THE ROLE OF INSULIN-LIKE GROWTH FACTOR AXIS COMPONENTS IN SMALL FOR GESTATIONAL AGE BIRTH

Thesis submitted for the award of

DOCTOR OF PHILOSOPHY

degree based on the research carried out in the department of

CELL BIOLOGY AND MOLECULAR GENETICS

Under the Faculty of Allied Health and Basic Sciences

by

Ms. Nithya M.N., M.Sc.

(Reg. No: 18PY7010)

Under the supervision of

Dr. Venkateswarlu Raavi., M.Sc., M.Phil., Ph.D.

Assistant Professor

Sri Devaraj Urs Academy of Higher Education and Research

(A Deemed to be University, declared under section 3 of UGC Act, 1956)

Tamaka, Kolar, Karnataka - 563 103, INDIA

April 2023

Declaration by the Ph.D. Scholar

I, **Ms. Nithya M.N.,** hereby declare that the work presented in the thesis entitled **"The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth"** is original and does not contain any fabrication. This research work was carried out by me under the supervision of **Dr. Venkateswarlu Raavi.,** Assistant Professor, Department of Cell Biology and Molecular Genetics under the faculty of Allied Health and Basic Sciences, Sri Devaraj Urs Academy of Higher Education and Research. Furthermore, I have not plagiarised any part of the thesis. Where necessary, I have borrowed the material from other authors/researchers and given appropriate credit to them through reference citation or acknowledgement. I also declare that the thesis has not been submitted for the award of any other degree/diploma to any other Higher Educational Institution.

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Date:

Place:

Signature of the Supervisor

Dr. Venkateswarlu Raavi., M.Sc., M.Phil., Ph.D.
Assistant Professor
Dept. of Cell Biology and Molecular Genetics,
SDUAHER, Tamaka, Kolar, Karnataka - 563 103

Co-supervisors Certificate

This is to certify that the thesis entitled **"The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth"** is an original work carried out by **Ms. Nithya M.N.,** under our co-supervision in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka - 563 103. A plagiarism check was carried out on the thesis, and the report was found to be acceptable.

Co-supervisor

Dr. Krishnappa J.,

Professor
Dept. of Paediatrics, SDUMC,
SDUAHER, Tamaka,
Kolar, Karnataka - 563 103

Date:

Place:

Co-supervisor

Dr. Sheela S. R.,

Professor
Dept. of Obstetrics and
Gynaecology, SDUMC,
SDUAHER, Tamaka,
Kolar, Karnataka - 563 103

Date:

Place:

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Head of the Department

Cell Biology and Molecular Genetics

Date:

Place:

Dean

Faculty of Allied Health and Basic Sciences

Date:

Place:

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

Tamaka, Kolar 563103

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Name of the Student	NITHYA M.N.
Registration Number	18PY7010
Name of the Supervisor / Guide	DR. VENKATESWARLU RAAVI
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Nithya MN 10/04/23

Signature of Student

R. venkateswarlu 10/04/23

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i/c Head of the Department

Head of Dept.
Cell Biology & Molecular Genetics
SDUAHER - Kolar

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LIST OF ABBREVIATIONS

AGA	Appropriate for Gestational Age
bp	Base Pairs
Ct	Cycle threshold
°C	Degree Celsius
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C
CO_2	Carbon dioxide
cDNA	Complementary DNA
CPT1A	Carnitine Palmitoyl Transferase 1A
DNA	Deoxyribonucleic Acid
dl	Decilitre
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic Acid
ELB	Erythrocyte Lysis Buffer
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FP	Forward Primer
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
g	Gram
GH	Growth Hormone
GPR120	G-protein Coupled Receptor 120
h	Hour
HCL	Hydrochloric acid
H19	Imprinted Maternally Expressed Transcript
HRP	Horseradish peroxidase
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding protein
IGFR	Insulin-like Growth Factor Receptor
INSR	Insulin Receptor
IPA	Isopropyl Alcohol

K_2HPO_4	Potassium Dihydrogen Phosphate
KCl	Potassium Chloride
$KHCO_3$	Potassium Bicarbonate
kg	Kilogram
LAF	Laminar Air Flow
L	Liter
LGA	Large for Gestational Age
LINE1	Long Interspersed Nuclear Elements
LSCS	Elective Lower Segment Caesarean Section
M	Molarity
$MgCL_2$	Magnesium Chloride
mg	Milligram
ml	Millilitre
µl	Microlitre
mM	Millimolar
min	Minutes
MI	Mitotic Index
mRNA	Messenger Ribonucleic acid
MS-PCR	Methylation Specific-PCR
Na_2HPO_4	Disodium Hydrogen Phosphate
n	Number of samples
ng	Nanogram
nm	Nanometre
N	Normality
NaCl	Sodium Chloride
NaH_2PO_4	Sodium Dihydrogen Phosphate
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NH_4Cl	Ammonium Chloride
NVD	Normal Vaginal Delivery
OD	Optical Density
PPARG	Peroxisome Proliferator-Activated Receptor Gamma

PBS	Phosphate Buffer Saline
%	Percentage
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
qPCR	Quantitative PCR
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RP	Reverse Primer
rpm	Resolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription PCR
SERINA3	Serpin Family A Member 3
SDS	Sodium Dodecyl Sulphate
SD	Standard Deviation
Sec	Seconds
SGA	Small for Gestational Age
SLC2A2	Solute Carrier Family 2, Facilitated Glucose Transporter Member 2
SPSS	Statistical Package for Social Sciences
TAE	Tris-Acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TMB	3,3′,5,5′-Tetramethylbenzidine
TSS	Transcription Start Site
WBC	White Blood Cells
WHO	World Health Organization

Hospital & Research Institute
Chettinad Academy
of Research and Education
(Deemed to be University)

CERTIFICATE OF PARTICIPATION

This is to certify that Dr/Mr/Mrs/Ms **NITHYA** from **Sri Devaraj Urs Medical College** has presented poster on the topic **Mitotic index difference between cord blood in SGA and AGA neonates** in the **National e conference "Genomics Conclave 2021"** conducted by Department OF Biochemistry, Chettinad Hospital and research Institute on 27th and 28th May 2021.

Dr. S. Sumathy
HOD, Biochemistry, CHRI

Dr.K. Pitchai Balashanmugam
Dean, CHRI

Prof. Dr. T. Balasubramaniam
Vice Chancellor, CARE

e - Certificate

Presentations

CERTIFICATE

OF PARTICIPATION

NATIONAL CONFERENCE ON MEDICAL AND HEALTH SCIENCES (NCMHS-22)

03rd December 2022 | Trivandrum, Kerala

This is to certify that **Nithya M.N**

of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India has done his/her excellence in presenting the research paper titled Expression levels of IGFR1 and IGFR2 mRNA in placental tissue of SGA and AGA neonates

on 03rd December 2022 at Trivandrum, Kerala.

Dr. Tara Srivastava
Director

Raj Kumar
Co-ordinator

Presentations

88th Annual Meeting of SBCI and conference on
"Advances at the interface of Biology and Chemistry" (SBCI-2019)
October 31- November 3, 2019

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This is to certify that Prof. /Dr. /Mr. /Ms. Nithya M.N

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Dr. A. Ballal
Convenor
SBCI-2019

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ABSTRACT

Gestation is a time gap between conception and birth of neonates. The age of the fetus or the number of weeks the pregnancy lasts is called as gestational age. Based on the number of gestational weeks and birth weight, the neonates can be classified as Small for Gestational Age (SGA), Appropriate for Gestational Age (AGA), and Large for Gestational Age (LGA). SGA neonates have a birth weight of less than the 10^{th} percentile, and it is a common problem in developing countries such as India, Pakistan, Nigeria, and Bangladesh. The prevalence of SGA varies from 5.3-41.5% (East-South Asia). The incidence of SGA is 30% in developing countries and 5-7% in developed countries. SGA is one of the leading causes of perinatal morbidity and mortality in neonates. About 85% of the neonates will catch-up with growth at the age of 6 months-2 years. If the SGA neonates do not attain catch-up growth, they remain short stature and have a risk of insulin resistance, high blood pressure, type 2 diabetes, cardiovascular diseases, and mortality in early life.

The incidence of SGA births depends on factors such as Intrauterine Growth Restriction (IUGR), preterm birth, environment, nutrition, growth factors, lifestyle, genetic composition, and pregnancy complications such as maternal age, low level of oxygen, low blood sugar, genetic makeup, etc. The Growth Hormone (GH)/Insulin-like Growth Factor (IGF)-axis plays a significant role in the growth and development of the fetus and neonates. Recent studies from the western world highlight that the growth of the fetus depends on the levels of insulin and IGF-axis components such as IGF1, IGF2, IGF binding proteins (IGFBP), IGF receptors (IGFR), gene expression, and epigenetic modification of IGF-axis genes. The altered levels of IGF-axis components may lead to abnormal fetus growth. The studies also highlighted the importance of the IGF-axis in

GH therapy/monitoring the growth of SGA neonates. The literature on SGA and GH/IGF-axis components revealed contrasting results (higher, lower, or no change), that might depend on the individuals' nutrition, hormone levels, environment, and genetic makeup. To the best of my knowledge, the role of IGF-axis components and the likely factors influencing it was not understood fully in the Indian population. Hence, the thesis aimed to investigate the role of IGF-axis components in SGA neonates born to south Indian women.

A total of 98 pregnant women undergoing labor in the department of Obstetrics and Gynecology, RL Jalapa Hospital, Kolar, Karnataka, were recruited in the present study (Ref No: SDUMC/KLR/IEC/32/2019-20). The pregnant women were grouped as AGA (n=49) and SGA (n=49) based on the gestational weeks and birth weight. After delivery, umbilical cord blood samples (5 ml) were collected and used to measure the levels of IGF1, IGF2, and IGFBP3 proteins using the ELISA method and cultured *in vitro* to calculate the mitotic index. In addition to cord blood, approximately 150-200 mg of placental tissue samples were collected using a sterile surgical blade. The placental tissues were used to measure the *IGFR1* and *IGFR2* gene expression using qPCR and methylation status of *IGF2*, *H19*, *IGF1*, and *IGFR1* genes using methylation-specific PCR.

The mean±SD (range) of IGF1, IGF2, and IGFBP3 proteins in AGA neonates is 118±33 (54-210), 124±10 (103-146), and 1606±277 (1032-2006), and in SGA neonates, is 118±47 (4-294), 123±12 (92-154), and 1432±387 (315-2137) (ng/ml) respectively. The levels of IGF1 (p=1) and IGF2 (p=0.69) protein did not differ between AGA and SGA neonates, whereas the IGFBP3 protein is significantly (p=0.023) less in SGA compared to AGA neonates. The plasma levels of IGF1, IGF2, and IGFBP3 proteins

did not correlate (r=0.01) with gestational weeks and birthweight of neonates. Further, the study was extended to find the expression of *IGFR1* and *IGFR2* genes in the placenta of AGA and SGA neonates. The mean±SD (range) of Δ cycle threshold (Ct) of the *IGFR1* gene obtained from AGA neonates is 5.06±4.28 (-4.82-10.63), and SGA neonates are 7.03±3.82 (1.44-13.20). The mean±SD (range) of ΔCt of the *IGFR2* gene obtained from AGA neonates is 2.45±3.56 (-7.67-6.04), and SGA neonates are 3.94±1.59 (1.66-7.96). The fold change expression of *IGFR1* and *IGFR2* genes in SGA neonates was calculated ($2^{-\Delta\Delta Ct}$ method) by subtracting the ΔCt of AGA from SGA neonates. The *IGFR1* gene is down-regulated 3.9-folds, whereas the *IGFR2* gene is down-regulated 2.8-folds in SGA compared to AGA neonates. Further, there is no correlation between *IGFR1* and *IGFR2* gene expression with gestational weeks and the birth weight of neonates.

The results indicate that the levels of IGFBP3 protein were significantly lower, and the expression of *IGFR1* and *IGFR2* genes were downregulated in SGA compared to AGA neonates. Further, the study was extended to find the methylation status of the IGF-axis of imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1*) gene promoters in the placenta of AGA and SGA neonates. The methylation of imprinted and non-imprinted genes is 2.5, 1.5, 5, and 7.5% lower in SGA compared to AGA neonates. The co-methylation of IGF-axis gene promoters [imprinted: 7.5% lower, non-imprinted: 15% lower, and both imprinted and non-imprinted: 20% lower] were lower in SGA compared to AGA neonates. The methylation of imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1*) genes significantly reduced ($p<0.05$) the levels of IGFBP3 protein, *IGFR1* and *IGFR2* gene expression, gestational weeks, and birth weight of SGA compared to AGA neonates. Further, the study was extended to find the difference in the intrinsic division capacity of the cord blood lymphocytes of AGA and

SGA neonates. The range (mean±SD) of the mitotic index obtained from AGA neonates is 12-22% (15.71±1.81), and SGA neonates are 10-16% (12.60±1.16). The mitotic index of SGA neonates is significantly ($p=0.0001$) less compared to AGA neonates. The mitotic index is significantly ($p<0.05$) less in methylated positive SGA compared to AGA neonates. The levels of IGF-axis proteins and gene expression, gestational weeks, and birth weight did not correlate with the mitotic index of cord blood lymphocytes.

Overall, the thesis generated evidence on the role of IGF-axis components (IGF1, IGF2, IGFBP3 proteins, *IGFR1* and *IGFR2* gene expression, methylation of *IGF2*, *H19*, *IGF1*, and *IGFR1* gene promoter, intrinsic division capacity of cord blood lymphocytes) in the SGA neonates born to Indian women. The information might be helpful for further studies to explore the potential markers to screen/monitor/diagnose/management of SGA neonates/babies born to Indian women.

Abstract

ABSTRACT

Gestation is a time gap between conception and birth of neonates. The age of the fetus or the number of weeks the pregnancy lasts is called as gestational age. Based on the number of gestational weeks and birth weight, the neonates can be classified as Small for Gestational Age (SGA), Appropriate for Gestational Age (AGA), and Large for Gestational Age (LGA). SGA neonates have a birth weight of less than the 10^{th} percentile, and it is a common problem in developing countries such as India, Pakistan, Nigeria, and Bangladesh. The prevalence of SGA varies from 5.3-41.5% (East-South Asia). The incidence of SGA is 30% in developing countries and 5-7% in developed countries. SGA is one of the leading causes of perinatal morbidity and mortality in neonates. About 85% of the neonates will catch-up with growth at the age of 6 months-2 years. If the SGA neonates do not attain catch-up growth, they remain short stature and have a risk of insulin resistance, high blood pressure, type 2 diabetes, cardiovascular diseases, and mortality in early life.

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Introduction

1. Introduction

1.1. Gestation

Gestation is a time gap between the origin and birth of the fetus developed in the uterus (Figure 1.1). The duration of gestation varies from species to species. The duration of a human pregnancy can be divided into three trimesters: the first trimester is from the last menstrual period through the 13th week, the second trimester is from the 14-28th/29th week, and the third trimester is from 29th/30th-42nd week. The time interval of gestation is known as gestational age (Sayers et al., 2017).

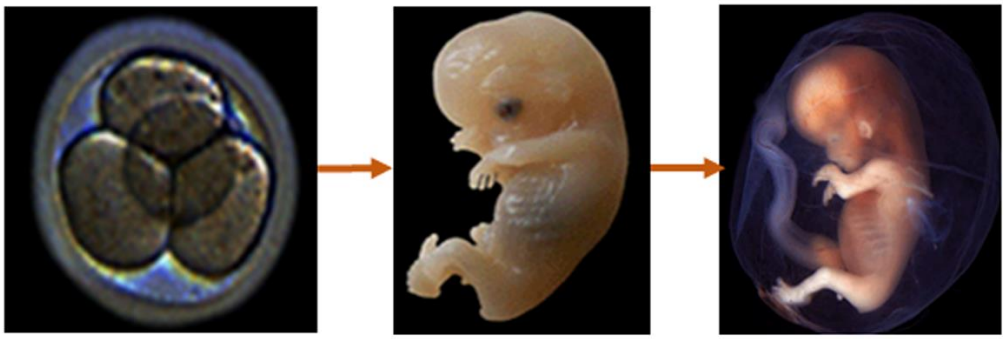

Figure 1.1: Gestation in human beings *(**Source:** Textbook of Clinical Embryology, 1st Edition, 2012)*

1.2. Gestational age

Gestational age is the number of weeks the pregnancy last or the age of the fetus. In general, the gestational age in human beings lasts for 38-42 weeks (term); if the gestational age is below 37 weeks, they are called pre-term, and after 42 weeks, they are called post-term. Further, the gestational age can be divided into two stages, i.e., embryonic and fetal. The embryonic stage ranges from the 5-10th week of pregnancy. The fetal stage ranges from the 10th week to the birth of the neonates (Sayers et al., 2017).

1.3. Classification of gestational age

Based on the number of gestational weeks and birth weight, the neonates were classified as Small for Gestational Age (SGA), Appropriate for Gestational Age (AGA), and Large for Gestational Age (LGA). SGA neonates have a birth weight of less than the 10th percentile, and AGA neonates have birth weight ranges from 10-90th percentile, whereas the LGA neonates have a birth weight of more than the 90th percentile (Figure 1.2) (Bakketeig et al., 1998). The AGA neonates will have a healthy life. In contrast, SGA and LGA neonates will have alterations in their metabolism and increased mortality and morbidity at the early stages of their life.

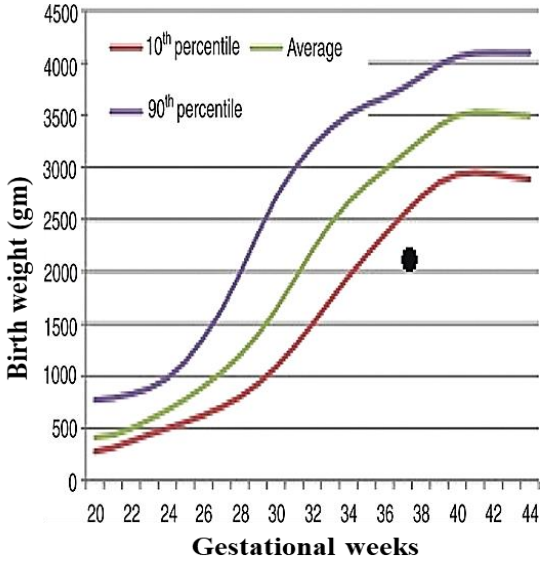

Figure 1.2: Schematic representation of the relation between gestational weeks and birth weight of the neonates *(**Source:** Bakketeig et al., 1998)*

1.3.1. Small for gestational age

SGA neonates have a birth weight less than the 10th percentile for their gestational age, and it is a major problem in developing countries such as India, Nepal, Bangladesh, etc. (Osuchukwu et al., 2021). SGA neonates can be classified into two groups: (i) normal neonates with SGA and (ii) growth restriction neonates with SGA. Neonates

with SGA have a normal birth weight less than the 10th percentile because of inherent factors such as maternal height, weight, ethnicity, parity etc., Growth-restricted neonates with SGA will have a birth weight greater than the 10th percentile and have a high risk of short- and long-term complications and death (Osuchukwu et al., 2021).

1.3.1.1. Etiology of SGA

The etiology of SGA is mainly categorized into two types (i) symmetric and (ii) asymmetric growth restriction. Symmetric growth restriction occurs during 1st trimester of gestation, which is mainly due to genetic conditions (achondroplasia, bloom syndrome, and chromosomal anomalies such as trisomy 18 and 21, etc.) and congenital infections (toxoplasmosis, rubella, cytomegalovirus, malaria, and HIV). Asymmetric growth restriction occurs during the late 2nd-3rd trimester of gestation, which is due to maternal factors (age of the mother, malnutrition, first pregnancy, low oxygen level, low APGAR score, and low blood sugar), and placental problems (single umbilical artery, placental hemangiomans, placenta previa, and placental abruption).

1.3.1.2. Epidemiology of SGA

SGA is one of the major problems in developing countries and has a high risk of perinatal mortality, morbidity, and metabolic syndromes such as obesity and type 2 diabetes mellitus in adult life (Cho et al., 2016). The prevalence of SGA varies from 5.3-41.5% in East-South Asia (Khadilka et al., 2016). The incidence of SGA in developing countries is 30%, and 5-7% in developed countries (Sebastian et al., 2015). About 85% of neonates undergo catch-up growth at 6 months-2 years. The majority of SGA neonates are born in developing countries such as India, Pakistan, Nigeria, and Bangladesh.

1.3.1.3. Complications associated with SGA

The SGA neonates who did not attain the catch-up growth may have several complications in their early and late childhood stages. In general, SGA neonates do not have a complication related to the organ system; however, they have the risk of prematurity, neonatal asphyxia, hypothermia, hypoglycemia, hypocalcemia, polycythemia, and sepsis. The low birth size of the neonates also increases the risk of neurodevelopmental problems and cardiometabolic diseases (Sharma et al., 2016).

1.4. Factors responsible for SGA

SGA results from combination of several factors, including external and internal factors. External factors such as environment, nutrition, etc., and internal factors such as intrauterine growth restriction, gestational hypertension, pregnancy complication, placental abruption, growth factors, epigenetics, etc., play an important role in the SGA outcomes (Figure 1.3).

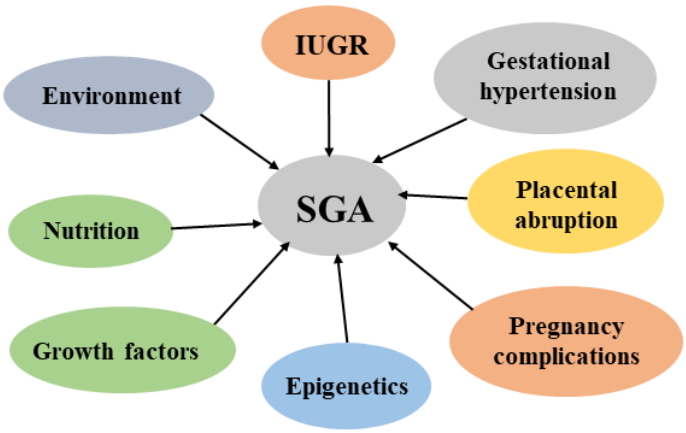

Figure 1.3: Schematic representation of the factors responsible for SGA outcomes *(**Source**: Salcedo-Bellido et al., 2017)*

1.4.1. External factors responsible for SGA

Environment: Environment is one of the external factors affecting the fetus growth. Women exposed to tobacco smoke, air pollution, pesticides, solvents, metals, water contaminants, etc., during pregnancy have a high risk of SGA birth (Nieuwenhuijsen et al., 2013; Kumar et al., 2019).

Nutrition: Nutrition is also an external factor affecting fetal growth. During pregnancy, inadequate intake of essential nutrients has a high/increased risk of SGA babies/newborns (Ricci et al., 2010; Salcedo-Bellido et al., 2017).

1.4.2. Internal factors responsible for SGA

Intrauterine growth restriction (IUGR): IUGR is an internal factor affecting fetal growth. Some maternal factors such as the mothers age, maternal health, inter-pregnancy interval, maternal infection, etc., affect the fetus growth and are responsible for SGA. The inadequate supply of nutrients to the placenta is also one-factor affecting fetal growth (Sharma et al., 2016).

Gestational hypertension: Epidemiological studies suggest that gestational hypertension and pre-eclampsia are the common hypertensive disorder of pregnancy that can increase the risk of SGA birth. Gestational hypertension is common in twin pregnancies, age above 35 years, lower education, factory workers, etc. In addition, some maternal conditions, such as kidney disease, diabetes, multiple fetuses, etc., also increase the risk of SGA (Laine et al., 2019; Longhitano et al., 2022).

Pregnancy complications: Complications such as anemia, anxiety, depression, diabetes, high blood pressure, etc., are the most common symptoms seen during pregnancy. These complications might increase the risk of physical and mental health problems in pregnant women and the growth of the fetus (Schetter et al., 2012).

Placental abruption: Placental abruption occurs when the placenta separates from the inner wall of the uterus before birth. It can decrease the baby oxygen level and nutrients and result in the fetus abnormal growth. Maternal high blood pressure and abdominal trauma are some of the risk factors which cause placental abruption. The major symptoms of placental abruption are vaginal bleeding, contractions, discomfort, and tenderness (Martinelli et al., 2018).

Growth factors: Growth factors are naturally occurring substances capable of stimulating cell proliferation, cell differentiation, and wound healing. The levels of growth factors play an important role in the growth and development of the fetus. Secretion of growth hormone (GH) and circulating insulin-like growth factor levels have decreased or increased in SGA infants (Saenger et al., 2012; Renes et al., 2019). The growth hormone therapy showed an improvement in the growth of neonates born as SGA (Hwang et al., 2014).

1.5. Growth hormone

Growth hormone is a regulatory factor that has a role in fetal growth and development. GH is secreted by the anterior pituitary gland under the acute stimulatory effects of hypothalamic peptide GH-releasing hormone (GHRH) and inhibited by somatostatin (Olarescu et al., 2019). Ghrelin is a naturally occurring GH-releasing peptide (GHRP) that is present in both the stomach and hypothalamus and is involved in the hormonal regulation of GH release. Ghrelin in the stomach plays a role in the nutritional regulation of the GH/IGF-axis. Once GH is released from the pituitary gland, it will increase the production of IGF1 in the tissues (Chatelain et al., 2007; Khatib et al., 2014). Short children born SGA usually do not have GH deficiency but have low GH secretion. The recombinant human GH (rhGH) administration to children with GH

deficiency effectively promotes growth and enhances height gain in non-GH deficiency disorders (Teran et al., 2016; Deodati et al., 2017).

1.6. Insulin-like growth factors axis

The GH/IGF-axis is involved in the growth and metabolism of the cells, and it plays an important role in the metabolism, cell proliferation, differentiation, and growth of the fetus/neonates. The IGF-axis consist of two types of ligands (Insulin-like growth factor (IGF) 1 and 2), two types of cell surface receptors (Insulin-like growth factor receptor (IGFR) 1 and 2), and six types of binding proteins (Insulin-like growth factor binding protein (IGFBP) 1-6 (Blum et al., 2018) (Figure 1.4).

1.6.1. Insulin-like growth factor: IGF is a single-chain polypeptide that shares homology with a similar structure of pro-insulin, which is expressed in the placenta and regulates fetal growth. There are two IGFs, IGF1 and IGF2; IGF1 is detectable in fetal tissue and plays a major role in early fetal development, whereas IGF2 plays a major role in the growth and development division of the cells (Netchine et al., 2011; Agrogiannis et al., 2014).

1.6.2. Insulin-like growth factor receptors: IGFR is a protein present on the surface of all cells. It is a transmembrane receptor that is activated by a hormone called IGF1. There are two types of IGFRs; IGFR1 and IGFR2, IGFR1 is a transmembrane glycoprotein with kinase activity, whereas IGFR2 is a single-chain protein without kinase activity. IGFRs play a major role in embryonic development (Agrogiannis et al., 2014).

1.6.3. Insulin-like growth factor binding protein: The bioavailability of IGFs is modulated by six binding proteins (IGFBP1-6). The IGFBP3 has more affinity for IGFs than other IGFBPs; hence, approximately 80-90% of IGFs are bound mainly to

IGFBP3. During the pregnancy, IGFBP2, 3, 4, and 5 are cleaved by proteases, which reduces the affinity for IGFs. IGFBP3 is the most common binding protein in the placenta (Blum et al., 2018).

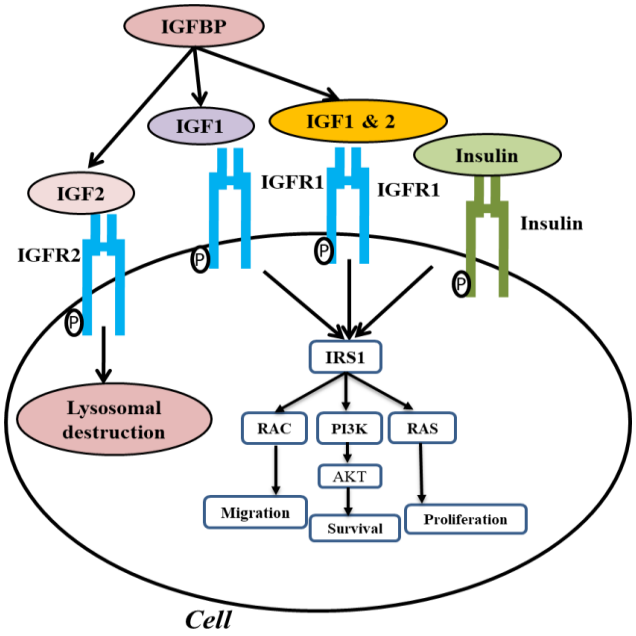

Figure 1.4: Schematic representation of the IGF-axis components and their response in cells *(**Source:** Blum et al., 2018)*

1.6.4. Physiological role of growth hormone/insulin-like growth factor axis

In children, the GH/IGF-axis is an endocrine system that is a crucial regulator of linear growth. The liver is the main target for GH action, where it reduces the release of glucose and IGF1. The production of IGFBP1 and 2 inhibits GH, whereas it is stimulated by the production of IGFBP3, 4, and 5. At the intravascular space, IGFBP3 is produced in the hepatic sinusoidal cells. IGF1 binds mainly to IGFBP3 to form a binary complex, and it binds to a protein called acid-labile subunit (ALS) to form a ternary complex in circulation (Blum et al., 2018). These ternary complexes inactivate IGF1 and IGFBP3 and prolong their half-life in circulation. The binding of IGF1 to IGFBP3 is not active; it can release through chemical equilibrium in tissues, and free IGF1 then binds to cell surface receptors to trigger the signaling cascade within the cell.

However, IGFBP3 acts as a hormone binding to specific receptors or importin-beta at the cell surface. It is translocated to the cell nucleus, which interacts with the receptors to form a complex regulating the transcription of genes responsible for proliferation, migration, survival, and apoptosis (Ranke et al., 2015).

1.7. Role of the placenta in the growth of a fetus

Placenta is an organ that develops in the uterus during pregnancy. The placenta plays a major role in the supply of nutrients to the baby in the uterus. It also removes the waste products generated by the baby (Garnica et al.,1996). The placental abruption, placenta previa, and placenta accrete are the major placental abnormalities that can affect the function of the placenta. In addition, factors such as maternal age, high blood pressure, twin pregnancy, previous uterine surgery, abdominal trauma, etc., are also responsible for placental abnormalities. Some common symptoms of placental abnormalities are vaginal bleeding, abdominal pain, back pain, and uterine contractions. Fetal nutrient supply also influences metabolic activity, hormone production, and growth factors (Díaz et al., 2014). In SGA, the volume of the placenta, surface area, and vascularization of the villi that mediate the maternal and fetal exchange is reported to be altered (Woods et al., 2018). Due to its important functions in fetal growth and development, several studies analyzed the levels of the IGF-axis components in the placenta and their association with birth weight and gestational weeks. The cross-sectional image of the placenta is shown in figure 1.5.

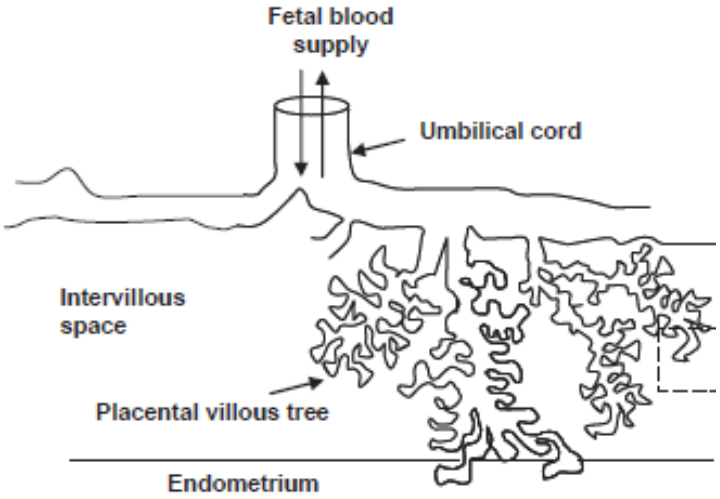

Figure 1.5: Cross section of human placenta *(**Source:** Forbes et al., 2008)*

1.8. Role of the umbilical cord in the growth of a fetus

The umbilical cord is a vital connection between the placenta and the fetus. Development of the umbilical cord starts at the embryonic stage around the 3rd week; by the 7th week, it is fully formed with connecting stalk, vitelline duct, and umbilical vessels surrounding the amniotic membrane. The umbilical cord plays a major role in circulating blood between the embryo and the placenta. The umbilical vein supplies fetal blood from the placenta to the fetus, providing the necessary oxygen and nutrients (Voskamp et al., 2013). Studies showed that a single umbilical artery is associated with SGA birth which is not related directly but affects the placenta (Battarbee et al., 2017). The umbilical cord plays an important role in the development of the fetus. Recent studies have analyzed the levels of the IGF-axis components in the cord blood and their association with demography details of mother and neonates.

The growth hormone and IGF-axis play an essential role in the growth of the fetus. Therefore, we did a thorough literature search on the levels of IGF-axis components in the placenta, cord blood, and blood samples and their association with AGA and SGA. The results of those studies have been summarized in the review of literature section.

Review of Literature

2. Review of literature

SGA neonates have a birth weight below the 10th percentile for their gestational age, and it is one of the leading causes of perinatal morbidity and mortality in developing countries. SGA neonates may exhibit a continuous growth delay, resulting in the childrens short stature (Osuchukwu et al., 2021). Most of the SGA neonates showed abnormal growth hormone secretion and insulin-like growth factor levels (Hawkes et al., 2013). The IGF-axis is commonly known as Growth Hormone (GH)/IGF-axis. IGF structure is similar to pro-insulin, which is mainly secreted by the liver after stimulation with GH. GH/IGF-axis plays an important role in the development of cells and growth in mammals (Holt et al., 2003). GH is a polypeptide hormone secreted by somatotrophs of the anterior pituitary that promotes cell division, regeneration, and growth (Steyn et al., 2016). The action of IGFR is mediated by the binding of IGF1 to IGFR1 and IGF2 to both IGFR1 and IGFR2. The binding of IGFs to these receptors is regulated by a family of IGF-binding proteins (IGFBP1-6). The IGFBPs prolong the half-life and serve as a carrier for IGFs (Allard et al., 2018). The long-term consequences of insulin resistance include type 2 diabetes mellitus, coronary heart disease, cerebrovascular accidents, and cancers (Martin-Timon et al., 2014). The imprinted and non-imprinted genes in IGF-axis are essential to placental growth, and functions, including pregnancy maintenance and parturition, are under epigenetic control (Steyn et al., 2016). Recent studies from different populations analyzed the levels of IGF-axis (IGFs, IGFBPs, IGFRs, methylation of genes, etc.) components in cord blood, placenta, and peripheral blood to understand the role of the IGF-axis in the SGA. We have reviewed the available literature and summarized the major findings of those studies in the following sections.

2.1. Levels of IGF-axis protein in cord blood, blood, and placenta of AGA and SGA neonates

The levels of IGF-axis proteins were altered (low, high, and no difference) in the cord blood, blood, and placenta of SGA and AGA neonates. The plasma levels of IGF2, IGFR2, IGF1, and IGFBP3 proteins in the cord blood plasma of AGA, SGA, and LGA neonates were lower in term SGA compared to term AGA infants, and also the levels of IGF2 were associated with the birth weight (Zhang et al., 2015). IGF1 and IGFBP3 in cord blood of AGA, SGA, and LGA infants were significantly lower in preterm compared to term babies. The levels of these proteins were positively correlated with birth weight and birth length of neonates (Yang et al., 2000). The levels of IGF1 in cord blood plasma did not show any difference between AGA and SGA neonates of the Mexican population (Martínez-Cordero et al., 2006). IGF1, IGF2, and IGFBP5 protein levels were positively associated with birth weight in the cord blood of AGA, SGA, and LGA infants. In contrast, IGFBP4, PAPP-A, and PAPP-A2 levels were negatively associated with birth weight (DiPrisco et al., 2019).

In addition to cord blood, a few studies also analyzed the levels of IGF-axis proteins in the blood sample of neonates/babies. The levels of IGF1, IGFBP3, and leptin were lower, and the levels of ghrelin and IGFBP1 were higher in SGA compared to AGA neonates, and the levels of ghrelin, IGFBP1, and IGFBP3 proteins were negatively associated with birth weight (Mendez-Ramirez et al., 2009). Similarly, a few studies also analyzed the levels of IGF-axis proteins in the placenta of AGA and SGA neonates. Nawathe *et al.,* (2016) observed that the level of IGF1 protein in the placenta was lower, whereas the IGFBP2, 3, 4, and 7 were higher in SGA compared to AGA and LGA neonates (Nawathe et al., 2016). The levels of IGF1 proteins were lower, and levels of IGFR1 were higher in the placenta of SGA compared to controls (Sandoval et

al., 2020). The placental levels of IGFBP1 protein were higher in SGA compared to the LGA group, and the level was positively correlated with the maternal and infant triceps skin-fold thickness. In addition to IGFBP1, the level of IGF1 protein was significantly less in SGA compared to the LGA group, and the levels were correlated significantly with maternal and newborn anthropometry (Akram et al., 2011). The levels of IGF1 and IGFR1 proteins were higher in SGA compared to AGA and LGA newborns (Iniguez et al., 2010).

The levels of the IGF-axis proteins in the GH treated SGA neonates were analyzed in recent studies. The serum IGF1 and plasma free IGF1 levels in the blood sample of growth hormone deficient (GHD) children were decreased after 6-12 months of treatment with recombinant human growth hormone. In contrast, the serum IGFBP3 did not alter (Wacharasindhu et al., 2002). The serum IGF1 and free IGF1 protein levels in the blood were increased after GH treatment (Juul et al., 1998). Table 2.1. shows the list of studies on the levels of IGF-axis protein components in cord blood, blood, and placenta of AGA, SGA, and LGA neonates.

Table 2.1: Summary of published reports on the levels of IGF-axis protein components in cord blood, blood, and placenta of AGA, SGA, and LGA neonates

S. no	Publication	Type of Study	Proteins	Findings
			Cord blood sample	
1	Zhang *et al.*, 2015	Case-control	IGF2, IGFR2, IGF1, and IGFBP3	IGF2 levels were lower in term SAG compared to term AGA
2	Yang *et al.*, 2000	Case-control	IGF1 and IGFBP3	IGF1 and IGFBP3 were lower in SGA compared to AGA

3	Martínez-Cordero *et al.*, 2006	Case-control	IGF1	IGF1 levels did not differ between SGA and AGA neonates
4	DiPrisco *et al.*, 2019	Case-control	IGF1, IGF2, IGFBP4, IGFBP5, PAPPA, and PAPPA2	IGF1, IGF2, and IGFBP5 were positively and IGFBP4, PAPPA, and PAPPA2 levels were negatively associated with birth weight
			Blood sample	
5	Mendez-Ramirez *et al.*, 2009	Cross-sectional and comparative	Ghrelin, leptin, IGF1, IGFBP1, 2, and 3	IGF1, IGFBP3, and leptin levels were lower, and IGFBP1 and ghrelin levels were higher in SGA babies
			Placental sample	
6	Nawathe *et al.*, 2016	Case-control	IGF1, IGFBP 2, 3, 4, and 7	The IGF1 protein levels were lower, and IGFBP2, 3, 4, and 7 protein levels were higher in SGA compared to AGA neonates
7	Sandoval *et al.*, 2020	Case-control	IGF1 and IGFR1	The level of IGF1 was lower, and IGFR1 was higher in SGA compared to controls
8	Akram *et al.*, 2011	Case-control	IGF1	The IGFBP1 level was higher, and IGF1 levels were lower in SGA and correlated with maternal and newborn anthropometry
9	Iniguez *et al.*, 2010	Case-control	IGF1, IGF2 and IGFR1	IGF1 and IGFR1 proteins were higher in SGA compared to AGA
10	Wacharasindhu *et al.*, 2002	Retrospective	IGF1, IGFBP3	Serum IGF1 and plasma free IGF1 levels decreased after the treatment with rhGH
11	Juul *et al.*, 1998	Retrospective	IGF1	The IGF1 levels were increased after the GH treatment

***AGA**=Appropriate for gestational age; **LGA**=Large for gestational age; **SGA**=Small for gestational age **IGF**=Insulin-like growth factor; **IGFBP**=Insulin-like growth factor binding protein; **IGFR**=Insulin-like growth factor receptors*

2.2. Expression of IGF-axis genes in the placenta and cord blood of AGA and SGA neonates

The expression of IGF-axis genes was altered (low, high, and no difference) in placenta and cord blood samples of SGA and AGA neonates. The expression of *IGF1* mRNA was lower, and the *IGFBP1, 2, 3,* and *4* were higher in SGA placenta than in LGA neonates. In contrast, the expression of *IGF1* was positive, and all binding proteins were negatively correlated with the birth weight of neonates (Nawathe et al., 2016). The expression of *IGF1* and *IGFR1* mRNA was higher in the placenta of SGA compared to AGA and LGA newborns (Iniguez et al., 2010). The expression of *WNT2, IGF2/H19, SERPINA3, HERVWE1, PPARG,* and *LINE1* genes did not differ in the placenta of SGA and AGA neonates (Leeuwerke et al., 2016). Few studies were analyzed in the expression of IGF-axis genes in Sprague-Dawley rats. The expression of *IGFR1* mRNA was lower, *IGFBP3* mRNA was higher, and *IGF1* mRNA was not altered after IGF1 treatment in the growth restricted fetus of rats (Shaikh et al., 2005). The expression of *IGFR1* mRNA in the placenta of Sprague-Dawley rats was significantly low in growth restricted fetuses, and no difference in the expression of the *INSR* gene (Reid et al., 2002).

In addition to the placenta, few studies also reported the alterations in the expression of IGF-axis genes in the cord blood of AGA and SGA neonates. Zhang *et al.,* (2015) observed that the expression of *IGF2* mRNA was lower in the cord blood of preterm SGA compared to preterm AGA neonates (Zhang et al., 2015). The expression of *ISR2* mRNA was higher, and the expression of *IGFR1, INSR, IRS1, SLC2A2,* and

SLC2A4 mRNA did not differ between SGA and AGA neonates; however, the *ISR2* mRNA was correlated with birth weight (Fujimoto et al., 2017).

The expression of *IGF2* and *CDKN1C* genes was higher in the human placenta of assisted reproductive technology (ART), and the birthweight of ART singletons was significantly low in naturally conceived SGA neonates (Chen et al., 2018). The expression levels of the *IGF1* gene were inversely associated with T2D in a dose dependent manner, and this association was not significant among the birth weights (Geng et al., 2021). Table 2.2. shows the list of studies on the expression of IGF-axis genes in the placenta and cord blood of AGA, SGA, and LGA neonates.

Table 2.2: Summary of the published reports on the expression of IGF-axis genes in placenta and cord blood of AGA, SGA, and LGA neonates

S. no	Publication	Type of Study	Genes	Findings
			Placental sample	
1	Nawathe *et al.*, 2016	Case-control	*IGF1, IGF2, IGFBP1, 2, 3, 4, 5 6,* and *7*	Expression of *IGF1* mRNA was reduced, and *IGFBP1, 2, 3, 4, 5, 6,* and *7* mRNA expression were higher in SGA compared to AGA neonates
2	Iniguez *et al.*, 2010	Case-control	*IGF1,* and *IGFR1*	The mRNA of *IGF1* and *IGF1R* was higher in SGA compared to AGA neonates
3	Leeuwerke *et al.,* 2016	Retrospective case-control	*WNT2, IGF2/H19, SERPINA3, HERVWE, PPARG,* and *LINE*1	No difference in the expression of all genes between SGA and controls

4	Shaikh *et al.*, 2005	Animal	*IGF1* and *IGFR1*	The expression of *IGFR1* mRNA was reduced in the fetus treated with *IGF1* when compared with saline
5	Reid *et al.*, 2002	Animal	*IGFR1* and *INSR*	*IGFR1* mRNA was significantly low in growth restricted fetus
			Cord blood sample	
6	Zhang *et al.*, 2015	Case-control	*IGF2*	The mRNA expression of *IGF2* was lower in preterm SGA and higher in term SGA
7	Fujimoto *et al.*, 2017	Case-control	*IGFR1*, *INSR*, *IRS1*, *SLC2A2*, and *SLC2A4*	The mRNA expression of *IRS2* was higher in SGA and did not differ in the expression of *IGFR1*, *INSR*, *IRS1*, *SLC2A2*, and *SLC2A4* mRNA between groups
8	Chen *et al.*, 2018	Case-control	*IGF2* and *CDKN1C*	The expression of *IGF2* mRNA was higher in ART singletons
9	Geng *et al.*, 2021	Case-control	*IGF1*	*IGF1* levels were inversely associated with the T2D risk in dose-dependent manner

***AGA**=Appropriate for gestational age; **SGA**=Small for gestational age; **LGA**=Large for gestational age; **IGF**=Insulin-like growth factor; **IGFR**=Insulin-like growth factor receptors; **IGFBP**=Insulin-like growth factor binding protein; mRNA=Messenger Ribonucleic acid; **PPARG**=Peroxisome proliferator-activated receptor gamma; **WNT2**=Wingless-related integration site; **SERINA3**=Serpin family A member 3; **INSR**=Insulin Receptor; **LINE1**=Long interspersed nuclear elements; **SLC2A2**=Solute carrier family 2, facilitated glucose transporter member 2; **CDKN1C**=Cyclin-dependent kinase inhibitor 1C*

2.3. Methylation of IGF-axis genes in the placenta and cord blood of AGA and SGA neonates

The alteration in the sequence of DNA is reported to be associated with several genetic disorders in humans. In addition to the alterations in the sequence of DNA,

epigenetic mechanisms are involved in altering the function of genes and are associated with genetic diseases. DNA methylation is one such epigenetic modification of the bases in the DNA molecule. DNA methylation occurs at C5 of the cytosine ring within the cytosine and guanine (CpG) dinucleotides. The methyl group on C5 of cytosine in the promoter is maintained and added by DNA methyltransferases (DNMTs). DNMTs 3a and DNMTs 3b as a new methylation pattern to unmodified DNA known as de novo DNMTs. DNA methylation can alter DNA segments activity without changing the sequence. At the gene promoter region, DNA methylation acts as a repressor for gene transcription methylation in CpG rich areas of promoters known as CpG islands. Several studies suggested that altered placental DNA methylation plays an important role in placental and fetal growth (Koukoura et al., 2012).

Recent studies analyzed the epigenetic alterations of IGF-axis genes in the placenta, cord blood, and the blood samples of SGA, AGA, and LGA neonates. The major epigenetic analysis performed was the methylation of the CpG islands in both imprinted and non-imprinted genes, such as *IGF2, H19, IGF1*, *IGFR1*, etc. The methylation of the *IGF2* gene in the placenta was lower in FGR compared to controls, and it is positively associated with the concentration of DEHP (Zhao et al., 2016). The methylation of the *IGFBP3* gene promoter was less, and the methylation of *IGF1* was 1.5 times higher in the placenta of SGA compared to AGA neonates (Nawathe et al., 2016). The methylation levels of *IGF2, GNASAS, INSIGF*, and *LEP* genes did not differ in the placenta of AGA and SGA neonates, and the methylation levels of *IGF2, GNASAS, INSIGF*, and *LEP* genes were not associated with birth weight (Tobi et al., 2011). The methylation levels of *IGF2* and *H19* genes in the placenta did not differ between SGA and AGA neonates (Leeuwerke et al., 2016).

In addition to the placenta, a few studies also reported the alteration in the methylation status of IGF-axis genes in the peripheral blood and cord blood of AGA and SGA neonates. The methylation of the *IGF2* gene is 2.7% lower in SGA children, and the levels of methylation of the *IGF2* gene did not correlate with birth weight (Murphy et al., 2014). The methylation of *IGF2* gene *DMR* in the blood was higher, and the *H19* gene *DMR* was lower in SGA compared to AGA (Zhang et al., 2015). The methylation levels of candidate genes (*GPR120*, *CPT1A*, and *IGFBP4*) in the peripheral blood were higher, and the methylation levels of these candidate genes were associated with body mass index (Daiz et al., 2022). The levels of IGF1 gene promoter were less in infants with IUGR and positively correlated with the neonates body weight and body length (Kantake et al., 2020). The methylation of the *IGF2* gene in cord blood was significantly lower in VLBW compared to the control (Wehkalampi et al., 2013). The *IGF2* gene *DMR* methylation in the placenta was lower in SGA compared to AGA neonates, and *IGF2* gene *DMR* methylation was associated with weight gain in the neonates (Bouwland et al., 2013). Table 2.3. shows the summary of published reports on the methylation of IGF-axis genes in the placenta, blood, and cord blood of AGA, SGA, and LGA neonates.

Table 2.3: Summary of the published reports on the methylation of IGF-axis genes in the placenta, blood, and cord blood of AGA, SGA, and LGA neonates

S. no	Publication	Type of study	Genes	Findings
			Placental sample	
1	Zhao *et al.,* 2016	Case-control	*IGF2*	The methylation of *IGF2* gene is lower in FGR and positively associated with concentration of DEHP
2	Nawathe *et al.,* 2016	Case-control	*IGF1* and *IGFBP1-7*	The methylation of *IGFBP 1, 2, 3, 4,* and *7* were lower and *IGF1* promoter

				were higher in SGA compared to AGA neonates
3	Tobi *et al.*, 2011	Case-control	*IGF2*, *GNASAS*, *INSIGF*, and *LEP*	The gene methylation did not show any difference between SGA and AGA neonates
4	Leeuwerke *et al.*, 2016	Case-control	*IGF2* and *H19*	The methylation of genes did not show any difference between SGA and AGA neonates
			Blood sample	
5	Murphy *et al.*, 2014	Case-control	*IGF2* and *H19*	The methylation of *IGF2* was lower in SGA compared to controls
6	Zhang *et al.*, 2015	Case-control	*IGF2* and *H19*	The methylation of *IGF2* were higher *H19 DMR* was lower SGA compared to AGA neonates
7	Daiz *et al.*, 2022	Case-control	*GPR120*, *CPT1A*, and *IGFBP4*	Methylation of *GPR120*, *CPT1A*, and *IGFBP4* were hypermethylated in SGA compared to AGA and methylation levels were associated with body mass index
8	Kantake *et al.*, 2020	Case-control	*IGF1*	*IGF1* promoter methylation levels were less in infants with IUGR *IGF1* gene promoter was positively associates with the body weight and body length
			Cord blood sample	
10	Wehkalampi *et al.*, 2013	Case-control	*IGF2*	The methylation of *IGF2* was low in VLBW than controls
11	Bouwland *et al.*, 2013	Case-control	*IGF2* and *H19*	*IGF2* methylation was lower in SGA compared to controls and associated with birth weight

***SGA**=Small for gestational age; **AGA**=Appropriate for gestational age; **LGA**=Large for gestational age; **IGF**=Insulin-like growth factor; **IGFR**=Insulin-like growth factor*

receptors; ***IGFBP****=Insulin-like growth factor binding protein;* ***mRNA****=Messenger Ribonucleic acid;* ***INSR****=Insulin Receptor;* ***CPT1A****=Carnitine palmitoyl transferase 1A;* ***GPR120****=G-protein coupled receptor 120;* ***WNT2****=Wingless-related integration site;* ***H19****=Imprinted maternally expressed transcript;* ***TSS****=Transcriptional start site*

2.4. Association of the IGF-axis gene methylation with the demography of the SGA and AGA neonates

The DNA methylation levels of *IGF2* and *AHRR* genes were positively associated with birth weight, and the methylation levels of *HSD11B2* and *WNT2* genes were negatively associated with fetal growth indicators (Xiao et al., 2016). The methylation level of the *IGF2* gene in the placenta was correlated with the newborn fetal growth and the maternal protein *IGF2* levels. However, the methylation level of the *H19* gene was correlated with cord blood IGF2 levels (St-pierre et al., 2012). The methylation of *IGF1* and *IGFR1* gene promoter in blood was positively correlated with the *IGF1* and *IGFR1* gene expression (Ye at al., 2016). The methylation of the levels of *IGF1, IGF2, IGFR2, IGFBP, PHLDA2,* and *PLAGL1* genes in the cord blood was positively correlated with birth weight (Ouni et al., 2015). The methylation levels of ICR1 and ICR regions of *the IGF2* gene in cord blood were not associated with birth weight (Burris et al., 2013). Table 2.4. shows the summary of published reports on the association of methylation of IGF-axis genes with birth weight and gestational weeks in the placenta, cord blood of AGA, SGA, and LGA neonates.

Table 2.4: Summary of the published reports on the association of IGF-axis genes methylation with birth weight and gestational weeks of AGA, SGA, and LGA neonates

S. no	Publication	Type of study	Genes	Findings
			Placental sample	
1	Xiao *et al.,* 2016	Case-control	*IGF2, AHRR,*	*IGF2* methylation was positively associated with birth weight

			HSD11B2, and *WNT2*	
2	St-pierre *et al.,* 2012	Case-series	*IGF2* and *H19*	The methylation level of *IGF2* gene were correlated with newborn fetal growth and with maternal protein IGF2 levels The methylation level of *H19* gene were correlated with IGF2 protein levels in cord blood
3	Ye *et al.,* 2016	Case-control	*IGF1* and *IGFR1*	Methylation levels of *IGF1* and *IGFR1* was positively correlated with the expression of *IGF1* and *IGFR1* genes
			Cord blood sample	
4	Ouni *et al.,* 2015	Case-control	*IGF1, IGF2, IGFR2, IGFBP, PHLDA2,* and *PLAGL1*	Methylation levels of IGF1, *IGF2, IGFR2, IGFBP, PHLDA2,* and *PLAGL1* was associated with birth weight
5	Burris *et al.,* 2013	Prospective cohort	*IGF2*	*IGF2* gene methylation was not associated with birth weight

***IGF**=Insulin-like growth factor; **IGFR**=Insulin-like growth factor receptors; **IGFBP**=Insulin-like growth factor binding protein; **DNA**=Deoxyribose nucleic acid; **H19**=Imprinted maternally expressed transcript; **PPARG**=Peroxisome proliferator-activated receptor gamma; **WNT2**=Wingless-related integration site; **PLAGL1**=PLAG1 like Zinc Finger 1*

2.5. Indian studies on the levels of IGF-axis components and its association with birth weight and gestational weeks

We have reviewed the studies on the IGF-axis components in the Indian population. We found very limited studies where they analyzed the IGF-axis protein levels or anthropometric measurements of the SGA and AGA neonates. Prakash *et al.,*

(2021) observed that the 10 and 90th percentiles were lower in all gestational ages compared to existing international standards. The mean birth weight of male and female in term babies born to primiparae was significantly higher compared with multiparae (Prakash et al., 2021)**.** Sebastian *et al.,* (2015) studied the risk factors over 15 years; they found that the incidence of SGA was significantly reduced by 26%; the women with the above modifiable risk factors need to be identified early and provided with health education on optimal birth weight (Sebastian et al., 2015). Prasad *et al.,* (2012) studied the growth of the SGA after treatment of GH for two years; the height and Z-score (HAZ) was higher in Group I (treated with growth hormone) compared to group II (not treated with growth hormone) ($p<0.05$). The percentage of pubertal children was less in cases compared to controls. GH increased the growth velocity Z-score during the first year than the second year of treatment. Thus, they conclude that GH progresses the height of short SGA children without accelerating pubertal progression (Prasad et al., 2012). Wacharasindhu *et al.,* (2002) observed that the serum level of IGF1 and plasma free IGF1 levels were increased before treatment and then after six months to one year. In contrast, the serum IGFBP3 levels did not significantly increase after treatment. In addition, the free/total IGF1 ratio decreased after treatment with rhGH. Therefore, they suggested that plasma free IGF1 levels could be a good predictor of growth hormone response in SGA neontaes/babies (Wacharasindhu et al., 2002).

2.6. The intrinsic division capacity of the lymphocytes

The mitotis plays an important role in the growth of development of neonates and children. The literature suggests that the IGF-axis components were altered in SGA neonates placenta, cord blood, and blood samples. The end response of the IGF-axis is the growth of the cells. Hence few studies analyzed the alteration in the division capacity of the cells. The mean of the mitotic index of the cord blood in mono-therapy

and poly-therapy groups did not show any statistically significant from mitotic index in the negative control of neonates whose mothers were exposed to anti-epileptic drugs (AED) therapy due to epilepsy (Witczak et al., 2010). The mitotic index of the blood lymphocytes of A, B, AB, and O groups was analyzed in healthy subjects. The percentage of mitotic index is altered among different blood groups and associated with the responsiveness to ionizing radiation (Viswanathan et al., 2018).

The literature review indicates that the expression levels of IGF-axis components such as IGF1 and 2, IGFBP1-7, IGFR1 and IGFR2, methylation of IGF2, H19, IGF1, IGFR1 etc., were altered (higher, lower, and no difference) in the SGA neonates born to different populations in the world. In addition, the levels of IGF-axis components were also reported to be altered by various factors such as the nutrition, environment, genetic composition, pregnancy complications etc. We found few studies on the IGF-axis components in SGA neonates of Indian population, where they analysed the growth hormone treatment induced alteration in IGFs/anthropometric measurement. After thorough review of literature, we did not come across any studies were they analysed various components (IGF levels, IGF receptor expression, methylation of imprinted and non-imprinted genes) of IGF-axis in the SGA neonates of Indian population. Hence, in the present study, we aim to analyze the levels of IGF-axis protein and mitotic index in cord blood, and the expression of IGF-axis genes and methylation of IGF-axis genes in the placenta of AGA and SGA neonates born to Indian women.

Materials and Methods

4. Materials and methods

4.1. Materials

The following list of chemicals, glassware, plastic ware, equipment's, and reagents were used in the present study.

4.1.1. List of chemicals

Agarose: Sigma-aldrich (Cat. #A9539)

Ammonium chloride: Amresco (Cat. #12125-02-9)

Antibiotic antimycotic solution: HiMedia (Cat. #A002)

cDNA conversion kit: Takara (Cat. #RR037A)

Chloroform: Spectrochem (Cat. #67-66-3)

Colchicine: Sigma (Cat. #C9754-1G)

Deoxynucleotide triphosphate (dNTPs): Genei (Cat. #61060250005130)

Disodium hydrogen phosphate (Na_2HPO_4): SD Fine-Chem limited (Cat. #20383K05)

DNA ladder: Genei (Cat. #6126526710017300)

Ethylenediamine tetraacetic acid (EDTA): Thermo Fisher Scientific (Cat. #17892)

ELISA kits (IGF1, IGF2, and IGFBP3): Krishgen Biosystem (Cat. #KBH0411, KBH0392, KBH0391 respectively)

Ethanol: Analytical reagent (Cat. #20180907)

Fetal bovine serum (FBS): Gibco (Cat. #42F1383K)

Gel loading dye **(**6X) Bromophenol blue: Thermo Fisher Scientific (Cat. #R0611)

Giemsa stain: Thermo Fisher Scientific (Cat. #Q38723)

Glacial acetic acid: Pure (Cat. #37013L25)

Hydrochloric acid (HCl): SDFCL (Cat. #20125L25)

Isopropanol: SD Fine-Chem limited (Cat. #39694L25)

Methanol: SD Fine-Chem limited (Cat. #89139L05)

Phytohemagglutinin (PHA): Gibco (Cat. #10576-015)

Positive and negative controls for Methylation PCR: Zymo research (Cat. #D5015)

Potassium bicarbonate ($KHCO_3$): Nice (Cat. #P1422910)

Potassium chloride (KCl): Merck (Cat. #M10D600110)

Potassium dihydrogen phosphate (K2HPO4): Merck (Cat. #F33294)

Primers for DNA methylation of promoter *IGF2*, *H19*, *IGF1*, and *IGFR1* genes: Zymo research (Cat. #D5001)

Proteinase K: Invitrogen (Cat. #2204622)

Sodium bicarbonate ($NaHCO_3$): Thermo Fisher Scientific (Cat. #25080094)

Sodium chloride (NaCl): HiMedia (Cat. #7647-14-5)

Sodium dihydrogen phosphate (NaH_2PO4): Merck (Cat. #7558-80-7)

Sodium dodecyl sulfate (SDS): Sigma-aldrich (Cat. #V800386)

Sodium hydroxide (NaOH): Thermo Fisher Scientific (Cat. #15895)

SYBR green master mix: Bio-Rad (Cat: #1725271)

Taq DNA polymerase: Genei (Cat. #610602500051730)

Tris-Base: SRL (Cat. #2044122)

Trizol reagent: Thermo Fisher Scientific (Cat. #15596018)

4.1.2. Glassware

Beakers: Borosil (Cat. #1002)

Coverslips: Blue star

Cuvette for spectrophotometer: Perkin Elmer UV spectroscopy

Duran Bottles: Borosil (Cat. #3321)

Glass rod: Pacific Star Corporation (Cat. #PFS1007711 3PK)

Measuring cylinders: Borosil (Cat. #579)

Microscopic glass slides: Lab tech (Cat. #S18105)

Reagent bottles: Borosil (Cat. #3321)

Standard flask: Borosil (Cat. #5640029)

4.1.3. Plastic ware

COMB (1.0 mm): Tarsons (Cat. #7087)

Couplin jar (50 ml): Abdos (Cat. #P90101)

Cryo box (1.8 ml): Tarsons (Cat. #524010)

Eppendroff tubes (1.5 ml): Tarsons (Cat. #22363204)

Eppendroff tubes (2 ml): Tarsons (Cat. #22363352)

Falcon tubes (15 ml): Merck (Cat. #CLS431470)

Falcon tubes (50 ml): Merck (Cat. #CLS431472)

Heparin vacutainer: Becton Dickinson (Cat. #367871)

K2 EDTA vacutainer: Becton Dickinson (Cat. #367856)

Microtips (0.2-10 µl): Abdos (Cat. #P10115)

Microtips (100-1000 µl): Abdos (Cat. #P10106)

Microtips (2-20 µl): Abdos (Cat. #P10130)

Parafilm: Bemis (Cat. #PM999)

Pasteur pipette: Tarsons (Cat. #940060)

PCR cooling pack: Merck (Cat. #Z606634)

PCR plates: Thermo Fisher Scientific (Cat. #AB0600)

PCR tube racks: Fisher scientific (Cat. #03-448-20)

PCR tubes (0.2 ml): Tarsons (Cat. #510051)

pH strip: Fisher Scientific (Cat. #HP-V0WD-7PP6)

Sealers: Bio-Rad (Cat. #MSB1001)

Syringe filters: Merck (Cat. #SLGVV255F)

Syringe filters: HiMedia (Cat. #SF14)

Syringes: BD (Cat. #26144)

Whatman filter paper: Merck (Cat. #GSWP04700)

4.1.4. Equipments

-80 °C Freezer: Cryo Scientific (Cat. #URC-V-700-4)

Autoclave: (Cat. #AV-104)

CO_2 incubator: Thermo Scientific (Cat. #371)

Cooling centrifuge (4 °C): Remi (Cat. #CM-12)

Electronic balances: Sartorious (Cat. #GE 612-1)

ELISA reader: Merilyzer (Cat. #EIAQuant)

Gel documentation system: Bio-Rad (Cat. #Et9970616AA)

Gel electrophoresis unit: Bio scientific (Cat. #18-1130-01)

Inverted microscope: (Cat. #3842000306)

Laminar air flow: Esco (Cat. #LAF 35)

Light microscope: Nikon (Cat. #E-200)

Media preparation unit: MRC laboratory instruments

Micro pipettes (1-10 µl, 2-20 µl, 200 µl, and 1 ml): Glassco

Millipore: Millipore system (Cat. #ZRXQ0031N)

Nanodrop: Implen (N60)

Normal centrifuge: Remi (Cat. #R8C)

PCR machine: Bio-Rad (Cat. #1851148)

PCR plate centrifuge: BR Biochem (Cat. #BIDH-100)

pH meter: Sartorious (Cat. #PB-11-P10)

Real-time PCR: Bio-Rad (Cat. #781BR16930)

Slide warmer: YORCO (Cat. #YSI-132)

UV spectrophotometer: Perkin Elmer (Cat. #LAMBDA 35)

Vortex: Remi (Cat. #CM 101)

Water bath: Julabo (Cat. #TW-8)

4.1.5. Preparation of reagents

Phosphate buffer saline (PBS): About 8 g of sodium chloride, 200 mg of potassium chloride, 1.44 g of disodium hydrogen phosphate, and 245 mg of potassium dihydrogen phosphate was dissolved in 1000 ml of double distilled water, and the pH was adjusted to 7.4 by adding 0.1 M HCl or 0.1 M NaOH, autoclaved, and stored at 4 °C.

Erthrolysis buffer (ELB): 155 mM of ammonium chloride (8.29 g), 10 mM of potassium bicarbonate (1.00 g), 0.1 mM of EDTA (200 µg) were dissolved in 1000 ml of double distilled water, and the pH was adjusted to 7.4 by adding 0.1 M HCl or 0.1 M NaOH.

Proteinase K: About 2 mg of proteinase K powder was dissolved in 1 ml of double distilled water.

20% Sodium dodecyl sulfate (SDS): About 20 g of sodium dodecyl sulfate was dissolved in 100 ml of double distilled water.

5 M NaCl: About 29.22 g of NaCl was dissolved in 100 ml of double distilled water.

Roswell Park Memorial Institute (RPMI 1640): RPMI 1640 powder, 2 g of sodium bicarbonate was dissolved in 1000 ml of double distilled water. The pH of the media was adjusted to 7.4 by adding 0.1 M HCl or NaOH. The prepared media was filtered using a 0.22 µm Whatman filter and stored at 4 ºC. 1% of antibiotics was added to the

media, kept the media for sterility check by incubating 24, 48, and 72 h at 37 °C before using it for culturing blood samples.

Fetal bovine serum (FBS): FBS was heat inactivated by incubating at 55 ºC for 30 min and stored at 4 ºC for further use.

Colchicine: About 5 mg of colchicine was dissolved in 5 ml of sterile water and filtered using a syringe filter (stock 1). 100 µl of stock 1 was added to 9.9 ml of sterile distilled water (stock 2). 10 µl (0.02 µg/ml) of stock 2 was added to 5 ml culture, and 20 µl (0.02 µg/ml) to 10 ml culture at 24^{th} h.

Carnoy's fixative: 3:1 ratio of methanol and glacial acetic acid was prepared in a reagent bottle, tightly capped, and stored at 4 ºC.

0.075 M Hypotonic solution: 0.56 g of potassium chloride was dissolved in 100 ml of double distilled water and incubated at 37 ºC for 30 min.

10% Giemsa: 5 ml of commercially available Giemsa solution was mixed with 45 ml of double distilled water.

70% Isopropyl alcohol (IPA): 70 ml of isopropyl alcohol was mixed with 30 ml of double distilled water.

Tris-Acetate EDTA buffer: About 4.85 g of tris base, 1.14 ml of glacial acetic acid, and 2 ml of 0.5 M EDTA were dissolved in 100 ml of double distilled water. The buffer pH was adjusted to 8 by adding 0.1 M HCl or 0.1 M NaOH, autoclaved, and stored at room temperature.

Ethanol (80 and 85%): 80 and 85 ml of ethanol were mixed with 20 and 15 ml of double distilled water.

Primer dilution: The primers used for both gene expression and methylation-specific PCR was diluted as per the manufacturer's instructions. The primers' concentration for gene expression and methylation-specific PCR ranges between 0.5-1 µM.

TE buffer: About 0.2 ml of 0.5 M EDTA, 1 ml of 1 M tris-HCl was added to the reagent bottle and made up to 100 ml using distilled water. The pH of the solution was adjusted to 8 and stored at room temperature.

RNA later buffer: About 175 g of ammonium sulfate, 10 ml of 0.5 M EDTA, and 6.25 ml of 1 M sodium citrate were dissolved in 233.75 ml of distilled water. The pH of the solution was adjusted to 5 and stored at room temperature.

1% Ethidium bromide: About 0.5 g of ethidium bromide was dissolved in 50 ml of distilled water and kept for stirring for 2-3 h.

4.2. Methods

The methods followed in the present study were described as follows.

4.2.1. Study participants and ethics approval

A case-control study was designed, and prior approval was obtained from the Institutional Ethics Committee, Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER), Tamaka, Kolar, Karnataka, India (Ref No: SDUMC/KLR/IEC/32/2019-20). Pregnant women (n=98; 49/group) attending the department of Obstetrics and Gynaecology, R. L. Jalapa Hospital (RLJH), SDUAHER, Tamaka, Kolar, Karnataka, India, were recruited for the study. The pregnant women who underwent both normal vaginal delivery (NVD) and elective lower segment cesarean section (LSCS) were considered for the study. The South Indian pregnant women gave birth to both AGA and SGA babies. The sample size for the present study was calculated based on the difference in levels of IGF-axis components in AGA and SGA groups (Zhang et al., 2015). The required sample size for the present study was determined as n=49/group**.** After obtaining written informed consent from pregnant women, the placental tissue and umbilical cord blood samples were collected following

the inclusion and exclusion criteria for mothers and neonates (Tables 4.1 and 4.2). Both placental tissue and umbilical cord blood samples were transported to the department of Cell Biology and Molecular Genetics, SDUAHER, for further analysis.

4.2.2. Inclusion and exclusion criteria

AGA and SGA neonates were classified based on the intrauterine chart, gestational weeks, and birth weight of neonates. The birth weight of neonates was measured using a Filizola digital scale; the minimum and maximum capacity of the scale is 250 g-15 kg (Bennini et al., 2010).

Table 4.1: The inclusion and exclusion criteria for the selection of pregnant women

Inclusion	Exclusion
➢ Mothers with AGA and SGA neonates	➢ Pre-eclampsia ➢ Gestational diabetes ➢ Gestational hypertension ➢ Placental abruption

Table 4.2: The inclusion and exclusion criteria for the selection of neonates

Inclusion	Exclusion
➢ Full-term neonates ➢ Both genders (AGA and SGA)	➢ Post-term ➢ Large for gestational age ➢ Twins ➢ Congenital anomaly

The overview of the methodology followed in the present study is shown in figure 4.1.

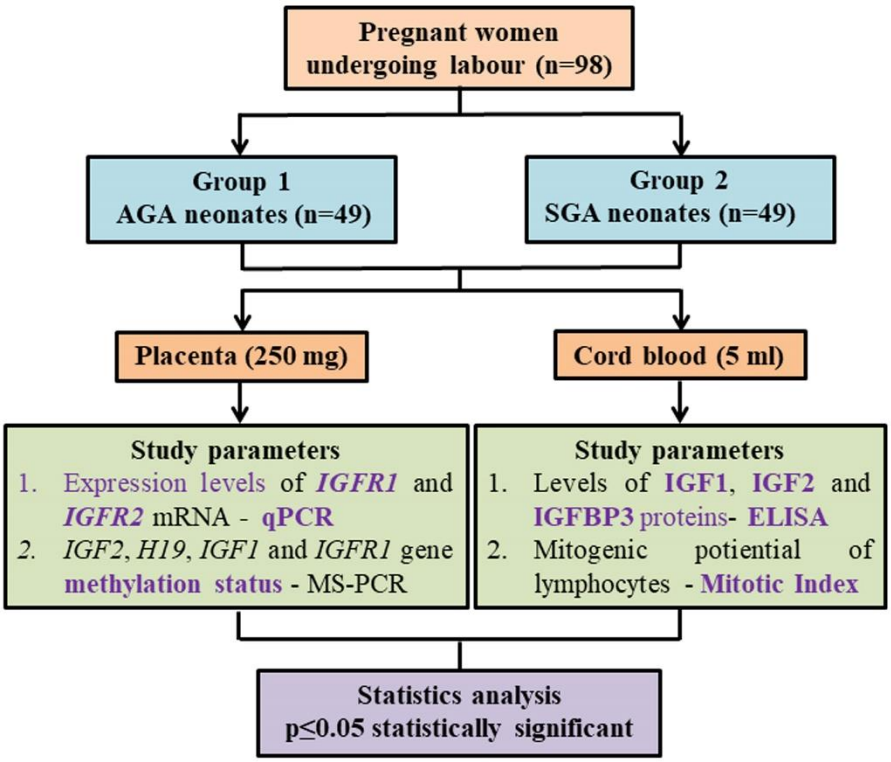

Figure 4.1: The overview of the methodology followed in the present study

4.2.3. Collection of placental tissue samples

After obtaining the written consent, the placental tissue from pregnant women (n=40/group) who underwent labor was collected following inclusion and exclusion criteria (Tables 4.1 and 4.2). After delivery, approximately 150-200 mg of placental tissue samples were collected using a sterile surgical blade and transferred to RNAs later buffer and PBS, stored at -80 ºC until further use. The placental tissue stored in RNA later was used to measure the IGF-axis gene expression. The placental tissue stored in PBS was used to find the methylation status of *IGF2*, *H19*, *IGF1*, and *IGFR1* genes.

4.2.4. Collection of umbilical cord blood samples

About 5 ml of umbilical cord blood samples were collected using a sterile syringe in EDTA and heparin-coated tubes. The samples in the EDTA tube were centrifuged at 3000 rpm for 15 min, and the plasma portion was separated and stored at -80 °C. The plasma samples were used to measure the levels of IGF1, IGF2, and IGFBP3 proteins using the ELISA method. The samples collected in heparinized tubes were cultured *in vitro* to calculate the mitotic index.

4.2.5. Hematological and biochemical parameters of a pregnant woman

The hematological and biochemical parameters data were collected from the medical reports of pregnant women (who participated in the present study) available in the Central Diagnostics Laboratory Services of RLJH, SDUAHER.

4.2.6. Quantification of IGF1, IGF2, and IGFBP3 protein levels in cord blood plasma

The levels of IGF-axis proteins such as IGF1, IGF2, and IGFBP3 proteins in the cord blood plasma (n=40/group) were measured using the ELISA method (Maya et al., 2018). The standards and samples (100 µl) were added to the antibody-coated wells of the microtiter plate, covered with sealer, and incubated at 37 °C for 90 min. After incubation, the contents were discarded and washed 4 times with 1X wash buffer. To the wells, 100 µl of biotinylated antibodies (IGF1, IGF2, and IGFBP3**)** were added to the respective wells and incubated at 37 °C for 60 min. After incubation, the contents were discarded and washed 4 times with 1X wash buffer. To the wells, 100 µl of Streptavidin: Horseradish peroxidase conjugate (HRP) were added and incubated at 37 °C for 30 min. After incubation, the contents were discarded, washed with 1X wash buffer, and 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate were added and

incubated at 37 °C for 10 min (Figure 4.2A). Finally, 50 µl of stop solution was added to the wells, and absorbance was read at 450 nm (Figure 4.2B) using a microplate reader.

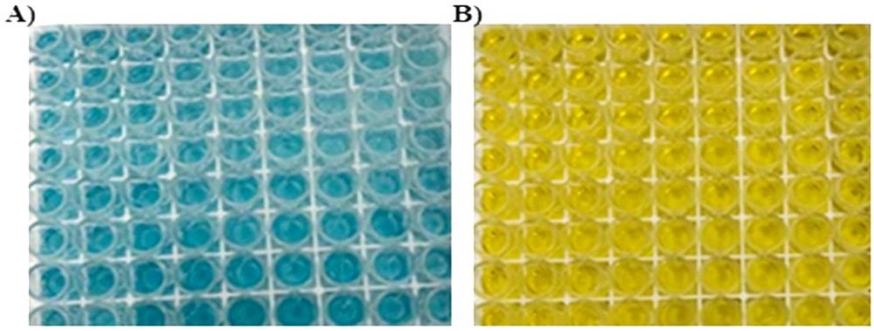

Figure 4.2: Representative image of ELISA plate used to measure the IGF-axis proteins in plasma of AGA and SGA neonates

IGF1, IGF2, and IGFBP3 protein levels in plasma samples were derived from the standard graphs and expressed as ng/µl. The levels of all these proteins between AGA and SGA groups were compared to find the significance.

4.2.7. Isolation of total RNA from placental tissue using the Trizol method

Total RNA from the placental tissue (n=20/group) of AGA and SGA was isolated using the Trizol method (Rio et al., 2010). About 25 mg of placental tissue samples were removed from RNA later buffer and washed with ELB to remove the RBC. To the tissue, 1 ml of Trizol was added and homogenized thoroughly. A representative image of placental tissue is shown in figure 4.3. The overview of the qPCR methodology followed in the study is shown in figure 4.4. The homogenate was transferred to Eppendorf, added 200 µl of chloroform was, mixed thoroughly, and incubated for 5 min at room temperature. The tubes were centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was collected in a fresh Eppendorf tube, added 500 µl of isopropanol alcohol, and incubated for 10 min at room temperature. The tubes were centrifuged, discarded the supernatant and the pellet was washed twice using 75% ethanol. The pellet was kept for air dry for 15 min, added 30 µl of RNAs free water mix well incubate at

55-60 ℃ for 15 min and stored at -80 °C. The concentration of RNA was quantified using a nanodrop (shown in figure 4.5). The quality of the RNA was assessed using denaturing agarose gel electrophoresis. The quality of the RNA sample above 1.8 was used to convert cDNA and analyze the gene expression using Syber green.

Figure 4.3: Representative image of placental tissue samples collected from AGA and SGA neonates

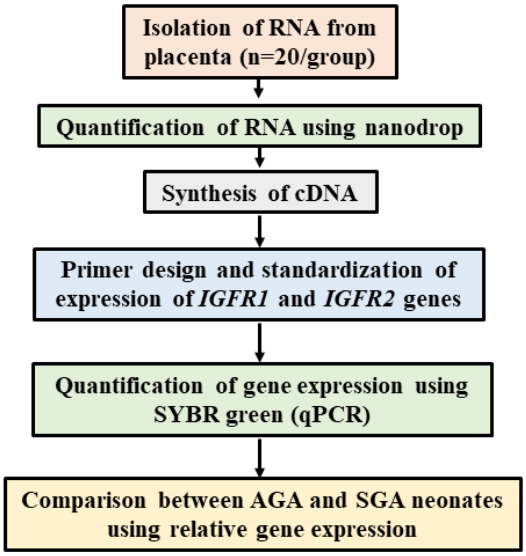

Figure 4.4: The overview of the methodology followed for the study of gene expression changes using qPCR

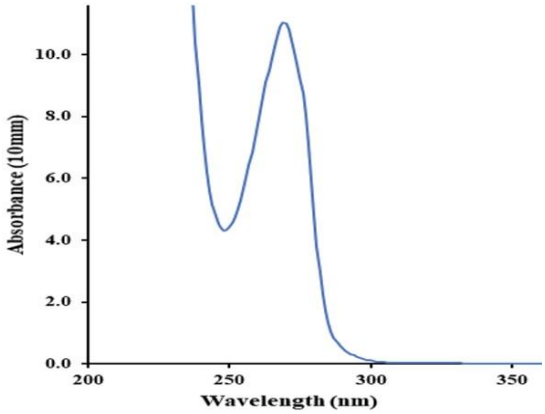

Figure 4.5: The concentration of RNA was quantified using nanodrop at 260 nm

4.2.8. Conversion of total RNA into cDNA

The total RNA (200 ng) samples were converted into cDNA using a prime script RT reagent kit (Tuo et al., 2015). The composition of the reverse transcription reaction is shown in table 4.3. The conditions used for reverse transcription PCR are shown in table 4.4.

Table 4.3: Composition of the reverse transcription reaction

Components	Volume (µl)
5Xprime script buffer	2.0
Prime script RT enzyme mix-I	0.5
Oligo dT primer	0.5
Random hexamers	0.5
RNAse free water	1.5
Total RNA	5.0
Total	**10**

Table 4.4: The conditions used for conversion of RNA to cDNA

Step	Incubation	Duration
1	37 ℃	15 min
2	85 ℃	5 sec
	Hold at 4 ℃	

The cDNA samples were stored at -20 ℃ and used to analyze IGF-axis gene expression using qPCR.

4.2.9. Quantification of *IGFR1* and *IGFR2* gene expression using qPCR

Real-time quantification of *IGFR1* and *IGFR2* gene expression was performed using SYBR green (Browne et al., 2020). The primers for *IGFR1, IGFR2,* and *GAPDH* genes were designed using Primer3 software (Untergasser et al., 2012). The primers were standardized in the laboratory before analyzing the gene expression. The reaction

mixture composition used for quantification is shown in table 4.5. The conditions used for the qPCR reaction are shown in table 4.6.

Table 4. 5: The composition of the qPCR reaction used to quantify gene expression

Components	Volume (µl)
Forward primer	1
Reverse primer	1
RNase free water	1
cDNA	2
SYBR green	5
Total	**10**

Table 4.6: The conditions used for qPCR reaction to quantify gene expression using SYBR green.

Step	No. of cycles	Cycles	Temperatures (°C)	Duration
1	1	Initial denaturation	95	3 min
2	40 cycles	Denaturation	95	10 sec
3*	(steps 2-4)	Annealing	57	30 sec
4*		Extension		

*Plate read at Steps 3 and 4. Green fluorescence is detected at 57 °C

The cycle threshold (Ct) values were obtained by adjusting the threshold in the software attached to qPCR. The representative images of the amplification graphs and melt curves were obtained after amplification and shown in figures 4.6 and 4.7. All the experiments were run as duplicates, and the average values were calculated. The *GAPDH* gene was used as an internal control to normalize the gene expression of interest. The relative expression of *IGFR1* and *IGFR2* genes was calculated using the $2^{-\Delta\Delta Ct}$ method **(**Raavi et al., 2019, Schmittgen et al., 2008). The difference in the expression of *IGFR1* and *IGFR2* genes between the AGA and SGA groups was represented as fold changes.

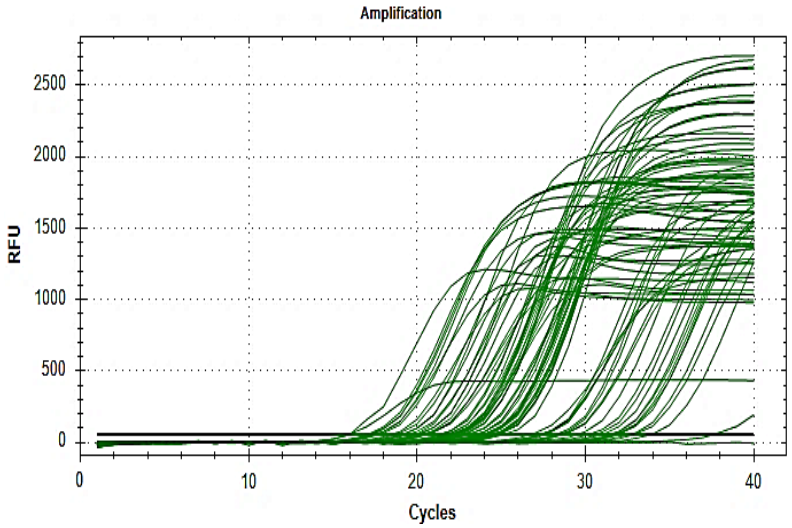

Figure 4.6: Representative graphs of the amplification of the genes in qPCR

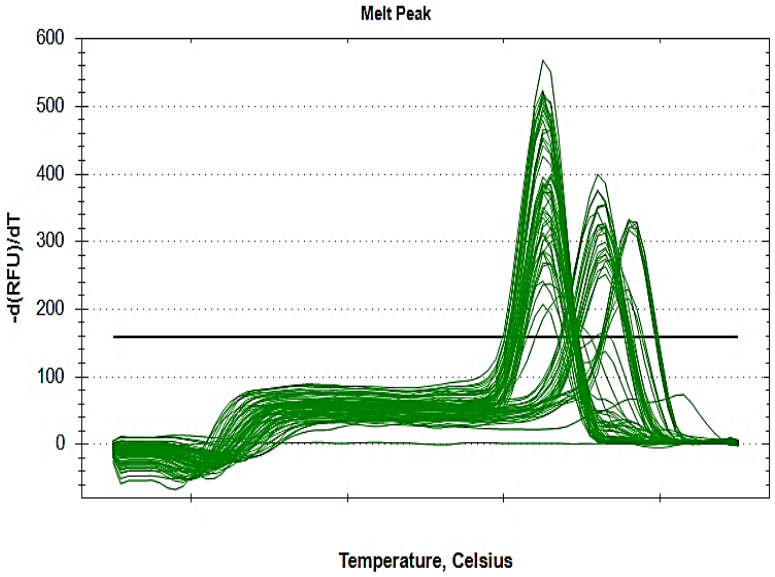

Figure 4.7: Melt cure analysis of the expression of the genes in qPCR

4.2.10. Isolation of DNA from placental tissue

The methodology for isolating DNA from placental samples (n=40/group) was followed as described in Gaaib *et al.*, (2011). The overview of the method followed for the analysis of DNA methylation in the placenta of AGA and SGA neonates is shown in figure 4.8. Briefly, 25 mg of placental tissue samples were rinsed with ELB, minced into small pieces, transferred to a 15 ml falcon tube containing 5 ml of ELB, added 33 µl proteinase-K, and incubated at 37 °C for 2 days. After 2 days, 270 µl of 20% SDS

was added and incubated at 37 ℃ overnight. To the contents, 500 µl of 5 M NaCl and an equal volume of isopropanol were added and mixed thoroughly until the thread-like structure was observed. The thread-like structure was transferred to a 1.5 ml Eppendorf tube, and 1 ml of 80% ethanol was added; the tubes were centrifuged at 3000 rpm for 10 min. The ethanol (80%) wash was repeated twice, and the pellet was dried at room temperature for 30 min. The DNA pellet was dissolved in 500 µl of TE buffer, incubated at 65 ℃ for 25 min, and stored at -20 ℃ until further use. The quality of DNA was analyzed using nanodrop (shown in figure 4.9). The quality of the DNA was assessed using denaturing agarose gel electrophoresis. The quality of the samples above 1.8-1.9 was used to treat with bisulfite.

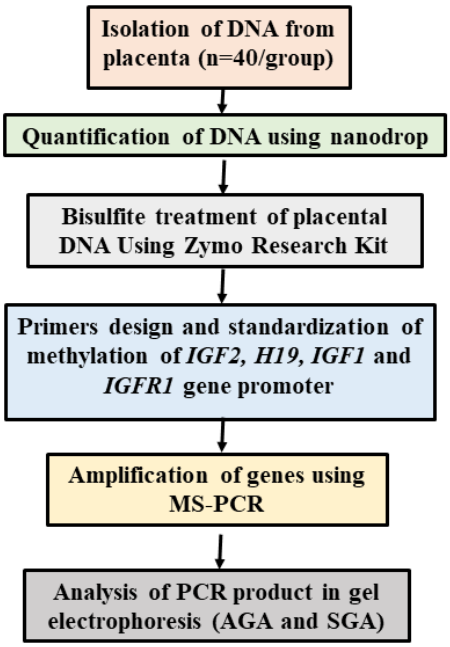

Figure 4.8: The overview of the methodology followed for the analysis of DNA methylation in the placenta of AGA and SGA neonates

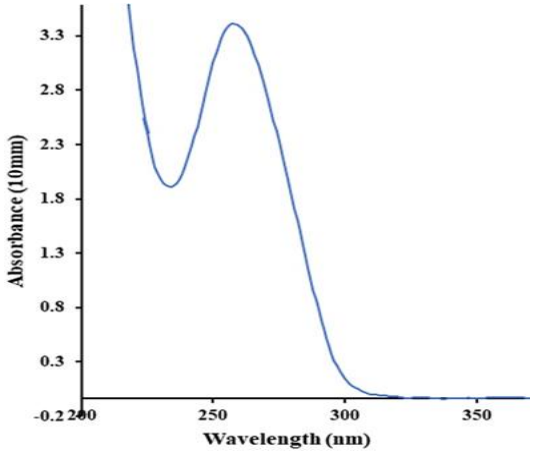

Figure 4.9: The concentration of DNA was quantified using nanodrop at 260 nm

4.2.11. Sodium bisulfite treatment of genomic DNA from the placenta

The bisulfite conversation of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research, USA) as per the manufacturer's instructions (Figure 4.10) *(*Tobi et al., 2011). Briefly, 14 µl of genomic DNA samples were mixed with 100 µl of CT conversion reagent and incubated at 50 °C in a dark area for 12 h. After incubation, the contents were added to Zymo-Spin™ IC Columns (already treated with 400 µl of M-binding buffer) and centrifuged. The columns were washed with M-wash buffer, added 200 µl of M-desulphonation buffer, incubated for 20 min, and centrifuged. Finally, the columns were washed with M-wash buffer, added 10-15 µl of M-elution buffer, and centrifuged. All centrifugations were performed at 10000 xg for 30 sec. The bisulfite-converted DNA was used to analyze the methylation status of *IGF2*, *H19*, *IGF1*, and *IGFR1* gene promoters using methylation specific-PCR (MS-PCR).

Figure 4.10: Bisulfite conversion of AGA and SGA placental DNA

4.2.12. Methylation-specific PCR for promoter regions of *IGF2, H19, IGF1,* and *IGFR1* genes

The methylation status of promoter regions of the genes was identified using MS-PCR described in Ku *et al.,* (2011). *IGF2, H19, IGF1,* and *IGFR1* gene promoter region sequences were obtained from the National Center for Biotechnology Information (NCBI) database. The TSS1 in the promoter region of *IGF1, H19,* and *IGFR1* genes and TSS11 in the *IGF2* gene were used to design the primers. The primers (methylated and unmethylated) were designed using meth primer software (Li and Dahiya 2002). The primer sets were standardized in the laboratory before analysis of the samples for methylation analysis. The composition of the MS-PCR reaction is shown in table 4.7. The conditions used for the MS-PCR are shown in table 4.8.

Table 4.7: The composition of the MS-PCR reaction for identification of methylation status of IGF-axis genes in AGA and SGA neonates

Components	Volume (µl)
10X PCR buffer	2.5
10 mM dNTP	2.5
Forward primer	1.0

Reverse primer	1.0
Autoclaved water	16.7
Taq enzyme	0.3
Bisulfite DNA	1.0
Total	**25**

Table 4.8: The conditions used in MS-PCR for identification of methylation status of IGF-axis genes in AGA and SGA neonates

Step	No. of cycles	Cycles	Temperature (°C)	Duration
1	1	Initial denaturation	94	5 min
2	45 cycles (Steps 2-4)	Denaturation	94	30 sec
3		Annealing	55-61	30 sec
4		Extension	72	30 sec
5	1	Final extension	72	5 min

4.2.13. Analysis of the MS-PCR products obtained from the placental samples of AGA and SGA neonates

The MS-PCR products were analyzed using agarose gel electrophoresis as described in Skiriute *et al.,* (2012). Briefly, the amplified samples using methyl and unmethyl primers were run on 2% agarose gels at 120 V. The gels were visualized using a gel documentation system, and the methylation percentage was calculated. If the samples were positive for both methylated and unmethylated in a gel, it was considered methylation positive. The DNA sample (treated with bisulfite) from healthy human blood lymphocytes was considered a negative control. Standard bisulfite converted universal methylated human DNA standard was used as a positive control. The difference in the percentage of the methylation of *IGF2, H19, IGF1,* and *IGFR1* gene promoters was compared between AGA and SGA neonates. The percent of co-methylation patterns was also determined from the methylation data.

4.2.14. *In vitro* culture of cord blood samples and calculation of the mitotic index

Blood culture setup: The procedure for calculating the mitotic index in the blood samples was followed by the IAEA 2011 manual. Briefly, the cord blood samples (n=98) collected from AGA and SGA neonates were cultured *in vitro* by adding 5 ml RPMI-1640 media, 20% heat-inactivated FBS, 1% antibiotics, 2% PHA, and 500 µl of umbilical cord blood. The blood cultures were incubated at 37 °C in a 5% CO_2 incubator. At 24^{th} h, the cells were arrested at metaphase by adding 10 µl of colchicine (0.02 µg/ml) and further incubated for 24 h. The representative image of the culture of the cord blood sample is shown in figure 4.11.

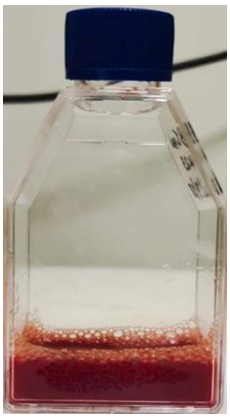

Figure 4.11: The representative image of the culture of the cord blood sample obtained from SGA neonates

Culture harvest: At 48^{th} h, the cultures were harvested by treating them with hypotonic solution (0.075 M KCl) for 30 min at 37 °C in a water bath. The cells were fixed using Carnoy's fixative (3:1 methanol and acetic acid), cast on a clean chilled glass slide, and air-dried overnight in a slide warmer at 37 °C. The slides were stained with 10% Giemsa solution for 5 min at room temperature, and 1000 cells (both blast cells and metaphases) were scored manually from each sample using a light microscope with 40X magnification (Viswanathan et al., 2019). The representative image of the metaphase obtained from the cord blood samples is shown in figure 4.12.

Calculation of the mitotic index: The mitotic index was calculated using the following formula: **MI** = Total number of metaphases/total number of cells scored X100.

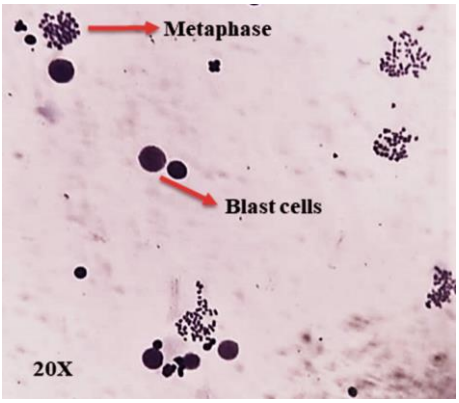

Figure 4.12. Representative image of the metaphase obtained from cord blood samples

4.3. Statistical analysis

The data were represented as mean±standard deviation (SD)/range/percentage. The difference in the variable between the two groups was tested using an unpaired 't-test and Mann-Whitney U test. Pearson's correlation analysis was used to find the association between the two variables. The p-value <0.05 was considered statistically significant. Statistical tests were performed using Microsoft Excel, a statistical package for social studies (SPSS) software. The representative graphs were prepared using Origin software.

Materials and Methods

4. Materials and methods

4.1. Materials

The following list of chemicals, glassware, plastic ware, equipment's, and reagents were used in the present study.

4.1.1. List of chemicals

Agarose: Sigma-aldrich (Cat. #A9539)

Ammonium chloride: Amresco (Cat. #12125-02-9)

Antibiotic antimycotic solution: HiMedia (Cat. #A002)

cDNA conversion kit: Takara (Cat. #RR037A)

Chloroform: Spectrochem (Cat. #67-66-3)

Colchicine: Sigma (Cat. #C9754-1G)

Deoxynucleotide triphosphate (dNTPs): Genei (Cat. #61060250005130)

Disodium hydrogen phosphate (Na_2HPO_4): SD Fine-Chem limited (Cat. #20383K05)

DNA ladder: Genei (Cat. #6126526710017300)

Ethylenediamine tetraacetic acid (EDTA): Thermo Fisher Scientific (Cat. #17892)

ELISA kits (IGF1, IGF2, and IGFBP3): Krishgen Biosystem (Cat. #KBH0411, KBH0392, KBH0391 respectively)

Ethanol: Analytical reagent (Cat. #20180907)

Fetal bovine serum (FBS): Gibco (Cat. #42F1383K)

Gel loading dye **(**6X) Bromophenol blue: Thermo Fisher Scientific (Cat. #R0611)

Giemsa stain: Thermo Fisher Scientific (Cat. #Q38723)

Glacial acetic acid: Pure (Cat. #37013L25)

Hydrochloric acid (HCl): SDFCL (Cat. #20125L25)

Isopropanol: SD Fine-Chem limited (Cat. #39694L25)

Methanol: SD Fine-Chem limited (Cat. #89139L05)

Phytohemagglutinin (PHA): Gibco (Cat. #10576-015)

Positive and negative controls for Methylation PCR: Zymo research (Cat. #D5015)

Potassium bicarbonate ($KHCO_3$): Nice (Cat. #P1422910)

Potassium chloride (KCl): Merck (Cat. #M10D600110)

Potassium dihydrogen phosphate (K2HPO4): Merck (Cat. #F33294)

Primers for DNA methylation of promoter *IGF2*, *H19*, *IGF1*, and *IGFR1* genes: Zymo research (Cat. #D5001)

Proteinase K: Invitrogen (Cat. #2204622)

Sodium bicarbonate ($NaHCO_3$): Thermo Fisher Scientific (Cat. #25080094)

Sodium chloride (NaCl): HiMedia (Cat. #7647-14-5)

Sodium dihydrogen phosphate (NaH_2PO4): Merck (Cat. #7558-80-7)

Sodium dodecyl sulfate (SDS): Sigma-aldrich (Cat. #V800386)

Sodium hydroxide (NaOH): Thermo Fisher Scientific (Cat. #15895)

SYBR green master mix: Bio-Rad (Cat: #1725271)

Taq DNA polymerase: Genei (Cat. #610602500051730)

Tris-Base: SRL (Cat. #2044122)

Trizol reagent: Thermo Fisher Scientific (Cat. #15596018)

4.1.2. Glassware

Beakers: Borosil (Cat. #1002)

Coverslips: Blue star

Cuvette for spectrophotometer: Perkin Elmer UV spectroscopy

Duran Bottles: Borosil (Cat. #3321)

Glass rod: Pacific Star Corporation (Cat. #PFS1007711 3PK)

Measuring cylinders: Borosil (Cat. #579)

Microscopic glass slides: Lab tech (Cat. #S18105)

Reagent bottles: Borosil (Cat. #3321)

Standard flask: Borosil (Cat. #5640029)

4.1.3. Plastic ware

COMB (1.0 mm): Tarsons (Cat. #7087)

Couplin jar (50 ml): Abdos (Cat. #P90101)

Cryo box (1.8 ml): Tarsons (Cat. #524010)

Eppendroff tubes (1.5 ml): Tarsons (Cat. #22363204)

Eppendroff tubes (2 ml): Tarsons (Cat. #22363352)

Falcon tubes (15 ml): Merck (Cat. #CLS431470)

Falcon tubes (50 ml): Merck (Cat. #CLS431472)

Heparin vacutainer: Becton Dickinson (Cat. #367871)

K2 EDTA vacutainer: Becton Dickinson (Cat. #367856)

Microtips (0.2-10 µl): Abdos (Cat. #P10115)

Microtips (100-1000 µl): Abdos (Cat. #P10106)

Microtips (2-20 µl): Abdos (Cat. #P10130)

Parafilm: Bemis (Cat. #PM999)

Pasteur pipette: Tarsons (Cat. #940060)

PCR cooling pack: Merck (Cat. #Z606634)

PCR plates: Thermo Fisher Scientific (Cat. #AB0600)

PCR tube racks: Fisher scientific (Cat. #03-448-20)

PCR tubes (0.2 ml): Tarsons (Cat. #510051)

pH strip: Fisher Scientific (Cat. #HP-V0WD-7PP6)

Sealers: Bio-Rad (Cat. #MSB1001)

Syringe filters: Merck (Cat. #SLGVV255F)

Syringe filters: HiMedia (Cat. #SF14)

Syringes: BD (Cat. #26144)

Whatman filter paper: Merck (Cat. #GSWP04700)

4.1.4. Equipments

-80 °C Freezer: Cryo Scientific (Cat. #URC-V-700-4)

Autoclave: (Cat. #AV-104)

CO_2 incubator: Thermo Scientific (Cat. #371)

Cooling centrifuge (4 °C): Remi (Cat. #CM-12)

Electronic balances: Sartorious (Cat. #GE 612-1)

ELISA reader: Merilyzer (Cat. #EIAQuant)

Gel documentation system: Bio-Rad (Cat. #Et9970616AA)

Gel electrophoresis unit: Bio scientific (Cat. #18-1130-01)

Inverted microscope: (Cat. #3842000306)

Laminar air flow: Esco (Cat. #LAF 35)

Light microscope: Nikon (Cat. #E-200)

Media preparation unit: MRC laboratory instruments

Micro pipettes (1-10 µl, 2-20 µl, 200 µl, and 1 ml): Glassco

Millipore: Millipore system (Cat. #ZRXQ0031N)

Nanodrop: Implen (N60)

Normal centrifuge: Remi (Cat. #R8C)

PCR machine: Bio-Rad (Cat. #1851148)

PCR plate centrifuge: BR Biochem (Cat. #BIDH-100)

pH meter: Sartorious (Cat. #PB-11-P10)

Real-time PCR: Bio-Rad (Cat. #781BR16930)

Slide warmer: YORCO (Cat. #YSI-132)

UV spectrophotometer: Perkin Elmer (Cat. #LAMBDA 35)

Vortex: Remi (Cat. #CM 101)

Water bath: Julabo (Cat. #TW-8)

4.1.5. Preparation of reagents

Phosphate buffer saline (PBS): About 8 g of sodium chloride, 200 mg of potassium chloride, 1.44 g of disodium hydrogen phosphate, and 245 mg of potassium dihydrogen phosphate was dissolved in 1000 ml of double distilled water, and the pH was adjusted to 7.4 by adding 0.1 M HCl or 0.1 M NaOH, autoclaved, and stored at 4 °C.

Erthrolysis buffer (ELB): 155 mM of ammonium chloride (8.29 g), 10 mM of potassium bicarbonate (1.00 g), 0.1 mM of EDTA (200 µg) were dissolved in 1000 ml of double distilled water, and the pH was adjusted to 7.4 by adding 0.1 M HCl or 0.1 M NaOH.

Proteinase K: About 2 mg of proteinase K powder was dissolved in 1 ml of double distilled water.

20% Sodium dodecyl sulfate (SDS): About 20 g of sodium dodecyl sulfate was dissolved in 100 ml of double distilled water.

5 M NaCl: About 29.22 g of NaCl was dissolved in 100 ml of double distilled water.

Roswell Park Memorial Institute (RPMI 1640): RPMI 1640 powder, 2 g of sodium bicarbonate was dissolved in 1000 ml of double distilled water. The pH of the media was adjusted to 7.4 by adding 0.1 M HCl or NaOH. The prepared media was filtered using a 0.22 µm Whatman filter and stored at 4 ºC. 1% of antibiotics was added to the

media, kept the media for sterility check by incubating 24, 48, and 72 h at 37 °C before using it for culturing blood samples.

Fetal bovine serum (FBS): FBS was heat inactivated by incubating at 55 ºC for 30 min and stored at 4 ºC for further use.

Colchicine: About 5 mg of colchicine was dissolved in 5 ml of sterile water and filtered using a syringe filter (stock 1). 100 µl of stock 1 was added to 9.9 ml of sterile distilled water (stock 2). 10 µl (0.02 µg/ml) of stock 2 was added to 5 ml culture, and 20 µl (0.02 µg/ml) to 10 ml culture at 24^{th} h.

Carnoy's fixative: 3:1 ratio of methanol and glacial acetic acid was prepared in a reagent bottle, tightly capped, and stored at 4 ºC.

0.075 M Hypotonic solution: 0.56 g of potassium chloride was dissolved in 100 ml of double distilled water and incubated at 37 ºC for 30 min.

10% Giemsa: 5 ml of commercially available Giemsa solution was mixed with 45 ml of double distilled water.

70% Isopropyl alcohol (IPA): 70 ml of isopropyl alcohol was mixed with 30 ml of double distilled water.

Tris-Acetate EDTA buffer: About 4.85 g of tris base, 1.14 ml of glacial acetic acid, and 2 ml of 0.5 M EDTA were dissolved in 100 ml of double distilled water. The buffer pH was adjusted to 8 by adding 0.1 M HCl or 0.1 M NaOH, autoclaved, and stored at room temperature.

Ethanol (80 and 85%): 80 and 85 ml of ethanol were mixed with 20 and 15 ml of double distilled water.

Primer dilution: The primers used for both gene expression and methylation-specific PCR was diluted as per the manufacturer's instructions. The primers' concentration for gene expression and methylation-specific PCR ranges between 0.5-1 µM.

TE buffer: About 0.2 ml of 0.5 M EDTA, 1 ml of 1 M tris-HCl was added to the reagent bottle and made up to 100 ml using distilled water. The pH of the solution was adjusted to 8 and stored at room temperature.

RNA later buffer: About 175 g of ammonium sulfate, 10 ml of 0.5 M EDTA, and 6.25 ml of 1 M sodium citrate were dissolved in 233.75 ml of distilled water. The pH of the solution was adjusted to 5 and stored at room temperature.

1% Ethidium bromide: About 0.5 g of ethidium bromide was dissolved in 50 ml of distilled water and kept for stirring for 2-3 h.

4.2. Methods

The methods followed in the present study were described as follows.

4.2.1. Study participants and ethics approval

A case-control study was designed, and prior approval was obtained from the Institutional Ethics Committee, Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER), Tamaka, Kolar, Karnataka, India (Ref No: SDUMC/KLR/IEC/32/2019-20). Pregnant women (n=98; 49/group) attending the department of Obstetrics and Gynaecology, R. L. Jalapa Hospital (RLJH), SDUAHER, Tamaka, Kolar, Karnataka, India, were recruited for the study. The pregnant women who underwent both normal vaginal delivery (NVD) and elective lower segment cesarean section (LSCS) were considered for the study. The South Indian pregnant women gave birth to both AGA and SGA babies. The sample size for the present study was calculated based on the difference in levels of IGF-axis components in AGA and SGA groups (Zhang et al., 2015). The required sample size for the present study was determined as n=49/group**.** After obtaining written informed consent from pregnant women, the placental tissue and umbilical cord blood samples were collected following

the inclusion and exclusion criteria for mothers and neonates (Tables 4.1 and 4.2). Both placental tissue and umbilical cord blood samples were transported to the department of Cell Biology and Molecular Genetics, SDUAHER, for further analysis.

4.2.2. Inclusion and exclusion criteria

AGA and SGA neonates were classified based on the intrauterine chart, gestational weeks, and birth weight of neonates. The birth weight of neonates was measured using a Filizola digital scale; the minimum and maximum capacity of the scale is 250 g-15 kg (Bennini et al., 2010).

Table 4.1: The inclusion and exclusion criteria for the selection of pregnant women

Inclusion	Exclusion
➢ Mothers with AGA and SGA neonates	➢ Pre-eclampsia ➢ Gestational diabetes ➢ Gestational hypertension ➢ Placental abruption

Table 4.2: The inclusion and exclusion criteria for the selection of neonates

Inclusion	Exclusion
➢ Full-term neonates ➢ Both genders (AGA and SGA)	➢ Post-term ➢ Large for gestational age ➢ Twins ➢ Congenital anomaly

The overview of the methodology followed in the present study is shown in figure 4.1.

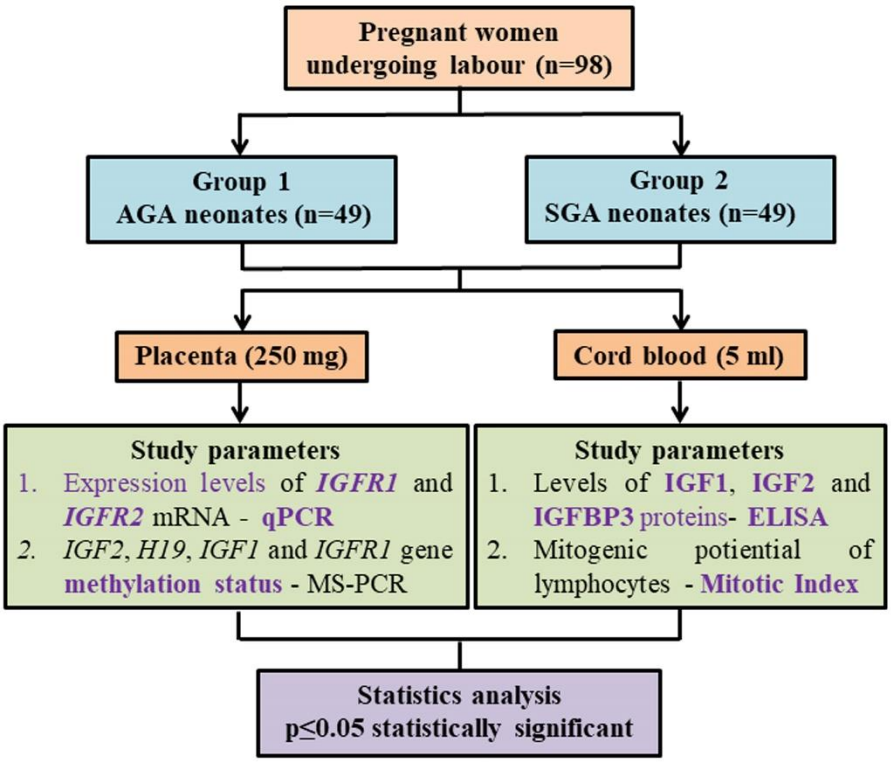

Figure 4.1: The overview of the methodology followed in the present study

4.2.3. Collection of placental tissue samples

After obtaining the written consent, the placental tissue from pregnant women (n=40/group) who underwent labor was collected following inclusion and exclusion criteria (Tables 4.1 and 4.2). After delivery, approximately 150-200 mg of placental tissue samples were collected using a sterile surgical blade and transferred to RNAs later buffer and PBS, stored at -80 ºC until further use. The placental tissue stored in RNA later was used to measure the IGF-axis gene expression. The placental tissue stored in PBS was used to find the methylation status of *IGF2*, *H19*, *IGF1*, and *IGFR1* genes.

4.2.4. Collection of umbilical cord blood samples

About 5 ml of umbilical cord blood samples were collected using a sterile syringe in EDTA and heparin-coated tubes. The samples in the EDTA tube were centrifuged at 3000 rpm for 15 min, and the plasma portion was separated and stored at -80 °C. The plasma samples were used to measure the levels of IGF1, IGF2, and IGFBP3 proteins using the ELISA method. The samples collected in heparinized tubes were cultured *in vitro* to calculate the mitotic index.

4.2.5. Hematological and biochemical parameters of a pregnant woman

The hematological and biochemical parameters data were collected from the medical reports of pregnant women (who participated in the present study) available in the Central Diagnostics Laboratory Services of RLJH, SDUAHER.

4.2.6. Quantification of IGF1, IGF2, and IGFBP3 protein levels in cord blood plasma

The levels of IGF-axis proteins such as IGF1, IGF2, and IGFBP3 proteins in the cord blood plasma (n=40/group) were measured using the ELISA method (Maya et al., 2018). The standards and samples (100 µl) were added to the antibody-coated wells of the microtiter plate, covered with sealer, and incubated at 37 °C for 90 min. After incubation, the contents were discarded and washed 4 times with 1X wash buffer. To the wells, 100 µl of biotinylated antibodies (IGF1, IGF2, and IGFBP3**)** were added to the respective wells and incubated at 37 °C for 60 min. After incubation, the contents were discarded and washed 4 times with 1X wash buffer. To the wells, 100 µl of Streptavidin: Horseradish peroxidase conjugate (HRP) were added and incubated at 37 °C for 30 min. After incubation, the contents were discarded, washed with 1X wash buffer, and 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate were added and

incubated at 37 °C for 10 min (Figure 4.2A). Finally, 50 µl of stop solution was added to the wells, and absorbance was read at 450 nm (Figure 4.2B) using a microplate reader.

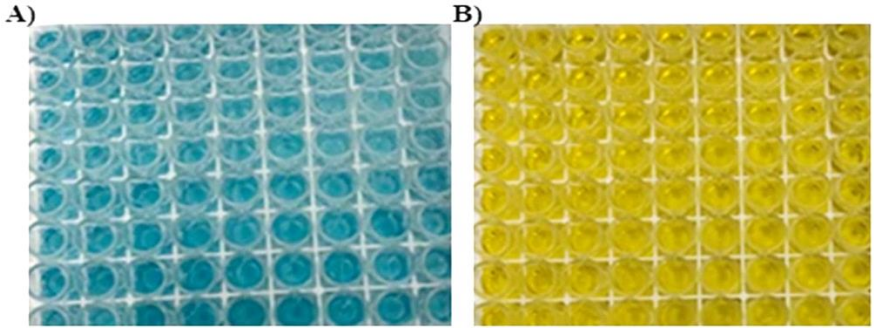

Figure 4.2: Representative image of ELISA plate used to measure the IGF-axis proteins in plasma of AGA and SGA neonates

IGF1, IGF2, and IGFBP3 protein levels in plasma samples were derived from the standard graphs and expressed as ng/µl. The levels of all these proteins between AGA and SGA groups were compared to find the significance.

4.2.7. Isolation of total RNA from placental tissue using the Trizol method

Total RNA from the placental tissue (n=20/group) of AGA and SGA was isolated using the Trizol method (Rio et al., 2010). About 25 mg of placental tissue samples were removed from RNA later buffer and washed with ELB to remove the RBC. To the tissue, 1 ml of Trizol was added and homogenized thoroughly. A representative image of placental tissue is shown in figure 4.3. The overview of the qPCR methodology followed in the study is shown in figure 4.4. The homogenate was transferred to Eppendorf, added 200 µl of chloroform was, mixed thoroughly, and incubated for 5 min at room temperature. The tubes were centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was collected in a fresh Eppendorf tube, added 500 µl of isopropanol alcohol, and incubated for 10 min at room temperature. The tubes were centrifuged, discarded the supernatant and the pellet was washed twice using 75% ethanol. The pellet was kept for air dry for 15 min, added 30 µl of RNAs free water mix well incubate at

55-60 ℃ for 15 min and stored at -80 °C. The concentration of RNA was quantified using a nanodrop (shown in figure 4.5). The quality of the RNA was assessed using denaturing agarose gel electrophoresis. The quality of the RNA sample above 1.8 was used to convert cDNA and analyze the gene expression using Syber green.

Figure 4.3: Representative image of placental tissue samples collected from AGA and SGA neonates

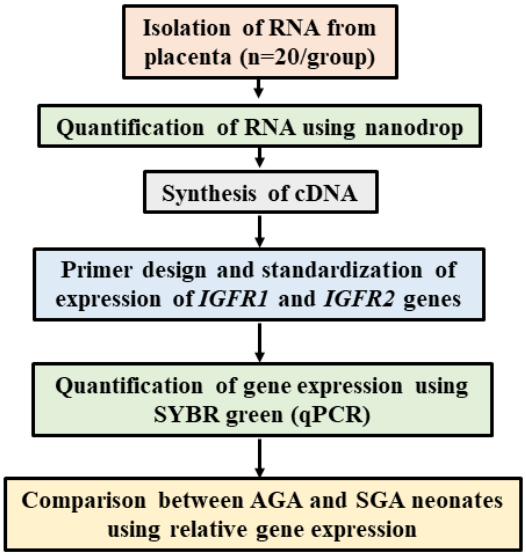

Figure 4.4: The overview of the methodology followed for the study of gene expression changes using qPCR

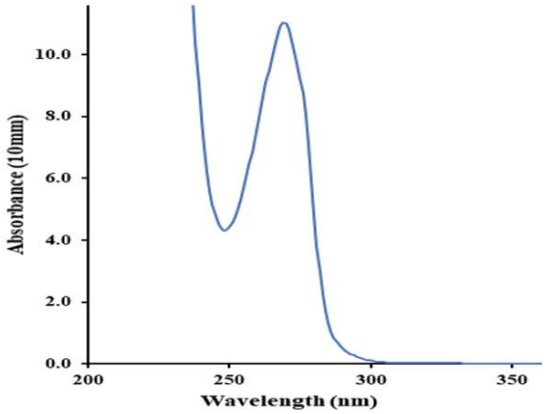

Figure 4.5: The concentration of RNA was quantified using nanodrop at 260 nm

4.2.8. Conversion of total RNA into cDNA

The total RNA (200 ng) samples were converted into cDNA using a prime script RT reagent kit (Tuo et al., 2015). The composition of the reverse transcription reaction is shown in table 4.3. The conditions used for reverse transcription PCR are shown in table 4.4.

Table 4.3: Composition of the reverse transcription reaction

Components	Volume (µl)
5Xprime script buffer	2.0
Prime script RT enzyme mix-I	0.5
Oligo dT primer	0.5
Random hexamers	0.5
RNAse free water	1.5
Total RNA	5.0
Total	**10**

Table 4.4: The conditions used for conversion of RNA to cDNA

Step	Incubation	Duration
1	37 ℃	15 min
2	85 ℃	5 sec
	Hold at 4 ℃	

The cDNA samples were stored at -20 ℃ and used to analyze IGF-axis gene expression using qPCR.

4.2.9. Quantification of *IGFR1* and *IGFR2* gene expression using qPCR

Real-time quantification of *IGFR1* and *IGFR2* gene expression was performed using SYBR green (Browne et al., 2020). The primers for *IGFR1, IGFR2,* and *GAPDH* genes were designed using Primer3 software (Untergasser et al., 2012). The primers were standardized in the laboratory before analyzing the gene expression. The reaction

mixture composition used for quantification is shown in table 4.5. The conditions used for the qPCR reaction are shown in table 4.6.

Table 4. 5: The composition of the qPCR reaction used to quantify gene expression

Components	Volume (µl)
Forward primer	1
Reverse primer	1
RNase free water	1
cDNA	2
SYBR green	5
Total	**10**

Table 4.6: The conditions used for qPCR reaction to quantify gene expression using SYBR green.

Step	No. of cycles	Cycles	Temperatures (°C)	Duration
1	1	Initial denaturation	95	3 min
2	40 cycles	Denaturation	95	10 sec
3*	(steps 2-4)	Annealing	57	30 sec
4*		Extension		

*Plate read at Steps 3 and 4. Green fluorescence is detected at 57 °C

The cycle threshold (Ct) values were obtained by adjusting the threshold in the software attached to qPCR. The representative images of the amplification graphs and melt curves were obtained after amplification and shown in figures 4.6 and 4.7. All the experiments were run as duplicates, and the average values were calculated. The *GAPDH* gene was used as an internal control to normalize the gene expression of interest. The relative expression of *IGFR1* and *IGFR2* genes was calculated using the $2^{-\Delta\Delta Ct}$ method **(**Raavi et al., 2019, Schmittgen et al., 2008). The difference in the expression of *IGFR1* and *IGFR2* genes between the AGA and SGA groups was represented as fold changes.

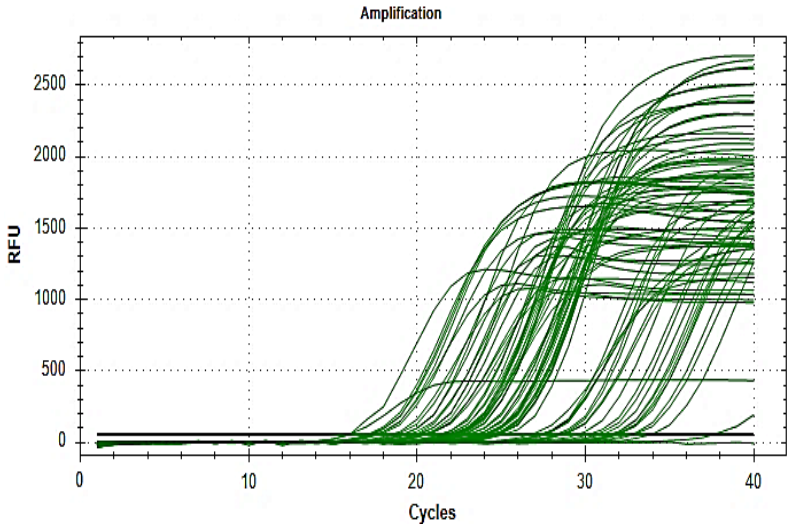

Figure 4.6: Representative graphs of the amplification of the genes in qPCR

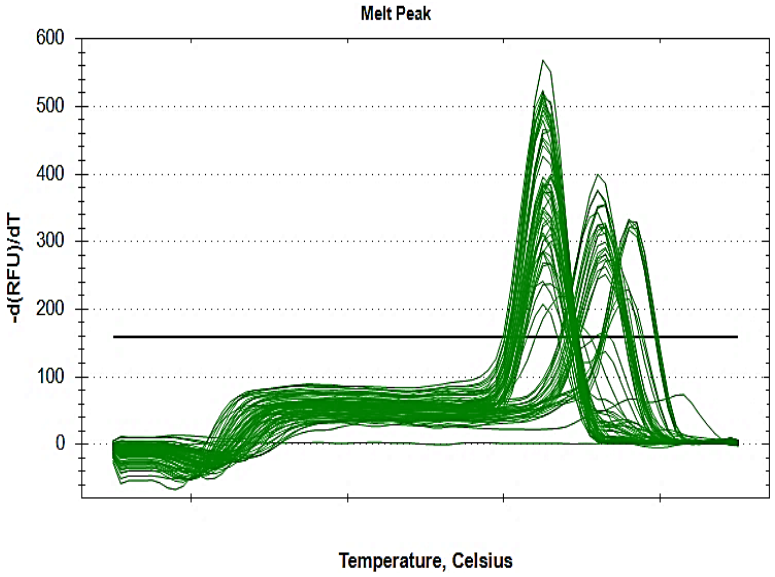

Figure 4.7: Melt cure analysis of the expression of the genes in qPCR

4.2.10. Isolation of DNA from placental tissue

The methodology for isolating DNA from placental samples (n=40/group) was followed as described in Gaaib *et al.*, (2011). The overview of the method followed for the analysis of DNA methylation in the placenta of AGA and SGA neonates is shown in figure 4.8. Briefly, 25 mg of placental tissue samples were rinsed with ELB, minced into small pieces, transferred to a 15 ml falcon tube containing 5 ml of ELB, added 33 µl proteinase-K, and incubated at 37 °C for 2 days. After 2 days, 270 µl of 20% SDS

was added and incubated at 37 ℃ overnight. To the contents, 500 µl of 5 M NaCl and an equal volume of isopropanol were added and mixed thoroughly until the thread-like structure was observed. The thread-like structure was transferred to a 1.5 ml Eppendorf tube, and 1 ml of 80% ethanol was added; the tubes were centrifuged at 3000 rpm for 10 min. The ethanol (80%) wash was repeated twice, and the pellet was dried at room temperature for 30 min. The DNA pellet was dissolved in 500 µl of TE buffer, incubated at 65 ℃ for 25 min, and stored at -20 ℃ until further use. The quality of DNA was analyzed using nanodrop (shown in figure 4.9). The quality of the DNA was assessed using denaturing agarose gel electrophoresis. The quality of the samples above 1.8-1.9 was used to treat with bisulfite.

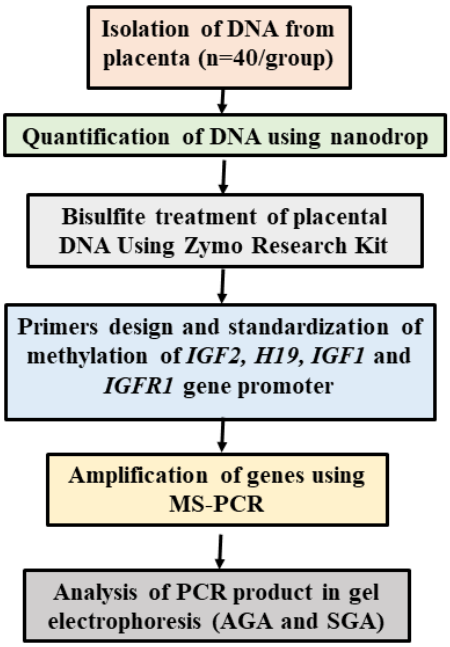

Figure 4.8: The overview of the methodology followed for the analysis of DNA methylation in the placenta of AGA and SGA neonates

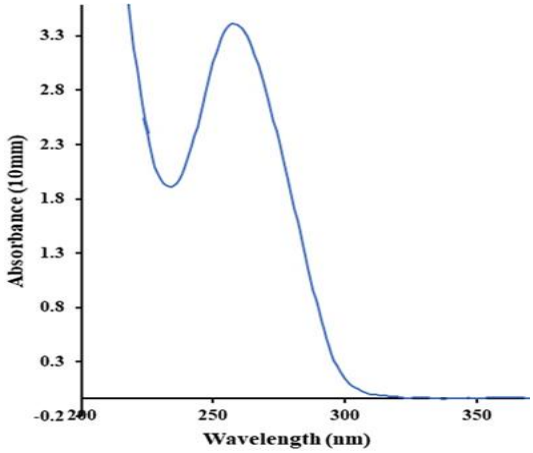

Figure 4.9: The concentration of DNA was quantified using nanodrop at 260 nm

4.2.11. Sodium bisulfite treatment of genomic DNA from the placenta

The bisulfite conversation of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research, USA) as per the manufacturer's instructions (Figure 4.10) *(*Tobi et al., 2011). Briefly, 14 µl of genomic DNA samples were mixed with 100 µl of CT conversion reagent and incubated at 50 °C in a dark area for 12 h. After incubation, the contents were added to Zymo-Spin™ IC Columns (already treated with 400 µl of M-binding buffer) and centrifuged. The columns were washed with M-wash buffer, added 200 µl of M-desulphonation buffer, incubated for 20 min, and centrifuged. Finally, the columns were washed with M-wash buffer, added 10-15 µl of M-elution buffer, and centrifuged. All centrifugations were performed at 10000 xg for 30 sec. The bisulfite-converted DNA was used to analyze the methylation status of *IGF2*, *H19*, *IGF1*, and *IGFR1* gene promoters using methylation specific-PCR (MS-PCR).

Figure 4.10: Bisulfite conversion of AGA and SGA placental DNA

4.2.12. Methylation-specific PCR for promoter regions of *IGF2, H19, IGF1,* and *IGFR1* genes

The methylation status of promoter regions of the genes was identified using MS-PCR described in Ku *et al.,* (2011). *IGF2, H19, IGF1,* and *IGFR1* gene promoter region sequences were obtained from the National Center for Biotechnology Information (NCBI) database. The TSS1 in the promoter region of *IGF1, H19,* and *IGFR1* genes and TSS11 in the *IGF2* gene were used to design the primers. The primers (methylated and unmethylated) were designed using meth primer software (Li and Dahiya 2002). The primer sets were standardized in the laboratory before analysis of the samples for methylation analysis. The composition of the MS-PCR reaction is shown in table 4.7. The conditions used for the MS-PCR are shown in table 4.8.

Table 4.7: The composition of the MS-PCR reaction for identification of methylation status of IGF-axis genes in AGA and SGA neonates

Components	Volume (µl)
10X PCR buffer	2.5
10 mM dNTP	2.5
Forward primer	1.0

Reverse primer	1.0
Autoclaved water	16.7
Taq enzyme	0.3
Bisulfite DNA	1.0
Total	**25**

Table 4.8: The conditions used in MS-PCR for identification of methylation status of IGF-axis genes in AGA and SGA neonates

Step	No. of cycles	Cycles	Temperature (°C)	Duration
1	1	Initial denaturation	94	5 min
2	45 cycles (Steps 2-4)	Denaturation	94	30 sec
3		Annealing	55-61	30 sec
4		Extension	72	30 sec
5	1	Final extension	72	5 min

4.2.13. Analysis of the MS-PCR products obtained from the placental samples of AGA and SGA neonates

The MS-PCR products were analyzed using agarose gel electrophoresis as described in Skiriute *et al.,* (2012). Briefly, the amplified samples using methyl and unmethyl primers were run on 2% agarose gels at 120 V. The gels were visualized using a gel documentation system, and the methylation percentage was calculated. If the samples were positive for both methylated and unmethylated in a gel, it was considered methylation positive. The DNA sample (treated with bisulfite) from healthy human blood lymphocytes was considered a negative control. Standard bisulfite converted universal methylated human DNA standard was used as a positive control. The difference in the percentage of the methylation of *IGF2, H19, IGF1,* and *IGFR1* gene promoters was compared between AGA and SGA neonates. The percent of co-methylation patterns was also determined from the methylation data.

4.2.14. *In vitro* culture of cord blood samples and calculation of the mitotic index

Blood culture setup: The procedure for calculating the mitotic index in the blood samples was followed by the IAEA 2011 manual. Briefly, the cord blood samples (n=98) collected from AGA and SGA neonates were cultured *in vitro* by adding 5 ml RPMI-1640 media, 20% heat-inactivated FBS, 1% antibiotics, 2% PHA, and 500 µl of umbilical cord blood. The blood cultures were incubated at 37 °C in a 5% CO_2 incubator. At 24^{th} h, the cells were arrested at metaphase by adding 10 µl of colchicine (0.02 µg/ml) and further incubated for 24 h. The representative image of the culture of the cord blood sample is shown in figure 4.11.

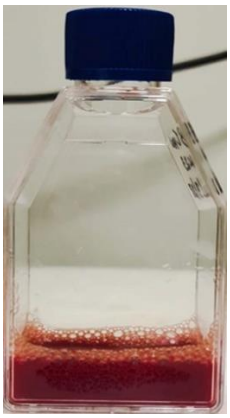

Figure 4.11: The representative image of the culture of the cord blood sample obtained from SGA neonates

Culture harvest: At 48^{th} h, the cultures were harvested by treating them with hypotonic solution (0.075 M KCl) for 30 min at 37 °C in a water bath. The cells were fixed using Carnoy's fixative (3:1 methanol and acetic acid), cast on a clean chilled glass slide, and air-dried overnight in a slide warmer at 37 °C. The slides were stained with 10% Giemsa solution for 5 min at room temperature, and 1000 cells (both blast cells and metaphases) were scored manually from each sample using a light microscope with 40X magnification (Viswanathan et al., 2019). The representative image of the metaphase obtained from the cord blood samples is shown in figure 4.12.

Calculation of the mitotic index: The mitotic index was calculated using the following formula: **MI** = Total number of metaphases/total number of cells scored X100.

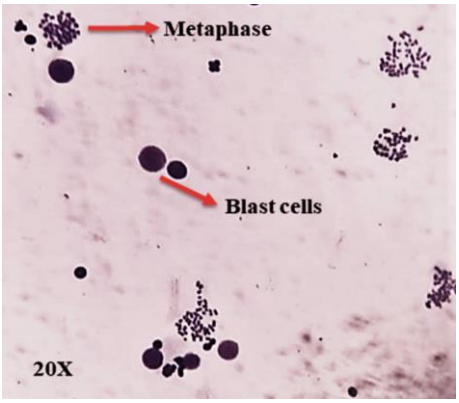

Figure 4.12. Representative image of the metaphase obtained from cord blood samples

4.3. Statistical analysis

The data were represented as mean±standard deviation (SD)/range/percentage. The difference in the variable between the two groups was tested using an unpaired 't-test and Mann-Whitney U test. Pearson's correlation analysis was used to find the association between the two variables. The p-value <0.05 was considered statistically significant. Statistical tests were performed using Microsoft Excel, a statistical package for social studies (SPSS) software. The representative graphs were prepared using Origin software.

Results

5. Results

5.1. Demographic details of the pregnant women and neonates

A total of 98 pregnant women undergoing labor in the department of OBG, RLJH, Kolar, were recruited in the present study based on the inclusion-exclusion criteria. The pregnant women were grouped as AGA (n=49) and SGA (n=49) based on the gestational weeks and birth weight of neonates using the Filizola scale. The demographic details were collected from all the study participants using the proforma. The age (mean±SD) of the mother with AGA neonates is 24.36±3.27, and SGA neonates are 25.16±4.32. There is no significant difference (p=0.914) in the age of the mother with AGA and SGA neonates. The gestational weeks (mean±SD) of the mother with AGA neonate is 35.79±3.71, and SGA neonates are 35.51±3.10. There is no significant difference (p=0.069) in the gestational weeks of the mothers with AGA and SGA neonates. The percentage of normal vaginal delivery and lower segment cesarean section delivery in AGA neonates is 59.1% and 40.9%, and in SGA neonates are 55.1% and 44.9%, respectively. The birth weight (kg) (mean±SD) of AGA neonates is 2.75±0.39, and SGA neonates are 2.16±0.40. The birth weight of SGA neonates is significantly less (p=0.0001) when compared to AGA neonates. The percentage of male and female neonates in AGA neonates is 42.8% and 57.2%, and in SGA neonates are 61.2% and 38.8%, respectively. The demographic details of the pregnant women and neonates was shown in table 5.1.

Table 5.1: Demographic details of the pregnant women and neonates who participated in the present study

Parameter	AGA (n=49)	SGA (n=49)	p-value
Age of the pregnant women (Years) (mean±SD)	24.36±3.27	25.16±4.32	0.914

Gestational weeks (mean±SD)	35.79±3.71	35.51±3.10	0.069
Mode of delivery (NVD/LSCS)	29/20	27/22	-
Birth weight (kg) (mean±SD)	2.75±0.39	2.16±0.40	0.0001
Gender of the neonate (M/F)	21/28	30/19	-

***M**=Male; **F**=Female; **AGA**=Appropriate for gestational age; **SGA**=Small for gestation age; **NVD**=Normal vaginal delivery; **LSCS**=Lower segment caesarean section; **n**=Number of study subjects; **kg**=Kilograms; **SD**=Standard deviation*

5.2. Hematological/biochemical parameters of mothers with AGA and SGA neonates

To find whether the hematological/biochemical parameters were different among the mothers with AGA (n=49) and SGA (n=49) neonates, we collected the information from the case sheets of the pregnant women undergoing labor. The RBC count (million/mm^3) (mean±SD) of the mother with AGA neonates is 4.78±0.40, and the mother with SGA neonates is 4.32±0.39. The WBC count (mean±SD) (thousands/mm^3) of mothers with AGA neonates is 13.73±1.24, and for mothers with SGA neonates is 13.24±1.34. The percentage of hemoglobin (mg/dl) (mean±SD) of mothers with AGA neonates is 11.99±1.0, and for mothers with SGA neonates is 12.31±0.81. The platelet count (T/mm^3) (mean±SD) of mothers with AGA neonates is 216.79±23.68, and mother with SGA neonates is 221.41±20.45. The blood urea (mean±SD) of a mother with an AGA neonate is 26.42±0.76, and the mother with SGA neonates is 24.86±0.68. The RBC (p=0.18), WBC (p=0.62), percentage of hemoglobin (p=0.109), platelet count (p=0.340), and blood urea (p=0.151) of the mother with AGA and SGA neonates did not show any significant difference. The serum creatinine (mg/dl) (mean±SD) of a mother with an AGA neonate is 0.48±0.09, and the mother with SGA neonates is

0.43±0.06. The serum creatinine is significantly (p=0.02) higher in mothers with SGA neonates compared to AGA neonates. The details of the hematological/biochemical parameters of mothers with AGA and SGA neonates are shown in table 5.2.

Table 5.2: Hematological/biochemical parameters of mothers with AGA and SGA neonates

Parameter	AGA (n=49)	SGA (n=49)	p-value
RBC count(mil/mm3)	4.78±0.40	4.32±0.39	0.186
WBC count (T/mm3)	13.73±1.24	13.24±1.34	0.62
% Hemoglobin (mg/dl)	11.99±1.0	12.31±0.81	0.109
Platelet count (T/mm3)	216.79±23.68	221.41±20.45	0.340
Serum creatinine (mg/dl)	0.43±0.06	0.48±0.09	0.024
Blood urea (mg/dl)	26.42±0.76	24.86±0.68	0.151

***AGA**=Appropriate for gestational age; **SGA**=Small for gestation age*; ***RBC**=Red blood cell; **WBC**=White blood cells; **mil/mm^3**=millions per cubic millimeter; **mg/dl**=milligrams per deciliter; **T/mm^3**=metric ton per millimeter*

5.3. The levels of IGF-axis components in AGA and SGA neonates

The IGF-axis components play an important role in the growth and development of the fetus and neonates. The levels of the IGF-axis components are reported to be altered in the SGA neonates. Monitoring the levels of the IGF-axis components might help better manage SGA neonates. In the present study, we have investigated the levels of major IGF-axis components such as IGF-axis proteins (IGF1, IGF2, and IGFBP3), IGF-axis gene expression (*IGFR1* and *IGFR2*), and the methylation pattern of the IGF-axis genes (*IGF2*, *H19*, *IGF1*, and *IGFR1*).

5.3.1. Quantification of the levels of IGF-axis proteins in the cord blood plasma of AGA and SGA neonates

To find the IGF-axis protein levels in AGA neonates (n=49) and SGA neonates (n=49), samples of umbilical cord blood plasma were collected, and IGF1, IGF2, and

IGFBP3 proteins were analyzed using the ELISA method. The concentration of IGF-axis proteins in the AGA and SGA plasma was quantified based on the standard graphs generated using an ELISA kit.

5.3.1.1. The levels of IGF1 protein in the cord blood plasma of AGA and SGA neonates

The levels of plasma IGF1 protein were measured using the equation obtained from the standard graph (y=0.0009x+0.1876) and expressed as ng/ml. The standard graph for IGF1 protein was shown in figure 5.1A (r=0.95). The plasma level of IGF1 protein (mean±SD; range) in AGA neonates is 118±33 (54-210), and SGA neonates are 118±47 (4-294) ng/ml. The IGF1 protein level in the cord blood plasma did not differ (p=1) between AGA and SGA neonates. The range of the plasma IGF1 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates is shown in figure 5.1B.

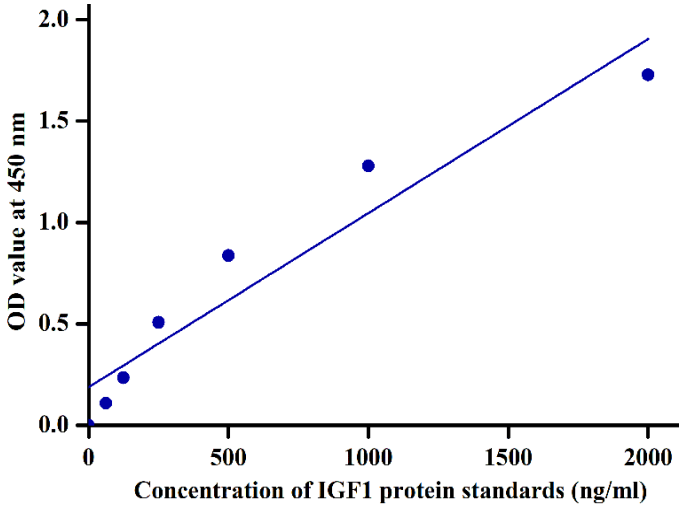

Figure 5.1A: The standard graph for the IGF1 protein was generated using standards provided in the ELISA kit

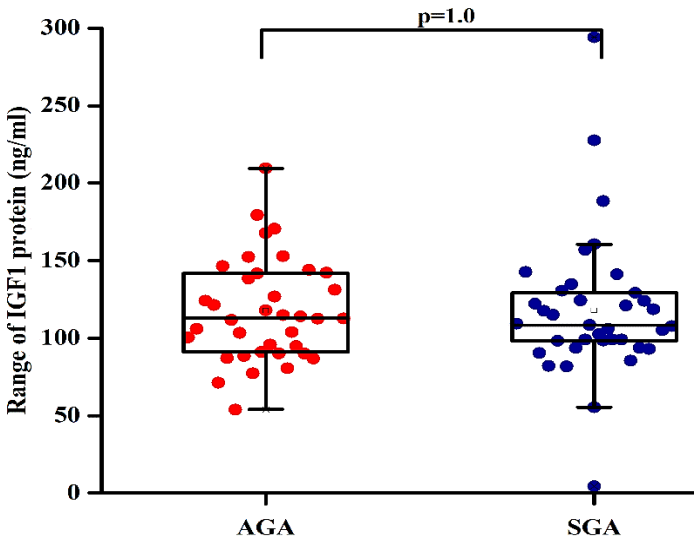

Figure 5.1B: The range of the plasma IGF1 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates

Correlation analysis was performed to find the association between the plasma level of IGF1 protein vs. gestational weeks (AGA and SGA). There is no correlation between the plasma level of IGF1 protein with gestational weeks (r=0.24) **(**Figure 5.1C).

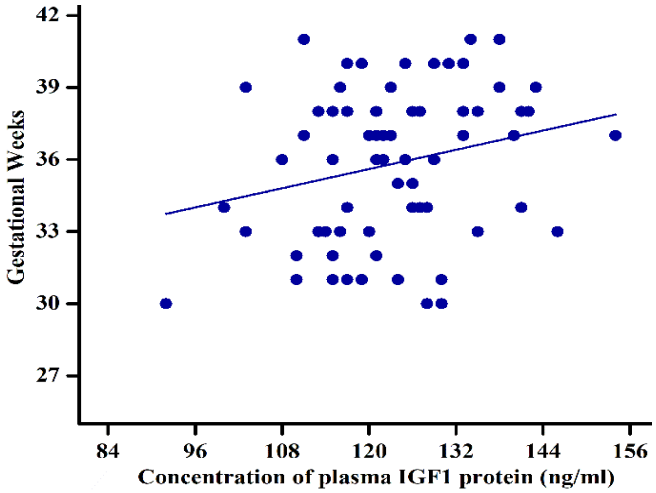

Figure 5.1C: Correlation between plasma level of IGF1 protein and gestational weeks of AGA and SGA neonates

Further, the correlation analysis was performed to find the association between the plasma level of IGF1 protein and the neonates birth weight (AGA and SGA). There

is no correlation between the plasma level of IGF1 protein and the birth weight of the neonates (r=-0.008) (Figure 5.1D).

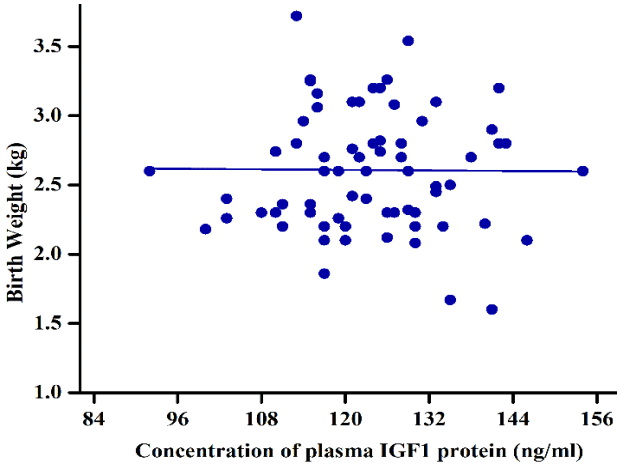

Figure 5.1D: Correlation between plasma level of IGF1 protein with a birth weight of AGA and SGA neonates

5.3.1.2. The levels of IGF2 protein in the cord blood plasma of AGA and SGA neonates

The levels of plasma IGF2 protein were measured using the equation obtained from the standard graph (y=0.0006x+0.1215) and expressed as ng/ml. The standard graph for the IGF2 protein is shown in figure 5.2A (r=0.98). The plasma levels (mean±SD; range) of IGF2 protein in AGA neonates is 124±10 (103-146), and in SGA neonates, they are 123±12 (92-154) ng/ml. The IGF2 protein levels in the cord blood plasma did not differ (p=0.69) between AGA and SGA neonates. The range of the plasma IGF2 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates is shown in figure 5.2B.

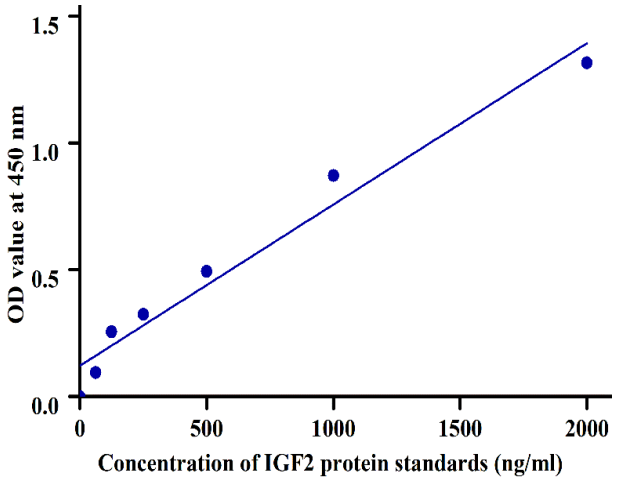

Figure 5.2A: The standard graph for the IGF2 protein was generated using standards provided in the ELISA kit

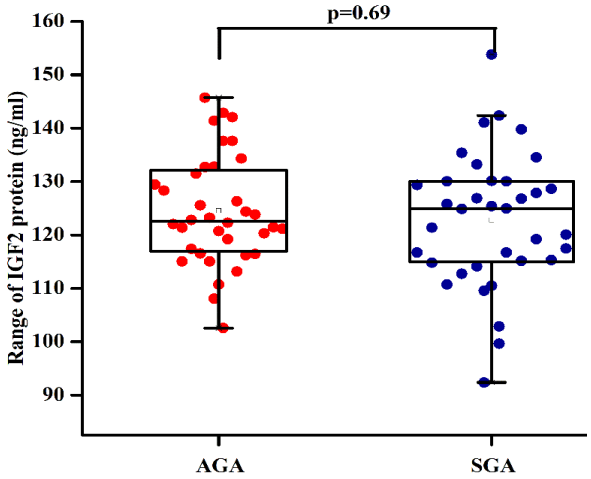

Figure 5.2B: The range of the plasma IGF2 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates

Correlation analysis was performed to find the association between the plasma level of IGF2 protein vs. gestational weeks (AGA and SGA). There is no correlation between the plasma level of IGF2 protein with gestational weeks (r=0.24) (Figure 5.2C).

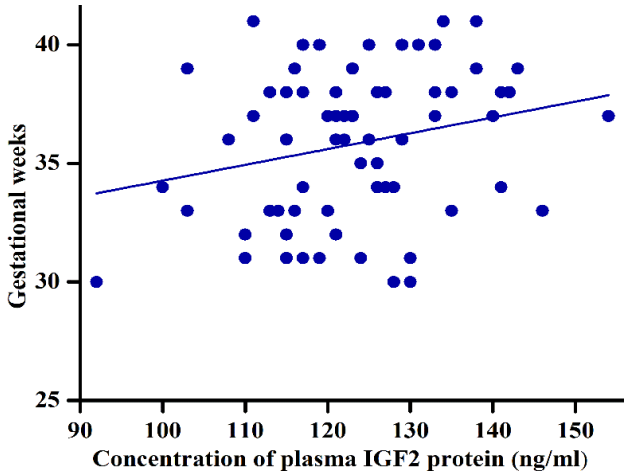

Figure 5.2C: Correlation between plasma level of IGF2 protein and gestational weeks of AGA and SGA neonates

Further, correlation analysis was performed to find the association between the plasma level of IGF2 protein vs birth weight of the AGA and SGA neonates. There is no correlation between the plasma level of IGF2 protein and the birth weight of AGA and SGA neonates (r=0.01) (Figure 5.2D).

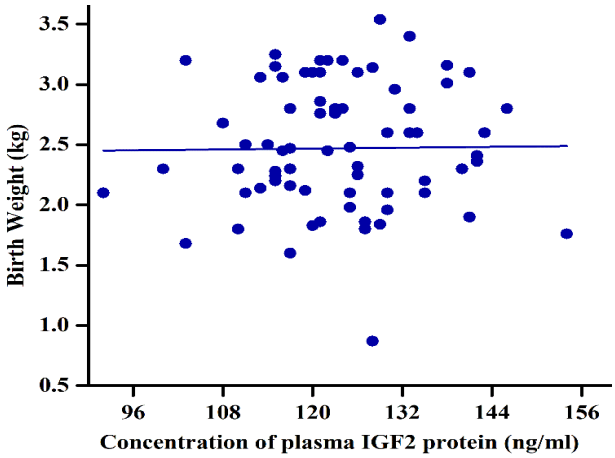

Figure 5.2D: Correlation between plasma level of IGF2 protein and birth weight of the AGA and SGA neonates

5.3.1.3. The levels of IGFBP3 protein in the cord blood plasma of AGA and SGA neonates

The levels of plasma IGFBP3 protein were measured using the equation obtained from the standard graph (y=0.1302x+0.0018) and expressed as ng/ml. The standard

graph for the IGFBP3 protein was shown in figure 5.3A (r=0.99). The plasma levels of IGFBP3 protein (mean±SD; range) in AGA neonates is 1606±277 (1032-2006), and SGA neonates are 1432±387 (315-2137) ng/ml. The IGFBP3 protein level in the plasma is significantly (p=0.02) less in SGA neonates compared to AGA neonates. The range of the plasma IGFBP3 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates is shown in figure 5.3B.

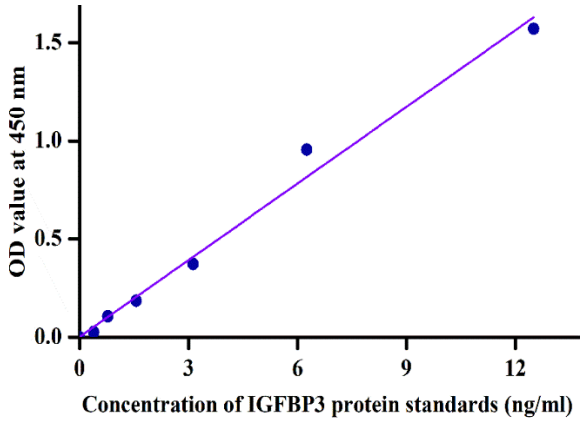

Figure 5.3A: The standard graph for the IGFBP3 protein was generated using standards provided in the ELISA kit

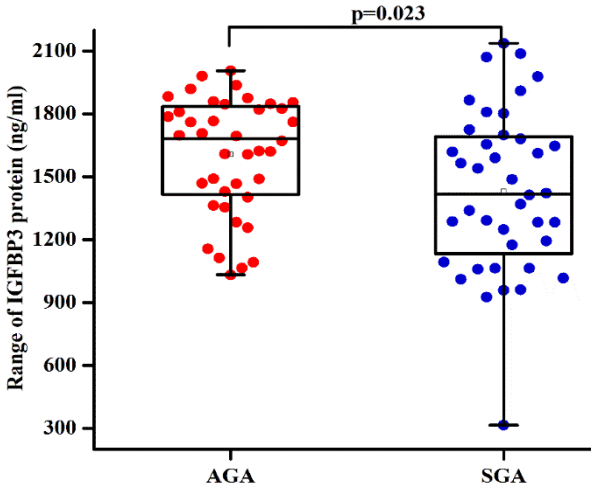

Figure 5.3B: The range of the plasma IGFBP3 protein (ng/ml) in the cord blood plasma of AGA and SGA neonates

Correlation analysis was performed to find the association between the plasma level of IGFBP3 protein vs. gestational weeks. There is no correlation between the plasma levels of IGFBP3 protein with gestational weeks (r=0.01) **(**Figure 5.3C).

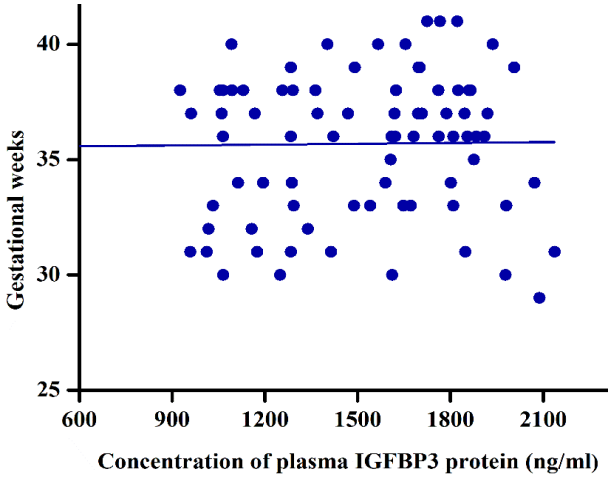

Figure 5.3C: Correlation between the plasma levels of IGFBP3 protein and gestational weeks of AGA and SGA neonates

Further, correlation analysis was performed to find the association between the plasma level of IGFBP3 protein vs. the birth weight of AGA and SGA neonates. There is no correlation between the plasma level of IGFBP3 protein and the birth weight of the AGA and SGA neonates (r=0.158) **(**Figure 5.3D).

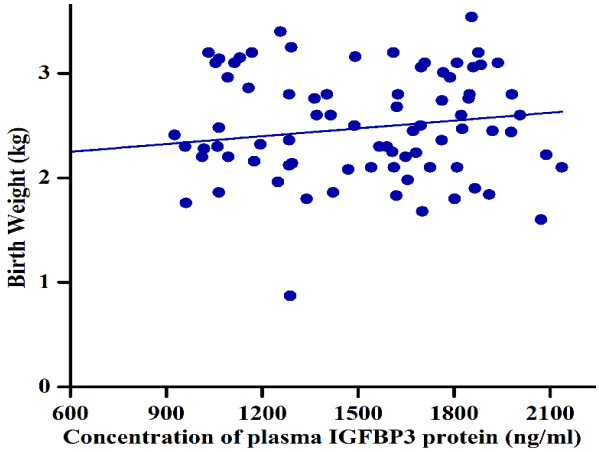

Figure 5.3D: Correlation between plasma level of IGFBP3 protein and birth weight of the AGA and SGA neonates

5.3.2. Quantification of IGF receptor gene expression in the placenta of AGA and SGA neonates

The IGF1 and IGF2 protein levels did not differ between AGA and SGA neonates, but the levels of IGFBP3 protein were significantly lower in SGA compared to AGA neonates. Further, the study was extended to find the difference in the expression levels of *IGFR1* and *IGFR2* genes in the placenta of AGA (n=20) and SGA (n=20) neonates. The expression of *IGFR1* and *IGFR2* genes was analyzed using qPCR (SYBR green chemistry). The primers for qPCR amplification of the two genes were designed using the "Primer 3" tool. The sequence of the primers used for the quantification of gene expression is shown in table 5.3. The primers were standardized for annealing temperature and specific amplification of the genes. The representative agarose gel image of the standardization of primers for *IGFR1* and *IGFR2* genes is shown in figure 5.4. The expression of *IGFR1* and *IGFR2* genes in the AGA and SGA samples was analyzed using relative gene expression. The *GAPDH* gene expression was used as an internal control to normalize the expression of target genes. The samples were run in duplicates and the fold change expression between two (ΔΔCt of SGA - ΔΔCt of AGA) groups was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 5.3: The list of primers used for the analysis of *IGFR1* and *IGFR2* gene expression in the placenta of AGA and SGA neonates

Gene	Primer sequences (5'-3')	Size of PCR product
IGFR1	*FP*: CTACGTGAAGATCCGCCATT *RP*: TGCAAGTTCTGGTTGTCGAG	128 bp
IGFR2	*FP*: ATGCACGACTTGAAGACACG *RP*: GTGATTTGTGCCTTGCTGGC	120 bp
GAPDH	*FP*: ACCCAGAAGACTGTGGATGG *RP*: CAGTGAGCTTCCCGTTCAG	121 bp

IGFR1*=Insulin like growth factor receptor1*; ***IGFR2****=Insulin like growth factor receptor2*; ***GAPDH****=Glyceraldehyde-3-phosphate dehydrogenase*; ***FP****=forward primer*; ***RP****=reverse primer*

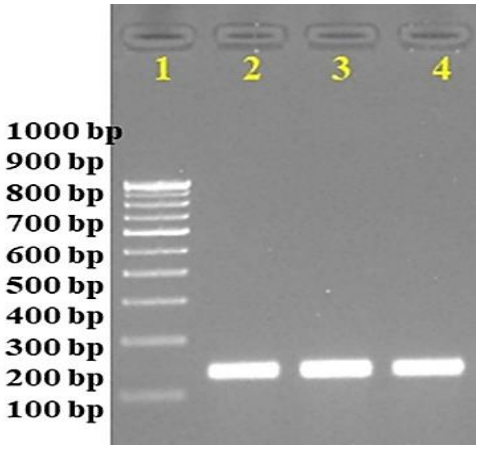

Figure 5.4: Representative agarose gel image of the standardization of primers used for quantification of *IGFR1* (lane 2), *IGFR2* (lane 3), *GAPDH* (lane 4) gene expression, and ladder (lane 1)

5.3.2.1. Expression of *IGFR1* gene in the placenta of AGA and SGA neonates

The normalized expression (ΔCt) of the *IGFR1* gene was obtained by subtracting the Ct value of the *IGFR1* gene with the *GAPDH* gene. The relative expression (ΔΔCt) for the *IGFR1* gene was calculated by subtracting the ΔCt value of SGA neonates from ΔCt value of AGA neonates. The ΔCt of *IGFR1* (mean±SD; range) gene obtained from placental tissue of AGA neonates is 5.06±4.28 (-4.82-10.63) and SGA neonates are 7.03±3.82 (1.44-13.20). The normalized expression of the *IGFR1* gene in the placenta of AGA and SGA neonates was shown in figure 5.5A.

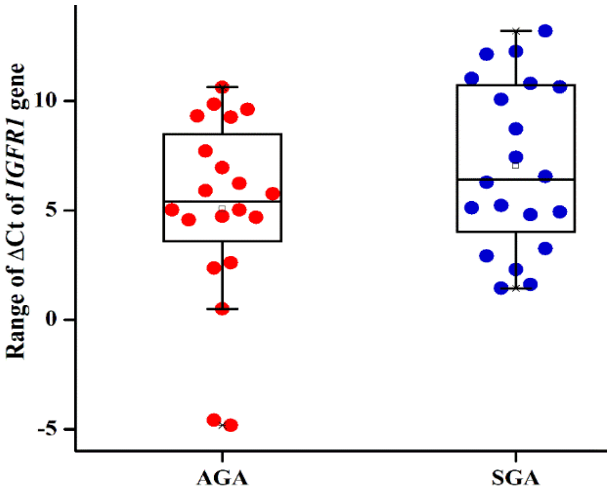

Figure 5.5A: Range of the normalized expression (ΔCt) level of *IGFR1* gene in the placenta of AGA and SGA neonates

The fold change expression between the two groups was calculated using $2^{-\Delta\Delta Ct}$ method. The *IGFR1* gene expression was 3.9 folds down-regulated in SGA compared to AGA neonates. The fold change expression of the *IGFR1* gene in the placenta of AGA and SGA neonates is shown in figure 5.5B.

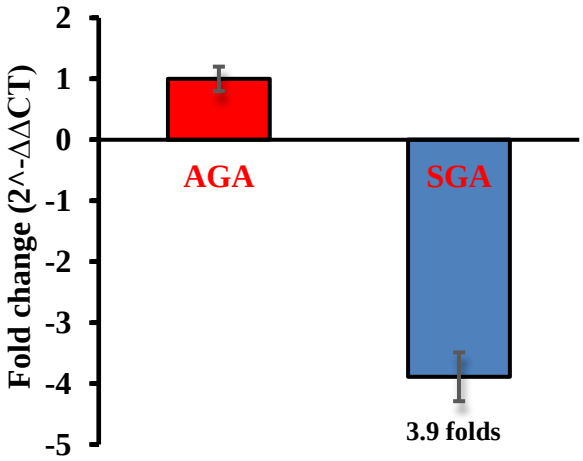

Figure 5.5B: Fold change expression of *IGFR1* gene in the placenta of AGA and SGA neonates

Correlation analysis was performed to find the association between the expression of the *IGFR1* gene vs. gestational weeks (AGA and SGA). The expression of the *IGFR1* gene did not correlate with gestational weeks (r=0.14) (Figure 5.5C).

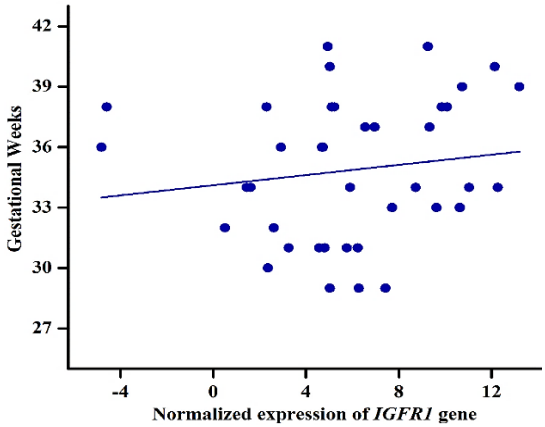

Figure 5.5C: Correlation between expression of *IGFR1* gene and gestational weeks of AGA and SGA neonates

Further, correlation analysis was performed to find the association between the expression of the *IGFR1* gene vs. the birth weight of AGA and SGA neonates. The expression of the *IGFR1* gene did not correlate with the birth weight of the AGA and SGA neonates (r=-0.046) (Figure 5.5D).

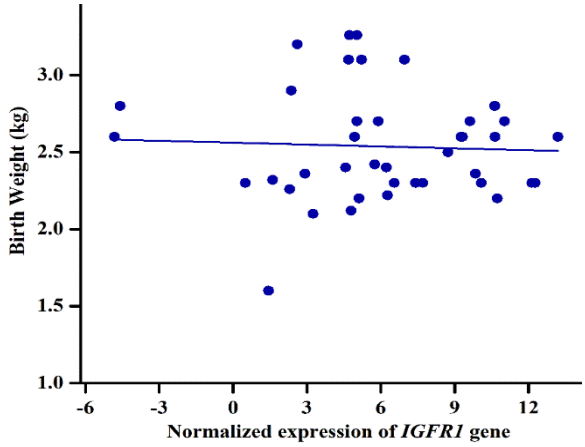

Figure 5.5D: Correlation between the expression of the *IGFR1* gene with the birth weight of AGA and SGA neonates

Further, correlation analysis was performed to find the association between the expression of the *IGFR1* gene vs. plasma levels of IGF1, IGF2, and IGFBP3 proteins. The expression of the *IGFR1* gene did not correlate with IGF1, IGF2, and IGFBP3 protein levels (r=0.01).

5.3.2.2. Expression of *IGFR2* gene in the placenta of AGA and SGA neonates

The normalized expression (ΔCt) of the *IGFR2* gene was obtained by subtracting the Ct value of the *IGFR2* gene with the *GAPDH* gene. The relative expression (ΔΔCt) for the *IGFR2* gene was calculated by subtracting the ΔCt value of SGA neonates from ΔCt value of AGA neonates. The ΔCt of the *IGFR2* (mean±SD; range) gene obtained from placental tissue of AGA neonates is 2.45±3.56 (-7.67-6.04), and SGA neonates are 3.94±1.59 (1.66-7.96). The normalized expression of the *IGFR2* gene in the placenta of AGA and SGA neonates was shown in figure 5.6A.

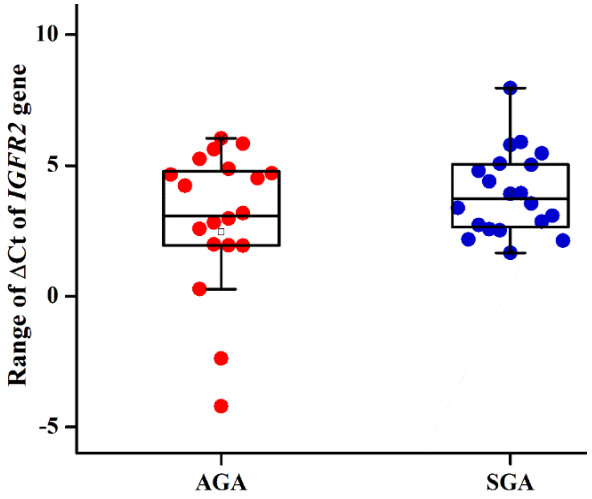

Figure 5.6A: Normalized expression (ΔCt) level of *IGFR2* gene in the placenta of AGA and SGA neonates

The fold change expression between the two groups was calculated using the $2^{-\Delta\Delta Ct}$ method. The *IGFR2* gene was 2.8 folds down-regulated in SGA compared to AGA neonates. The fold change expression of the *IGFR2* gene in the placenta of AGA and SGA neonates is shown in figure 5.6B.

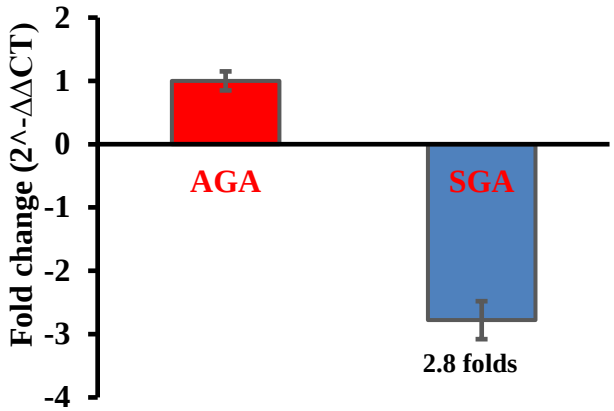

Figure 5.6B: The fold change expression of the *IGFR2* gene in the placenta of AGA and SGA neonates

Correlation analysis was performed to find the association between the expression of the *IGFR2* gene and gestational weeks (AGA and SGA). The expression of the *IGFR2* gene did not correlate with the gestational weeks of AGA and SGA neonates (r=0.12) (Figure 5.6C).

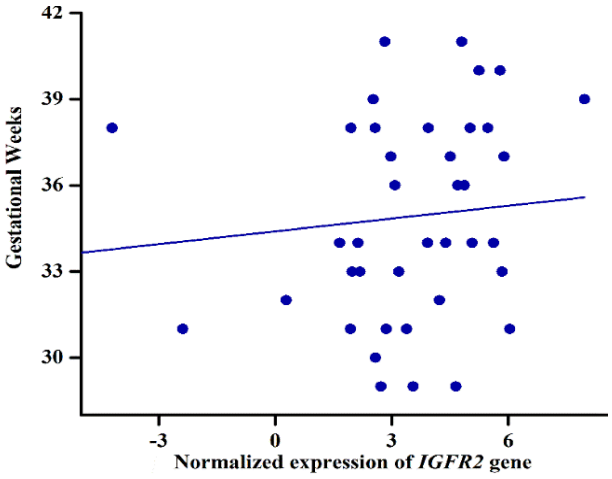

Figure 5.6C: Correlation between the expression of the *IGFR2* gene in the placenta and gestational weeks of AGA and SGA neonates

Further, correlation analysis was performed to find the association between the expression of the *IGFR2* gene vs. the birth weight of AGA and SGA neonates. The

expression of the *IGFR2* gene did not correlate with the birth weight of AGA and SGA neonates (r=0.125) (Figure 5.6D).

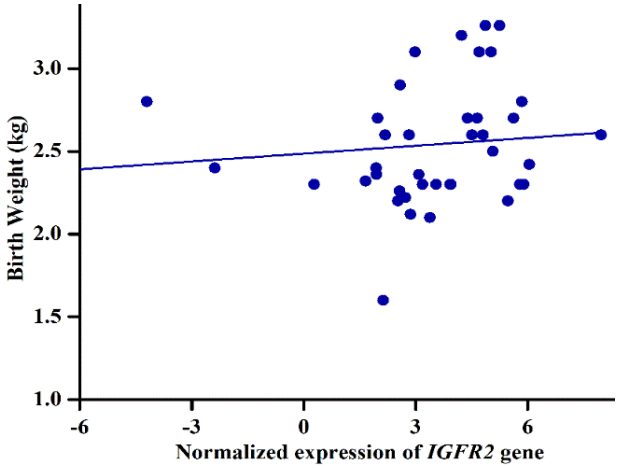

Figure 5.6D: Correlation between the expression of the *IGFR2* gene in the placenta and birth weight of AGA and SGA neonates

Correlation analysis was performed to find the association between the expression of the *IGFR2* gene vs. plasma levels of IGF1, IGF2, and IGFBP3 proteins. The expression of the *IGFR2* gene did not correlate with IGF1, IGF2, and IGFBP3 protein levels in AGA and SGA neonates (r=0.02).

5.3.3. Methylation status of the promoter regions of IGF-axis genes in the placenta of AGA and SGA neonates

The IGFBP3 protein levels were significantly lower in the cord blood plasma, and the expression of *IGFR1* (3.9-folds) and *IGFR2* (2.8-folds) genes was downregulated in the placenta of SGA compared to AGA neonates. Further, the study was extended to find the epigenetic alternation, such as the methylation of promotors of both imprinted (*IGF2* and *H19*) and non-imprinted genes (*IGF1* and *IGFR1)* in AGA and SGA neonates. The methylation status of promoter regions of IGF-axis genes in the placenta of AGA and SGA neonates was analyzed using MS-PCR. The sequences of *IGF2, H19, IGF1,* and *IGFR1* gene promoter regions were obtained from the NCBI database. The

transcription starts site (TSS)1 in the promoter region of *IGF1, H19,* and *IGFR1* genes and TSS11 in the *IGF2* gene was used to design primers. The primers (methylated and unmethylated) specific to *IGF2, H19, IGF1,* and *IGFR1* gene promoter regions were designed using meth primer software. The sequence of primers, number of CpG islands, and the length of PCR product used to analyze imprinted and non-imprinted genes were presented in table 5.4. The primers were standardized for annealing temperature and specific amplification of the genes.

Table 5.4: The list of primers used for the analysis of methylation status of promoters of *IGF2, H19, IGF1,* and *IGFR1* genes

Gene	Region	No. of CpG islands	Primer sequences (5'-3')	Size of PCR product
IGF2	TSS11	1	*MF: TATTGGGGTTTTTTAAATGTAGAC*	289 bp
			MR: CCTAAACGCTAACAACAACCG	
			UF: GGGGTTTTTTAAATGTAGATGA	287 bp
			UR: AACCTAAACACTAACAACAACCAAA	
H19	TSS1	1	*MF: GATCGACGGATTTATAGCGG*	174 bp
			MR: TCTAAAAACCGCATATTACCGAA	
			UF: GATTGATGGATTTATAGTGGGG	175 bp
			UR: ATCTAAAAACCACATATTACCAAAC	
IGF1	TSS1	1	*MF: GTAATTGGGATAAGGGGTTATTC*	228 bp
			MR: TACTAAACATAAAAACACAAACGTC	
			UF: TAATTGGGATAAGGGGTTATTTGA	227 bp
			UR: TACTAAACATAAAAACACAAACATC	
IGFR1	TSS1	2	*MF: CGTATTAGGCGAATTCGAGA*	176 bp
			MR: GCTAAAAATCACAACCGAAAC	
			UF: GAGTGTATTAGGTGAATTTGAGA	183 bp
			UR: ACTCACTAAAAATCACAACCAAAAC	

IGF*=Insulin-like growth factor;* ***IGFR****=Insulin-like growth factor receptor;* ***TSS****=Transcription start site;* ***MF****=Methylated forward primer;* ***MR****=Methylated reverse primer;* ***UF****=Unmethylated forward primer;* ***UR****=Methylated reverse primer;*

CpG*=Cytosine nucleotide followed by Guanine in a DNA sequence;* ***PCR****=Polymerase chain reaction;* ***H19****=Gene coding the noncoding RNA;* ***bp****=Base pair*

5.3.3.1. Methylation status of *IGF2* gene promoter in the placenta of AGA and SGA neonates

The percentage of methylation-positive samples for the paternally imprinted gene (*IGF2*) in the placental of AGA and SGA neonates were calculated from the MS-PCR agarose gel images. The representative agarose gel image of methylation of the imprinted *IGF2* gene was shown in figure 5.7A. The methylation percentage in AGA neonates is 70% (28/40 samples), and in SGA neonates was 67.5% (27/40 samples). The methylation of the *IGF2* gene was 2.5% lower in SGA neonates compared to AGA neonates. The percentage of *IGF2* methylation in the placenta of AGA and SGA is shown in figure 5.7B.

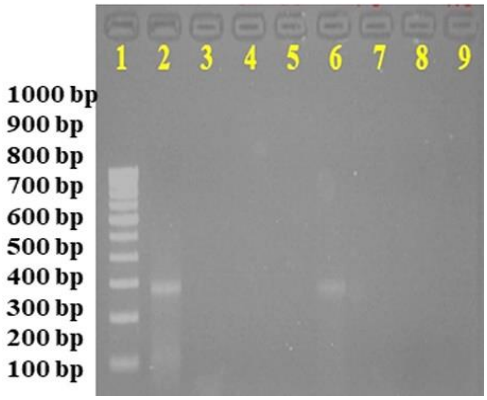

Figure 5.7A: Representative agarose gel image of imprinted (*IGF2)* gene methylation in AGA and SGA neonates. ***1****=DNA ladder (100 bp);* ***2, 3****=AGA methylation and unmethylation;* ***4, 5****=SGA methylation and unmethylation;* ***6, 7*** *=Positive methylation and unmethylation;* ***8, 9****=Negative methylation and unmethylation*

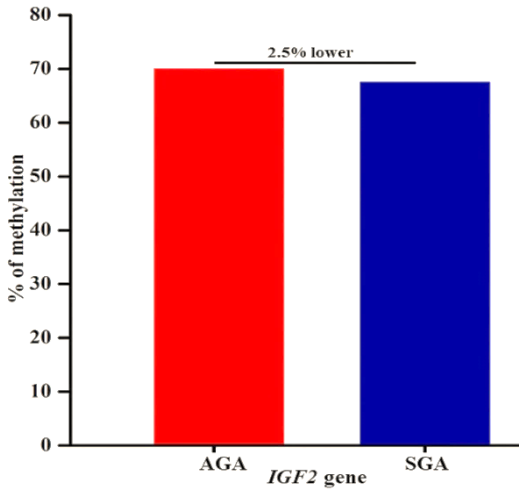

Figure 5.7B: The percentage of methylation of the promoter of the *IGF2* gene in the placenta of AGA and SGA neonates

To find the influence of methylation of *IGF2* on IGF-axis components, the levels of IGF1, IGF2, and IGFBP3 protein, gene expression (*IGFR1* and *IGFR2*), mitotic index, birth weight, gestational weeks and age of the mother obtained from the IGF2 methylated positive samples of AGA and SGA neonates were compared using t-test. The IGF1 and IGF2 protein levels did not differ, whereas the levels of IGFBP3 protein were significantly ($p<0.05$) less in *IGF2* methylated positive SGA compared to AGA neonates. The expression of the *IGFR1* gene is 1.8, and the *IGFR2* gene is 3-fold downregulated in *IGF2* methylated positive SGA compared to AGA neonates. The levels of the mitotic index were significantly ($p<0.05$) less in *IGF2* methylated positive SGA compared to AGA neonates. The birth weight was significantly less ($p<0.05$), whereas the age of the mother and gestational weeks did not differ in the *IGF2* methylated positive SGA compared to AGA neonates. The details of the influence of methylation of the *IGF2* gene on IGF-axis components in AGA and SGA neonates are shown in table 5.5.

Table 5.5: The effect of methylation of *IGF2* gene on IGF-axis components, mitotic index, gestational weeks, and birth weight of AGA and SGA neonates

Gene	Parameter (mean±SD)	AGA	SGA	p-value
	IGF1 (ng/ml)	119.22±31.73	127.08±47.60	0.476
	IGF2 (ng/ml)	124.45±10.66	122.69±10.89	0.901
	IGFBP3 (ng/ml)	1513±318.6	1316±339.3	0.035
	IGFR1 gene expression	1.8-fold downregulated in SGA compared to AGA		
IGF2	*IGFR2* gene expression	3-fold downregulated in SGA compared to AGA		
	Age of the mother	24.75±3.40	24.88±4.22	0.903
	Birth weight (kg)	2.90±0.29	2.07±0.34	0.0001
	Gestational weeks	36.46±2.91	34.40±3.29	0.065
	Mitotic index (%)	16.06±1.92	12.87±1.17	0.0001

IGF*=Insulin-like growth factor;* ***H19****=Gene coding the noncoding RNA;* ***IGFR****= Insulin-like growth factor receptor;* ***IGFBP****=Insulin-like growth factor binding protein;* ***AGA****=Appropriate for gestational age;* ***SGA****=Small for gestation age;* ***kg****=Kilograms;* ***SD****=Standard deviation;* ***ng/ml****=Nano gram/milliliter;* ***%****=Percentage*

5.3.3.2. Methylation status of *H19* gene promoter in the placenta of AGA and SGA neonates

The methylation of the *IGF2* gene promotor was less in the placenta of SGA compared to AGA neonates. Further, the percentage of methylation of the promoter region of the maternally imprinted gene (*H19*) in the placental samples of AGA and SGA neonates was calculated from the MS-PCR agarose gel images. The representative agarose gel image of methylation of the imprinted *H19* gene was shown in figure 5.8A. The methylation percentage in AGA neonates was 97.5% (39/40 samples), and in SGA neonates was 96% (38/40 samples). The methylation of the *H19* gene was 1.5% lower in SGA neonates compared to AGA neonates. The percentage of *H19* methylation in the placenta of AGA and SGA neonates is shown in figure 5.8B.

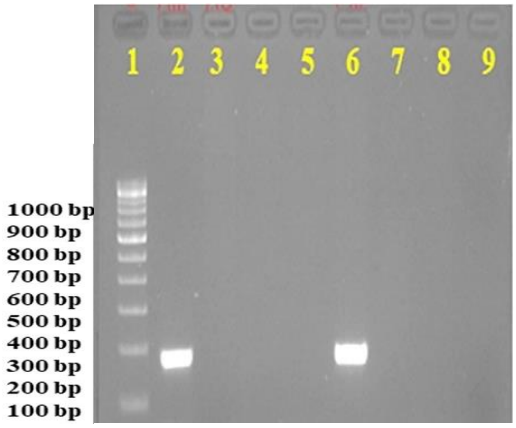

Figure 5.8A: Representative agarose gel image of imprinted (*H19)* gene methylation in AGA and SGA neonates. ***1****=DNA ladder (100 bp);* ***2, 3****=AGA methylation and unmethylation;* ***4, 5****=SGA methylation and unmethylation;* ***6, 7****=Positive methylation and unmethylation;* ***8, 9****=Negative methylation and unmethylation*

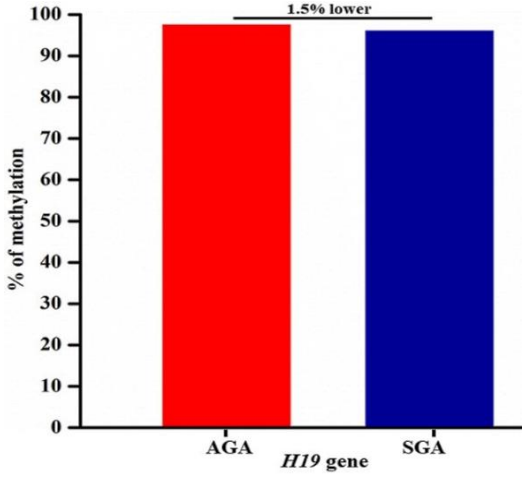

Figure 5.8B: The percentage of methylation of *H19* gene promoter in the placenta of AGA and SGA neonates

To find the influence of methylation of *H19* on IGF-axis components, the levels of IGF1, IGF2, and IGFBP3 protein, gene expression (*IGFR1* and *IGFR2*), mitotic index, birth weight, gestational weeks and age of the mother obtained from the *H19* methylated positive samples of AGA and SGA neonates were compared using t-test. The IGF1 and IGF2 protein levels did not differ, whereas, the levels of IGFBP3 protein were significantly ($p<0.05$) less in *H19* methylated positive SGA compared to AGA neonates. The expression of the *IGFR1* gene did not differ, whereas, the *IGFR2* gene is

1-fold downregulated in *H19* methylated positive SGA compared to AGA neonates. The levels of the mitotic index were significantly ($p<0.05$) less in *H19* methylated positive SGA compared to AGA neonates. The birth weight and gestational weeks were significantly less ($p<0.05$), whereas the age of the mother did not differ in the *H19* methylated positive SGA compared to AGA neonates. The details of the influence of methylation of the *H19* gene on IGF-axis components in AGA and SGA neonates was shown in table 5.6.

Table 5.6: The effect of methylation of the *H19* gene on IGF-axis components, mitotic index, gestational weeks, and birth weight of AGA and SGA neonates

Gene	Parameter (mean±SD)	AGA	SGA	p-value
	IGF1 (ng/ml)	115.92±29.75	119.04±47.85	0.751
	IGF2 (ng/ml)	124.14±10.41	123.17±12.79	0.721
	IGFBP3 (ng/ml)	1581±309.6	1420±378.4	0.045
	IGFR1 gene expression	0-fold downregulated in SGA compared to AGA		
H19	*IGFR2* gene expression	1-fold downregulated in SGA compared to AGA		
	Age of the mother	24.87±3.31	24.81±3.89	0.941
	Birth weight (kg)	2.87±0.31	2.04±0.41	0.0001
	Gestational weeks	36.89±3.32	34.60±3.25	0.003
	Mitotic index (%)	15.95±1.80	12.46±1.00	0.0001

IGF*=Insulin-like growth factor;* ***H19****=Gene coding the noncoding RNA;* ***IGFR****= Insulin-like growth factor receptor;* ***IGFBP****=Insulin-like growth factor binding protein;* ***AGA****=Appropriate for gestational age;* ***SGA****=Small for gestation age;* ***kg****=Kilograms;* ***SD****=Standard deviation;* ***ng/ml****=Nano gram/milliliter;* ***%****=Percentage*

5.3.3.3. Co-methylation status of *IGF2* and *H19* gene promoter in the placental tissue of AGA and SGA neonates

The percentage of co-methylation of *IGF2* and *H19* genes in the placental tissue of AGA and SGA neonates was calculated from the agarose gel images. The percentage of co-methylation of *IGF2* and *H19* genes was 70% (28/40 samples) in AGA neonates

and 62.5% (25/40 samples) in SGA neonates. The co-methylation of *IGF2* and *H19* genes was 7.5% lower in SGA neonates compared to AGA neonates. The percentage of co-methylation of *IGF2* and *H19* genes in AGA and SGA neonates is shown in figure 5.9.

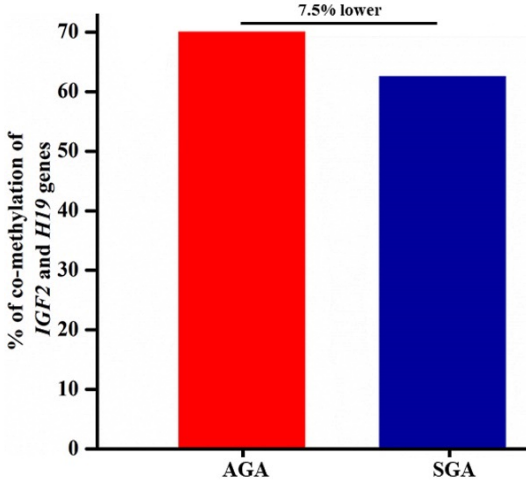

Figure 5.9: The percentage of co-methylation of *IGF2* and *H19* gene promoter in AGA and SGA neonates

5.3.3.4. Methylation status of *IGF1* gene promoter in the placenta of AGA and SGA neonates

The percentage of methylation of promoters of imprinted genes (*IGF2* and *H19*) was less in the placenta of SGA compared to AGA neonates. Further, to find the alteration in the methylation of non-imprinted genes (*IGF1* and *IGFR1*), the methylation of the promoter of the *IGF1* gene in the placental tissue of AGA and SGA neonates was calculated from the MS-PCR agarose gel images. The representative agarose gel image of methylation of the non-imprinted gene (*IGF1*) is shown in figure 5.10A. The percentage of the *IGF1* gene in AGA neonates was 60% (24/40), and in SGA neonates was 55% (22/40). The methylation of the *IGF1* gene was 5% lower in SGA compared to AGA neonates. The percentage of methylation of the *IGF1* gene in the placenta of AGA and SGA neonates is shown in figure 5.10B.

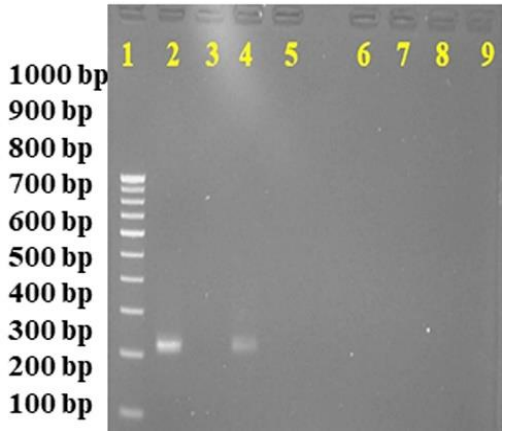

Figure 5.10A: Representative agarose gel image of the non-imprinted (*IGF1)* gene methylation in AGA and SGA neonates. ***1****=DNA ladder (100 bp);* ***2, 3****=AGA methylation and unmethylation;* ***4, 5****= Positive methylation and unmethylation;* ***6, 7*** *=SGA methylation and unmethylation;* ***8, 9****=Negative methylation and unmethylation*

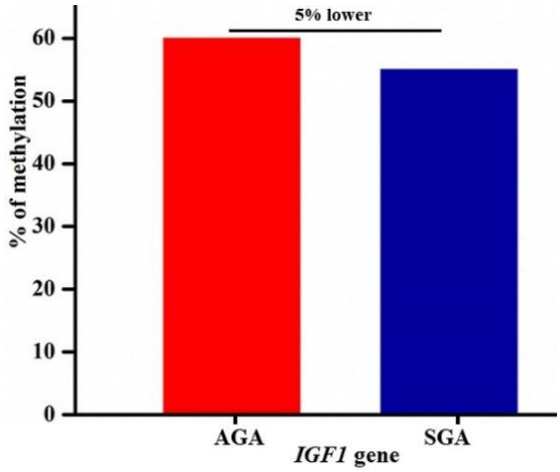

Figure 5.10B: The percentage of methylation of *IGF1*gene promoter in the placenta of AGA and SGA neonates

To find the influence of methylation of *IGF1* on IGF-axis components, the levels of IGF1, IGF2, and IGFBP3 protein, gene expression (*IGFR1* and *IGFR2*), mitotic index, birth weight, gestational weeks and age of the mother obtained from the *IGF1* methylated positive samples of AGA, and SGA neonates were compared using t-test. The IGF1 and IGF2 protein levels did not differ, whereas the levels of IGFBP3 protein were significantly ($p<0.05$) less in *IGF1* methylated positive SGA compared to AGA neonates. The expression of the *IGFR1* gene is 1.9-fold, and the *IGFR2* gene is 2.7-fold

downregulated in *IGF1* methylated positive SGA compared to AGA neonates. The levels of the mitotic index were significantly ($p<0.05$) less in *IGF1* methylated positive SGA compared to AGA neonates. The birth weight and gestational weeks were significantly less ($p<0.05$), whereas the age of the mother did not differ in the *IGF1* methylated positive SGA compared to AGA neonates. The details of the influence of methylation of the *IGF1* gene on IGF-axis components in AGA and SGA neonates was shown in table 5.7.

Table 5.7: The effect of methylation of *IGF1* gene on IGF-axis components, mitotic index, gestational weeks, and birth weight of AGA and SGA neonates

Gene	Parameter (mean±SD)	AGA	SGA	p-value
IGF1	IGF1 (ng/ml)	124.12±33.80	114.40±52.00	0.446
	IGF2 (ng/ml)	124.89±10.11	122.28±14.95	0.433
	IGFBP3 (ng/ml)	1526±291.15	1284±367.61	0.023
	IGFR1 gene expression	1.9-folds down-regulated in SGA compared to AGA		
	IGFR2 gene expression	2.7-folds down-regulated in SGA compared to AGA		
	Age of the mother	25.20±3.33	24.04±2.55	0.192
	Birth weight (kg)	2.91±0.32	2.07±0.44	0.0001
	Gestational weeks	37.37±3.58	35.22±3.61	0.048
	Mitotic index (%)	15.92±1.97	12.82±1.16	0.040

IGF*=Insulin-like growth factor;* ***IGFR****=Insulin-like growth factor receptor*; ***IGFBP****=Insulin-like growth factor binding protein;* ***AGA****=Appropriate for gestational age;* ***SGA****=Small for gestation age;* ***kg****=Kilograms;* ***SD****=Standard deviation;* ***ng/ml****=Nano gram/milliliter;* ***%****=Percentage*

5.3.3.5. Methylation status of *IGFR1* gene promoter in the placenta of AGA and SGA neonates

The percentage of methylation of the promoter of *IGF1* gene methylation was less in SGA compared to AGA neonates. Further, the percentage of methylation in the promoter region of the *IGFR1* gene in the placental tissue of AGA and SGA neonates

was calculated from the MS-PCR agarose gel images. The representative agarose gel image of methylation of the non-imprinted gene (*IGFR1*) is shown in figure 5.11A. The percentage of the *IGFR1* gene in AGA neonates was 85% (34/40 samples), and in SGA neonates was 77.5% (31/40 samples). The methylation of the *IGFR1* gene was 7.5% lower in SGA compared to AGA neonates. The percentage of methylation of the *IGFR1* gene in the placenta of AGA and SGA neonates is shown in figure 5.11B.

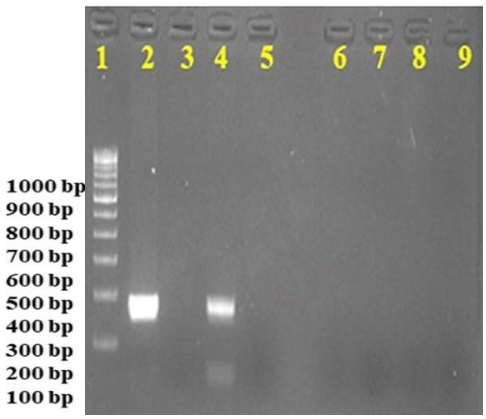

Figure 5.11A: Representative agarose gel image of the non-imprinted (*IGFR1)* gene methylation in AGA and SGA neonates. ***1****=DNA ladder (100 bp);* ***2, 3****=AGA methylation and unmethylation;* ***4, 5****= Positive methylation and unmethylation;* ***6, 7*** *=SGA methylation and unmethylation;* ***8, 9****=Negative methylation and unmethylation*

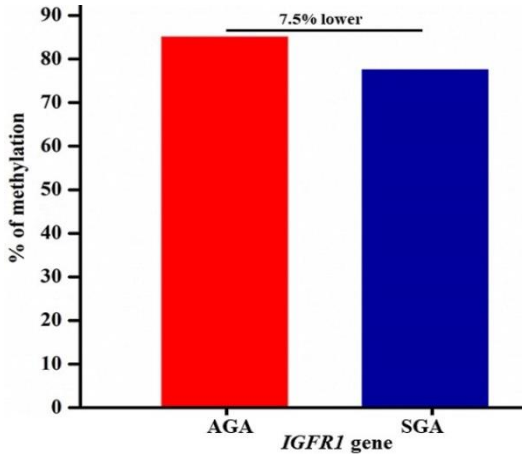

Figure 5.11B: The percentage of methylation of *IGFR1* gene placenta of AGA and SGA neonates

To find the influence of methylation of *IGFR1* on IGF-axis components, the levels of IGF1, IGF2, and IGFBP3 protein, gene expression (*IGFR1* and *IGFR2*), mitotic index, birth weight, gestational weeks and age of the mother obtained from the *IGFR1* methylated positive samples of AGA, and SGA neonates were compared using t-test. The IGF1 and IGF2 protein levels did not differ, whereas, the levels of IGFBP3 protein were significantly ($p<0.05$) less in *IGFR1* methylated positive SGA compared to AGA neonates. The expression of the *IGFR1* gene is 2.2-fold, and the *IGFR2* gene is 1.8-fold downregulated in *IGFR1* methylated positive SGA compared to AGA neonates. The levels of the mitotic index were significantly ($p<0.05$) less in *IGFR1* methylated positive SGA compared to AGA neonates. The birth weight and gestational weeks were significantly less ($p<0.05$), whereas the age of the mother did not differ in the *IGFR1* methylated positive SGA compared to AGA neonates. The details of the influence of methylation of the *IGFR1* gene on IGF-axis components in AGA and SGA neonates was shown in table 5.8.

Table 5.8: The effect of methylation of *IGFR1* genes on IGF-axis components, mitotic index, gestational weeks, and birth weight of neonates

Gene	Parameter (mean±SD)	AGA	SGA	p-value
IGFR1	IGF1 (ng/ml)	115.21±34.29	116.24±49.12	0.999
	IGF2 (ng/ml)	125.28±10.71	120.59±13.08	0.210
	IGFBP3 (ng/ml)	1557±313.19	1389±349.5	0.052
	IGFR1 gene expressior	2.2-folds down-regulated in SGA compared to AGA		
	IGFR2 gene expressior	1.8-folds down-regulated in SGA compared to AGA		
	Age of the mother	24.94±3.32	25.29±4.94	0.732
	Birth weight (kg)	2.87±0.33	2.03±0.44	0.0001
	Gestational weeks	36.67±3.44	34.38±3.41	0.009
	Mitotic index (%)	16.09±1.79	12.51±1.19	0.0001

IGF*=Insulin-like growth factor;* ***IGFR****=Insulin-like growth factor receptor;* ***IGFBP****=Insulin-like growth factor binding protein;* ***AGA****=Appropriate for gestational*

age; ***SGA****=Small for gestation age;* ***kg****=Kilograms;* ***SD****=Standard deviation;* ***ng/ml****=Nano gram/milliliter;* ***%****=Percentage*

5.3.3.6. Co-methylation status of *IGF1* and *IGFR1* gene promoter in the placenta of AGA and SGA neonates

The percentage of co-methylation of non-imprinted (*IGF1* and *IGFR1*) genes in the placental tissue of AGA and SGA neonates was calculated from the MS-PCR agarose gel images. The percentage of co-methylation of *IGF2* and *H19* genes was 52.5% (21/40 samples) in AGA neonates and 37.5% (15/40 samples) in SGA neonates. The co-methylation of *IGF1* and *IGFR1* genes was 15% lower in SGA neonates compared to AGA neonates. The percentage of co-methylation of *IGF1* and *IGFR1* genes in the placenta of AGA and SGA neonates is shown in figure 5.12A.

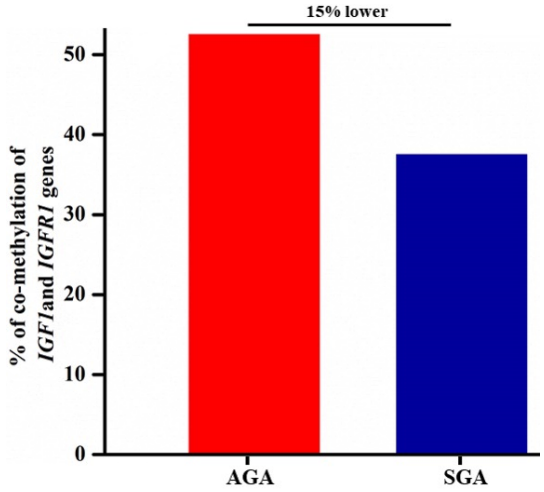

Figure 5.12A: The percentage of co-methylation of *IGF1* and *IGFR1* genes in the placenta of AGA and SGA neonates

The percent of co-methylation of both imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1)* genes in the placenta of AGA and SGA neonates was calculated from the MS-PCR agarose gel images. The percent of co-methylation of imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1)* genes was 40% (16/40 samples) in AGA and 20% in SGA neonates. The co-methylation of imprinted (*IGF2*

and *H19)* and non-imprinted (*IGF1* and *IGFR1*) genes were 20% lower in SGA compared to AGA neonates. The percentage of co-methylation of both imprinted (*IGF2* and *H19)* and non-imprinted (*IGF1* and *IGFR1*) genes in the placenta of AGA and SGA neonates is shown in figure 5.12B.

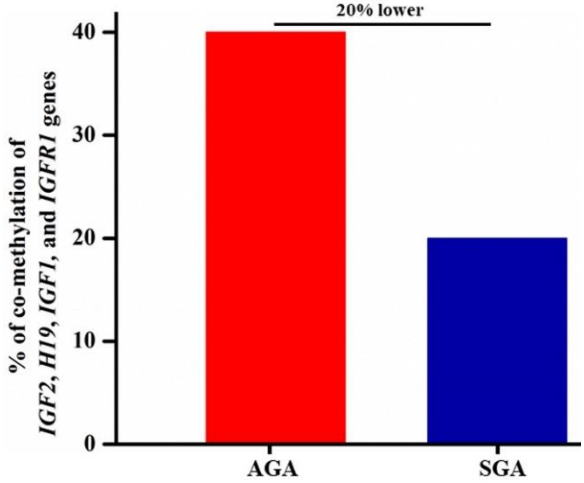

Figure 5.12B: The percentage of co-methylation of imprinted (*IGF2* and *H19)* and non-imprinted (*IGF1* and *IGFR1)* genes in the placenta of AGA and SGA neonates

5.4. Mitogenic potential of cord blood lymphocytes of AGA and SGA neonates

The results indicate that the IGF-axis components were significantly less in the SGA neonates when compared to AGA neonates. Further, to find whether the intrinsic division capacity of the cord blood lymphocytes is altered, we have cultured the cord blood from AGA and SGA neonates, cultured *in vitro* by stimulating with PHA, and calculated the percent of the mitotic index. The representative images of the metaphase spread obtained from the cultured cord blood samples of AGA and SGA neonates was shown in figure 5.13A.

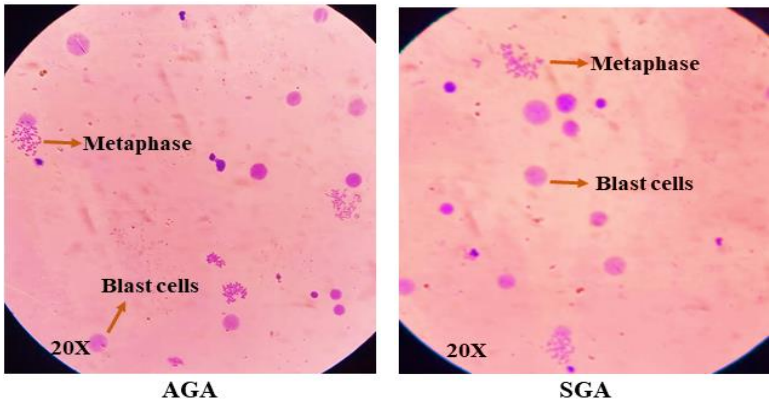

Figure 5.13A: Representative images of the metaphase spread obtained from the cord blood samples of AGA and SGA neonates

The range (mean±SD) of mitotic index obtained from AGA neonates is 12-22% (15.71±1.81), and SGA neonates are 10-16% (12.60±1.16). The mitotic index of SGA neonates is significantly less (p=0.001) compared to AGA neonates. The comparison of the range of mitotic index of cord blood obtained from AGA and SGA neonates was shown in figure 5.13B.

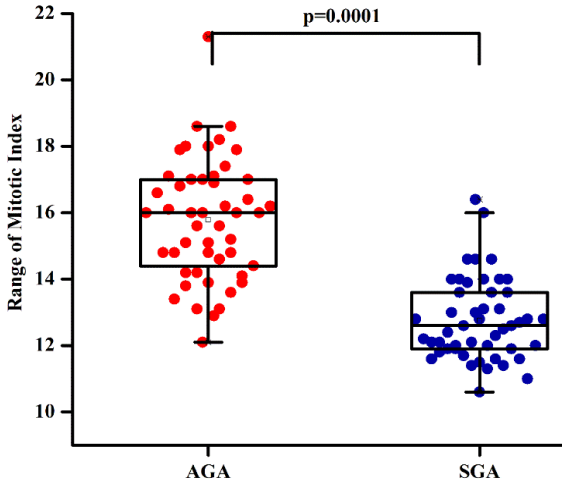

Figure 5.13B: The range of mitotic index in cord blood lymphocytes of AGA and SGA neonates

Correlation analysis was performed to find the influence on hematological/biochemical parameters such as (RBC, WBC, Platelet, hemoglobin, serum creatinine, and blood urea) of the pregnant women on the mitotic index. The

mitotic index of cord blood lymphocytes did not correlate with the hematological/biochemical parameters of the pregnant women with AGA and SGA neonates (r=0.023).

Further, to find the influence of IGF-axis (IGF1, IGF2, and IGFBP3) proteins, and expression of IGF-axis (*IGFR1* and *IGFR2)* genes on the mitotic index, correlation analysis was performed. The levels of IGF-axis components did not correlate with the mitotic index of the cord blood lymphocytes (r=0.02).

Further, to find the association between the mitotic index with gestational weeks and birth weight (AGA and SGA), correlation analysis was performed. There is no correlation between the mitotic index and gestational weeks (r=-0.08) and birth weight (r=0.347) of AGA and SGA neonates (Figure 5.13C and D).

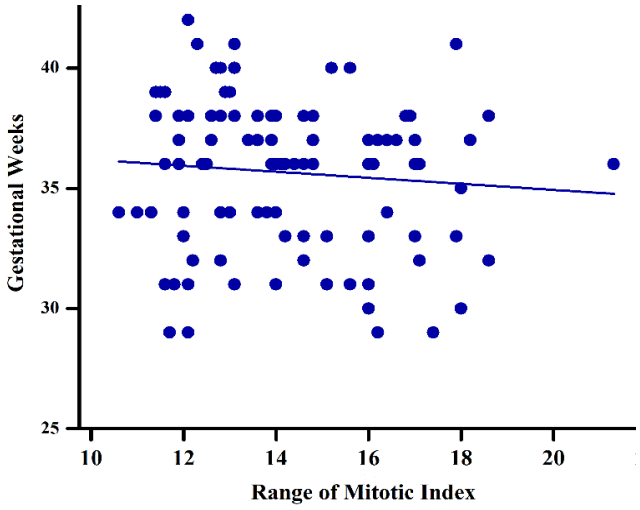

Figure 5.13C: Correlation between the mitotic index and gestational weeks of AGA and SGA neonates

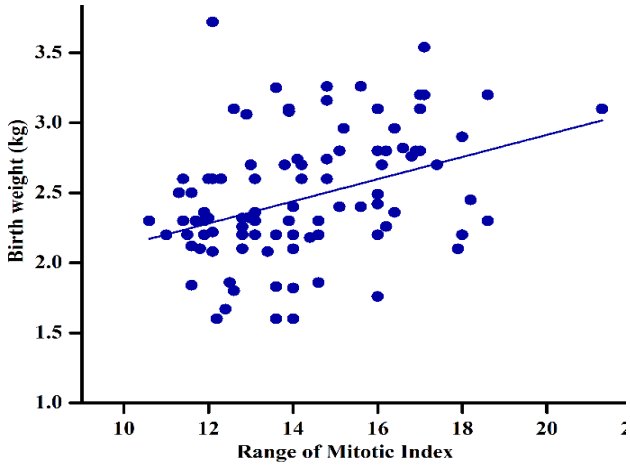

Figure 5.13D: Correlation between the mitotic index and birth weight of AGA and SGA neonates

Discussion

6. Discussion

Development of the human fetus is an important stage during gestation where the interaction between fetal, placental, maternal, growth factors, nutrition, hormones, environmental, and genetic factors determine the pregnancy outcomes (Nieuwenhuijsen et al., 2013). Recent studies found that the growth hormone (GH)/IGF-axis plays an essential role in the fetal growth and development and the growth of the children (Wang et al., 2020). Gene knockout gene studies in animals have also proven the importance of the GH/IGF axis in fetal growth and SGA. The imbalance in any of those IGF-axis components might affect the growth of the fetus and result in low birth weight/SGA. Recent studies also revealed contrasting results (higher, lower, or no difference in the levels of IGF-axis components) and the impact of nutrition, environment, genetic makeup, etc., on low birth weights and SGA. Measuring the levels of IGF-axis components at different SGA stages might help better manage and adequately care for SGA-affected babies. There were no reports on the role of IGF-axis components in the regional population (Kolar, Karnataka). Hence, in the present study, I have investigated the role of IGF-axis components in the cord blood and placenta of the AGA and SGA neonates born to south Indian women (Kolar District, Karnataka). In specific, the levels of IGF1, IGF2, and IGFBP3 proteins and mitotic index in the cord blood, and the expression of *IGFR1* and *IGFR2* genes and methylation status of imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1*) gene promoter in the placenta was analyzed. The results obtained from the present study and their significance were discussed with the already available literature from various populations in the world.

Inference 1: IGFBP3 protein levels were lower, and the IGF1 and IGF2 were unaltered in SGA compared to AGA neonates

The present study results showed that the levels of IGF1 and IGF2 proteins in cord blood plasma did not differ between AGA and SGA neonates. In contrast, the levels of IGFBP3 protein were significantly lower in SGA compared to AGA neonates. The levels of IGF1, IGF2, and IGFBP3 proteins in the present study showed more variation within the groups, possibly due to various factors such as nutritional status, environment, individual genetic makeup, etc. The levels of IGF1, IGF2, and IGFBP3 proteins obtained in the present study were comparable with published reports from different parts of the world (Iniguez et al., 2010; Akram et al., 2011; Rustogi et al., 2018; Motte-Signoret et al., 2021). Similar to published reports, the IGF1 levels in cord blood plasma did not differ between AGA and SGA neonates of the Mexican population (Martínez-Cordero et al., 2006). The IGF1 protein levels in the present study were in contrast to a published report, where they found that the IGF1 in cord blood is significantly low in SGA compared to LGA and AGA (Yalinbas et al., 2019). IGF1 and IGFBP3 in the cord blood are significantly lower in preterm compared to term babies (Yang et al., 2000).

Similar to cord blood, the plasma levels of IGF1, IGF2, and IGFBP3 in infants peripheral blood also showed lower in term SGA compared to term LGA and term AGA (Zhang et al., 2015). A similar study also showed that the levels of IGF1 and IGFBP3 proteins in peripheral blood were significantly low, whereas the IGFBP1 proteins were higher in SGA compared to AGA neonates (Mendez-Ramírez et al., 2009). In addition to blood samples, few studies have analyzed the levels of IGF1, IGFBP3, and IGFR1 proteins in the placenta; they found that the levels of IGF1 protein are lower and IGFR1

protein was higher in SGA compared to controls (Sandoval et al., 2020). A similar study also found that the levels of IGF1 and IGFBP1, 2, 3, 4, and 7 proteins in the placenta of AGA, SGA, and LGA neonates suggest that the IGF1 is lower and IGFBPs are higher in SGA compared to AGA neonates (Nawathe et al., 2016). Recent studies also found the alteration of the IGF-axis components after treatment of SGA babies with growth hormone. Juul *et al.,* (1998) observed that the serum IGF1 and free IGF1 protein levels were increased after GH treatment (Juul et al., 1998). Liu *et al.,* (2019) observed that the Z-score of IGF-1 level after one month of GH treatment was significantly increased and positively correlated with the dosage of GH in short stature (Liu et al., 2019).

In addition to measuring the IGF axis components, the association of the levels with birth weight and gestational weeks was analyzed. The levels of IGF1 were significantly positively associated with birth weight and birth length of babies born in Boston compared with Shanghai (Lagiou et al., 2009). Daniel *et al.,* (2005) observed that IGFBP3 levels were positively associated with the birth weight of LGA and macrosomia (Daniel et al., 2005). Luo *et al.,* (2012) observed that maternal IGF1 levels were correlated with birth weight and placental weight in gestational diabetics than in nondiabetic pregnancies (Luo et al., 2012). Liu *et al.,* (2017) observed that the IGF1 level of neonates was positively correlated with neonatal weight (Liu et al., 2017). However, in the present study, the levels of IGF1, IGF2, and IGFBP3 were not correlated with the birth weight and gestation weeks of neonates.

Inference 2: The expression of *IGFR1* and *IGFR2* genes were downregulated in SGA compared to AGA neonates

The expression of *IGFR1* and *IGFR2* genes in the placenta of AGA and SGA neonates was measured since the IGF-axis receptors play an important role in

transducing the signal from outside to inside the cell and are responsible for the growth of the fetus. The results showed that the expression of *IGFR1* and *IGFR2* genes were downregulated by 3.9-folds and 2.8-folds in the placenta of SGA compared to AGA neonates. Similar to the present study results, the lowered expression of *IGFR1* and *IGFR2* genes was observed in the placenta of macrosomia when compared to AGA neonates (Jiang et al., 2009). The expression of *IGFR1* mRNA was reduced in the fetus treated with *IGF1* compared to treatment with saline (Shaikh et al., 2005). The expression of *IGFR1* mRNA in the placenta was also lower in the growth-restricted fetuses of rats (Reid et al., 2002).

In contrast to the present study, higher mRNA levels of *IGF1*, *IGF2*, and *IGFR1* genes in the placenta were observed in SGA compared to AGA neonates *(Iniguez et al., 2010).* A similar study also found that the expression of *IGF2* mRNA in cord blood plasma was higher in term SGA compared to term AGA neonates (Zhang et al., 2015). Fujimoto *et al.,* (2017) observed that the levels of the expression of *IGFR1* in the cord blood did not show any significant difference between AGA and SGA neonates (Fujimoto et al., 2017). In addition to the gene expression patterns, the association studies also revealed that the expression of IGF-axis genes was correlated with birth weight and gestation weeks of neonates. The expression of the *IGFR1* gene is negatively correlated with the birth weight of neonates (Blum et al., 2018). The expression of the *IGFR1* gene was negatively correlated with the birth weight, birth length, and weight of the placenta of SGA compared to AGA neonates (Iniguez et al., 2010). However, in the present study, the expression of *IGFR1* and *IGFR2* genes did not correlate with neonates birth weight and gestation weeks.

The IGF-axis proteins and the expression of genes analyzed in the present study were altered in SGA; however, the levels did not correlate with the birth weight and gestational weeks of neonates. We assume that the significant variation in the levels of these IGF-axis proteins and gene expression and less sample size might be one of the reasons for the lack of an association with birth weight and gestational weeks. However, measuring the levels of the IGF-axis components might be important to monitor the growth hormone treatment of neonates born with SGA neonates.

Inference 3: The percent of methylation of *IGF2, H19, IGF1,* and *IGFR1* gene promoters were lower in SGA compared to AGA neonates

Imprinted genes *(IGF2* and *H19):* Recent studies highlight that the methylation of the IGF-axis genes (imprinted genes) has been shown to be associated with SGA (Leeuwerke et al., 2016). In the present study, the levels of IGFBP3 protein and *IGFR1* and *IGFR2* gene expression were lower in SGA compared to AGA neonates. Further, to find the alteration in the methylation status and its influence on the IGF-axis components, we have measured the methylation status of IGF-axis [imprinted: *IGF2* and *H19*, and non-imprinted: *IG12* and *IGFR1*] genes were analyzed in the placenta of AGA and SGA neonates. The percentage of methylation in the promoter region of imprinted [*IGF2;* 2.5% lower, and *H19;* 1.5% lower] genes was lower in SGA compared to AGA neonates. The results of the present study align with published reports where the methylation of *IGF2* and *AHRR* genes was significantly lower in the placenta of FGR compared to the control (Zhao et al., 2016). In contrast to the present study results, the levels of methylation of *IGF2* and *H19* in the placenta did not show differences between SGA and AGA neonates (Leeuwerke et al., 2016). In addition to the placenta, studies also analyzed the methylation in cord blood; they found that *IGF2*

DMR methylation was lower in SGA compared to AGA neonates (Wehkalampi et al., 2013). A similar study also showed the percentage of methylation of *IGF2* DMR was lower in cord blood of preeclampsia when compared to normal pregnancy (Jung et al., 2008). In contrast, few studies showed higher *IGF2* gene methylation in SGA cord blood than in AGA (Zhang et al., 2015). In addition to cord blood, the peripheral blood samples showed lower methylation of the paternally imprinted (*IGF2)* gene in SGA compared to AGA neonates (Murphy et al., 2014).

Non-imprinted genes *(IGF1* and *IGFR1):* In line with the imprinted genes, the percent of methylation of the promoter region of non-imprinted (*IGF1;* 5% lower, and *IGFR1;* 7.5% lower) genes was also lower in SGA compared to AGA neonates. The results of the present study were similar to published reports where the CpG methylation of the promoter regions of *IGFBP1, 2, 3, 4,* and *7* genes was lower in the placenta of SGA compared to AGA neonates (Nawathe et al., 2016). In contrast to the present study, few studies showed an alteration in the percentage of methylation; Li *et al.,* (2020) reported that the CpG site at the promoter region of *IGF1* gene was highly methylated in placental tissue of IUGR compared to normal (Li et al., 2020). In addition to the placenta, few studies analyzed the methylation in blood; they found that the methylation of the *IGFBP1* gene was significantly higher in Type II diabetes compared to controls (Hafez et al., 2021). Further, we have also analyzed the percent of co-methylation of imprinted, non-imprinted, and both imprinted and non-imprinted genes in the placenta of AGA and SGA neonates. The percent of co-methylation of imprinted (*IGF2* and *H19*; 7.5% lower), non-imprinted (*IGF1* and *IGFR1*; 15% lower), and both imprinted and non-imprinted (*IGF2, H19, IGF1,* and *IGFR1*; 20% lower) genes were lower in SGA compared to AGA neonates. The results indicate that both imprinted and non-imprinted

genes in the IGF-axis showed an alteration in the methylation status in the placenta of SGA.

Inference 4: The methylation of IGF-axis genes was associated with birth weight and gestational weeks of neonates

To find the effect of the altered methylation status of the imprinted and non-imprinted genes on IGF-axis components and demography, we have compared the levels of IGF1, IGF2, IGFBP3 proteins, expression of *IGFR1* and *IGFR2* genes, mitotic index, and demography details obtained from the methylation positive samples of AGA and SGA neonates. The percent of methylation of imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1)* genes significantly ($p<0.05$) reduced the levels of IGFBP3 protein, birth weight, percentage of mitotic index, gestational weeks, and downregulation of *IGFR1* and *IGFR2* gene expression in SGA when compared to AGA neonates. Recent studies also suggest that the alteration in the methylation status of IGF-axis genes can impact the expression of IGFs, IGFBPs, and *IGFR*s, gestational weeks, and birth weight of SGA neonates. The methylation of the *IGF2* gene in the cord blood samples was positively associated with the birth weight adjusted to gestational age and had no association with gestational age (Haggarty et al., 2013). The methylation of *IGF2 DMR* and *H19* genes in cord blood was positively associated with fetal and infant growth (Bouwland et al., 2013). Kantake *et al*., (2020) observed that the methylation of CpG sites in *IGF1* P2 in whole blood was positively correlated with body weight and length of preterm infants with IUGR (Kantake et al., 2020). There was no correlation between *IGF1* gene expression and DNA methylation in the placenta of IUGR compared to normal (Li et al., 2020). The CpG -137 methylation of the *IGF1* gene was negatively correlated with birth weight (Stunff et al., 2018). The methylation of *IGF1*

and *IGFR1* gene promoters in peripheral blood was positively associated with the expression of *IGF1* and *IGFR1* genes, whereas the *IGFBP3* methylation was negatively correlated with the expression of the *IGFBP3* gene (Ye et al., 2016). Ouni *et al.,* (2015) observed that the methylation of *IGF1 DMR* was lower in the liver and growth plates of children, and the methylation of the *IGF1* gene promoter showed a negative association with serum IGF1 and growth of the children (Ouni et al., 2015). Turan *et al.,* (2012) observed the methylation of the CpG sites in *IGF1, IGF2, IGF2R, IGFBP, PHLDA2,* and *PLAGL1* genes in placenta and cord blood samples were positively correlated with the birth weight (Turan et al., 2012). In the present study, the percent (number of positives/total number of samples analyzed) of methylation of imprinted and non-imprinted genes were analyzed using MS-PCR instead of pyrosequencing, where specific CpG sights were analyzed. In addition to measuring the levels of IGF-axis components, understanding the methylation patterns of IGF-axis genes might provide information that can be used to better management of SGA neonates in the Indian population (Kolar, Karnataka).

Inference 5: Mitotic index (intrinsic division capacity) of the cord blood lymphocytes was lower in SGA compared to AGA neonates

The IGFBP3 protein levels in cord blood plasma, expression of *IGFR1* and *IGFR2* genes, and methylation of IGF-axis imprinted (*IGF2* and *H19*) and non-imprinted (*IGF1* and *IGFR1*) genes in the placenta were reduced in SGA compared to AGA neonates. To find the altered IGF-axis influence on the cells' division capacity, we have investigated the mitotic index in the cord blood lymphocytes of SGA neonates. The obtained mitotic index ranges between 12-22% and 10-16% for AGA and SGA neonates, respectively. The mitotic index of cord blood lymphocytes of SGA neonates

was significantly less when compared to AGA neonates. An altered mitotic index (4.1-24.6%) was also observed in healthy individuals (n=90), and it varied among blood groups (A, B, AB, and O) and responsiveness to different doses of ionizing radiation (Viswanathan et al., 2019). The mitotic index of cord blood lymphocytes did not correlate with maternal age, gestational weeks, birth weight, hematological parameters of the mother, or IGF-axis protein, and gene expression levels of AGA and SGA neonates (r=0.347). To the best of our knowledge, this is the first study where we measured the mitotic potential difference of lymphocytes of cord blood obtained from AGA and SGA neonates. Further studies need to explore the potential links (signaling pathways) between the altered IGF-axis components and mitosis of the cord blood lymphocytes of SGA neonates.

Summary

7. Summary

- The levels of IGFBP3 protein in the cord blood plasma were significantly less ($p=0.02$) in SGA neonates compared to AGA neonates, whereas the levels of IGF1 ($p=1$) and IGF2 ($p=0.69$) proteins did not differ between AGA neonates and SGA neonates.
- The expression levels of *IGFR1* (3.9-folds) and *IGFR2* (2.8-folds) genes in the placenta were down-regulated in SGA neonates compared to AGA neonates.
- The altered levels of IGF-axis proteins (IGF1, IGF2, and IGFBP3) and the expression of the genes (*IGFR1* and *IGFR2*) did not correlate ($r=0.1$) with gestational weeks and birth weight.
- The percentage of methylation of IGF-axis gene promoter [imprinted: *IGF2* (2.5% lower) and *H19* (1.5% lower) and non-imprinted genes: *IGF1* (5% lower) and *IGFR1* (7.5% lower)] in the placenta were lower in SGA neonates compared to AGA neonates.
- The percentage of co-methylation of the IGF-axis gene promoter [imprinted (7.5% lower), non-imprinted (15% lower), and both imprinted and non-imprinted (20% lower)] in the placenta was lower in SGA neonates compared to AGA neonates.
- The methylation of the IGF-axis gene promoter (both imprinted and non-imprinted) significantly ($p<0.05$) reduced the levels of IGFBP3 protein, *IGFR1* and *IGFR2* genes expression, mitotic index, gestation weeks, and birth weight of the SGA neonates compared to AGA neonates.
- The percent of the mitotic index of the cord blood lymphocytes (cultured *in vitro*) was significantly less ($p=0.0001$) in SGA neonates compared to AGA neonates.

New knowledge generated

8. New knowledge generated

The present thesis generated new knowledge on the levels of IGF-axis components such as IGF1, IGF2, and IGFBP3 proteins and mitotic index of lymphocytes in the cord blood, and the expression of *IGFR1* and *IGFR2* genes and methylation of *IGF2*, *H19*, *IGF1*, and *IGFR1* genes promoter in the placenta of SGA and AGA neonates born to Indian women. The results indicates that the IGF-axis components were significantly altered in SGA when compared to the AGA neonates born to Indian women.

Limitations of the study

9. Limitations of the study

- The present study did not collect information on the mothers nutritional status, educational status, and environmental conditions. The information of these factors might be helpful for a better understanding of the role of the IGF-axis in SGA neonates.
- The present study used MS-PCR to analyze the percentage of methylation of imprinted and non-imprinted genes instead of pyrosequencing. Pyrosequencing can help to analyze the specific CpG site in the targeted sequence of IGF-axis genes.

Institutional Ethics Committee Approval

SDUAHER

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

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13. Dr. Mamata Kale, Asst. Professor of Microbiology, SDUMC

No. SDUMC/KLR/IEC/32/2019-20 **Date:06-06-2019**

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph.D study entitled **"Understanding the role of Insulin-like growth factor axis in small for gestational age birth"** being investigated by **Ms. Nithya.M.N**, Dr. A.V. M. Kutty , Dr.Sharath.B, Dr. Krishnappa.J[1] & Dr. Sheela.S.R[2] in the Department of Cell Biology and Molecular Genetics, Paediatics[1] & OBG[2] at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study.**

Sujatha.M.P
Member Secretary
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

Note: The same Institutional Ethics Clearance No. can be used for Presentation & Publication. However Presentation to be preceded before Publication.

Proforma, Patient Information Sheet and Written Consent Form

PROFORMA

Small for Gestational age						**Sample no.**		**SGA /AGA**
Mother's Name:						IP no.**:**		
Address:						Mobile**:**		
Mother's age (yrs)		Height		Delivery mode		LSCS		NVD
Gravida				Parity				
Gestational age at delivery (wks)				Fetal number				
Obst. complications	Preeclampsia			Gestational diabetes		Gestational hypertension		
	Placental abruption			Oligohydromnios		Polyhydromnios		
	Others (specify):							
Medical history	M	F		Mat GM	Mat GF	Pat GM		Pat GF
T2DM								
CVD								
Others (specify)								
RBC count (mil / µL)				WBC count (mil / µL)				
Platelet Count (T/ µL)				Haemoglobin (mg/dl)				
Blood Urea				Serum creatinine				
Long-term medications								
Diagnosis								
Neonate data				IP no.**:**				
Date of Birth		Birth weight			Birth length			
Head circumference		APGAR Score		1''	5''	10''		
Systemic examination								
CVS	Heart rate			Murmurs				
	Peripheral pulses			CFT				
RS	Respiratory rate			RDS score				
	Chest retractions			Grunting				
CNS	Convulsions			Abnormal movement				
	Spontaneous eye opening			Spontaneous limb movement				
P/A	Abdominal distension			Umbilical arteries/veins				
IUGR/FGR		Ponderal index - Weight in gms/ (Length in cm)^3 X 100 =						

PART I: PATIENT INFORMATION SHEET

Name of the project	The role of Insulin-like growth factor-axis components in small for gestational age birth
Name of the Research Scholar	Ms. Nithya M.N.
Name of Organization	Sri Devaraj Urs Academy of Higher Education and Research, Kolar

Small for gestational age (SGA) is a condition in which the weight and length of the newborn is less than expected for their gestational age. It is one of the major causes for perinatal mortality and morbidity. Infant birth weight is strongly associated with maternal birth weight, indicating that birth weight is a heritable trait through maternal line. Insulin-like growth factor (IGF) axis is an endocrine system expressed in placenta and helps to regulate the fetal growth. The IGF axis plays a major role in the promotion of cell proliferation, aging and the inhibition of cell death.

Participant Selection: This study comprises of two groups. Group one will comprise of AGA neonates and groups two will comprise of SGA neonates. Neonates will be recruited from the Department of Obstetrics and Gynaecology OBG of R. L. Jalapa Hospital and Research Centre, Tamaka, Kolar, attached to Sri Devaraj Urs Medical College. Neonates will be provided with standard care available at R. L. Jalapa Hospital and Research Centre.

Voluntary Participation: Your participation in this study is entirely voluntary. There is no compulsion to participate in this study. You will be no way affected if you do not wish to participate in the study. You are required to sign only if you voluntarily agree to participate in this study. Further you are at a liberty to withdraw from the study at any time. We assure that your withdrawal will not affect your treatment by the concerned physician in any way.

Procedure: We will be collecting a small volume of cord blood (5 ml) and placental tissue (100-250 mg).

Duration: The research takes place over 3 years in total.

Risks: No drug will be tested on you. 5ml of cord blood and placenta will be collected. Fresh sample will not be taken.

Benefits: Participation in this study does not involve any cost for you. Also, no monetary compensation will be paid for your participation in this study.

Confidentiality: All information regarding personal identification will be kept confidential & will not be disclosed to anyone except if it is required by the law. Only the project investigators will have access to the identification details.

Storage of samples: Genetic material prepared from your cord blood sample and placenta may be stored for future research projects. In such an event, permission from the Ethics Committee will be obtained prior to use.

Sharing the Results: The results obtained from this study will be published in scientific or Medical Journals or Medical conferences. We would not compel you any time during this process; also, we would greatly appreciate your cooperation to the study. We would like to get your consent to participate in the study.

For any information you are free to contact investigator. This study has been approved by the Institutional ethical committee & has been started only after their formal approval. The sample collected will be stored in the institute and I request you to permit us to store and use this sample for any future study.

This document will be stored in the safe locker in the Dept. of Cell Biology and Molecular Genetics & a copy given to you for information.

Name of the Clinical Investigators:

Dr. Krishnappa J.
Prof. of Pediatrics
Mob No: 9448587233

Dr. S.R. Sheela
Prof. of OBG
Mob No: 9845217277

PART 2: WRITTEN INFORMED CONSENT FORM

Title of the study: The role of Insulin-like growth factor-axis components in small for gestational age birth

Declaration by the participant:

- This research study has been explained to me; I have been given the chance to discuss it and ask questions. All of my questions have been answered to my satisfaction.
- I understand that this research project involves drawing and testing of cord blood and placenta sample.
- I have read each page of Information Sheet or it has been read to me.
- I agree to allow access health information as explained in the information sheet.
- I agree to allow collection of 5 ml cord blood sample and 100-250 mg of placental tissue from me and data for the research purposes explained in the Information Sheet.
- I understand that all the information collected will be kept confidentially.
- I voluntarily consent to take part in this research study.

	Name	**Signature**	**Date**
Participant			
Witness 1			
Witness 2			
Person taking consent			

ರೋಗಿಯಮಾಹಿತಿಹಾಳೆ

ಅಧ್ಯಯನದಶೀರ್ಷಿಕೆ :	ಗರ್ಭಾವಸ್ಥೆಯ ವಯಸ್ಸಿನ ಜನನಕ್ಕೆ ಇನ್ಸುಲಿನ್ ತರಹದ ಬೆಳವಣಿಗೆಯ ಅಂಶದ ಅಕ್ಷದ ಅಂಶಗಳ ಪಾತ್ರವು ಚಿಕ್ಕದಾಗಿದೆ
ಸಂಶೋಧಕರಹೆಸರು :	ನಿತ್ಯ ಎಂ. ಎನ್.
ಸಂಸ್ಥೆಯಹೆಸರು :	ಶ್ರೀ ದೇವರಾಜ್ ಅರಸು ಅಕಾಡೆಮಿ ಆಫ್ಪೈಯರ್ಎಜುಕೇಶನ್ ಅಂಡ್ರಿ ಸರ್ಚ್. ಕೋಲಾರ

ಗರ್ಭಾವಸ್ಥೆಯ ವಯಸ್ಸಿಗೆ ಚಿಕ್ಕದು (SGA) ನವಜಾತ ಶಿಶುವಿನ ತೂಕ ಮತ್ತು ಉದ್ದವು ಅವರ ಗರ್ಭಾವಸ್ಥೆಯ ವಯಸ್ಸಿಗೆ ನಿರೀಕ್ಷಿಸಿದ್ದಕ್ಕಿಂತ ಕಡಿಮೆ ಇರುವ ಸ್ಥಿತಿಯಾಗಿದೆ. ಇದು ಪ್ರಸವಪೂರ್ವ ಮರಣ ಮತ್ತು ಅಸ್ವಸ್ಥತೆಗೆ ಪ್ರಮುಖ ಕಾರಣಗಳಲ್ಲಿ ಒಂದಾಗಿದೆ. ಶಿಶು ಜನನ ತೂಕವು ತಾಯಿಯ ಜನನ ತೂಕದೊಂದಿಗೆ ಬಲವಾಗಿ ಸಂಬಂಧಿಸಿದೆ . ಇದು ತಾಯಿಯ ರೇಖೆಯ ಮೂಲಕ ಜನನ ತೂಕವು ಆನುವಂಶಿಕ ಲಕ್ಷಣವಾಗಿದೆ ಎಂದು ಸೂಚಿಸುತ್ತದೆ. ಇನ್ಸುಲಿನ್ ತರಹದ ಬೆಳವಣಿಗೆಯ ಅಂಶ (IGF) ಅಕ್ಷವು ಜರಾಯುಗಳಲ್ಲಿ ವ್ಯಕ್ತವಾಗುವ ಅಂತಃಸ್ರಾವಕ ವ್ಯವಸ್ಥೆಯಾಗಿದೆ ಮತ್ತು ಭ್ರೂಣದ ಬೆಳವಣಿಗೆಯನ್ನು ನಿಯಂತ್ರಿಸಲು ಸಹಾಯ ಮಾಡುತ್ತದೆ. ಜೀವಕೋಶದ ಪ್ರಸರಣ, ವಯಸ್ಸಾಗುವಿಕೆ ಮತ್ತು ಜೀವಕೋಶದ ಸಾವಿನ ಪ್ರತಿಬಂಧವನ್ನು ಉತ್ತೇಜಿಸುವಲ್ಲಿ IGF ಅಕ್ಷವು ಪ್ರಮುಖ ಪಾತ್ರ ವಹಿಸುತ್ತದೆ.

ಭಾಗವಹಿಸುವವರ ಆಯ್ಕೆ: ಈ ಅಧ್ಯಯನವು ಎರಡು ಗುಂಪುಗಳನ್ನು ಒಳಗೊಂಡಿದೆ. ಗುಂಪು ಒಂದು SGA ಜನ್ಮ ಒಳಗೊಂಡಿರುತ್ತದೆ. ಗುಂಪು ಎರಡು AGA ಜನ್ಮವನ್ನು ಒಳಗೊಂಡಿರುತ್ತದೆ. ನವಜಾತ ಶಿಶುಗಳನ್ನು ಆರ್.ಎಲ್. ಜಲಪ್ಪ ಆಸ್ಪತ್ರೆ ಮತ್ತು ಸಂಶೋಧನಾ ಕೇಂದ್ರದ ಪ್ರಸೂತಿ ಮತ್ತು ಸ್ತ್ರೀರೋಗ ಶಾಸ್ತ್ರ ಇಲಾಖೆಯಿಂದ ನೇಮಕ ಮಾಡಲಾಗುತ್ತದೆ, ತಮಕಾ, ಕೋಲಾರ, ರೀ ದೇವರ ಯುರ್ಸ್ ವೈದ್ಯಕೀಯ ಕಾಲೇಜು. ಆರ್. ಎಲ್.ಜಲಪ್ಪ ಆಸ್ಪತ್ರೆ ಮತ್ತು ಸಂಶೋಧನಾ ಕೇಂದ್ರದಲ್ಲಿನಿ ನವಜಾತ ಶಿಶುಗಳುನ್ನು ಪ್ರಮಾಣಿತ ಆರೈಕೆಯಲ್ಲಿ ಒದಗಿಸಲಾಗುತ್ತದೆ.

ಸ್ವಯಂಪ್ರೇರಿತ ಭಾಗವಹಿಸುವಿಕೆ: ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನಿಮ್ಮ ಭಾಗವಹಿಸುವಿಕೆ ಸಂಪೂರ್ಣವಾಗಿ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿರುತ್ತದೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಯಾವುದೇ ಕಡ್ಡಾಯವಿಲ್ಲ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಬಯಸದಿದ್ದರೆ ನೀವು ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನೀವು ಸ್ವಯಂ ಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸಿದರೆ ಮಾತ್ರ ನೀವು ಸಹಿ ಮಾಡಬೇಕಾಗುತ್ತದೆ. ಮತ್ತಷ್ಟು ನೀವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆಗೆದುಕೊಳ್ಳಲು ಸ್ವಾತಂತ್ರ್ಯದಲ್ಲಿದ್ದಾರೆ. ನಿಮ್ಮ ಹಿಂತೆಗೆದುಕೊಳ್ಳುವಿಕೆಯು ನಿಮ್ಮ ಚಿಕಿತ್ಸೆಯನ್ನು ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಯಾವುದೇ ವೈದ್ಯರ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ ಎಂದು ನಾವು ಭರವಸೆ ನೀಡುತ್ತೇವೆ.

ಕಾರ್ಯ ವಿಧಾನ: ನಾವು ಒಂದು ಸಣ್ಣ ಪ್ರಮಾಣದ ಬಳ್ಳಿಯ ರಕ್ತ (5 ಮಿಲಿ) ಮತ್ತು ಜರಾಯು ಅಂಗಾಂಶವನ್ನು (100-250 ಮಿಗ್ರಾಂ) ಸಂಗ್ರಹಿಸುತ್ತೇವೆ

ಅವಧಿ: ಸಂಶೋಧನೆಯು ಒಟ್ಟು 3 ವರ್ಷಗಳು ನಡೆಯುತ್ತದೆ.

ಅಪಾಯಗಳು: ನಿಮ್ಮ ಮೇಲೆ ಯಾವುದೇ ಔಷಧಿ ಪರೀಕ್ಷಿಸಲಾಗುವುದಿಲ್ಲ. 5ml ನಷ್ಟು ಬಳ್ಳಿಯ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ. ತಾಜಾ ಮಾದರಿಯನ್ನು ತೆಗೆದುಕೊಳ್ಳಲಾಗುವುದಿಲ್ಲ.

ಪ್ರಯೋಜನಗಳು: ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸುವಿಕೆಯು ನಿಮಗೆ ಯಾವುದೇ ವೆಚ್ಚವನ್ನು ಒಳಗೊಂಡಿರುವುದಿಲ್ಲ. ಅಲ್ಲದೆ, ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನಿಮ್ಮ ಭಾಗವಹಿಸುವಿಕೆಗೆ ಯಾವುದೇ ವಿತ್ತೀಯ ಪರಿಹಾರವನ್ನು ಪಾವತಿಸಲಾಗುವುದಿಲ್ಲ.

ಗೌಪ್ಯತೆ: ವೈಯಕ್ತಿಕ ಗುರುತಿನ ಬಗ್ಗೆ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನೂ ಗೌಪ್ಯವಾಗಿರಿಸಲಾಗುವುದು ಮತ್ತು ಕಾನೂನಿನ ಅಗತ್ಯವಿದ್ದರೆ ಹೊರತುಪಡಿಸಿ ಯಾರಿಗೂ ಅದನ್ನು ಬಹಿರಂಗಪಡಿಸಲಾಗುವುದಿಲ್ಲ. ಯೋಜನೆಯ ಶೋಧಕರು ಮಾತ್ರ ಗುರುತಿನ ವಿವರಗಳಿಗೆ ಪ್ರವೇಶವನ್ನು ಹೊಂದಿರುತ್ತಾರೆ.

ಮಾದರಿಗಳಶೇಖರಣೆ: ನಿಮ್ಮ ಬಳ್ಳಿಯ ರಕ್ತದ ಮಾದರಿ ಮತ್ತು ಜರಾಯುಗಳಿಂದ ತಯಾರಾದ ಆನುವಂಶಿಕ ವಸ್ತುಗಳನ್ನು ಭವಿಷ್ಯದ ಸಂಶೋಧನಾ ಯೋಜನೆಗಳಿಗಾಗಿ ಸಂಗ್ರಹಿಸಬಹುದು. ಅಂತಹ ಸಂದರ್ಭದಲ್ಲಿ, ಬಳಕೆಗೆ ಮೊದಲು ನೈತಿಕ ಸಮಿತಿಯಿಂದ ಅನುಮತಿಯನ್ನು ಪಡೆಯಲಾಗುತ್ತದೆ.

ಫಲಿತಾಂಶಗಳನ್ನುಹಂಚಿಕೆ: ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಫಲಿತಾಂಶಗಳು ವೈಜ್ಞಾನಿಕ ಅಥವಾ ವೈದ್ಯಕೀಯ ನಿಯತಕಾಲಿಕಗಳಲ್ಲಿ ಅಥವಾ ವೈದ್ಯಕೀಯ ಸಮ್ಮೇಳನಗಳಲ್ಲಿ ಪ್ರಕಟವಾಗುತ್ತವೆ. ಈ ಪ್ರಕ್ರಿಯೆಯಲ್ಲಿ ನಾವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ನಿಮ್ಮನ್ನು ಒತ್ತಾಯಿಸುವುದಿಲ್ಲ; ಸಹ ಅಧ್ಯಯನದ ನಿಮ್ಮ ಸಹಕಾರವನ್ನು ನಾವು ಬಹಳವಾಗಿ ಶ್ಲಾಘಿಸುತ್ತೇವೆ. ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಿಮ್ಮ ಒಪ್ಪಿಗೆಯನ್ನು ಪಡೆಯಲು ನಾವು ಬಯಸುತ್ತೇವೆ. ಯಾವುದೇ ಮಾಹಿತಿಗಾಗಿ ನೀವು ತನಿಖೆದಾರರನ್ನು ಸಂಪರ್ಕಿಸಲು. ಈ ಅಧ್ಯಯನವು ಸಾಂಸ್ಥಿಕನೈತಿಕಸಮಿತಿಯಿಂದ ಅನುಮೋದಿಸಲ್ಪಟ್ಟಿದೆ ಮತ್ತು ಅವರ ಔಪಚಾರಿಕ ಅನುಮೋದನೆಯ ನಂತರ ಮಾತ್ರ ಪ್ರಾರಂಭಿಸಲ್ಪಟ್ಟಿದೆ. ಸಂಗ್ರಹಿಸಿದ ಮಾದರಿಯನ್ನು ಇನ್ಸ್ಟಿಟ್ಯೂಟ್ನಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುವುದು ಮತ್ತು ಈ ಮಾದರಿಯನ್ನು ಯಾವುದೇ ಭವಿಷ್ಯದ ಅಧ್ಯಯನಕ್ಕಾಗಿ ಸಂಗ್ರಹಿಸಲು ಮತ್ತು ಬಳಸಲು ನಮಗೆ ಅನುಮತಿಸಲು ನಾನು ವಿನಂತಿಸುತ್ತೇನೆ. ಈ ಡಾಕ್ಯುಮೆಂಟ್ಅನ್ನು ಸೆಲ್ಬಯಾಲಜಿ ಮತ್ತು ಮಾಲಿಕ್ಯುಲರ್ಜೆನೆಟಿಕ್ಸ್ವಿ ಭಾಗದಲ್ಲಿ ಸುರಕ್ಷಿತಲಾಕರ್ನಲ್ಲಿ ಮತ್ತು ಮಾಹಿತಿಗಾಗಿ ನಿಮಗೆ ನೀಡಿದನಕಲನ್ನು.

ಕ್ಲಿನಿಕಲ್ ಇನ್ವೆಸ್ಟಿಗೇಟರ್ಗಳ ಹೆಸರು:

1. ಡಾ. ಕೃಷ್ಣಪ್ಪ ಜೆ.
ಮಕ್ಕಳ ವಿಭಾಗದ ಪ್ರೊ
ಮೊಬೈಲ್: 9448587233

2. ಡಾ. ಶೀಲಾ ಎಸ್.ಆರ್.
ಪ್ರಸೂತಿ ಮತ್ತು ಸ್ತ್ರೀರೋಗ ಶಾಸ್ತ್ರದ ಪ್ರೊ
ಮೊಬೈಲ್: 9845217277

ತಿಳುವಳಿಕೆಯ ಅನುಮೋದನೆ ರೂಪ

ಅಧ್ಯಯನದ ಶೀರ್ಷಿಕೆ: ಗರ್ಭಾವಸ್ಥೆಯ ವಯಸ್ಸಿನ ಜನನಕ್ಕೆ ಇನ್ಸುಲಿನ್ ತರಹದ ಬೆಳವಣಿಗೆಯ ಅಂಶದ ಅಕ್ಷದ ಅಂಶಗಳ ಪಾತ್ರವು ಚಿಕ್ಕದಾಗಿದೆ

ಪಾಲ್ಗೊಳ್ಳುವವರ ಘೋಷಣೆ:

- ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನವನ್ನು ನನಗೆ ವಿವರಿಸಲಾಗಿದೆ; ಇದನ್ನು ಚರ್ಚಿಸಲು ಮತ್ತು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶ ನೀಡಲಾಗಿದೆ. ನನ್ನ ಎಲ್ಲಾ ಪ್ರಶ್ನೆಗಳಿಗೆ ನನ್ನ ತೃಪ್ತಿಗೆ ಉತ್ತರ ನೀಡಲಾಗಿದೆ.
- ಈ ಸಂಶೋಧನಾ ಯೋಜನೆಯು ಬಳ್ಳಿಯ ರಕ್ತ ಮತ್ತು ಜರಾಯು ಮಾದರಿಯ ರೇಖಾಚಿತ್ರ ಮತ್ತು ಪರೀಕ್ಷೆಯನ್ನು ಒಳಗೊಂಡಿರುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಮಾಹಿತಿ ಶೀಟ್ನ ಪ್ರತಿ ಪುಟವನ್ನು ನಾನು ಓದಿದ್ದೇನೆ ಅಥವಾ ಅದನ್ನು ನನಗೆ ಓದಿದೆ.
- ಮಾಹಿತಿ ಹಾಳೆಯಲ್ಲಿ ವಿವರಿಸಿದಂತೆ ಪ್ರವೇಶ ಆರೋಗ್ಯ ಮಾಹಿತಿಯನ್ನು ಅನುಮತಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.
- ನನ್ನಿಂದ 5 ಮಿಲಿ ಬಳ್ಳಿಯ ರಕ್ತದ ಮಾದರಿ ಮತ್ತು 100-250 ಮಿಗ್ರಾಂ ಜರಾಯು ಅಂಗಾಂಶವನ್ನು ಸಂಗ್ರಹಿಸಲು ಮತ್ತು ಮಾಹಿತಿ ಹಾಳೆಯಲ್ಲಿ ವಿವರಿಸಿದ ಸಂಶೋಧನಾ ಉದ್ದೇಶಗಳಿಗಾಗಿ ಡೇಟಾವನ್ನು ಸಂಗ್ರಹಿಸಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.
- ಸಂಗ್ರಹಿಸಿದ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನು ಗೌಪ್ಯವಾಗಿರಿಸಲಾಗುವುದು ಎಂದು ನಾನು ಅರ್ಥ ಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.
- ನಾನು ಈ ಸಂಶೋಧನಾ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸುತ್ತೇನೆ.

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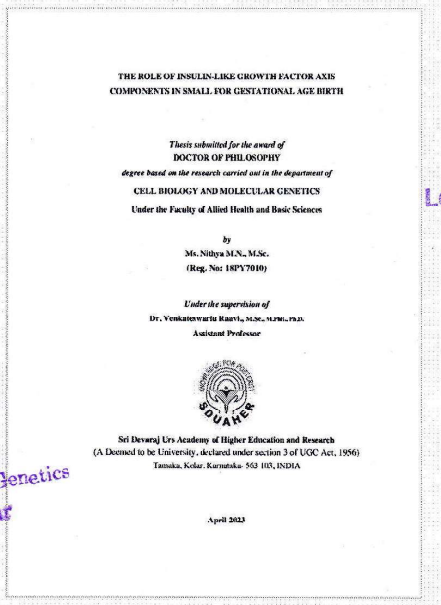

THE ROLE OF INSULIN-LIKE GROWTH FACTOR AXIS COMPONENTS IN SMALL FOR GESTATIONAL AGE BIRTH

Thesis submitted for the award of
DOCTOR OF PHILOSOPHY
degree based on the research carried out in the department of
CELL BIOLOGY AND MOLECULAR GENETICS
Under the Faculty of Allied Health and Basic Sciences

by
Ms. Nithya M.N., M.Sc.
(Reg. No: 18PY7010)

Under the supervision of
Dr. Venkateswarlu Raavi, M.Sc., M.Phil., Ph.D.
Assistant Professor

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THE ROLE OF INSULIN-LIKE GROWTH FACTOR AXIS COMPONENTS IN SMALL FOR GESTATIONAL AGE BIRTH Thesis submitted for the award of DOCTOR OF PHILOSOPHY degree based on the research carried out in the department of CELL BIOLOGY AND MOLECULAR GENETICS Under the Faculty of Allied Health and Basic Sciences by Ms. Nithya M.N., M.Sc. (Reg. No: 18PY7010) Under the supervision of Dr. Venkateswarlu Raavi., M.Sc., M.Phil., Ph.D. Assistant Professor Sri Devaraj Urs Academy of Higher Education and Research (A Deemed to be University, declared under section 3 of UGC Act, 1956) Tamaka, Kolar, Karnataka- 563 103, INDIA April 2023 Declaration by the Ph.D. Scholar I, Ms. Nithya M.N., hereby declare that the work presented in the thesis entitled "The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth" is original and does not contain any fabrication. This research work was carried out by me under the supervision of Dr. Venkateswarlu Raavi., Assistant Professor, Department of Cell Biology and Molecular Genetics under the faculty of Allied Health and Basic Sciences, Sri Devaraj Urs Academy of Higher Education and Research. Furthermore, I have not plagiarised any part of the thesis. Where necessary, I have borrowed the material from other authors/researchers and given appropriate credit to them through reference citation or acknowledgement. I also declare that the thesis has not been submitted for the award of any other degree/diploma to any other Higher Educational Institution. Date: Place: Ms. Nithya M.N. (Reg. No: 18PY7010) Supervisor Certificate This is to certify that the research work presented in this thesis entitled "The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth" is original and does not contain any fabrication or plagiarism. Furthermore, this thesis has not been submitted for the award of any other degree/diploma to any other Higher Educational Institution. Date: Place: Signature of the Supervisor Dr. Venkateswarlu Raavi., M.Sc., M.Phil., Ph.D. Assistant Professor Dept. of Cell Biology and Molecular Genetics, SDUAHER, Tamaka, Kolar, Karnataka - 563 103 Co-supervisors Certificate This is to certify that the thesis entitled "The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth" is an original work carried out by Ms. Nithya M.N., under our co-supervision in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karanataka-563 103. A plagiarism check was carried out on the thesis, and the report was found to be acceptable. Co-supervisor Dr. Krishnappa J., Professor Dept. of Paediatrics, SDUMC, SDUAHER, Tamaka, Kolar, Karnataka - 563 103 Co-supervisor Dr. Sheela S. R., Professor Dept. of Obstetrics and Gynaecology, SDUMC, SDUAHER, Tamaka, Kolar, Karnataka - 563 103 Date: Date: Place: Place: Institutional Endorsement This is to certify that the thesis entitled "The role of Insulin-like Growth Factor axis components in Small for Gestational Age Birth" is a bonafide research work carried out by Ms. Nithya M.N., in the Department of Cell Biology and Molecular Genetics, under the guidance and supervision of Dr. Venkateswarlu Raavi., Assistant Professor, Department of Cell Biology and Molecular Genetics, and co-supervision of Dr. Krishnappa J., Professor, Department of Paediatrics, and Dr. Sheela S. R., Professor, Department of Obstetrics and Gynaecology, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnatak- 563 103. Head of the Department Cell Biology and Molecular Genetics Dean Faculty of Allied Health and Basic Sciences Date: Date: Place: Place: ACKNOWLEDGEMENT It is with profound respect and gratitude I wish to express my gratefulness to my Ph.D. supervisor Dr. Venkateswarlu Raavi., Assistant Professor, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education, Tamaka, Kolar, for his able guidance, valuable suggestions, and encouragement throughout my work. I express my gratitude to my co-supervisors Dr. Krishnappa J., Professor, Department of Pediatrics, and Dr. Sheela S. R., Professor, Department of Obstetrics and Gynaecology, SDUAHER, for having allowed me to collect the samples for the study. I sincerely thank Dr. Manojit Debnath., Additional Professor, Department of Human Genetics, National Institute of Mental Health and Neuroscience, Bangalore, for his invaluable suggestions as an external member of the Doctoral Advisory Committee. 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Recommendations

10. Recommendations

The thesis generated basic information on the alternation in the IGF-axis components in SGA neonates. The thesis recommends that the analysis of IGF-axis components might be helpful in understanding the molecular and cellular level alteration in SGA, which can be used for better management of neonates/children born to the Indian population.