TIN2 that lacks a N-terminal

domain still can bind to TRF1 and causes elongation, which suggests the role of TIN2 as a negative regulator of telomere length. In addition, the loss of TIN2 triggers a DNA damage response at the telomere (Kim *et al.* 2004).

1.1.3.4. POT1

POT1 was first identified due to its sequence similarity to the alpha subunit of the TEBP alpha/beta telomeric binding complex in *Oxytricha nova* (Baumann and Cech 2001). POT1 contains a conserved pair of oligonucleotide/oligosaccharide (OB) folds in its N-terminus which makes possible its binding to the single-stranded G-strand telomeric sequence (Baumann *et al.* 2002). Through the C-terminal region, POT1 binds TPP1, establishing in this way connection with TIN2. It has been reported that TPP1 is essential for the association of POT1 with telomeres (Hockemeyer *et al.* 2007). A reported function of human POT1 is to bind the displacement G-strand localised in the D-loop thus guaranteeing the stability of the T-loop (Loayza *et al.* 2004). It has also been revealed that a mutant form of POT1 which lacks the DNA binding domain leads to a lack of telomere length control mediated by TRF1 and induces telomere elongation via telomerase (Loayza and de Lange 2003).

Studies in animals, plants and humans support an essential role for POT1 in maintaining telomere integrity (Baumann and Cech 2001). Repression of ATR signalling cascade by POT1 is likely to be the result of POT1 ability to bind single stranded DNA and to inhibit ATR activation by blocking access of the single-stranded binding replication protein A (RPA), mechanism by which ATR is recruited to the telomere (Denchi and de Lange 2007). TPP1-POT1 heterodimer has been postulated to modulate telomerase access to the telomeres (Xin *et al.* 2008).

POT1 is also suggested to prevent the telomere from activating a catastrophic DNA damage response as reported in animal studies where the cells were knockout (Hockemeyer *et al.* 2006).

1.1.3.5. TPP1

TPP1 is the heterodimeric partner of POT1 (Wang *et al.* 2007). TPP1 connects POT1 with TIN2 through its centrally located POT1 interaction domain and its C-terminal TIN2 interaction domain (Liu *et al.* 2004). At the N-terminus of TPP1 there is an OB-fold domain that interacts with telomerase, suggesting a role of TPP1 in recruitment of telomerase (Abreu *et al.* 2010). POT1 recruitment to telomeres mainly occurs through the TPP1/TIN2 connection of POT1 to the dsDNA binding proteins TRF1 and TRF2 (Hockemeyer *et al.* 2007).

Impaired TPP1 expression results in loss of telomeric POT1 and subsequent telomere deprotection (Liu *et al.* 2004). TPP1 is critical for the association of POT1 with telomeric DNA and is also responsible of POT1 subcellular localisation (Chen *et al.* 2007).

1.1.4. Telomere function

1.1.4.1. Chromosome capping and DNA damage response

The end of linear chromosomes is very similar to DNA double-stranded breaks. One of the main functions of the telomeres is to protect chromosome ends from those signalling pathways activated by double-strand breaks that arrest cell division cycle or induce DNA repair. This end-protection function is achieved by several mechanisms including telomere binding proteins (shelterin complex) that bind to single and double-stranded telomeric DNA and forms a superstructure, the T-loop. Cell cycle arrest responses occur via the ATM and ATR kinase pathways. While the ATM kinase pathways is directly activated by DNA ends, the ATR kinase pathway is activated in presence of single-stranded DNA resulting from the resection of the 5'end of a double-strand break. Likewise, DNA repair responses occur via homology-directed repair (HDR) or NHEJ (de Lange 2009). When telomeres fail to adequately protect chromosomal termini, the latter can be recognised as double-strand breaks and all the above DNA damage responses can lead to telomere degradation or chromosome fusions (de Lange 2002).

1.1.4.2. TPE

Apart from chromosomal end protection, telomeres have the ability of inhibit expression of nearby genes through a processed called telomere position effect (TPE) (Baur *et al.* 2004; Robin *et al.* 2014). However, this effect is very limited in range supported by substantial decrease in TPE 100 kb from the telomere (Kulkarni *et al.* 2010). Telomere position effect was first described in human cells by Baur *et al.* (2001).

They not only demonstrate the presence of TPE in mammalian cells, but also that the strength of silencing is proportional to telomere length. It is thought that changes arisen just before the cell enters senescence could have been programmed by continuous telomere attrition division after division, altering gene expression patterns that could affect cell and organ function.

1.1.4.3. Telomeric repeat-containing RNA (TERRA)

Telomeric DNA is transcribed by RNA polymerase II into long non-coding telomeric repeat-containing RNA, TERRA (Azzalin *et al.* 2007). The size of this transcript varies between 100 bases to 100 kb and contains UUAGGG repeats, in addition to sequences unique to the subtelomeric region of each chromosome (Chu *et al.* 2017). Several studies have reported the role of TERRA as a regulator of telomerase and telomere length (Sandell *et al.* 1994; Redon *et al.* 2010; Pfeiffer and Lingner 2012). It has also been suggested its role regulating the recombination between telomere ends (Balk *et al.* 2013). An additional function of TERRA is its function acting as scaffold for the recruitment of chromatin factors (Deng *et al.* 2009).

Depletion of TERRA results in dysregulation of subtelomeric genes and internal genes apart from loss of telomeric repeats, insertion, duplication and fusion events. It displays an important role maintaining chromosome integrity (Chu *et al.* 2017).

1.1.5. Telomere length maintenance pathways

Telomeres are essential to protect genome integrity. In the absence of telomere maintenance mechanisms, every time a cell divides losses telomeric repeats, ultimately entering senescence (Cerone *et al.* 2001). The failure to efficiently preserve telomeres can promote certain diseases, such as dyskeratosis congenita, idiopathic pulmonary fibrosis and cancer (Stewart *et al.* 2012).

1.1.5.1. Telomerase

Telomerase is a ribonucleoprotein with reverse transcriptase activity, which functions as the major regulator of telomere length. Since its discovery in *Tetrahymena thermophila* (Greider and Blackburn 1985), its existence has been later confirmed in many other organisms including humans.

1.1.5.1.1. Structure

In humans, telomerase is a dimeric structure that consist of a catalytic subunit with reverse transcriptase activity (TERT) and a telomerase RNA component (TERC), which provides the template from which TERT adds the telomeric DNA repeats to the ends of chromosomes in a process called repeat addition processivity (RAP) (Blackburn 2000).

TERT is the most conserved subunit of telomerase and contains four domains: telomerase essential N-terminal (TEN) domain with the ability to bind single stranded telomeric DNA and also to interact with TPP1 recruiting telomerase to telomeres, telomerase RNA binding domain (TRBD) responsible for the interaction with TERC, reverse transcriptase domain (RT) which is the catalytic domain of the enzyme and C-terminal (CTE) extension domain (Sandin and Rhodes 2014).

TERC is the most versatile component of telomerase and contains three domains: the pseudoknot/template core domain which is essential for telomerase activity and contains the template for telomere addition, the CR4/CR5 domain and a box H/ACA domain which serve as binding site for those proteins that are involved in RNA processing, stability and subcellular localisation (Wyatt *et al.* 2010).

There are various telomerase accessory proteins, which are species-specific, and are associated with the complex. They regulate telomerase biogenesis, its subcellular localisation and function (Wyatt *et al.* 2010). Using mass spectrometric analysis in HeLa cells, the proteins dyskerin, NHP2, NOP10, pontin/reptin and TCAB1 (telomerase cajal body protein 1) were identified as integral protein components of human telomerase (Fu and Collins 2007; Cohen *et al.* 2007; Venteicher *et al.* 2008; Venteicher *et al.* 2009). Dyskerin, NHP2 and NOP10 are required for the stability and accumulation of TERC (Fu and Collins 2007). Pontin and reptin are two related members of the ATPases superfamily with diverse cellular activities, which are necessary for the stability of dyskerin and TERC (Venteicher *et al.* 2008).

A model has been proposed where dyskerin, pontin and reptin form a scaffold with the ability of recruiting and stabilising TERC, and also assembling the telomerase ribonucleoprotein particle. Once the complex is shaped, pontin and reptin dissociate and produce the activation of the catalytic enzyme (Venteicher *et al.* 2008). Furthermore, TCAB1 regulates telomerase affecting its subcellular localisation (Venteicher *et al.* 2009).

Defects in telomere maintenance as caused by mutations in telomerase or telomere protein components, are present in germ cell and affects multiple organs. As a result, the disorders generated can be classified as childhood or adulthood-onset. In the first group are included disorders such as dyskeratosis congenita (DC) (mainly mutations in TERT, TERC, DKC1 (Dyskeratin Pseudouridine Synthase 1) or TIN2 gene) with mucocutaneous manifestations; Hoyerall-Hreidarsoon syndrome (HHS) (mainly mutations in DKC1) with developmental delay, immunodeficiency and cerebellar hypoplasia phenotype; and Revesz syndrome (mutations in TIN2) characterised by bilateral exudative retinopathy. Some of the manifestations are overlapping between the different disorders and it is common the presence of immunodeficiencies and bone marrow failure in the most severe cases of telomere shortening. Idiopathic pulmonary fibrosis (IPF) (mutations in TERT and TERC) constitutes the most common manifestation of telomere-mediated disorders with onset in adulthood. In addition, mutations in TERT and TERC genes have been described in patients with aplastic anemia (AA), a disease manifestation exhibited in humans with

telomere syndromes, where the bone marrow is affected (Armanios and Blackburn 2012).

1.1.5.1.2. Mechanism of action

Telomerase is responsible of *de novo* telomeric DNA addition onto the telomere end. The enzyme is capable of synthesizing approximately 60 nucleotides with one or two binding and extension events that are added to a human telomere (Zhao *et al.* 2011). TERT performs reverse transcription adding dNTPs onto the 3' overhang using the complementary RNA molecule contained in the TERC subunit as a template. When the 5'-template boundary is reached, the newly synthesized DNA in the 3' is translocated within the template. Another round of nucleotide addition is then initiated. Finally, a primer is synthesized at the complementary strand and usual DNA replication produce double-stranded DNA completing the elongation of the telomeres (Blackburn and Collins 2011) (**Figure 1.3**).

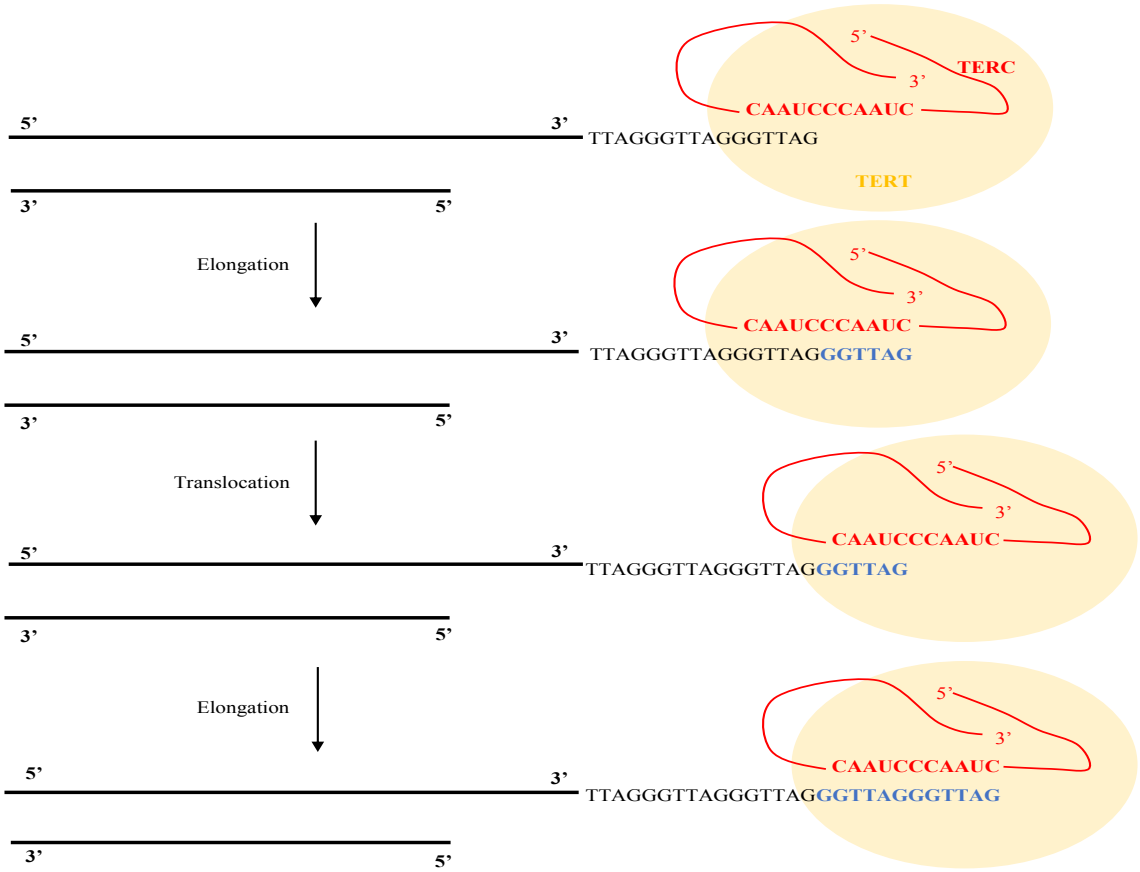


Figure 1.3. Telomere maintenance by telomerase. The catalytic subunit of the telomerase, TERT, synthesises a new telomeric sequence adding nucleotides onto the 3'overhang using the TERC component as a template. After DNA synthesis and when the 5'-template boundary is reached, the newly synthesis DNA is translocated within the template. This exposes additional template sequences and enables further rounds of telomeric elongation. Adapted from (Greider and Blackburn 1989).

1.1.5.1.3. Regulation

Telomerase activity seems to be highly regulated. Its activity is restricted to cells with extended proliferative capacity such as germline, embryonic tissues and self-renewing stem cell populations of the hematopoietic system and skin. For the rest of tissues, telomerase is inactivated in the womb (Wright *et al.* 1996). Conversely, it has been shown that approximately 90% of tumours have reactivated telomerase (Prescott *et al.* 2012).

Telomerase expression and activity is regulated at multiple levels. Enzyme activity is mainly determined by transcriptional regulation of the TERT subunit. However, new studies suggest that the catalytic subunit TERT, also undergoes post-translational control. Furthermore, the TERC subunit has been reported to suffer post-transcriptional regulation. In some cases changes in genes dosage and alternative isoforms of TERT and TERC subunits change telomerase activity. Further ways of control include telomere-associated proteins and telomeric RNA transcripts (Cifuentes-Rojas and Shippen 2012).

Transcriptional regulation of TERT is influenced by the general transcription factor Sp1. Mutations in this factor result in repression of hTERT promoter activity (Kyo *et al.* 2008). Telomerase expression can also be controlled by oncogenes and tumour suppressors. For instance, c-Myc binds to the hTERT promoter. Overexpression of Myc has been reported in cancer cells and results in greater telomerase activity (Greenberg 2005). A negative regulator of TERT expression is the Wilm's tumor suppressor protein (WT1) (Oh *et al.* 1999).

TERT subunit can also be regulated in a post-translational manner. MKRN1 ubiquitin ligase E3 interacts with TERT and its overexpression triggers TERT degradation with the subsequent decrease in telomerase activity and telomere shortening (Kim 2005).

Likewise, TERC subunit suffers transcriptional and post-transcriptional regulation. While Sp1 and HIF1 induce transcription of TERC subunit, Sp3 is involved in its repression (Cairney and Keith 2008).

Lastly, shelterin components have been implicated in telomerase regulation. TPP1 interacts directly with the TEN domain within TERT and forms a subcomplex with POT1, another shelterin component, reproducing the contact of POT1 with the single-stranded 3'overhang at the chromosome termini. TPP1 is involved in telomerase recruitment (Xin *et al.* 2007).

1.1.5.2. Alternative Lengthening of Telomeres (ALT)

ALT constitutes a telomerase-independent mechanism by which a cell can elongate its telomeres. This mechanism was first described in immortalised human cell lines, which exhibited very long and heterogeneous telomeres, whose length was maintained for many population doublings in the absence of telomerase (Bryan *et al.* 1995). In about 10% of cancers, in particular those of mesenchymal origin, use ALT pathway to prevent telomere attrition (Cesare and Reddel 2010). Telomere elongation in ALT cells involves homologous recombination and two models have been proposed to explain it. The first model suggests that unequal telomere sister chromatid exchanges (T-SCEs) generate one daughter cell that has a lengthened telomere and subsequent prolonged proliferative capacity, and another daughter cell with a shortened telomere and diminished proliferative capacity. This mechanism for segregation of telomeres can provide cells with the capacity of extensive proliferation. The second model suggests that lengthening of telomeres could be the result of recombination-mediated synthesis of new telomeric DNA using an existing telomeric sequence from an adjacent chromosomal telomere as a copy template (Cesare and Reddel 2010).

1.2. Role of telomere biology in ageing and disease susceptibility

Ageing is characterised by a progressive loss of physiological integrity that leads to damaged function and increased susceptibility to death (Lopez-Otin *et al*. 2013).

1.2.1. Telomere attrition is a hallmark of ageing

Telomere attrition is a hallmark of ageing, along with genomic instability, epigenetic alteration, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. They all are present during normal process of ageing, their experimental aggravation can accelerate ageing processes and their experimental improvement delay ageing and subsequently increase lifespan (Lopez-Otin *et al.* 2013).

Telomeres, which are guanine rich structures, are considered to be especially sensitive to age-related processes such as oxidative damage that can occur as a consequence of increased reactive oxygen species (ROS) levels (von Zglinicki 2000).

1.2.2. Telomere attrition as an adaptive strategy

Despite its potentially detrimental effects on later-life performance, telomere attrition could constitute an adaptive strategy. Telomere maintenance involves costs from resource expense and from interactions between biological and physical mechanisms. Telomere preservation in those cells in which telomere exhaustion represents a substantial fitness cost occurs at the cost of tolerating telomere attrition in somatic cells, even if that has an effect on later-life performance (Young 2018).

Telomere attrition constitutes a tumour suppressive strategy in long-lived species, such as humans. Compared to short-lived species, long-lived species, who reproduce after several years require more effective DNA repair and improved protection against a possible tumour growth. In this regard, loss of telomeric repeats after replication or damage limits the proliferative capacity of abnormal cells (Landsdorp 2009). The flip side of this is potentially ageing.

1.2.3. Telomeres and disease susceptibility (age-related diseases)

Loss of telomeric repeats in human cells with age differ greatly between cells and tissues (Aubert and Lansdorp 2008). Loss of cell viability secondary to telomere attrition may contribute to the onset of degenerative diseases that occur during natural human ageing. In addition, an accelerate rate of telomere shortening has been reported in several human premature ageing syndromes, in which ageing-related pathologies present an early onset (Blasco 2005).

Patients who suffer a short-telomere syndrome, such as DC, HHS, Revesz syndrome, AA, Ataxia telangiectasia (ATM), Hutchinson-Gilford progeria syndrome (HGPS), Werner syndrome (WRN), Bloom syndrome (BLM), Fanconi anaemia (FANC), Nijmegen breakage syndrome, and ataxia telangiectasia-like disorder (Blasco 2005; Aubert and Lansdorp 2008), have an increased risk of suffering cancer. Defects in telomere function lead to genomic instability and contribute to cancer development. In some tissues where the turnover is constant, stem cell failure triggers errors in replication that provides the stem cell a clonal advantage, as occurs in stem cell bone marrow. Additionally, telomere dysfunction drives senescence in the immune system causing impaired cancer surveillance (Stanley and Armanios 2015).

In epidemiological studies, individuals with shorter telomeres have higher mortality rates. An increased risk for later age-related diseases (**Figure 1.4**) such as cardiovascular diseases (CVD), type 2 diabetes mellitus (T2DM), neurodegenerative diseases and osteoporosis is correlated with a reduction in leukocyte telomere length (Herrmann *et al.* 2018). Animal studies where the mice were genetically modified, telomere manipulation lead to replicative ageing in the osteoblast precursors cells and as a result, bone loss was promoted and caused osteoporosis (Saeed *et al.* 2011). However, this relationship has not been shown in humans (Sanders *et al.* 2009).

Inflammation together with oxidative stress are the principal pathways underlying CVD, which hasten the rate of telomere attrition and lead to cellular senescence as occurs with senescent endothelial cells in atherosclerosis (Yeh and Wang 2016). Individuals with shorter telomeres are at higher risk of being diagnosed with T2DM (Testa *et al.* 2011). Alzheimer's but no other neurodegenerative

diseases has been correlated with reduction in telomere length as assessed in blood leukocyte (Thomas *et al.* 2008).

Regarding reproduction, loss of telomere function in oocytes has been reported to contribute to meiotic dysfunction in ageing women (Keefe *et al.* 2006). Biron-Shental *et al.* (2016) found that telomeres in placental trophoblasts of pregnancies complicated with intrauterine growth restriction, secondary to placental insufficiency, were shorter than those found in uncomplicated pregnancies. It is therefore possible that telomere attrition leads to loss of cell viability and organ dysfunction, in this case placental dysfunction. Senescence as a result of losing telomeric repeats with each division, ultimately leading to limited cell division, is a natural process called lifespan. It corresponds to the length of time during which a person or an animal lives. Loss of telomeric DNA tracts limit cell lifespan by 2 pathways: acting as a mitotic clock and also depriving the chromosome of protective telomeric DNA essential for cell viability (Counter 1996).

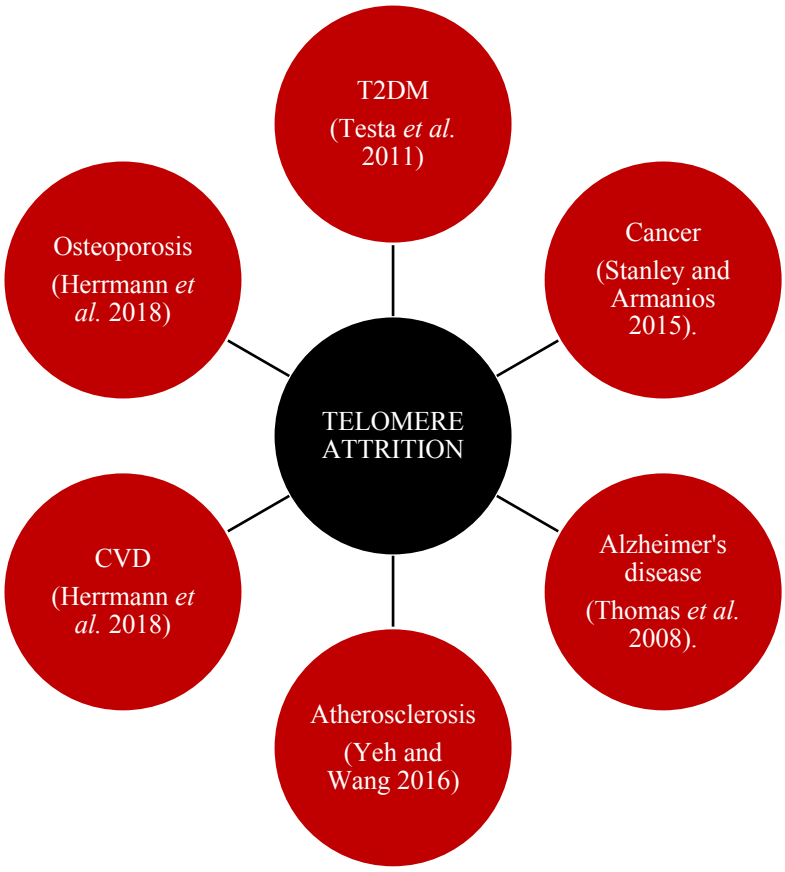


Figure 1.4. Age-related diseases associated with a reduction in telomere length.

1.3. Role of telomere biology in foetal programming

It is hypothesised that adjustments in response to the maternal environment including metabolic and growth responses affect foetal development in order to optimise survival. The problem is that in a non-adverse environment, such adaptive and permanent adjustments in foetal physiology become maladaptive predisposing the adult to disease later in life. This is called foetal programming (Johnson 2007). *In utero* exposure to different forms of physiological stress such as alterations in immune, endocrine, inflammatory and oxidative processes in the maternal-placental-foetal interface, can impact or program telomere biology of the foetus accelerating dysfunction, ageing and disease risk (Entringer *et al.* 2012) (**Figure 1.5**). An example of this are the female offspring of mothers with GDM. Shorter telomeres in childhood may predict or contribute to the increased risk of developing disease later in life (Hjört *et al.* 2018).

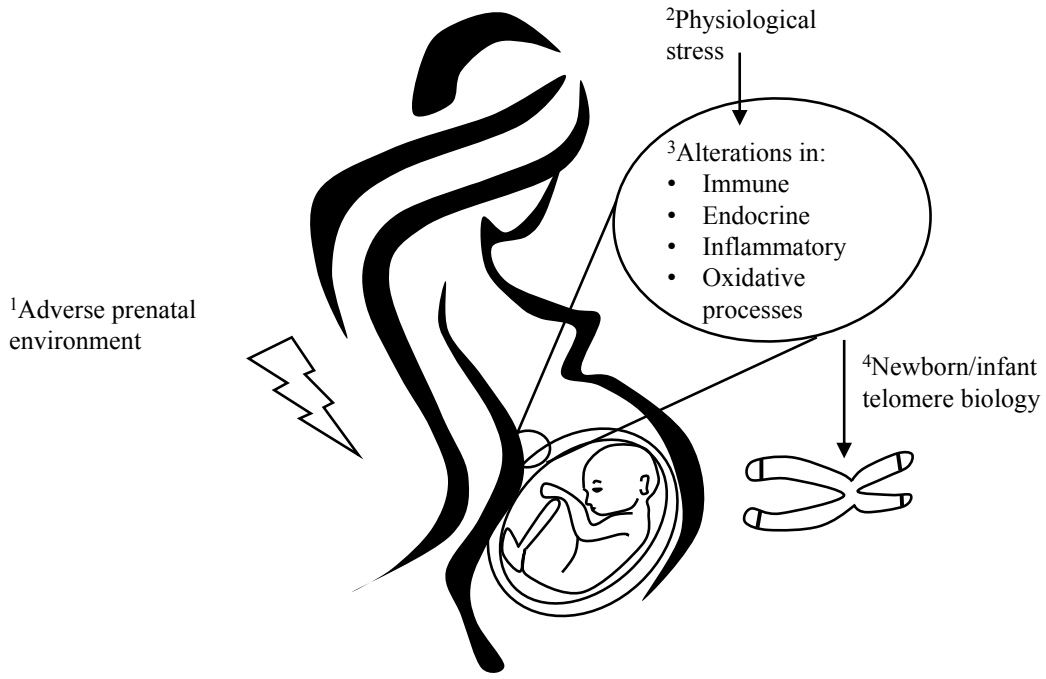


Figure 1.5. Outline of the foetal programming of telomere biology hypothesis.

1.3.1. Maternal health status and environmental exposures

Acquired diseases during pregnancy as gestational diabetes mellitus (GDM) may have a great impact during foetal development. Foetuses exposed to maternal diabetes have a higher risk of abnormal glucose later in life, CVD, hypertension and T2DM (Monteiro *et al.* 2016).

1.3.1.1. Maternal diet

Balanced diet contains all 3 groups of macronutrients: complex carbohydrates, essential amino acids and fatty acids, which are essential for normal foetal growth and development. Micronutrients in the form of iron supplementation and maternal folic acid are also positively correlated with birth weight (Godfrey *et al.* 1996; Mitchell *et al.* 2004). Deficiency of these nutrients in the mother causes changes in placental nutrient transport and reduced body weight of the foetus (Belkacemi *et al.* 2011). In addition, there are periods, such as during peri-implantation and first trimester of pregnancy, when placental and foetal growth are especially vulnerable, since this is a stage of rapid placental development (Wu *et al.* 2004).

Maternal undernutrition increases placental vascular resistance, which in turns increases workload in the foetal heart. Alterations in placental morphology is a feature of some intrauterine growth restricted (IUGR) foetuses who are at risk of developing cardiovascular diseases later in life. Alterations in the maternal environment as in the case of nutrient restriction, may also modify imprinted genes, a group of genes expressed in a parent-of-origin specific manner with essential functions in foetal growth and development, such as IGF-1. In addition, reduction in the activity of the placental enzyme 11β-Hydroxysteroid Dehydrogenase-2 (11 beta-HSD2), which converts excess cortisol to inactive cortisone, leads to an increase in glucocorticoids levels. Excess glucocorticoids in the foetus may program it to be at higher risk of suffering metabolic diseases in the adulthood (Belkacemi *et al.* 2011). Animal studies suggest that maternal undernutrition will impact differently depending on whether the mothers entered pregnancy with high or low nutritional stores. In this way, mothers with low pre-pregnancy stores exposed to a further period of undernutrition, will suffer

remarkable impairment of foetal and placental growth, compared with the mothers who were nourished around conception and then underwent a stage of dietary restriction. These will have placental hypertrophy (Godfrey *et al.* 1996).

Maternal overnutrition or overeating during pregnancy may result in foetal growth restriction and also increase the risk of neonatal mortality and morbidity. Maternal undernutrition as well as maternal overnutrition represent the two sides of dysfunctional nutrition with the same pregnancy outcome (Wu *et al.* 2004). It is speculated that impaired placental syntheses of nitric oxide and polyamines may be the underlying reason for IUGR in response to these two antagonistic nutritional problems (Wu *et al.* 2004).

1.3.1.2. Exercise

During pregnancy a minimum of 150 minutes of moderate intensity activity per week is recommended by the Royal College of Obstetricians and gynaecologists. Exercising is beneficial for maternal and foetal health. Moderate exercise throughout pregnancy has been correlated with a reduction in more than 36% risk of suffering GDM and also a reduction in excessive weight gain (Sanabria-Martinez *et al.* 2015).

Benefits for the babies are noticeable in body weight and composition, development of the nervous system and cardiovascular health. Children of mothers who exercise, may have a decrease risk of developing chronic diseases such as obesity, diabetes and cardiovascular diseases (Moyer *et al.* 2016). Conversely, Juhl *et al.* (2010) found a negative correlation between the number of times mothers work out per week and birth weight, as well as other signs of foetal growth. The propose mechanism for this association is a decreased in placental blood supply as a result of the exercise (Clapp 2003; Juhl *et al.* 2010).

1.3.1.3. Smoking

Intrauterine exposure to nicotine increases the incidence of obesity, cardiovascular diseases and non-insulin dependent diabetes mellitus in the adulthood. Although the mechanisms by which nicotine programmes the foetuses for adult diseases is still unclear, the potential mechanisms include the effect of nicotine on foetal growth and on central regulatory circuits. Nicotine is a harmful

substance that reduces foetal growth. It is responsible of maternal food intake inhibition and energy expenditure increase, which results in poor/under nutrition.

It is also responsible of causing alterations in foetal metabolism and in placental structure and function, where these perturbations impact oxygen and nutrients delivery across the placenta. Likewise, perturbations in central regulatory circuits imply disruptions in brain neurotransmitters causing long-term changes in the regulation of energy balance (Bergen 2006).

1.3.1.4. Alcohol

Intrauterine exposure to alcohol induces endocrine imbalances that alter maternal-foetal hormonal interaction. The hypothalamic–pituitary–adrenal (HPA) axis results particularly affected by prenatal alcohol consumption. Alcohol also has an effect on the immune function, altering the adequacy of the immune system response (Zhang *et al.* 2005). The neuroadaptive mechanisms that mediate stress response are compromised and sensitise the organism to future stressors, increasing the risk of suffering stress, depression and anxiety disorders later in life (Hellemans *et al.* 2010).

1.3.1.5. Illegal drugs

Drugs can act directly on the foetus because of their capacity to cross the placenta. They can also exert their effect in an indirect manner through the placenta, altering the utero-placental blood flow or affecting maternal physiology and thus affecting the foetus, as in the case of alterations of secretion stress hormones (Ross *et al.* 2015; Behnke and Smith 2013). Drugs can also exert their function early in gestation when they have a teratogenic potential or in the foetal period, when they have a more subtle effect contributing to abnormal foetal growth and alterations in the neurotransmission process (Behnke and Smith 2013).

Illegal Drugs effects can range from low birth to developmental deficits, affecting behaviour and cognition. Long-lasting changes in brain structure and function are caused by drugs. While cocaine and marijuana have been related with impaired attention, language, learning skills and behavioural problems (Cressman *et al.* 2014; Fried 1995), methamphetamine exposure *in utero* has been correlated with

foetal growth restriction, decreased length and head circumference (Ross *et al.* 2015), a decrease in arousal and poor quality of movement in infants. Likewise, heroin has been linked with low birth weight but, how its use during pregnancy affect the foetus and its development it is still unclear as stated by the national institute on drug abuse.

1.3.2. Impact of prenatal adversity on infant's development

Early adversity *in utero* such as drugs, nutrient restriction and different types of psychosocial stress can lead to metabolic and neurobiological abnormalities in the offspring (Monk *et al.* 2013).

A well-known example of famine consequences is the Dutch Hunger winter of 1944-1945 in which, caloric restriction, especially in the 2nd trimester was found to be associated with several neurodevelopmental abnormalities linked with an increased risk of suffering schizoid personality or schizophrenia in later life (Susser and Lin 1992, Hulshoff Pol *et al.* 2000). Roseboom *et al.* (2006) also reported other effects of the Dutch famine on reproduction and metabolism, which has been related with an increased prevalence of obesity and cardiovascular diseases in the offspring later in life (Scholte *et al.* 2015).

Experiencing stress during pregnancy is associated with negative birth outcomes including decreased birth weight and length of gestation. Low birth weight and premature babies have been associated with an increased risk for several negative neurodevelopmental outcomes such as poorer cognitive ability, behavioural problems such as hyperactivity, lower academic ability and an increased risk of developing psychosocial distress and major depressive disorder. It is suggested that these outcomes may be affected via changes in the function of the HPA axis (Lazinski *et al*. 2008, Monk *et al.* 2013).
In this context, it is likely that the placenta as nutrient and hormones supplier to the foetus, plays a central role mediating the programming effects of deleterious maternal environment during development.

1.3.3. The placenta

The placenta, a transient organ of pregnancy, is responsible for the bidirectional signalling that occurs between the maternal and the foetal system. It functions to supply oxygen, nutrients and hormones to support foetal growth and development, as well as flooding the maternal system with hormones which have an effect on maternal metabolism to support a successful pregnancy. Correct functioning of the placenta to achieve optimal foetal growth is also essential for later life health (John and Hemberger 2012).

1.3.3.1. Placental development and structure

After fertilisation, the zygote divides several times during which the size of the conceptus remains unchanged. At around 8- to 16-cell stage, the cleaving conceptus undergoes compaction to give rise to a morula which later results in a blastocyst at the 32- to 64-cell stage. The human blastocyst is composed of an inner cell mass (ICM) and a layer of mononucleated trophoblast that surrounds the blastocoelic cavity. While the embryo arises from the inner cell mass, the trophoblast cells are the first extra-embryonic lineage, which is essential in the nutrition and support of the growing foetus during the first trimester through the uptake of oviductal and uterine secretions, called histiotrophic nutrition (Johnson 2007).

The origin of the placenta begins 6-7 days after fertilisation, when the implantation of blastocyst into the uterine epithelium occurs. At this stage, polar trophoblast undergoes syncytial fusion to generate an invasive syncytiotrophoblast layer, which is capable of penetrating the uterine epithelium.

The remaining trophoblast cells form the underlying cytotrophoblast layer, which are not in contact with maternal tissues. Cytotrophoblast cells proliferate and fuse with the syncytiotrophoblast layer, contributing to the expansion of this layer, and invasion of the uterine wall (Huppertz 2008).

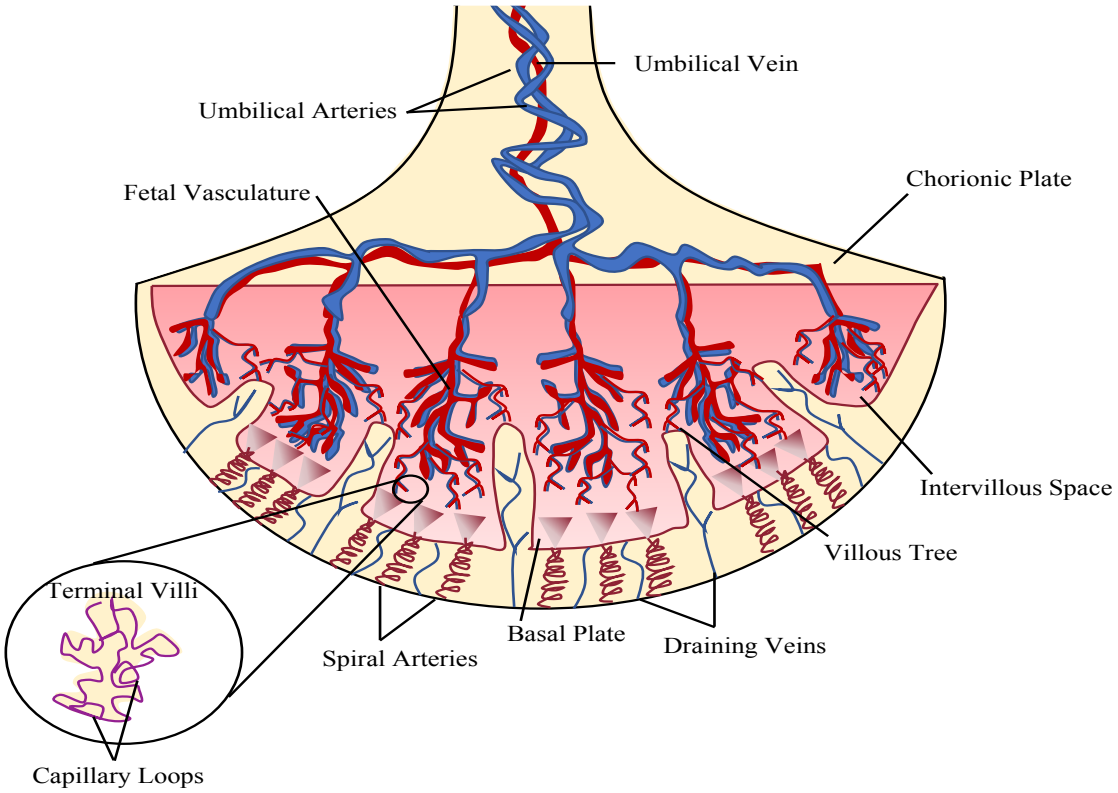


Figure 1.6. Structure of the human placenta. Foetal blood flows to the placenta from the umbilical arteries and returns oxygenated via the umbilical vein. Maternal blood enters the placenta through the spiral arteries and contacts with the intervillous space, then percolates between the villous tree and returns deoxygenated to the maternal circulatory system via the draining veins. Adapted from (Plitman Mayo *et al*. 2016).

Fluid-filled spaces appear in the syncytiotrophoblast. These join to form the lacunae, which are filled with tissue fluids and uterine secretions. The syncytiotrophoblast surrounding the lacunae are called trabeculae. The invasive phenotype of the syncytiotrophoblast leads to its expansion into the uterine wall resulting in maternal blood vessels erosion and allowing maternal blood flow into the vacuoles. The basic structure at this stage includes the following areas: the early chorionic plate (embryo facing); the lacunar-trabeculae system which derives into intervillous space and villous trees and the basal plate (endometrium facing) (Huppertz 2008) (**Figure 1.6**).

Cytotrophoblast cells of the chorionic plate gain access to the endometrium penetrating through the trabeculae mass of the syncytiotrophoblast. These cytotrophoblasts differentiate into villous trophoblasts which form the basic structure of the cotyledon, the chorionic villi. This major structure is responsible

of maternal-foetal exchange (Rampersad *et al*. 2011). Other subset of cytotrophoblasts differentiate into interstitial trophoblasts, which invade the decidua basalis and endovascular trophoblasts, which are essential in the invasion and remodelling of the maternal spiral arteries, thereby establishing the uteroplacental circulation at the end of the first trimester (Huppertz 2008).

Maternal spiral arteries adaptations are key to a successful pregnancy. The delicate foetal villi receive large amounts of blood to the placental intervillous space at a low resistance pace maximising maternal blood flow as a result of arterial remodelling (Burton *et al.* 2009). Maternal Spiral arteries deliver oxygenated blood and nutrients to the intervillous space where it contacts with the terminal villi, specialised treelike structures whose small diameter provide a high surface exchange area (Rampersad *et al.* 2011). Maternal and foetal circulations are separated by only a thin layer of syncytiotrophoblast (Huppertz 2008). Oxygen and nutrient rich blood is then transported to the foetal systemic circulation by chorionic veins and the umbilical vein (Wang and Zhao 2010).

Likewise, carbon dioxide and nutrient depleted foetal blood is carried via umbilical arteries and villous core foetal vessels to the terminal villi where they diffuse to the endometrial and uterine veins back to the maternal circulatory system (Wang and Zhao 2010).

1.3.3.2. Placental function

Adequate foetal growth and development rely on placental capacity to transport and release metabolic products (1), flood maternal and foetal circulations with hormones (2) and protect the foetus acting as a barrier (3) (Gude *et al.* 2004).

The placenta is an organ which requires oxygen and nutrients to grow and function (Illsley 2011). It also allows a rapid exchange of oxygen via diffusion from maternal to foetal blood and of carbon dioxide from foetal to maternal blood. This exchange is facilitated by differences in concentration and pressure in the feto-maternal circulation, higher affinity of foetal haemoglobin for oxygen, as well as the Bohr effect (Gude *et al.* 2004).

Glucose is the main carbohydrate and source of energy carry across the placenta. It is transferred by glucose transporters (GLUTs) located on the syncytiotrophoblast membrane from the mother to the foetus (Gude *et al.* 2004). The placenta is responsible of more than half of the consumption of uterine glucose delivery (Illsley 2011). Other nutrients cross the placenta as smaller molecules that support foetal protein and lipids synthesis (Gude *et al.* 2004).

The placenta acts as a super endocrine organ producing placental hormones that flood maternal and foetal circulation with endocrine and paracrine functions. These hormones are mainly produced by cytotrophoblasts and syncytiotrophoblasts and to a lesser extent by villous stromal cells and macrophages, also known as Hofbauer cells. Human chorionic gonadotropin (hCG) produced by the placenta stimulates the corpus luteum to produce progesterone. Progesterone is responsible of uterine quiescence, blocking uterine contraction. It is also involved in the immune response to the feto-placental allograft. The placenta is also a source of oestrogen, which influence uterine blood flow and guarantee a steady production of progesterone and steroid metabolism, as well as priming maternal breast for lactation. Moreover, the placenta synthesises human placental lactogen (hPL) and placental growth hormone (PGH). These hormones induce maternal food intake and nutrient storage promoting foetal and placental growth through its major role regulating insulin levels and calcium absorption (McNamara and Kay 2011).

The placenta protects the foetus from eventual xenobiotics that circulates in maternal blood with mechanisms, such as export pumps, which are located in the maternal facing membrane of the syncytiotrophoblast. However, some xenobiotic substances including alcohol and certain drugs can cross the placenta and exert teratogenic effects. The placenta also constitutes a barrier against microorganism transmission from the mother to the foetus. However, some bacteria, protozoa and a number of virus can pass through the placenta leading in some cases to poor pregnancy outcomes (Gude *et al.* 2004).

1.3.3.3. Placental dysfunction

Placental dysfunction or insufficiency refers to the condition in which either the placenta does not develop properly or becomes progressively dysfunctional. This leads to a reduction in maternal blood supply to the placenta, which is unable to deliver an adequate supply of oxygen and nutrients to the growing foetus. Under these conditions, the placenta cannot fully support the needs of the developing baby and some pregnancy complications may arise (Gagnon 2003).

Some of these complications include IUGR and pre-eclampsia (PE), which increase preterm birth risk, which is the leading cause of perinatal morbidity and mortality (Hunt *et al.* 2016).

Intrauterine growth restriction is defined as the failure of the foetus to achieve its genetically optimal growth potential. A foetal growth restricted baby is at increased risk of developing CVD as well as diabetes mellitus (DM) in adulthood (Crocker 2011).

Although the aetiology of IUGR remains unclear, failure in trophoblast transformation of uterine spiral arteries and the resulting impaired placental perfusion represents the first step in the development of the disease. Different responses by the mother, the placenta or the combination of both to the suboptimal utero-placental blood flow will determine the different pathogenic manifestations (Crocker and Lyall 2011).

1.3.4. Determinants of variation in telomeres

Numerous factors including age, sex, race/ethnicity, paternal age at birth, mutations in telomerase and telomere maintenance-related genes all impact on constitutive telomere length (Starkweather *et al.* 2014). Additionally, there are other psychosocial, environmental and behavioural factors that impact of somatic length during the life course. For example, telomere length has been correlated with chronic (Epel *et al.* 2004) and acute psychological stress (Epel *et al.* 2006), prenatal stress (Entringer *et al.* 2011), childhood maltreatment (Tyrka *et al.* 2010), mental health (Hartmann *et al.* 2010; Lung *et al.* 2007), socioeconomic status (Cherkas *et al.* 2006), smoking (Valdes *et al.* 2005), physical activity (Ludlow *et al.* 2008), obesity (Brouilette *et al.* 2008) and sleep quality and duration (Liang *et al.* 2011) among others.

1.3.4.1. Stress processes and telomere variation

Stress may contribute to telomere shortening through stress-biological processes such as oxidative stress, inflammation, dysregulation of stress hormones and metabolic changes.

1.3.4.1.1. Oxidative stress and telomeres

In vitro studies suggest that oxidative stress may be the main cause of telomere shortening. Oxidative stress is the result of an imbalance between the antioxidant mechanisms of the body and the production of ROS. These species can be exogenous or the product of aerobic metabolism and are highly reactive, causing oxidative damage in various molecules including the DNA telomeric sequence. This damage results in the eventual loss of telomeric tracts through double stranded breaks and during DNA replication as a consequence of single-strand DNA damage (Reichert and Stier 2017). An increase in ROS associated with age, has also been reported to impact the telomerase enzyme. TERT, the telomerase catalytic subunit is translocated from the nucleus to the cytosol and into the mitochondria, thereby decreasing telomerase nuclear bioavailability and total telomerase activity (Haendeler *et al.* 2004). Ahmed and Lingner (2018) suggest that the mechanisms by which oxidative stress induced damage in non-modified

fibroblasts, which do not express telomerase, is by enhancing the end replication problem interrupting the preservation of the distal ends of telomeres.

1.3.4.1.2. Inflammation and telomeres

It has been reported in mice that chronic low-grade inflammation increases telomere dysfunction by ROS-mediated DNA damage. Telomere dysfunction intensify the accumulation of senescent cells, which in turn stimulate the production of ROS and the secretion of bioactive pro-inflammatory peptides, the so-called senescence-associated secretory phenotype (SASP) contributing to accelerate ageing. It draws a positive feedback loop (Jurk *et al.* 2014).

C-reactive protein (CRP), an inflammatory marker, is produced in the liver in response to inflammation. This pro-inflammatory molecule is elevated in obese and overweight individuals whose condition represents an inflammatory state. High CRP levels in plasma of these individuals have been correlated with short telomere length. CRP might be the link between high body mass index (BMI) and short telomeres through a low-grade inflammation pathway. CRP stimulates white blood cells (neutrophils) to release oxygen radicals (Prasad 2004). In this regard, Ogawa *et al.* (2008) suggested that ageing is associated with low-grade inflammation and an increase in CRP production, which may lead to spontaneous ROS production by neutrophils. CRP production is stimulated by other cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), contributing to cell senescence (Rode *et al.* 2014).

The cumulative effect of high levels of IL-6 and TNF-alpha increases the risk for short telomere length. Those patients with high levels of inflammatory molecules in plasma are at risk of accelerate leukocyte telomere attrition and those with short telomeres are at risk of suffering diseases with an immunological aetiology (O'Donovan *et al.* 2011).

Active immune cells express high levels of the telomerase enzyme. Telomerase upregulation avoid immune cell senescence which is induced by antigen exposure and oxidative stress. The competence of the immune system depends on cell renewal and clonal expansion of the main cellular types: T and B cells (Kaszubowska 2008).

There is a very close relationship between nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), a master regulator of inflammation and the telomerase enzyme. TERT, the catalytic subunit of the enzyme, binds the p65 subunit of NF-κβ and is recruited to a subset of NF-κβ target gene promotors such as IL-6 and TNF-α, providing a feed-forward loop where telomerase regulates NF-κβ expression and NF-κβ can transcriptionally upregulate telomerase levels. This could explain the cohabitation of inflammation and maintained telomerase activity in the human cancer scenario (Ghosh *et al.* 2012).

ROS production via mitochondrial or non-mitochondrial pathways enhances telomere attrition. When replicative senescence occurs in a system with continuous cell expansion as the immune system (immunosenescence), it loses competence and an imbalance between inflammatory and anti-inflammatory responses arises. This state is characterised by low-grade chronic inflammation and an increase in oxidative stress, which in turns leads to telomere dysfunction. As a result, senescent cells accumulate and pro-inflammatory and oxidative signals are produced, contributing to increase inflammation and mitochondrial dysfunction, thereby spreading DNA damage and senescence towards surrounding cells (Kaszubowska 2008; Correia-Melo, Hewitt and Passos 2014; Jose *et al.* 2017 and Jurk *et al.* 2014).

1.3.4.1.3. Dysregulation of stress hormones and telomeres

An adequate regulation of the HPA axis is essential to avoid sustained activation of the axis and subsequent long-term health issues. For this purpose, there is a negative feedback loop to control this system. In response to stress, the HPA axis stimulates the adrenal glands to produce cortisol, the main glucocorticoid found in humans with a systemic function on immune, metabolic, and vascular processes (Stephens and Wand 2012). In animal studies, it has been shown that embryonic exposure to corticosteroids causes an increase in oxidative stress levels and shorter telomeres later in life (Haussmann *et al.* 2012). The plasticity of the neuroendocrine system to adapt to different threats reveal the complexity in individual cortisol responses. Some studies have demonstrated that greater cortisol responses to mental stress are linked with telomere attrition in adults and

children (Epel *et al.* 2006; Steptoe *et al.* 2017; Gotlib *et al.* 2015; Kroenke *et al.* 2011).

1.3.4.1.4. Metabolism and telomeres

Lipid and glucose-insulin related metabolism may have a role in telomere maintenance regulation. In terms of fat intake, a study in postmenopausal women revealed an inverse association between small to medium chain saturated fatty acids (SMSFAs) intake and telomere length. Dairy is the major source of this type of fatty acids, which are present in butter, fat-containing milk and cheese (Song *et al.* 2013).

Interestingly, it has also been reported lower levels of oxidative stress, inflammation, apoptosis and telomere attrition in human umbilical cells in relation with the Mediterranean diet (Marin *et al.* 2012). It could be that high levels of oxidative stress and inflammation mediate the effect of SMSFAs on telomeres (Song *et al.* 2013). A recent study has correlated some biomarkers of cardiovascular disease risk such as high-density lipoprotein (HDL) cholesterol, and triglycerides with leukocyte telomere length (Rehkopf *et al.* 2016).

Previous studies also found a correlation between reduced HDL-cholesterol levels and shorter telomere length (Dei Cas *et al.* 2013; Chen *et al.* 2009) and further evidence suggest that there is a positive correlation between high levels of HDL-C and a slower rate of Leukocyte telomere length shortening, possibly explained by the antioxidant and anti-inflammatory effects of this lipoprotein (Chen *et al.* 2009).

Regarding glucose-insulin metabolism, insulin resistance has been inversely correlated with shorter telomere length (Aviv *et al.* 2006; Al-Attas *et al.* 2010). Individuals who inherit short telomere length are prone to insulin resistance, which is a sign of diabetes because their glucose homeostasis might be altered at an earlier age compared with their counterparts. Insulin resistance constitutes a chronic state of inflammation and oxidative stress, conditions that exacerbate telomere erosion (Verhulst *et al.* 2016).

A study in mice suggested the role of telomerase in glucose metabolism, as TERC deficient mice exhibit impaired glucose tolerance. The cause is altered glucose-stimulated insulin secretion from the islets of the pancreas. Beta cells of TERC deficient mice have a limited replicative capacity which compromised pancreatic islet size and threaten insulin secretion capacity (Kuhlow *et al.* 2010).

1.3.5. Determinants of constitutive telomere length

The initial setting of telomere length at conception is a critical element in an individual telomere biology complex. For any given individual at any age, telomere length depends on the initial setting of telomere length at conception and the rate of telomere attrition during lifetime. Telomere attrition is, in turn, the result of cell replicative activity, long-term exposure to stress-related biological agents such as oxidative stress, inflammation, dysregulation of stress hormones and metabolic changes, and the activity of the telomerase enzyme to mitigate telomere exhaustion (Aviv 2008).

Studies in animals and humans support the important role of telomere biology in lifespan and disease risk. The facility to track animals from birth onwards makes them a great model to study the impact of early life telomere length on lifespan. A study in zebra finches measured telomere length at different points in the bird's life and found that individuals with longer telomere length at 25 days, had longer lives. Early life telomere length constitutes a great lifespan predictor due to the contribution of those telomeres on subsequent tissue function and homeostasis. The effect of early life telomere length on longevity may be a consequence of inherited variation in telomere length and/or variation in telomere loss in the early growth period (Heidinger *et al.* 2012).

In humans there is considerable interindividual variation in leukocyte telomere length. This variation among adults arises early in life and doesn't change that much during adulthood. It has been reported that the rate of leukocyte telomere shortening in the adulthood is about 30 bp/year. It is speculated that telomere length in the adult life depends mainly on the leukocyte telomere length at conception, and attrition during the first 20 years of life (Benetos *et al.* 2013). A study using same-sex twins reported that leukocyte telomere length during adult life has a heritable component (Hjelmborg *et al.* 2015). The heritability of telomere

length is defined as the proportion of variance in leukocyte telomere length or leukocyte telomere length attrition attributable to genetic factors (Hjelmborg *et al.* 2015). It also suggested that environmental factors that occur during development and early childhood have a greater contribution to telomere length variation over the life of the individual than those that affect telomere attrition during the adulthood in an age dependent manner (Hjelmborg *et al.* 2015). These observations suggest that telomere length measured at birth can be considered as a risk factor for developing cancer or age-related diseases later in life. In a recent human study, it has been demonstrated that new-borns with short telomeres showed higher levels of genetic damage (Moreno-Palomo *et al.* 2014). Finally, a synchrony exists within the organs, whose telomeres shorten at an age-dependent rate independent of their proliferative capacity. Therefore, existing differences between highly and minimally proliferative tissues should be established early in life (Daniali *et al.* 2013).

1.3.6. Developmental ontogeny of the telomere biology system

The telomerase enzyme is active in stem cells, most cancers and immortalised cell lines except for normal somatic cells (Holt and Shay 1999). To guarantee the transmission of full-length chromosomes to the next generation, the telomerase enzyme must be activated in germ cells (Dahse *et al.* 1997; Hastie *et al.* 1990).

An experiment in mice reported shortening of telomeres in mature oocytes and how this phenomenon was overcome. Telomere length in individual mouse oocytes, zygotes and 2 cell embryos were measured using quantitative fluorescence *in situ* hybridisation (Q-FISH). Although telomeres were short in mature oocytes, they lengthened significantly in the 1-2 cell stage embryo; a model that was even observed in telomerase knockout mice. This suggest that recently fertilised eggs counter the short telomeres inherited from mature oocytes though mechanisms independent of the telomerase such as telomere sister chromatid exchange, which operates in the early-cleavage-stage embryo. In the blastocyst stage, the telomerase becomes highly active again (Liu *et al.* 2007). Conversely, Meerdo *et al.* 2015 found that between the oocyte and cleavage stages, there is a significant reduction in telomere-to-centromere ratio in bovine

animals. In human cleavage stage embryos, telomerase presented a lower activity compared with oocytes or blastocysts as reported by Wright *et al.* 2001.

Telomerase is necessary for the cells forming the human embryo as they need it to proliferate rapidly during the embryogenesis process. It has been also demonstrated that the amount of telomerase is not correlated with the potential for embryonic growth (Wright *et al.* 2001). Just after birth the body regulates and repressed telomerase activity except in some tissues (Wright *et al.* 1996).

1.3.7. Factors that contribute to constitutive telomere length

Stress-related processes may constitute a mechanism to explain foetal programming of telomere biology as:

1. They are vulnerable to environmental exposures.
2. They are key signalling molecules between foetal and maternal interface.
3. They can exert long-term effects via epigenetic on the developing telomere biology system.

1.3.7.1. Heritability

Initial telomere length is inherited (Blackburn 1991). An individual telomere length is the result of many genes of small effect at any point and depend on the initial length of the zygote's telomeres, the amount of attrition and the amount of restoration (Hill 2010).

Multiple genetic effects could alter telomere length in various ways: acting on the initial telomere length in the fertilised egg or on the resistance to telomere attrition or on extension of telomerase expression. The phenotype is the result of the cumulative effect of all these genes which have a small effect (Lynch and Walsh 1998).

It is important to differentiate between leukocyte telomere length, which is heritable, with a reported heritability ranging from 36-82% and the rate of telomere attrition during adult life which is also heritable but to a lesser extent compared with the initial setting of telomere length, indicating little environmental effect. Therefore, heritability and early-life environment constitute the main determinants of lifetime leukocyte telomere length (**Figure 1.7**) (Hjelmborg *et al.* 2015). In this context, there are environmental factors that influence leukocyte

telomere length at birth, during growth and developmental periods, as well as other environmental factors that have an effect on age-dependent attrition during the adulthood (Hjelmborg *et al.* 2015). The first referred to those maternal stress and conditions around conception and across pregnancy that has an impact on the growing foetus, while the latter, concerned the impact of unique-environmental factors such as lifestyle or socioeconomic status on the rate of telomere exhaustion. However, the effect of those unique factors is very small compared with the joint action of leukocyte telomere length of heritability and *in utero* environment, which accounts for about 87% (Hjelmborg *et al.* 2015).

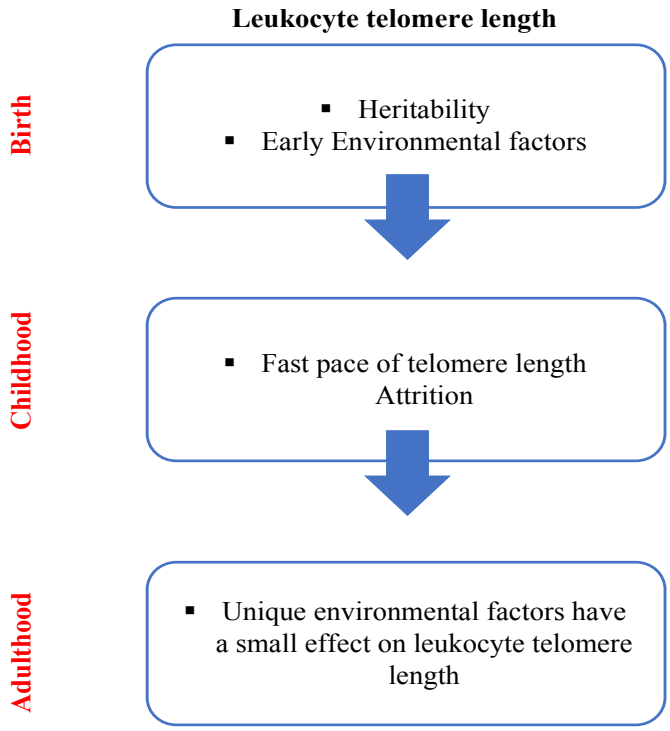


Figure 1.7. Diagram that illustrates the determinants of lifetime leukocyte telomere length.

1.3.7.2. Prenatal environment

There are a many environmental factors that are likely to produce chronic stress and have been linked to telomere attrition in adults (Haussmann and Marchetto 2010). Some experimental studies in rodents have introduced changes in growth rate, infection status and social crowding, which have been associated with shorter telomeres. Illmonen *et al.* 2008 demonstrated in mice that exposure to infection diseases can cause telomere attrition. In rats, a low protein diet was shown to influence growth and longevity in male offspring. Foetal growth restriction was associated with shorter life span and age-related telomere attrition in liver and kidney (Jennings *et al.* 1999). Fast growth has been associated with decreased lifespan. Studies on penguins reported that the chicks who underwent catch-up growth exhibited higher levels of oxidative stress molecules and accelerated telomere loss (Geiger *et al.* 2012). A previous study in rats, showed similarly, that poor maternal nutrition can lead to low birth weight offspring. Low birth weight followed a postnatal catch up growth was associated with telomere shortening in pancreatic beta cells and islet dysfunction, which has been associated with a reduction in the lifespan (Tarry-Adkins *et al.* 2009). Stress in the form of overcrowding was experimentally induced in male and female wild-caught house mice (*Mus musculus)*, which were found to have shorter telomeres than mice that were not stressed (Kotrschal *et al.* 2007). When corticosterone levels were manipulated *in ovo* in chickens (Haussmann *et al.* 2012) there was an increase in the proportion of short telomeres in red blood cells accompanied by high levels of oxidative stress. Early-life competition also constitutes an adverse environmental exposure. For example, a recent study in meerkats demonstrated that early-life competition and maternal nutrition had an effect on the telomere biology of their pups. It was suggested that the reduction in telomere length in the pups is a consequence of food competition and those pups with shorter telomeres have a lower probability of survival to adulthood (Cram *et al.* 2017). In addition to these studies, it has been reported *in vitro* that human T lymphocytes exposed to cortisol exhibit a reduction in telomerase activity (Choi *et al.* 2008).

There are several human studies that have reported the effects of maternal conditions and exposures during pregnancy such as obesity, hypertension, BMI, poor nutrition, diabetes and stress on offspring telomere dynamics. Leukocyte telomere length at birth has been correlated with birth weight (Lee *et al.* 2017; Tellechea *et al.* 2015). In this context, it has been demonstrated that women who are underweight pre-pregnancy have an increased risk of delivering a small for gestational age or low birth weight baby. Macrosomic or large for gestational age babies are more likely to occur with pre-pregnancy overweight (Yu *et al.* 2013).

Consistent with this, higher pre-pregnancy BMI has been linked to reduced cord blood and placental telomere length (Martens *et al.* 2016). Telomere shortening may play an important role in ageing of the placenta as suggested by placental telomere length reduction during the third trimester of gestation in a twin study (Gielen *et al.* 2014). Pregnancies complicated with IUGR have been linked with shorter telomeres and a decrease in the activity of the telomerase enzyme in the human placenta (Toutain *et al.* 2013; Biron-Shental *et al.* 2010, 2010, 2011, 2014; Izutsu *et al.* 1998). Impaired telomere biology might play a role as an underlying mechanism in the development of this condition, contributing to placental dysfunction that ultimately causes FGR.

There are susceptible stages as *in utero*, in which environmental factors are more harmful than in other stages (Entringer *et al.* 2015). Send *et al.* 2017 reported a significant association between maternal prenatal stress and shorter telomeres.

These findings were supported by other studies (Entringer *et al.* 2013; Marchetto *et al.* 2016). The first study on the long-term consequences of maternal stress on infant telomere biology revealed that leukocyte telomere length as a biomarker of cellular ageing, was reduced in young adulthood exposed to *in utero* psychosocial stress (Entringer *et al.* 2011).

As a serious obstetric complication, gestational diabetes implies short and long-term risks for the mother and offspring health. With reference to telomere length, Xu *et al.* (2014) found that offspring exposed to GDM exhibited reduced leukocyte telomere length compared with babies of healthy pregnant women. In addition, a study in 9-to 16-year-old girls exposed to gestational diabetes *in utero* revealed significant telomere reduction consistent with extensive levels of oxidative stress

and inflammation, which is believed to be a potential cause of telomere attrition (Hjort *et al.* 2018). Similarly, arterial hypertension appears to be a critical factor in the modulation of the newborn telomere length, which was found significant and inversely correlated with maternal history of arterial hypertension (Tellechea *et al.* 2015).

It is possible that prenatal adversity has a direct effect on those stress-related molecules that ultimately, impact telomere function. These studies have assessed telomere length in placenta and cord blood mainly, and some in children, young adults and adults. In a recent study, folate, an essential nutrient for DNA synthesis, is specially needed when cell turnover is increased as occurs during foetal development. Umbilical cord RBC folate levels and foetal telomere length at birth were found directly proportional (Louis-Jacques *et al.* 2016). In a study that interrogate the effect of prenatal smoking status on foetal telomere length, it was found that foetal telomere length was inversely correlated with smoking status of the mother (Salihu *et al.* 2015). The syncytiotrophoblast layer of the placenta exhibited significant DNA double-strand breakage in those mothers who smoke during pregnancy (Slatter *et al.* 2014). In pregnant women living in areas with poor air quality, exposure to high levels of air pollution was associated with telomere reduction at birth (Martens *et al.* 2017). A summary listing different organismal studies, environmental exposures, telomere outcomes, tissue and techniques including quantitative polymerase chain reaction (q-PCR), Southern blot (SB), real time PCR (RT-PCR), Terminal restriction fragment (TRF), Q-FISH, Fluorescence *in situ* hybridisation **(**FISH) and telomeric repeat amplification protocol (TRAP) is depicted in **Table 1.1**.

Table 1.1. Table summarising prenatal environmental exposures reported to contribute to the initial setting

Studies		**Environmental exposures**	**Telomere length outcome**	**Tissue and tech**
Animals	Mice	Exposure to infection diseases	Telomere attrition	White blood cell
	Rat	Low protein diet	Age-related telomere attrition	Liver and Kidne
		Poor maternal nutrition	Telomere attrition	Pancreas β (Sou
	Penguin	Fast growth (catch-up)	Telomere attrition	Red blood cells
	Wild-caught house mice	Overcrowding stress	Telomere attrition	White blood cell
	Chicken	Corticosterone *in ovo*	Telomere attrition	Red blood cells
	Meerkats	Early-life competition & maternal nutrition	Telomere attrition	Skin samples (q-
In vitro		Cortisol exposure	Decrease in telomerase activity	Human T-cells
Humans		Birth weight	Associated with telomere length at birth	White blood cell
		Higher pre-pregnancy BMI	Telomere attrition	Cord blood & Pl
		IUGR during pregnancy	Telomere attrition	Placenta (q-FISH
				(FISH)
			Decrease in telomerase activity	(TRAP
		Maternal prenatal stress	Telomere attrition	Cord blood (q-P
		Psychosocial stress	Telomere attrition	White blood cell
		GDM	Telomere attrition	Cord blood (q-P
				White blood cell
		Hypertension	Telomere attrition	Cord blood (q-P
		Folate levels	Telomere attrition	Cord blood (q-P
		Smoking	Telomere attrition	Cord blood (q-P
		Air pollution	Telomere attrition	Cord blood & Pl

1.3.8. Mechanisms underlying foetal programming of telomere biology

The same stress-related molecules that are able to impact telomere biology during the adulthood may be also implicated in the foetal programming of the telomere biology system. It is possible that stable epigenetic alterations in embryonic and foetal tissue contribute to such programming, explaining the enduring effects of adverse foetal, infant and childhood environment (Wadhwa *et al.* 2009). The development of complex and chronic diseases later in life cannot be just attributed to genomic heritability on its own. Epigenetics adjust expression pattern of genes in a site and specific way. It represents an adaptive response to insults during sensitive phases, as in the developmental period. Two types of genes are modified epigenetically: imprinting genes and genes with metastable epialleles. The mechanisms of epigenetic action include DNA methylation, post-translational histone modification and microRNA-mediated repression/activation. The result is different levels of expression. The reason why epigenetics can explain long-lasting effects is because it influences long-term expression of a gene by altering the accessibility of transcription factors to the DNA sequence. In this way, epigenetic mechanisms can influence heritable changes in gene expression that do not involve changes in the genetic sequence of an organism (Vo and Hardy 2012).

1.3.8.1. Epigenetics and the growing foetus

Mammalian telomeres and subtelomeric regions are rich in epigenetic marks that are characteristic of heterochromatin. Suppression of master epigenetic regulators, such as histone methyltransferases and DNA methyltransferases leads to loss of telomere length control and acute telomere attrition, which impacts the epigenetic status of telomeres and subtelomeric regions (Blasco 2007). It is possible that environmental exposures affect foetal epigenetics, and this, in turn, determines telomere length and disease development later in life. An example of this model is illustrated under normal and abnormal oxygen supply. Under adequate foetal oxygen supply subtelomeric regions and hTERT gene regulatory regions are massively methylated, which stabilises telomeres and keep the activity of the telomerase enzyme low. However, hypoxia caused by

uteroplacental insufficiency increases expression of HIF-1 alpha. This transcriptor factor binds hTERT promoter and enhances hTERT activity.

Additionally, increased expression of HIF-1 alpha leads to DNA methyltransferases (DNMTs) downregulation causing hypomethylation in subtelomeric DNA and hTERT regulatory region ultimately affecting telomere conformation and stability in addition to increase telomerase activity. Higher telomerase activity leads to telomere elongation but also causes telomere instability and a faster rate of telomere shortening in the adulthood (Ravlic *et al.* 2018).

1.4. Telomere length analysis

Since telomere length is considered a biomarker for ageing and can be used in the prognosis of age-related diseases, developing methods that quantify telomere length in an accurate and consistent manner has become a priority **(Table 1.2)**. This responds to the need to detect those telomeres within the length ranges that can lead to senescence, apoptosis and telomere fusion (Hemann *et al.* 2001).

Table 1.2. Table detailing the advantages and disadvantages of each telomere-measurement technique (Aubert *et al.*2012; Vera and Blasco 2012; Montpetit 2014, Aviv *et al.* 2011).

Method	Advantages	Disadvantages
TRF	-Widely used and cheap -No special requirements	-Low overall sensitivity -Requires big amounts of DNA -Time consuming -Provides average telomere length per sample -Cannot detect shorter telomeres
Q-FISH	-High sensitivity, specificity and resolution -Provides individual telomere length measurements -Only needs a small number of cells -Can detect critically shortened telomeres	-Low throughput -Only can be used in proliferating cells
Flow-FISH	-High throughput -Fast and accurate	-Requires cell in suspension -Provides a mean value for telomere length of the whole population of telomeres within a cell
qPCR	-High throughput -Low-cost -Requires only small amounts of DNA	-Quantifies the average telomere length per sample and cannot provide individual telomere length measurements -High measurement error (coefficient of variation)
STELA	-Most accurate technique -Detects telomere length from specific chromosome ends -It only requires very limited starting material	-Low throughput and labour intensive -Restricted to XpYp, 2p, 11q and 17p chromosome ends -Cannot detect telomeres >20kb

1.4.6.1. TRF Analysis

TRF analysis involves the digestion of genomic DNA using restriction enzymes, which are not able to digest telomeric repeats. As a result, the majority of genomic DNA is degraded except the telomeric tracts, leaving terminal restriction fragments. These fragments are then separated by resolving them on an agarose gel and after Southern blotting, a telomere-repeat containing labelled probe is required to hybridise to the telomeric tracts. Hence, a smear is produced containing the labelled telomeric DNA but also containing subtelomeric and telomere repeat variants that shows a high heterogeneity of the terminal restriction fragments (Baird, Jeffreys and Royle 1995). TRF analysis advantages include: it is a widely used technique to assess telomere length, it can be used with genomic DNA, cultured cells and tissues and is relatively cheap. Likewise, the disadvantages of using this technique include: low overall sensitivity, it requires large amounts of DNA to be performed, is time consuming and more importantly, the technique doesn't generate individual telomere lengths measurements, just average telomere length within the sample. TRF analysis is unable to detect small telomeric molecules, which are less concentrated and therefore give a lower hybridization signal (Aubert *et al.* 2012).

1.4.6.2. Q-FISH

Q-FISH is a technique based on the measurement of fluorescence arising from the hybridization of a fluorescent peptide nucleic acid (PNA) probe with denatured single-stranded DNA of metaphase chromosomes. To visualize the chromosomes a fluorescence microscope is necessary, where the average telomere length is estimated comparing the recorded of the telomeric fluorescent signal to that of known standards. The intensity of the fluorescence signal is directly proportional to telomere length (Landsdorp *et al.* 1996) and the images provided also revealed information on telomere length distribution for each chromosomal pair and arm (Poon *et al.* 1999). Thus, in contrast to TRF analysis, Q-FISH possess higher sensitivity, specificity and resolution. It only needs a small number of cells compared with TRF and allows telomere length detection of specific chromosome ends. It also represents an improvement for critically shortened telomeres detection (up to 0.15 kb). However, only small data sets can

be generated, and it can only be implemented in proliferating cells in culture, so it cannot be applied to senescence cells (Montpetit *et al.* 2014; Aubert *et al.* 2012).

1.4.6.3. Flow-FISH

This high throughput technique combines telomere FISH and flow cytometry to provide telomere length measurements (Rufer *et al.* 1998). It requires cells in suspension, which are fixed before the probe is added. Then, they are treated with a telomeric fluorescent-conjugated PNA probe and finally flow cytometry is used for the analysis. Despite this the technique represents a fast and accurate method. It cannot reveal the telomere length of individual chromosomes; just provides mean value for the telomere length of the whole population of telomeres within a cell (Aubert *et al.* 2012). It has become the most used technique to analyse telomere length dynamics in the immune system (Baird 2005). Its usefulness lies in its ability to measure telomere length in cell subtypes within a sample using antibody staining (Aubert *et al.* 2012).

1.4.6.4. Q-PCR

This low-cost and high throughput technique was developed by Cawthon *et al.* in 2002 and is the most commonly used method. It measures telomere (T) signals and single copy gene (S) signals, and compared it with a reference DNA, to obtain a relative T/S ratios that are proportional to average telomere length. In order to avoid primer dimerization, the designed primers only bind to G and C-rich strands within the telomeres and contain mismatches along their length. Only relative telomere length is provided by q-PCR unless coupled with a standard oligomer, which allows measurement of absolute telomere length (O'Callaghan and Fenech 2011). Like TRF, uses genomic DNA, but it requires only small amounts of DNA. A shortcoming of the qPCR technique is that it can only provide mean telomere length per cell sample, not being able to determine telomere length per cell or per individual telomere. Lacking the possibility of detecting small telomeric molecules (Vera and Blasco 2012). Aviv *et al.* (2011) reported a high measurement error (coefficient of variation), when using this technique.

1.4.6.5. STELA

STELA was developed by Baird *et al.* 2003 (**Figure 1.8**), it is a single-molecule PCR based telomere length analysis technology that can determine the full spectrum of telomere lengths from specific chromosome ends. STELA targets to specific chromosomes ends where the sequence telomere-adjacent is known. The technique involves annealing an oligonucleotide linker (telorette) to the C-rich strand using the G-rich 3'overhang as a template. Long PCR is then used to amplify between the specific telomere-adjacent primer and another primer called teltail whose sequence is complementary to the telorette linker. The PCR products are resolved using agarose gel electrophoresis and then detected by southern hybridization using a telomere-specific probe. The resultant blots show a pattern of bands, where each band constitutes a single telomere from a single chromosome end (Baird *et al.* 2003).

STELA measures telomere length in a highly accurate manner and despite it is low throughput, it is high-resolution and can detect critically shortened telomeres, in addition to subtle changes in telomere length. It doesn't require specialised equipment and can be performed with very limited starting material (Vera and Blasco 2012). However, it is restricted to several well characterised chromosomal ends including XpYp, 2p, 11q and 17p, for which a specific sub-telomeric primer should be designed (Britt-Compton *et al.* 2006). Additionally, it is labour intensive and cannot be used on fixed or degraded material as it requires high quality DNA (Baird *et al.* 2003). A further disadvantage is that telomeres longer than 20kb cannot be detected by STELA limiting its use in animals whose telomeres are long such as *Mus musculus* (Vera and Blasco 2012).

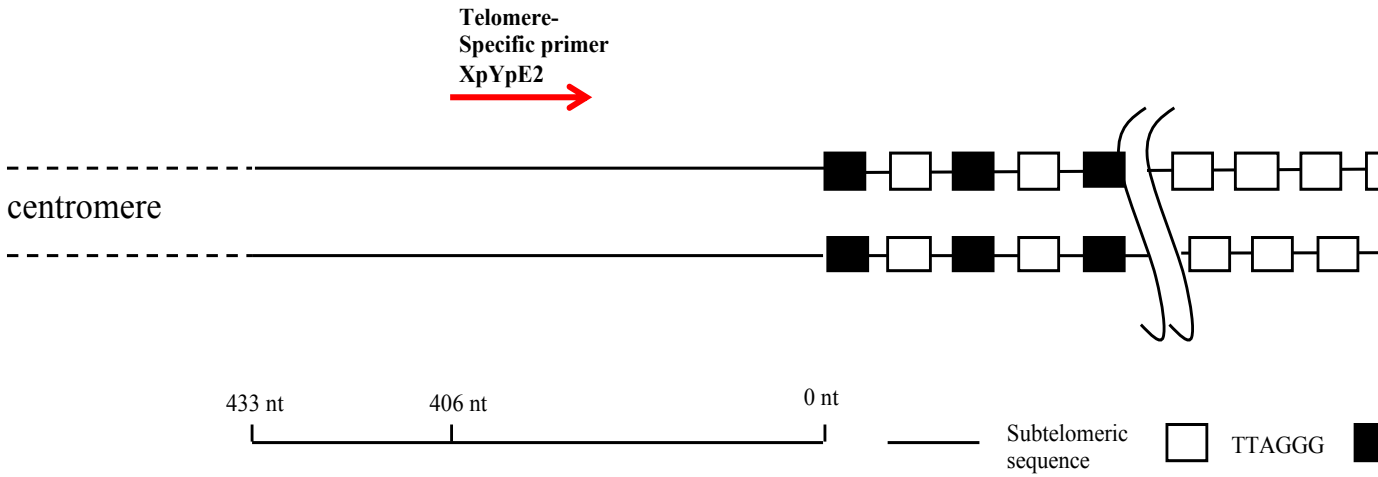


Figure 1.8. Single Telomere Length Analysis (STELA) at XpYp. Diagram of the assay with oligonucleotide primers
et al. 2003).

1.5. Study aims and hypotheses

Data from both human and mouse studies suggest that environmental exposures, such as GDM and prenatal depression, which are associated with high oxidative stress levels, can impact or program telomere biology of the foetus accelerating dysfunction, ageing and disease risk. The placenta is exposed to the same insults as the foetus and may provide a readily available tissue to assess the effects of different exposures during pregnancy on telomere length. In addition, few studies have examined placental telomere in relation with these exposures and none have applied higher resolution assays. The aims of this study were to:

1. Optimise STELA, a high resolution technique which provides length distribution at individual chromosome ends, for use in the human placenta.
2. Measure telomere length profiles in placenta from healthy pregnancies with respect to sampling site, mode of delivery and foetal sex.
3. Measure telomere length profiles in placenta from pregnancies complicated by prenatal depression and gestational diabetes.
4. Identify environmental factors associated with telomere dysfunction in the human placenta.

The hypotheses to be tested were as follows:

1. After optimisation, STELA will provide high resolution telomere length measurements in the human placenta.
2. Sampling site, mode of delivery and foetal sex will not impact telomere length or telomere length distributions in the placenta from healthy pregnancies.
3. Prenatal depression and gestational diabetes will be associated with telomere attrition in the human placenta.
4. Adverse maternal lifestyles (such as alcohol, poor diet and smoking) will be associated with telomere shortening in the human placenta.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Reagents and equipment

Reagents used in this study were obtained from different sources including: Amersham, Bio-Rad, Fisher, GE Healthcare, PerkinElmer, Roche, Thermo Fisher Scientific and Sigma.

Plastic and glass lab equipment used was obtained from different sources including: Eppendorf, Gilson, Starstedt and Thermo Scientific.

The equipment used in this study was obtained from different sources including: Amersham, Bio-Rad, EPS, Flowgen, GE Healthcare, Grant Instruments, MSE, Promega, Teche, Thermo Scientific.

2.1.2. Placental samples

The placental samples used in this study were obtained from two different cohorts: Welsh Pilot study and Grown in Wales (GIW) study. The Welsh Pilot Study is a maternal lifestyle study developed as part of Anna Janssen PhD (**Thesis of A.B. Janssen**). The Grown in Wales Study is an MRC funded study interrogating the relationship between placental gene expression and antenatal and postpartum maternal mood disorders (Janssen *et al.* 2018). Ethical approval and written informed consent were obtained for all the participants as shown in **Table 2.1**. Research was carried in accordance with the principles of the Declaration of Helsinki as revised in 2008.

Table 2.1. Cohort name, hospital origin and ethical approval for each cohort.

Cohort	Hospital origin	Ethical approval
Welsh Pilot Study	University Hospital Wales (UHW) and Royal Gwent Hospital (RGH)	South East Wales Research Ethics Committee Panel B in 2010, REC number 10/WSE02/10. Sponsor: NHS – Cardiff and Vale ULHB.
Grown in Wales (GIW) Study	University Hospital Wales (UHW)	Wales Research Ethics Committee 2 2015, REC number 15/WA/0004.

Detailed information about the cohort and number of samples used in each Chapter is included in **Table 2.2**.

Table 2.2. Cohort origin and number of samples of each individual chapter.

Chapter	Cohort origin	Number of samples
Chapter 3: Characterisation of the human placenta: optimization of STELA	Welsh Pilot Study + Grown in Wales (GIW) Study	N=42
Chapter 4: Impact of gestational diabetes mellitus on telomere length	Welsh Pilot Study + Grown in Wales (GIW) Study	N=100
Chapter 5: Impact of prenatal depression on telomere length	Grown in Wales (GIW) Study	N=109

2.1.2.1. Cohort descriptions

A comparison between the two cohorts regarding study design, participant recruitment and participant questionnaires is summarised in **Table 2.3**.

Table 2.3. Comparison between the two cohorts.

Characteristics	Welsh Pilot Study	Grown in Wales (GIW) Study
Participants recruited	271	355
Recruitment dates	From 20 weeks up till delivery	1 day prior to an Elective caesarean
Inclusion criteria Exclusion criteria	Healthy singleton pregnancy Foetal anomalies and infectious diseases	Singleton term pregnancy Foetal anomalies and infectious diseases
Study design	Control Growth restriction Macrosomic Gestational diabetes Preeclampsia	Control Women with antenatal and/or postnatal mood disorders
Self-administered questionnaire	Physical information Socio-economic characteristics of the mother Pregnancy information Maternal lifestyle factors Food frequency questionnaire (FFQ)	Physical information Socio-economic characteristics of the mother Pregnancy information Maternal lifestyle factors Food frequency questionnaire (FFQ) A1 questionnaire consisted of two assessments of perceive mood disorders
Obstetric covariates (medical notes)	Obstetric history Current pregnancy Maternal habits Birth outcomes Infant wellbeing	Obstetric history Current pregnancy Maternal habits Birth outcomes Infant wellbeing
Other variables studied		Saliva Maternal serum Mental health history

A participant information sheet with all the necessary information (**see Appendix 1a and 1b**) was used to explain the study to the participants. Written informed consent (**see Appendix 2a and 2b**) was obtained from healthy singleton pregnancies, unless otherwise stated. Mothers with infectious diseases or those whose infants have any foetal anomalies were excluded from this study.

A self-administered participant questionnaire (**see Appendix 3a and 3b**) was distributed between the participants. The information collected included: maternal physical characteristics (BMI, age and birth weight), socio-economic characteristics (maternal education and family income), and specific pregnancy information (gestational age, *in vitro* fertilization (IVF) or natural conception). It also included information on maternal lifestyle factors before and during pregnancy, such as smoking, alcohol, drugs, exercise and diet. The variables selected for inclusion in the questionnaire are factors known to be associated with foetal growth restriction. The questionnaire was designed to be completed within reasonable time.

A food frequency questionnaire (FFQ) was used to assess diet patterns before and during pregnancy. It is composed of a list of different foods and the participant should indicates how often they consumed each food over a limited period of time (Cade *et al*. 2004). It has been reported that FFQs are commonly used to assess diet patterns during pregnancy (Venter *et al.* 2006).

Despite the availability of pre-existing FFQ, the length, target population and diet components of those were an issue and a new FFQ was designed for this study. In this case, the new FFQ was designed considering the usual diet of the target population and those food items that have been previously shown to be related with foetal growth restriction. In addition, the FFQ was evaluated by an experienced Cardiff-based nutritionist, J. Crovini, and by a group of women who take part in a questionnaire trial. Based on the nutritionist and questionnaire trial feedback, the definitive questionnaire (**see Appendix 3a and 3b**) was submitted for ethical approval.

In the Grown in Wales (GIW) study, an additional section was added to the self-administered participant questionnaire: questionnaire (A1). This questionnaire was completed at recruitment and consisted of two assessments of perceived

mood symptoms (EPDS and STAI). While, the Edinburgh Postnatal Depression Scale (EPDS) (**only in Appendix 3b**) was used to assess depression symptoms, State-Trait Anxiety Inventory (STAI) test (**only in Appendix 3b**) was used to assess trait anxiety. Participants repeated the EPDS and STAI questionnaires at different times as shown in **Figure 2.1**.

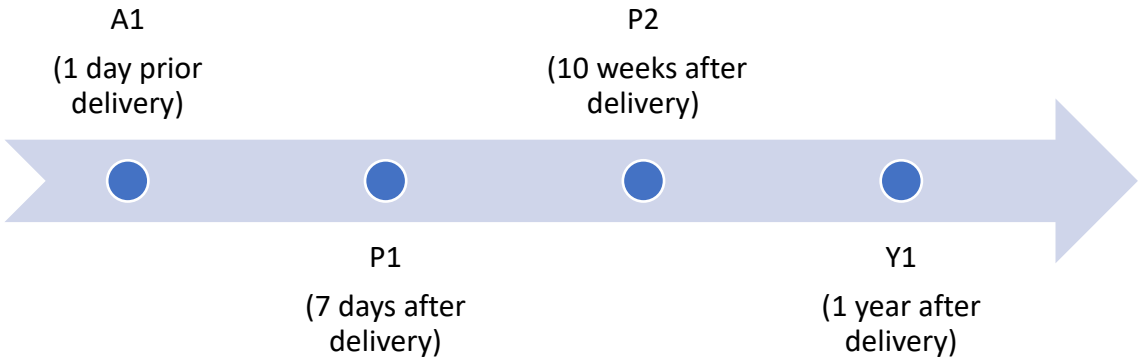


Figure 2.1. Timeline showing the different time points in which the questionnaires were completed. EPDS and STAI questionnaires were completed 1 day prior delivery (A1) and within 7 days (P1), 10 weeks (P2) and 1 year (Y1) postpartum.

For additional information including participant's medical notes, a data collection proforma was produced (**see Appendix 4**) in order to guarantee consistency across study sites. Data collection was carried out by N. Savory, A. Homes and personnel from the National Institute for Social Care and Health Research Clinical Research Centre (NISCHR CRC) at UHW and J. Beasley at RGH. Obstetric history included parity, previous stillbirths and previous low birth weight or macrosomic pregnancies. Information on the current pregnancy was also obtained including prescribed medication, intrauterine infection or complications, such as antepartum haemorrhage. Information regarding smoking, alcohol and drugs before and during pregnancy was recorded in the self-report questionnaire and compared with medical notes to counteract inconsistencies in reporting. Information about birth outcomes was also obtained including mode of delivery, complications during delivery, birth weight, foetal sex and gestational age. Lastly, infant wellbeing measurements were taken such as Apgar scores, arterial cord blood pH and NICU admission.

There are multiple parameters used to describe foetal growth adequacy. Apart from birth weight, statistic growth, SGA abdominal, head circumference measurements on scan, IUGR diagnosis, amniotic fluid index (AFI), Doppler anomalies and ultrasound growth centiles during pregnancy were growth variables recorded from the participant's medical notes. To calculate birth weight centiles it was necessary to consider maternal height, weight, parity and ethnicity in addition to infant birth weight, gestational age and foetal sex using the GROW bulk centile calculator (UK), version 6.7.5 (Gardosi and Francis 2014).

In relation to gestational diabetes mellitus, participant's medical notes included gestational age at which the first abnormal glucose tolerance test (GTT) was detected and the point at which GDM was diagnosed. Glucose tolerance test was compromised of a fasting glucose test and 2-hour glucose test. Previous GDM pregnancy was also recorded. Finally, GDM management pathway was recorded (lifestyle intervention and/or medication). Women at UHW and RGH are not normally screened for GDM unless they meet one or more risk factors such as previous family history of diabetes, previous history, ethnicity or BMI ≥ 30. Therefore, it was necessary to include a number of control participants known to have a normal GTT to the study cohort.

Preeclampsia diagnosis and the gestational age at diagnosis were recorded from the participant's notes. It was also recorded a previous PE pregnancy. First occurrence of hypertension and the appearance of proteinuria were key to differentiate between pregnancy-induced hypertension, which it is referred to hypertension without proteinuria, and preeclampsia, in which hypertension and proteinuria are both present. Treatment for preeclampsia as well as the gestational age at which the treatment began was noted.

Additional variables were studied in the Grown in Wales cohort including: saliva, maternal serum and mental health history. A sample of saliva was provided by the participants in the morning, a minimum of 30 minutes after their last meal. Cortisol concentration was determined in these samples as some studies have reported that mothers with depression show higher antenatal cortisol levels whereas other studies did not find such association (Seth *et al.* 2016).

In relation to maternal health history, previous mood disorders as well as time of diagnosis, medication type and whether SSRI were taken previously and in the present was recorded.

2.1.2.2. Placental dissection

Placental dissection was carried out by Anna Janssen as part of her PhD (**Thesis of A.B. Janssen**) and by trained midwives. Complications during or just after delivery were the main causes for failing to dissect a study participant's placenta.

Placental collection and dissection took place within 2 hours of delivery. Placenta were weighed and checked on both (maternal and foetal) sides for abnormalities. Chorionic villous samples were taken from the maternal side of the placenta, 1cm below the surface, midway between the medial and lateral edge at five different sampling sites (**Figure 2.2**), except when intraplacental variation of telomere length was analysed (**see Chapter 3**). Contamination with maternal decidual cells can introduce a significant confounder when study the placenta. To avoid this, the top cotyledon surface was removed and the villous trophoblast tissue bellow sampled.

Placental samples were washed in phosphate buffered saline, PBS (Life technologies) and stored in RNAlater (Sigma) at −80°C until needed. A placenta proforma was designed to record details of each placental dissection (**see Appendix 5**).

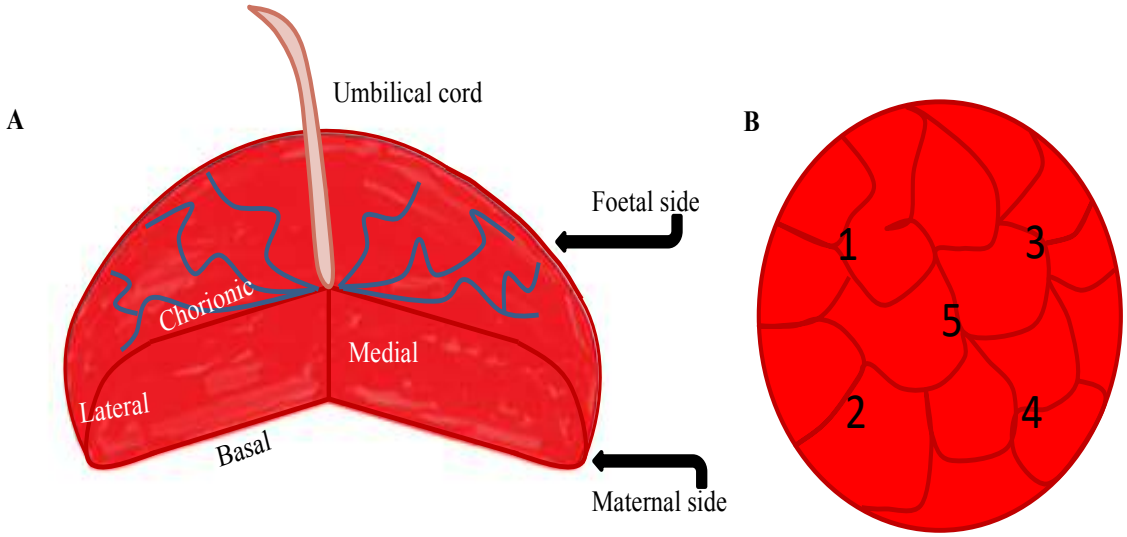


Figure 2.2. Placenta dissection protocol. A. Foetal and maternal sides of the placenta pointed. B. Maternal side of the placenta showing characteristic cotyledon surface where five evenly spaced sites were biopsied.

2.2. Methods

A flow diagram of STELA protocol using placental samples is depicted in **Figure 2.3**.

2.2.1. DNA extraction: proteinase K and phenol/chloroform protocol

Genomic DNA was isolated from approximately 40 mg of placental tissue by a standard proteinase K and phenol/chloroform protocol (Hogan *et al.* 1994). Placental tissue slices in a 2 ml Eppendorf were lysed in 1 ml lysis buffer (100 mM Tris pH 7.8, 50 mM EDTA, 0.2% SDS and 200 mM NaCl), followed by addition of 1/50 th volume 10 mg/ml proteinase K (final concentration 100 µg/ml). To lyse the cells it was necessary to pipetted up and down with a 1 ml blue Gilson tip and incubate overnight at 55°C. An equal volume of phenol (Sigma) was added to each tube and mixed thoroughly for 10 minutes at room temperature, left for 5 minutes and mixed again for another 10 minutes. The samples were then centrifuged at 12,000 rpm for 10 minutes. The aqueous phase was removed to a fresh tube and an equal volume of chloroform was added (Fischer), mixed thoroughly for 10 minutes at room temperature and centrifuged at 12,000 rpm for 10 minutes. The aqueous phase was removed to a fresh tube and 1/30 th volume of 3 M NaOAc (pH 6.0) and one volume of 100% ethanol was added and then mixed a few times at room temperature. At this stage, the DNA was seen as a flocculent white precipitate which could be scooped out with a yellow tip before rinsing the DNA in 70% ethanol, leaving the DNA in the tube and spinning down to remove ethanol traces. Immediately after that, the DNA was resuspended in 10 mM Tris-HCl (pH 8; Sigma), left at room temperature for 30 minutes and then stored at 4°C.

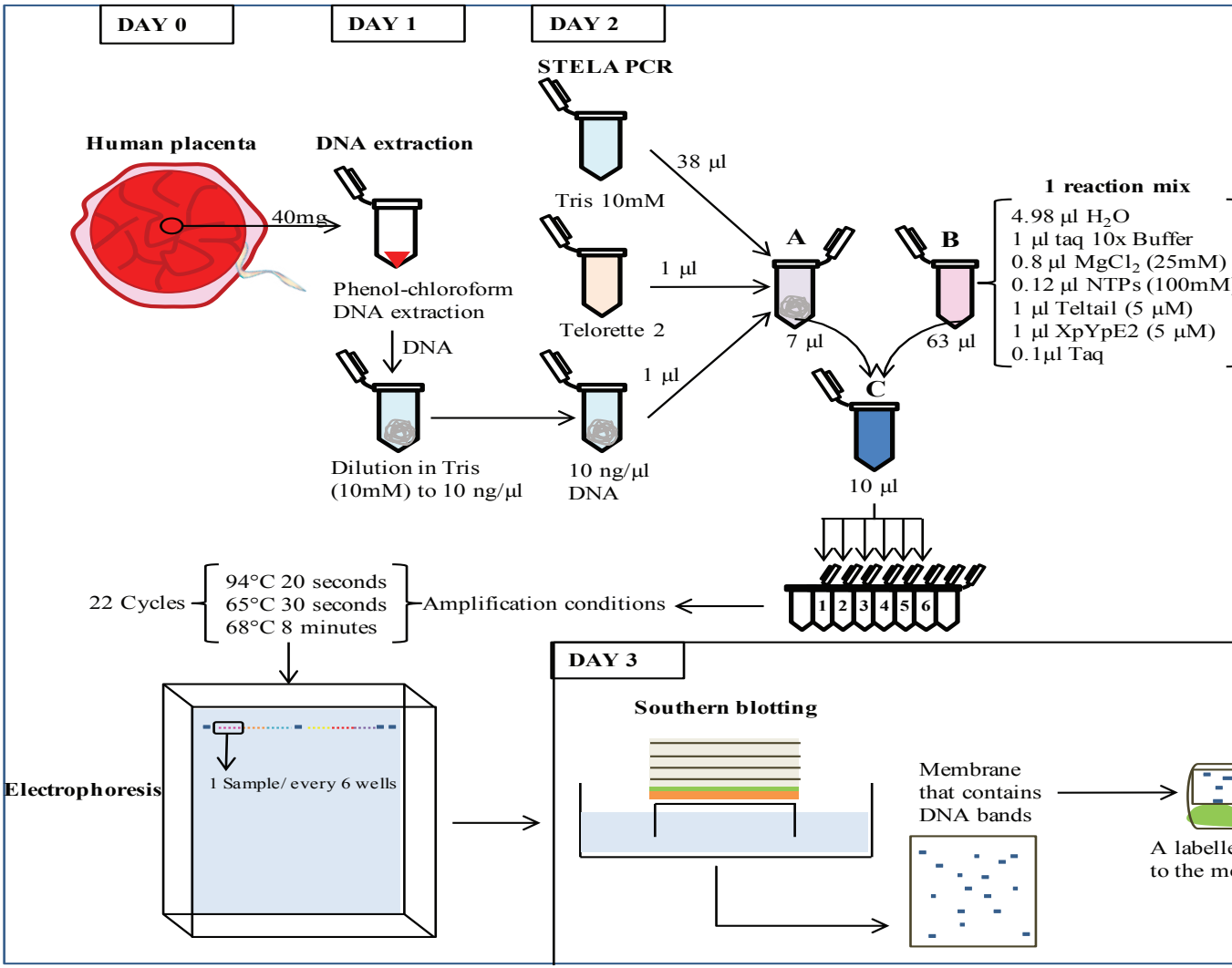


Figure 2.3. Flow diagram of STELA protocol using placental samples. Timeline to perform STELA technique

2.2.2. DNA quantification

To quantify DNA concentration, the Fluorescent DNA Quantitation Kit (BioRad) was used in triplicate for each sample. 1xTEN buffer (10x stock; 100 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.4) solution was made with double-distilled water. After that, a DNA intercalator fluorescent dye called Hoechst 33258 (1 mg/ml stock) was added to a final concentration of 0.1 µg/ml. To calibrate the fluorometer it was necessary to prepare a blank and a standard solution. For this purpose, 2 ml of 1xTEN buffer containing Hoechst was added to a cuvette and read (blank). For the standard, 10 µl of a reference tube with known DNA concentration (100 µg/ml) was added to a cuvette with 2 ml 1XTEN buffer containing Hoechst. DNA samples which are to be measured, were thawed at 37°C to guarantee that the DNA was homogenously dissolved in Tris-HCl. When this was done, 2 µl of DNA were added to 2 ml of TEN buffer containing Hoechst and mixed thoroughly before being quantified. In the case that DNA concentrations were over 250 µg/ml, DNA was further diluted using 10 mM Tris-HCl (pH 8) and measured again. The mean for the triplicate was calculated.

2.2.3. Oligonucleotides

A summary of the primers that were used can be found in **Table 2.4**. The oligonucleotides were synthesized by MWG-Biotech.

Table 2.4. A summary of the primers used during the study.

Application	Primer name	Oligonucleotide sequences
STELA	XpYpE2	5′-TTGTCTCAGGGTCCTAGTG-3′
	Telorette2	5′-TGCTCCGTGCATCTGGCATCTAACCCT-3′
	Teltail	5′-TGCTCCGTGCATCTGGCATC-3′
	17pSeq1rev	5′-GAATCCACGGATTGCTTTGTGTAC-3′

2.2.4. STELA PCR

STELA PCR was applied to examine telomere length distributions from specific chromosome ends in human placental samples. Firstly, a Telorette 2 (tel2)/DNA mix (3 µl DNA (10 ng), 1 µl Tel2 (10 µM) and 36 µl Tris-HCl (10 mM, pH 8.0)) was prepared. Then, 7 µl Tel2/DNA mix was mixed with 63 µl PCR reaction mix (**Table 2.5**), made for 6 reactions of 10 µl each. To avoid evaporation through the program cycles 10 µl of mineral oil was pipetted on the top of each reaction.

Table 2.5. STELA PCR reaction mix.

Component [Stock]	**1x reaction (µl)**	**[1x reaction]**
Double-distilled water	4.98	
Taq buffer 10x	1	1x
$MgCl_2$ (25 mM)	0.8	2 mM
dNTPs (100 mM)	0.12	1.2 mM
Telomere specific primer (5 µM)	1	0.5 µM
Teltail primer (5 µM)	1	0.5 µM
DNA/Tel2 mix	1	750 pg
Taq/PWO (10:1)	0.1	0.5
Total	10	

Afterwards, reactions were cycled using a Tetrad thermal cycler (Bio-Rad) as shown in **Table 2.6**.

Table 2.6. STELA PCR cycling conditions. *In the case of 17p the annealing temperature was 59°C. In addition, the temperature for short-term storage is 10°C.

Cycles	Temperature	Time
22	94°C	20 seconds
	*65°C	30 seconds
	68°C	8 minutes

For each batch of STELA analysis two positive controls for the STELA PCR were include comprising of DNAs from samples that were previously demonstrated to provide robust telomere length profiles. In addition, as a positive control for the Southern hybridisation STELA PCR products from a previous sample were included on the STELA gel.

2.2.5. Gel electrophoresis

2.2.5.1. Gel electrophoresis for STELA

STELA PCR products were resolved on 0.5% 40 cm Tris-acetate-EDTA (1xTAE) agarose gel. To make this gel, 2 g agarose (Roche) was dissolved in 400 ml boiling Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) and was cooled down. After that, 40 µl EtBr (10 mg/ml) was added to the solution. Gels were then submerged in 4°C TAE buffer and cooling for approximately 30 minutes before loading. Each STELA reaction was added 2 µl of a 6x Ficoll-based loading dye (5% bromophenol blue, 5% xylene, 15% Ficoll) and then 5 µl DNA marker mix (0.5 µl 1 kb (Stratagene), 0.5 µl 2.5 kb ladder (Bio-Rad), 1 µl 6x Ficoll and 4 µl H_2O per lane) or 5 µl STELA reaction was loaded into the gel and run at 120 V for 17 hours.

2.2.5.2. Visualisation of PCR products

The fluorescent dye EtBr is able to intercalate within the DNA and therefore under the UV light it is possible to visualise stained bands. The gel was cut above the largest visual band that correspond with the largest DNA ladder (25 kb) and bellow the smallest band that correspond with the smallest DNA ladder (250 bp).

2.2.6. Southern blotting

Agarose gels with the STELA PCR products were first washed twice (6 minutes/wash) in depurination buffer (0.25 M HCl) and then washed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 15 minutes. Then, a positively charged nylon membrane (Hybond-XL, GE Healthcare) was placed on the top of the agarose gel. This, allows the transference of negative-charged DNA fragments from the gel to the membrane by alkaline Southern blotting with denaturation buffer during at least 3 hours.

2.2.7. Probe synthesis and hybridisation

2.2.7.1. Probe synthesis

Radioactively labelled DNA probe was generated by random-hexaprime labelling. 25 ng of DNA probe was diluted to a total volume of 45 µl with TE buffer (10 mM Tris-HCl and 1 mM EDTA) and then heated to 96°C for 5 minutes to denature the DNA. The mix was then snap-cooled on ice for 5 minutes before added to the Rediprime Labelling system (GE Healthcare). In addition to this, 4 µl of [^{33}P]dCTP (3000 Ci/mmol) (Perkin Elmer) were added to the mix and heated at 37°C for a minimum of 1 hour. After that, 1 µl of radiolabeled DNA ladder (1/2.5 kb) was added along with 50 µl of double-distilled water. Radiolabelled probes were stored in the fridge at 4°C for a maximum of 2 weeks.

2.2.7.2. Hybridisation

Membranes were washed with water and placed into a pre-warmed hybridisation bottle (Thermo Scientific) with 15 ml church buffer (0.5 M Sodium phosphate dibasic (Na_2HPO_4), 7% SDS, 1% Bovine Serum Albumin (BSA), 1 mM EDTA) at 56°C in a hybridisation oven for 20 minutes. Meanwhile, the radiolabeled probe was denatured by heating it at 95°C for 5 minutes and 25 µl were added to each hybridisation bottle. The membranes were hybridised at 56°C overnight.

2.2.7.3. Removing unbound probe

To remove excess unbound probe membranes were washed using a high-stringency washing buffer (0.1x sodium chloride sodium citrate and 0.1% sodium dodecyl sulphate) several times and incubated at 56°C. Then, washed membranes were placed in a hybridisation oven at 56°C for 20 minutes to dry.

2.2.7.4. Visualisation of Radiolabelled Blots

Radiolabelled southern blots were placed in a cassette with a phosphoimager screen (GE healthcare) on top and left for 24 hours. Then, to detect the hybridised molecules, the phosphoimager screen was scanned using the Typhoon FLA 9500 biomolecular imager (GE Healthcare).

Radiolabelled probes were removed from the membranes using boiling 0.1% SDS for 1 h before hybridising with a different probe.

2.2.8. Gel analysis and statistics

Individual telomere lengths were determined using the ImageQuant software (GE Healthcare) and descriptive statistics about the telomere length distribution were generated. This analysis included the subtraction of the distance between the STELA primer binding site and the telomere repeating region: 408 bp for the XpYpE2 primer and 311 bp in the case of the 17pSEq1rev primer.

Statistical analysis from the data obtained from STELA was performed using SPSS 23.0 and GraphPad Prism 7. Specific statistical analysis are further detailed in each individual chapter.

Chapter 3: Characterisation of the human placenta: optimisation of STELA

3.1. Abstract

Telomeres are tandem repeat sequences located at the ends of eukaryotic chromosomes which are essential for genomic stability. Telomeres become shorter with each cell division or as a result of certain lifestyle factors. Shorter telomeres have been correlated with cellular dysfunction, senescence and ageing. In the placenta, several studies have used low-resolution techniques to assess telomere length. In this study, STELA was applied to provide high-resolution chromosome individual telomere length profiles. This technique was applied to ask whether more accurate and detailed information could be obtained when measuring individual telomere lengths in the human placenta. This work was published in 2017 (Garcia-Martin *et al.* 2017).

3.2. Introduction

The main determinants of an individual's telomere lengths are the initial setting of telomere length at conception and its rate of attrition during *in utero* development and the first decades of life (Benetos *et al.* 2013). Understanding the factors that determine telomere length at birth will help to clarify the relation between telomere length and disease in later life. Telomere length is considered to contribute to the development of cancer, several age-related diseases and premature ageing syndromes (Blasco *et al.* 2005). As discussed in the main introduction, studies in animals and humans suggest that *in utero* exposure to adverse conditions impact telomere length at birth (Entringer *et al.* 2011).

The placenta is a fetally derived organ that can be easily collected at birth. Placental integrity and its appropriate performance are essential for foetal growth, development and survival (Palma-Gudiel *et al.* 2018). Hence the suitability of this tissue, which has been exposed to the same environmental insults as the foetus, may provide a tool to assess the impact of environmental exposures on telomeres during pregnancy (Shammas *et al.* 2011). In the placenta, critically shortened and therefore dysfunctional telomeres may be caused by intrauterine exposure to specific maternal states and conditions (**Figure 3.1**).

Several methods have been applied to assess telomere length (Montpetit *et al.* 2014). Some of those, have been already used to assess telomere length in the placenta, including the two most widely used methods TRF and q-PCR. However, these methods provide relatively low-resolution telomere length information and thus subtle differences in telomere length distributions may not be apparent (Aubert *et al.* 2012). STELA, a single-molecule PCR based telomere length analysis technology can determine the full spectrum of telomere length from specific chromosome ends (Baird *et al.* 2003). Although STELA is relatively low throughput, it provides high-resolution telomere length distribution that can detect the presence of telomeres within the length ranges that can lead to senescence, apoptosis and telomere fusion (Abdallah *et al.* 2009; Hemann *et al.* 2001).

Figure 3.1. Suboptimal intrauterine conditions that have been correlated with telomere dysfunction. Preeclampsia, GDM, IUGR, high maternal pre-pregnancy BMI and air pollution exposure are examples of maternal states and conditions that contribute to telomere attrition in the placenta.

STELA has the potential to provide a richer and more detailed view of telomere length distributions in the placenta in a similar way that previously obtained in other cellular types or tissues (**Figure 3.2**).

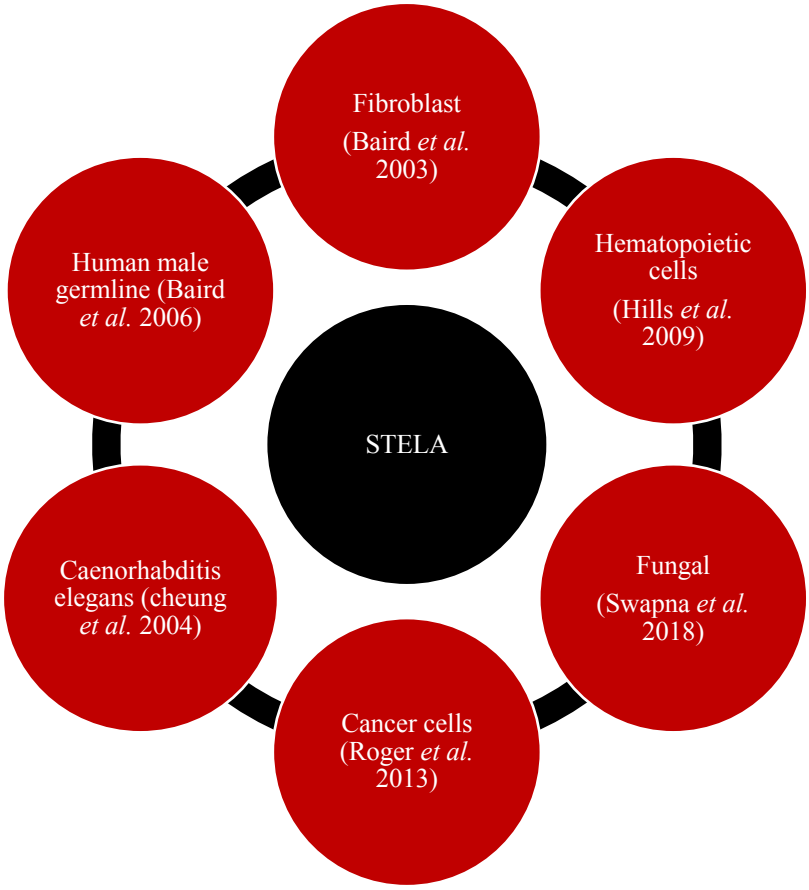


Figure 3.2. Applications of Single Telomere Length Analysis (STELA) in different cells, tissues and organisms.

3.3. Aims

The purpose of this chapter was to optimise STELA for placental analysis and to examine whether this technique might provide more detailed information on telomere homeostasis in the human placenta. STELA XpYp was used to examined telomere length with respect to sampling site, mode of delivery and foetal sex in the placenta from healthy singleton pregnancies.

3.4. Chapter specific methods

General methods are described in **Chapter 2**. Specific methods related to the optimisation of STELA in human placental samples are described below. In this Chapter, telomere length was determined in human placentas from two cohorts: the Welsh Pilot Study and the Grown in Wales Study.

3.4.1. STELA protocol optimisation

Genomic DNA was isolated from approximately 40 mg of placental tissue (same quantity used in **A.B. Janssen thesis** to isolate RNA from human placental samples) and STELA was performed afterwards. For one participant, DNA was extracted from placental tissue and STELA was used with two different DNA concentrations (**Figure 3.3**). Subsequent STELA blots were conducted with 30 ng, as this concentration gives a minimum of 36 bands (minimum threshold quantity previously established).

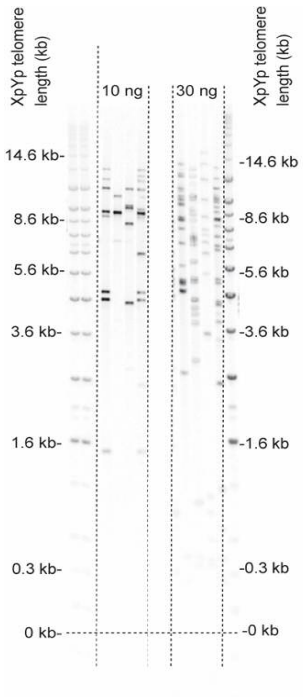


Figure 3.3. XpYp STELA on human placental samples. DNA was obtained from one placental sample and STELA was performed using two different concentrations (10 and 30 ng). The sample consist of four STELA PCR reactions.

Only placenta from healthy singleton pregnancies with no recorded medical disorders and babies within the normal weight range were used in this study. To analyse intraplacental variation of telomere length, three placental samples (near to the cord insertion, middle and lateral edge) were biopsied from each of the foetal, middle and maternal layers as described by Janssen *et al.* (2015) (**Figure 3.4**). This approach was applied to five term placentas from elective C-section deliveries (N = 5 samples; 9 individual biopsies per sample).

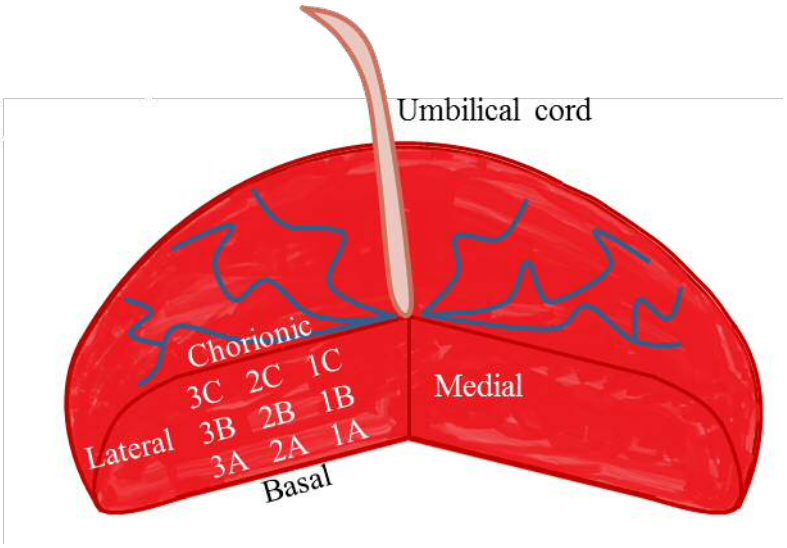


Figure 3.4. Sampling site dissection. Adapted from "correlation between sampling site and gene expression" by Wyatt *et al.* Placenta (2005). From the medial to the lateral edge the tissue was divided into three fractions and each of these fractions was in turn divided into three sections from the chorionic plate to the basal plate, obtaining 9 sampling sites per placenta for a total of 5 placentas.

To analyse the effect of labour and foetal sex on telomere length, the standard approach of taking chorionic villous samples from the maternal side of the placenta at 3-5 different locations midway between the medial and lateral edge was used, and genomic DNA prepared from combined samples to minimise variation introduced by single site sampling. Placental telomere length was compared between 10 labour (either vaginal delivery or emergency C-section) and 11 non-labour (elective C-section) samples to determine whether telomere length vary with mode of delivery. Telomere length was compared between 17 female and 20 male placentas from elective C-section deliveries.

3.4.2. Statistical analysis

All analysis was carried out using GraphPad Prism 7.02 (2016) for Windows with a P value < 0.05 considered statistically significant. Normal distribution was assessed using D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov Test. Correlation between gestational age at delivery and placental telomere length was assessed using a simple linear regression analysis and comparison of mean placental telomere length between smokers and non-smokers was assessed by unpaired t-test. For non-normally distributed data, associations between telomere length and sampling site, mode of delivery or foetal sex were assessed using a nonparametric Mann-Whitney two tailed test.

3.5. Results

3.5.1. Participants demographics

XpYp STELA was applied to a total of 42 individual placenta to assess whether the telomere length distributions varied according to intra-placental variation, mode of delivery and foetal sex differences. A summary of participant demographics is given in **Table 3.1**.

Table 3.1. Main characteristics of the study participants. Mean (SD)/Range or number (%) is shown. Note: due to missing values, some numbers do not add up to 100%.

	Study Participants (N=42)
Maternal characteristic	
Ethnicity	
Caucasian	39 (93%)
Parity	
Primiparous	14 (33.3%)
Multiparous	24 (57.1%)
Maternal age	30 (5.31) / 20-40
Maternal BMI	26 (5.15) / 17-38
Birth Outcome	
Mode of Delivery	
Vaginal	7 (16.7 %)
Emergency C-Section	3 (7.1 %)
C-Section	32 (76.2 %)
Birth weight (g)	3525 (368) / 3010-4580
Gestational age (weeks)	39 (1.15) / 37-42
Placental weight (g)	686 (146) / 396-1138
Gender	
Female	22 (52.4%)
Male	20 (47.6%)

Participants were mainly Caucasian with a BMI just above of what is considered normal during pregnancy (18.5-24.9) and babies within the normal birth weight range. Gestational age and smoking during pregnancy were assessed in relation to placental telomere length. There was no significant correlation between gestational age at delivery and placental telomere length (β = 0.210, P = 0.18) (**Figure 3.5A**). In addition, there was no significant differences in placental telomere length between smokers and non-smokers (p = 0.08) (**Figure 3.5B**).

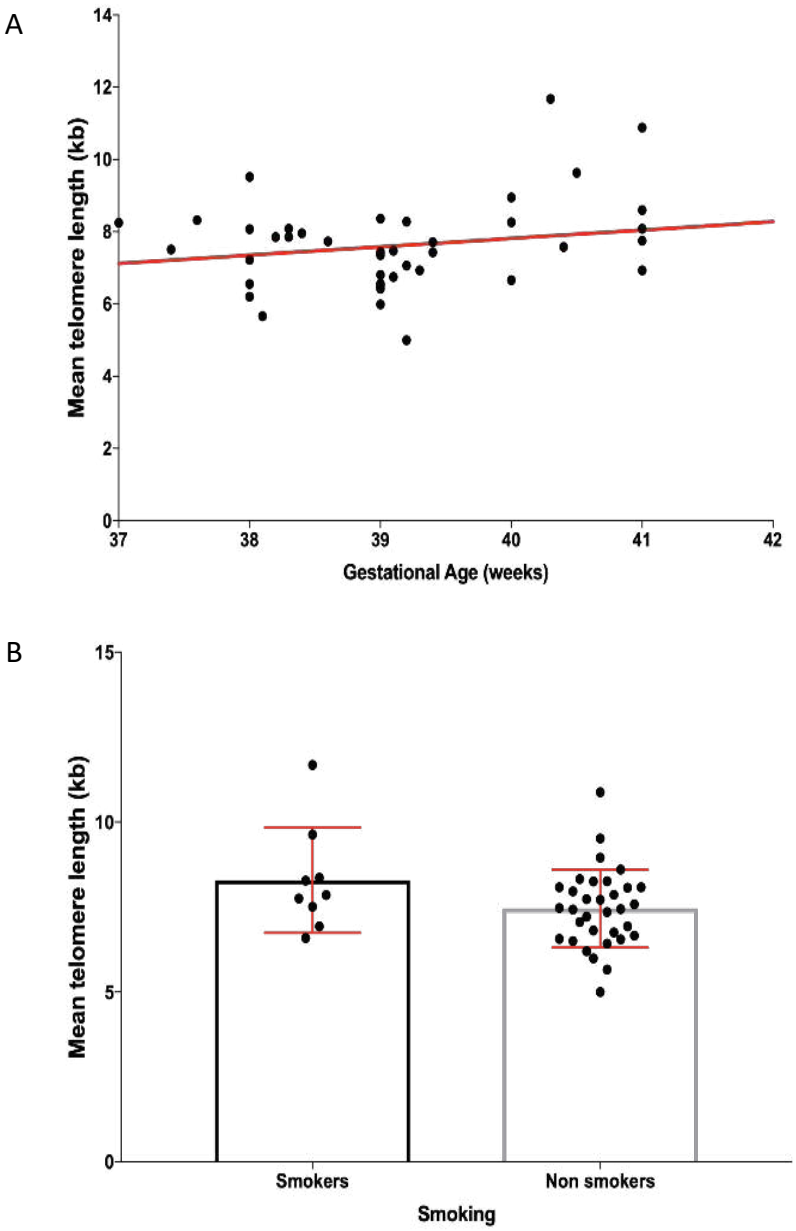


Figure 3.5. Gestational age and smoking in relation to placental telomere length. (**A**) **Correlation between** gestational age at delivery and placental telomere length (β = 0.210, P = 0.18). Simple linear regression analysis was used to assess statistically significant differences. *P < 0.05. (**B**) Comparison of mean placental telomere length between smokers and non-smokers (P = 0.08). Mean telomere is presented (±SD). Unpaired t-test was used to assess statistically significant differences. *P < 0.05.

3.5.2. Placental telomere length by STELA

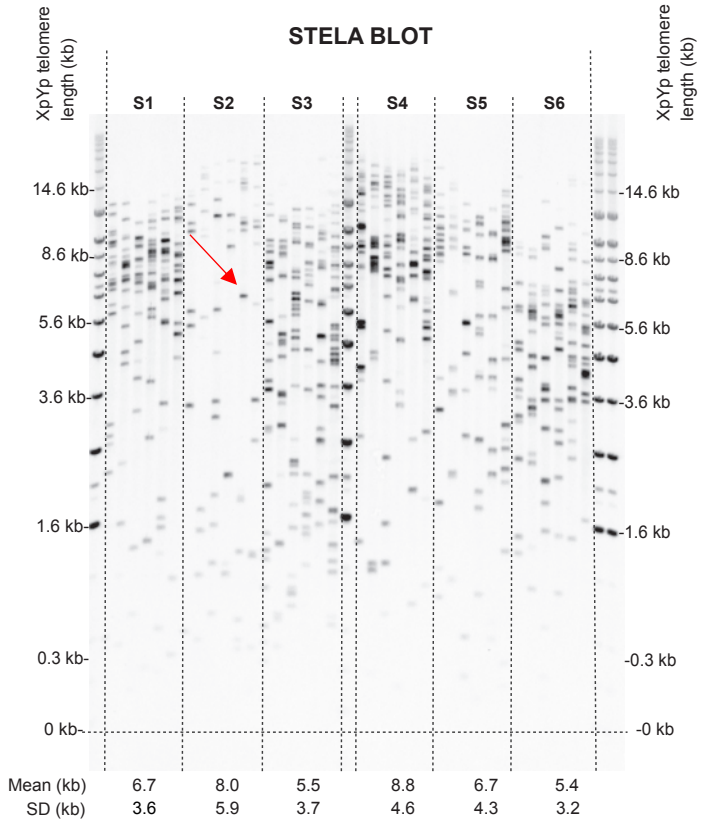


Figure 3.6. **Representative STELA blot.** STELA of six genomic DNA samples from six placentas from six participants (S1-S6). DNA was extracted from placental tissue, which is composed by several types of cells and each single input DNA molecule (red arrow) was amplified at a single chromosome end. An estimated of 70 amplifiable molecules were added to the master mix and aliquoted into 6 separate PCR tubes to give a representative sample of the telomere length distribution. Mean telomere and SD are presented below each sample (±SD). For instance, S1 mean and SD (6.7 ± 3.6) is the result of sum of each individual telomere molecule divided by the number of molecules calculated once the distance between the binding site of the telomere adjacent primer to the start of the telomere was subtracted.

3.5.3. Intraplacental variation in telomere length

It has been previously shown that site of dissection can impact observed gene expression profiles (Janssen *et al.* 2015). Telomere length variation with dissection site could introduce a significant confounder when comparing across different cohort studies.

For five participants, DNA was prepared from tissue biopsies from 9 different sites across the placenta (**Figure 3.4**) and telomere length was compared using STELA to determine whether telomere length varied between sampling sites. Intraplacental variation of mean telomere length is shown in **Figure 3.7**.

Results from one participant are shown in **Figure 3.8A** with a graphical representation shown in **Figure 3.8B**. There were no significant differences in mean telomere length between the inner (medial) and the outer (lateral) sampling sites (7.58 kb vs. 7.52 kb; $p > 0.99$) (**Figure 3.8C**) or between the maternal (basal) and the foetal (chorionic) side (7.39 kb vs. 7.52 kb; $p > 0.99$) (**Figure 3.8D**).

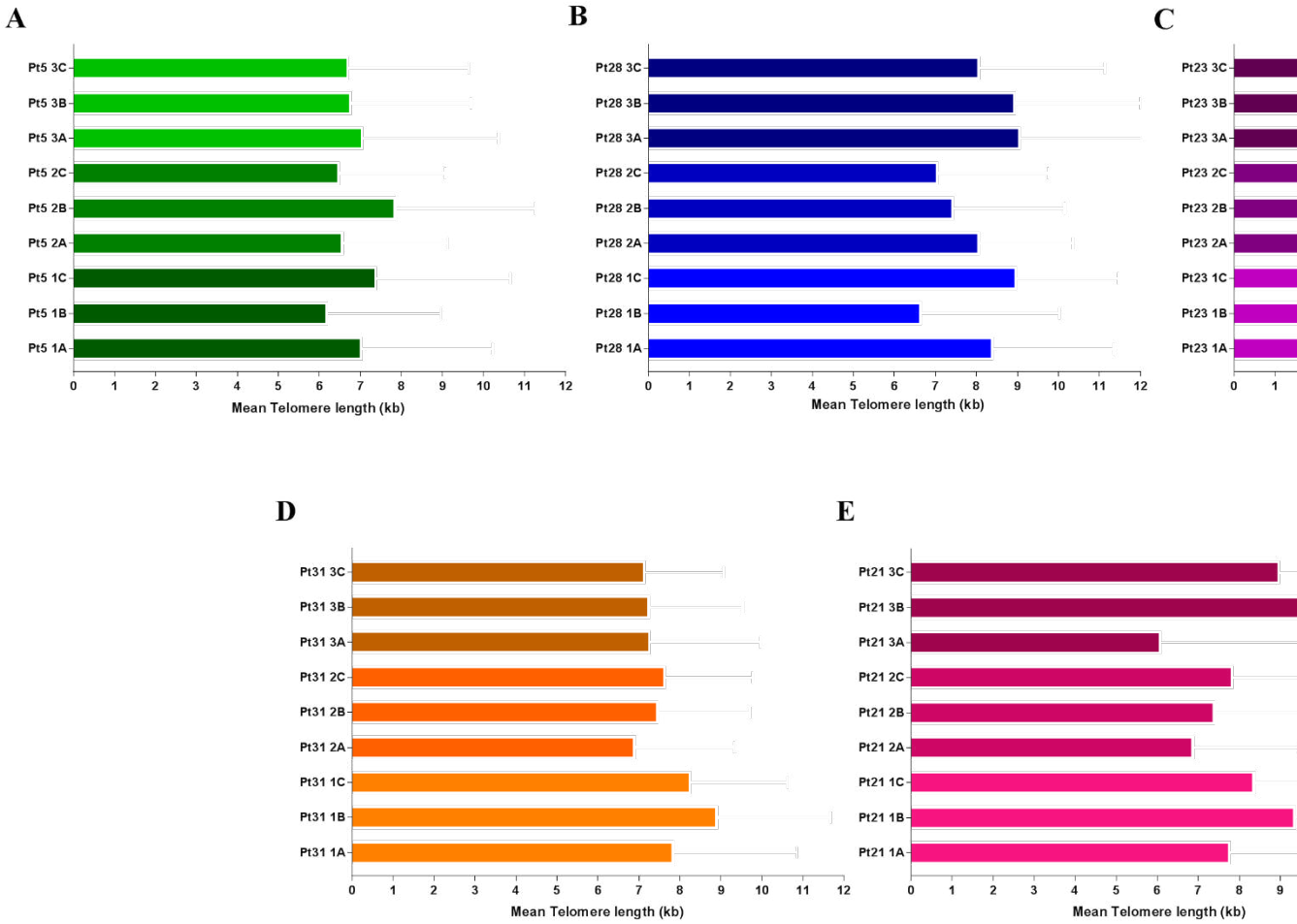


Figure 3.7. Intraplacental variation of telomere length. Following the protocol for sampling site dissectio were taken from the medial to the lateral edge (1-3) and from the basal to chorionic plate (A-C). Graphs sh each sampling site.

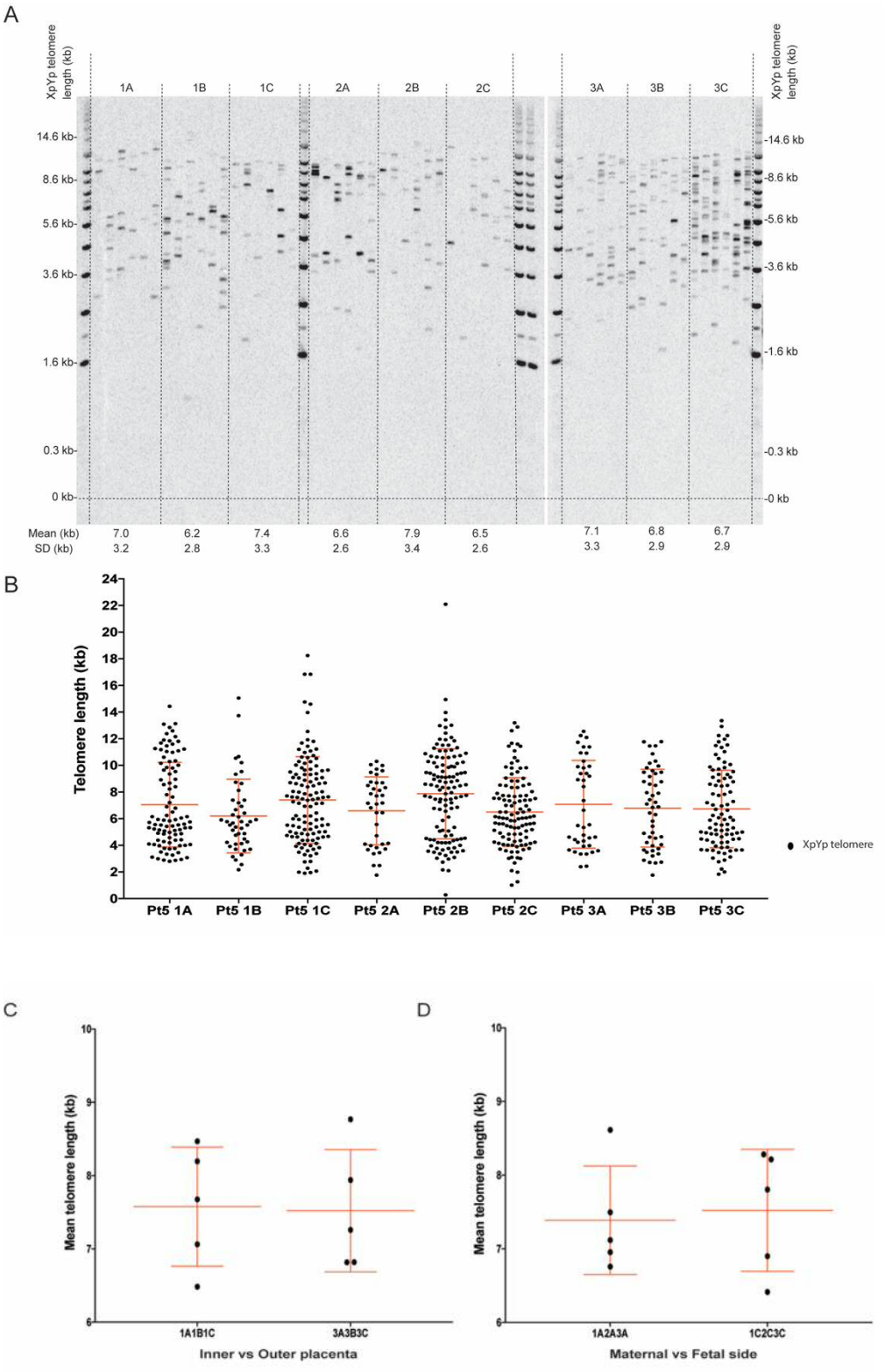


Figure 3.8. Intraplacental variation of telomere length. (**A**) STELA of nine genomic DNA samples from one placenta from one participant. (**B**) Graphical representation of data in **A**. (**C**) Comparison of mean telomere length between inner (1A,1B,1C) and the outer (3A,3B,3C) sampling sites ($P > 0.99$). (**D**) Comparison of mean telomere length between the maternal (1A,2A,3A) and the foetal side (1C,2C,3C) ($P > 0.99$). Mean telomere length is presented (±SD). Five placentas were used for this study (N=5). Mann-Whitney two-tailed test was used to assess statistical significant differences. *$P < 0.05$.

3.5.4. Effect of mode of delivery on telomere length

Beside site of dissection, it has been previously reported that gene expression obtained from placental samples can vary with mode of delivery (Janssen *et al*. 2015). Importantly, a recent study found using qPCR, that term labour amniotic fluid samples exhibited a higher telomere-repeat content than those not in labour at the point of sampling (Polettini *et al*. 2015). We applied STELA to labour (either vaginal delivery or emergency C-section; N = 10) and non-labour (elective C-section; N = 11) samples (**Figures 3.9A and 3.9B**). There was no significant effect of labour on mean placental telomere length (7.85 kb vs. 7.61 kb; P = 0.97) (**Figure 3.9C**).

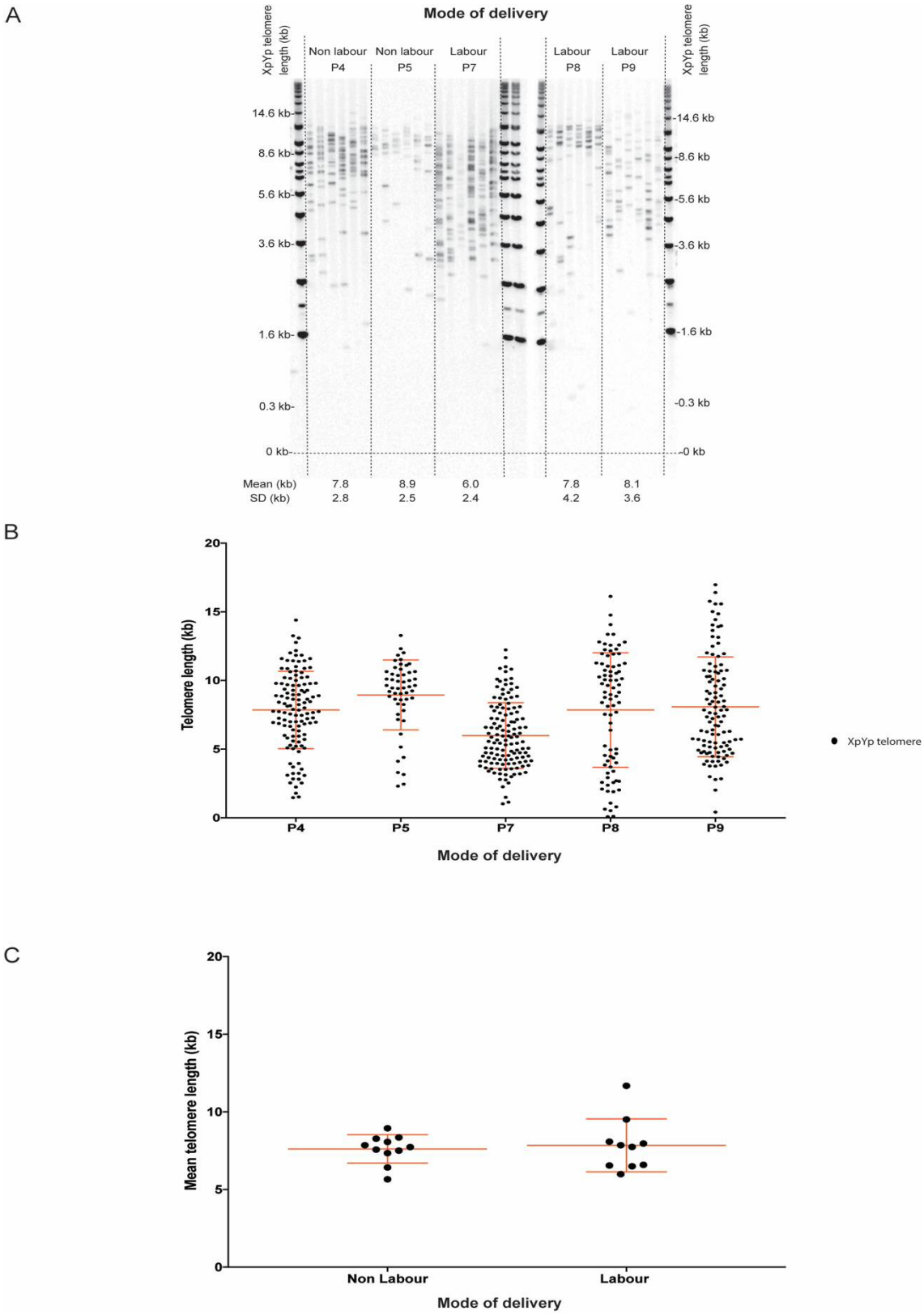


Figure 3.9. Effect of labour on telomere length. (**A**) STELA of five genomic DNA samples from five placentas from five participants. (**B**) Graphical representation of data in **A**. (**C**) Comparison of mean telomere length between non-labouring and labouring placental samples (P = 0.97). Mean telomere length is presented (±SD). Twenty one participants were used for this study (N = 11+10). Mann-Whitney two-tailed test was used to assess statistical significant differences. *P < 0.05.

3.5.5. Effect of foetal sex on telomere length

To examine whether foetal sex impacts telomere length, STELA was applied to N = 17 female and N = 20 male placenta to ask whether there were any sex differences (**Figure 3.10A and 3.10B**). Consistent with observations in the adult population (Gardner *et al.* 2014), mean telomere length of female placenta was 300 bp longer than those observed in male placenta, but this difference was not significant (7.85 kb vs. 7.55 kb; P = 0.75) (**Figure 3.10C**). Then, telomere length was compared between male and female placenta, but only in elective C-section samples. No significant difference was found (N = 13 female and N = 14 male; 7.58 kb vs. 7.68 kb; P = 0.79) (**Figure 3.10D**).

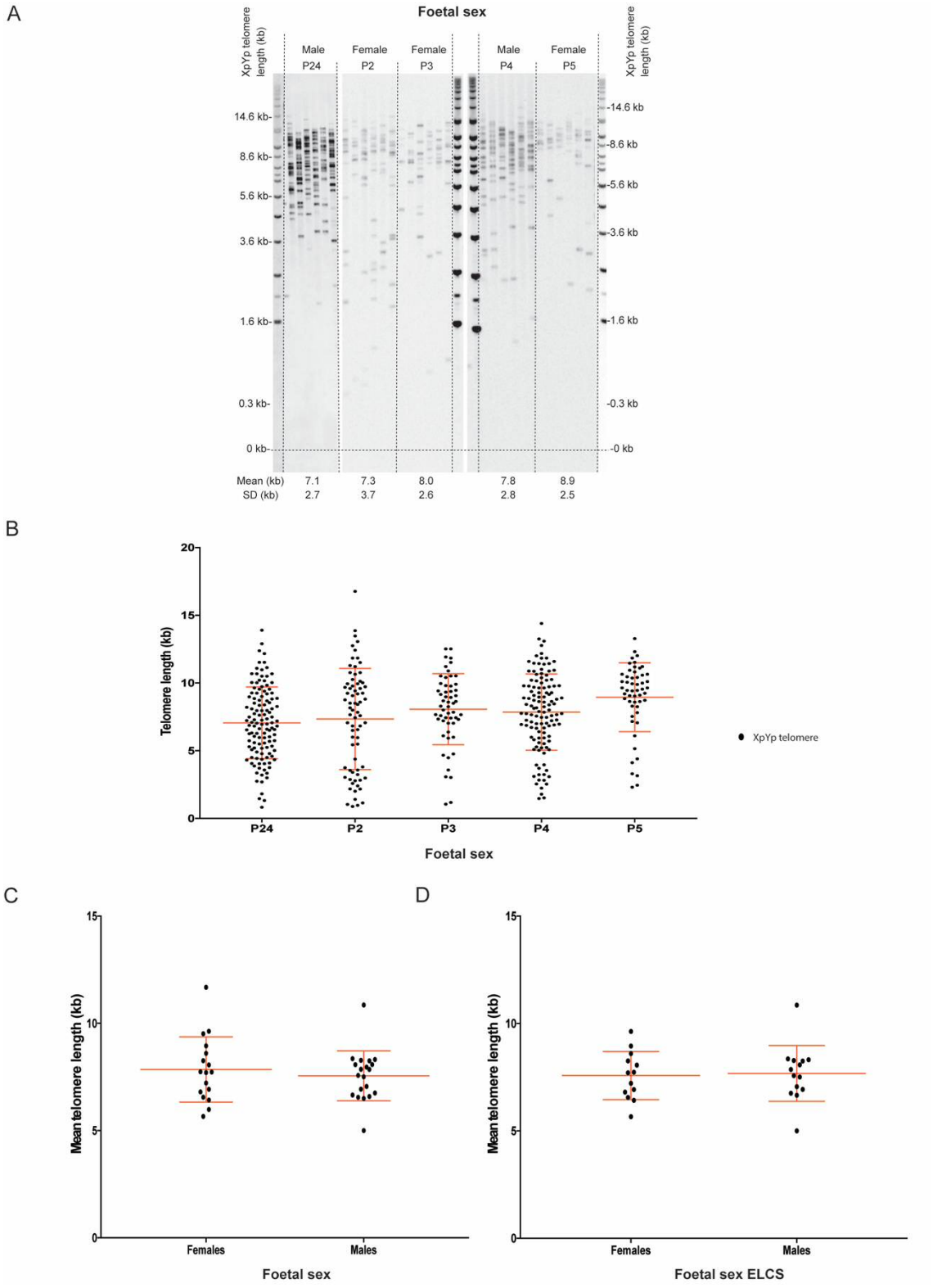


Figure 3.10. Effect of foetal sex on telomere length. (**A**) STELA of five genomic DNA samples from five placentas from five participants. (**B**) Graphical representation of data in **A**. (**C**) Mean telomere length by foetal sex (P = 0.75). (**D**) Mean telomere length for only the elective C-section placentas (P = 0.79). Mean telomere length is presented (±SD). Thirty seven participants were used for this study (N = 17+20) and twenty seven when only elective C-section samples were analysed (N = 13+14). Mann-Whitney two-tailed test was used to assess statistical significant differences. *P < 0.05.

3.5.6. Telomere length heterogeneity

The mean telomere length was 7.7 kb ranging from 5.0 to 11.7 kb across all the samples (N = 42) and SD was 1.3. This data was used to define short (μ − 1σ) and long telomeres (μ + 1σ) (**Figure 3.11**).

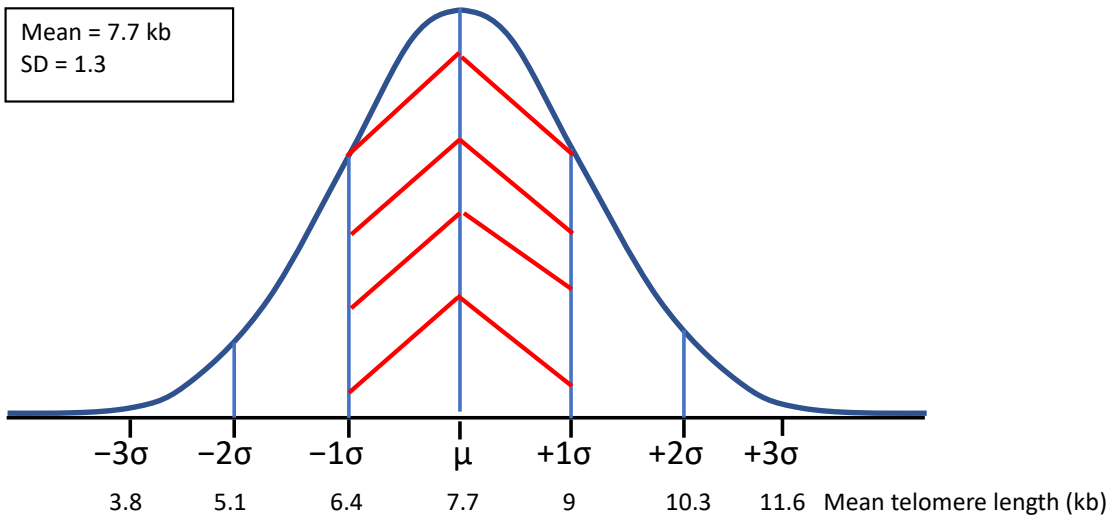


Figure 3.11. Normal distribution representation of the data used in this chapter. Mean telomere length and derived values were used to define short (μ − 1σ) and long telomeres (μ + 1σ).

In addition to determining the absolute length of specific telomeres, STELA revealed considerable telomere length heterogeneity within the placental samples, a feature that had not been previously reported (**Figure 3.12**). In contrast, Lin *et al.* (2010) performed STELA in patients at different stages of chronic lymphocytic leukaemia (CLL). XpYp STELA showed different telomere distributions in B cells from normal (SD ≈ 3 kb), and stage A (SD ≈ 2 kb), stage B (SD ≤ 2 kb) and stage C (SD ≤ 1 kb) of CLL samples. This data showed a reduction in mean telomere length profiles and SD as the disease advanced, suggesting that telomere erosion contributes to disease progression.

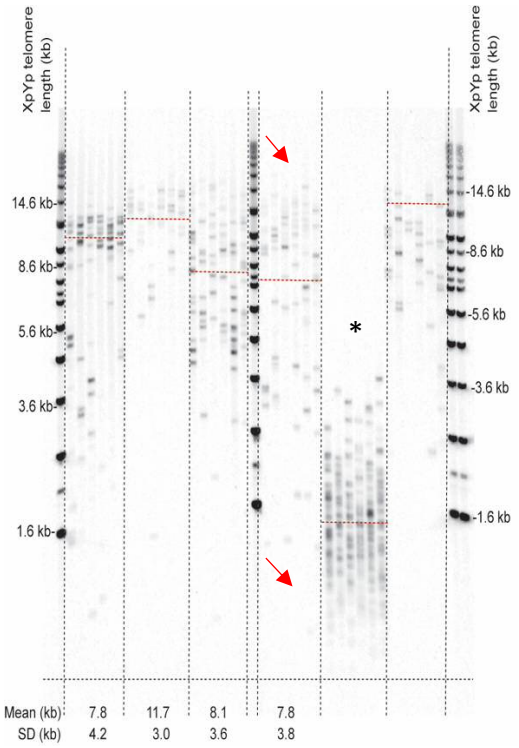


Figure 3.12. XpYp STELA on human placental samples. Red arrows points towards the longest (TL>15 kb) and the shortest telomere (TL<1 kb) for a specific sample. The asterisk (*) indicates telomere distributions from cancer cells used as a control.

STELA enables the detection of those telomeres within the length ranges that can lead to senescence as depicted in **Figure 3.12**, where the shortest telomere is approaching a dysfunctional length (<500 bp).

3.6. Discussion

3.6.1. The placenta as a potential diagnostic tool

Environmental insults and/or stress molecules present during the 9 months of pregnancy may impact both the foetus and the placenta. This makes the placenta a unique tissue for the non-invasive study of the impact of prenatal exposures. This transient organ is the perfect tool to study the signatures left by those environmental insults that threaten a healthy pregnancy.

Since telomere length is considered a biomarker of the replicative history of human cells and shorter telomeres in the placenta have been correlated with some complications of pregnancy (**Figure 3.1**), understanding inter-individual variation in telomere length distributions at birth and how these may be modulated by environmental insults and stress will contribute to a greater understand of factors that influence telomere biology.

A study in adults reported that telomere length was proportional across different cell types, such as leukocytes, muscle, skin and fat within an individual. All constitute highly and minimally proliferative tissues but they appeared to display similar age-dependent telomere attrition. Differences in telomere length between high and minimally proliferative tissues are probably establish early in life. Intraindividual synchrony in telomere length across the somatic tissues have been described in humans and other mammals (Daniali *et al.* 2013).In the foetus, it has been previously reported synchrony in telomere length among multiple organs (Youngren *et al.* 1998). Consistent with these findings, Okuda *et al.* 2002 found that telomere length within organs in the newborn, such as white blood cells, umbilical artery and skin were highly correlated. They also hypothesised that environmental and genetic determinants, which begin exerting their effect *in utero*, are the main source of telomere length variation among adults. Since placental tissue consist of cells of foetal origin and has previously been reported that foetal tissues exhibit telomere length synchrony, the placenta may provide a tool to study the effect of environmental exposures on the foetus.

3.6.2. STELA XpYp

Some of the most widely used techniques are not able to provide highly accurate telomere length measurements or detect subtle changes in telomere dynamics and distribution (Vera and Blasco 2012). This is the first time on which STELA has been used to determine placental telomere length in a number of samples providing high-resolution chromosome specific telomere length profiles in this tissue.

This study measured telomere length with respect to sampling site, mode of delivery and foetal sex. Factors such as gestational age and smoking, were also assessed in relation to telomere length as previous studies showed an association. Unlike Gielen *et al.* (2014), who reported a 25% reduction in placental telomere length during the third trimester, this study found no association between gestational age and placental telomere length. In addition, several studies have found an association between maternal smoking and double-strand DNA break damage in the syncytiotrophoblast of the placenta (Slatter *et al.* 2014) and foetal telomere shortening (Salihu *et al.* 2015; Mirzakhani *et al.* 2017). In this study no significant differences in placental telomere length were found when comparing smokers and non-smokers. STELA was applied to the XpYp telomeres, located at the end of the 'pseudoautosomal' region. This region has an obligate crossover in each meiosis, and thus segregates independently of sex. STELA assays for other unique telomeres such as 2p, 9q, 11q and 17p exist (Garcia-Martin *et al.* 2017). However, previous work did not report differences in telomere distributions between sex and autosomal chromosomes (Roger *et al.* 2013) and thus this work was restricted to the XpYp telomere only. This is a potential limitation of the study since it is possible, although unlikely, that the sex chromosome telomeres are behaving differently to autosomal telomeres. Using STELA, high-resolution telomere length profiles were generated from placental biopsies. No significant difference was observed in telomere length in response to sampling site, mode of delivery or foetal sex.

3.6.3. Intra-placental variation of telomere length

Previous research showed intra-individual synchrony in telomere length across different somatic tissues in an adult individual (Daniali *et al.* 2013) and in newborn (Youngren *et al.* 1998; Okuda *et al.* 2002). However, these studies concerned inter-organ synchrony, but within an organ there are numerous cell types, which despite of their developmental connection, have specific signalling pathways and distinct replicative histories. In this context, it has been previously reported cell type-specific intra-cellular responses in the form of changes in telomere length to common external factors, as exhibited by peripheral blood mononuclear cell (PBMC) (Lin *et al*. 2016). Another study in humans showed telomere length variation across five different regions on the brain (Mamdani *et al.* 2015). This arises the intriguing possibility of a variable rate of cellular ageing of the same cell types within the same organ.

Martens *et al.* 2016 using quantitative real-time PCR method found that intra-placental variability for telomeres was of 11%. Another study using TRF technique assessed mean telomere length to control to examine variability within the placenta (Allsopp *et al.* 2007), they reported no significant differences; our study is consistent with these findings.

3.6.4. Telomere length variation with respect to mode of delivery

The natural and physiological process of senescence also affects the cells that compose the foetal membrane. When this happens, telomere fragments are released into the amniotic fluid. In this regard, Polettini *et al.* (2015) reported that term labour amniotic fluid (AF) samples had a higher telomere-repeat content than term/not in labour AF as assed by qPCR. The presented in this chapter showed no correlation between mode of delivery and telomere length by STELA. Mode of delivery was further divided into two different categories: labour which includes vaginal delivery, emergency caesarean and forceps, and non-labour which refers exclusively to elective caesareans. Labour is the natural and physiological end of a pregnancy whilst an elective caesarean is a planned procedure where labour itself has not yet initiated. Consequently, none of the signalling cascades which normally occur prior to the birth have started. It has

been hypothesised (Phillippe 2015) that the loss of telomeric repeat sequences could lead to placental trophoblast and chorion cell apoptosis, which in turn results in cell-free foetal DNA release. This induces an inflammatory response that ultimately trigger the onset of parturition. However, our data does not support this hypothesis. It is remarkable to note that the telomere length distributions observed across the samples showed that the cells within placenta contain telomeres within the length ranges observed in senescent cells (Baird *et al.* 2003). These short and heterogeneous telomeres support a major proliferative history of the placenta to generate this organ in a brief period of time.

3.6.5. Telomere length variation with respect to foetal sex

No significant differences in placental telomere length between males and females was detected, either for the full set or just within the placenta from elective caesareans. This data supports a previous study which shown no difference in placental telomere length between female and male in third trimester live-born twins (Gielen *et al.* 2014). Okuda *et al.* (2002) also observed no correlation between mean TRF and foetal sex when examining different foetal tissues such as umbilical cord, foreskin or white blood cells. However, a few studies have reported that telomere length varies between sexes in several tissues using Southern blotting (Cherif *et al.* 2003; Benetos *et al.* 2014). Cherif *et al.* (2003) using rat as a model, observed that males had shorter telomeres in all organs except the brain. A similar trend has been reported for human monozygotic, dizygotic and opposite-sex twins where females showed longer leukocyte telomere length compared to males (Benetos *et al.* 2014). In line with these findings, a more recent study (Wilson *et al.* 2016) using qPCR, reported a correlation between placental TL and sex, with the telomeres in term female placenta being longer compared to male placentas. Adult women can have longer telomeres as assessed with Southern blot but this divergence has not been universally reported in all studies using different techniques. Cell types used included whole blood, peripheral blood mononuclear cells, lymphocytes or granulocytes (Gardner *et al.* 2014).

Nevertheless, evidences suggest that differences in growth and survival of the foetus may be mediated by sex-specific function of the placenta, which response

differently to an adverse environment (Clifton 2010). Therefore, although no differences in placental telomere length was noticed in the control placentas analysed with respect to foetal sex, it is possible that placental telomere length may be impacted to different extents in male and female placenta by pregnancy complications such as gestational diabetes or prenatal depression.

3.6.6. Telomere heterogeneity in the placenta

We observed a very high level of telomere length heterogeneity within the samples not previously reported for the placenta. Telomere-induced replicative senescence seems to be a heterogeneous process. It is just necessary the presence of the shortest telomere to signal senescence (Bourgeron *et al.* 2015). As a result of the end replication problem, intra- and intercellular heterogeneity is generated (Tomaska and Nosek 2009). It is important to note that the replication of the parental telomere produces two new telomeres with different lengths. Sources of telomere heterogeneity include those mechanisms responsible of telomere length, such as shortening and lengthening by telomerase, which occurs independently in each telomere, and stochastic events (Tomaska and Nosek 2009). Stochastic events has been previously singled out as the cause of single telomere length variation in fibroblast cells (Baird *et al.* 2003). These events can elucidate why human fibroblast replicative life span is so heterogeneous (Martin-Ruiz *et al.* 2004). Although telomeres shorten with every cell division, ultra-short telomeres seldom occur. It is believed that it is the effect of massive stochastic events on very few telomeres what cause senescence, rather than progressive telomere shortening (Baird *et al.* 2003). Telomere length heterogeneity is suggested to be an adaptive value that allows cells to maintain viable and flexible populations (Tomaska *et al*. 2009).

In the context of the placenta, a transient organ that last only 9 months, it reflects the replicative history of this tissue. The placenta ages as pregnancy advances and replicative senescence is a consequence of this physiological phenomenon (Sultana *et al.* 2018; Kalmbach *et al.* 2013). In this line, Gielen *et al.* (2014) reported in a twin study a 25% reduction in placental telomere length during the 3rd trimester from around 14 kb at 28 weeks to around 11 kb at 42 weeks (term). A progressive telomere shortening up to 42 weeks suggests ongoing cell division

and indicates that telomere attrition may play a role in ageing of the placenta. In our study all the placentas were term placentas and differences in telomere length throughout pregnancy could not be evaluated. A reduction in telomerase activity during maturation of the placenta has also been described by Kyo *et al.* (1997).

Telomere shortening has been correlated with oxidative damage and ageing in syncytiotrophoblast cells as pregnancy advances. Likewise, labour has also been associated with senescence-related pathways induced by telomere shortening (Sultana *et al.* 2018). During 9 months the placenta will suffer approximately 36 round of cell division (Simpson *et al.* 1992). Assuming no telomerase activity and a rate of telomere erosion of about 90 bp per cell division, that will equates to 3 kb or so of telomere attrition.

3.6.7. Summary

This is the first study applying STELA to measure placental telomere length profiles in relation to sampling site, mode of delivery and foetal sex. In this study we found no significant differences by these principles, which indicates that this technique has a wider application in pregnancy cohort studies irrespective of study design.

A limitation of this study is the sample size. Although comparable to the term placenta sample size in the Wilson *et al.* (2016) study, may be small-scale to discover very subtle changes in telomere length. It is likely that other methodological differences may account for these observations. Nevertheless, this study only examines telomeres within the X and Y chromosomes and it may be possible that sex chromosomes lack a sex difference regarding telomere length. Rather, there may be disparities between populations. Further statistical analysis and additional modelling considering other variables, such as gestational age, in a bigger sample is required to confirm these conclusions.

A very high level of heterogeneity was observed within samples not previously described for the placenta. Given that this is an ephemeral endocrine organ of pregnancy that lasts a mere 9 months, it is remarkable that such heterogeneity is generated. This technique reveals a much richer picture of telomere dynamics which will be important for future studies exploring the relationship between telomere length with birth outcomes and maternal lifestyles that will be discussed in the following chapters.

Chapter 4: Impact of gestational diabetes mellitus on placental telomere length

4.1. Abstract

Telomere attrition has been reported in cases of GDM and T2DM suggesting that uncontrolled hyperglycaemia may be a cause or consequence of telomere impairment. In this study, STELA was applied to examine whether the prenatal adversity GDM was associated with telomere shortening in the human placenta.

In this chapter, 100 term placentas (37-42 weeks) obtained after an elective caesarean from healthy singleton pregnancies were used. STELA was applied to genomic DNA obtained from combined placental biopsies. Differences in telomere length were assessed between the control group (Glucose tolerance test negative) (N = 69), GDM lifestyle intervention group (diet and exercise as treatment) (N = 14) and GDM treated group (metformin and/or insulin as treatment) (N = 17).

There were no significant influences of GDM on telomere length or telomere length heterogeneity in placenta from female infants. However, placental telomeres from male infants exposed to GDM in medically untreated pregnancies were significantly shorter than placental telomeres from control male infants (P = 0.02) and infants whose mothers were diagnosed with GDM and treated with metformin and/or insulin (P = 0.003).

Our data support a foetal origin for shortened telomeres in males exposed *in utero* to poorly controlled hyperglycaemia and further suggest that treatment with metformin may provide protection in males. Targeted treatment of GDM pregnancies where the foetus is known to be male may be an effective strategy for alleviating adverse outcomes.

4.2. Introduction

GDM is a form of diabetes that can be defined as any degree of glucose intolerance with onset or first recognition during pregnancy. This includes both the cases in which the condition is resolved with delivery, as well as those where the condition persisted after pregnancy as stated by the American Diabetes Association (2014). Currently, one in seven births are affected by gestational diabetes and these numbers are likely to grow in the upcoming years, as reported by the International Diabetes Federation (2017).

Insulin resistance occurs during normal pregnancy as a physiological adaptation of the mother to ensure nutrients supply to the developing foetus (**Figure 4.1**) (Sonagra *et al.* 2014). This refers to a reversible resistance to the action of insulin on glucose uptake and use thereof. Tissues such as liver, adipose tissue and muscle lose efficacy to respond to normal circulating concentrations of insulin. As the pregnancy progresses to the third trimester so does insulin resistance, with a fall of 50% in insulin sensitivity with respect to the normal expected value (Sonagra *et al.* 2014). This drop is thought to be influenced by the action of placental production of TNFα, placental lactogen, growth hormone, oestrogen, progesterone and cortisol (Sonagra *et al.* 2014; Agha-Jaffar *et al.* 2016). All of this causes increased maternal glucose levels. Thus, to maintain normoglycaemia in the mother, beta cells of the islets of Langerhans in the pancreas increase insulin production restricted to the first phase insulin response in early pregnancy stages (about 120% increase) and 3-3.5 times higher first and second phase insulin responses by 36 weeks of pregnancy (Agha-Jaffar *et al.* 2016). The resultant changes in maternal carbohydrate and lipid metabolism includes more use of fats for energy by the mother and spares carbohydrates for the foetus (Sonagra *et al.* 2014).

In some pregnancies, beta cells cannot cope with the new great insulin demands leading to a persistent hyperglycaemia state and subsequently to diabetes mellitus (Sonagra *et al.* 2014).

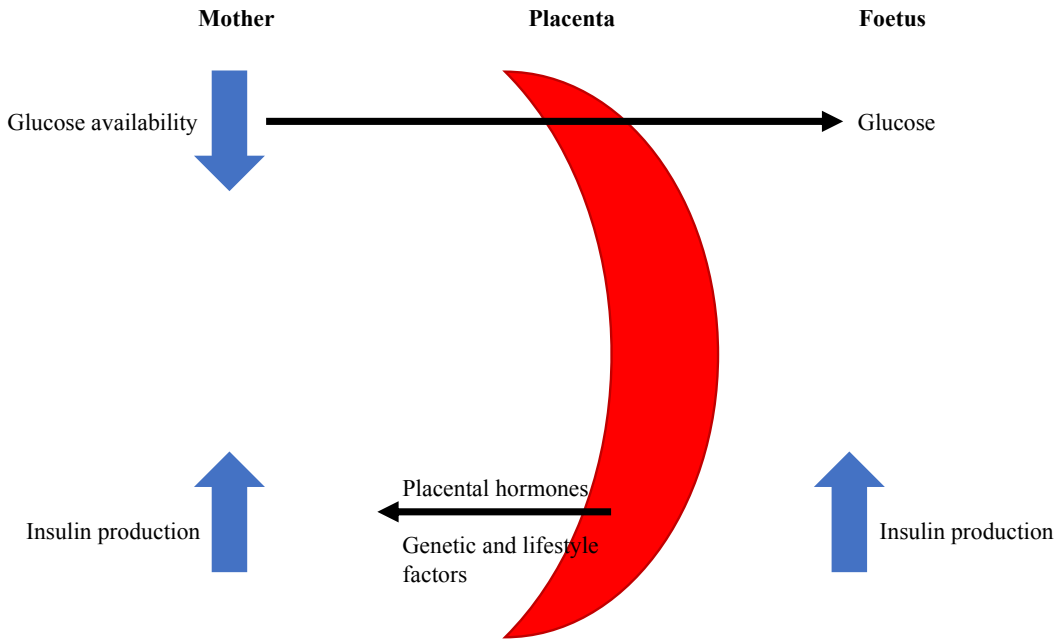


Figure 4.1.Insulin resistance as a physiological adaptation.

GDM constitutes a risk factor for developing diabetes mellitus. In line with this, around 30-50 % of women with a previous history of GDM develop diabetes within five to ten years after delivery (Buchanan *et al.* 2012).

The development of GDM involves genetic factors such as certain polymorphisms and lifestyle factors, with overweight and obese women at high risk (Chu *et al.* 2007). Risk factors for developing GDM include: non-modifiable risk factors (non-white ethnicity, advance maternal age, hormonal unbalance, previous GDM pregnancies and family history of T2DM) and modifiable factors (pre-pregnancy obesity, dietary factors and physical activity levels) (Agha-Jaffar *et al.* 2016).

A diabetic pregnancy has consequences for both maternal and foetal outcomes (**Table 4.1**). Beyond perinatal complications, there are also long-term effects of the exposure to high levels of glucose *in utero*.

Table 4.1. Summary of perinatal and maternal outcomes. Foetal and maternal complications of a diabetic pregnancy (Fraser *et al.* 2014; Reece 2010).

Perinatal outcomes	**Maternal outcomes**
Macrosomia	Gestational hypertension
Hypoglycemia	Cardiovascular disease
Respiratory distress syndrome	Metabolic syndrome
Polycythemia	Preterm birth
Hyperbilirubinemia	Caesarean
Cardiomyopathy	
Congenital abnormalities	
Sudden infant death	

Uncontrolled GDM increases the likelihood of delivering a macrosomic or large for gestational age (LGA) baby (birth weight above the 90th percentile) along with complications at delivery including caesarean section (Brown *et al.* 2017). Offspring of mothers with GDM have been reported to be developmentally harmed, as they are less likely to reach timely developmental milestones (Girchenko *et al.* 2018). They are also more likely to be large for gestational age, as during pregnancy have been receiving excess glucose, which in turns increase insulin production and stimulates foetal fat deposition and skeletal growth (Fraser *et al.* 2014). Furthermore, offspring exposed to *in utero* hyperglycaemia have a five-fold increased risk of suffering T2DM later in life (Holder *et al.* 2014).

As well as a problem for the foetus, GDM has been associated with health issues in the mother. Women diagnosed with GDM are also seven times more likely to develop diabetes mellitus type 2 later in life (Kim 2014). In addition, a recent study (Hinkle *et al.* 2016) suggested a bidirectional relationship between GDM and depression. Women with depression symptoms early in pregnancy have an increased risk of suffering GDM and women diagnosed with GDM have an

increased risk of postnatal depression. Postpartum is a very sensitive period in which depressive symptoms may arise. It is suggested that activation of the sympathetic nervous system along with the HPA axis results in increased cortisol levels (Hinkle *et al.* 2016). Cortisol acts at tissue level, ultimately leading to insulin resistance and GDM.

There are a number of risk factors for developing GDM. One is obesity, is also a risk factor for the development of T2DM (Law and Zhang 2017). Generally, women who develop GDM have a higher BMI and this is a well-known trigger of low-grade inflammation, which contributes to the onset of hyperglycaemia. Hyperglycaemia is known to also increase the production of superoxide molecules, which may worsen the condition (Law and Zhang 2017). Given the increasing prevalence of maternal obesity worldwide, understanding the consequences of GDM has even greater significance.

As stated by The National Institute for Health and Care Excellence (NICE, 2018), for the diagnosis of GDM, women with one or more risk factors for developing GDM (see **Figure 4.3**) are offered a test. There are two types of test: a fasting glucose test and a 75 g 2-hour OGTT (oral glucose tolerance test). A fasting plasma glucose of 5.6 mmol/l or above or a 2-hour plasma glucose of 7.8 mmol/l or above is considered a positive test for GDM. For the treatment of GDM, glucose monitoring, dietary modification, lifestyle intervention including practicing regular exercise and when necessary pharmacotherapy can be prescribed. Provided that fasting plasma glucose does not exceed 7 mmol/l, changes in diet and exercise will be suggested. However, the addition of metformin to the treatment pathways is recommended when changes in diet and exercise within 1-2 weeks are insufficient to meet the blood glucose target. In case metformin is contraindicated or not well tolerated, insulin is used instead. Combination of metformin and insulin along with changes in lifestyle (diet and exercise) are recommended in those cases in which blood glucose levels are not levelled.

Telomeres are considered as a biological clock, suggestive of cumulative cell damage within an organ (Blackburn, 2000). Several studies showed that individuals with T2DM had shorter telomeres (Nan, Ling and Bing 2015). This implies the possibility that diabetes contributes to telomere erosion and

impairment. In this line, a recent study in the 9-to 16-year old offspring of mothers with GDM, revealed a negative association between blood leukocyte telomere length and offspring insulin levels and HOMA-IR, which is a method for assessing β-cell function and insulin resistance. This suggests that short telomere length may represent a risk factor and could anticipate the development of T2DM and associated metabolic disorders. GDM creates an *in utero* environment that exhibits remarkable inflammation and oxidative stress, the major cause of telomere attrition (Hjort *et al.* 2018). Consistent with this, it is possible that such telomere attrition found in blood leukocytes, it is also shown by other cells such as pancreatic β-cells.

Be born with shorter telomeres represents an additional risk in the disease progression of diabetes (Kirchner *et al.* 2017). Telomere attrition may lead to premature β-cell senescence. When this happen, there is a decreased in the β-cell mass within the islet of Langerhans in the pancreas and the secretion of insulin and glucose tolerance may be jeopardized (Elks and Scott 2014; Kuhlow *et al.* 2010). Interestingly, Zhao *et al.* (2014) also reported that short telomere length is associated with later in life development of T2DM, regardless of other known T2DM risk factors. Telomere attrition as a result of prenatal adversities, such as GDM, may contribute to the increased incidence of chronic diseases later in life such as T2DM (Entringer *et al.* 2018) (**Figure 4.2**).

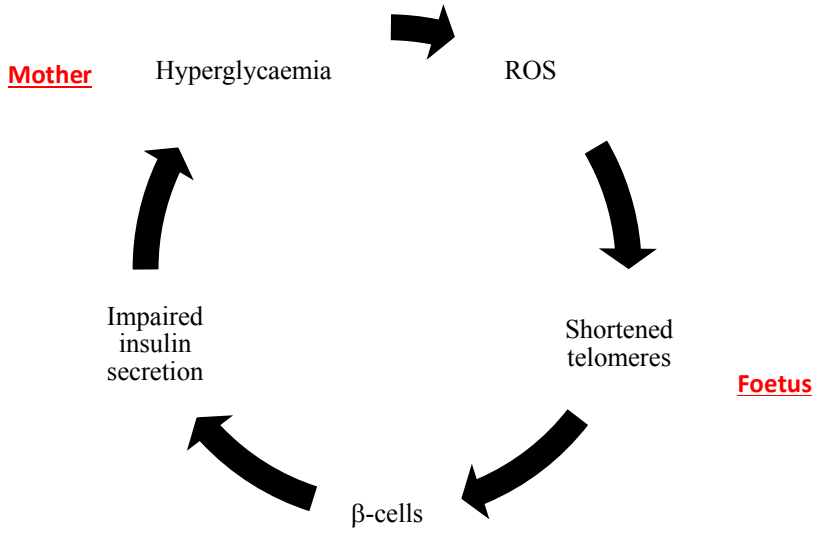


Figure 4.2. A plausible hypothesis relating GDM and T2DM.

Despite the clinical relevance of GDM, only few studies have studied the impact of this disease on placental telomeres, and none has applied high-resolution techniques to assess it. A summary of the studies reported or not a link between GDM and telomere length is depicted in **Table 4.2.**

Table 4.2. Associations between telomere length and GDM. Related information with respect to category study, number of samples, utilised tissue, technique, telomere outcome and author is shown.

Exposure	Study	Nº	Tissue	Technique	Telomere Outcome	Author
Offspring of GDM mothers	Human	N=439 GDM N= 469 Control	Peripheral blood DNA	q-PCR	Telomere attrition	Hjort *et al.* 2018
Offspring of GDM mothers	Human	N= 82 GDM N= 65 Control	Foetal leukocytes	q-PCR	Telomere attrition	Xu *et al.* 2014
Offspring of GDM mothers	Human	N= 16 GDM N=16 control	Placenta Cord blood leukocytes	Q-FISH Immunochemistry and RT-PCR Q-FISH	Telomere attrition Decrease telomerase expression No correlation found	Biron-Shental *et al.* 2015, 2016
Offspring of GDM mothers	Human	N=31 GDM N=69 Control	Placenta	STELA	Telomere attrition	Garcia-Martin *et al.* 2018
GDM mothers	Human	N=25 GDM N= 50 Control	Leukocytes	q-PCR	No correlation found	Harville *et al.* 2010
Offspring of GDM mothers	Human	N= 71 GDM N= 81 Control	Cord blood mononuclear cells	Flow-FISH Telomerase PCR-ELISA	No correlation Increase telomerase activity	Cross *et al.* 2010

4.3. Aims

The purpose of this chapter was to apply STELA for measuring telomere length profiles in placenta from pregnancies complicated by gestational diabetes.

4.4. Chapter specific methods

Methods have been described in **Chapter 2**. For helping with the interpretation of the results presented at the end of this chapter, methods related to participant recruitment (**Figure 4.3**), placental biopsies and statistical analysis are described below. In this chapter, telomere length was measured in human placenta from two cohorts, the Welsh Pilot Study and Grown in Wales Study.

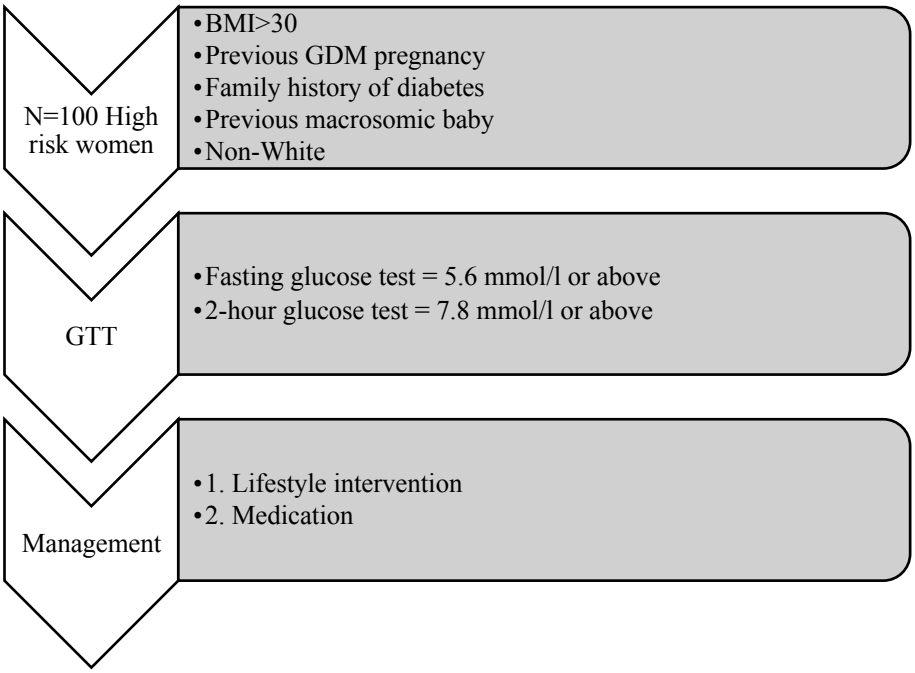


Figure 4.3. Summary of study design.

High-risk women (N=100) were identified through standard clinical practice and offered glucose tolerance testing, usually at 24-28 weeks of pregnancy. This glucose tolerance test consisted of two measurements: a fasting glucose test and a 2-hour glucose test. A fasting plasma glucose level of 5.6 mmol/l or above, or a 2-hour plasma glucose level of 7.8mmol/l or above was considered hyperglycaemia and therefore those women were offered medical advice.

Changes in lifestyle were first offered including dietary advice and a recommended 150 minutes of moderate exercise, such as brisk walking or swimming, per week. If the hyperglycaemia persisted, women were prescribed medication, which was usually metformin but, in some cases, involved insulin injection when metformin was not well tolerated, or blood sugar levels were

dangerously high. From the N = 100 participants who were offered the glucose tolerance test, 31 were classified as GDM pregnancies with a note being made of treatment pathways. The 69 remaining women were classified as non-GDM pregnancies and used as controls for this study.

To analyse the effect of GDM on telomere length, XpYp STELA was applied to genomic DNA prepared from placental biopsies combined from several biopsies of the same placenta, taken from the maternal side of term placentas (37-42 weeks) 1 cm below the surface and at five different locations midway between the medial and lateral edge.

4.4.1. Statistical analysis

All statistical analysis was performed using SPSS 23.0 for Windows. Data are expressed as means with standard deviation, or as numbers (%). Differences between groups were assessed using one-way analysis of variance (ANOVA) for normally distributed characteristics (maternal age, BMI, weight gain, birth weight and placental weight) and the Kruskal-Wallis test for characteristics that followed a non-normal distribution (gestational age in weeks, fasting glucose and 2 hours glucose). $\chi 2$ test was used for categorical data. Relationships between the main dependent variable and other variables were analysed by simple linear regression, or by hierarchical regression allowing the entry of multiple independent variables if significant at $P \leq 0.05$. Differences in telomere length between the control group, the GDM lifestyle intervention group and the GDM mothers treated with metformin and/or insulin were tested by Analysis of Covariance (ANCOVA). ANCOVA was conducted to adjust for maternal ethnicity and $P \leq 0.05$ was considered statistically significant. This was followed by group comparisons using Fisher's least significant difference (LSD) method.

4.5. Results

4.5.1. Maternal, birth and metabolic characteristics of the study participants

Mothers diagnosed with GDM (N = 31) and control mothers (N = 69) were mainly White, representative of the local population (**Table 4.3**). Selected controls were matched for ethnicity (Yuen *et al.* 2015), maternal age (Lao *et al.* 2006) and obesity (Sebire *et al.* 2001), as these are risk factors for GDM. Apart from fasting plasma glucose and 2 hours plasma glucose levels, only gestational age was significantly different between the groups (P = 0.034) with mothers diagnosed with GDM, on average, giving birth one week earlier than the control mothers.

Table 4.3. Maternal, birth and metabolic characteristics in the control, lifestyle intervention and medication group. Mean (SD) or number (%) is shown. Note: due to missing values, some numbers do not add up to 100%. *P* values were assessed using ANOVA, Kruskal-Wallis test or χ^2 test.

Study participants	**Lifestyle intervention group (GDM)** **N=14**	**Medication group (GDM+M)** **N=17**	**Control group** **N=69**	***P* VALUE**
Maternal characteristics				
• Ethnicity (White)	13(93%)	13(76%)	62(90%)	0.26
• Parity ➢ Primiparous ➢ Multiparous	3(21.4%) 5(35.7%)	0(0%) 12(70.6%)	7(10.1%) 51(74%)	0.42
• Maternal age	34(3.6)	32(3.9)	31(5.8)	0.23
• Maternal BMI	31(7.8)	33(6.3)	31(6.3)	0.26
• Weight gain	11.2(13)	10.8(5.5)	13.3(8.3)	0.65

• Mode of delivery ➢ Elective C-section	14(100%)	17(100%)	69(100%)	NA
• Smoking	0(0%)	1(5.88%)	8(11.6%)	0.43
• Socio-economic status ➢ Lowest income	0(0%)	2(11.8%)	9(13%)	0.1
Birth outcome ➢ Birth weight (g)	3734(503)	3697(382)	3734(562)	0.96
• Gestational age (weeks)	38(0.6)	38(0.6)	39(0.7)	**0.034**
• Placental weight (g)	691(149.8)	709(119.1)	723(133.4)	0.69
• Gender ➢ Male ➢ Female	 10(71.4%) 4(28.6%)	 10(58.8%) 7(41.2%)	 38(55.1%) 31(44.9%)	0.53
Metabolic characteristics • Fasting plasma glucose (mmol/l) • 2 hours glucose (mmol/l)	 5.2(1.2) 8.1(2.6)	 5.9(1.1) 9.5(1.4)	 4.55(0.5) 5.46(1.3)	 **<0.0001** **<0.0001**

4.5.2. Relationship between telomere length and potential confounders

It has been previously demonstrated using XpYp STELA, foetal sex, mode of delivery or sampling site does not impact on telomere length and/or telomere distributions in a healthy pregnancy (Garcia-Martin *et al.* 2017) (**see Chapter 3**). While there was no significant association (–) ($P > 0.1$) between placental telomere length and variables including maternal BMI, weight gain, socio-economic status, maternal age, gestational age, birth weight and smoking, within the control group a significant association (+) was found between placental telomere length and maternal ethnicity ($P = 0.02$) (**Figure 4.4**). This relationship has been previously reported (Jones *et al.* 2017).

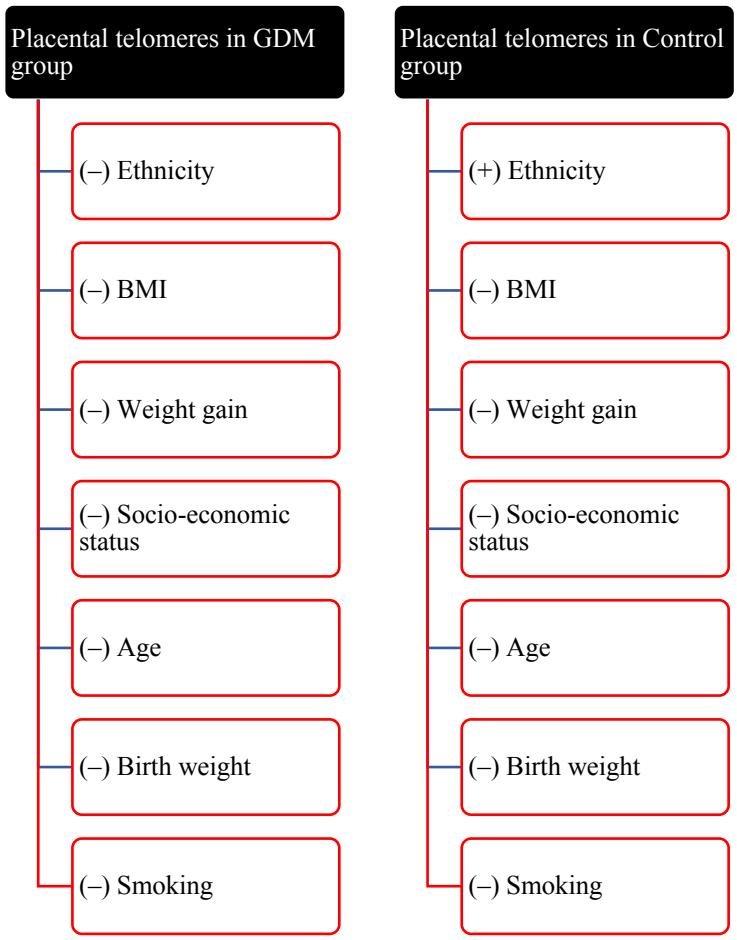


Figure 4.4. Relationship between placental telomere length and potential confounders in GDM and control mothers. Relationships between the main dependent variable and other variables were analysed by simple linear regression, or by hierarchical regression allowing the entry of multiple independent variables if significant at $P \leq 0.05$.

4.5.3. Telomere length is associated with intrauterine exposure to poorly controlled hyperglycaemia in male placenta

A comparison between the control group and the GDM group did not show significant differences in telomere features. However, direct examination of the telomere-length profiles identified GDM participants with remarkable shortened placental telomeres (**Figure 4.5**). These samples exclusively came from women diagnosed with GDM who were not prescribed medication (lifestyle intervention group).

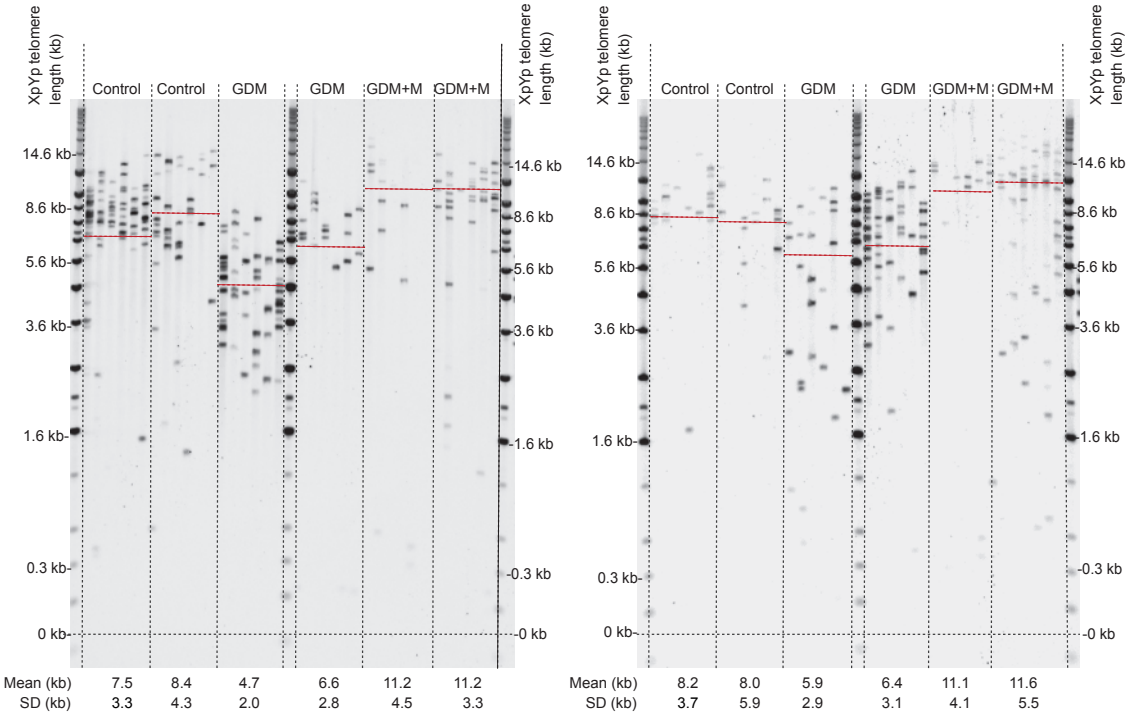


Figure 4.5. Representative STELA comparing placental samples from control, GDM lifestyle intervention (labelled GDM) and GDM medication (labelled GDM+M) groups. Red dashed line across the STELA profiles indicates the mean. Each sample consist of six STELA PCR reactions. Mean telomere length are represented below each sample (±SD).

When the GDM group was further divided into GDM mothers who undertook dietary and exercise changes (lifestyle intervention group) versus those prescribed insulin and/or metformin (medication group) (**Table 4.3**), placental telomeres in the lifestyle intervention group were found to be approximately, 1 kb shorter than those in either the control or medication groups. However, this was not significant as assessed by ANCOVA ($F(2,96) = 1.66$, $P = 0.2$) (**Figure 4.6a**).

Likewise, the lifestyle intervention group exhibited 7% more telomeres below 5 kb but again, without significance (**Figure 4.6b**). Further division of the samples taking into account foetal sex revealed significant mean telomere length differences in male placenta between control, GDM lifestyle intervention and medication groups by ANCOVA ($F(2,54) = 4.89$, $P = 0.01$). Telomeres in the GDM lifestyle intervention group were shorter compared to both the control group ($P = 0.02$) and the GDM medication group ($P = 0.003$) (**Figure 4.6c**). In line with this observation, the lifestyle intervention group exhibited one-third higher percentage of telomeres under 5 kb compared to the control group ($P = 0.03$) and a greater than two-fold compared to the GDM medication group ($P = 0.004$) as assessed by ANCOVA ($F(2,54) = 4.65$, $P = 0.01$) (**Figure 4.6d**). Fewer placental samples were available from female infants and no significant differences in telomere length were found between the three groups analysed (**Figure 4.6e and f**). In addition, percentages of short ($7.7 - 1\sigma$) and long telomeres ($7.7 + 1\sigma$) (mean telomere length in kb) were calculated (**Table 4.4**) using the lengths derived from **Chapter 3**. Placental samples from male infants in the GDM lifestyle intervention group showed a high percentage of short telomeres (80%), while those samples from male infants in the GDM medication group exhibited a few percentage points of short telomeres (10%).

Table 4.4. Proportion of women with short/long placental telomeres across all the groups. Number (%) is shown.

		Short telomeres	Long telomeres
Control	Male	39	61
	Female	39	61
GDM	Male	80	20
	Female	25	75
GDM+M	Male	10	90
	Female	57	43

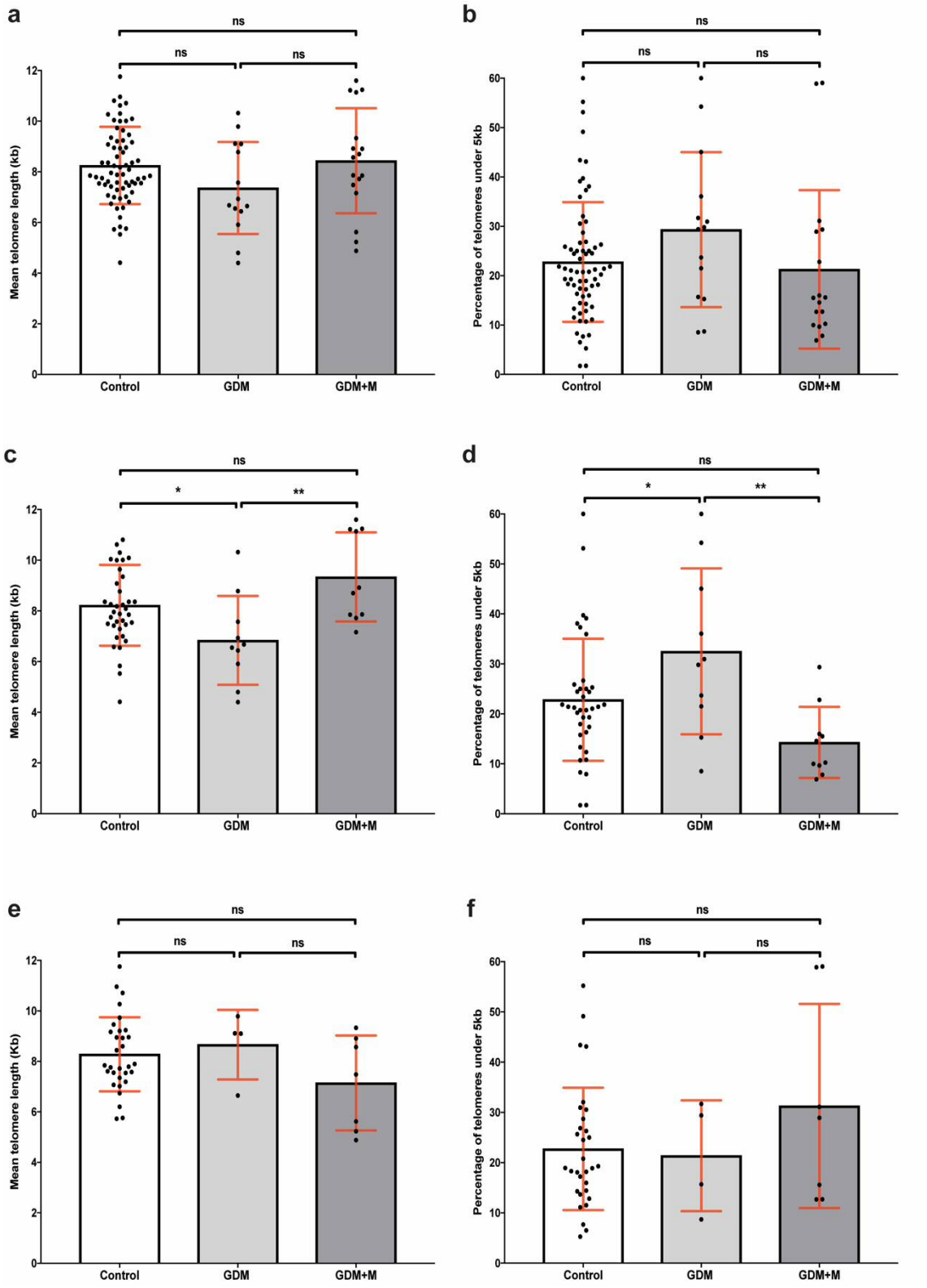


Figure 4.6. Placental telomere length differences between control, GDM and GDM+M groups. Comparison of mean telomere length between control, GDM and GDM+M groups (N=69+14+17) (ns) (a), in male placenta (N=38+10+10) (P = 0.01) (c), and in female placenta (N=31+4+7) (ns) (e). Comparison of percentage of telomeres below 5 kb between control, GDM and GDM+M groups (N=69+14+17) (ns) (b), in male placenta (N=38+10+10) (P = 0.01) (d), and in female placenta (N=31+4+7) (ns) (f). Mean telomere length and percentage below 5 kb is presented (±SD). ANCOVA test was used to identify statistical significant differences *P < 0.05.

4.6. Discussion

4.6.1. GDM contributes to telomere shortening

In this study, high-resolution telomere length analysis was used to examine placental telomeres in relation to the prenatal adversity, GDM, to provide further evidence to support the hypothesis that GDM may exacerbate telomere shortening. Placenta from women whose GDM was managed by changes in lifestyle, such as diet and exercise (lifestyle intervention group), exhibited shortened telomeres. To the contrary to what was expected, women with a more severe presentation of the disease and therefore under medical prescription of insulin and/or metformin, exhibited telomeres in the normal range for a non-diabetic pregnancy.

4.6.2. Metformin and/or insulin protects against telomere erosion

Metformin is a biguanide compound used to treat diabetes that works decreasing hepatic glucose output and increasing insulin sensitivity (**Figure 4.7**). The primary target of metformin is the mitochondria. It exerts a mild and transient inhibition of the mitochondrial respiratory-chain complex 1. This prevents mitochondrial ATP production, and thus increases cytoplasmic ADP:ATP and AMP:ATP ratios, ultimately activating AMPK. (Rena, Hardie and Pearson 2017). Therefore, AMP-K activation is secondary to the effect of metformin on the mitochondria. It has also been proposed to inhibit intracellular signaling pathways that drive cellular ageing, thus becoming a gerosupressant agent (Rena, Hardie and Pearson 2017). This drug crosses the placenta easily, showing the foetus similar concentrations to that of the maternal circulation. It has become the treatment of choice for GDM patients when changes in lifestyle are insufficient to maintain euglycaemia due to its many advantages. It lowers glucose level without the risk of hypoglycaemia, hyperinsulinemia or weight gain (Feig and Moses 2011).

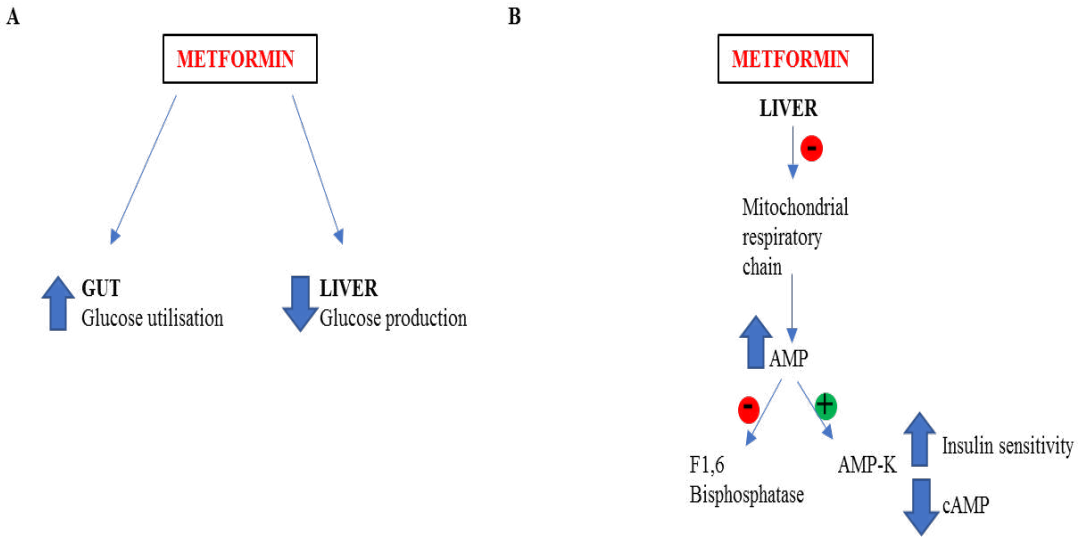


Figure 4.7. Mechanism of action of metformin. (A) Metformin has multiple sites of action: in the liver reduces glucose production and in gut increases glucose utilisation. (B) It can act through adenosine monophosphate-activated protein kinase (AMP-K) dependent and independent pathways. Metformin inhibits the mitochondrial respiratory chain in the liver, leading to an increase in the adenosine monophosphate (AMP) levels. AMP can block the F1,6 Bisphosphatase enzyme or activates AMP-K, which subsequently leads to an increase in insulin sensitivity and reduction in cyclic adenosine monophosphate (cAMP). Enhanced insulin sensitivity is the consequence of changes in fat metabolism and the reduction in cAMP results in a reduction in glyconeogenic enzymes (Rena, Hardie and Pearson 2017).

Experiments in rodents indicated that the drug exerts a direct effect on several phenotypes, that altogether define the senescent state, therefore reducing cellular senescence (Anisimov *et al.* 2011). This drug mitigates age-related consequences of tissue inflammation because of senescent cell accumulation. It is though that metformin may enhance the immune system responsiveness to senescent cells, inhibiting production of new senescent cells and its aggregation. It also plays an important role in control of oxidative damage and regulation of lifespan extension, which is likely to happen via inhibition of the mammalian target of rapamycin (mTOR) signaling, a signaling pathway generally referred to as 'caloric restriction-like strategy' (Menendez *et al.* 2011).

It has been reported that activation of AMP-K upregulates TERRA, which is possibly function as a shield protecting telomeric repeats from ROS. It is possible that metformin function via activation of AMP-K pathway, stimulating telomere transcription, thereby preventing telomere dysfunction (Diman *et al.* 2016). An alternative explanation would be that metformin acts dampening the characteristic low-graded chronic inflammation associated with GDM condition.

A direct anti-inflammatory effect of metformin includes its role as an activator of AMP-K, which inhibits NF-κβ, a master regulator of inflammation and immune responses. Indirectly, metformin beneficial effects are linked to metabolic outcomes, such as improvement of hyperglycemia and weight loss. These are variables that play a role in the development of GDM and contribute to worsening inflammation (Saisho 2015). Further work is required both to validate our preliminary results and to explore the underlying mechanism in detail.

It would have been interesting to know how the action of insulin compared to metformin, but due to the small number of samples within the GDM group it was not possible to assess this.

4.6.3. Sexual dimorphism in telomere length in response to GDM

Although no differences in placental telomere length was noticed in the control placentas analysed with respect to foetal sex, placental telomere length may be affected differently in male and female placenta by different pregnancy complications, such as GDM.

A comparison between GDM mothers and control mothers did not initially reveal a significant difference in telomere characteristics. However, direct observation of the telomere-length profiles by foetal sex identified placental telomeres from male infants considerably shortened, which were found to be exclusively from women who did not take medication for GDM (lifestyle intervention group). This finding highlights the importance of considering sex differences when studying the effect of environmental exposures on newborn telomere length.

The placenta appears to function in a sex specific manner. It responds differently to the same changes in the maternal environment exhibiting a very unique approach to guarantee survival. In the case of the male placenta, it undergoes minimal adjustments, which are associated with a greater risk of adverse outcomes (Clifton 2010). Carrying a male foetus has been previously associated with an increased risk of developing GDM in the mother (Retnakaran *et al.* 2015). In this study, pregnant women underwent metabolic characterisation, which included oral glucose tolerance test and β-cell function evaluation. They found that carrying a male foetus was associated with poorer β-cell function and higher

postprandial glycaemia, suggesting that foetal sex may impact maternal glucose metabolism. β-cell compensation in response to insulin resistance is necessary to cope with the new insulin demands and is induced by the secretion of placental lactogens and prolactin. It is therefore possible, that being a male foetus and consequently carrying the Y chromosome, affects the placental secretion of hormones that leads to β-cell compensation (Retnakaran *et al.* 2015). Gabory *et al.* (2013) suggested that those perinatal complication in male foetuses attributed to placental dysfunction may be associated to the abundance of X-linked genes involved in the process of placentogenesis. Although there are compensatory mechanism to equal the dosage, some X-linked genes can escape inactivation. Different chromosomes have different transcription profiles at the embryo stage in response to environmental insults, and it has been suggested that reduced maternal-foetal compatibility to the male placenta forced this tissue to upregulate immune-related transcripts to avoid rejection (Perez-Cerezales *et al.* 2018). These findings are consistent with other studies that have noted the particular vulnerability to prenatal exposures of male foetuses, as in the case of GDM, which revealed to be a risk factor for childhood overweight in boys, but not in girls (Le Moullec *et al.* 2018; Li *et al.* 2017). However, in contrast to our findings, Hjort *et al.* (2018) found that telomere length in blood leukocytes was reduced in girls exposed to GDM *in utero* but not in boys. These differences could be adjusted if women carrying a male foetus are more likely to take medication, which has been previously reported (Giannubilo *et al.* 2018). Further research is required in this area.

4.6.4. STELA provides a tool to quantify exposure to GDM

Telomere length in the newborn depends on the telomere length inherited from the maternal and paternal gametes. As reported before (Garcia-Martin *et al.* 2017), the remarkable telomere heterogeneity revealed in the term placenta is the result of an estimated 36 rounds of cell division during the 9 months of gestation (Simpson *et al.* 1992), suggesting that the placenta undergoes a highly dynamic process of telomere maintenance. Consistent with this, placental telomeres might be considered a sensitive tool for testing prenatal adversity and,

prospectively, measuring the effectiveness of intervention strategies, which it has been demonstrated here with respect to GDM.

This study has not shown whether the impaired telomeres were inherited by the offspring. Xu *et al.* (2014) and Hjort *et al.* (2018) reported that offspring born to GDM mothers exhibited shortened telomeres in blood leukocytes, suggesting that intrauterine exposure to gestational diabetes causes foetal telomere attrition and may programming the foetus for metabolic disease in the adulthood. However, not all the studies reported such a link in cord blood (Harville *et al.* 2010; Cross *et al.* 2010; Biron Shental *et al.* 2016). Biron-Shental *et al.* (2015) demonstrated that placenta exposed to GDM showed shorter telomere length using the FISH assay on 16 samples. In this study, shortened telomere were defined by a weak signal intensity, and when further analysis was performed, a significantly lower telomerase expression was found in these same samples (Biron-Shental *et al.* 2016). Inconsistency in these studies may be due to the technical challenges imposed by the use of leukocyte DNA. Nevertheless, this study demonstrated that STELA provides an alternative, sensitive and precise tool to quantify exposure to an adversity, GDM.

4.6.5. Summary

In this study, telomere length was measured in placental samples from women diagnosed with diabetes, taking into consideration foetal sex and treatment pathways. Using STELA, high-resolution telomere length profiles from placental samples were obtained. The main finding was that placental telomere shortening associated with GDM was rescued by maternal treatment with metformin and/or insulin.

A limitation of this study is the sample size. Due to the smaller number of placental samples from girls, it cannot be concluded that the relationship found between telomere length, GDM and the anti-diabetic drugs (insulin and/or metformin) is limited to boys. Although the statistical analysis controlled for a number of factors which may impact telomere length, it must be considered that questionnaire-based studies are naturally subjective and there may be other environmental factors apart from GDM and medical treatment affecting telomere length. As term placenta was analysed in individual pregnancies and there is no way to know when telomeres changes happened, it may be possible that the telomere length variations exhibited by the placenta were inherited from the parents.

Although a better understanding of the underlying mechanism and long-term outcomes of different treatment pathways is necessary, it may be speculated that early adoption of targeted medical treatment of GDM mothers where the baby is known to be male may be an effective strategy for alleviating adverse outcomes in the offspring.

Chapter 5: Impact of prenatal depression on placental telomere length

5.1. Abstract

Shortened telomeres have been reported in cases of depression and anxiety suggesting that telomere attrition may either be a consequence of certain psychiatric disorders, or a contributor to psychopathology, or a reflection of common mediators. STELA was applied to investigate the effects of intrauterine exposure to prenatal depression and anxiety on placental telomere length and telomere length distributions.

In this study, 109 term placentas (37-42 weeks) obtained from elective caesarean deliveries of healthy singleton pregnancies were examined. Depression and trait anxiety symptoms were assessed one day prior to delivery using the EPDS and the STAI test. STELA was applied to genomic DNA obtained from combined placental biopsies. A hierarchical multiple regression was used that included the effect of pregnancy status, lifestyle factors, socio-economic status and maternal depression and anxiety symptoms.

A significant negative association between prenatal depression symptoms and XpYp telomere length in the female placenta was found ($\beta = -0.347$, $P = 0.026$), as compared with male placenta ($\beta = 0.082$, $P = 0.586$). Female babies whose mothers experienced moderate to severe depression symptoms during pregnancy, as evidenced by higher EPDS, had significantly shorter placental telomere length compared to female babies of mothers who scored lower in the EPDS questionnaire.

5.2. Introduction

Prenatal depression describes active depression symptoms in pregnancy and is one of the strongest predictors of postpartum depression (Stowe, Hostetter and Newport 2005). Worldwide it has been estimated that about 10% of women experience depression during pregnancy, rising to 15% in developing countries as reported by the World Health Organisation (2015), and recent data suggest that these rates are increasing (Janssen *et al.* 2018; Pearson *et al.* 2018). Despite being a different disorder, symptoms of depression are commonly found to occur alongside symptoms of anxiety, and these two mood disorders are highly comorbid during gestation (Sartorius *et al.* 1996, Heron *et al.* 2004). Women with a previous history of psychiatric illness are at higher risk of develop anxiety and/or depression during pregnancy (Biaggi *et al.* 2016), as well as women from a lower socioeconomic status, less education, non-marital status, non-employment, with less social support, smokers, with sleep disturbances and other physiological factors (Field 2017). Maternal mood disorders in pregnancy are associated with a number of negative perinatal and maternal outcomes (**Table 5.1**).

Table 5.1. Summary of perinatal and maternal outcomes. Foetal and maternal outcomes including higher risk of different complications (Field *et al.* 2006; Choi *et al.* 2014; Shivakumar *et al.* 2011).

Perinatal outcomes	**Maternal outcomes**
Intensive care required	Placental abnormalities
Bronchopulmonary dysplasia	Preeclampsia
Intraventricular haemorrhage	Spontaneous abortion
LBW and SGA	Premature delivery
FGR	Postnatal depression
Increased heart rate	Less likely to attend antenatal examinations
Less optimal performance on Brazelton Neonatal Behavioural Scale	Compromised mother-child bonding
Show inferior mental, motor and emotional development	
Social and emotional problems during childhood	

In addition to short term indicators of wellbeing, there is evidence to suggest that maternal stress, anxiety and depression during pregnancy impacts foetal neurodevelopment contributing to longer term difficulties for offspring (O'Donnell *et al.* 2009). Some of these outcomes include effects on attention regulation, cognitive and motor development, apprehensive temperament, negative responsiveness to novelty in the first year of life, behavioural and emotional problems, impulsivity, externalizing and processing speed in adolescents. It has also been reported that there is a lower gray matter (brain cell) density in childhood linked to exposure to poor maternal mood *in utero* (Dunkel Schetter and Tanner 2012). Potential mechanisms underlying the effect of prenatal depression on foetal development are depicted in **Figure 5.1**.

Blood cortisol levels increase over pregnancy peaking at term (Phocas *et al.* 1990). In humans, cortisol is the main glucocorticoid, a class of steroid hormone made in the cortex of the adrenal glands in response to the secretion of the adrenocorticotropic hormone (ACTH) in the pituitary gland (Stephens 2012). Normal daily functions of this hormone include various homeostatic maintenance actions: blood pressure, immune system, metabolism of protein, carbohydrates and adipose tissue, anti-inflammatory action and foetal development. Cortisol has an important role helping the body respond to stress, which can be of a physical or psychological nature (Hannibal and Bishop 2014). A normal HPA axis follows a negative feedback loop where cortisol production inhibits the action of the cortisol-released hormone (CRH). However, in depressive patients, this axis is dysregulated and there is an altered response to stress and inability to maintain the regulation, they present a hyperactive HPA axis (Meltzer-Brody 2011). Cortisol is essential for normal brain and lung development in the foetus, however, too much cortisol could be harmful for the foetus (St-Pierre *et al.* 2016). High levels of cortisol in the mother may affect foetal development either by promoting changes in the placenta environment or potentially crossing the placenta exposing the foetus. The placenta produces CRH in response to cortisol, stimulating the production of more cortisol in the mother (Field *et al.* 2006). CRH is associated with uterine artery constriction and a decrease in blood flow to the foetus, thus restricting oxygen and nutrient supply, which is related to some birth complications, such as foetal growth restriction (Field *et al.* 2006). It

is thought that about 20% of the maternal cortisol can cross the placenta and reach the foetus, where higher levels of cortisol might lead to dysregulation of the foetal autonomic nervous system and by activation of glycogenolysis (Field et al. 2006; St-Pierre et al. 2016). However, some very recent data suggests that, with normal placental function, very little maternal cortisol crosses over to the foetus (Stirrat *et al.* 2018).

Depression during pregnancy can be treated with antidepressants including serotonin reuptake inhibitors (SSRI) (Mason and Weiner 2011). SSRIs including fluoxetine, citalopram, paroxetine, and sertraline block monoamine transporters, noradrenaline transporter (NET) and serotonin transporter (SERT), which help to alleviate the symptoms associated with depression as proposed by Mason and Weiner (2011). Current data suggest that some of these drugs can cross the placenta (Erwing *et al.* 2015) and there is considerable debate as to whether women should be treated during pregnancy, or not (Kott and Brummelte 2019).

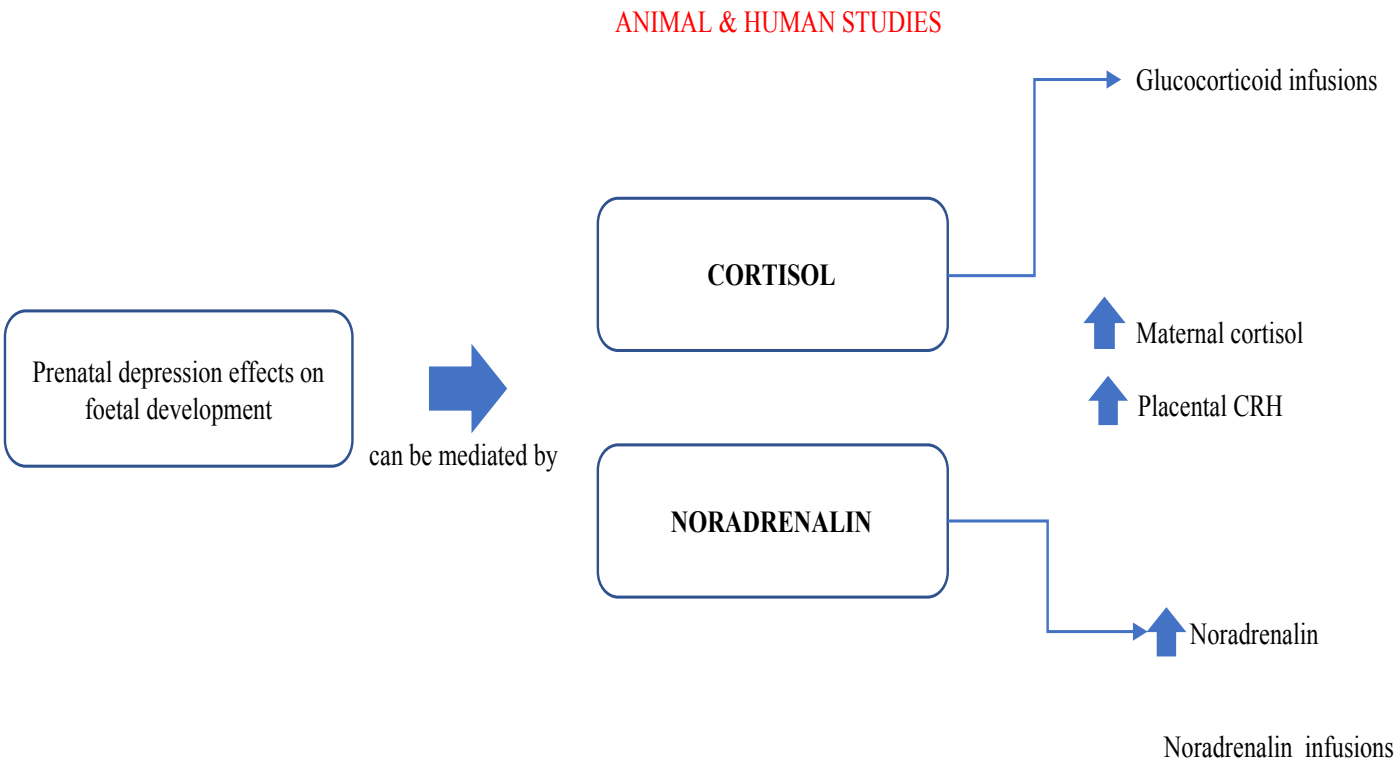


Figure 5.1. Potential mechanisms underlying the effect of prenatal depression on foetal development (Field *et a*

Some age-related diseases such as cardiovascular diseases, obesity or T2DM, occur concomitantly with psychiatric disorders such as depression (Palmos *et al.* 2018). A link between depression and a faster rate of biological ageing has been speculated (Palmos *et al.* 2018). One possibility is that impaired telomere biology impacts brain function either by early replicative senescence or by increased oxidative stress (Epel and Prather 2018) contributing to mental health disorders. In this regard, Mamdani *et al.* (2015) reported that post-mortem brains from depressed individuals showed evidence of decreased neurogenesis and a reduction in the hippocampal volume, a region of the brain implicated in depression and dementia. Those patients also exhibited a reduction in telomere length in the hippocampus. In this context, several studies have reported associations between telomere length and different types of stress: stress-induced depression, chronic mild stress, temporary stress and stress-induced depression during pregnancy (**Table 5.2**).

Biochemical changes as a result of stress and depression, such as inflammation, oxidative stress, insulin resistance, and autonomic and neuroendocrine stress reactivity, may impair telomere function (Epel and Prather 2018). In a recent study, cortisol responsivity was associated with a greater rate of telomere attrition in leukocytes over a period of time. Participants exposed to psychological stress responded differently to cortisol, exhibiting different rates of cellular ageing (Steptoe *et al.* 2017).

Whether telomere length can be used to predict psychiatric disorders, such as depression, is still unclear. In humans, Wei *et al.* (2016) found that telomere length was shorter in individuals with depression, and that those who carried a genetic variation in the hTERT subunit of the telomerase enzyme were more susceptible to depression, using DNA obtained from saliva samples. In addition, a study in rodents showed that chronic mild stress provoked a decrease in TERT and telomerase activity in the hippocampus. In this region, experimental inhibition of the telomerase enzyme induced depressive-like behaviours and impaired hippocampus neurogenesis (Zhou *et al.* 2011). Overexpression of the telomerase enzyme triggered the opposite (Zhou *et al.* 2011). These findings suggest that the telomerase enzyme may modulate depression-like behaviours in the hippocampus through regulating neurogenesis.

Despite the clinical relevance of prenatal depression and anxiety, few studies have reported on the impact of prenatal stress on newborn leukocyte telomere length (Entringer *et al.* 2011, 2013; Marchetto *et al.* 2016 and Send *et al.* 2017). No study has reported on placental telomere characteristics in pregnancies impacted by these mood disorders. This is important because, as previously discussed, the placenta is both a proxy for the foetal exposures during pregnancy and placental dysfunction may contribute to adverse outcomes.

5.3. Aims

The purpose of this chapter was to apply STELA for measuring telomere length profiles in placenta from pregnancies complicated by prenatal depression and anxiety.

Table 5.2. Associations between telomere length and different types of stress: stress-induce depression, ch and stress-induced depression during pregnancy. Related information with respect to category study, number telomere outcome and author is shown.

Exposure	Study	Number	Tissue	Technique	Telomere
Chronic mild stress	Mice	NA	Hippocampus	TRAP-Assay	Decreased telom
Stressors during 1 year	Blackbird	49	RBC	TRF	Shorter telomer
Early-life competition	Bird	132	RBC	q-PCR	Telomere attriti
Chronic stress	Mice	16	Saliva and blood	q-PCR	Telomere attriti
Chronic stress	Rats	30	PBMCs	TRAP-Assay	Increased telom
Depression	Human	662	Saliva	qRT-PCR	Shorter telomer
Childhood maltreatment	Human	31	Leukocytes	q-PCR	Shorter telomer
Psychological stress	Human	411	Leukocytes	qRT-PCR	Telomere attriti
Chronic psychological stress	Human	58	PBMCs	q-PCR	Telomere attriti
Offspring of depressed mothers	Human	97	Saliva	q-PCR	Shorter telomer
Maternal psychosocial stress	Human	27 mothers-newborn dyads	CB PMCs	q-PCR	Shorter telomer
Offspring of stressed mothers	Human	94 young adults	Leukocytes	q-PCR	Shorter telomer
Offspring of stressed mothers	Human	24 mother-newborn dyads	CB PMCs	TRF	Shorter telomer
Offspring of stressed mothers	Human	273 newborns 274 mothers	CB PMCs	q-PCR	Shorter telomer

5.4. Chapter specific methods

Methods have been described in **Chapter 2**. To support the interpretation of results presented at the end of this chapter, methods related to selection criteria for participants and maternal measurements of depression and anxiety (**Figure 5.2**), placental biopsies and statistical analysis are described below. In this chapter, telomere length was measured in human placenta from the Grown in Wales Study.

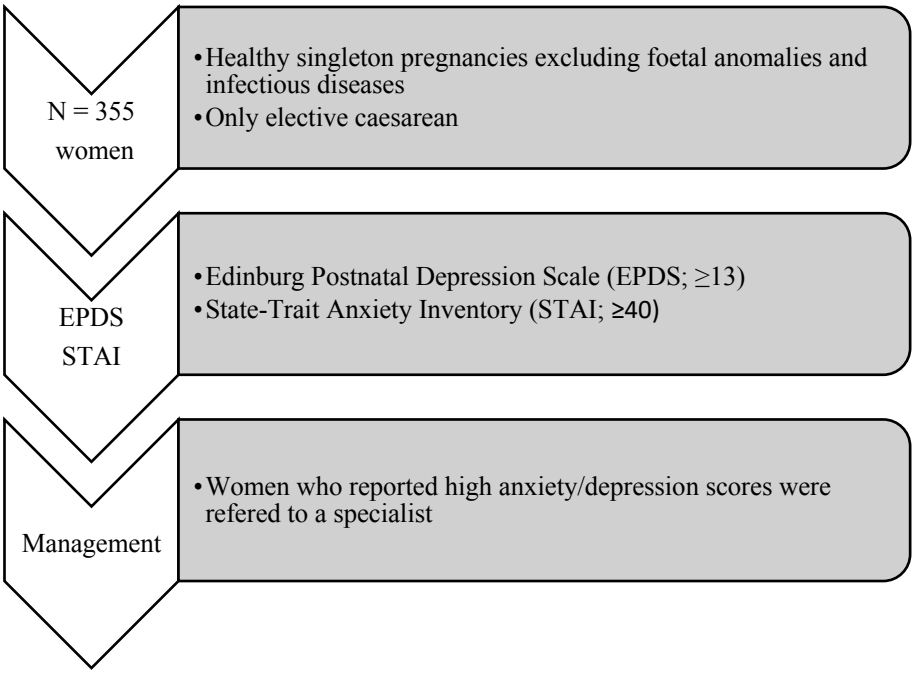


Figure 5.2. Summary of study design.

Participants were pregnant women enrolled in the Grown in Wales (GIW) study, a prospective pregnancy cohort which recruited N = 355 women to interrogate the relationship between placental gene expression and antenatal and postpartum mood disorders (Janssen *et al.* 2018). Women were recruited the morning before a booked elective caesarean delivery. They were asked to complete a self-administered participant questionnaire (A1), which included a section consisting of two assessments of perceived mood symptoms (EPDS and STAI; **see Appendix 3b**). In addition, they completed a lifestyle questionnaire and a food frequency questionnaire. Data was also retrieved from their medical

records including treatment for depression with antidepressants and mental health history, and a note made by the research midwives on birth weight and other characteristics. The EPDS and STAI questionnaires were completed again within 7 days (P1), 10 weeks (P2) and 1 year (Y1) postpartum (**see Figure 2.1**).

EPDS was used to assess maternal depression. This self-report questionnaire has been previously validated (Cox *et al.* 1996) and consists of ten questions ranging from 0 (low depression) to 30 (high depression). An EPDS score of $\geq$ 13 is considered depression (Cox *et al.* 1987).

STAI was used to assess maternal anxiety. This self-report questionnaire has been previously validated and describes general feeling of the participants. It consists of 20 questions, where every question score 4 ranging from 20 (low anxiety) to 80 (high anxiety). STAI score ≥ 40 has been used to indicate anxiety (Grant *et al.* 2008).

In the initial study design (Phase 1) 60 placental samples were selected based on their EPDS scores from the N = 355 participants. 30 samples were from pregnancies where women were classified as non-depressed controls with an EPDS score of < 13 and a STAI score of < 40 and 30 were from pregnancies where women reported significant depression and anxiety symptoms with an EPDS score of $\geq$ 13 and a STAI score of $\geq$ 40. These two groups were matched for the biological characteristics shown in **Table 5.3**.

To analyse the effect of prenatal depression and/or anxiety on telomere length, XpYp and 17p STELA was applied to genomic DNA prepared from combined placental biopsies taken from the maternal side of term placentas (37-42 weeks) 1 cm below the surface and at five different locations midway between the medial and lateral edge.

To analyse the effect of cortisol on telomere length, participants provided a sample of saliva in the morning the day before their surgery, more than 30 min after their last meal. Then, samples were kept at −80ºC until concentration was determined.

5.4.1. Statistical analysis

Placental XpYp telomere length was normally distributed as determined by D'Agostino & Pearson normality test (K2 = 2.584, P = 0.27), Shapiro-Wilk test (W = 0.98, P = 0.25) and Kolmogorov-Smirnov test (KS distance = 0.08, P = 0.06). Likewise, placental 17p telomere length was normally distributed as determined by D'Agostino & Pearson normality test (K2 = 0.86, P = 0.64), Shapiro-Wilk test (W = 0.98, P = 0.61) and Kolmogorov-Smirnov test (KS distance = 0.05, P > 0.1). Differences between groups (control and depressed) were assessed using $\chi 2$ test for categorical data and t-test, as all characteristics followed a normal distribution.

To examine the association between maternal depression/anxiety during pregnancy and placental telomere length adjusted for the effects of other possible determinants, a linear regression model was used that included the effects of: maternal age, gestational age, parity, smoking and alcohol during pregnancy, BMI, WIMD (Welsh Index of Multiple deprivation) score and EPDS/STAI score. To assess whether or not there was an association between XpYp and 17p telomeres, a simple linear regression was used. This type of analysis was also performed to evaluate the association between maternal salivary cortisol and mean placental XpYp telomere length. Differences on placental telomere length in control, untreated prenatal depression and treated prenatal depression groups were tested by one-way ANOVA. All statistical analysis was performed using SPSS 23.0 for Windows and GraphPad Prism 7.03.

5.5. Results

5.5.1. First phase

5.5.1.1. Participants demographics

Table 5.3. Main characteristics of the study participants. Mean (SD)/Range or number (%) is shown. Note: due to missing values, some numbers do not add up to 100%. *P* values were assessed using independent samples T- test or χ^2 test.

	Control group (N = 30)	Depressed group (N = 30)	P value
Maternal characteristic			
Ethnicity			
Caucasian	30 (100%)	30 (100%)	NA
Parity			NA
Primiparous	7 (23.3%)	7 (23.3%)	
Multiparous	23 (76.7%)	23 (76.7%)	
Maternal age	32 (5.56) / 19-41	31 (5.15) / 21-39	0.47
Maternal BMI	25 (4.88) / 17-36	28 (4.64) / 20-39	0.12
Birth Outcome			
Mode of Delivery			
C-Section	30 (100%)	30 (100%)	NA
Birth weight (g)	3169 (480) / 2260-3930	3301 (391) / 2460-3930	0.24
Gestational age (weeks)	39 (0.37) / 38-40	38 (0.71) / 37-41	0.49
Placental weight (g)	589 (117) / 376-850	626 (113) / 455-940	0.22
Gender			NA
Female	30 (50%)	30 (50%)	
Male	30 (50%)	30 (50%)	

STELA was applied to a total of 60 placental samples to assess whether intrauterine exposure to prenatal depression and anxiety impact telomere length in the placenta. A summary of participant demographics is given in **Table 5.3**.

5.5.1.2. STELA analysis

5.5.1.2.1. XpYp STELA

Mean placental telomere length did not statistically differ between male (mean = 7.77, SD = 1.43) and female (mean = 7.77, SD = 1.42) infants, P = 0.97.

A comparison of the SD of placental telomere length between depressive women and control women did not revealed a significant difference (P = 0.77). However, dividing the samples by foetal sex revealed a significant difference in placental telomere length in the female placenta (P = 0.036), but not in male placenta (P = 0.143) (**Figure 5.3**). It was also noted that there was an increased level of heterogeneity in the telomere length profiles exhibited by the female placenta in mothers that reported depression symptoms, from a SD of 3.15 in samples from control mothers to 3.70 in samples from depressive mothers. In contrast, male placenta exhibited a SD of 3.76 in control mothers and 3.24 in depressive mothers, but this was not significant. In addition, a linear regression model showed a significant positive association of EPDS score with the percentage of telomeres below 4 kb in length in female placenta (β = 0.378, P = 0.046), but not in male placenta (β = – 0.082, P = 0.71). EPDS score was not associated with mean telomere length neither in female placenta (β = – 0.154, P = 0.41), nor in male placenta (β = 0.063, P = 0.77). Telomeres in these length ranges (< 4 kb) are consistent with those observed in senescent cell populations, and it seemed that the percentage of shorter telomeres increased with EPDS score.

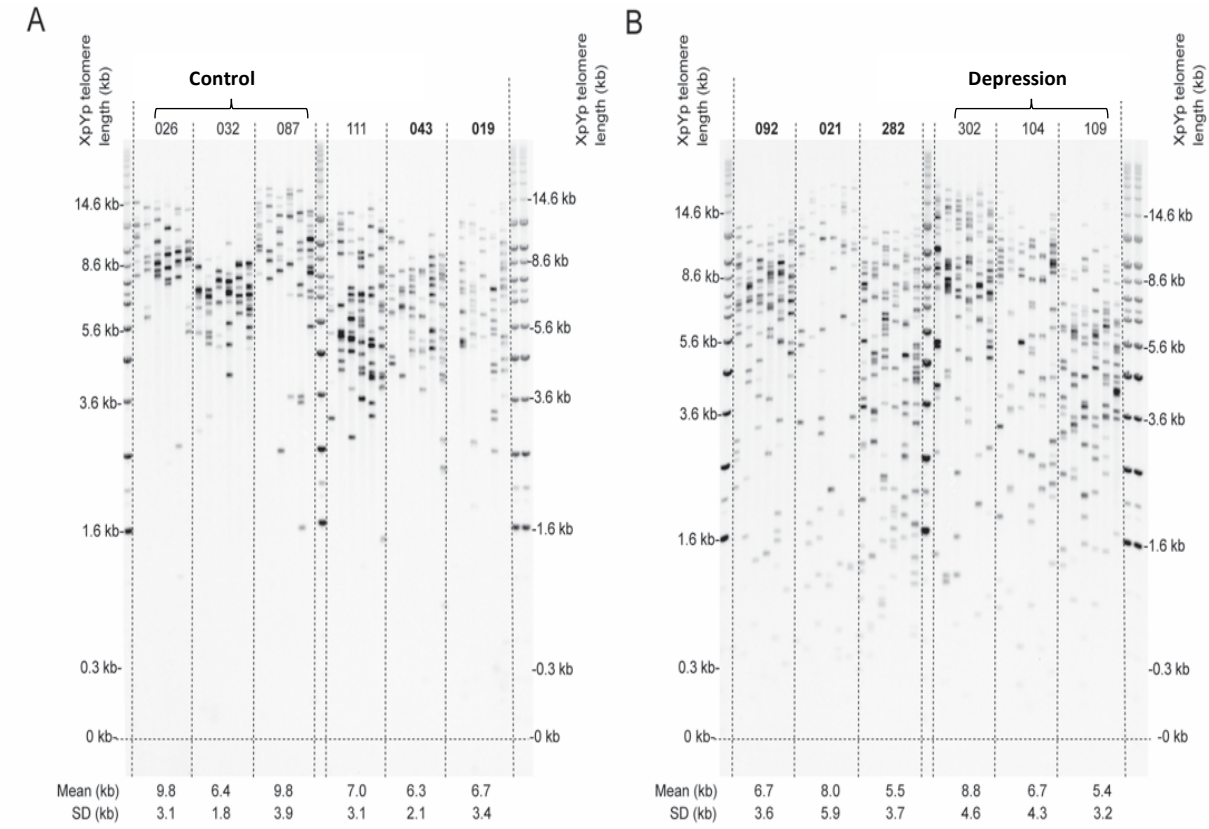


Figure 5.3. Representative STELA illustrating female placenta samples from control (≤ 13 EPDS score) and depression (≥ 13 EPDS score) groups. Each sample consist of six STELA PCR reactions. Mean telomere length are represented below each sample (±SD).

Following these initial observations we sort to further examine the association between maternal depression during pregnancy and placental telomere length, by adjusting for the effect of other possible determinants. A hierarchical multiple regression was used that included the effect of pregnancy status, lifestyle factors, socio-economic status and maternal depression and anxiety symptoms (**Figure 5.4)**.

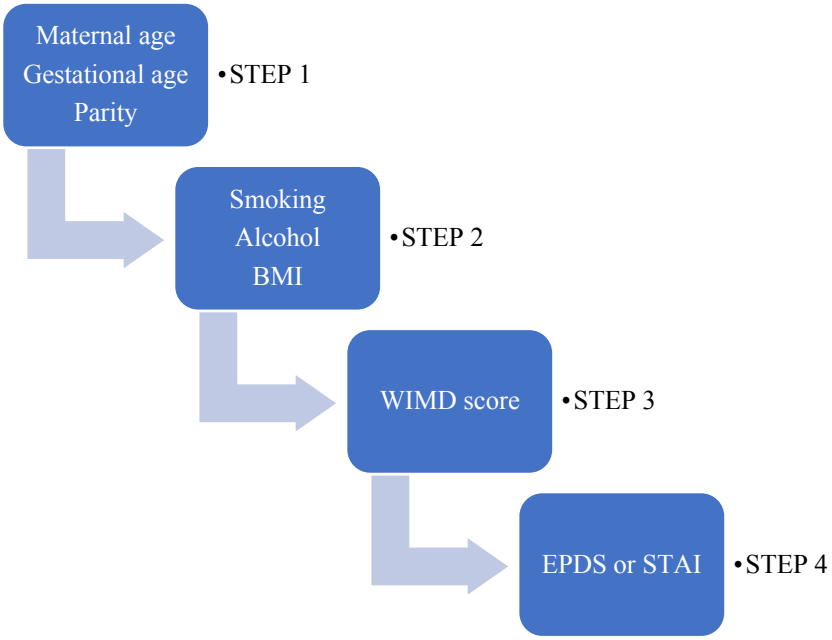


Figure 5.4. Hierarchical multiple regression with entry of different predictors at 4 successive steps.

The hierarchical multiple regression increased confidence as it controlled for several predictor variables. After adjusting for all the variables above described the relationship between the percentage of telomeres below 4 kb and the EPDS score was no-longer significant ($\beta = 0.532$, $P = 0.063$).

5.5.1.2.2. 17p STELA analysis

One possibility was that the ability to detect differences between the control and depression-exposed placenta might be confounded by studying the sex chromosome telomeres. Therefore, an additional study was carried out to analyse the length distributions of an autosomal telomere, the 17p was chosen for this purpose as a robust STELA assay has previously been developed for this chromosome end. Thus the same 60 samples as analysed at XpYp were subjected to 17p STELA.

It was first found that the mean length of telomeres at 17p were shorter compared to XpYp telomeres. The mean telomere length of 17p was around 2 kb shorter in males (5.87 kb vs 7.97 kb) and in females (6.41 kb vs 8.13 kb) compared to XpYp. Direct observation of the raw data revealed a very high level of telomere length heterogeneity at 17p (SD = 3.44), similar to XpYp (SD = 3.46), with some samples having telomeres ranging from <1 kb to >15 kb (**Figure 5.6**). Mean placental telomere length at 17p did not statistically differ between male (mean = 5.87, SD = 1.22; N = 30) and female (mean = 6.41, SD = 1.24; N = 30) infants, P = 0.09. In addition, a simple linear regression analysis was performed to assess whether or not there is an association between XpYp and 17p telomeres (**Figure 5.5**). There was no significant correlation between XpYp and 17p mean telomere length (β = 0.178, P = 0.174).

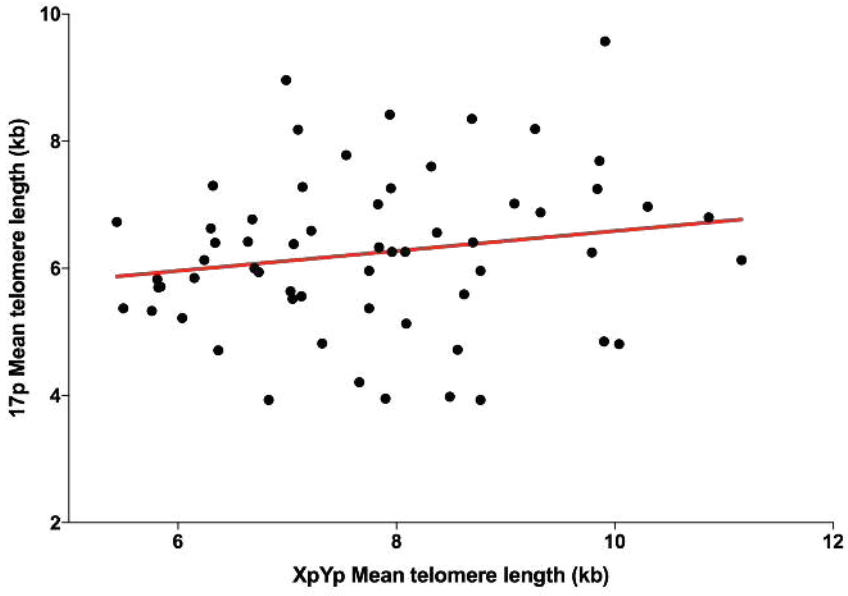


Figure 5.5. XpYp and 17p telomeres correlation. No significant correlation was found between XpYp and 17p mean telomere length (β = 0.178, P = 0.174). Simple linear regression analysis was used to assess statistical significant differences. *P < 0.05.

As previously described, a hierarchical multiple regression was used that included the effect of pregnancy status, pregnancy lifestyle, socio-economic status and maternal depression and anxiety symptoms (**Figure 5.4**). Associations between the predictors and mean 17p telomere length were examined in the sample as a whole and stratified by infant sex. As a whole, mean 17p telomere length was not significantly associated with EPDS score ($\beta = 0.094$, $P = 0.56$) nor with STAI score ($\beta = 0.171$, $P = 0.26$). When the samples were stratified by infant sex (**Figure 5.7**), no significant correlations were found between placental telomere length and several predictors, including EPDS and STAI. This was the case for male and female placenta.

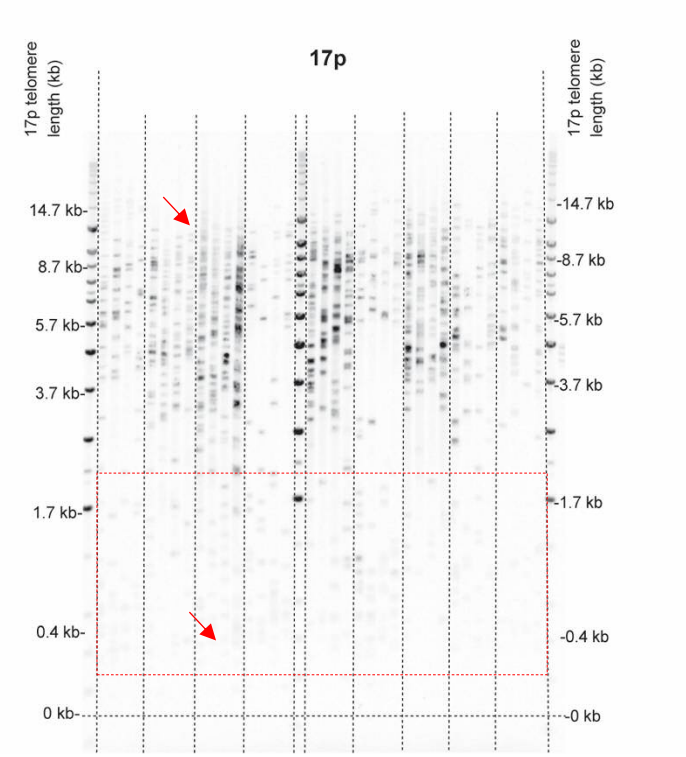
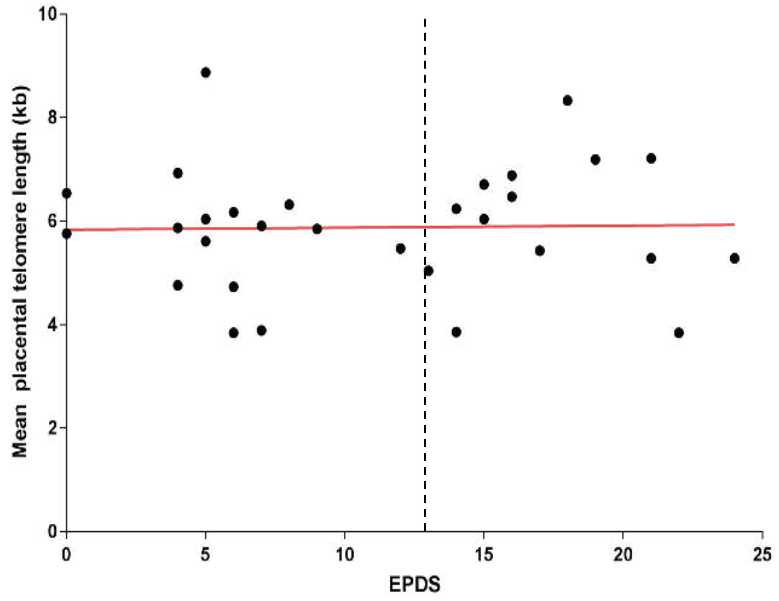


Figure 5.6. 17p STELA on human placental samples. Red arrows points towards the longest (TL > 15 kb) and the shortest telomere (TL < 1 kb) for a specific sample. The square includes all single telomeres within the length that leads to senescence and apoptosis, being overall shorter than those on the XpYp region.

A



B

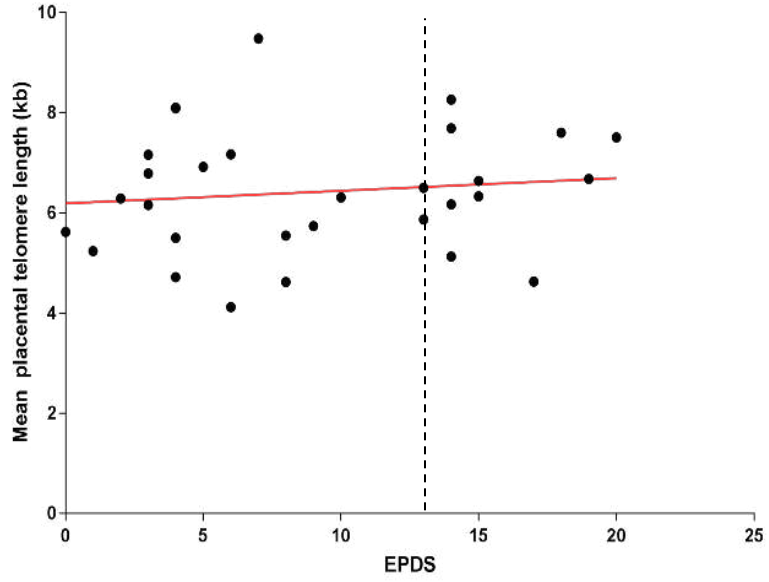


Figure 5.7. Linear regression model showing the association between maternal perceived depression symptoms (EPDS score) and mean placental telomere length in the 17p region. The model showed no association of prenatal depression exposure (EPDS score) with mean telomere length on male placenta (N = 30) (ns) (A), or on female placenta (N = 30) (ns) (B). Dotted line divides study population into placenta from control (< 13) and highly depressed (≥ 13) mothers. Depression symptoms were assessed using an EPDS questionnaire. A hierarchical linear analysis was used to identify statistical significant differences *P < 0.05.

5.5.2. Second phase

5.5.2.1. Participants demographics

This preliminary analysis of the original 60 samples suggested shorter telomeres at XpYp in the depression group, but this was not found to be statistically significant once other factors were controlled for and there was no evidence of telomere length differentials at 17p.

Whilst the data on XpYp was not statistically significant at a stringent probability ($P \geq 0.05$), the P value was 0.063. Therefore, it was decided to examine a larger number of samples to increase the power of the study by including data from the control samples analysed in the GDM discovery set where there was EPDS and STAI data (**see Chapter 4**). The samples chosen were from the same cohort study, collected in exactly the same way. This approach allowed existing data on an additional 49 samples from normal euglycemic women to be added to the 60 existing ones, making a total of 109 samples (second phase). From these N = 109 participants, 79 were classified as non-depressed controls with an EPDS score of < 13 and a STAI score of < 40 and 30 were from pregnancies where women reported significant depression and anxiety symptoms with an EPDS score of ≥ 13 and a STAI score of ≥ 40 (**Table 5.4**).

Table 5.4. Main characteristics of the study participants. Mean (SD)/Range or number (%) is shown. Note: due to missing values, some numbers do not add up to 100%. *P* values were assessed using independent samples T- test or χ^2 test.

	Control group (N = 79)	Depressed group (N = 30)	P value
Maternal characteristic			
Ethnicity			
Caucasian	30 (100%)	29 (96%)	0.54
Parity			0.82
Primiparous	14 (17.7%)	7 (23.3%)	
Multiparous	65 (82.2%)	23 (76.7%)	
Maternal age	32 (5.51) / 19-44	30 (5.68) / 20-39	0.31
Maternal BMI	29 (6.41) / 18-51	28 (4.92) / 20-39	0.51
Birth Outcome			
Mode of Delivery			
C-Section	79 (100%)	30 (100%)	NA
Birth weight (g)	3491 (620) / 2260-5080	3488 (499) / 2460-5110	0.98
Gestational age (weeks)	39 (0.61) / 38-41	38 (0.68) / 37-41	0.12
Placental weight (g)	663 (129) / 376-941	671 (150) / 455-1060	0.76
Gender			0.42
Female	38 (48%)	17 (56%)	
Male	41 (52%)	13 (44%)	

5.5.2.2. XpYp STELA analysis

Using the combined data, the mean placental telomere length did not statistically differ between male (mean = 7.97, SD = 1.66) and female (mean = 8.13, SD = 1.47) placenta, P = 0.60.

A hierarchical multiple regression was used that included the effect of pregnancy status, lifestyle factors, socio-economic status and maternal depression and anxiety symptoms (**Figure 5.4; Tables 5.5, 5.6, 5.7 and 5.8**).

Table 5.5. Summary of hierarchical multiple regression model for maternal variables predicting placental telomere length among male infants. The model showed no association of prenatal depression exposure (EPDS score) with mean telomere length on male placenta (β = 0.082, P = 0.586). Hierarchical multiple regression was used to assess statistical significant differences. *P < 0.05. Independent variables entered the regression model included: maternal age, gestational age, parity, smoking during pregnancy, alcohol during pregnancy, BMI, WIMD score and EPDS**.** Mean telomere length entered the regression model as the dependent variable.
[a] Unstandardised coefficients: represents the amount of change in a dependent variable due to a change of 1 unit in the independent variable.
[b] Standardised coefficient: compares the strength of the effect of each individual variable to the dependent variable.

	Model 1				Model 2			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	.041	.044	.133	.361	.033	.047	.109	.477
Gestational Age	.295	.297	.140	.326	.269	.307	.127	.386
Parity	-.033	.305	-.016	.913	.219	.412	-.104	.598
Smoking during pregnancy					.753	.868	.167	.390
Alcohol during pregnancy					.180	.926	.029	.847
BMI at booking					-.006	.035	-.027	.855
WIMD Score								
EPDS								
	Model 3				Model 4			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	-.030	.052	-.100	.558	-.026	.053	-.084	.628
Gestational Age	.263	.293	.124	.373	.285	.298	.135	.343
Parity	.485	.407	.230	.240	.460	.413	.218	.272
Smoking during pregnancy	.630	.828	.139	.451	.637	.834	.141	.449
Alcohol during pregnancy	-.380	.911	-.062	.679	-.271	.940	-.044	.775
BMI at booking	.006	.034	.023	.869	.004	.034	.018	.901
WIMD Score	.001	.000	.419	.020*	.001	.000	.422	.020*
EPDS					**.022**	**.040**	**.082**	**.586**

Table 5.6. Summary of hierarchical multiple regression model for maternal variables predicting placental telomere length among female infants. **.** The model showed an association of prenatal depression exposure (EPDS score) with mean telomere length on female placenta (β = - 0.347, P = 0.026). Hierarchical multiple regression was used to assess statistical significant differences. *P < 0.05. Independent variables entered the regression model included: maternal age, gestational age, parity, smoking during pregnancy, alcohol during pregnancy, BMI, WIMD score and EPDS**.** Mean telomere length entered the regression model as the dependent variable.
[a] Unstandardised coefficients: represents the amount of change in a dependent variable due to a change of 1 unit in the independent variable.
[b] Standardised coefficient: compares the strength of the effect of each individual variable to the dependent variable.

	Model 1				Model 2			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	.025	.040	.105	.532	.060	.041	.251	.154
Gestational Age	1.003	.478	.310	.042*	1.171	.478	.362	.019*
Parity	-.254	.270	-.158	.353	-.305	.265	-.191	.256
Smoking during pregnancy					-1.288	.768	-.262	.101
Alcohol during pregnancy					1.387	.796	.247	.089
BMI at booking					-.037	.040	-.133	.354
WIMD Score								
EPDS								
	Model 3				Model 4			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	.045	.045	.188	.327	.055	.043	.230	.212
Gestational Age	1.271	.495	.393	.014*	.999	.484	.309	.046*
Parity	-.222	.285	-.139	.439	-.369	.277	-.230	.191
Smoking during pregnancy	-1.344	.774	-.273	.091	-1.456	.735	-.296	.055
Alcohol during pregnancy	1.424	.801	.253	.083	1.814	.777	.323	.025*
BMI at booking	-.038	.040	-.137	.342	-.052	.038	-.184	.183
WIMD Score	.000	.000	.136	.415	.000	.000	.040	.806
EPDS					**-.098**	**.042**	**-.347**	**.026***

Table 5.7. Summary of hierarchical multiple regression model for maternal variables predicting placental telomere length among male infants. The model showed no association of anxiety (STAI score) with mean telomere length on male placenta (β = - 0.023, P= 0.879). Hierarchical multiple regression was used to assess statistical significant differences. *P < 0.05. Independent variables entered the regression model included: maternal age, gestational age, parity, smoking during pregnancy, alcohol during pregnancy, BMI, WIMD score and STAI**.** Mean telomere length entered the regression model as the dependent variable.

[a] Unstandardised coefficients: represents the amount of change in a dependent variable due to a change of 1 unit in the independent variable.

[b] Standardised coefficient: compares the strength of the effect of each individual variable to the dependent variable.

	Model 1				Model 2			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	.051	.046	.162	.274	.044	.048	.141	.365
Gestational Age	.246	.303	.115	.420	.215	.314	.101	.496
Parity	-.059	.307	-.028	.849	.196	.413	.094	.637
Smoking during pregnancy					.770	.870	.171	.381
Alcohol during pregnancy					.223	.929	.037	.812
BMI at booking					-.003	.036	-.015	.922
WIMD Score								
STAI								
	Model 3				Model 4			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	-0.20	.053	-.064	.708	022	.055	-.070	.693
Gestational Age	.209	.298	.098	.488	202	.305	.095	.511
Parity	.463	.408	.221	.263	470	.415	.225	.264
Smoking during pregnancy	.647	.829	.144	.439	661	.843	.147	.437
Alcohol during pregnancy	-.337	.913	-.055	.714	375	.956	-.062	.697
BMI at booking	.009	.034	.036	.801	009	.035	.036	.802
WIMD Score	.001	.000	.419	.020*	001	.000	.419	.022*
STAI					**004**	**.025**	**-.023**	**.879**

Table 5.8. Summary of hierarchical multiple regression model for maternal variables predicting placental telomere length among female infants. The model showed no association of anxiety (STAI score) with mean telomere length on female placenta (β = -0.111, P= 0.462). Hierarchical multiple regression was used to assess statistical significant differences. *P < 0.05. Independent variables entered the regression model included: maternal age, gestational age, parity, smoking during pregnancy, alcohol during pregnancy, BMI, WIMD score and STAI**.** Mean telomere length entered the regression model as the dependent variable.
[a] Unstandardised coefficients: represents the amount of change in a dependent variable due to a change of 1 unit in the independent variable.
[b] Standardised coefficient: compares the strength of the effect of each individual variable to the dependent variable.

	Model 1				Model 2			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	0.25	.039	.105	.526	.060	.041	.253	.145
Gestational Age	1.004	.455	.324	.033*	1.150	.457	.371	.016*
Parity	-.254	.265	-.160	.344	-.312	.259	-.196	.236
Smoking during pregnancy					-1.289	.759	-.261	.097
Alcohol during pregnancy					1.383	.786	.246	.086
BMI at booking					-.036	.039	-.130	.356
WIMD Score								
STAI								
	Model 3				Model 4			
	B[a]	SE B[a]	β[b]	P	B[a]	SE B[a]	β[b]	P
Maternal Age	.046	.045	.191	.312	.049	.045	.206	.281
Gestational Age	1.244	.473	.401	.012*	1.178	.484	.380	.020*
Parity	-.232	.278	-.146	.410	-.277	.286	-.174	.339
Smoking during pregnancy	-1.344	.765	-.273	.087	-1.318	.770	-.267	.095
Alcohol during pregnancy	1.419	.791	.252	.081	1.439	.796	.256	.078
BMI at booking	-.037	.039	-.133	.348	-.042	.039	-.149	.300
WIMD Score	.000	.000	.135	.415	.000	.000	.099	.567
STAI					**-.016**	**.021**	**-.111**	**.462**

When the samples were divided by infant sex, significant correlations with placental telomere length emerged for several predictors. In male infants, higher WIMD score (i.e. less deprived) was correlated with longer placental telomere length. Among female infants, higher gestational age and alcohol was correlated with longer placental telomere length, With female infants only, maternal depressive symptoms during pregnancy were significantly associated with shorter mean placental telomere length (β = - 0.347, P = 0.026) (**Figure 5.8 and Figure 5.9 (B)**.

A hierarchical regression tested the independent effects of several predictors on placental telomere length among male and female infants. The entry of multiple independent variables in four steps was performed as follows: the first step included the pregnancy status variables (maternal age, gestational age and parity), the second step included the pregnancy lifestyle variables (smoking, alcohol and BMI), the third step included WIMD score and the fourth and last step included EPDS or STAI. Because EPDS and STAI are highly associated, raising concerns about multi-collinearity, either EPDS or STAI were included in this step. Using this approach, EPDS but not STAI was correlated with placental telomere length. In fact, adding EPDS to the model resulted in an overall prediction for female infants of 35% (r^2 = 0.35), but not for males 16% (r^2 = 0.16). This means that 35% of the variance in female placental telomere length can be explained by the final model.

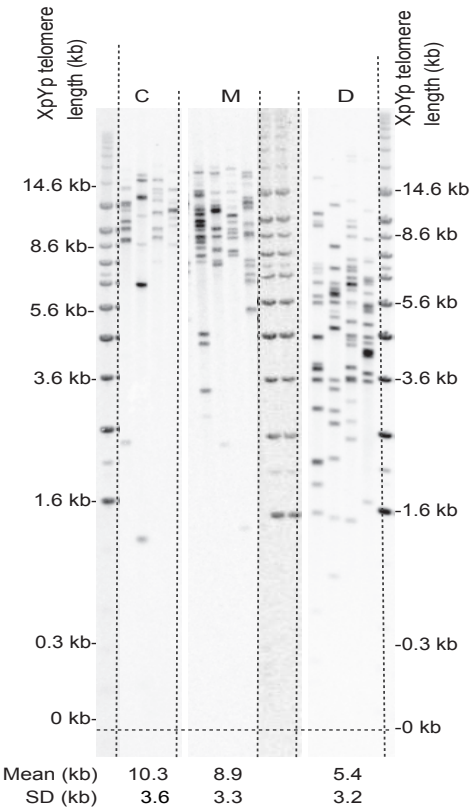
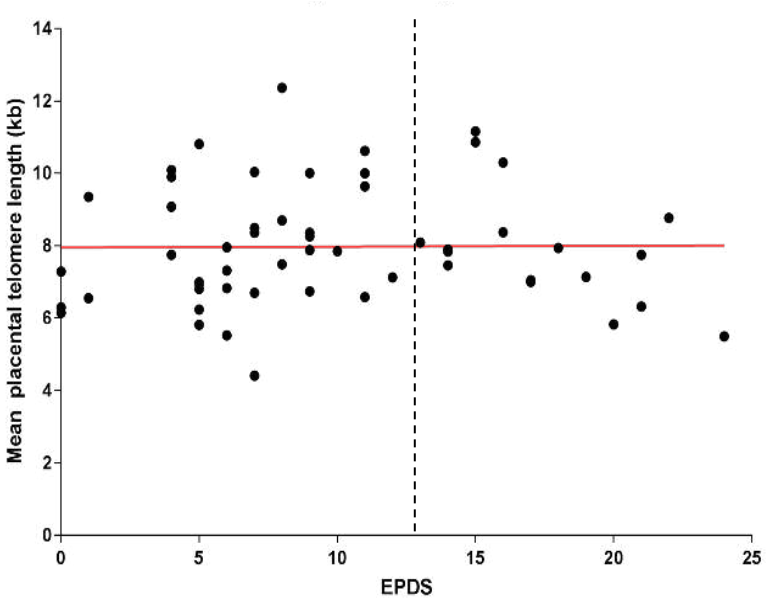


Figure 5.8. Composite figure. Representative STELA illustrating female placental samples from control (0-6 EPDS score) (labelled C), Mild depression (7-12 EPDS score) (labelled M) and Depression (> 13 EPDS score) (labelled D) groups. Each sample consist of four STELA PCR reactions. Mean telomere length are represented below each sample (±SD).

A



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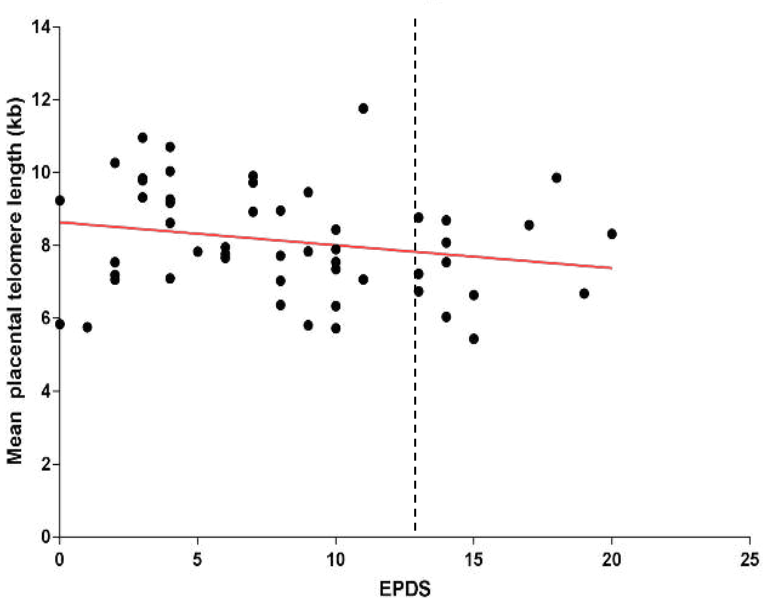

Figure 5.9. Linear regression model showing the association between maternal perceived depression symptoms (EPDS score) and mean placental XpYp telomere length. The model showed no association of prenatal depression exposure (EPDS score) with mean telomere length on male placenta (N = 55) (β = 0.082, P = 0.586) (A), and a significant negative association on female placenta (N = 54) (β = - 0.347, P= 0.026) (B). Dotted line divides study population into placenta from control (< 13) and highly depressed (≥ 13) mothers. Depression symptoms were assessed using an EPDS questionnaire. A hierarchical linear analysis was used to identify statistical significant differences *P < 0.05.

5.5.2.3. XpYp telomere length and cortisol

A negative, but not statistically significant linear association (**Figure 5.10**), was found between maternal salivary cortisol collected the morning prior to the ELCS and mean placental XpYp telomere length (β = - 0.123, P = 0.21) without any adjustment and when controlling for the variables depicted in **Figure 5.4** (β = - 0.074, P = 0.48). Salivary cortisol levels ranged between 0.02 to 1.98 µg/dL, and Median was 0.28 µg/dL. Obel *et al.* (2005) reported similar values on late pregnancy samples collected during the morning, in a study that determined whether exposure to stressful life events was associated with variation in cortisol levels in pregnant women.

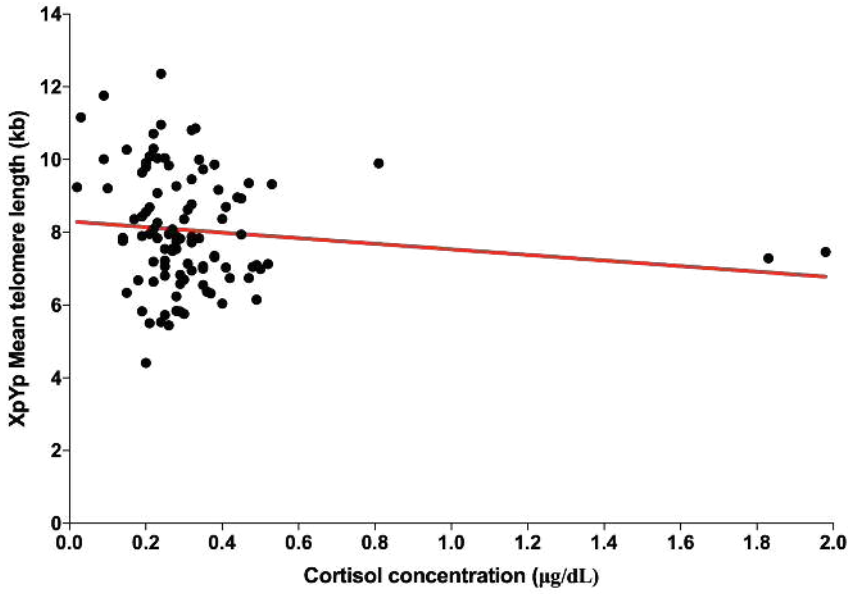

Figure 5.10. Linear regression showing the association between maternal salivary cortisol and mean placental XpYp telomere length. No association was found between maternal cortisol exposure and placental telomere length (β = - 0.123, P = 0.21) without any adjustment and when controlling for the variables depicted in **Figure 5.4** (β = - 0.074, P = 0.48). Simple linear regression was used to identify statistical significant differences *P < 0.05.

5.5.2.4. XpYp telomere length and SSRI

From the N = 109 participants available, 10 were recorded to be taking SSRI medication. The sample was divided into three groups: control participants with no signs of depression (C) (N = 42), participants with mild depression and depression not receiving SSRI treatment (M+D) (N = 57) and participants with mild depression and depression receiving SSRI treatment (M+D+SSRI) (N=10) (**Figure 5.11**). Telomeres in the M+D+SSRI group were, on average, 1 kb shorter than those in either the control or M+D groups. Multiple comparisons between the three groups showed that telomeres were shorter in the M+D+SSRI group compared to both the control group (P = 0.055) and M+D group (P = 0.034) as assessed by ANOVA.

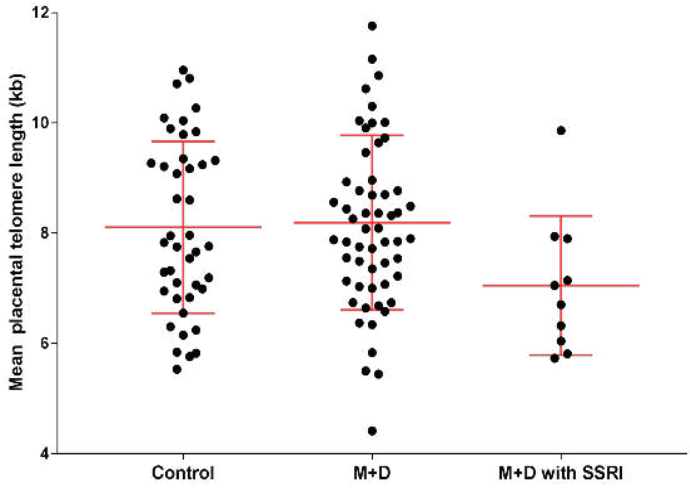

Figure 5.11. Telomere length differences in placenta from control, M+D and M+D+SSRI groups. Mean telomere length is presented (±SD). ANOVA test was used to assess statistical significant differences *P < 0.05.

5.6. Discussion

5.6.1. Prenatal depression associated with shorter placental telomeres

Our study is the first to identify a link between intrauterine exposure to maternal depression and telomere shortening in the placenta. Moreover, this association was present only in the placenta from female foetuses and not male foetuses. After accounting for the potential effects of maternal age, gestational age, parity, smoking, alcohol, BMI and WIMD score, prenatal depression was significantly associated with mean placental telomere length. All these variables along with EPDS accounted for 35% of the variance in placental telomere length among female infants. However, examination of an autosomal telomere, 17p, did not reveal a similar association.

A negative association between depression and telomere length in various tissues including buccal cells, leukocytes and nervous system cells, has been previously reported in a human meta-analysis (Ridout *et al.* 2016). Likewise, Wei *et al.* (2016) found shorter telomeres in the salivary DNA of individuals with depression using qRT-PCR. A number of studies have reported associations between different intrauterine exposures and offspring telomere length (Entringer *et al.* 2011,2013; Marchetto *et al*. 2016 and Send *et al.* 2017). To the best of our knowledge, only Gotlib *et al.* (2015) have reported an effect of maternal depression on offspring telomeres. In this study, the 10- to 14-year-old girls at familiar risk of developing depression exhibited shorter telomere length in their salivary DNA as assessed by q-PCR.

5.6.2. Sexual dimorphism in response to prenatal depression

Our results showed a significant reduction on telomere length in female placenta from women who reported elevated depressive symptoms just prior to delivery. In this regard, it has been previously reported that carrying a female baby compared with male baby increases the odds of A1 EPDS $\geq$ 13 by a factor of 2.86 (Janssen *et al.* 2018).

Foetal sex has already been highlighted as an important factor to take into account when analysing the response of the foetus to an insult (Doyle *et al.* 2015).

The placenta, like the foetus, has sex-related characteristics which include differences in placental gene expression. These differences are not limited to sexual chromosomes, but also autosomal genes. For instance, genes involved in immune pathways, which differentially expressed, may contribute to sex differences in the foetal response to an infection (Clifton 2010). It has been reported that, unlike male placenta, the female placenta is responsive to changes in glucocorticoid concentration (Clifton 2010). This study reported that pathways responsive to cortisol were affected in the presence of increased cortisol levels. In response to an insult, the female placenta generates multiple adaptations in placental gene and protein expression, which trigger a decrease in growth to ensure livelihood (Clifton 2010). Doyle *et al.* (2015) found that male and female foetuses had different responses when exposed to *in utero* maternal distress. While negative mood, cortisol and diastolic blood pressure in males showed accelerated foetal development, females were variously responsive. It is hypothesized that acceleration in foetal development may occur to reduce exposure time to an adverse *in utero* environment. These results support the relevance of the maternal HPA axis in foetal development. In contrast to Doyle *et al.* (2015), we assessed maternally reported symptoms or anxiety and depressive symptoms, whereas they used an index of EMA (Ecological momentary assessment of emotional experiences), which represents the negative mood of an individual based on ratings of 18 mood states. In our published study on prevalence of depressive and anxiety symptoms in the GIW cohort, we compared salivary cortisol to maternally-reported depression symptoms and found no correlation (Janssen *et al.* 2018). Salivary cortisol range levels detected in this study were similar to those found by Obel *et al.* (2005) in late pregnancy samples collected during the morning. We found no correlation between maternal salivary cortisol and telomere length. It is therefore possible that other stress-related physiological mechanisms apart from the HPA axis are involved, such as alterations at the level of neuropeptides, monoamines, and steroid and the autonomic nervous system (Savolainen *et al.* 2015). Our results are supported by Savolainen *et al.* (2015) who, using a Trier Social Stress Test, did not find associations between leukocyte telomere length and salivary and plasma cortisol or plasma ACTH in response to stress.

A recent study noted, for the first time, sex-specific differences in the maternal factors that influence newborn telomere length (Bosquet Enlow *et al.* 2018). They reported differences in the pattern of association between the predictor and telomere length, being some variables only associated with telomere length in male infants (education, depressive symptoms, sexual abuse and familial emotional support) and others in female infants (maternal age). In contrast to our results, they found among males a correlation between elevated depressive symptoms in pregnancy and shorter newborn telomere length. According to their results, male foetuses were more susceptible to maternal exposure than females, at least with respect to leukocyte DNA.

5.6.3. Antidepressants and telomere length

Previously, telomere length has been inversely associated with oxidative stress and inflammation in individuals with depression. This could be interpreted as telomere shortening not preceding depression, but rather the consequence of inflammation and oxidative stress processes. This study reported that a lifetime exposure to depression was inversely correlated with telomere length in leukocytes but those taking antidepressants did not show this correlation (Wolkowitz *et al.* 2011). In this context, Zhou *et al.* (2011) showed in mice that fluoxetine, an antidepressant drug, was able to reverse changes in TERT and telomerase activity induced by chronic mild stress, produced by the sequential application of a variety of mild stressors during 3 weeks. Needham *et al.* (2015) did not find any effect of antidepressant medication use on telomere length. In this study, we found a correlation between the use of SSRI during prenatal depression and reduced placental telomere length. However, the latter correlation should be interpreted cautiously as the group is very small, and 9 from those 10 women who were under SSRI treatment had a mental health history of depression. Duration of depression has been previously reported as a major determinant of telomere length attrition in leukocytes from patients with depression (Wolkowitz *et al.* 2011).

Reduced psychiatric responses to medications have been previously reported in association with shorter leukocyte telomere length (Hough *et al.* 2016). In a study assessing SSRI response in MDD (major depressive disorder) patients, they

found that those patients whose telomeres were shorter before the treatment exhibited poorer response to SSRI treatment. It is therefore a possibility that telomere length could be used as a biomarker to predict SSRI treatment response (Hough *et al.* 2016). Similarly, Rasgon *et al.* (2016) found that telomere length acted as a predictor in response to another antidepressant drug, pioglitazone.

5.6.4. Disparities in results between the XpYp and 17p telomeres

Prenatal depression was associated with a lower mean telomere length only in the female placenta. This was the case for the XpYp telomere but not for the 17p telomere. The caveat is that fewer samples were present in the 17p group.

Whilst previous work indicates that telomere distributions are similar between sex chromosomes and autosomal chromosomes (Roger *et al.* 2013), this was not support by our findings. There was no correlation between XpYp and 17p mean telomere length in the human placenta. We found that 17p telomeres were approximately 2 kb shorter that the XpYp telomeres. It may be possible that 17p telomere is more sensitive to placental exposures. The placenta also showed a very high level of heterogeneity within samples at 17p with telomeres lengths ranging from < 1 kb to > 15 kb (**Figure 5.6**). When a comparison was made between mood scores and 17p telomeres length, no association was found in either male or female placenta. This could suggest that only the sex chromosomes are impacted by maternal mood. However, an important caveat should be noted: while 109 samples were analysed at the XpYp telomere, only 60 from the 109 available were analysed for the 17p telomere. It is therefore possible that differences between the samples precluded the identification of an association. However, differences with respect to demographics when comparing control and depressed participants were not found between the preexisting 60 participants and the new 109 added to the study.

A further limitation of the study is that self-reported questionnaires were used. Although self-report questionnaire have a high acceptance by women who feel less constrained in responding as stated by the WHO (2008), these questionnaire are not a substitute for a full clinical evaluation. Another limitation of this study was the insufficient data available with respect to mental health treatments. This is particularly important as some drugs may influence telomere length. Finally,

while the statistical analysis controlled for some factors, there may be other factors such as paternal factors contributing to placental telomere length.

5.6.5. Summary

In this study, telomere length was measured in placental samples from pregnancies where women reported depression and anxiety symptoms, taking into consideration foetal sex in addition to the effect of pregnancy status, lifestyle factors and socio-economic status. STELA was applied to the XpYp and 17p telomere and detailed telomere-length profiles from placental samples were generated. The main finding was that placental telomere shortening was associated with prenatal depression, but that this was specific to female placenta and only observed at XpYp telomere. However due to different sample sizes when comparing XpYp and 17p telomeres, it cannot be concluded that the relationship found between telomere length and prenatal depression is limited to the XpYp chromosomes.

Further work is needed to discover the molecular underpinnings that associate prenatal depression and telomere shortening in the placenta. If telomere length is considered as a biomarker for prenatal depression and placenta a proxy of the foetus, early intervention strategies for pregnancies where the baby is known to be a female may impact offspring lifetime health outcomes.

Chapter 6: General discussion and future directions

6.1. Key findings

The work described in this thesis has been individually discussed in each chapter. Accordingly, this chapter will serve as a general overview of the results, implications of such results, strength and limitations and further directions. Key findings are outlined below:

Chapter 3	• Sampling site, mode of delivery or foetal sex do not impact telomere length distributions in the placenta. • STELA revealed remarkable telomere length heterogeneity within samples.
Chapter 4	• Placental telomere length is affected by GDM. • Placental telomeres from male infants exposed to GDM in medically untreated pregnancies were shorter than placental telomeres from control male infants and infants whose mothers were diagnosed with GDM and treated with metformin and/or insulin.
Chapter 5	• Placental telomere length is affected by prenatal depression. • A significant negative association was found between prenatal depression symptoms and XpYp telomere length in the female placenta.

6.2. Implications of results obtained from chapter 3

Telomere length at birth is heritable and is also influenced by environmental factors (Hjelmborg *et al.* 2015). Therefore, studying how environmental exposures shape telomere length *in utero* can help to understand the observed telomere length variability in early life, which may contribute to developmental programing. The placenta is a fetally derived organ that can be used as a proxy for the foetal exposures during pregnancy. Placental dysfunction may also contribute to adverse outcomes. Some studies have studied telomere length in the placenta in relation to various pregnancy adversities: IUGR and PE (Biron-Shental *et al.* 2010), GDM (Biron-Shental *et al.* 2015), pre-pregnancy BMI (Martens *et al.* 2016) and air pollution exposure (Martens *et al.* 2017). However, none have used a high-resolution telomere analysis technique. We have applied for the first time STELA to measure placental telomere length profiles taking into account: sampling site, mode of delivery and foetal sex.

Dissection site did not impact telomere length in the placenta. Martens *et al.* (2016) have previously demonstrated a minimal intra-placental variability of telomere length. Alssopp *et al.* (2007) similarly reported no differences in telomere length variability within the placenta. In this study, mode of delivery did not impact telomere length in the placenta. In contrast, Polettini *et al.* (2015) reported a higher telomere-repeat content in term labour amniotic fluid. Previous studies showed a correlation between telomere length and foetal sex (Cherif *et al.* 2013, Benetos *et al.* 2014 and Wilson *et al.* 2016). The current study demonstrated no effect of foetal sex on telomere length. Gielen *et al.* (2014) and Okuda *et al.* (2002) support our findings.

In summary, the findings of the current study indicate that STELA has a wide application in pregnancy cohort studies regardless of study design and provides a new tool to interrogate the link between telomere length and pregnancy complications secondary to placental dysfunction.

This study also provided the first evidence that the term placenta exhibits remarkable telomere heterogeneity. This organ is thought to be generated by about 36 rounds of cell divisions (Simpson *et al.* 1992). These results provide support for the hypothesis that the placenta ages as pregnancy advances and

replicative senescence is a consequence of this phenomenon (Sultana *et al.* 2018; Kalmback *et al.* 2013). Gielen *et al.* (2014) also reported a correlation between telomere attrition and gestational age, which suggests a possible role of telomere attrition in ageing of the placenta. Kyo *et al.* (1997) has also shown a reduction in telomerase activity during maturation of the placenta. To conclude, our application of STELA revealed a richer picture of the telomere dynamics in the placental tissue than previously recognised.

6.3. Implications of results obtained from chapter 4

The fact that one in seven births are affected by gestational diabetes (International Diabetes Federation 2017), makes it one of the most common complications of pregnancy. A diabetic pregnancy implies maternal and foetal outcomes. Exposure to high levels of glucose *in utero* compromises the long-term health of those babies (Fraser *et al.* 2014; Reece 2010).

Telomeres are indicative of cumulative cell damage within an organ (Blackburn, 2000). In addition, shorter telomeres have been found in individuals with T2DM (Nan, Ling and Bing 2015) and offspring exposed to GDM *in utero* (Hjort *et al*. 2018; Xu *et al.* 2014, Biron-Shental *et al*. 2015), suggesting that hyperglycaemia may be a cause or consequence of telomere dysfunction.

In this study, a preliminary analysis only comparing GDM and control mothers did not reveal a significant different in telomere features. However, after splitting the samples by sex, placental telomeres from male infants exposed to GDM in medically untreated pregnancies were found shorter than placental telomeres from control male infants and infants whose mothers were diagnosed with GDM and treated with metformin and/or insulin. These findings have 2 ramifications: firstly, the need to consider sex differences when studying the effect of environmental insults on infant telomere length and secondly, our data suggest that treatment with metformin and/or insulin may rescue placental telomere attrition associated with GDM. Based on these results, it may be speculated that where the baby is known to be male, adoption of targeted medical treatment of GDM pregnancies may alleviate adverse outcomes in the infants.

These findings suggest a sex-specific response of the placenta to an insult: GDM. The strategy to cope with an adverse maternal environment in the case of the male placenta involves minimal changes in gene and protein expression to ensure continued growth, which are associated with a greater risk of adverse outcomes (Clifton 2010). The mechanisms underlying sex-specific responses in the placenta are still unclear. Retnakaran *et al.* (2015) have reported that carrying a male foetus is associated with an increased risk of developing GDM in the mother. This association is based on the fact that carrying a male foetus was linked to poorer beta cell function in the pancreas and higher postprandial

glycaemia, suggesting that foetal sex may have a role in maternal glucose metabolism. It is speculated that carrying the Y chromosome could influence the placental secretion of hormones that leads to beta cell compensation. This compensation arises in response to insulin resistance and is essential to cope with the new insulin demands. Gabory *et al.* 2013 have proposed that the abundance of X-linked genes involved in the complex process of placentogenesis, may be linked to perinatal complications shown by male foetuses secondary to placental dysfunction. It is also possible that the infants inherited shorter telomeres from mothers who were already predisposed to develop GDM.

6.4. Implications of results obtained from chapter 5

Recent data suggests that depression during pregnancy is a global problem that is increasing (Janssen *et al.* 2018; Pearson *et al.* 2018) and represents about 15% of the women in developing countries (WHO 2015). These women have an increased risk to develop postpartum depression (Stowe, Hostetter and Newport 2005). Maternal mood disorders during pregnancy are correlated with a number of negative perinatal and maternal outcomes (Field *et al.* 2006; Choi *et al.* 2014; Shivakumar *et al.* 2011).

Telomere function may be impacted by biochemical changes resulting from stress and depression, such as inflammation, oxidative stress, insulin resistance, and autonomic and neuroendocrine stress reactivity (Epel and Prather 2018). In addition, shorter telomeres have been found in individuals with depression (Wei *et al.* 2016), as a result of childhood maltreatment (Tyrka *et al.* 2010), psychological stress (Steptoe *et al.* 2017), chronic psychological stress (Epel *et al.* 2014) and offspring of depressed mothers (Gotlib *et al.* 2015) and stressed mothers (Entringer *et al.* 2011, 2013; Marchetto *et al.* 2016 and Send *et al.* 2017).

This study provides the first evidence that intrauterine exposure to maternal depression is correlated with telomere shortening in the placenta. A significant negative association was found between prenatal depression symptoms and XpYp telomere length in the female placenta. Based on these results, it may be speculated that where the baby is known to be female, early intervention strategies may contribute to offspring later in life health.

These findings suggest a sex-specific response of the placenta to an insult: prenatal depression symptoms. The strategy to cope with an adverse maternal environment in the case of the female placenta involves multiple changes in gene and protein expression that leads to a minor reduction in growth, to ensure survival (Clifton 2010). Unlike male placenta, the female placenta is responsive to changes in glucocorticoid concentration (Clifton 2010). Even though we have found no correlation between maternally-reported depression symptoms and salivary cortisol (Janssen *et al.* 2018), it may be speculated that the female placenta could be responsive to other stress-related physiological mechanisms apart from the HPA axis, which have not been tested here in this study

(Savolainen *et al.* 2015). It is also possible that the offspring inherited shorter telomeres from women at risk of depression.

A summary of the findings obtained from this PhD added to previous studies that correlated telomere attrition and different prenatal adversities is depicted in **Figure 6.1.**

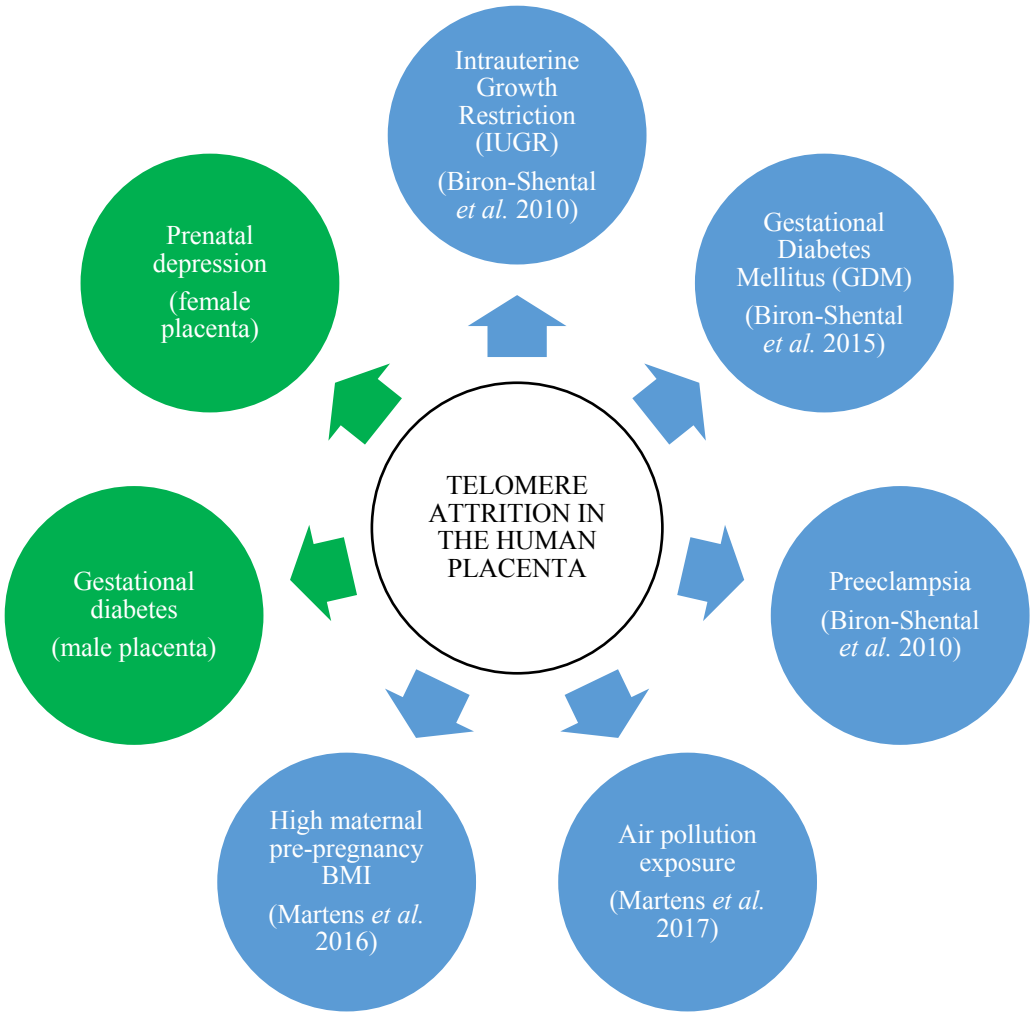

Figure 6.1. Suboptimal intrauterine conditions that have been correlated with telomere dysfunction. Preeclampsia, GDM, IUGR, high maternal pre-pregnancy BMI and air pollution exposure are examples of maternal states and conditions that contribute to telomere attrition in the placenta. In green colour: contribution from this PhD.

6.5. Strengths and limitations

Chapter 3

For the first time STELA was optimised to measure placental telomeres. Telomere length has been previously measured in the placenta in relation to IUGR, GDM, PE, air pollution exposure and high maternal pre-pregnancy BMI using respectively Q-FISH, q-PCR and RT-PCR (Biron-Shental *et al.* 2010, 2010, 2015; Martens *et al.* 2016 and 2017). These methods provide relatively low-resolution compared to STELA and therefore, very subtle differences in telomere distributions may not be as obvious (Aubert *et al.* 2012). STELA has been successfully applied in several cellular types or tissues as depicted in **Figure 3.2**. An advantage of using this technique was that a more detailed information on telomere homeostasis in the human placenta was provided. As a result, remarkable heterogeneity not previously reported before was observed in the placenta using this technique. In addition, this study demonstrated that sampling site, mode of delivery or foetal sex did not affect the telomere distributions, indicating that this technique has a potentially broader application in pregnancy cohort regardless of study design. The placenta, an easily accessible foetal derived tissue, constitutes a proxy for the foetal exposures during pregnancy. Malfunction of this organ may contribute to adverse outcomes.

One limitation of this study was the sample size. The majority of the participants were from a Caucasian origin, which can be interpreted as a strength in terms of variable control. However, larger samples were ethnic diversity is greater may provide further information of the role that ethnicity plays in the risk of suffering certain pregnancy complications. Although in the results above described only the Xp and Yp chromosomal telomeres were examined, Roger *et al.* 2013 reported no differences in telomere length between sex and autosomal chromosomes, and thus the XpYp telomere is representative of the other chromosome ends. Another limitation is the sole use of placenta as foetal origin source. Although previous research has reported intra-individual synchrony in telomere length across different somatic tissues in the newborn (Youngren *et al.* 1998; Okuda *et al.* 2002), it would be of interest to reproduce the study also in CB as some studies have reported differences between these two tissues (Biron-

Shental 2015, 2016). It is also important to note that the heterogeneous telomere length profiles revealed in the placenta in some individuals appears as bimodal or trimodal distributions. It is therefore possible that differentiated distributions correspond to same cell type with different replicative history or different cell types with different replicative history. A culture of placenta may give the answer. It would be also of interest to perform a more detailed cell fractionation of the placental tissue prior to STELA. Finally, the analysis did not control for any variable and further statistical analysis controlling for other variables including gestational age, would be advantageous.

Chapter 4

This is the first time telomere length has been measured with respect to GDM using a high resolution technique. There is only one other study which observed telomere attrition in the placenta from babies exposed to GDM *in utero* (Biron-Shental *et al.* 2015). Other studies have explored the role of telomere length in GDM using q-PCR, Q-FISH and Flow-FISH (Hjort *et al.* 2018, Xu *et al.* 2014, Biron-Shental *et al.* 2016, Harville *et al.* 2010 and Cross *et al.* 2010). A strength of this study was that the groups made up of controls, untreated GDM participants and treated GDM participants only differ in gestational age and the metabolic characteristics linked to GDM. A rigorous statistical analysis identified a significant association between maternal ethnicity and telomere length. Further comparison of telomere length between the groups controlled for this variable. Another strength was that telomere length analysis was presented as average telomere length but also as a percentage of telomeres below 5 kb, covering the range of telomeres within the lengths that leads to senescence. In this study, a sexual dimorphism in telomere length in response to GDM was described. This highlights the importance of taking into account foetal sex when analysing the effect of environmental insults on newborn telomere length. Finally, placental telomeres have emerged as a sensitive tool for testing prenatal adversity and measuring effectiveness of intervention strategies, such as metformin/insulin use.

Although this study found a correlation between intrauterine GDM exposure and placental telomere length, it failed to stablish causality. While it seems more probably that hyperglycaemia caused the telomere shortening, it is possible that

the offspring inherited shorter telomeres from mothers predisposed to develop GDM. It would be necessary to examine the telomere length in oocytes to address this question. Placental telomere attrition associated with GDM appeared to be prevented by maternal treatment with metformin and/or insulin in male placenta. This protective role may not be limited to boys which was not possible to assess in this study due to the smaller number of placental samples from girls. Another limitation is the fact that the placenta is a foetal derived tissue obtained at birth. These patterns of telomere length may not be present in the foetus, telomeres at this time has been reported as more vulnerable to being affected by several confounders compared to telomere length in the adolescence (Hjort *et al.* 2018). A further limitation was the small number of samples on treatment and it was not possible to explore how the action of insulin compared to metformin.

Chapter 5

This study is the first to report how prenatal depression and anxiety impact placental telomere features. To the best of our knowledge, only Gotlib *et al.* 2015 have reported an effect of maternal depression on offspring telomeres. Other studies have noted the impact of prenatal stress on new-born leukocyte telomere length (Entringer *et al.* 2011, 2013; Marchetto *et al.* 2016 and Send *et al.* 2017). A strength of this study was that female placenta exhibited shorter telomeres in female offspring exposed to maternal depression even after accounting for the potential effects of several variables. A hierarchical multiple regression that includes maternal age, gestational age, parity, smoking, alcohol consumption and WIMD made the analysis more robust. Another strength is the data available with respect to maternal salivary cortisol, mental health history and antidepressant treatment which could be factored into the analysis. In particular, it enables controlling for the variable of SSRI treatment when analysing the association between telomere length and depression. It is also important to note that association between placental telomere length and prenatal depression was assessed in two chromosomes: the sex chromosomes (XpYp) and an autosomal chromosome (17p).

Although this study found a correlation between intrauterine prenatal depression exposure and placental telomere length, it was not possible to establish causality. Whether the telomere impairment was inherited by offspring from women at risk of depression potentially contributing to depression or whether depression directly or indirectly caused the telomere dysfunction is unknown. Another limitation of this study is the use of self-reported questionnaires, the reliance on maternal self-report data on symptoms of depression and anxiety. The classification of women in depressed or not depressed can be subjective as EPDS cut-off can vary. In this regard, while using a cut-off point of ≥ 13, a woman with EPDS 12 would be control, a cut off by range: 0-6, 7-12 and ≥13 would categorised this woman as mild depressed. Another limitation is that no correlation was found between salivary cortisol and telomere length, indicating that other stress related physiological mechanism should be explored. There was no correlation between cortisol and maternally reported mood symptoms which suggest the relationship between maternal mood and cortisol is not a simple one. The data available with respect to antidepressants was not identical between the medical notes and the maternal questionnaires (Janssen *et al.* 2018). Some drugs may influence telomere length (Wolkowitz *et al.* 2011) and it would be important to increase sample size and validate compliance of taking the drugs in a future study. These factors mean that analysing the effect of cortisol and antidepressant use on telomere length has to be interpreted cautiously. Finally, the behaviour of sex chromosomes was different to the autosomal chromosome analysed. This may be due to differences in sample size (109 vs 60), which would mean the study of the autosome was underpowered. However, there may be inherent differences in telomere biology which would be interesting to explore in a larger dataset.

6.6. Future directions

Arising from the findings of this thesis, there are a number of future studies that could be done.

Firstly, it is important to note the remarkable telomere heterogeneity revealed in the term placenta. While the number of divisions have been estimated as 36 (Simpson *et al.* 1992), future work could explore this in more detail - perhaps using a culture system to look at the variance of the distributions after specific numbers of cell divisions for different cell types.

Secondly, placental telomeres by STELA is a sensitive tool for testing different prenatal adversities, as proven here with GDM and prenatal depression. If these findings are validated in other cohorts, STELA could provide a tool for measuring the effectiveness of intervention strategies or the impact of certain drugs in the foetus as it did with metformin (Garcia-Martin *et al.* 2018). During pregnancy, there is a characteristic fall in insulin sensitivity. This drop is thought to be influenced by the action of certain placental hormones and cytokines (Sonagra *et al.* 2014; Agha-Jaffar *et al.* 2016). Analysis of these molecules in relation to GMD and insulin resistance would shed light on their potential contribution as regulators of placental function. In addition, a characteristic low-grade inflammation is associated with GDM and therefore, testing of inflammatory markers in maternal plasma would be of interest (Korkmazer and Solak *et al.* 2015). Whilst the gold standard for the diagnosis of diabetes mellitus is the glycosylated haemoglobin (HbA1c) test, the standard screening test for gestational diabetes is the oral glucose tolerance test (OGTT) (Khalafallah *et al.* 2016). HbA1c test refers to the average blood sugar level for the past 2-3 months, showing the percentage of blood sugar bonded to Hb. It is a simple, non-fasting, less time consuming test that with proper optimisation could be an alternative to OGTT (Khalafallah *et al.* 2016).

Long term follow-up of mothers and children exposed to GDM would be beneficial as both are at risk of suffering long-term effects of the exposure to hyperglycaemia (Fraser *et al.* 2014; Reece 2010). This study found that placental telomeres from male infants exposed to GDM in medically untreated pregnancies were shorter. However, this study has not shown whether the impaired telomeres

were inherited by the offspring. In this regard, Hjort *et al.* 2018 and Xu *et al.* 2014 reported shortened telomeres in blood leukocytes of offspring exposed to GDM. It would be interesting to apply STELA in matched cord blood samples to provide further evidence that GDM impacts telomere length in the newborn and that metformin protects against telomere shortening also in foetal cells. The mechanisms of this gerosupressant agent, which suppresses the ageing process, are still unclear. Different explanations to its action include: the inhibition of new senescent cells and its aggregation (Anisimov *et al.* 2011), inhibition of mTOR signalling (Mendendez *et al.* 2011), activation of AMP-K which in turns upregulates TERRA (Diman *et al.* 2016) and indirectly inhibits NF-κβ, dampening the inflammation (Saisho 2016). No study to date has aimed to identify disrupted processes in relation to metformin action by interrogating telomerase, senescence and apoptosis pathways in existing RNAseq data, along with western blot technique in placental samples. Moreover, it would of interest to establish a primary cell culture from placental tissue or a trophoblast cell line. Regarding primary cell culture, a disadvantage of this method is that it should be performed when the tissue is still fresh (less than 1 h) and it also has a finite lifespan and limited expansion capacity. In our study all the samples were frozen with RNA later, reason why new samples would be required to conduct this approach. On the other side, primary cell culture mimics *in vivo* environment of the normal or abnormal placenta (Sagrillo-Fagundes *et al.*2016). In addition, it would useful to establish a culture system using choriocarcinoma cell line BeWo and the recently identified human trophoblast stem cell lines (Okae *et al.* 2018) to begin to test the mechanism of action of metformin *in vitro*. In this study, human trophoblast cells gave rise to the 3 major trophoblast lineages and presents similar transcriptomes and methylomes than primary trophoblast cells. It represents an important tool for molecular and functional characterisation of human trophoblast cells, and to investigate the pathogenesis of developmental disorders that entails trophoblast defects.

Finally, a central role has been given to the HPA axis in relation to depression (Meltzer-Brody 2011). We did not find any correlation between cortisol and telomere length in this study or between cortisol and maternal mood symptoms in our prevalence study (Janssen *et al.* 2018). It is important to note that samples

of saliva were collected in the morning at least 30 minutes after the last meal by the women themselves. To avoid confounders, the sample could be taken in the hospital at the same time for all the women in the same conditions. In this study, prenatal depression symptoms were associated with telomere shortening in the female placenta but not male placenta (P = 0.026). It would be interesting to apply STELA in matched cord blood samples to provide further evidence that prenatal depression impacts telomere length in the female newborn, and then relate these measurements to infant neurodevelopmental outcomes at 12 months. There is follow up data for the Grown in Wales Study cohort with questionnaires and videos with information regarding cognition, expressive and receptive language to evaluate infant neurodevelopmental outcomes. STELA could be used to ask whether shortened telomeres at birth predict infant outcomes. In this regard, Gotlib *et al.* 2015 provided evidence that daughters of depressed mothers had shorter telomeres and proposed that accelerate biological ageing may predispose them to develop depression and other age-related medical illnesses. Future work using STELA could include the development of this technique for buccal cells which could be collected less invasively than blood cells. Buccal cells are derived from the same germ cell layer as the nervous system, the ectoderm, (François *et al.* 2014), and may be more representative of telomere length in the brain. Optimising STELA for human stem cells would open the potential for *in vitro* approaches to explore mechanisms. Together these developments would address whether the shorter telomere identified at birth are still present in the children when they are 4-5 years old, and whether they predict any neurodevelopmental characteristics of these children, complimented by *in vitro* studies on telomere biology.

To conclude, Vera and Blasco (2012) pointed out the limitation of STELA in the analysis of long telomeres (typically greater than 20 kb). Mice, whose telomeres are 5 to 10 times longer than human (50-150 kb) (Calado and Dimitriu 2014) could not be applied STELA unless they are knock out for telomerase. This opens a new opportunity for obtaining high resolution telomere length analysis in mice experiments regardless of the above mentioned limitation. STELA could also be coupled with fluorescence-activated cell sorting (FACs) or magnetic-activated cell sorting technology (MACs) for measuring telomere length in different cell types.

Unlike MACs, FACs keeps cell purity at the expense of high speed, which can actually damage the cells. Conversely, MACs is beneficial for maintaining better cell viability (Liu *et al.* 2018).

6.7. Concluding remarks

In conclusion, this thesis has optimised the high-resolution technique STELA to measure placental telomeres, providing a richer picture of the telomere dynamics in the placenta. This study has demonstrated that placental telomeres can be used as a sensitive tool for testing prenatal adversities and measuring the effectiveness of intervention strategies. An interesting sexual dimorphism has arisen from two different environmental exposures: GDM and prenatal depression. It seems that these pregnancy-related complications impact telomere length in a sex-specific manner in the placenta. This thesis has not shown whether the shorter telomeres found in the placenta were inherited by the offspring which will be important to determine in future studies.

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Appendix

Appendix 1A: Participant information sheet

Version 4.1
Date: 14.05.13
REC ref no. 10/WSE02/10

Study title: Examining imprinted gene expression in human placenta.

Part 1 of information sheet

Invitation paragraph:

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will give you the information sheet and answer any questions you have. We'd suggest this should take about 10 minutes. Talk to others about the study if you wish.

Ask us if there is anything that is not clear.

What is the purpose of the study?

We want to look at the factors in the placenta that effect growth of the baby. We know of a group of genes (instructions) that are important for building the placenta. If these "instructions" are incorrect or misread, then normal growth and development does not happen. We will look at these instructions in a large number of placentae, including yours, so that we can understand them better. This will help us predict which pregnancies might need closer monitoring and more support from our Obstetrics team to ensure a safe delivery and a long and healthy life for babies born in the future.

What is the placenta?

The placenta is the afterbirth. After your baby is born, more contractions push the placenta out through the vagina. The placenta at this stage is also called the afterbirth. Normally it is destroyed.

What does the placenta do?

The placenta is the organ that links you to your baby via the umbilical cord. The job of the placenta during pregnancy is to pass oxygen and food from your blood supply to the baby. Waste products from the baby, such as carbon dioxide, are returned along the umbilical cord back to the placenta and then into your bloodstream.

Why do we need to collect the placenta?

Following the birth of your baby your placenta is normally destroyed. By collecting placentae and studying them, we can hope to learn what is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy. For example, we would like to learn more about why some babies are born smaller or larger than average or why some women develop pre-eclampsia or gestational diabetes.

Why have I been invited?

Any pregnant women can participate. As of April 2007 a new legislation came into effect (The EU Directive for Tissue and cells), which states that consent for the use and testing of your placenta must be obtained prior to the birth of your baby or before labour is so advanced that you do not have time to make an informed decision(which includes active labour).

This ensures that you have had plenty of time to ask us any questions you have relating to the collection, testing and storage of your placenta. We encourage you to make an informed decision about donating your placenta.

Do I have to take part?

It is up to you to decide to join the study. We describe the study in this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

You will be asked to complete a short questionnaire about your pregnancy. Any questions that you feel unhappy answering can be left blank. This information will be given a unique identification number so that your details are kept confidential.

Once your baby has been born, your midwife will confirm that consent has been given and will pass your placenta to our staff, along with your signed consent form. Small pieces of the placenta are then placed in collection tube. We need to collect only a few small pieces the size of a thumb tip. The rest will be destroyed by the normal Hospital procedures.

Your donation is given a unique identification number. All the information provided to us uses this number only. Your details are therefore kept confidential. We will not know your name or address, just the babies weight and a few details about your pregnancy i.e head and abdominal measurements of baby and any medical problems you have experienced. We will collect the details about the pregnancy and newborn at the time of birth and then the details will be anonymised therefore we will not be able to collect any further information and cannot identify you after wards.

We expect study to last approximately 1 year.

Expenses and payments?

Your placental sample will be collected at the time of birth. Therefore it will not cost you any money. We do not pay for your donation.

What are the side effects of any treatment received when taking part?

The procedures we use to collect a placental sample are safe and risk-free for both you and your baby. This is because the collection of the placenta happens AFTER the baby is born, AFTER the cord is cut and AFTER the placenta has been delivered. Donating samples of your placenta does not interfere with the management of labour and delivery, or with the aftercare of you or your baby.

What are the possible disadvantages and risks of taking part?

There are no disadvantages of taking part in the research as we will only take the sample from the placenta after the delivery of placenta before it is sent for destroying destruction as per hospital policy.

What are the possible benefits of taking part?

We cannot promise the study will help you in current pregnancy, but the information we get from this study we can learn what genetic activity in the placenta is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy. For example, we would hope to learn more about why some babies are born smaller or larger than average or why some women develop pre-eclampsia or gestational diabetes.

What if there is a problem?

Any complaint about the way you have been dealt with during the study you will be addressed. The detailed information on this is given in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2 of the information sheet

What if relevant new information becomes available?

As this study is anonymous we will not be able to contact you.

What will happen if I don't want to carry on with the study?

If you withdraw from the study by just informing your midwife and we will not use your placental sample for the study. You can withdraw from the study before the placenta is passed on to the research team, after which the samples will not be identifiable.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number at the end). If you remain unhappy and wish to complain formally, you can do this [e.g. NHS Complaints Procedure].

What will happen to any samples I give?

The placenta will be stored for a short time (weeks) and then we will prepare RNA and DNA (in order to look at the genes) from it. This will be stored for the duration of the study. These samples will be anonymous and we cannot identify you or your baby from them.

What If a participant, who has given informed consent, loses capacity to consent during the study?

No further data or tissue will be collected once the placental samples have been taken. It will not be possible to identify the tissue related to the participant if she loses the capacity to consent during the study after the samples have been taken. If between giving consent and the donation of the tissue sample, the woman loses capacity to consent she will be withdrawn from the study and placental tissue samples will not be taken. as the samples will be anonymised after they have been taken.

What will happen to the results of the research study?

This is an anonymous study. We will not know who you are. We will not have your name or address. This means we cannot contact you to tell you anything about your placenta. We aim to publish the results of our research in scientific journals, and will also provide feedback via the hospital newsletter, and local media.

Who is organising and funding the research?

University Hospital Wales & University of Cardiff (Biosciences)

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by South East Wales Research Ethics Committee.

6.2.11 Further information and contact details

Dr Sajitha Parveen (Principal Investigator, Royal Gwent Hospital)

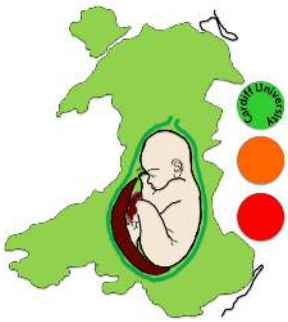
Email: sajitha.parveen@wales.nhs.uk

Study Chief Investigator:

Mr R Penketh (Consultant Obstetrician & Gynaecologist)

Email: Richard.Penketh@wales.nhs.uk

Appendix 1B: Participant information sheet



Participant Information Sheet

Version 1.3
Date: 22.05.15
REC ref no: 15/WA/0004

Title of Project: Grown in Wales

We would like to invite you to take part in our research study. This information sheet explains why the research is being done and what it would involve for you. A member of our research team will answer any questions you may have.

What is the purpose of the study?

We will look at factors in the placenta that affect growth of the baby and health of the mother. We know that genes (instructions) are important for building the placenta. If these "instructions" are incorrect, then normal growth and development does not happen. We will look at these instructions in placentas, including yours, so that we can understand them better. This will help us predict which future pregnancies might need closer monitoring and more support from our Obstetrics team. We also may be able to use this information to predict future outcomes for mothers and their children. We will also collect cord blood and your saliva so we can measure factors like hormones that are also important for healthy pregnancy.

What is the placenta and cord blood?

The placenta is also called the afterbirth. During pregnancy, the placenta is the organ that links you to your baby via the umbilical cord. It passes oxygen and food from your blood supply to the baby via the umbilical cord. After your baby is born, more contractions push the placenta out through the vagina. Normally it is destroyed.

Cord blood is found in the umbilical cord which links you to your baby during pregnancy. After your baby is born, the umbilical cord and cord blood is normally destroyed.

Why do we need to collect the placenta, cord blood and saliva?

After your baby is born, your placenta and the umbilical cord are normally destroyed. By collecting them and studying them, we can learn what is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy. For example, we would like to learn more about why some babies are born smaller than average and why some women develop postnatal depression.

Why have I been invited?

Any pregnant women can participate but consent for the use and testing of these samples must be obtained prior to the birth of your baby or before labour is so advanced that you do not have time to make an informed decision. This ensures that you have had plenty of time to ask us any questions relating to the research study.

Do I have to take part?

It is up to you to decide to join the study. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

Before you have your baby, you will be asked to complete a short questionnaire about you and your pregnancy. Any questions that you feel unhappy answering can be left blank. At this time, we will also ask for a sample of your saliva - this is done easily and painlessly by spitting in to a plastic tube provided by the researcher.

If you have given a blood sample for a clinical test and there is unused blood, we will also collect this for our study.

Once your baby has been born, your midwife will confirm that consent has been given and will pass your placenta with the umbilical cord to a trained researcher. Small pieces of the placenta (about the size of a pea) are then placed in collection tubes. We will also extract blood from the umbilical cord. The rest of the tissue will be destroyed.

In the first week after you have had your baby, we will ask you to complete another short questionnaire about how you are feeling and provide another sample of your saliva.

Two months after your baby is born we will contact you to ask if you wish to continue in the study. If you do, we will send you another short questionnaire about how you are feeling and provide a tube for you to collect your saliva. You will return these to us in the stamped addressed envelope provided.

When your child is around 1 year old we will contact you again to ask if you wish to continue in the study. If you do, we will invite you and your child to carry out play tests to assess your child's development. This should not be distressing for your child and should take no more than 2 hours.

As part of this study we will also collect details about your pregnancy and newborn at the time of birth such as birth weight. We will also collect data on any relevant medical problems you or you baby have experienced.

Your donations and all your data will be given a unique identification number that will be used to identify your samples. Your identity and that of your child will be recorded on a secure database under password protected and accessible only by members of the Grown in Wales research team, Cardiff University. The samples and your data will therefore be anonymous to anyone outside of the team who may wish to look at your samples in the future.

We expect study to last approximately 1 year but we may contact you in the future to ask if you want to participate in future studies on you or your child. This would require a separate consent form. If you consent to taking part in the follow up or to be contacted about future studies, your contact details will be stored on a password protected secure database only accessible to members of the research team and you will only be contacted by a Grown in Wales research team member.

Expenses and payments?

Donating your samples will not cost you any money and we do not pay for your donation. You will be reimbursed any travel costs incurred if you and your child take part in the 1-year follow up visit and you will be given a £20 voucher or equivalent to compensate you for any inconvenience.

What are the side effects of any treatment received when taking part?

The procedures we use to collect the placenta, saliva and cord blood samples are safe and risk-free for both you and your baby. This is because the collection of the placenta happens AFTER the baby is born, AFTER the cord is cut and AFTER the placenta has been delivered. Donating these samples does not interfere with the management of labour and delivery, or with the aftercare of you or your baby.

What are the possible disadvantages and risks of taking part?

There are no disadvantages to taking part in the research as we will only take the samples after the delivery of placenta and umbilical cord before they are sent for destruction as per hospital policy.

What are the possible benefits of taking part?

We cannot promise the study will help your current pregnancy, but with the information we get from this study we can learn more about what is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to one of our trained researchers who will do their best to answer your questions. Contact details are provided below. If you remain unhappy and wish to complain formally, you can do this through NHS Complaints Procedures.

What if relevant new information becomes available?

We will not contact you directly but relevant new information will be communicated to the medical team caring for you or your GP as necessary.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time, without giving a reason. If you want to withdraw from the study, simply inform your midwife or one of the research team. If you do not wish to take part after your baby is born, you can inform us when we contact you at 2 and 12 months.

What if a participant, who has given informed consent, loses capacity to consent during the study?

If between giving consent and the collection of samples, the woman loses capacity to consent she will be withdrawn from the study and samples will not be taken. If the woman loses capacity to consent after the samples have been taken i.e. after delivery but before discharge from hospital, no further data or tissue will be collected but any samples that have been taken will be included in the study unless the participant withdraws from the study.

What will happen to any samples I give?

The placenta tissue will be used to prepare RNA, protein and DNA (in order to look at the genes). The cord blood, maternal blood and saliva will be processed for measuring hormones. Cord blood will also be used to prepare DNA (to look at genes). These samples will have a unique reference number and only researchers directly involved with the study will have access to the code that reveals your identity. If any placental tissue, cord blood, maternal blood or saliva is left at the end of the study, it will be kept and may be used in future research by the Grown in Wales research team or other researchers within the UK or abroad. Other researchers using these samples will not be supplied with your name or any other identifiable information and will not be able to identify you from the tissue. Your tissue will not be sold and will not be used in genetic research, animal research or the commercial sector.

What will happen to the results of the research study?

We aim to publish the results of our research in scientific journals, and will also provide feedback via the hospital newsletter, and local media. We may work with other researchers but your identity will be anonymised for this work. We will not provide direct feedback to you on your specific data or that of your baby.

Who is organising and funding the research?

Professor Rosalind John is the chief investigator for this study and curator of the samples. Mr Richard Penketh is the clinical lead. Professor John and Mr Penketh will run the study. The work will be supported by University Hospital Wales & University of Cardiff (Biosciences) with additional funding from charities and research councils.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by South East Wales Research Ethics Committee.

For Further information and contact details:

Professor Ros John (Chief Investigator)
Email: JohnRM@cf.ac.uk

Mr R Penketh (Consultant Obstetrician & Gynaecologist),
Email: Richard.Penketh@wales.nhs.uk

Appendix 2A: Written informed consent

Centre Number:

REC reference number: 10/WSE02/10

Study of Imprinted Gene Expression in the Placenta

CONSENT FORM VERSION 4

Date: 15.12.11

Title of Project: Examining imprinted gene expression in human placenta.

Name of Researcher

Please initial box

1. I confirm that I have read and understand the information sheet dated **15.12.11 (version 4)** for the above study, have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that only relevant sections of my medical notes and data collected during the study may be looked at by research team members, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records, but the data retrieved will be anonymised. ☐
4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file: 1(original) to be kept in medical notes.

Appendix 2B: Written informed consent

Participant Consent Form

Title of Project:
Grown in Wales

Version 1.3, Date 22.05.15

REC reference number: 15/WA/0004

Please initial the relevant box

	YES	NO
1. I confirm that I have read and understand the information sheet dated 22.05.15 (version 1.3), have had the opportunity to consider the information, ask questions and have has these answered satisfactorily.	☐	☐
2. I am willing to complete the participant questionnaires for this study	☐	☐
3. I am willing to donate my blood sample, placenta, cord blood and saliva samples	☐	☐
4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by research team members (Cardiff University), from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in research.	☐	☐
5. I agree to be contacted at 2 and 12 months after delivery to complete additional questionnaires and provide a sample of my saliva.	☐	☐
6. I agree that, if possible, my infant and I will come back when my child is approximately 12 months for a simple assessment of their development.	☐	☐
7. I agree to be contacted until my child is 12 years old about participating in future studies. I understand that this may involve tracing through NHS databases and GP records.	☐	☐
8. I agree to my anonymised samples and data being used in future studies by the GIW research team or other institutions in the UK and abroad.	☐	☐
9. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐	☐
10. I agree to take part in the above study.	☐	☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Appendix 3A: Self-administered participant questionnaire

Study ID:

Date:

Study of Imprinted Gene Expression in the Placenta

Participant Questionnaire

Version 1

15.12.11

REC Ref no: 10/WSE02/10

Thank you for answering these questions. It would help us if you could answer all the questions, however if you feel unhappy about answering any of them, please feel free to leave them blank. All information is kept in the strictest confidence and will not affect the standard of care you receive.

Where known, please provide us with the answers to the following questions :

About You

- **To which of these ethnic groups do you consider you belong?** *(Please tick)*

  Caucasian (White) ☐ Indian/Pakistani/Bangladeshi ☐ Middle Eastern ☐

  Far Eastern ☐ African/Afro-Caribbean ☐

  South American/Hispanic ☐ Mixed (*please specify*) ☐ ……

  Do not wish to say ☐ Other *(Please specify)* ☐ ……

- **What is your country of birth?** *(Please tick)*

  Wales ☐ England ☐ Ireland ☐ Scotland ☐ Other ☐

- **What is your age?** ……

- **What was your weight before pregnancy?** …… **kg or** …… **lb**

- **Towards term (37 weeks), we will ask for your final weight** which will be recorded here by yourself or your midwife as …….... **kg or** …….... **lb at** …….. **weeks**

- **Do you know your birthweight?** *(Please tick)*

  Yes ☐ it was ……..

  No ☐

- **What is your highest level of education?** *(Please tick)*

  Left before GCSE ☐ GCSE/O levels ☐ A levels ☐ University ☐

About your family

- **To which of these ethnic groups does your baby's father belong?** *(Please tick)*

  Caucasian (White) ☐ Indian/Pakistani/Bangladeshi ☐ Middle Eastern ☐

  Far Eastern ☐ African/Afro-Caribbean ☐

  South American/Hispanic ☐ Mixed (*please specify)* ☐……

  Do not wish to say ☐ Other *(Please specify)* ☐……

- **What is your baby's father's country of birth?** *(Please tick)*

  Wales ☐ England ☐ Ireland ☐ Scotland ☐ Other ☐

- **What is your family income per year before deductions?** *(Please tick)*

  <£18,000 ☐ £18,000 - £25,000 ☐ £25,000 - £43,000 ☐

  >£43,000 ☐ Do not wish to say ☐

About your pregnancy

- **How many weeks pregnant are you today?** ……..…..

- **Is this pregnancy a natural or assisted conception?** *(Please tick)*

  ☐ ☐ ☐

- **Did you smoke in the three months <u>before</u> you found out you were pregnant?**

  (Please tick) Yes (daily) ☐ Yes (Occasionally but not every day) ☐ No ☐

- **Have you smoked <u>during</u> your pregnancy?** *(Please tick)*

  During the **first 12 weeks**: Yes (daily) ☐ Yes (Occasionally but not every day) ☐

  No ☐

  After 12 weeks: Yes (daily) ☐ Yes (Occasionally but not every day) ☐

  No ☐

- **Did you drink alcohol in the three months <u>before</u> you found out you were pregnant?**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

- **Have you had alcohol <u>during</u> your pregnancy?**

  During the **first 12 weeks:**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

  After 12 weeks:

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

- **Have you taken any illicit drugs during your pregnancy? Yes / No**

  If yes, what kind of drugs did you take? ……

- **Have you done any strenuous exercise during your pregnancy? Yes / No**

After you found out you were pregnant, how often did you eat the following foods? For each food type, please tick one box that best describes how often you ate this food. If this is different to before you found out you were pregnant, please circle in the right hand column whether you ate more or less of this food before. If this was not different, please leave the right hand column blank.

Example: If you typically ate chocolate 4 times each week during your pregnancy (but ate less before you found you were pregnant) you would complete the row as shown:

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Chocolate			✔			More / (Less)

Fruit and Vegetables

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Fresh Fruit						More / Less
Dried fruit e.g. raisins, dried apricots						More / Less
Salad and Cooked vegetables						More / Less

Dairy and Carbohydrates

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Milk						More / Less
Cheese, yoghurt						More / Less
Bread, cereals, potatoes, rice, pasta						More / Less

Meat, Meat alternatives and Fish

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Meat alternatives e.g. beans, peas, tofu, soy						More / Less
Meat (unprocessed) e.g. steak, ham, chicken slices						More / Less
Meat (processed) e.g. Burgers, sausages, fried chicken						More / Less
Fish/shellfish						More / Less

Snacks

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Chocolate						More / Less
Chips, Crisps						More / Less
Cakes, biscuits, ice cream						More / Less
Take away meals e.g. Chinese food, Curry						More / Less

Drinks and Supplements

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Soft drinks						More / Less
Caffeine e.g. tea, coffee, power drinks						More / Less
Supplements e.g. iron, folate						More / Less

Thank you for your help with this study, all the information you have provided us with is much appreciated. This will be kept in the strictest confidence and will not affect the standard of care you receive.

Appendix 3B: Self-administered participant questionnaire

Study ID:

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

ant Questionnaire

: Grown in Wales

GIG CYMRU NHS WALES | Bwrdd Iechyd Prifysgol Caerdydd a'r Fro | Cardiff and Vale University Health Board

Version 1.1

22.05.15

Thank you for answering these questions. It would help us if you could answer all the questions, however if you feel unhappy about answering any of them, please feel free to leave them blank. All information is kept in the strictest confidence and will not affect the standard of care you receive.

QUESTIONNAIRE PART 1

Where known, please provide us with the answers to the following questions:

About You

- **To which of these ethnic groups do you consider you belong?** *(Please tick)*

Caucasian (White)	☐	Indian/Pakistani/Bangladeshi	☐
Far Eastern	☐	African/Afro-Caribbean	☐
South American/Hispanic	☐	Middle Eastern	☐
Other	☐	Do not wish to say	☐

- **What is your country of birth?** *(Please tick)*

Wales	☐	Scotland	☐
England	☐	Ireland	☐
Other *(please specify)*	☐	……	

- **What is your age?** …….....

- **What is <u>your</u> height?** ……..... **m <u>or</u>** ……..... **ft**

- **If you are towards term (37 weeks), please record your final weight:**

  ……..... **kg <u>or</u>** …….. **st** ..... **lbs at** …….. **weeks**

- **Do you know <u>your</u> birthweight?** *(Please tick)*

  Yes ☐ it was ……..

  No ☐

- **What is <u>your</u> highest level of education?** *(Please tick)*

Left before GCSE	☐	GCSE/O levels	☐
A levels	☐	University	☐
Postgraduate Education	☐	Vocational Training	☐

<u>About your family</u>

- **To which of these ethnic groups does your baby's father belong?** *(Please tick)*

Caucasian (White)	☐	Indian/Pakistani/Bangladeshi	☐
Far Eastern	☐	African/Afro-Caribbean	☐
South American/Hispanic	☐	Middle Eastern	☐
Other	☐	Do not wish to say	☐

- **What is your <u>baby's father's</u> country of birth?** *(Please tick)*

Wales	☐	Scotland	☐
England	☐	Ireland	☐
Other *(please specify)*	☐	……	

- **What is your family income per year before deductions?** *(Please tick)*

<£18,000 ☐ £18,000 - £25,000 ☐ £25,000 - £43,000 ☐

>£43,000 ☐ Do not wish to say ☐

About your pregnancy

- **How many weeks pregnant are you today?** …….....

- **Is this pregnancy a natural or assisted conception?** *(Please tick)*

Natural ☐ Assisted *(IVF)* ☐ Do not wish to say ☐

- **Did you smoke in the three months before you found out you were pregnant?**

Yes (daily) ☐ Yes (Occasionally) but not every day) ☐

No ☐

- **Did you smoke during the first 12 weeks of your pregnancy?**:

Yes (daily) ☐ Yes (Occasionally) but not every day) ☐

No ☐

- **Did you smoke after the first 12 weeks of your pregnancy?**:

Yes (daily) ☐ Yes (Occasionally) but not every day) ☐

No ☐

- **Did you drink alcohol in the three months before you found out you were pregnant?**

Yes, almost every day ☐ Yes, once or twice a week ☐

Yes, once or twice a month ☐ Yes, once every couple of months ☐

No, ever ☐

- **Did you drink alcohol <u>during</u> the first 12 weeks of your pregnancy?**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐

  No, ever ☐

- **Did you drink alcohol <u>after</u> the first 12 weeks of your pregnancy?**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐

  No, ever ☐

- **Have you taken any illicit drugs during your pregnancy? Yes / No**

  If yes, what kind of drugs did you take?

  ……

- **Have you done any strenuous exercise during your pregnancy? Yes / No**

  (Strenuous exercise could be for example: running, jogging, squash, swimming, aerobics, cycling or football done for at least 30 minutes, at least once a week)

- **Have you previously suffered from a diagnosed mood disorder (e.g. depression, bipolar disorder, pre or postnatal depression)?** *(Please tick)*

  Yes ☐ No ☐ Do not wish to say ☐

  <u>If Yes:</u>

  Which Mood Disorder?.....

QUESTIONNAIRE PART TWO

DIRECTIONS: **After you found out you were pregnant, how often did you eat the following foods?** For each food type, please tick one box that best describes how often you ate this food. If this is different to before you were pregnant, please circle in the right hand column whether you ate more or less of this food after you found out you were pregnant. If this was not different, please leave the right hand column blank.

Example: If since finding out you were pregnant you ate more chocolate, usually around 2 times per week, you would complete the row as shown:

	More than once/day	Once /day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Chocolate			✔			(More) / Less

Fruit and Vegetables

	More than once/day	Once/ day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Fresh Fruit						More / Less
Dried fruit e.g. raisins, dried apricots						More / Less
Salad and Cooked vegetables						More / Less

	More than once/day	Once /day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Milk						More / Less
Cheese, yoghurt						More / Less

Meat, Meat alternatives and Fish

	More than once/day	Once /day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Meat alternatives e.g. beans, peas, tofu, soy						More / Less
Meat (unprocessed) e.g. steak, ham, chicken slices						More / Less
Meat (processed) e.g. Burgers, sausages, fried chicken						More / Less
Fish/shellfish						More / Less

Snacks

	More than once/day	Once /day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Chocolate						More / Less
Chips, Crisps						More / Less
Cakes, biscuits, ice cream						More / Less
Take away meals e.g. Chinese food, Curry						More / Less

Drinks and Supplements

	More than once/day	Once /day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Since I found out I was pregnant I ate..
Soft drinks						More / Less
Caffeine e.g. tea, coffee, power drinks						More / Less
Supplements e.g. iron, folate						More / Less

QUESTIONNAIRE PART THREE

DIRECTIONS: Please UNDERLINE the answer which comes closest to how you have felt in the **past week**, not just how you feel today. Here is an example already completed:

I have felt happy:

Yes, all the time
Yes, most of the time
No, not very often
No, not at all

This would mean: I have felt happy most of the time in the past few days.
Please complete the other questions in the same way. Do not take too long over it and make sure you answer all the questions.

IN THE PAST WEEK

1. I have been able to laugh and see the funny side of things:
   As much as I always could
   Not quite so much now
   Definitely not so much now
   Not at all

2. I have looked forward with enjoyment to things
   As much as I ever did
   Rather less than I used to
   Definitely less than I used to
   Hardly at all

3. I have blamed myself unnecessarily when things went wrong
   Yes, most of the time
   Yes, some of the time
   Not very often
   No, never
4. I have been anxious or worried for no good reason
   No, not at all
   Hardly ever
   Yes, sometimes
   Yes, very often

5. I have felt scared or panicky for no very good reason
   Yes, quite a lot
   Yes, sometimes
   No, not much
   No, not at all

6. Things have been getting on top of me
   - Yes, most of the time I haven't been able to cope at all
   - Yes, sometimes I haven't been coping as well as usual
   - No, most of the time I have coped quite well
   - No, I have been coping as well as ever

7. I have been so unhappy that I have had difficulty sleeping
   - Yes, most of the time
   - Yes, sometimes
   - Not very often
   - No, not at all

8. I have felt sad or miserable
   - Yes, most of the time
   - Yes, quite often
   - Not very often
   - No, not at all

9. I have been so unhappy that I have been crying
   - Yes, most of the time
   - Yes, quite often
   - Only occasionally
   - No, never

10. The thought of harming myself had occurred to me
   - Yes, quite often
   - Sometimes
   - Hardly ever
   - Never

DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then tick in the appropriate box on the right to indicate how you **generally** feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you **generally** feel, even before pregnancy.

	In general….	Almost never	Sometimes	Often	Almost always
1.	I feel pleasant				
2.	I feel nervous and restless				
3.	I feel satisfied with myself				
4.	I wish I could be as happy as others seem to be				
5.	I feel like a failure				
6.	I feel rested				
7.	I am "calm, cool and collected"				
	In general….	Almost never	Sometimes	Often	Almost always
8.	I feel that difficulties are piling up so that I cannot overcome them				
9.	I worry too much over something that really doesn't matter				
10.	I am happy				
11.	I have disturbing thoughts				
12.	I lack self-confidence				
13.	I feel secure				
14.	I make decisions easily				
15.	I feel inadequate				
16.	I am content				
17.	Some unimportant thought runs through my mind and bothers me				
18.	I take disappointments so keenly that I can't put them out of my mind				
19.	I am a steady person				
20.	I get in a state of tension or turmoil as I think over my recent concerns and interests				

Thank you for answering these questions. It would help us if you could answer all the questions, however if you feel unhappy about answering any of them, please feel free to leave them blank. All information is kept in the strictest confidence and will not affect the standard of care you receive.

Appendix 4: Data collection proforma

Mother Information: General		Mother Information: C	
Maternal weight at booking	..... (week:) BMI ..... (week:)	**Prescribed medication (incl. week)**	
Maternal age at delivery		**Bleeding**	Y
Mother Information: Obstetric History		**Concern over fetal growth**	Y
Previous no. pregnancies		**IUGR/SGA Diagnosis**	Y
Previous no. live births		**Ultrasound: Amniotic Fluid Index**	N
Previous Stillbirth	Yes / No	**Ultrasound anomalies**	Y
Previous LBW baby (<2.5kg)	Yes / No	**Doppler Anomalies**	Y
Previous Macrocosmic baby (>4kg)	Yes / No	**Ultrasound: SGA centile**	Y C
Illegal Drug use	Yes / No (Week)	**Ultrasound: LGA centile**	Y C
Smoking **Alcohol**	Yes / No (Week Number/day..... Yes / No (Week) Units/week.....		

Current Pregnancy: Preeclampsia		Delivery Time	
Diagnosis PET	Yes / No (Week)	**Maternal Weight at Delivery**	(Week)
Previous PET Pregnancy	Yes / No (Week)	**Mode of Delivery (& Indication)**	
Hypertension	Yes / No (Week)	**Complications during Delivery**	
Proteinuria	Yes / No (Week)	**Baby Information**	
Treatment for PET	Yes / No (Week) Treatment:	**Gestational Age**	
		Sex of Baby	
Current Pregnancy: Gestational Diabetes		**Arterial Cord Blood pH**	
Abnormal Glucose Tolerance Test	Yes / No (Week)	**Apgar Scores**	1 min…… 5 min ……
Diagnosis GDM	Yes / No (Week)	**Birthweight**	BW: Centile:
Previous GDM Pregnancy	Yes / No	**Head Circumference**	
Management	Diet Yes / No Exercise Yes / No Medication Yes / No	**NICU admission (and indication)**	
		Maternal mental health, e.g. previously diagnosed mood disorder, medication/treatment	
Delivery Information			
Delivery Date			

Appendix 5: Placenta proforma

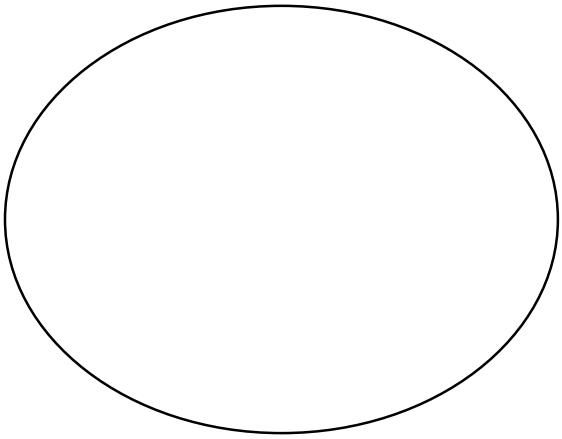
Study ID:

Date:

Time of Delivery: **Time dissection completed:**

Type of Delivery:

Please note sampling sites:



Total placental weight:

Gross placental abnormalities:

Date and time collected and stored in Biosi 3: