University of Tripoli
Faculty of Medicine
Department of Histology and Medical Genetics

Effect of advanced maternal age on human placental structure (Quantitative microscopical and histochemical study)

Yusra Ramadan Erfifi
(M.B.B.Ch)

Supervisors:

Prof. Dr. Bassem Saad Ahmed
Professor of Histology

Dr. Lubna Fathi Almaghur
Associate Professor of Obstetrics and Gynaecology

Thesis was submitted in partial fulfilment of the requirements for the degree of Master of Science in Histology (24/11/2022)

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Declaration

I am Yusra Ramadan Erfifi the undersigned hereby confirm that the work contained in this thesis, unless otherwise referenced is the researcher's work, and has not been previously submitted to meet the requirements of an award at this University or any other higher education or research institution, I furthermore, cede copyright of this thesis in favour of the University of Tripoli.

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Approval sheet

Effect of advanced maternal age on human placental structure

(Quantitative microscopical and histochemical study)

Yusra Ramadan Erfifi, University of Tripoli, MSc candidate, 2022

Supervisors: Dr. Bassem Saad Ahmed and Dr. Lubna Fathi Almaghur

Abstract

Background: The growing trend of delaying pregnancy creates new challenges for obstetric care, as older mothers have an increased risk of obstetric complications and perinatal morbidity and mortality. Quantitative histological studies of the placenta will be of clinical significance as well as advance the knowledge about the placental function and help to reveal the causes of these complications. **Study design**: A case-control study was conducted between June 2018 and April 2019 at the Department of Histology and Genetics, Faculty of Medicine, University of Tripoli and the Department of Obstetrics and Gynaecology, Ali Omer Asker hospital, Libya. **Aim of the study:** This study was carried out to evaluate the microscopic structure of the full-term human placenta and neonatal outcome in relation to advanced maternal age. (AMA). **Materials and Methods**: A total of 40 full-term human placentae of primiparous healthy Libyan women were obtained from the Department of Obstetrics and Gynaecology in Ali Omer Asker hospital. The placentae were divided into two groups; the control group (30 placentae from pregnant women of age between 20 and 35 years) and the study group (10 placentae from pregnant women 35 years of age and older). Whole-thickness placental section of size 1cm × 1cm was taken from the central part of each placenta and after proper fixation in 10% neutral buffered formalin, the tissues were dehydrated in ascending graduated concentrations of alcohol 50%, 70%, 80%, 96%, and 100%, followed by clearing in xylene and embedding in paraffin. Five-micron serial sections were generated with the help of a rotator microtome. The tissue sections were stained with hematoxylin and eosin stain and Mallory's trichrome then were examined for morphological changes. Furthermore, a quantitative analysis of placental parenchyma (fetal blood capillaries, intervillous space, collagen, and syncytiotrophoblast) was done using Leica Quantitative Image Analysis System. **Results**: The median age of mothers was 25 years with an Inter Quartile Range of (23–35.5). There was an increasing demand for assisted reproductive techniques with increasing age. Women at AMA had a higher risk for caesarean section. In addition, there was no significant difference in neonatal birth weight, Appearance; Pulse; Grimace; Activity; Respiration (APGAR) score at the 5^{th} minute, Neonatal Intensive Care Unit (NICU) admission rate, and placental weight between the two study groups ($P>0.05$). However, the placental weight/birthweight ratio in elderly mothers was significantly higher with respect to younger mothers ($P=0.021$). The histomorphometry study showed a highly significant reduction ($P=0.009$) in the lumen area of fetal blood capillaries in mothers at AMA compared to the control group. The area of intervillous space of placentae in older pregnant women compared to younger ones was significantly increased ($P=0.003$) while the syncytiotrophoblast thickness and collagen expressed in chorionic villi of two examined groups of pregnant women were not significantly different ($P>0.05$). **Conclusion:** AMA was associated with histological changes in the full-term human placenta and these changes explain the capability of older pregnant women's placenta to induce compensational mechanisms, whose main role is maintaining normal fetal growth and development.

Keywords: Advanced Maternal Age, histology of placenta, Histomorphometry study, Leica Image Analysis System.

Dedication

I would like to dedicate this work to my lovely daughter and my family.

Acknowledgement

First and foremost, thanks and praise be to Allah.

I would like to express my deepest gratitude to my supervisor **Prof. Dr. Bassem Kotob** for his expertise, support, and guidance throughout my work. He is very approachable and easy to talk to. His extremely fast responses to review my data with a big encouragement and motivation while doing this work. His attention to details always made me feel that I was his only MSc student.

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List of Abbreviations

AMA	Advanced Maternal Age
AOR	Adjusted Odd Ratio
APGAR	Appearance; Pulse; Grimace; Activity; Respiration
ART	Assisted Reproductive Techniques
BWT	Birth Weight
CS	Caesarean Section
CI	Confidence Interval
CTBs	Cytotrophoblasts
dCCTs	distal Cell Column Trophoblasts
DLK1	Delta Like Non-Canonical Notch Ligand 1
DPX	Distyrene, Plasticizer, Xylene
eCTBs	endovascular Cytotrophoblasts
ECM	Extra Cellular Matrix
EVTs	Extra Villous Trophoblast
FIGO	International Federation of Gynecology and Obstetrics
GDM	Gestational Diabetes Mellitus
g	gram
H&E	Haematoxylin and Eosin
HCG	Human Chorionic Gonadotrophin
HLA	Human leukocyte Antigen
ICM	Inner Cell Mass
IQR	Inter Quartile Range
iCTBs	interstitial Cytotrophoblasts
Kg	Kilogram
LGA	Large for Gestational Age
LBW	Low Birth Weight
µm	Micrometer
mg	Milligram
M.T	Mallory's Trichrome
MVM	Maternal Vascular Malformation
NICU	Neonatal Intensive Care Unit
NVD	Normal Vaginal Delivery
OR	Odd Ratio
PTB	Preterm Baby
PS	Primitive Syncytium
P-value	Probability
pCCTs	proximal Cell Column Trophoblasts
SCT	Syncytiotrophoblast
SGA	Small for Gestational Age
SPSS	Statistical Package for Social Science
SD	Standard Deviation
TE	Trophectoderm
uNK	uterine Natural Killer
U.S	United States
VSM	Vasculosyncytial Membrane
VAMA	Very Advanced Maternal Age
VCT	Villous Cytotrophoblast

Chapter 1:

1 Introduction

Over the last decades, the trend toward delaying childbearing has been increased dramatically worldwide irrespective of race and economic status. In the United States (U.S), the mean maternal age at delivery has grown from 24.9 years in 2000 to 27.0 years in 2019. Similar trends have been observed in England and Wales where the average maternal age at childbirth was 30.7 years in 2019 and has been gradually increasing since 1973 when it was 26.4 years. In Libya, the frequency of elderly parturient was increased from 18% in 2007 to 23.2% in 2014 (Information and Document Center-Ministry of Health 2007, 2014; Mathews and Hamilton, 2016; ONS, 2020 and Martin *et al.*, 2021).

Delayed motherhood may be attributed to a variety of reasons. In developed countries, the most significant reason is related to the advance in assisted reproductive techniques (ART), such as In vitro fertilization (IVF), and oocyte donation, which may offer childbearing in the 5^{th} and 6^{th} decade of life. In addition, academic and career opportunities, awareness of contraception, late marriage and remarriage, and delayed conception due to infertility are also among the contributing factors (Kahveci *et al.*, 2018; Wu *et al.*, 2019). On the contrary, the concept of large family size, ineffective or lack of family planning, male preference, together with religious issues can be held responsible for the increase in maternal age in developing countries (Giri *et al*., 2013; Shams *et al.*, 2021).

Advanced maternal age (AMA) is considered as women aged 35 years or older at an estimated time of delivery according to the International Federation of Gynaecology and Obstetrics (FIGO). Very advanced maternal age (VAMA) is referring to mothers delivering at age 45 years and older (Leader *et al.*, 2018).

Several studies have demonstrated the increased risk of obstetric morbidity and adverse pregnancy outcome, as well as infertility problems among elderly mothers. They are more prone to develop gestational diabetes mellitus (GDM), miscarriage, ectopic pregnancy, antepartum, and postpartum haemorrhage. In addition, AMA is associated with a higher frequency of gestational hypertension, preeclampsia, eclampsia, malpresentation, caesarean section, and lower instrumental vaginal delivery, therefore prolonged hospitalisation (Cavazos-Rehg *et al.*, 2015; Kahveci *et al.*, 2018 and Rydahl *et al.*, 2019).

Among the full-term new-borns to elderly mothers, incidences of chromosomal foetal abnormality such as Down syndrome (trisomy 21), large for gestational age (LGA), multiple births, foetal growth restriction, stillbirth, and neonatal death were reported to be higher. Furthermore, recent evidence suggests that adverse pregnancy outcomes are more pronounced among women aged 45 years or more (Schimmel *et al.*, 2015; Lean *et al.*, 2017; Ogawa *et al.*, 2017 and Radon-Pokracka *et al.*, 2019).

Normal foetal growth and maternal health involve the proper development and functioning of the placenta. The placenta is a highly specialised fetomaternal organ with a short life span facilitating metabolic and gas exchange as well as foetal waste disposal between a foetus and a mother. It is composed of the embryonic part (chorion) which is derived from the former trophoblast and the maternal part which is from the decidua basalis. The two trophoblast layers together differentiate and invade the endometrium to form chorionic villi that project into the blood-filled spaces. During pregnancy, the villi structure changes to meet the increased needs of foetal growth. Terminal villi are the main functional components of the placenta, suspended in the pools of maternal blood. Each villus branches several times to provide a larger surface for nutrients and O_2 absorption (Sadler, 2018).

Exchange of materials occurs between foetal blood in the capillaries and maternal blood bathing the villi, with diffusion occurring across the following structures: capillary endothelial cells and their basement membrane, villus connective tissue, trophoblastic basement membrane, and trophoblast cells. By the end of pregnancy, areas of syncytiotrophoblast nuclei are aggregated together to form clusters or knots on the surfaces of the villi. Additionally, disseminated fibrinoid deposits can be identified as a homogeneous, eosinophilic material inside and outside the villi as a common phenomenon observed in the full-term placenta (Mescher, 2018 ; Baergen *et al*., 2021).

The placenta of older women showed an increase in syncytiotrophoblast knots, and volume densities of peri villus fibrinoid which is often incompatible with normal foetal growth, while the total volume, surface density, and total capillary surface area in terminal villi were significantly lower. These changes might have a direct effect on fetomaternal transfusion, therefore the course and the outcome of pregnancy (Zigic *et al*., 2010).

On the other hand, it has been reported that the mean placental barrier thickness increased in women giving birth at an advanced age as a result of the increase in proliferative activity of the trophoblast layer and the reduction in apoptotic signals. Moreover, mature human placentae of older women showed significant morphological and quantitative differences in terminal villi and intervillous space in comparison to younger mothers. All of these might indicate a compensatory mechanism to overcome age-related hypofunction of the placenta and provides nutrition for foetal normal growth and development (Ramic *et al*., 2006; Markovic *et al*., 2010 and Jawad, 2014).

The influence of AMA on maternal and foetal outcomes has been extensively studied in the literature from clinical and macroscopical perspectives and they have been shown conflicting results. On the other hand, a few studies have focused on the placental histopathological findings in these women. Therefore, to clarify this doubt, the current study addresses the hypothesis that AMA is associated with histological changes and underlying pathological alterations in the placentae from these women that may account for, or correlate with, the reported influence on the maternal and foetal outcome.

1.1 Aim of study

This study has been undertaken to clarify the influence of AMA on the histological structure of full-term human placenta and neonatal outcomes.

1.2 Objectives of study

- To evaluate the histological and histochemical changes of full-term human placenta in AMA and compare them to younger aged women.
- To provide quantitative microscopical data on the parenchyma of full-term placenta of women in AMA with the aid of the Leica image analysis system and compare it with that of younger mothers.
- To evaluate the correlation between histomorphometry data and neonatal outcome of pregnancy.

Chapter 2:

2 Literature review

2.1 Human placental development

The placenta is a haemochorial organ that plays a vital role in facilitating nutrients, gas, and waste exchange between maternal and foetal circulation. Recent advances in 3-dimensional organoids, stem cell culture systems, and immunohistochemically studies have brought new insights into understanding human placental growth and the molecular mechanism of trophoblast development (Knofler *et al*., 2019).

Failure in placental development is considered an underlying cause of major pregnancy complications such as pre-eclampsia, foetal growth restriction, recurrent miscarriage, and stillbirth (Fisher, 2015; Tang *et al*., 2017). In addition, underlying placental abnormalities increase susceptibility to a variety of chronic diseases, for example, cardiovascular disease, type 2 diabetes, and psychiatric disorders in adulthood (Burton *et al*., 2016).

2.1.1 Pre-implantation

After fertilization, the zygote migrates through the fallopian tube and undergoes a series of mitotic divisions, creating a morula (mulberry). Once the morula enters the uterine cavity on the 3rd or the 4th day after fertilisation, a single cavity begins to appear which marks the development of a spherical structure (blastocyst). By the 4th to the 5th day after fertilisation, the blastocyst is segregated into two lineages; outer trophectoderm (TE), the precursor of trophoblast cells, and inner cell mass (ICM). The latter is also termed as embryoblast. The trophoblast cells create the placenta while the ICM develops into the embryo proper and other extraembryonic tissues such as allantois, amnion, and yolk sac (Sadler, 2018).

2.1.2 Implantation (nidation)

The development of the human placenta involves attaching and eventually embedding the blastocyst into the maternal endometrium which starts around the 5th or the 6th day from fertilisation and after the outer zona pellucida has disappeared. The trophoblast cells then differentiate into two layers. The underlying layer is composed of mononucleated and

actively proliferating cytotrophoblasts (CTBs). The overlaying layer has multinucleated amoeba-like cells, known as a primitive syncytium (PS) or syncytiotrophoblast. Simultaneously, the ICM differentiates into the hypoblast layer and the epiblast layer (Gamage *et al*., 2016).

Once intimate contact is made, a highly adhesive and invasive syncytiotrophoblast penetrates deep into the endometrium by sending out projections and releasing proteolytic enzymes which erode the maternal gland to meet the nutritional needs of the embryo. By the 9^{th} day, fluid-filled spaces (lacunae) appear within the enlarging syncytiotrophoblast, signifying the lacunar stage development as shown in Figure (1). These lacunae anastomose with maternal vascular sinusoids form the primitive uteroplacental circulation and maternal blood enters the lacunar system by the end of the second week. Eventually, lacunae coalesce into the intervillous space (Sadler, 2018).

Meanwhile, the cytotrophoblast rapidly proliferates and projects through the syncytiotrophoblast, thereby forming primary villi (a cytotrophoblast core with an outer layer of syncytiotrophoblast). Soon afterwards, extraembryonic mesenchymal cells grow inside the villous core transforming the primary villi into secondary villi. Finally, foetal capillaries form within the core, marking the development of tertiary villi or definitive placental villi that project in all directions in the intervillous space (Kojima *et al*., 2022). Concurrently, endometrial stroma undergoes several histological changes. The fibroblast becomes larger, polygonal, and more active in protein production. They become loaded with glycogen and lipids, and they are now called decidual cells. Furthermore, extravasation into intercellular spaces leads to stromal oedema. The endometrium is now changed into specialised tissue known as the decidua (Mescher, 2018).

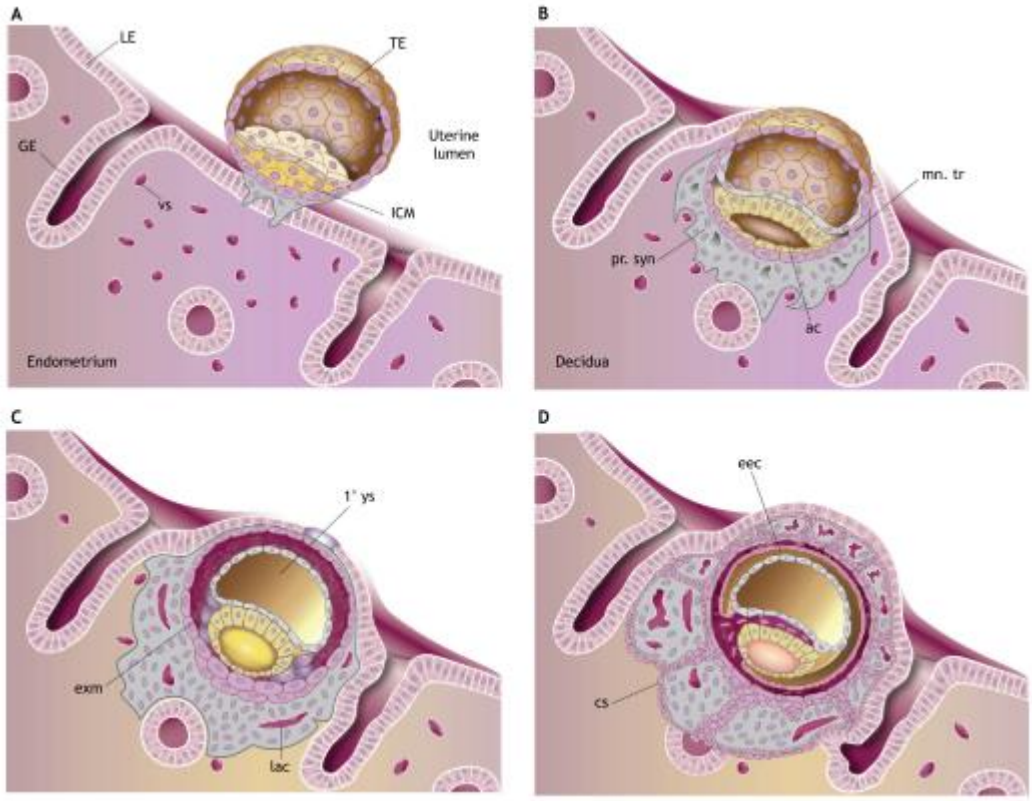


Figure (1). The early stages of human placental development. Diagram depicting the early steps in placenta formation following blastocyst implantation. (A, B) the pre-lacunar stages, (C) the lacunar stage, and (D) the primary villous stage. 1°ys, primary yolk sac; ac, amniotic cavity; cs, cytotrophoblastic shell; eec, extra-embryonic coelom; exm, extra-embryonic mesoderm; GE, glandular epithelium; ICM, inner cell mass; lac, lacunae; LE, luminal epithelium; mn. tr, mononuclear trophoblast; pr. syn, primary syncytium; TE, trophectoderm; vs, blood vessels (Turco and Moffett., 2019).

At the distal side, a local breakdown of the syncytium occurs. Columns of proliferative cytotrophoblast invade maternal stroma forming anchoring villi. On the other hand, some cytotrophoblasts migrate laterally and merge with neighbours forming a trophoblast shell. Cytotrophoblast cells in the tips of anchoring villi leave the shell to invade the decidua in a form of extravillous trophoblasts (EVTs) during the 2nd and the 3rd week of the pregnancy. Clusters of proliferative proximal cell column trophoblasts (pCCTs) appear in these villi indicating the progenitor cell population of differentiated EVTs, while distal cell column trophoblasts (dCCTs) cease mitosis but do not leave the cell cycle to enter a quiescent state. Alternatively, these cells undergo endoreduplication cycles and differentiate into EVTs (Burton and Jauniaux,2017; Velicky *et al*., 2018).

Two distinct populations of extravillous trophoblasts, namely, interstitial cytotrophoblasts (iCTBs), and endovascular cytotrophoblasts (eCTBs) are established fifteen to sixteen days after conception. The iCTBs infiltrate the uterine stroma and play an important role with other stromal cells to control immunological recognition of the placental/foetal allograft while the eCTBs colonise and remodel the maternal spiral arteries (Moffetti *et al*., 2017; Liu *et al*., 2022).

Another critical stage in human placentation is the migration of EVTs into the maternal spiral arteries as shown in Figure (2). To achieve this, iCTBs are attracted to the spiral arteries by uterine natural killer (uNK) cells and macrophages. After invading the spiral arteries, iCTBs develop into eCTBs which travel along the lumen and establish vascular adhesion. The eCTBs interdigitate through the endothelial layer, causing apoptosis of endothelial cells and the smooth muscle layer is replaced with a fibrinoid-rich matrix. Later in the pregnancy, the remodelling process makes the vessel larger, and low resistant to ensure optimal placenta blood flow which is necessary for the maximal perfusion and the protection of delicate villous trees (Knofler *et al*., 2019; Liu *et al*., 2022).

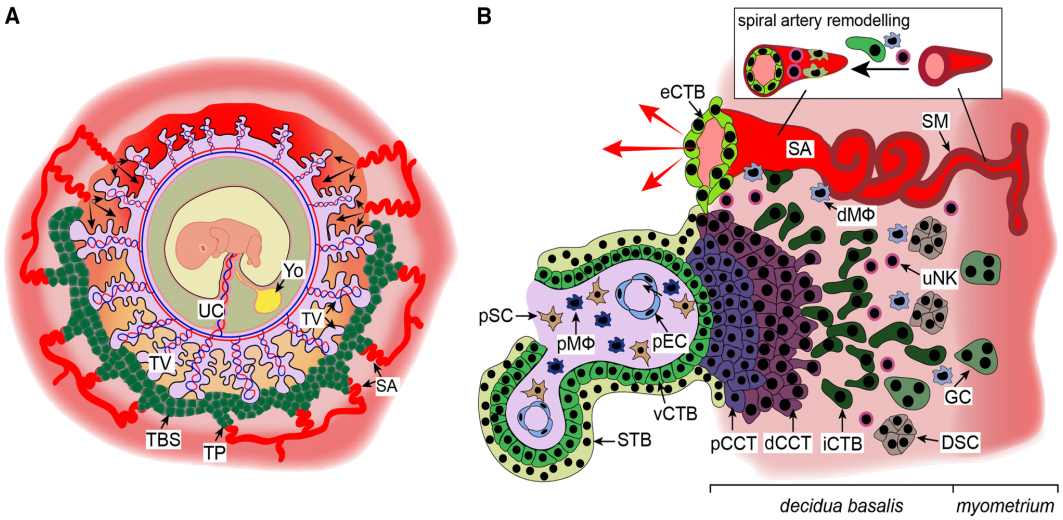


Figure (2). Development of the trophoblastic shell and formation of placental anchoring villi. (A) structure of the human trophoblastic shell and its surrounding arterial vessels. (B) depiction of a placental anchoring villus, spiral artery (SA) remodelling, and interaction of extravillous trophoblasts (EVTs) with different decidual cell types. dCCT distal cell column trophoblast, dMΦ decidual macrophage, DSC decidual stromal cell, eCTB endovascular cytotrophoblast, GC giant cell, iCTB interstitial cytotrophoblast, pCCT proximal cell column trophoblast, pEC placental endothelial cell, pMΦ placental macrophage, pSC placental stromal/mesenchymal cell, SM smooth muscle layer, STB syncytiotrophoblast, TBS trophoblastic shell, TP trophoblast plug, TV tertiary villi, UC umbilical cord, uNK uterine NK cell, vCTB villous cytotrophoblast, YO yolk sac (Knofler *et al*., 2019).

In addition to remodelling the spiral arteries, eCTBs accumulate and develop cellular plugs preventing the maternal arterial blood from flowing into the intervillous space until the 6–7 weeks of pregnancy. This provides a low-oxygen environment for early placental development. Finally, these plugs disappear completely to incite significant maternal blood flow into the intervillous space (Cindrova-Davies *et al.*,2020). Failure of remodelling and inappropriate adaption predisposes to hypoperfusion oxidative stress leading to severe foetal growth restriction, early onset pre-eclampsia, miscarriage, and premature delivery(Burton *et al*., 2019 and Knofler *et al*., 2019) .

2.1.3 The placental villous tree

Chorionic villi are crucial structures involved in the exchange between a mother and a foetus. These villi undergo dynamic morphological changes throughout pregnancy to improve maternal-fetal exchange. According to the size of the villus, vascular structure, and stromal characteristics, the placental villi are categorised into mesenchymal villi, immature intermediate villi, stem villi, mature intermediate villi, and terminal villi (Figure 3).

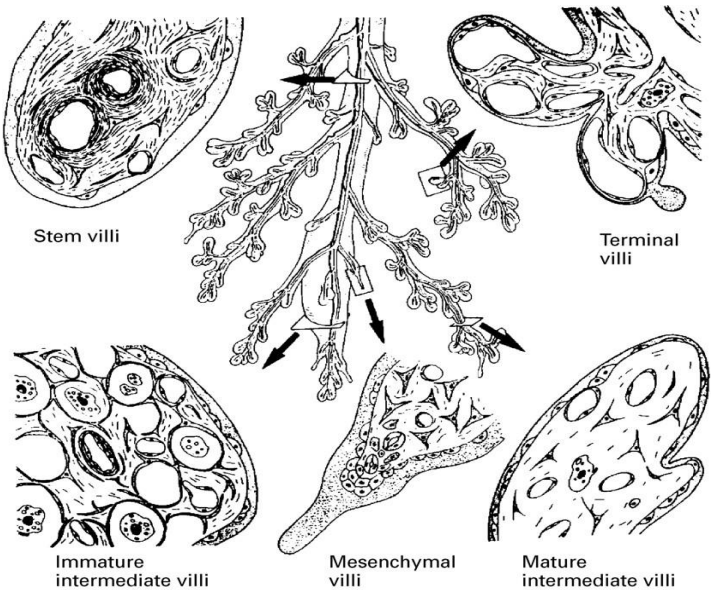


Figure (3). Representation of the peripheral branches of a mature villous tree together with typical cross-sections of the five villous types. The figures are reproduced from Haines & Taylor. Textbook of Obstetrical and Gynaecological Pathology. 4th Ed. 1995, by P Kaufmann.

2.1.3.1 Mesenchymal villi

The primitive type of chorionic villi seen during the first weeks of pregnancy is characterised by abundant loose stroma containing mesenchymal cells, poorly developed foetal capillaries, and two complete trophoblast layers encircling the villous core. The cytotrophoblast layer encloses the villous core while the syncytiotrophoblast layer is located on the villous surface. Mesenchymal villi are the only kind of villi that form in the placenta during the first six weeks of pregnancy with a diameter ranging between 100 and 250 μm. Later on, the mesenchymal villi differentiate into all other specialised villus types. They account for less than 1% of total villi volume at full term (Baergen *et al*., 2021).

2.1.3.2 Immature intermediate villi

The stroma of this kind of villi has a reticular structure created by matrix-free channels running parallel to the long axis of the villi. Numerous placental macrophages are found in these stromal channels and are known as Hoffbauer cells. From the 8th to the 22nd week of pregnancy, the immature intermediate villi are generated from mesenchymal villi and eventually converted into stem villi. They have a diameter of 100-400 μm and are thought to be the main sites of maternal-foetal interactions during the first and second trimesters (Baergen *et al*., 2021).

2.1.3.3 Stem villi

The stem villi of the human placenta signify the central branches of the villous trees with a diameter of 100-3000 μm. They are the largest villi of the placenta that extend from the chorionic plate to decidua basalis providing mechanical support to the feto-maternal interface. They have a condensed fibrous stroma occupied by central arteries and veins (Aplin *et al*., 2018).

2.1.3.4 Mature intermediate villi

The peripheral ramifications of the villous stems give rise to the majority of mature intermediate villi. They have a considerable diameter (60–150μm). Mature intermediate villi

are thought to be the first essential structure for foetal-maternal exchanges because of their extensive foetal vascularisation (Ortega *et al*., 2022).

2.1.3.5 Terminal villi

Terminal villi are the final branches of the placental villous tree developed from mature intermediate villi. They have a grape-like structure that floats in the intervillous space. Terminal villi are the main functional components of the placenta, they exhibit a high degree of vascularisation and highly dilated sinusoids. They branch repeatedly from the chorionic plate and subsequently generate an overall epithelial surface area of 12-14 m^2 by term to promote the exchange between fetal and maternal circulation (Mescher, 2018).

2.2 Macroscopic anatomy of the placenta

The placenta has two components: an embryonic part derived from the chorion and a maternal part which is formed by the decidua basalis. The mature human placenta is a discoid structure that is about 15-25 cm in diameter, 2-3 cm thick at the centre and the average weight is 500g. In addition, the human placenta has a maternal surface and a foetal surface. The maternal surface has a rough and spongy appearance that is divided by a series of non-complete placental septa into 15-20 lobes known as cotyledons. Each lobe is composed of densely branching villous trees. Each tree emerges from the chorionic plate through a stem villous and forms a lobule that is centred above the entrance of a maternal spiral artery through the basal plate. Calcium deposition may be seen on the maternal surface of the term placenta. On the other hand, the inner foetal surface is covered with amnion and appears smooth and transparent with visible branches and tributaries of umbilical vessels beneath it. The umbilical cord is usually inserted at or near its centre (Burton *et al*., 2016; Mayo, 2018).

2.2.1 Placental circulation

2.2.1.1 Uteroplacental circulation

The maternal blood flowing through the intervillous space is provided by spiral arteries. The pressure/concentration gradient between the intervillous and the foetal capillary facilitates the metabolic exchange between maternal and foetal circulation. At full term, the

intervillous blood flows at a rate of 500-600 ml/min. During gestation, spiral arteries remodelling is essential to the physiologic regulation of maternal uterine blood flow required for a successful pregnancy and foetus development. (Griffiths and Campbell, 2015; Degner *et al*., 2017).

2.2.1.2 Fetoplacental circulation

Deoxygenated blood from the foetus is carried to the placenta by two umbilical arteries. Within the villi, the umbilical arteries branch into chorionic arteries and terminate as capillaries. Substances in maternal blood flow from the intervillous space to the foetal capillaries. The foetal capillaries drain into chorionic veins which empty into a single umbilical vein (Griffiths and Campbell, 2015).

2.2.2 Umbilical cord

The umbilical cord is thought to be the physical and emotional connection between the mother and the foetus. It is a soft, convoluted cord made of a smooth outer amnion coating and a gelatinous ground substance called Wharton's jelly. The latter contains paired umbilical arteries and one umbilical vein. The oxygenated blood is carried to the foetus by the umbilical vein while the deoxygenated blood is returned to the placenta by umbilical arteries. At full term, the umbilical cord varies from 30 to 90 cm. The umbilical blood flow rate is ~350 ml/min (Basta and Lipsett, 2020).

2.3 Placental histology

Various techniques such as light and electron microscope, morphometry, immunohistochemistry, and in-situ hybridisation provide better knowledge about placenta villus tree development and structure. Generally, cross-sections of the human placenta exhibit different chorionic villi, however, these villi have the same following basic structures:

2.3.1 The villous stroma

In early pregnancy, each chorionic villous has a core occupied by small, undifferentiated mesenchymal cells. The stromal core of the villi is surrounded by a complete layer of cytotrophoblast and syncytiotrophoblast. The mesenchymal cells are a rich source of stem cells that allow the placenta to develop and remodel throughout the pregnancy. Furthermore, the collagenous matrix, which appears to occupy most of the intercellular spaces of the tertiary villous core, is also developed and secreted by large reticulum cells and fibroblasts. Collagen types I, III, IV, VI, and fibronectin have been observed as part of the villous stroma structure (Baergen *et al.*, 2021).

There are at least two types of fibroblasts identified by the presence or absence of the imprinted gene DLK1 (Delta Like Non-Canonical Notch Ligand 1). The fibroblasts with the DLK1 gene have pericyte-like characteristics and may be involved in placental vascular development. Hofbauer cells, also known as villous macrophages, are found in the chorionic villi of an 18^{th} day embryo and are most abundant early in the pregnancy, even before angiogenesis occurs. Immature or intermediate Hofbauer cells may be identified in the core of secondary villi, and they are the numerically dominating cell type in the villous mesenchyme throughout the first half of gestation. They are thought to protect the foetus against vertical infections, influence trophoblast and placental vascular development, and transport nutrients to the extra-embryonic coelom (Turco and Moffett, 2019).

As shown in Figure (4), a cross-section of full-term chorionic villous has a prominent syncytiotrophoblast layer, but fewer cytotrophoblasts are to be found. Clusters of syncytiotrophoblasts nuclei are seen and referred to as syncytial knots. It has been suggested that the knots are apoptotic and shed into the maternal circulation. The foetal capillaries

elongate and form loops against the SCT layer of terminal villi as pregnancy progresses, forming "vasculosyncytial membranes" that reduce the exchange gap between the maternal and foetal circulations. Thereby, optimising oxygen and nutrients transfer to the foetus (Lahti-Pulkkinen *et al.*, 2018 ; Mescher, 2018 and Gauster *et al*., 2022). Placental fibrinoids are cellular, homogeneous, eosinophilic, deposited materials that can be found in every normal and pathological placenta throughout pregnancy. Two types of fibrinoid have been identified: the fibrin-type fibrinoid is composed of a meshwork of fibres with fibrin-type cross- striations and the matrix-type fibrinoid consists of extracellular components such as collagen IV, fibronectin, laminin, heparan sulphate, tenascin and embedded extravillous cytotrophoblast cells (Zhang, 2021).

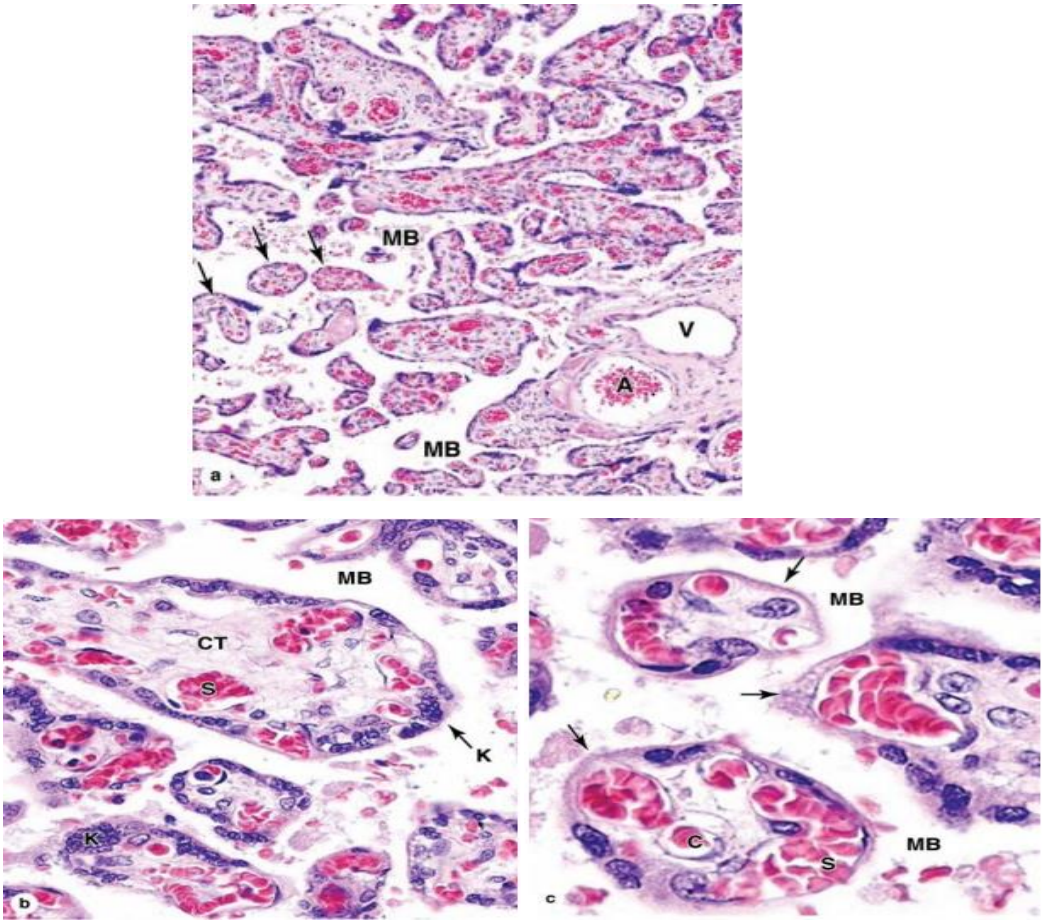


Figure (4). Full Term placenta. The placenta contains chorionic villi of the foetus and maternal blood pooled in spaces of the decidua. (a): At low magnification, a full-term placenta includes sections of many villus stems, containing arteries (A) and (V) of the extraembryonic vasculature, and hundreds of smaller villus branches (arrows) which contain connective tissue and microvasculature. Maternal blood (MB) normally fills the space around all the villi. X50. H&E. (b): At higher magnification, the villus connective tissue (CT) can be seen to still resemble mesenchyme and to be surrounded by epithelial cells of the trophoblast, including both the inner cytotrophoblast epithelium and the overlying syncytial trophoblast. In many areas nuclei of the syncytiotrophoblast layer have formed clusters or knots (K) on the surfaces of villi. The trophoblast separates the sinusoids (S) and other vessels containing foetal blood from the maternal blood (MB) in the intervillous space. X200. H&E. (c): Still higher magnification of the same section shows that the villus branches each contain several capillaries (C) and wide sinusoids (S) filled with foetal blood. By the end of pregnancy cells of the cytotrophoblast have greatly decreased in number in many areas of the villi and only a thin syncytiotrophoblast underlain by basement membrane surrounds the villus in these regions (arrows).X400. H&E (Mescher, 2018).

2.3.2 The trophoblast cell lineage

Trophoblast cells perform the majority of the placental functions. The term trophoblast was first described by the Dutch embryologist Ambrosius Arnold Willem Hubrecht in 1889. He also pointed out that the trophoblast cells are highly invasive or corrosive by nature and dependent on decidua to maintain their development (Pijnenborg and Vercruysse, 2013). There are many different phenotypes of human trophoblast cells that have been identified. The syncytiotrophoblast (SCT), the villous cytotrophoblast (VCT), and the extravillous trophoblast (EVT) subtypes are among them.

2.3.2.1 The syncytiotrophoblast (SCT)

The SCT is an outer uninterrupted epithelial layer that covers all villous tree surfaces. Unlike the other epithelia, it is made up of a continuous multinucleated surface layer with no cell borders. Moreover, the SCT is in close contact with maternal blood flowing into the intervillous space hence, the human placenta is identified as a haemochorial type. To amplify the efficacy of maternal/foetal exchange of gases and nutrients, the SCT apical surface bears abundant microvilli which increase the surface area by five to seven folds. These microvilli are rich in receptors for growth factors and hormones (Tashev *et al*., 2022).

The syncytial cytoplasm is more strongly basophilic and densely packed with organelles including rough endoplasmic reticulum, ribosomes, Golgi complex, numerous lysosomes, phagosomes, and mitochondria, indicating its high synthetic and metabolic function. In addition, the syncytial cytoskeleton plays a role in maintaining the shape of the villus (Baergen *et al.*, 2021). At the surface of terminal villi, the syncytial nuclei aggregate to form syncytial knots. The vast majority of syncytial knots within the placenta are believed to be artefacts from tangential sectioning. Syncytial knots are regularly present, increasing with gestational age, and can be used to assess villous maturity. Moreover, they are thought to be associated with conditions of uteroplacental hypoperfusion. The SCT is an important endocrine structure, secreting hormones and proteins into the maternal bloodstream and modulating the maternal environment necessary for proper foetal growth. In addition, SCT functions as a protective immunological barrier because it never expresses any human leukocyte antigen (HLA) molecules (Moffett and Colucci, 2015 ; Gauster *et al*., 2022).

2.3.2.2 The villous cytotrophoblast (VCT)

The VCT has historically been viewed as the "germinative" layer of the trophoblast because of its mitotic figures and expressed proliferative markers. In early pregnancy, VCT appears as a complete layer on a basement membrane beneath the SCT. As the villous trees grow, the VCT layer becomes discontinuous, covering only 25% of the villous surface by the full term. Throughout the pregnancy, the majority of VCT have light microscopic and ultrastructural characteristics of undifferentiated, proliferating stem cells, that distinctly identify them from overlying syncytiotrophoblast, A few cytotrophoblast cells exhibit a higher degree of differentiation, as seen by significant numbers of free ribosomes, rough endoplasmic reticulum, and mitochondria. This observation has typically been viewed as evidence of differentiation into a later syncytial state (Kolahi *et al*., 2017; Baergen *et al*., 2021).

2.3.2.3 Extravillous trophoblast (EVT)

The EVT lineage can be classified into three cell populations: a proliferative population known as extravillous cytotrophoblast cells; non-proliferative differentiated mononuclear trophoblast cells known as extra villous mononuclear trophoblast cells; and multinucleated differentiated population referred to as extra villous syncytial trophoblast cells. In addition, to certain endocrine roles, extravillous trophoblast cells are involved in the construction of the uteroplacental interface (Knofler *et al*., 2019; Turco and Moffett, 2019).

2.3.3 Trophoblastic basement membrane

The villous stroma is separated from the trophoblastic epithelium by the trophoblastic basement membrane. It serves as a supporting matrix for the trophoblast cells. Collagen IV, laminin, heparan sulphate, and fibronectin are the main components of the trophoblast basement membrane. Furthermore, the average thickness of the trophoblast basement membrane varies between 20 and 50 nm under normal conditions (Baergen *et al.*, 2021).

2.3.4 Maternal-foetal interface

Traditionally, the terms maternal-foetal barrier and placenta barrier are used interchangeably to denote the tissue layers separating the maternal and foetal circulation in the placenta. However, as some substances pass freely through it, the term barrier is, therefore, best avoided. Early in pregnancy, the maternal blood in the intervillous space is separated from the foetal blood by the following six components: the syncytium; the cytotrophoblast; the trophoblastic basement membrane; the connective tissue constituting the villus core; the basement membrane of the foetal capillaries; and the endothelium of foetal capillaries (Baergen *et al.*, 2021).

Later on, the cytotrophoblast layer is reduced in many areas covering the villus leaving a thin syncytiotrophoblast and basement membrane. The foetal capillaries are extended and then come in direct opposition to the terminal villi surface, their two basement membranes often join and subsequently constitute part of the vasculosyncytial membrane (VSM) to aid a maximum metabolite exchange as shown in Figure (5) (Sadler, 2018; Baergen *et al*., 2021).

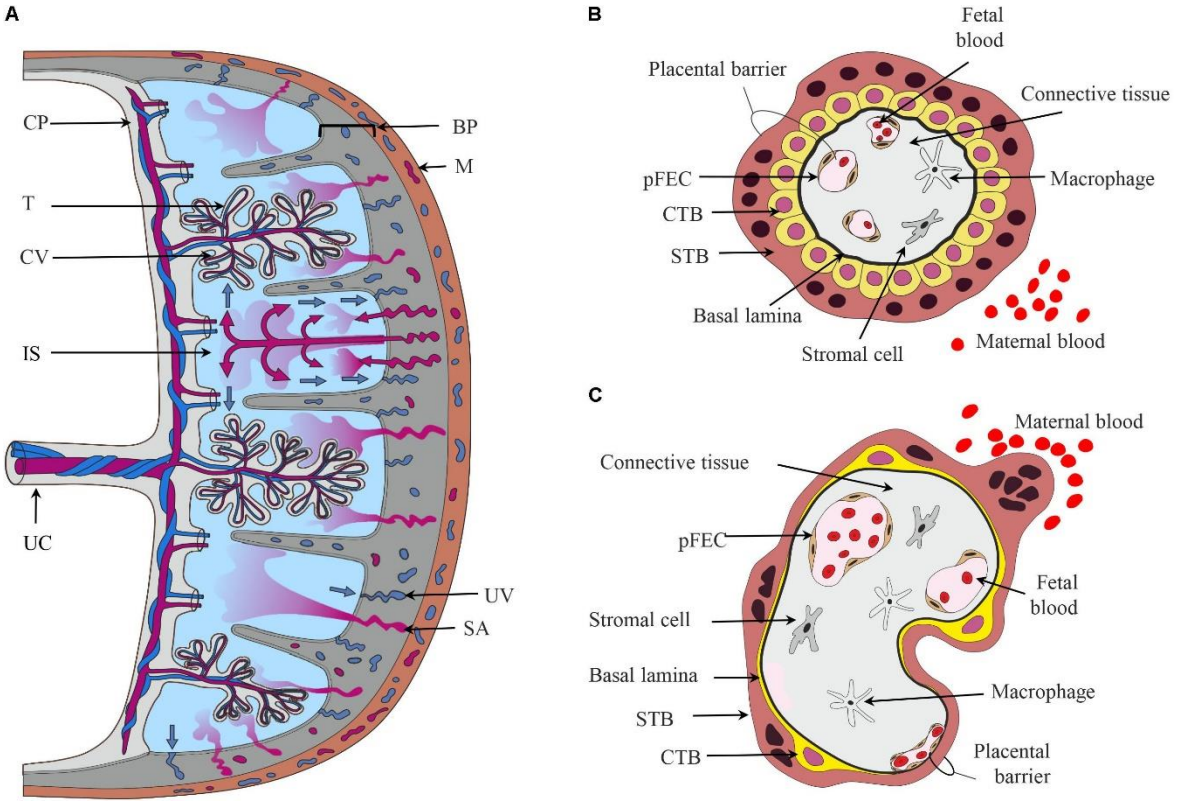


Figure (5). The placental barrier. (A) schematic depiction of the main structural elements of the human placenta. From the chorionic plate (CP), the umbilical cord (UC), and the chorionic villi (CV) originate. The umbilical vein carries oxygen- and nutrient-rich blood from the placenta to the foetus, while two arteries transport deoxygenated blood and waste products from the foetus to the placenta. The intervillous space (IS) is filled with maternal blood that enters this cavity via remodelled and opened maternal spiral arteries (SA) and leaves via uterine veins (UV). Cells in direct contact with maternal blood are the villous trophoblasts (T). The basal plate (BP) contains extravillous trophoblasts and decidual cells. (B, C) schematic representations of the first trimester (B) and term trimester chorionic villi (C) depicting the major cell types and the placental barrier. CTB, cytotrophoblast; M, myometrium of the maternal uterus, pFECs, placental-foetal endothelial cells; STB, syncytiotrophoblast (Chatuphonprasert *et al*., 2018).

2.4 The physiology of the placenta

The placenta is a multifunctional organ that is vital for successful human pregnancy and optimal foetal development. It holds several important roles such as facilitating respiratory gas exchange, delivering nutrients, removing foetal waste products, protecting the foetus against toxins and microbial infection, and providing peptides and hormones that control both maternal metabolism and foetal growth. Throughout pregnancy, several placental functional modifications arise to meet the growing physiological demands of the developing foetus (Kazma *et al.*, 2020).

2.4.1 The placenta as an endocrine organ

The SCT functions as a major endocrine organ, secreting numerous hormones and proteins into the maternal bloodstream which play several roles in pregnancy establishment and maintenance, foetal development, and labour. Abnormal levels of these hormones have been linked to a variety of diseases, including chromosomal abnormalities, ectopic pregnancy, and pre-eclampsia (Teasdale and Morton, 2018; Knofler *et al*., 2019).

2.4.1.1 Human Chorionic Gonadotrophin (HCG)

One of the most essential pregnancy hormones is the Human Chorionic Gonadotrophin (HCG). It is a hormone that belongs to the glycoprotein family which is produced by the human embryo. The detection of beta-HCG in blood and urine is a standard pregnancy test. HCG can be detected in maternal serum from the 8^{th} day after fertilisation, with its concentration peaking by the 10^{th} week of pregnancy before gradually declining towards the end (Forstner *et al.,*2020). According to Vitro studies, HCG is essential for immunotolerance since it suppresses the maternal immune system. In addition, HCG has a relaxant effect on human myometrial tissue, implying that it plays a direct role in myometrial quiescence during pregnancy (Motomura *et al*., 2021).

2.4.1.2 Progesterone

Progesterone is a steroid hormone that is important for the health of pregnant women. It promotes embryo attachment and implantation and plays a vital role in immunotolerance,

and reducing myometrium contractility throughout pregnancy. In addition, progesterone promotes lactation by preparing the mammary gland. Owing to the development of the syncytial layer, the placenta gradually becomes the main source of progesterone after 6–8 weeks of gestation until the end of pregnancy, as HCG concentration decreases (Kolatorova *et al*., 2022).

2.4.1.3 Oestrogens

The corpus luteum produces oestrogens during the first weeks of pregnancy later on the placenta synthesises these steroids. oestradiol is the most abundant oestrogen during pregnancy. Throughout, pregnancy, oestradiol promotes endometrial development and embryo implantation. Furthermore, oestradiol enhances the contraction of human myometrial cells by facilitating the development of gap junctions, implying its role in labour initiation. In addition to these local effects, oestradiol aids mammary epithelial proliferation, which prepares the breast for breastfeeding (Berkane *et al*., 2022).

Placental lactogen, placental growth hormone, and leptin are additional placenta-related hormones that influence different gestational events including implantation, placentation, immunomodulation, breastfeeding, and labour. Unbalanced production of these hormones could indicate major changes in these mechanisms, which could harm the gestational path and foetal growth (Napso *et al*., 2018; Kodogo *et al*., 2019).

2.5 Impact of advanced maternal age on maternal and neonatal outcome

Worldwide, pregnancy at AMA has become increasingly common in the past decades. The advanced progress in ART constitutes the most significant reason for delayed childbearing which allows patients in their 40s, 50s, or even 60s of life to become pregnant, especially in high-income countries (Kahveci *et al*., 2018; Molina-Garcia *et al.*, 2019).

Motherhood at or beyond the edge of reproductive age is believed to be associated with a higher risk of potentially life-threatening obstetrical and perinatal complications although the association has been a subject of debate in several studies. Richard Naeye was the first to investigate the relationship between AMA and poor pregnancy outcomes in 1983 (Attali and Yogev, 2021; Smithson *et al*., 2022).

Advanced maternal age is one of the suggested risk factors for preeclampsia. The latter is a primary cause of placental insufficiency and poor perinatal outcome. Lamminpaa *et al*. (2012) found that pre-eclampsia was more frequent in women of AMA and the adverse pregnancy outcomes among pre-eclamptic women were more likely to be in women of AMA. Furthermore, Chronic diseases such as diabetes mellitus and hypertension, with a possible influence on the course of pregnancy, were increasing with a growing maternal age (Fayed *et al*., 2017; Li *et al*., 2020). Several studies have reported that AMA was significantly associated with a higher rate of operative deliveries including caesarean sections, as well as instrumental vaginal deliveries (Rydahl *et al*., 2019; Fonseca *et al*., 2020).

Infants born to older mothers are at higher risk of congenital and acquired health concerns including Down syndrome, low birth weight, Alzheimer's disease, hypertension, and diabetes (Hviid *et al*., 2017). On the other hand, Sutcliffe *et al*. (2012) found that children born to elderly mothers demonstrated better language development and fewer social and emotional difficulties. Age alone might not be enough to clarify poor obstetric outcomes. Confounding factors including the heterogeneity of sample groups, variations in the concept of pregnancy outcomes, and inadequate control of variables such as maternal diseases, assisted conception, obesity, multiple pregnancies, and parity may all explain the differences in these observations.

Some studies have been conducted to re-evaluate the association between AMA and adverse pregnancy outcomes after adjustment of confounding factors in maternal characteristics and obstetric history. Khalil *et al*. (2013) found that elderly mothers are more prone to increased risk for a wide range of adverse pregnancy outcomes, including miscarriage, pre-eclampsia, SGA, GDM, and caesarean section, but not stillbirth, gestational hypertension, spontaneous preterm delivery, or LGA. In addition, Kahveci *et al.* (2018) demonstrated that pre-eclampsia, GDM, spontaneous late preterm delivery, and caesarean delivery were more common in advanced maternal age nulliparous women with no previous chronic diseases. However, the risk for spontaneous preterm delivery before 34 weeks, prolonged rupture of membranes, placenta previa, and operative vaginal delivery was similar in both elderly and young mothers. Furthermore, Berger *et al*. (2021) found that the risk of

having a preterm birth (PTB) and low birth weight (LBW) baby increased among elderly women regardless of pre-existing pregnancy-related health conditions and parity.

2.6 Histological changes of the human placenta due to the advance in maternal age

The advance in maternal age is associated with structural placental changes that affect the placental vascular perfusion and efficacy which might contribute to poor foetal outcomes including intrauterine foetal demise, intra-uterine growth restriction, oligohydramnios, and stillbirth. Therefore, studying the underlying placental histopathology is considered essential (Lean *et al*., 2017; Torous and Roberts, 2020).

Apoptosis is a normal physiological phenomenon in the placenta that increases significantly as pregnancy progresses. Many of the pathological abnormalities in the placenta are usually accompanied by an increase in apoptosis of trophoblasts. However, placentae from older healthier mothers had a lower incidence of apoptosis compared with those from younger mothers (Yamada *et al*., 2001). Additionally, the proportion of fibrinoid and intervillous space in the placenta of older pregnant women was found to be higher than younger pregnant women (Ramic *et al*., 2006). Moreover, the volume density and the absolute volume of syncytiotrophoblast in resorption villi of placentae were also significantly higher in older pregnant women. These histological changes help the placenta of older women to compensate for the decreased metabolic exchange between a mother and a foetus (Markovic *et al*., 2010).

Terminal villi are essential for the fetomaternal transfer of substances. The structural components of terminal villi (capillaries, stroma, and trophoblast) of placentae in older pregnant women undergo extensive morphological alterations to meet the demands of the developing foetus in comparison to the placentae of younger pregnant women. However, the volume density, total volume, surface density, and total capillary surface area are significantly lower suggesting that nutritional availability in the foetus is reduced, thus compromising foetal growth and development (Zigic *et al*., 2010).

Jawad (2014) observed an increase in the thickness of the placental barrier, a decrease in vascularisation of the terminal villi, and an increase in stoma and fibrin deposition in the placenta of older women. In addition, the mean placental weight was increased although the

neonatal weight was not significantly decreased. These findings were in agreement with a recent study conducted by (Meteeb and Al-Dhalimy, 2020).

Miremerg *et al.* (2020) studied the placental histopathology of 110 AMA patients that were matched with controls. Chronic maternal diseases and pregnancy complications including chronic hypertensive disorders, diabetes mellitus, preeclampsia, and placental abruption were excluded in an attempt to isolate the effect of maternal age. The study revealed that the maternal vascular malperfusion (MVM) included: placental haemorrhagic vascular changes (acute atherosis and mural hypertrophy), and villous changes (increased syncytial knots, increased intervillous fibrin deposition, distal villous hypoplasia, and villous infarcts) were higher among elderly women. Furthermore, Chen *et al*. (2021) found that AMA was associated with decreased α- Klotho expression(ageing suppressor gene) in placental trophoblasts, which initiates premature senescence and loss of invasion, both of which have a negative impact on neonatal outcomes and placental development.

Chapter 3:

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

1. Acid fuchsin, GURR (England).
2. Aniline blue, Park-scientific Ltd (England).
3. Celestin blue B, Raymond A. Lamb (England).
4. Charcoal (Decolorizing), Park-scientific Ltd (England).
5. DPX mount, Park-scientific Ltd (England).
6. Eosin, Park-scientific Ltd (England).
7. Ethanol absolute, Park-scientific Ltd (England).
8. Ferric ammonium sulphate, Park-scientific Ltd (England).
9. Formalin, Park-scientific Ltd (England).
10. Glacial acetic acid, Koch -light Lab Ltd (England).
11. Glycerol, BDH chemicals Ltd (England).
12. Haematoxylin, Park-scientific Ltd (England).
13. Hydrochloric acid, Riedel-de (England).
14. Paraffin wax, Park-scientific Ltd (England).
15. Phosphomolybdic acid, Park-scientific Ltd (England).
16. Potassium alum, Park-scientific Ltd (England).
17. Red mercuric oxide, T-baker Lab (England).
18. Xylene (xylol), Park-scientific Ltd (England).

3.1.2 Equipment and Instruments

1. Beakers, SCHOTT DURAN (Germany).
2. Containers, SCHOTT DURAN (Germany).
3. Copelin jar, Pyrex (England).
4. Coverslips, Knittel (Germany).
5. Crump filter, CRUMA-670G/GS (Spain).
6. Cylinders, SCHOTT DURAN (Germany).
7. Dark bottles, SCHOTT DURAN (Germany).
8. Filter and sheet papers, SCheicher & Schuell GmbH (Germany).
9. Flasks, SCHOTT DURAN (Germany).
10. Glass slides, Knittel (Germany).
11. Hot plate, MEDAX (Germany).
12. Leica plastic funnel, Leica (Germany).
13. Light microscope, Leica CME 1349522x (Germany).
14. Microtome blades, Leica 818 &819 (Germany).
15. Oven, Emmert (Germany).
16. Paraffin embedding centre, Leica EG1160 (Germany).
17. Photographer microscope, Leica (Germany).
18. Plastic cassettes, Knittel (Germany).
19. Rotary microtome, Leica RM2165 (Germany).
20. Stainless forceps, (Germany).
21. Water bath, Leica HI1210 (Germany).

3.2 Methods

3.2.1 Subjects and settings

The case-control study was conducted between June 2018 and April 2019 on a total of 40 Libyan women in a singleton pregnancy, primiparous with no previous chronic diseases. Their deliveries were at 37+0 to 40+6 weeks of gestation, either vaginally or by caesarean section in the labour ward and obstetric operation theatre at Ali Omer Asker hospital, Libya. The examined women were categorized into two age groups:

1. Group A (Control group): consisted of 30 pregnant women between the age of 20 and 35 years old.

2. Group B (Study group): consisted of 10 pregnant women of age 35 years and older.

AMA was defined as the maternal age of 35 years and older at an estimated time of delivery according to the definition of the International Federation of Gynaecology and Obstetrics. AMA was calculated according to the maternal age when women gave birth. Gestational age was calculated from the first day of the last period of pregnant women with a regular menstrual cycle, which was confirmed by the ultrasound in the first trimester. A histological study of the human placenta was carried out in the Department of Histology and Genetics at the Faculty of Medicine in University of Tripoli, Libya.

3.2.2 Data collection

A short questionnaire (Appendix 1) was ascertained to collect detailed information including obstetric and medical history, mode of delivery, neonatal birth weight and APGAR score, placental weight, and histomorphometry of placental structures. Mothers were enrolled according to the following inclusion criteria:

- AMA ≥35 years old.
- Younger age between 20 and 35 years old.
- Primiparous singleton pregnancy.
- Full-term pregnancy.
- Absence of any chronic diseases such as diabetes, high blood pressure, etc.
- Haemoglobin >10.5 g /dl.

- No history of aspirin or anticoagulant treatment.

3.2.3 Ethical statements

The present study was approved by the Ethics and Research Committee at the Faculty of Medicine, University of Tripoli, Libya. All participants were fully informed about the study. Written consent was obtained from them (Appendix II). The placental tissues have been buried immediately after completing the study with the help of Ali Omer Asker hospital staff.

3.2.4 Sample collection

A total of 40 full-term human placentae were collected from the labour ward and obstetric theatre at Ali Omer Asker hospital. The umbilical cord was clamped immediately after birth to preserve the vascular architecture. Membranes and umbilical cord were first removed from each placenta, then the placenta was washed gently in tap water to remove excess blood clots and amniotic debris. Placental weight was measured using a digital scale and the weight was expressed in grams. A whole-thickness placental section of size (1 cm ×1cm) was taken from the central part of each placenta and fixed in 10% neutral buffered formalin for 3-4 days at room temperature. All specimens were labelled with a numbered sticker and carried to the histology and genetics department at the faculty of medicine, then routinely processed for further microscopical study.

3.2.5 Paraffin blocks preparation

Tissue samples from the placenta were processed for paraffin blocks and prepared for histological studies according to the methods of (Suvarna *et al*., 2013) as follows:

1. **Fixation**: Each tissue was cut into a small fragment of about 1cm × 1cm before fixation to facilitate penetration of fixative and preservation of the tissue. The fixative used was 10% neutral buffered formalin for 3-4 days.
2. **Dehydration:** The tissue to be embedded was dehydrated by bathing them in grades of ascending alcohol as follows:

- 50% alcohol……...30 minutes.
- 70% alcohol……overnight.
- 80% alcohol…….30 minutes.
- 96% alcohol…….30 minutes.
- Absolute alcohol I……40 minutes.
- Absolute alcohol II……...40 minutes.

3. Clearing: Alcohol was then replaced by xylene.

- Xylene I –30 minutes.
- Xylene II – 30 minutes.

4. Embedding: The tissue was placed in melted paraffin at 58 to 60 Celsius degrees as shown in Figure (6).

- Paraffin I – 30 minutes.
- Paraffin II – 30 minutes.

Blocks were prepared and 5-micron serial sections were generated with the help of a rotator microtome and then transferred to the glass slide for staining (Figure 7)

Figure (6). Tissue embedding and cooling system.

Figure (7). Microtome, hot plate, and water bath.

3.2.6 Histological and histochemical techniques

3.2.6.1 Haematoxylin and Eosin stain preparation

H&E stain was used for the study of general histological features. Haematoxylin is a basic dye that stains the nucleus of the cell and eosin is an acidic dye that stains the components of the cytoplasm.

1. Eosin stain:

Dissolve 1g of eosin Y into 100ml of 70% alcohol then add 5ml of glacial acetic acid and mix well.

2. Harris's haematoxylin stain:

2.5 g of haematoxylin powder was dissolved in 50 ml of absolute alcohol and then added to the 50 g of potassium alum, which has previously been dissolved in 500 ml of warm distilled water in a 2-litre flask. The mixture was rapidly brought to a boil then 1.25g of mercuric oxide was added slowly and carefully. Plugging the flask into cold water or a sink with chipped ice to rapidly cool down the stain. When the solution was cold, 20 ml of acetic acid was added, and the stain became ready for immediate use.

3. Technique

1. Deparaffinize the sections with two changes of xylene.

- Xylene I …..3-5 minutes.
- Xylene II ….3-5 minutes.

2. The graded hydration process started by passing the slides through the following alcohol concentration and water cycles:
   - Absolute alcohol I ….....1 minute.
   - Absolute alcohol II …...1 minute.
   - 96 % alcohol ……... 1 minute.
   - 80 % alcohol……1 minute.
   - 70% alcohol …… 1 minute.
   - 50 % alcohol …….…. 1 minute.
   - Distilled water ……..5 minutes.
3. Stain with haematoxylin for 10 minutes, then wash in running tap water for 5-10 minutes.
4. Observe the slides under the light microscope for proper staining. If stained excessively, a dip in acid alcohol was given, then wash in running tap water for 10 minutes.
5. Stain the sections with eosin for 4 minutes.
6. Dehydrate the sections in the following series of alcohol.
   - 50 % alcohol …… 30 seconds.
   - 70% alcohol ……..30 seconds.
   - 80% alcohol ……..30 seconds.
   - 96% alcohol ……..30 seconds.
   - Absolute alcohol ……...30 seconds.
7. Clearing and Mounting: clear the slides in xylene, and mount them using DPX.

Results:

Cytoplasm……... Pink

Nuclei ……. Blue

3.2.6.2 Mallory Trichrome stain preparation:

This stain was used for the demonstration of collagen fibres in villus stroma.

1. Mallory I: (0,5 % aqueous acid fuchsin).

Dissolve 1.0g of Acid fuchsin into 100.0 ml distilled water then mix well.

2. Mallory II: (Orange G - aniline blue solution).

Dissolve 2 g aniline blue,1 g orange G and 1 g phosphomolybdic acid in 100 ml distilled water, mix well, filter, and use immediately for better results.

3. Celestine blue solution:

The ferric ammonium sulphate (25 g) is dissolved in the cold distilled water (500ml) with stirring, 2.5 g celestine blue is added to this solution, and the mixture is boiled for a few minutes, after cooling, the stain is filtered and 70 ml of glycerine is added. The final stain should be used for over 5 months. Filter before use.

4. Technique:

1) Deparaffinize section, hydrate through graded alcohol to water.
2) Treat with celestine blue for 10 minutes.
3) Rinse in distilled water for 5 minutes.
4) Stain in Harrison haematoxylin for 10 minutes.
5) Wash in running tap water for 5 minutes.
6) Differentiate in 1% acid alcohol one dip.
7) Wash in running tap water for 10 minutes.
8) Stain in 0.5 %acid fuchsin solution for 5 minutes.
9) Drain and stain with Orange G-aniline blue solution for 15-20 minutes.
10) Differentiate in 95% alcohol, dehydrate, clear, and mount in DPX.

Result:

Red blood cells……. yellow.

Collagen……. blue.

Elastic fibres……...red.

3.2.7 Histomorphometry study of full-term human placenta

The sections were examined under the light microscope at different magnifications to assess the quality of the villi and intervillous spaces. Five sections were taken from each block based on their technical quality. The fields were photographed at two levels of magnifications: (X200) was applied to estimate the intervillous space, collagen, and the total foetal blood capillary and intervillous space areas. A higher magnification (X1000) was used to estimate the thickness of the syncytiotrophoblast (SCT). A final sample of 400 microscopic fields was saved as PowerPoint images for quantitative morphometric studies which were done with the aid of the Leica Quantimeter 500+ Image Analyser system (Heidi Soft Corporation™ - 2000) software as shown in Figure (8). The image analyser was adjusted and calibrated into a micrometre measuring unit and the area of the foetal blood capillary was calculated by subtracting the total area of the foetal blood capillary and intervillous space from the area of intervillous space.

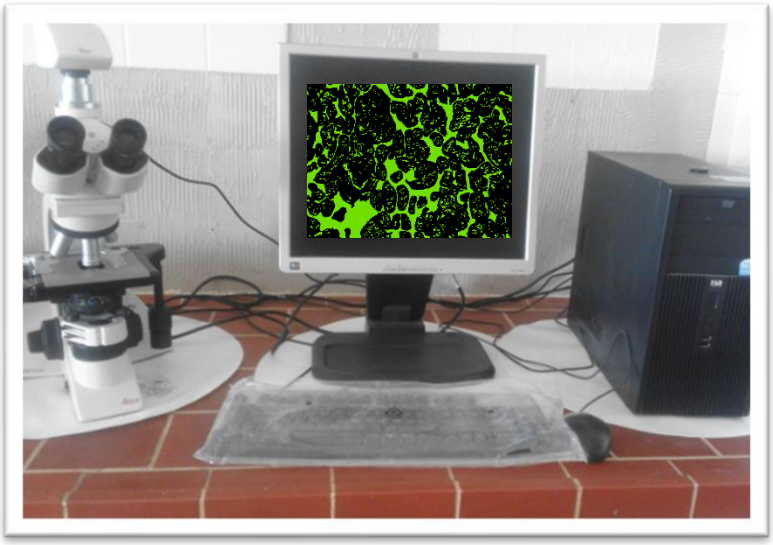


Figure (8). Image analysing system.

3.2.8 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (IBM SPSS Statistics, version 26.0) software. Shapiro-Wilk test and Q-Q plots were used to assess the normal distribution of data. Qualitative data were presented as frequency and percentage. The maternal age was expressed as the median and interquartile range (IQR) while the other data were normally distributed and expressed as mean and standard deviation. Quantitative normally distributed data were analysed statistically using Student's t-test to assess differences between the AMA group and control group. Correlation analysis was measured using the Pearson correlation coefficient test and Spearman correlation coefficient test. P-values of < 0.05 were considered statistically significant.

Chapter 4:

4 Results

4.1 Clinical aspect of the study

4.1.1 Maternal characteristics

This study included 40 mothers, of whom 25% were aged 35 years or more at childbirth. 75% of women were aged between 20 and 35 years at delivery. The median maternal age was 25 years with an IQR of 23–35.5 years. The age of all pregnant women ranged from 21 to 44 years.

4.1.1.1 Maternal age and fertility

Figure (9) shows that 93.3% of mothers aged less than 35 years conceived spontaneously while 6.7% of them needed medical therapy to conceive. On the other hand, 60% of advanced-age women needed assisted reproductive technology (ART) and 40% of them became pregnant naturally.

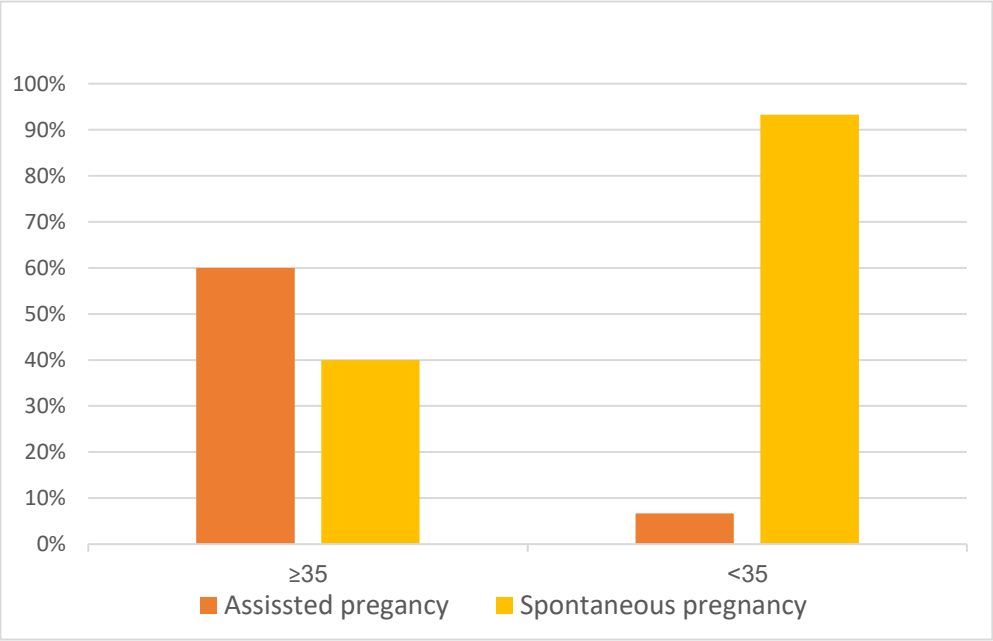


Figure (9). Distribution of pregnant women according to the method of conception.

4.1.1.2 Maternal age and mode of delivery

Table (1) shows that majority of younger mothers were delivered normally through the vagina with a percentage of 80%, while mothers of advanced age were more frequently delivered by C/S (70%). Overall, 67% of all mothers in this study were delivered normally.

Table (1). Frequency of mothers delivered by normal vaginal delivery (NVD) or caesarean section (C/S).

Parameter		MODE OF DELIVERY	
		NVD	**C/S**
Maternal age	**<35**	24	6
		80%	20%
	≥35	3	7
		30%	70%
Total		27	13
		67%	33%

4.1.2 Neonatal characteristics

4.1.2.1 Neonatal birth weight

All mothers in this study gave birth to babies with normal birth weights. The birth weight of neonates ranged from 2.660 kg to 4.00 kg with a mean and standard deviation of 3.270 ± 0.35 kg. As shown in Figure (10), the average weight of babies born to older women was 3.200 ± 0.47 kg. However, babies born to younger mothers had a marginally higher average birth weight of 3.300 kg and a standard deviation of 0.30 kg. The statistical analysis gives a p-value of 0.451, suggesting that the mother's age does not have a significant effect on the birth weight of babies.

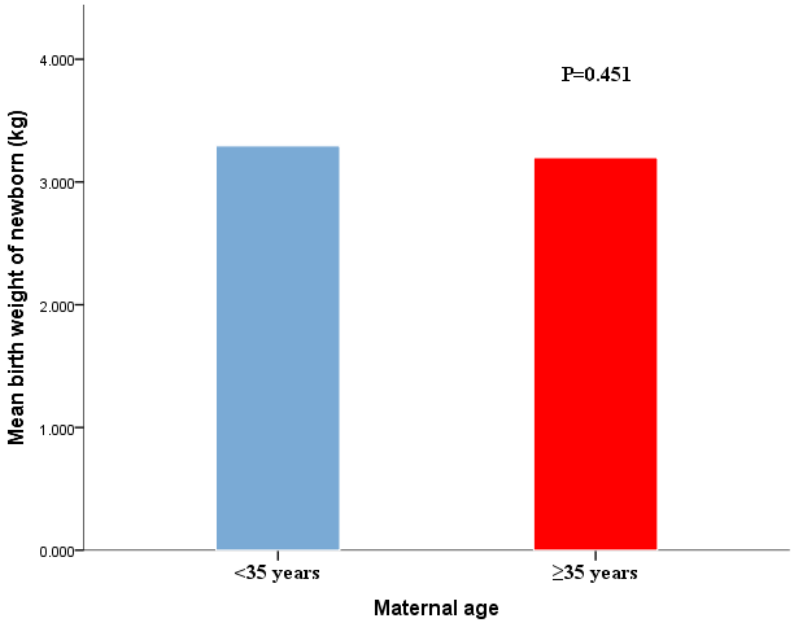


Figure (10). Statistical difference in neonatal birth weight between two age groups.

4.1.2.2 Maternal age and APGAR score

Figure (11) shows all babies of mothers under study were born with a normal APGAR score >7 at the 5th minute (A: Appearance; P: Pulse; G: Grimace; A: Activity; R: Respiration). There were no significant differences in the mean APGAR score of newborns of elderly women and women of the non-elderly group (M ± SD), (8.9 ± 0.74v/s 9.0 ± 0.72, P = 0.610**)**, respectively.

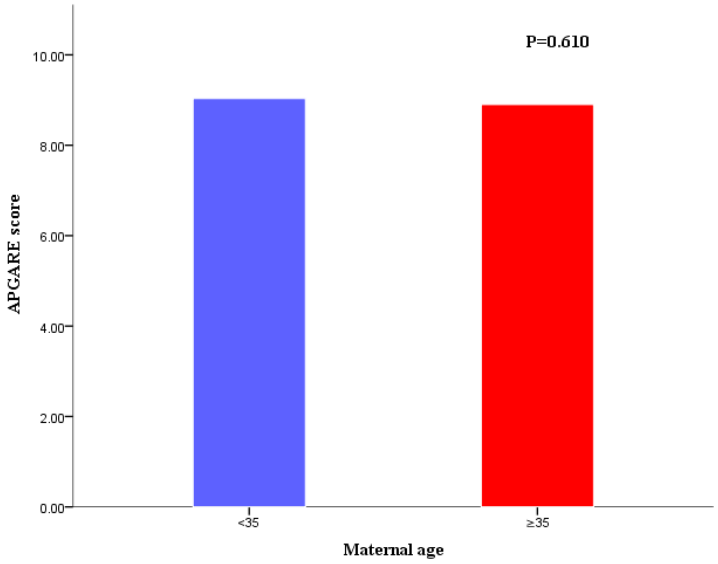


Figure (11). Statistical difference between two age groups regarding 5th minute APGAR score of new-borns.

4.1.2.3 Maternal age and admission to NICU

72.5% of newborns were given to their mothers and they did not need any clinical observation while admission to the neonatal intensive care unit was needed for 27.5% of all newborn babies. Only 3 newborn babies to elderly mothers were admitted to NICU as shown in Table (2). Additionally, the current study demonstrated that babies born to older women were at similar odds of neonatal intensive care unit admission (OR =1.17, 95% CI 0.24–5.70).

Table (2). Frequency of neonatal admission in NICU.

		NICU admission		Total
		YES	NO	
Maternal age	**<35**	8	22	30
		26.7%	73.3%	100.0%
	≥35	3	7	10
		30.0%	70.0%	100.0%
Total		11	29	40
		27.5%	72.5%	100.0%

4.1.3 Anthropometric measurement of the human placenta

In the current study, the placental weight of all mothers ranged from 300 g to 700 g with a mean ± SD of 467.5 ± 35.78 g. In addition, 14 placentae weighed less than 450 g (below the 10th Percentile), and 3 placentae weighed greater than 600 g (above the 90th Percentile) while 23 placentae fell in the normal range (Table 3).

Table (3). Frequency of placental weight.

Placental weight (g)	Frequency	Percent
<450 g	14	35
450 g - 600 g	23	57.5
>600 g	3	7.5

4.1.3.1 Placental weight and maternal age

Figure (12) shows that there was an increase in the mean placental weight of mothers aged ≥35 years (515 ± 113.16 g) in comparison to that of the control group (452 ± 85.58 g) although the difference between the placental weight of the two groups was not significant (P = 0.075).

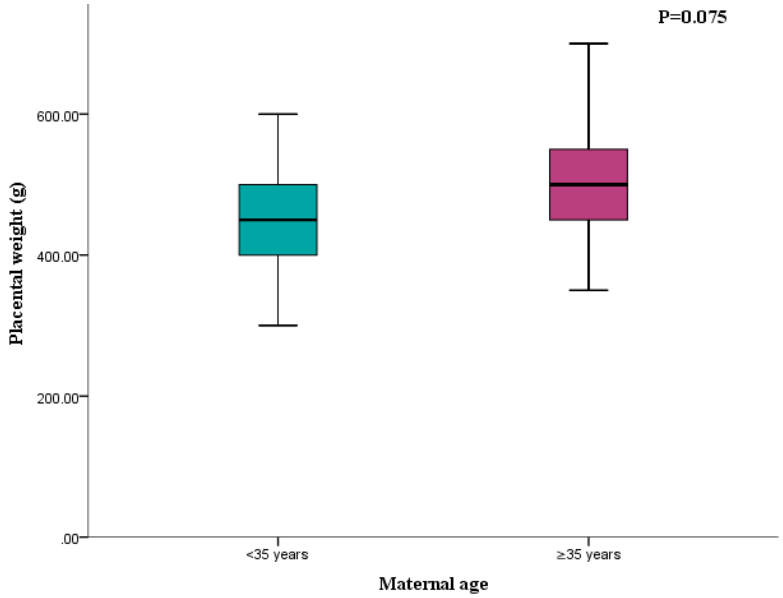


Figure (12). Statistical difference between two age groups regarding placental weight.

In addition, Figure (13) shows a non-significant and weak positive correlation between maternal age and placental weight for all mothers in this study (Spearman coefficient (r) =0.283, P = 0.08).

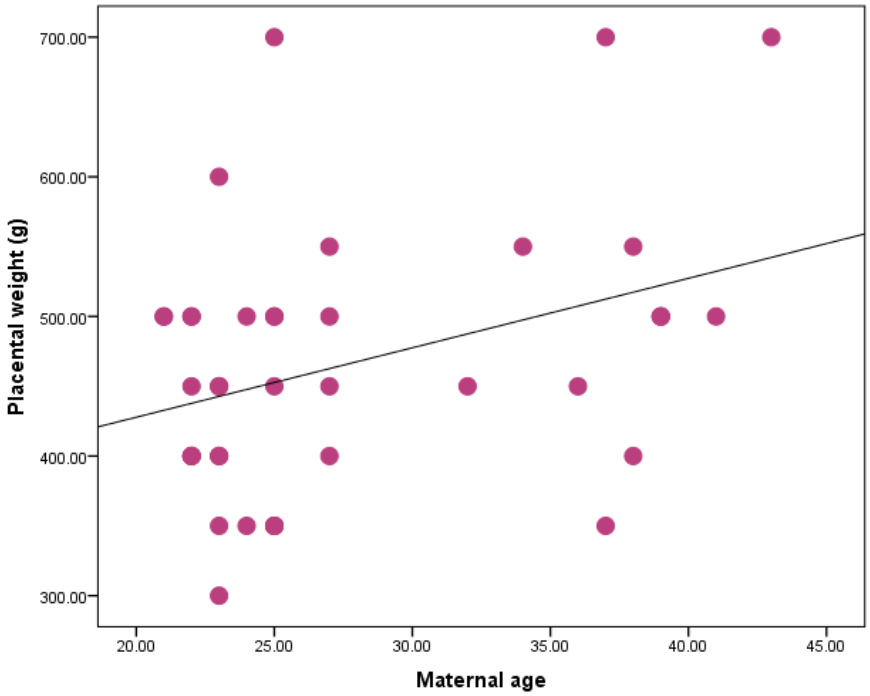


Figure (13). Correlation between maternal age and placental weight.

4.1.3.2 Placental weight and relation with birth weight

Figure (14) shows that there was a non-significant and weak positive relationship between the placental weight of all mothers and the birth weight of all neonates in this study (Pearson coefficient (r) = 0.279, P = 0.081).

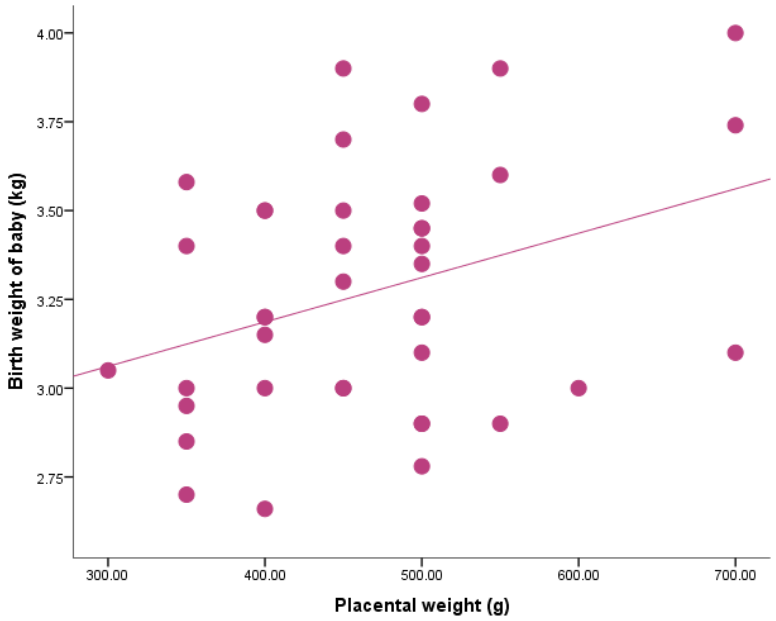


Figure (14). Correlation between placental weight and birth weight.

Moreover, the results demonstrated a significant rise in the mean placental weight/birthweight ratio in elderly mothers concerning controls (0.161 ± 0.024) and (0.138 ± 0.027; P =0.021), respectively (Table 4).

Table (4). Statistical difference of placental weight/birthweight ratio between two age groups.

Parameters	Maternal age		P-value
	<35	≥35	
	M ± SD	M ± SD	
Placental weight/birthweight ratio	0.138 ± 0.027	0.161 ± 0.024	0.021

4.2 Histological aspect of the study

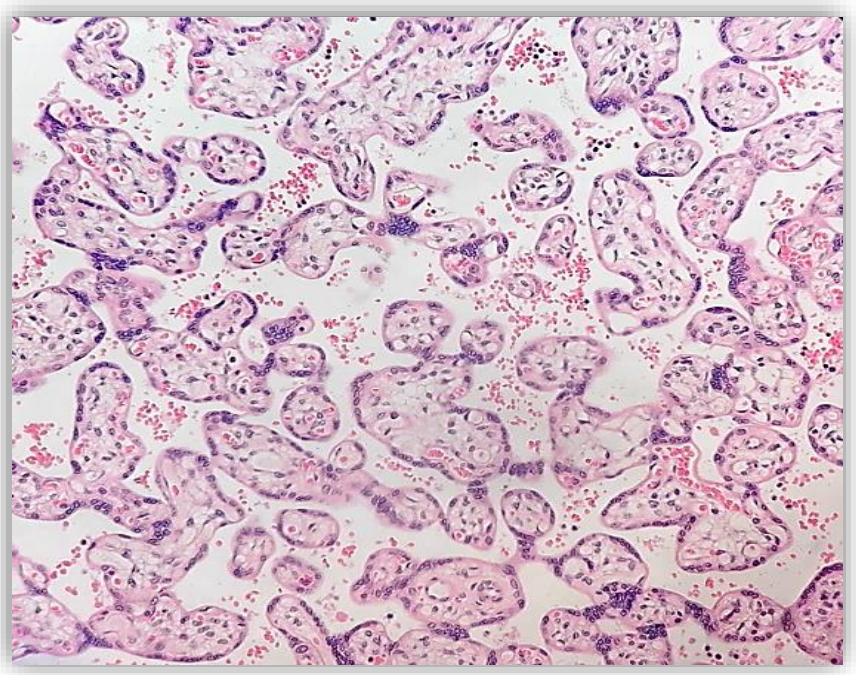


Figure (15). A photomicrograph of full-term human placenta (H&E. X 200).

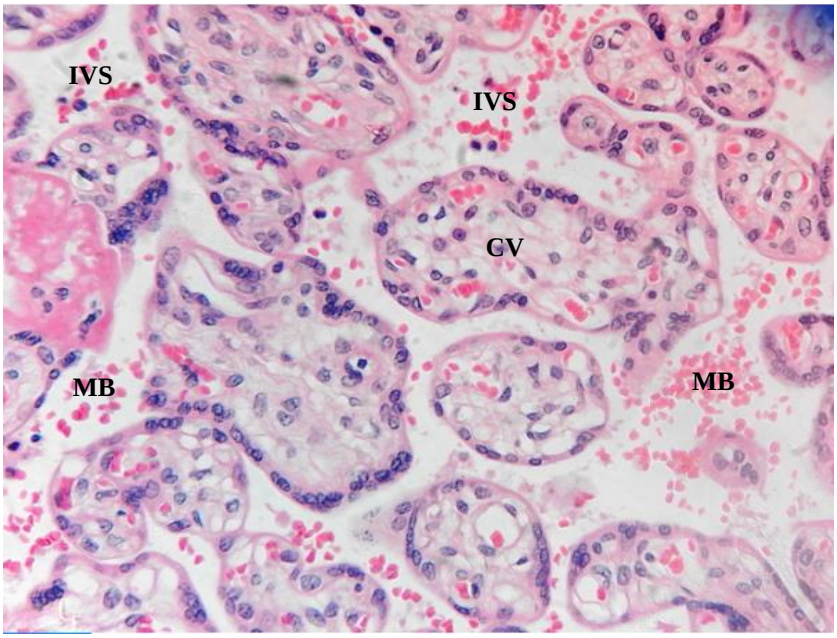


Figure (16). A photomicrograph of the full-term human placenta showing many chorionic villi (CV) floated in the intervillous space (IVS) which is pooled with maternal blood (MB) (H&E. X 400).

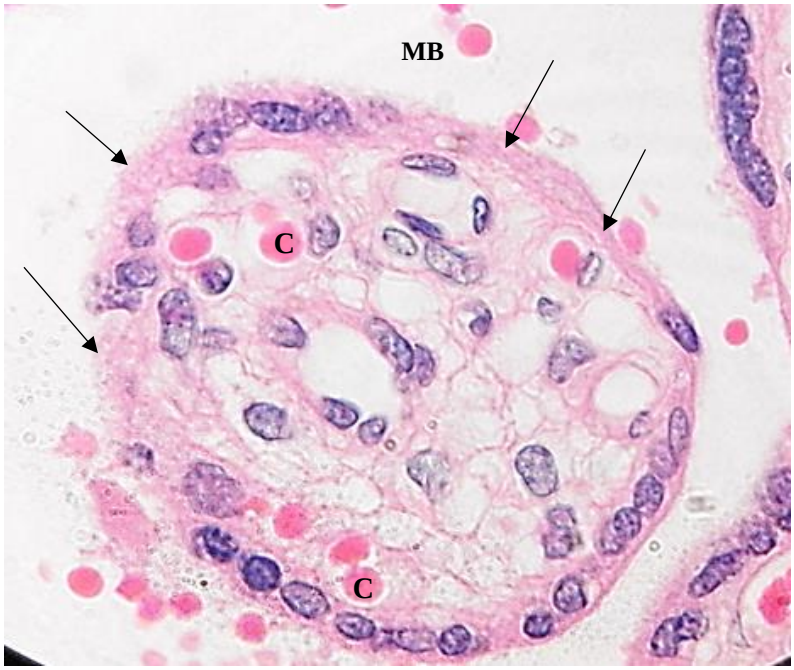


Figure (17). A photomicrograph of a full-term human placenta contains terminal villi surrounded by maternal blood (MB). Each villus has a core of connective tissue containing foetal blood capillaries (C). The cytotrophoblast cells have significantly decreased in many regions leaving a thin syncytiotrophoblast and basement membrane covering these areas as indicated by the arrows (H&E. X 1000).

4.2.1 Quantitative histomorphometry results

4.2.1.1 Maternal age and foetal blood capillary

The mean lumen area of the foetal blood capillaries in the placentae of all mothers under study was 74.708×10^3 µm² ± 18.045×10^3 and had a range of 2.750×10^3µm² to 232.461×10^3 µm² as shown in Table (5).

- **Mothers <35years of age**

The mean lumen area of the foetal blood capillaries was $93.843\times10^3 \pm 23.363\times10^3$ µm² and ranged from 2.750×10^3µm² to 232.461×10^3µm² as shown in Table (6) and Figures: 23,24,25.

- **Mothers ≥35years of age**

The mean lumen area of the foetal blood capillaries was $17.301\times10^3 \pm 4.240\times10^3$ µm² and ranged from 7.585×10^3 µm² to 42.325×10^3 µm² as shown in Table (6) and Figures: 26,27,28.

In addition, this study exhibited that the lumen area of foetal blood capillaries in the terminal villi of placentae is significantly lower in older pregnant women (P= 0.009) in comparison to the younger pregnant women as shown in Table (6) and Figure (18).

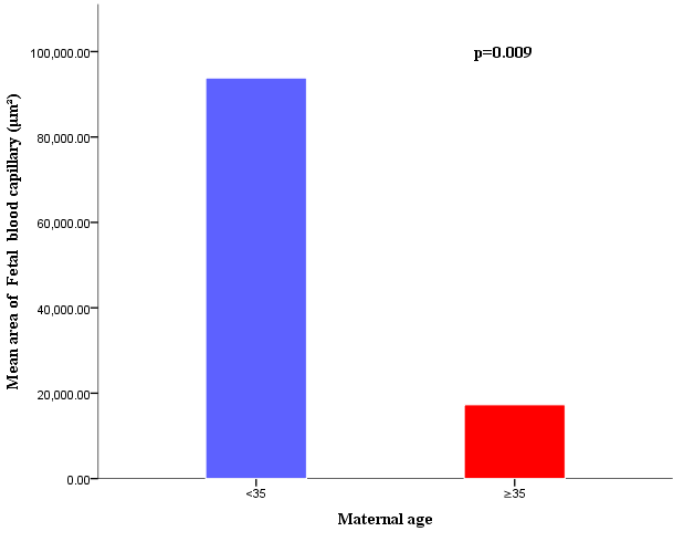


Figure (18). A comparison between mothers ≥35 years of age and the control group regarding the lumen area of foetal blood capillary.

4.2.1.2 Maternal age and intervillous space

As shown in Table (5), the area of maternal space in the placentae of all mothers ranged from 9.687×10^3 µm² to 315.295×10^3 µm² with an average value of $135.935\times10^3 \pm 33.578\times10^3$ µm².

- **Control group**

The mean area of maternal space in mothers <35 years old was $110.331\times10^3 \pm 27.174\times10^3$ µm² with a range (9.687×10^3 µm² - 254.997×10^3 µm²) as shown in Table (6) and Figures: 29,30,31.

- **Advanced maternal age group**

The mean area of maternal space in older pregnant mothers was $212.745\times10^3 \pm 56.318\times10^3$ µm² with a range (154.078×10^3µm² - 315.295×10^3µm²) as shown in Table (6) and Figures: 32,33,34.

Furthermore, the results showed that the mean area of intervillous space in the placenta of older pregnant mothers is significantly higher in comparison to the placenta of younger ones (P= 0.003) as shown in Table (6) and Figure (19).

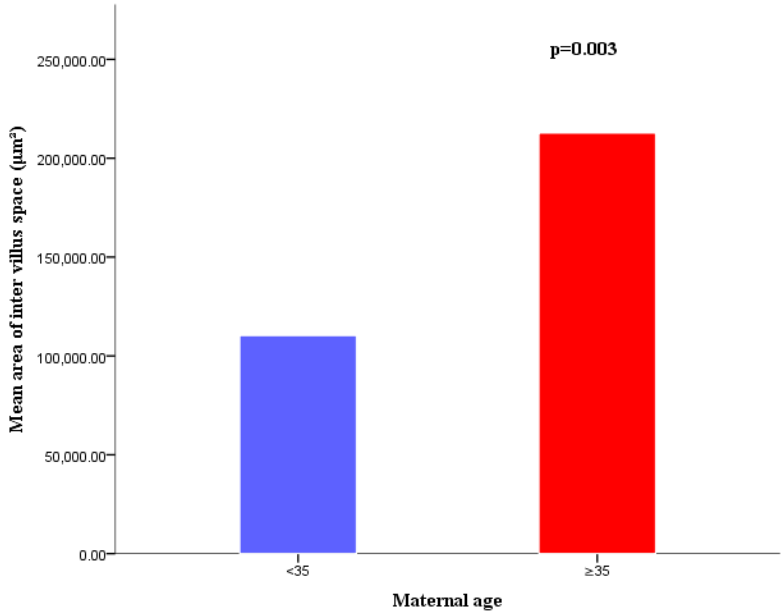


Figure (19). A comparison between mothers ≥35 years of age and the control group regarding the area of intervillous space.

4.2.1.3 The Correlation between the lumen area of foetal blood capillaries and intervillous space area of full-term human placentae

This study found that the capacity area of foetal blood capillaries had a significant and strong negative correlation with the area of intervillous space in the placenta of all mothers (Pearson coefficient (r) = - 0.668, P<0.001) as shown in Figure (20).

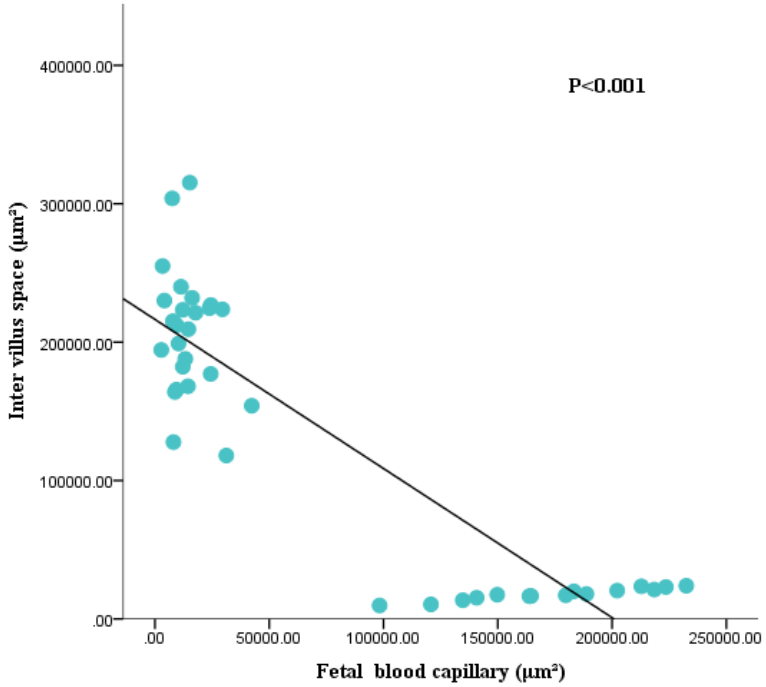


Figure (20). Correlation between intervillous space and foetal blood capillary.

4.2.1.4 Maternal age and placental collagen

The average area of collagen expressed in chorionic villi of all mothers was $68.420\times10^3 \pm 16.919\times10^3$ µm² and ranged from 29.046×10^3µm² to 137.131×10^3 µm² as shown in Table (5).

- **Mothers <35years of age**

The mean area of the collagen expressed in chorionic villi of younger mothers was $71.412\times10^3 \pm 16.754\times10^3$ µm² and ranged from 29.046×10^3 µm² to 137.131×10^3 µm² as shown in Table (6) and Figures: 35, 36, 37.

- **Mothers ≥35years of age**

The mean area of the villus collagen in placentae of older pregnant women was $59.446\times10^3 \pm 13.283\times10^3$ µm² and ranged from 34.475×10^3 µm² to 110.878×10^3 µm² as shown in Table (6) and Figures: 38, 39, 40.

Furthermore, the results exhibited no significant difference in the amount of collagen expressed in the chorionic villi of both groups (P=0.228) as shown in Figure (21).

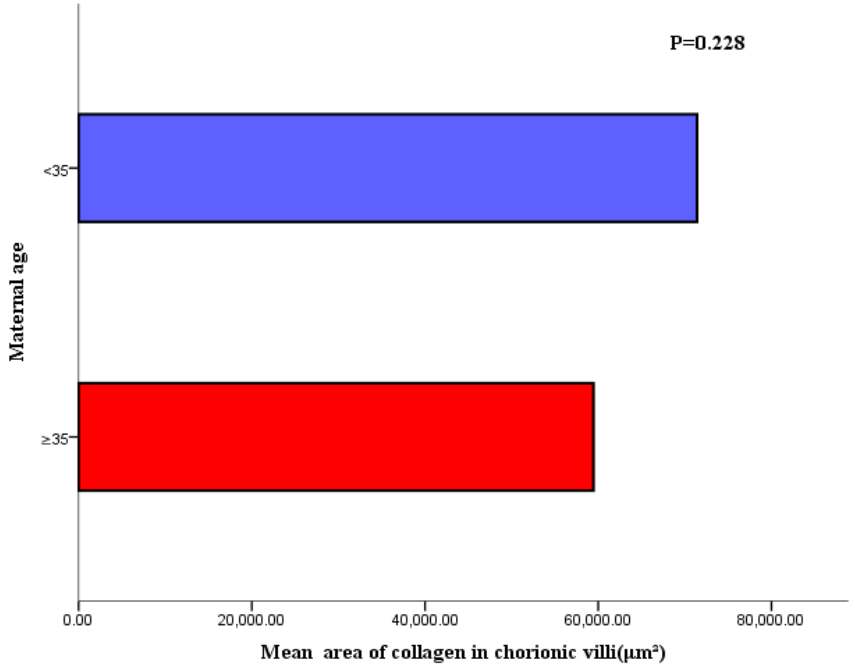


Figure (21). A comparison between mothers ≥35years of age and the control group regarding the area of collagen deposition in the chorionic villi.

4.2.1.5 Maternal age and syncytiotrophoblast layer thickness

It was observed in this study that the syncytial layer thickness in all mothers ranged from 5.90 µm to 9.39 µm with (M±SD) =7.22± 0.74 µm (see Table 5).

- **Control group**

The mean thickness of syncytiotrophoblast in mothers <35 years old was7.19 ± 0.83 µm with a range of (6.13 µm-8.45 µm) (see Table 6 and Figure 41)

- **Advanced maternal age group**

The mean thickness of syncytiotrophoblast in older pregnant mothers was 7.33 ± 0.37 µm with a range of (5.91µm- 9.39 µm) (see Table 6 and Figure 42).

In addition, the results showed that the syncytiotrophoblast layer in placentae of mothers ≥ 35years was thicker compared to mothers <35years although this difference was not statistically significant (P=0.616) (see Figure 22).

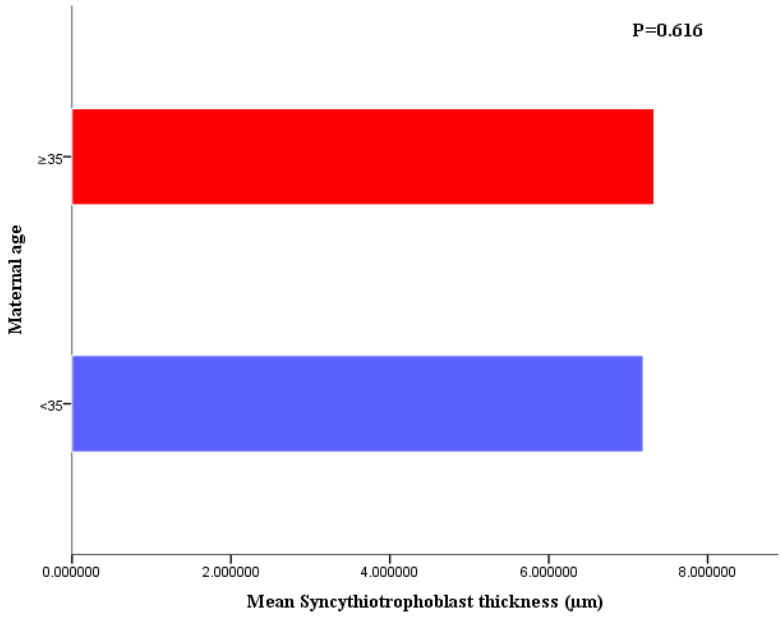


Figure (22). A comparison between mothers ≥35years of age and the control group regarding the thickness of syncytiotrophoblast.

Table (5). Descriptive statists of histological structures of full-term human placenta in all mothers: intervillous space; foetal blood capillary; villus collagen, and syncytiotrophoblast thickness.

Parameters	Minimum	Maximum	Mean	SD
Area of foetal blood capillary (µm²)	2.750×10^3	232.461×10^3	74.708×10^3	18.045×10^3
Area of intervillous space (µm²)	9.687×10^3	315.295×10^3	135.935×10^3	33.578×10^3
Area of villus collagen (µm²)	29.046×10^3	137.131×10^3	68.420×10^3	16.919×10^3
Syncytiotrophoblast thickness(µm)	5.90	9.39	7.22	0.74

µm²=micrometre². SD= Standard deviation.

Table (6). Statistical difference between mothers aged ≥35 years and mothers younger than 35 years regarding the histological structures of the human placenta: intervillous space; foetal blood capillary; villus collagen, and syncytiotrophoblast thickness.

Parameters	Maternal age <35years M ±SD	Maternal age ≥35 years M ±SD	P-value	Significance
Area of foetal blood capillary (µm²)	$93.843\times10^3 \pm 23.363\times10^3$	$17.301\times10^3 \pm 4.240\times10^3$	0.009	Decrease
Area of intervillous space (µm²)	$110.331\times10^3 \pm 27.174\times10^3$	$212.745\times10^3 \pm 56.318\times10^3$	0.003	Increase
Area of villus collagen (µm²)	$71.417\times10^3 \pm 16.754\times10^3$	$59.446\times10^3 \pm 13.283\times10^3$	0.228	Not-significant
syncytiotrophoblast thickness(µm)	7.19 ± 0.83	7.33 ± 0.37	0.616	Not-significant

M=Mean. P-value=Probability. SD= Standard deviation

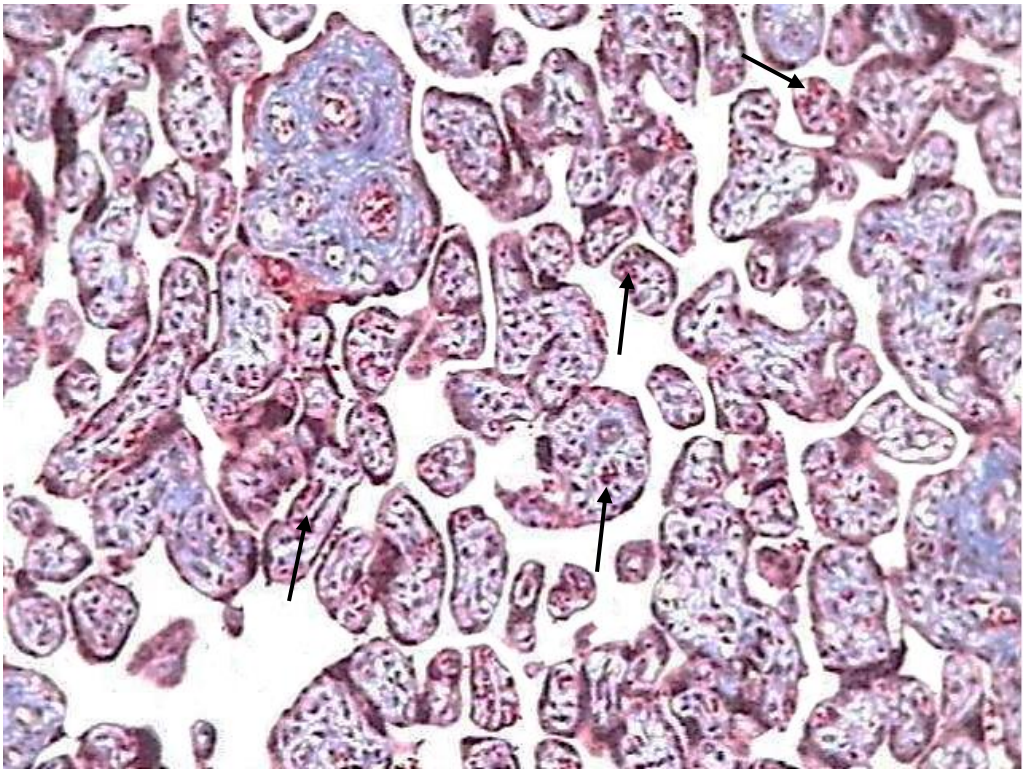


Figure (23). Computerised photomicrograph of full-term human placenta of the control group including sections of many terminal villi which contain connective tissue and microvasculature filled with foetal blood (arrows) (M.T. X200).

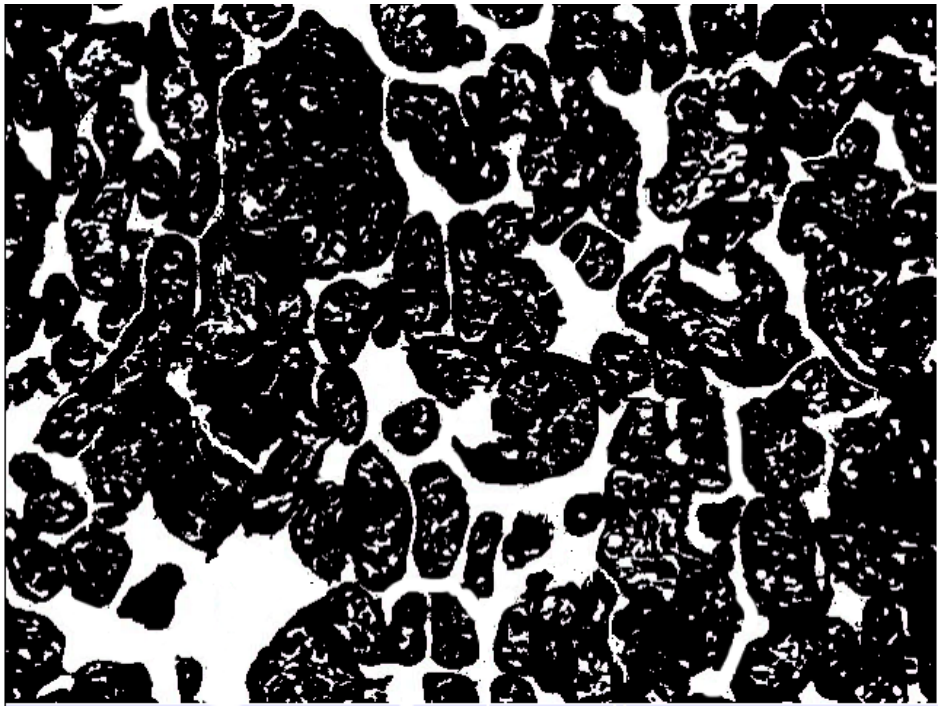


Figure (24). Computerised photomicrograph of full-term human placenta of control group showing the area of foetal blood capillaries within the chorionic villi (X200).

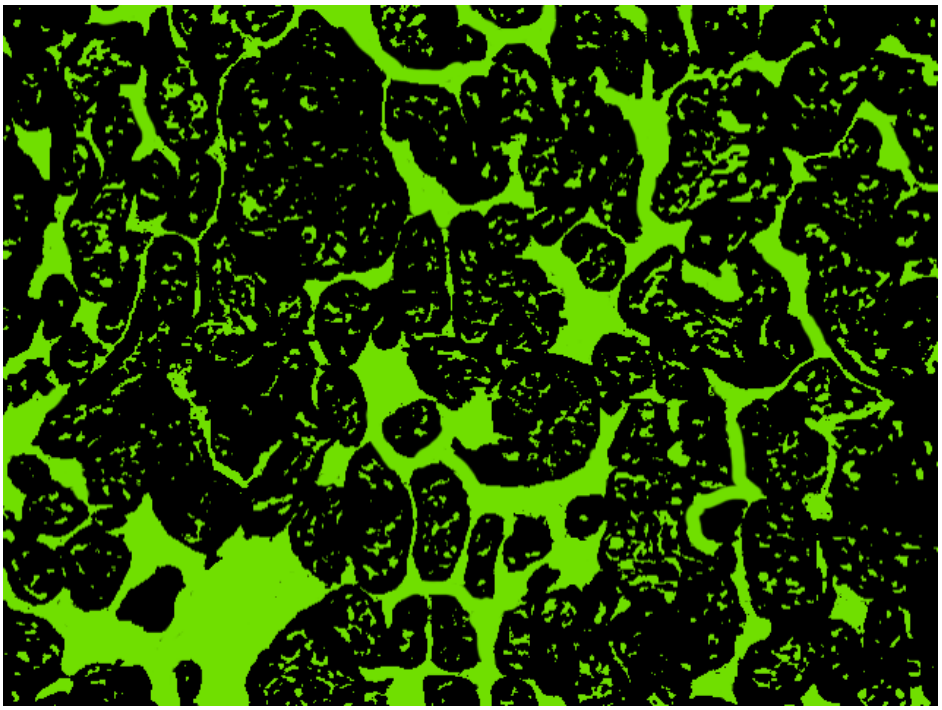


Figure (25). Computerised photomicrograph of full-term human placenta of control group showing the area of foetal blood capillaries within the chorionic villi (X200).

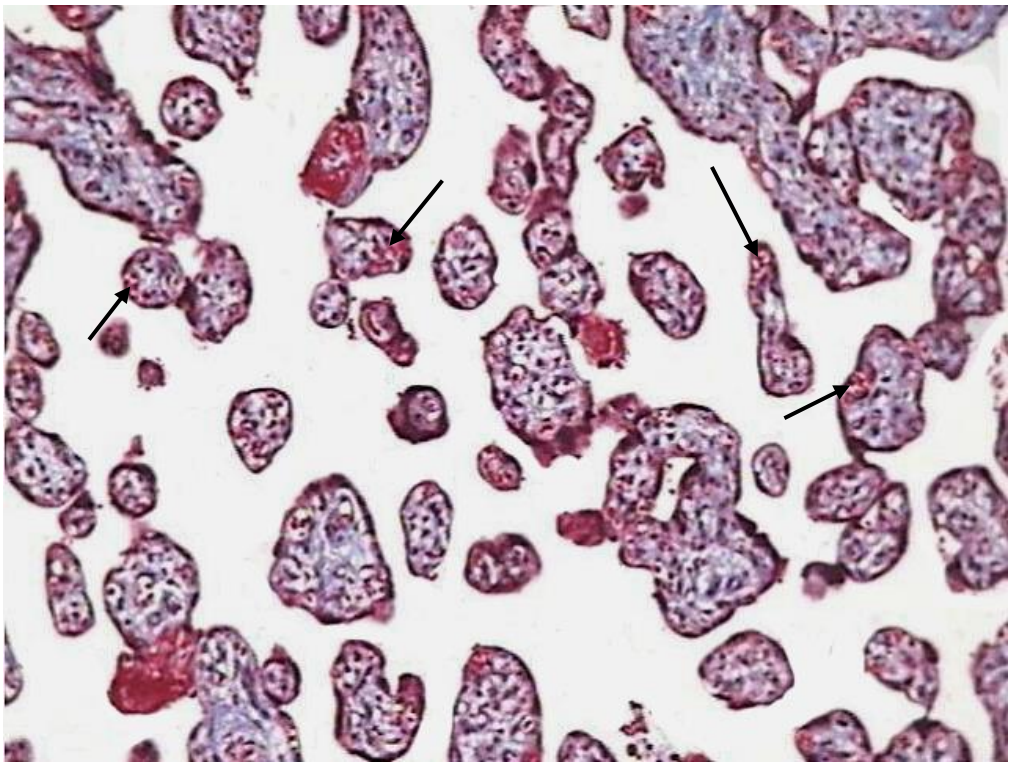


Figure (26). Computerised photomicrograph of full-term human placenta of advanced age group including sections of terminal villi floated in a wide intervillous space and each villus contains connective tissue and a few foetal blood capillaries (arrows) (M.T. X200).

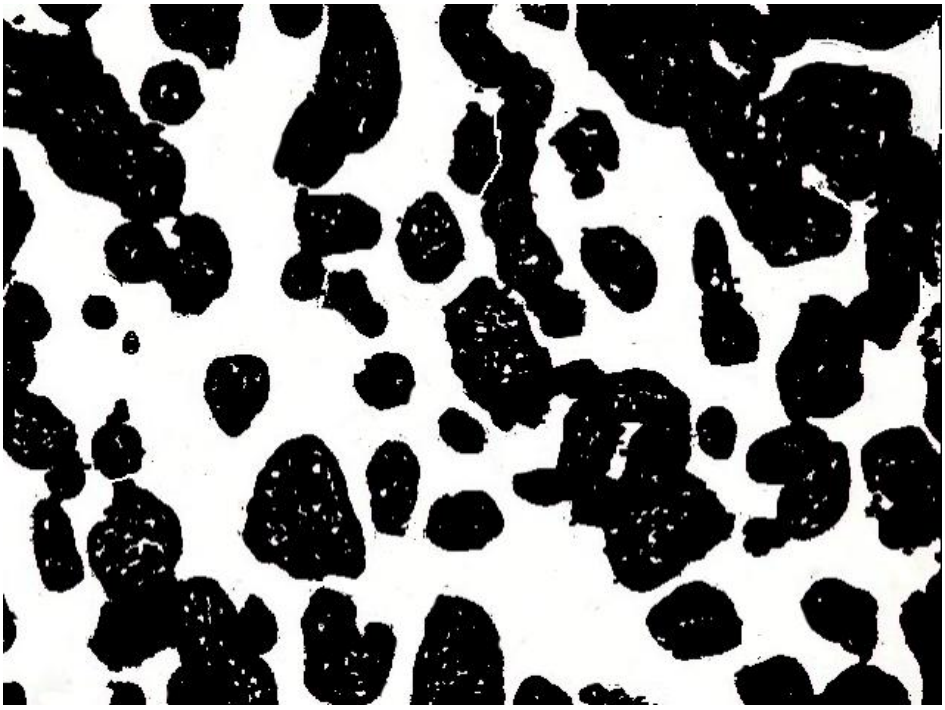


Figure (27). Computerised photomicrograph of full-term human placenta of advanced age group showing the area of foetal blood capillaries within the terminal villi (X 200).

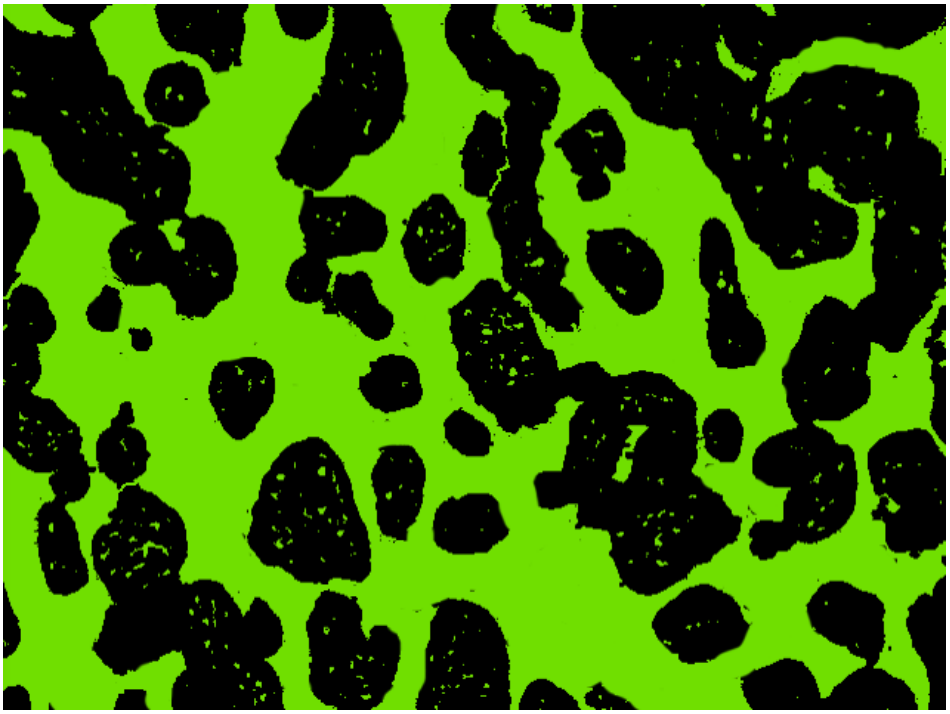


Figure (28). Computerised photomicrograph of full-term human placenta of advanced age group showing the area of foetal blood capillaries within the terminal villi (X 200).

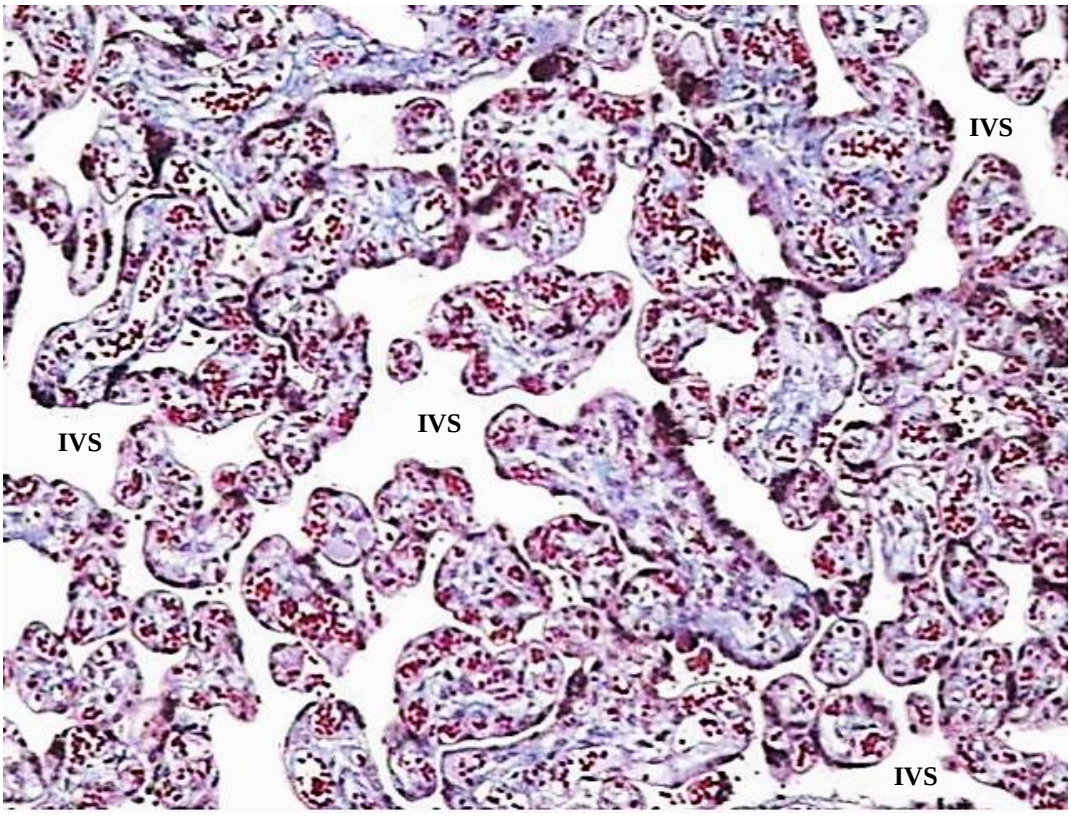


Figure (29). Computerised photomicrograph of full-term human placenta of the control group showing sections of chorionic villi floated in the intervillous space (IVS) (M.T. X200).

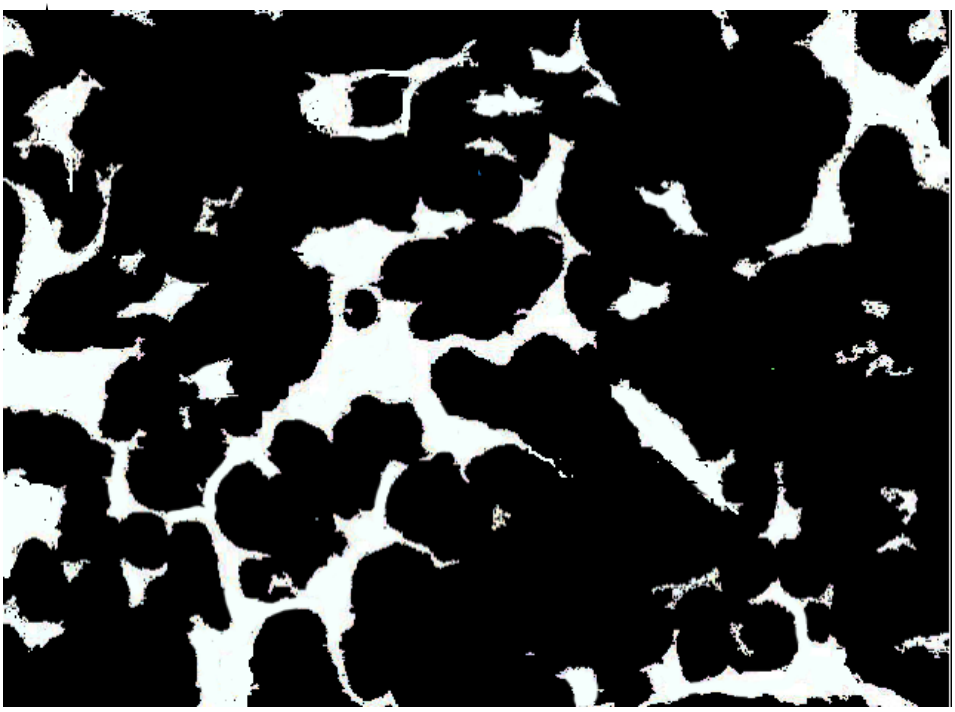


Figure (30). Computerised photomicrograph of full-term human placenta of control group showing the area of the intervillous space (X 200).

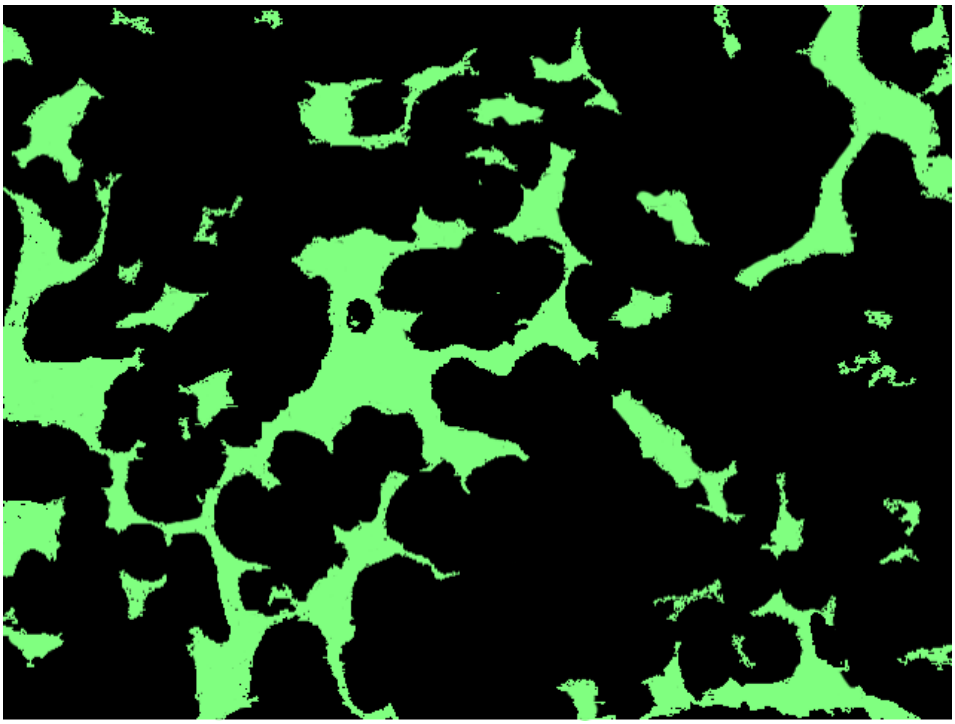


Figure (31). Computerised photomicrograph of full-term human placenta of control group showing the area of the intervillous space (X200).

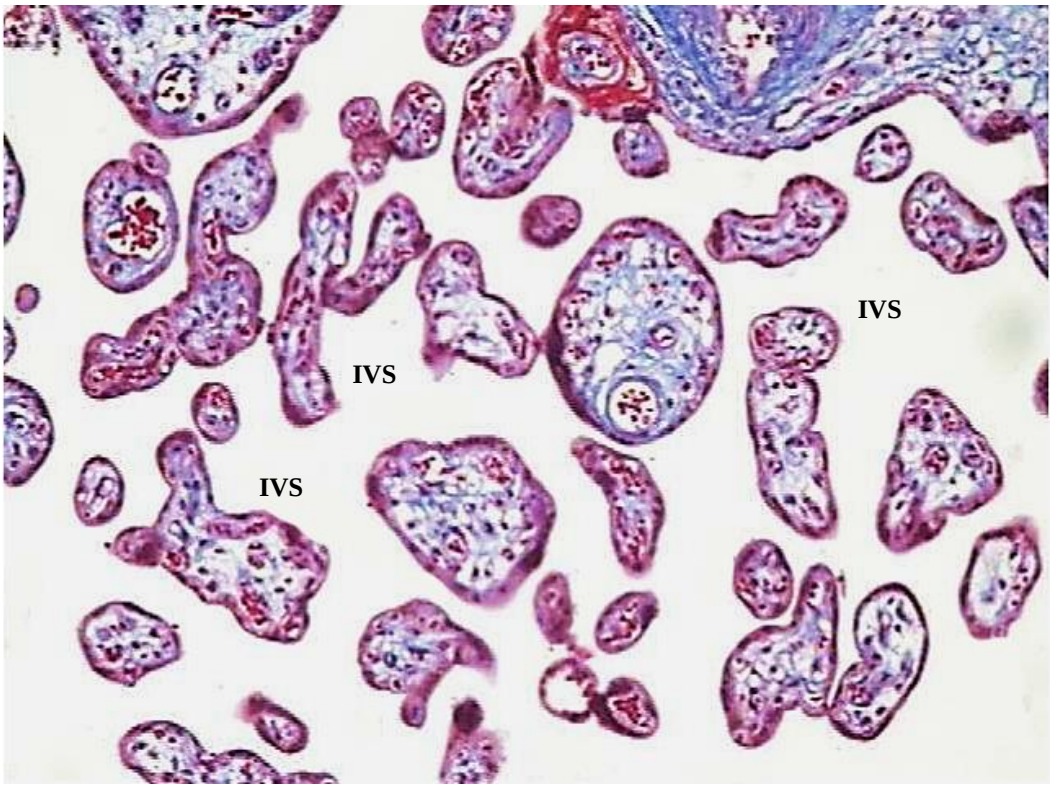


Figure (32). Computerised photomicrograph of full-term human placenta of advanced maternal age group showing sections of terminal villi floated in a wide intervillous space (IVS) (M.T. X200).

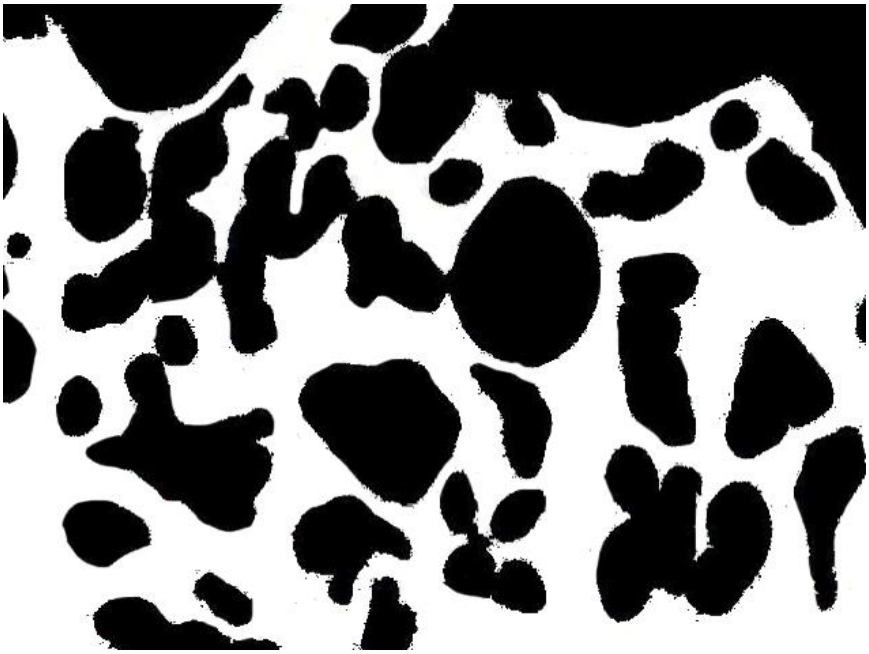
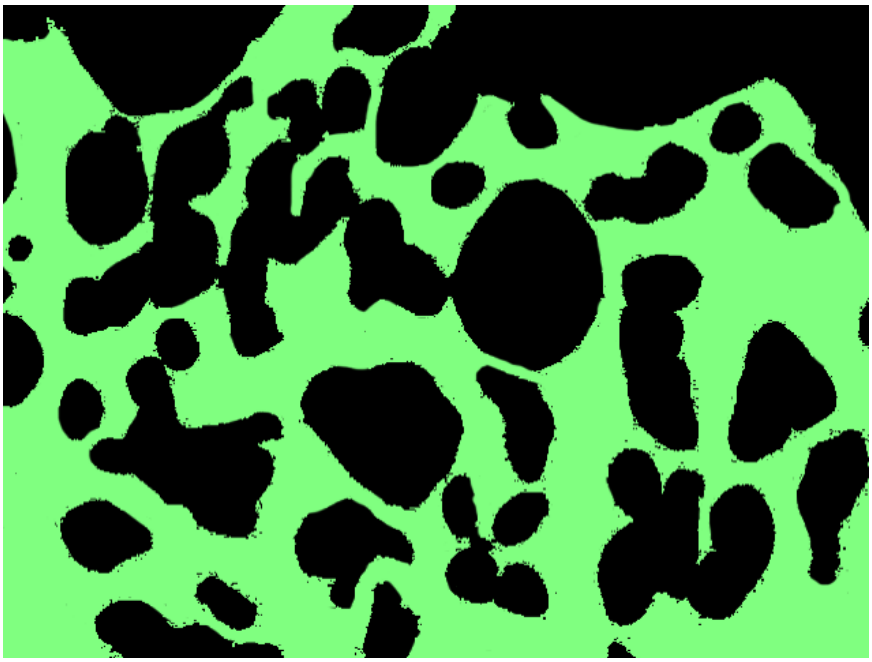


Figure (33). Computerised photomicrograph of full-term human placenta of advanced maternal age group showing the area of the intervillous space (X200).



Figure(34). Computerised photomicrograph of full-term human placenta of advanced maternal age group showing the area of the intervillous space (X200).

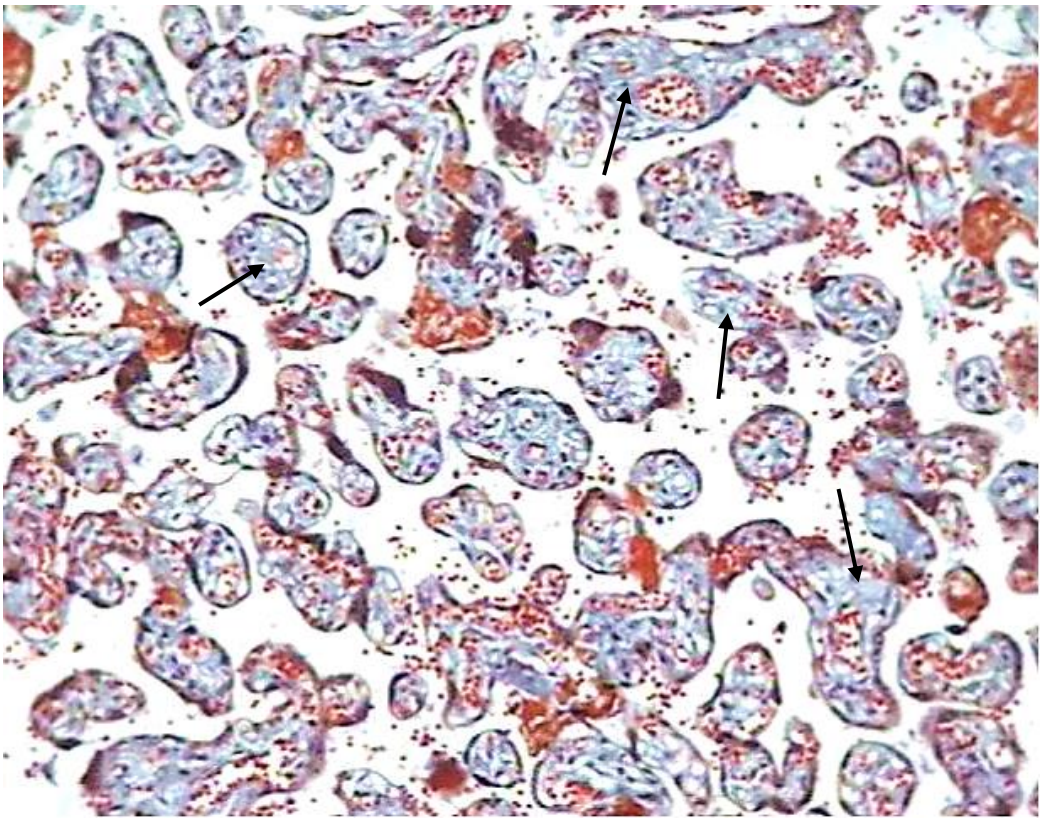


Figure (35). Computerised photomicrograph of full-term human placenta of control group showing the normal distribution of collagen fibres inside the chorionic villi (arrows) (M.T. X 200).

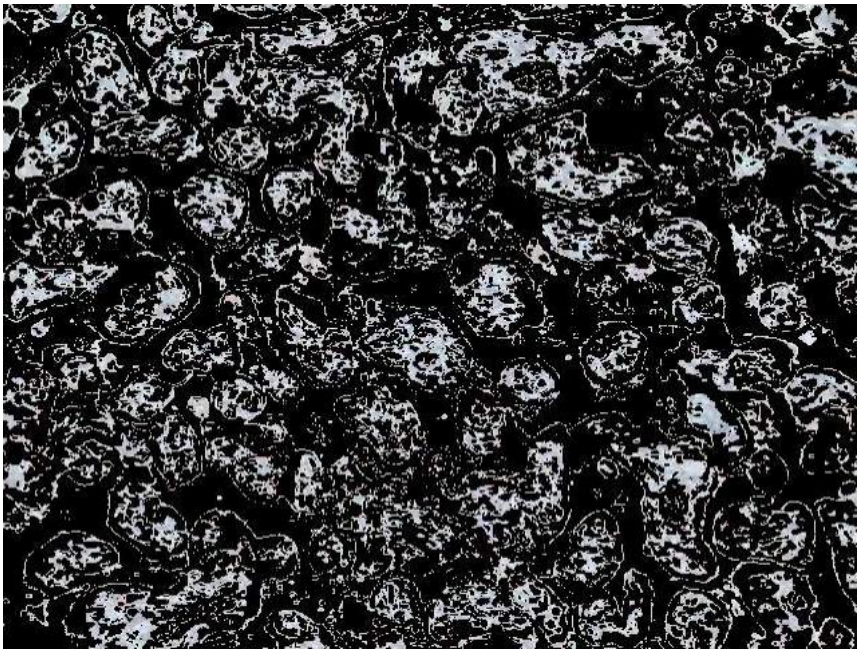


Figure (36). Computerised photomicrograph of full-term human placenta of control group showing the area of collagen fibres deposition inside the chorionic villi (white colour) X 200.

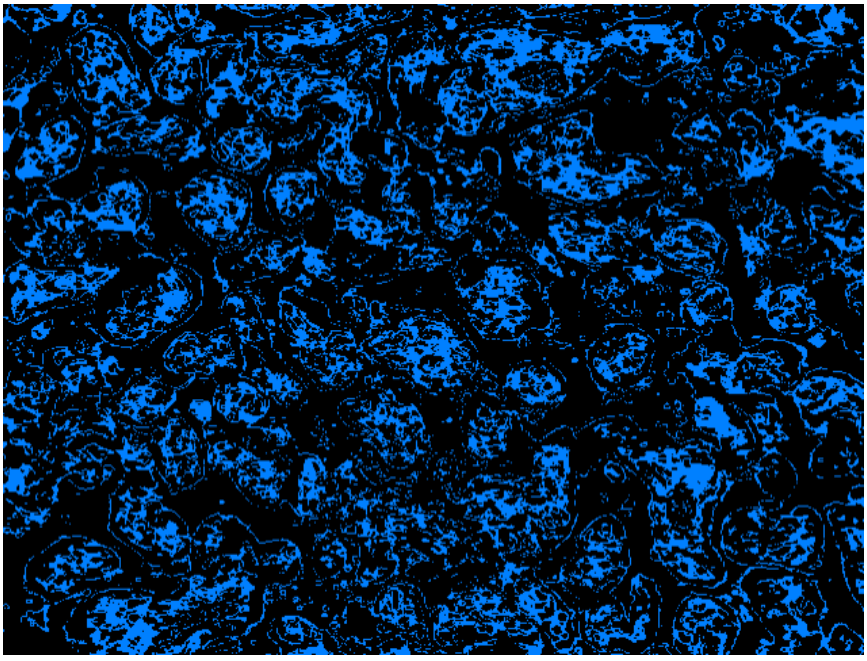


Figure (37). Computerised photomicrograph of full-term human placenta of control group showing the area of collagen fibres deposition inside the chorionic villi (blue colour) X 200.

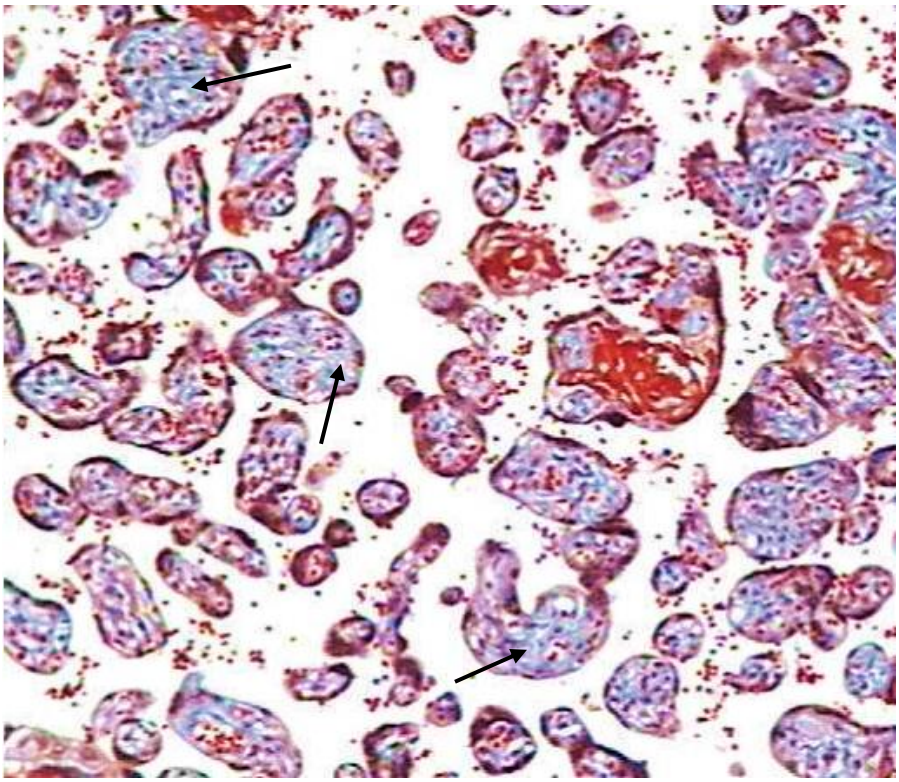


Figure (38). Computerised photomicrograph of full-term human placenta of mothers ≥35 years showing collagen fibres deposition within the villous connective tissue (arrows) (M.T. X 200).

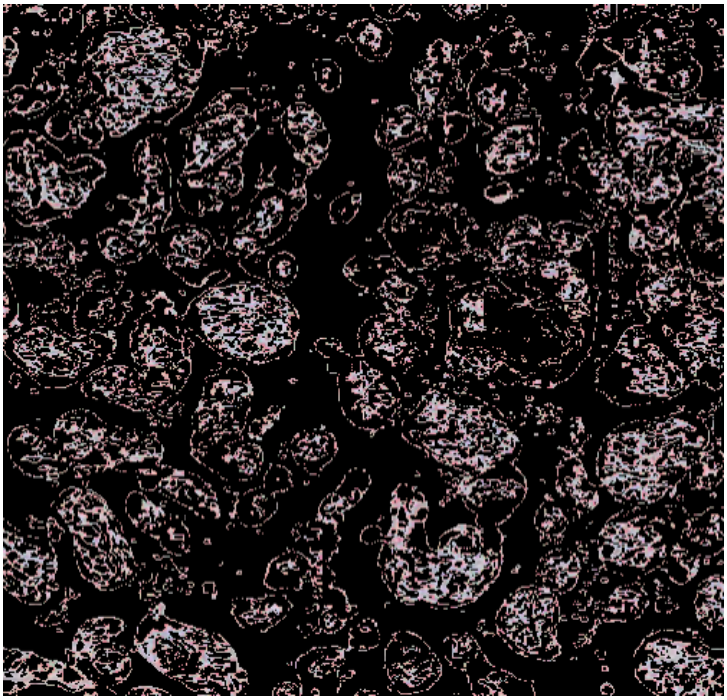


Figure (39). Computerised photomicrograph of full-term human placenta of mothers ≥35 years showing the area of collagen fibres deposition within the villous stroma (white colour) X 200.

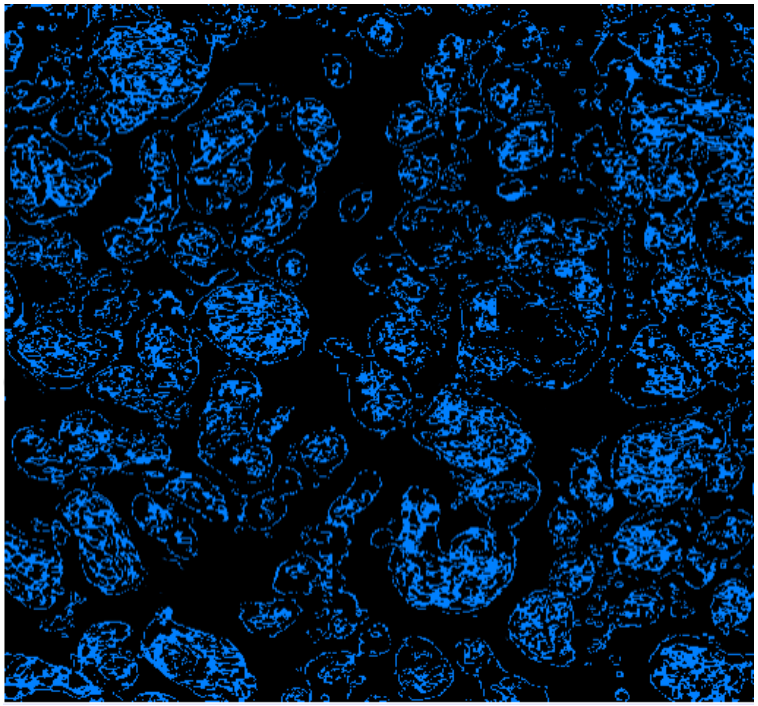


Figure (40). Computerised photomicrograph of full-term human placenta of mothers ≥35years showing the area of collagen fibres deposition within the villus stroma (blue colour) X 200.

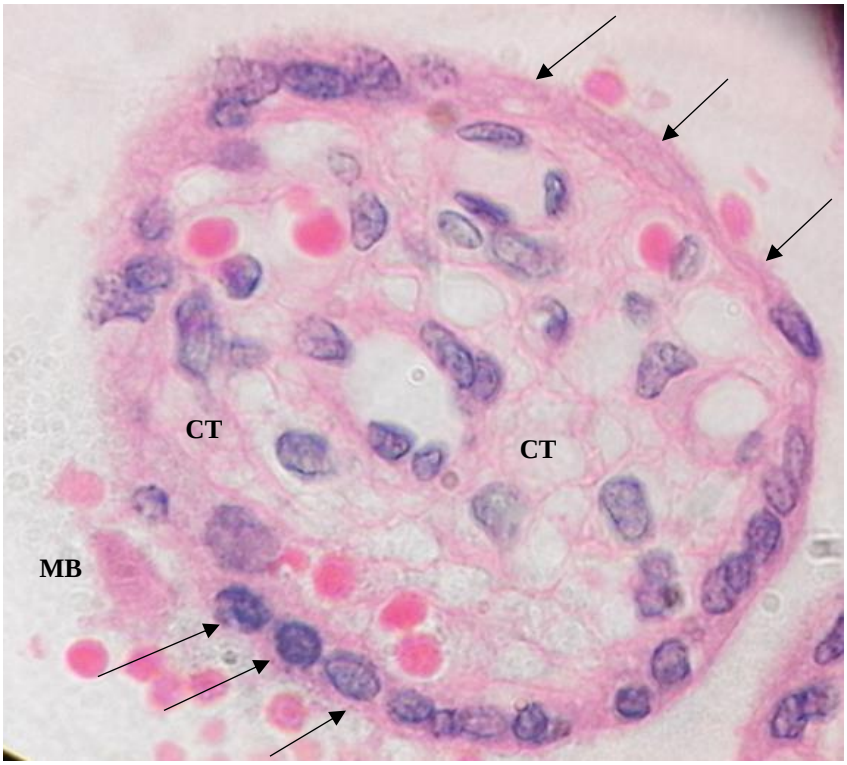


Figure (41). A photomicrograph of full-term human placenta of mothers <35years showing terminal villi surrounded by maternal blood (MB), and each villous has a connective tissue core (CT) covered by complete syncytiotrophoblast layer (arrows). (H&E. X 1000).

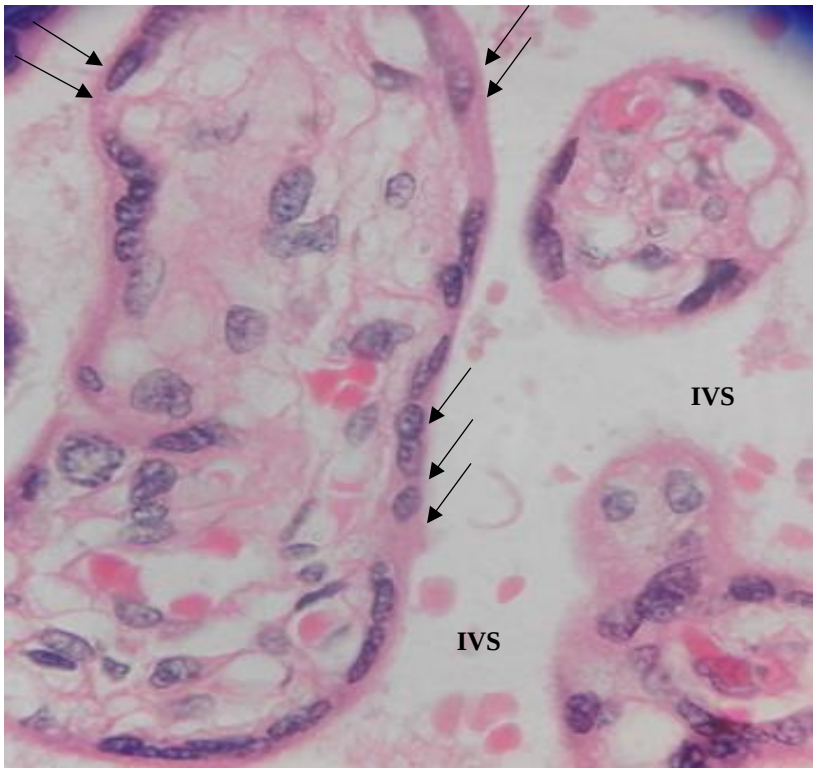


Figure (42). A photomicrograph of full-term human placenta of mothers ≥35 years showing the terminal villi are floated in intervillous space (IVS) which is lined by the syncytiotrophoblast (arrows) (H&E. X 1000).

4.2.2 Histological and histochemical appearance of the placenta

- **Control group**

Cross-sections of the full-term placenta in this group showed normal histological architecture. Many chorionic villi are seen surrounded by maternal blood that fills the space around them. Each villus has a connective tissue core with microvasculature and is surrounded by inner cytotrophoblast cells and outer syncytiotrophoblast (Figures: 15,16,17,43,44). In addition, a strong Mallory trichrome reaction was observed indicating a normal distribution of collagen fibres within the villi as shown in Figure (45).

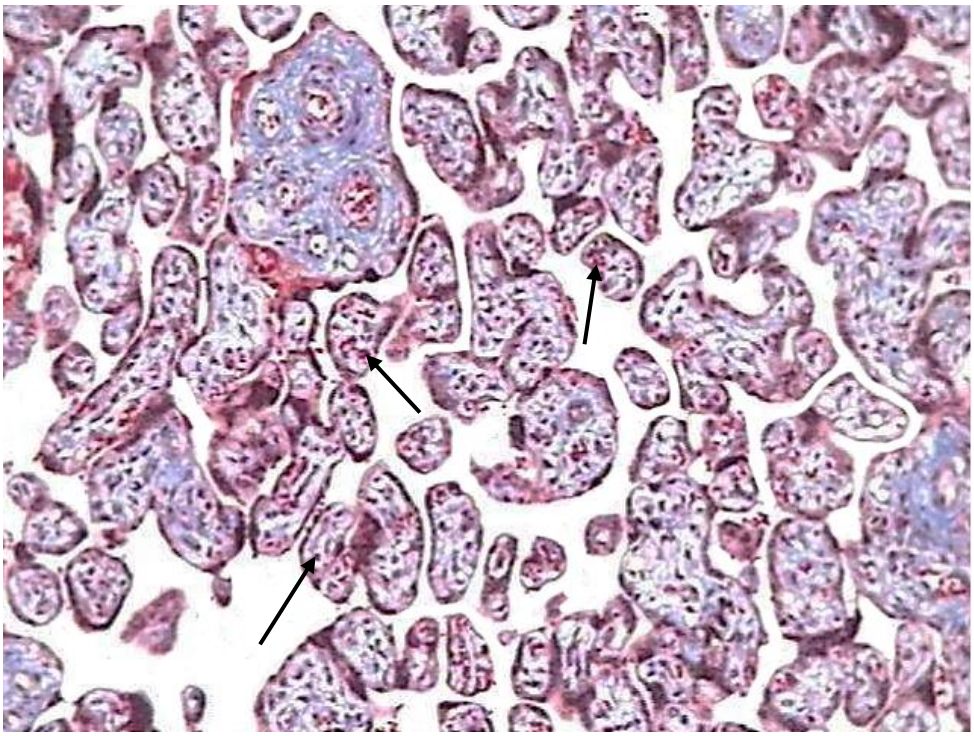


Figure (43). Computerised photomicrograph of full-term human placenta of the control group including sections of many terminal villi which contain connective tissue and microvasculature filled with foetal blood (arrows) (M.T. X200).

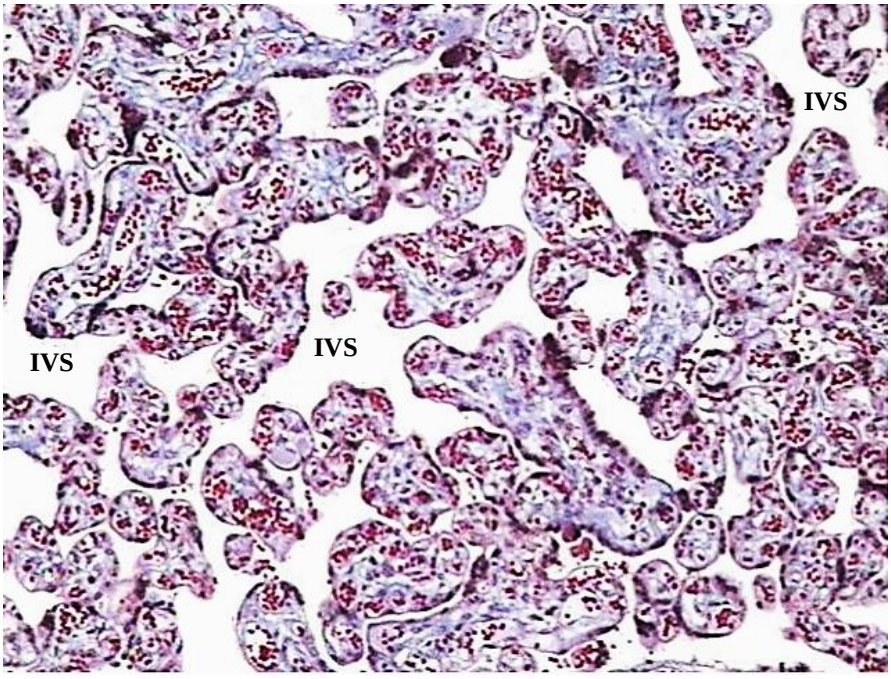


Figure (44). Computerised photomicrograph of full-term human placenta of the control group showing sections of chorionic villi floated in the intervillous space (IVS) (M.T. X200).

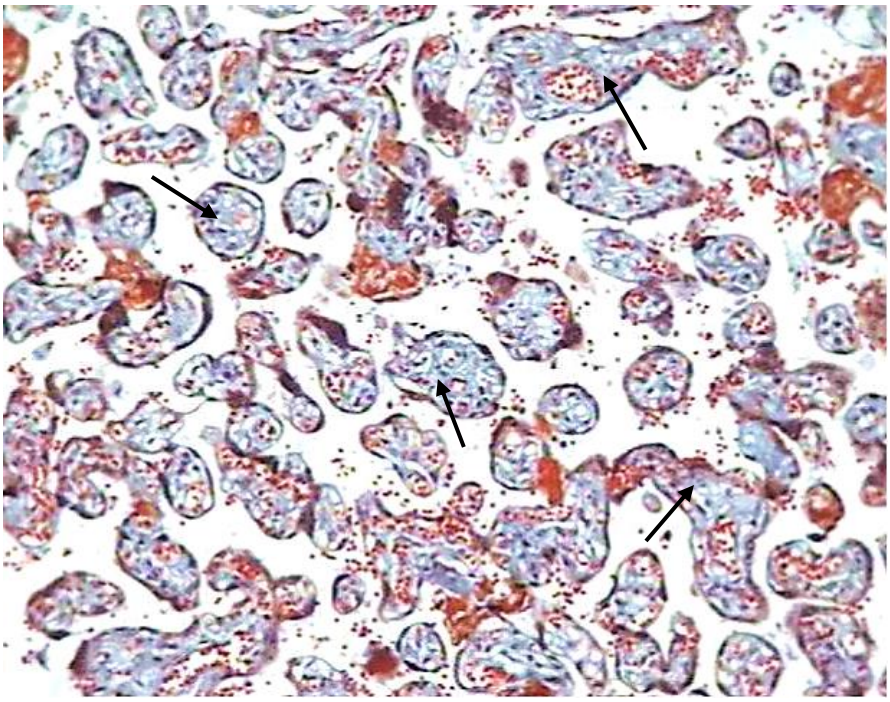


Figure (45). Computerised photomicrograph of full-term human placenta of control group showing the normal distribution of collagen fibres inside chorionic villi(arrows) (M.T. X 200).

- **AMA group**

On microscopic examination of the placenta in mothers ≥35 years, many morphological changes were observed. The chorionic villi were suspended in a wide intervillous area that had been filled with maternal blood (Figure 46). Furthermore, the terminal villi showed poor vascularization (Figure 47). In addition, chorionic villi showed a positive Mallory trichrome stain indicating the deposition of collagen fibres in the villus stroma as shown in Figure (48)**.**

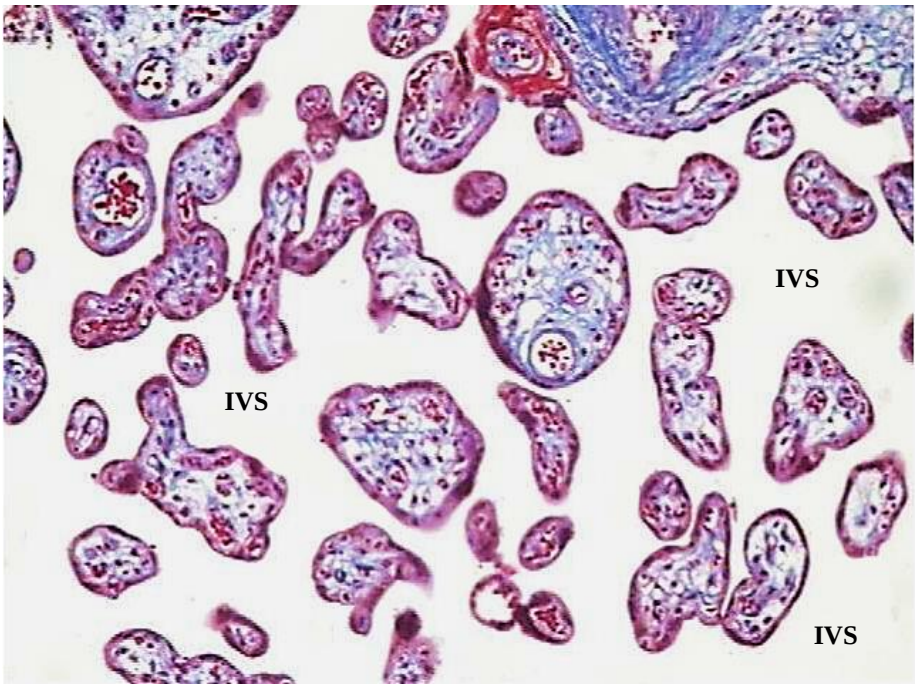


Figure (46). Computerised photomicrograph of full-term human placenta of advanced maternal age group showing sections of terminal villi floated in a wide intervillous space (IVS) (M.T. X200).

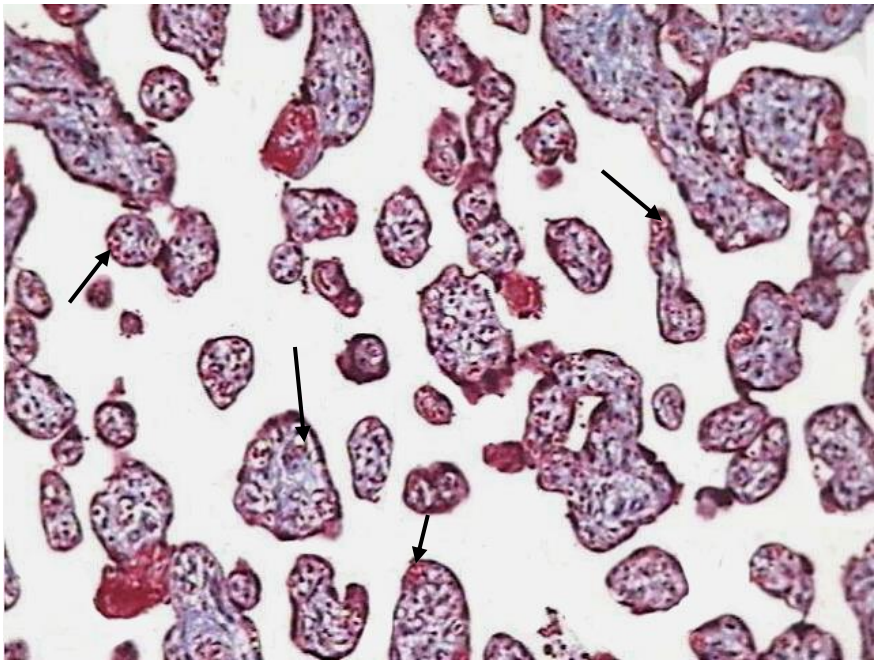


Figure (47). Computerised photomicrograph of full-term human placenta of advanced age group including sections of terminal villi floated in a wide intervillous space and each villus contains connective tissue and few foetal blood capillaries (arrows) (M.T. X200).

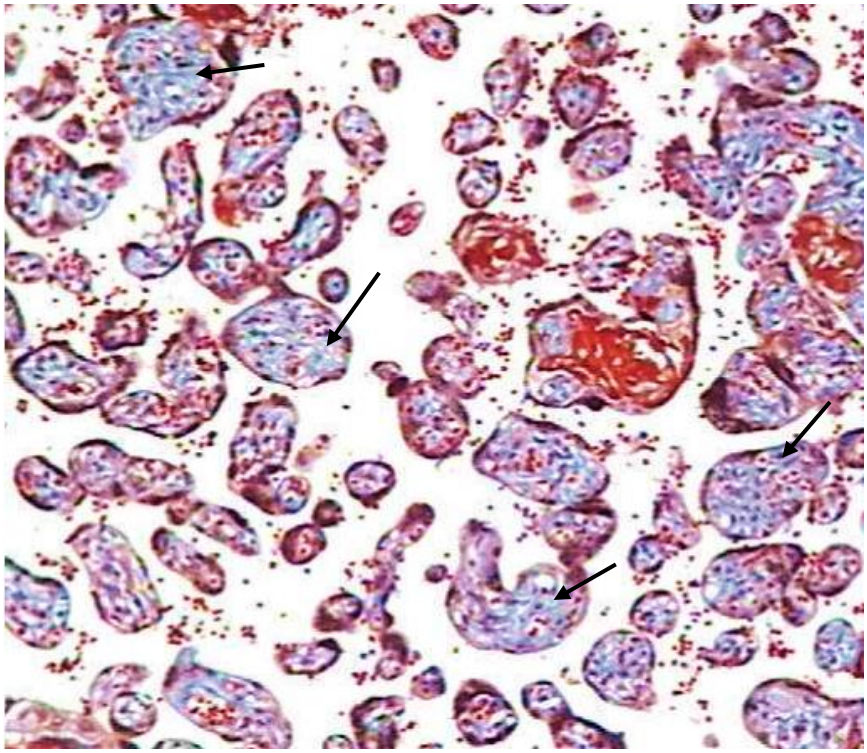


Figure (48). Computerised photomicrograph of full-term human placenta of mothers ≥35 years showing collagen fibres deposition within the villus connective tissue (arrows) (M.T. X 200).

Chapter 5:

5 Discussion

In recent times, the prevalence of advanced maternal age is increasing with changing trends in lifestyle, although the relationship between AMA and pregnancy outcome is still a matter of controversy. This delay in childbearing has been associated with an increased risk of both adverse maternal and foetal outcomes. However, the mechanism for the excess risk in AMA pregnancies is still unknown (Berger *et al*., 2021).

The findings of this study showed that women who had delayed childbearing beyond 35 years old for a variety of reasons were frequently associated with increased demand for ART services. These findings are consistent with the work of (Pawde *et al*., 2015) which revealed that the advanced maternal age group had higher assisted conception rates: 12.6%, compared to 3.5% for women less than 35 years of age (P< 0.001). Similar findings from Japanese and Chinese studies found that increasing maternal age was associated with increased rates of assisted reproductive technology (ART) pregnancies (Ogawa *et al*., 2017; Shan *et al*., 2018). This similarity might be mainly attributed to the fact that the women's fecundity and fertility progressively decline with advanced age due to the depletion of the ovarian follicular reserve and poorer oocyte quality(Wang *et al.*, 2020).

In addition, the increased likelihood of caesarean section among elderly mothers was observed in the present study. Similarly, a comparative cross-sectional study was conducted on 752 pregnant women in northern Ethiopia indicated that advanced-age mothers were 2.7 times more likely to undergo caesarean section than their adult counters (AOR 2.722, (95% CI 1.777– 4.170), p < 0.001) (Mehari *et al*., 2020). Other studies also report that advanced maternal age predisposes to caesarean delivery (Fayed *et al.*, 2017; Kahveci *et al*., 2018). This rising in CS rates among women at advanced maternal age could be contributed to increased rates of obstetric complications, maternal systemic disease, and mode of conception. In this study, age and mode of conception were the main factors attributed to the high CS rates.

Birth weight is an indicator of newborn health and a strong predictor of infant mortality and morbidity (Kiserud *et al*., 2018). In the current study, all new-borns had normal birth weight and there was a decrease in the mean birth weight of new-borns to mothers aged ≥35 years in comparison to that of the control group although the difference between the birth

weight of the two groups was not statistically significant (P>0.05). These findings are consistent with the work of Meteeb and Al-Dhalimy (2020) who found that no significant difference was shown in the mean birth weight between babies born to mothers aged 35 years and above and women aged less than 35 years (P>0.05). This has also been supported by studies conducted in Malaysia and Finland which showed no significant association between advanced maternal age and low birth weight (Rashed *et al*., 2016; Goisis *et al*., 2017). Nevertheless, this finding is contradicted by the studies done in northern Ethiopia which showed that advanced-age mothers had a 3.14 times higher adjusted odds ratio to have their babies born with a low birth weight than adult mothers P= 0.009 (Mehari *et al*., 2020). Similar reports also showed that the AMA was a major risk factor for low birth weight (Pinheiro *et al*., 2019; Wang *et al*., 2020). This observation could be ascribed to the abundance of adverse pregnancy complications and iatrogenic prematurity in advanced age which is controlled in the current study.

All babies of mothers in this study were born with normal APGAR scores at the 5th minute and there were no significant differences in APGAR scores between the two age groups (P= 0.616). The results are in agreement with Spanish and Turkish studies which revealed no association between advanced maternal age and low APGAR score (Kahveci *et al*., 2018; Guarga Montori *et al.*, 2021). However, the finding of this study is inconsistent with studies done in India and northwest Ethiopia which stated that the 5th minute APGAR score of new-borns of elderly women was significantly lower than those women of the non-elderly group (Nagarwal *et al*., 2015; Getaneh *et al*., 2021). This could be due to inadequate control for variables such as maternal diseases, multiple pregnancies, prematurity, and parity. All of these variables were controlled in this study.

In addition, it was observed in this study that the risk of NICU admission was almost similar in both age groups (OR =1.17, 95% CI 0.24–5.70). This is in line with a prospective case-control study presented 110 AMA singleton pregnancies that were delivered at completed 37 to 40 weeks and matched in a 1:1 ratio to the control group. The study showed that 9 babies were born to mothers of AMA and 10 newborns to mothers <35 years old needed NICU admission, P=0.99 (Miremerg *et al*., 2020). Another study conducted in India demonstrated that the rate of NICU admissions was similar in both age groups (8.7% vs 8.68%) (Pawde *et al*., 2015). However, the finding of this study is inconsistent with a study

done in Turkey which revealed that babies born to older mothers had a greater risk of being admitted to a neonatal intensive care unit (OR 1.69, 95% CI 1.02–2.76; OR 1.54, 95% CI 1.13–2.12 in the 35–39 and > 40 years old groups respectively (Kahveci *et al*., 2018). In another observational study that was carried out on 24,674 pregnancies in France, the rate of newborn transfer to a neonatal intensive care unit was found to be higher in the advanced maternal age group (Vandekerckhove *et al*., 2021). This variation could be attributed to the differences in sample size and the control of confounding factors represented in chronic maternal disease and obstetric complications.

The placenta is essential for the continuation of pregnancy as well as the growth and development of the foetus. Because the placenta involvement in pregnancy complications caused by maternal age is not well defined, and in an attempt to deepen the understanding of this effect, quantitative histological studies of the normal placenta will be of clinical importance as well as gaining advanced information about the placental function (Abdalla *et al*., 2016).

Placental weight is the most common way to describe its growth and it was reported to have a strong association with birth weight, abnormal placental weight was linked with increased perinatal complications like intrauterine foetal demise, congenital abnormalities and growth restriction (Adesina *et al*., 2016).

In the present study, it was observed that the mean placental weight was greater in AMA mothers although the difference was not significant (P=0.075). This result is in line with a study done by Miremerg *et al*.(2020) which showed no differences in the mean placental weight of the AMA group compared to the control group (P=0.860). In contrast, the study conducted by Meteeb and Al-Dhalimy (2020) showed a significant increase in the mean placental weight as maternal age increased (P<0.01). This may be accounted for by the time needed for placental delivery after normal vaginal delivery since the delay in placental delivery could result in a lower weight produced by the leakage of blood from the placenta. On the other hand, the placenta is removed immediately in caesarean delivery, this could result in an overestimation of placental weight among the oldest gravida in the group. Furthermore, it might be argued that the enlargement of the placenta in elderly mothers is a biological compensatory mechanism for placental dysfunction.

The placenta weight/birth weight ratio has been used as an indicator of neonatal outcomes. Abnormal placental weight/birthweight ratio was associated with an increased risk of adverse neonatal outcomes (Radan *et al*., 2022). In the current study, the placental weight/birthweight ratio is based on maternal age, with mothers of advanced maternal age having a significantly higher ratio (P=0.021) compared to mothers<35 years. This is in agreement with a large population cohort study done in Norway in which mothers of advanced maternal age had a higher placental weight relative to birth weight as compared with younger mothers (Haavaldsen *et al.,* 2011). On the contrary, another cohort study of 739 singleton births with very low birth weight (VLBW) in Christiana revealed mothers of advanced maternal age had a lower placental weight/birthweight ratio (P<0.01) compared to mothers<35 years (De Jongh *et al*., 2015). The reasons for the conflicting findings are likely related to the specific maternal populations studied. In the current study and Norway, the population was homogenous but in Christiana, the population comprised a diverse race/ethnicity background. In addition, the increase in the placental weight of elderly mothers could be contributing to the functional capacity of the placenta and its compensatory mechanisms needed for optimal foetal growth and development.

Histomorphometry study of the parenchyma of the human placenta (chorionic villi and intervillous space) allows structural data to be quantified for clinical implication as well as gain advanced knowledge of placental physiology (Abdalla *et al.*, 2016). This study noted that the lumen area of foetal blood capillaries in the terminal villi of placentae is significantly lower in older pregnant women (≥35 years) in comparison to the reference group (20–34 years) (P=0,009). This is in agreement with the quantitative study performed on 60 human placentae of a term pregnancy to determine the impact of maternal age on the structure of terminal villi. The study found that the mean value of surface density and total area of terminal villi microvasculature in women 35 years of age and older was significantly lower in comparison to the placentae in younger pregnant women (P<0,005) (Zigic *et al.*, 2010). Another study conducted in Iraq showed a decrease in vascularization of the terminal villi of pregnant women aged 35 years or older compared to that of the placenta of the control group (Meteeb and Al-Dhalimy, 2020).

Since foetal blood capillary is one of the structural components of placental terminal villi that have a critical role in enhancing the fetomaternal transfer of substances. These

studies also showed that there is a decreased metabolic transfer between the mother and the foetus and to meet the functional demands of the foetus for its normal growth and development, the placenta of older pregnant women induces compensational mechanisms.

In addition to these findings, the chorionic villi of older pregnant women were floated in a wider maternal space in comparison to that in the younger age group (P=0.003). Consistent with the current results, Ramic *et al*.(2006) stated that placentae in the volume unit of older pregnant women had a statistically significant higher proportion of intervillous space in comparison to the placenta of the younger pregnant mother (P<0,05). This observation could be attributed to a reduction in the number of villi or their size, or a disruption in the villous tree's space organization in one cm^3 of placental volume. On the other hand, the increase in intervillous space which constitutes the maternal blood volume favours the process of exchange of substances between mother and foetus as the large volume of maternal blood delivers large amounts of nutrients and oxygen per unit time for exchange with foetal blood, especially when coupled with a large exchange surface area for diffusion.

Collagen is one of the most predominant components of the extracellular matrix (ECM), controlling cellular biological activity and providing the scaffolding to maintain tissue integrity and cell adhesion. The chorionic villi stroma of normal full-term human placenta has a highly fibrous extracellular matrix. The amount of fibrous tissue is increasing as gestation advances. Moreover, the regulation of the expression of collagen is quite complex and any abnormal expressions of collagen and its fragments are associated with common obstetric disorders including recurrent miscarriage, gestational diabetes mellitus, and preeclampsia (Shi *et al*., 2020).

To the best of our knowledge, there are no previous studies published that investigate collagen fibres in the full-term human placenta of AMA. However, the most well-known mechanism of fibrosis and fibroblast activation is hypoxia (Rimon *et al*., 2008; Liu *et al*., 2017). Furthermore, Chen *et al.* (2005) revealed that the production of extracellular matrix by placental fibroblasts can be stimulated independently by hypoxia. Additionally, fibrosis of the villous stroma is one of the most prominent changes in the placentae of preeclamptic mothers. It has been suggested that ischemia and hypoxia are involved in the mechanism of

fibrosis in the villous stroma through a high expression of fibrosis-related genes in fibroblasts of the preeclamptic placenta (Ohmaru-Nakanishi *et al*., 2018). Since the placenta of older mothers has expressed a compensatory mechanism, whose main role is enhancing maternal-fetal exchange for optimal fetal growth and development (Ramic *et al.*, 2006), the current study revealed no significant difference in the amount of collagen expressed in the chorionic villi of placentae in both elderly and younger mothers (P=0.228).

The syncytiotrophoblast is a multinuclear epithelial layer that lines the intervillous space and plays important role in the foetal-maternal transfer of substances throughout the pregnancy. When the foetal blood capillaries are in close contacting the syncytiotrophoblast, a vasculosyncytial membrane (VSM) is created. The most crucial characteristics of the vasculosyncytial membrane are the maintenance of exchange surface area and sufficient diffusion. Undoubtedly there is a clear-cut inverse relation between villous VSM and foetal hypoxia. The hypoxic injury disrupts the syncytial architecture, which results in thicker VSM and impaired foetal-maternal exchange, both of which increase the risk of pregnancy problems (Azim *et al*., 2020).

In the present study, there was no significant difference in the thickness of the syncytiotrophoblast of terminal villi between advanced-aged mothers and adult mothers (P=0.616). Markovic *et al*. (2010) found that the volume density and the absolute volume of syncytiotrophoblast in terminal villi of older pregnant women were significantly higher than in pregnant women of age 20 to 34 (P< 0,005). Furthermore, Jawad (2014) indicated that increased trophoblastic layer thickness in pregnant mothers aged 35 years or more occurred as a compensatory mechanism to meet the functional demands of the fetus for its normal growth and development. This variation could be attributed to the differences in sample size. In addition, in this study, only syncytiotrophoblast thickness was measured while in other studies cytotrophoblast and trophoblast basement membrane were included.

Chapter 6:

6 Conclusion

The histomorphometry results of the current study revealed poor vascularisation of chorionic villi in the placenta of elderly mothers in comparison with younger mothers. To ensure optimal foetal growth and development, the placenta of older mothers induces compensational mechanisms. One of them is the increase in the intervillous space which is pooled with maternal blood. Therefore, providing a sufficient maternal-foetal exchange. In addition, the amount of collagen deposition in the chorionic villi of the elderly mother's placentae was not significantly different from that of the younger mother's placentae. This may explain the ability of the placenta to compensate for the hypoxia that induces chorionic villi fibrosis. On the other hand, the syncytiotrophoblast thickness was also found to be not affected by advanced maternal age.

AMA was associated with histological changes in the full-term human placenta and these changes explain the capability of older pregnant women's placenta to induce compensational mechanisms, whose main role is maintaining normal foetal growth and development.

Chapter 7:

7 Recommendations

- As the trend in AMA continues, care providers need to be aware of increased obstetrical and perinatal complications associated with delayed childbearing and obstetricians should adjust obstetrical management protocol to account for these age-related risk factors to ensure optimal maternal and perinatal outcomes in women of advanced maternal age.
- All adults of reproductive age should be aware of the obstetrical and perinatal risks of advanced maternal age so they can make decisions about the timing of childbearing.
- Comprehensive clinical studies with strong designs are required to establish the effect of maternal age on obstetric and perinatal outcomes in Libyan mothers supported by histological and immunohistochemical studies on their placentae.
- Promote more studies on the expression of the ageing-suppressor gene by the human placental trophoblast of elderly parturient.
- Future electro-microscopical studies on the placenta of mothers ≥35 years.

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Appendices

Appendices

Appendix I: Form of questionnaire and interview

Effect of advanced maternal age on human placental structure

(Quantitative microscopical and histochemical study).

Personal history

Name:	Age**:**	Gravida**:**	Para**:**	Blood Group:
Address**:**	Occupation:	LNMP:	G.A:	Nationality:

Obstetric history:

Is it a spontaneous pregnancy?	♦ Yes	♦ No
Did you get pregnant by IVF?	♦ Yes	♦ No
Did you do a booking visit in the first trimester?	♦ Yes	♦ No
Are you on a regular follow-up?	♦ Yes	♦ No
Did you have any hospital admissions during this pregnancy?	♦ Yes	♦ No
Do you have any chronic diseases?	♦ Yes	♦ No
Does your Hb level > 10.5mg?	♦ Yes	♦ No
Mode of delivery	♦ NVD	♦ C/S

Neonatal data:

Birth weight (Kg)		
Gender	♦M	♦ F
APGAR Score		
NICU admission	♦ Yes	♦ No

Placenta data:

Placental weight(g)	
Area of intervillous space(μm²)	
Area of fetal blood capillary(μm²)	
Area of placental collagen(μm²)	
Syncytiotrophoplast thickness(μm)	

Appendix II:

INFORMED CONSENT FORM:

I ……. Age…… give my full, free, and voluntary consent for participating in the study entitled "**Effect of advanced maternal age on human placental structure (Quantitative microscopical and histochemical study)**". The nature and significance of the study have been explained to me in the language I understand

انا..... العمر..... .اعطي موافقتي الكاملة ,الحرة والطوعية للمشاركة في الدراسة بعنوان "تأثير تقدم عمر الأم على بنية المشيمة البشرية" دراسة مجهرية كمية وهستوكيميائية" وقد تم شرح طبيعة وأهمية الدراسة بالنسبة لي باللغة التي أفهمها.

Sign of the patient:

Sign of witness:

Sign of investigator:

Place: Date

تأثير تقدم عمر الأم على بنية المشيمة البشرية

دراسة مجهرية كمية وهستوكيميائية

يسرى رمضان عبد العاطي الرفيفي

جامعة طرابلس كلية الطب البشري2022

المشرفان: د. باسم سعد قطب د. لبنى فتحي المقهور

المستخلص

الخلفية: يخلق الاتجاه المتزايد لتأخير الحمل تحديات جديدة لرعاية الام اثناء الولاده ، حيث أن الأمهات الأكبر سنا لديهن خطر متزايد لمضاعفات الحمل والولاده. الدراسات النسيجية الكمية للمشيمة ستكون ذات أهمية سريرية وكذلك تعززالمعرفة حول وظيفة المشيمة و الكشف عن أسباب هذه المضاعفات **تصميم الدراسة:** تم توظيف دراسة التحكم في الفتره الزمنيه ما بين يونيو 2018 وابريل 2019م في قسم علم الأنسجة و الوراثة ، كلية الطب البشري،جامعة طرابلس و قسم أمراض النساء والتوليد، مستشفى علي عمر عسكر، ليبيا **هدف الدراسة**: أجريت هذه الدراسة لتقييم التركيب المجهري للمشيمة البشرية للامهات المتقدمات في العمر وانعكاسها على صحة المولود **المواد والأساليب**: تم الحصول على مجموعه مكونه من 40 مشيمة بشرية كاملة النمو لامهات اصحاء و بكريه من قسم أمراض النساء والتوليد في مستشفى علي عمر عسكر ، ليبيا. تم تقسيم العينات إلى مجموعتين. المجموعة الضابطة (30 مشيمة من النساء الحوامل اللواتي تتراوح أعمارهن بين 20 و 35 عاما) ومجموعة الدراسة (10 مشيمة من النساء الحوامل في سن 35 عاما فما فوق)). تم أخذ عينه كاملة السماكة بحجم 1 سم ×1 سم من الجزء المركزي من كل مشيمة وبعد التثبيت المناسب في الفورمالين المحايد بنسبة 10٪. مررت الأنسجة في تركيزات متدرجة تصاعدية من الكحول 50٪ ، 70٪ ، 80٪ ، 96٪ ، و 100٪ ، تم طمرت في البارافين. وبمساعدة الميكروتوم تم الحصول على اشرطه شمعيه تسلسلية من خمسة ميكرون.وبعد ذلك صبغت بالهيماتوكسيلين والإيوسين وصبغة مالوري ثلاثي كروم و تم فحصها بحثا عن التغيرات المورفولوجية وقد تم ايضا إجراء تحليل كمي لمكونات المشيمة (الشعيرات الدموية الجنينية ، كولاجين, الفضاء البيني ، والأرومة الخلوية) باستخدام نظام لايكا للتحليل الكمي للصور. **النتائج**: كان وسيط عمر الأمهات 25 سنة مع معدلIQR (23-35.5). وكان هناك طلب متزايد على طرق المساعده على الانجاب مع زيادة العمرو كانت النساء المتقدمات في العمرأكثر عرضة للولادة القيصرية. بالإضافة إلى ذلك ، لم يكن هناك فرق ذو دلاله احصائيه في اوزان المواليد، ودرجة APGAR في الدقيقةالخامسه بعد الولاده ، ومعدل دخول المواليد لوحدة العناية المركزة لحديثي الولادة ، ووزن المشيمة بين مجموعتي الدراسة ($P>0.05$). ومع ذلك، كان معدل وزن المشيمة إلى وزن المولود لدى الأمهات المسنات أعلى بكثير فيما يتعلق بالأمهات الأصغر سنا ($P = 0.021$). وقدأظهرت الدراسة النسيجية المورفومترية انخفاضا كبيرا للغاية ($p=0.009$) في تجويف الشعيرات الدموية الجنينية لدى الأمهات في عمر متقدم مقارنة بالمجموعة الضابطة. وزياده في الفضاء البيني للزغابات المشيمية لدى النساء الحوامل الأكبر سنا مقارنة بالنساء الأصغر سنا زيادة كبيرة ($p=0.003$) في حين أن سمك الأرومة الخلوية والكولاجين المعبر عنهما في الزغابات المشيمية لمجموعتى النساء الحوامل اللتين تم فحصهما لم يختلفا اختلافا كبيرا ($p>0.05$). **الاستنتاج**: ارتبط تقدم عمر الام بتغيرات نسيجية في المشيمة البشرية الكاملة النمو وتفسر هذه التغييرات قدرة مشيمة النساء الحوامل الأكبر سنا على تحفيز الآليات التعويضية ، التي يتمثل دورها الرئيسي في الحفاظ على نمو الجنين وتطوره الطبيعي.

الكلمات الاستدلاليه: عمر الأم المتقدم، التركيب النسيجي للمشيمة، دراسة الأنسجة المورفومترية، نظام تحليل الصور.

وَامْرَأَتُهُ قائِمَةٌ فَضَحِكَتْ فَبَشَّرْناها بِإِسْحاقَ وَمِنْ وَراءِ إِسْحاقَ يَعْقُوبَ (71) قالَتْ يا وَيْلَتى أَأَلِدُ وَأَنَا عَجُوزٌ وَهذا بَعْلِي شَيْخًا إِنَّ هذا لَشَيْءٌ عَجِيبٌ (72) قالُوا أَتَعْجَبِينَ مِنْ أَمْرِ اللَّهِ رَحْمَتُ اللَّهِ وَبَرَكاتُهُ عَلَيْكُمْ أَهْلَ الْبَيْتِ إِنَّهُ حَمِيدٌ مَجِيد (73).

[سورة هود : الآية 71]

جامعة طرابلس

كلية الطب البشري

قسم الأنسجة والوراثة الطبية

تأثير تقدم عمر الأم على بنية المشيمة البشرية

دراسة مجهرية كمية وهستوكيميائية

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أستاذ مشارك في قسم أمراض النساء والولادة

قدمت هذه الرسالة إستكمالا لمتطلبات الإجازه العاليه "الماجستير" في علم الانسجة و الوراثه

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