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RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR FOR UNEXPLAINED RECURRENT MISCARRIAGE: A RANDOMISED PLACEBO CONTROLLED MULTI-CENTRE STUDY

by

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Abstract

Immune mediated mechanisms are thought to contribute to recurrent pregnancy losses. A number of treatment options with limited evidence are being used in clinical practice to treat women with recurrent miscarriages.

The objectives of this thesis was

a. To summarise the available evidence for granulocyte colony stimulating factor (G-CSF) in reproductive medicine.
b. To perform a randomised controlled study (RCT) to evaluate the efficacy and safety of recombinant human granulocyte colony stimulating factor (rhG-CSF) in women with unexplained recurrent miscarriages.

The main conclusions from this thesis are:

a. The systematic narrative review found that available evidence is of poor quality, but suggestive of benefit with granulocyte colony stimulating factor in women with recurrent miscarriages.
b. The RCT concluded that administration of rhG-CSF does not improve pregnancy outcomes among women with a history of unexplained recurrent miscarriages. RhG-CSF appears to be safe for both mothers and their offspring/s.

Dedication

I would like to dedicate this thesis to my parents, Eapen Verghese and Jolly Eapen, who sacrificed their whole life for their children's upbringing.

I also dedicate this thesis to Anita, Amelia, Abram, Aaron and Aidan – my wife, daughter and sons without whom this journey wouldn't have been possible.

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Abbreviations

Abbreviation	Definition
ABS	Absolute
ACA	Anti-cardiolipin Antibodies
ADA	Anti-Drug Antibodies
AE	Adverse Event
ALI	Acute Lung Injury
ALT	Alanine Aminotransferase
AMH	Anti Mullerian Hormone
ANA	Antinuclear antibody
ANC	Absolute Neutrophil Count
ARDS	Acute Respiratory Distress Syndrome
ART	Assisted Reproductive Technology
ASRM	American Society for Reproductive Medicine
AST	Aspartate Aminotransferase
ATC	Anatomical Therapeutic Classification
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
°C	Degrees Celsius
FBC	Full Blood Count
C4M2	Chromosome 4 Mutation 2
CD	Cluster of Differentiation
CIN	Cervical Intraepithelial Neoplasia
CMH	Cochran Mantel Haenszel
CRF	Case Report Form

CRO	Contract Research Organization
CSR	Clinical Study Report
CTCAE	Common Toxicity Criteria for Adverse Events
DC	Dendritic Cell
DMC	Data Monitoring Committee
DVT	Deep Vein Thrombosis
E2	Oestradiol
E. coli	Escherichia coli
ECG	Electrocardiogram
eCRF	Electronic Case Report Form
ELISA	Enzyme-linked Immunosorbent Assay
ESHRE	European Society of Human Reproduction and Embryology
FAS	Full Analysis Set
FBC	Full Blood Count
FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone
g	Gram
GA	Gestational Age
G-CSF	Granulocyte Colony Stimulating Factor
GGT	Gamma-glutamyl Transferase
hCG	Human Chorionic Gonadotropin
Hep	Hepatitis
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HSIL	High-grade Squamous Intraepithelial Lesion
IB	Investigator's Brochure

ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IEC	Independent Ethics Committee
IND	Investigational New Drug
INR	International Normalised Ratio
IRB	Institutional Review Board
IVF	In Vitro Fertilization
IVIG	Intravenous Immunoglobulin
IVIL	Intravenous Intralipid
IWRS	Interactive Web Response System
kg	Kilogram
L	Litre
LA	Lupus Anticoagulant
LAD	Leucocyte Antigen Detection
LDH	Lactate Dehydrogenase
LH	Luteinising Hormone
LIT	Leucocyte Infusion Therapy
LPD	Luteal Phase Defect
m2	Square Meter
MAIT	Mucosa-Associated Invariant T Cells
mcg	Microgram
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MDSC	Myeloid Derived Suppressor Cells
MedDRA	Medical Dictionary for Regulatory Activities

mg	Milligram
mL	Millilitre
μL	Microlitre
mM	Millimolar
mol	Mole
MTHFR	Methylene Tetra Hydro Folate Reductase
N	Number
NAB	Neutralizing Antibody
NCI	National Cancer Institute
NK	Natural Killer Cells
PAI	Plasminogen Activator Inhibitor
PCOS	Polycystic Ovarian Syndrome
PD	Pharmacodynamic
pg	Picograms
PIS	Participant Information Sheet
PK	Pharmacokinetic
pH	Negative Logarithm of the Hydronium Ion Concentration
PRL	Prolactin
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
OD	Once a Day
rDNA	Recombinant DNA
rhG-CSF	Recombinant Human Granulocyte Colony Stimulating Factor
RIF	Recurrent Implantation Failure
RM	Recurrent Miscarriage
RPL	Recurrent Pregnancy Loss

SAB	Spontaneous Abortion
SAE	Serious Adverse Event
SC	Subcutaneous
SRC	Study Review Committee
t	Time
TORCH	Toxoplasmosis, Rubella, Cytomegalovirus and Herpes
Th	T Helper Cells
TNF	Tumour Necrosis Factor
TPO	Thyroid Peroxidase
Treg	Regulatory T Cells
TSH	Thyroid Stimulating Hormone
TVU	Transvaginal Ultrasound
U/S	Ultrasound
UA	Urinalysis
ULN	Upper Limit of Normal
uNK	Uterine Natural Killer Cells
WBC	White Blood Cell
WHO	World Health Organization

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CHAPTER 1

RECURRENT MISCARRIAGE - BACKGROUND AND UNCERTAINTIES

Introduction

Loss of pregnancy prior to viability is defined as a miscarriage. Approximately 1 in 5 pregnancies sadly end in these sporadic events, the risks of which increases with advancing maternal age. The most common reason for miscarriage is chromosomal abnormality in the embryo. Repeated episodes of sporadic miscarriages result in recurrent miscarriages (RM). Many terms have been used in the past to describe repeated miscarriages such as repeated abortions, repeated pregnancy failure and repeated embryo demise. Women prefer clinicians to use more sensitive terminologies as some of the above terms may otherwise imply blame[1].

Royal College of Obstetricians and Gynaecologists (RCOG) define recurrent miscarriage as 3 or more pregnancy loss prior to 24 weeks of gestation[2]. RM affects 1% of couples. The prevalence increases to 3% if biochemical pregnancies were added and accounted for[3]. There is considerable debate regarding the definition, incidence and management of recurrent miscarriages. American

Society for Reproductive Medicine (ASRM) defines recurrent miscarriage as 2 or more documented pregnancy loss prior to 20 weeks of gestation[4]. These are pregnancies either confirmed by ultrasonography or by histology. Based on this definition the incidence ranges from 3-5%. Jaslow et al suggested that outcomes of index pregnancy following 2 or 3 miscarriages were similar[5] and therefore, a clinical plea to revisit the true definition of RM and to initiate investigations after 2 miscarriages.

Even though the terms recurrent pregnancy loss and recurrent miscarriage were used interchangeably, recent studies suggest they represent two different entities[6].

Recurrent pregnancy loss entails loss of any type of pregnancy irrespective of its location and gestational age (including molar, ectopic or biochemical) and RM is exclusively used for pregnancy failure following a clinically confirmed pregnancy i.e. a visualised pregnancy. Biochemical pregnancies are also termed as non-visualised pregnancies. Clinical miscarriages may be classified as early (less than 12 weeks of gestation and late clinical pregnancy losses (over 12 weeks of gestation). RM can be consecutive or non-consecutive.

Furthermore, based on previous livebirth/s, RM can be classified into:

a. Primary – is loss of 3 or pregnancies without any livebirth
b. Secondary – is loss of 3 or more pregnancies following a livebirth or
c. Tertiary – is loss of 3 or more pregnancies; however, in no particular order with livebirth/s in between.

It is imperative to distinguish these conditions to offer reliable and accurate opinion to counsel women regarding the long-term prognosis.

Impact of RM

RM can result in significant emotional, physical and financial implications for a couple. About a third of couples experience depression and anxiety while suffering RM[7]. This may result in a strain in their relationship. Most of the studies linked recurrent miscarriages with only the female partners[8,9]. The female partner may have emotional disturbances after the episode which may last for days or months. It is also quite normal to feel anxious about the following pregnancy and its outcome. Recent studies suggested that male partners also undergo same emotional trauma as their female partners[7].

Established RM clinics should have facilities to organise behavioural therapy, counselling support and also support groups for couple with RM.

Clinical Evaluation

RM is a multifactorial condition and in spite of comprehensive investigation pathway followed by clinicians, only about 50% of RM are attributed to an identifiable cause. The remaining cases are considered to be unexplained.

Importance of a detailed history

Epidemiological and life style factors can play a role in recurrent miscarriage. Niekerk et al reported increasing risk of miscarriage with earlier gestation, majority of which occurs under 12 weeks of gestation[10]. Advancing maternal age which causes decline in oocyte number and quality also increases the risk of miscarriage. Previous pregnancy loss increases the risk of miscarriage in index pregnancy[11]. Increase in paternal age was associated with autism in offspring until now, but new research suggests increase in miscarriage related to sperm DNA fragmentation and sperm aneuploidy. Consanguineous marriages can also increase the risk of RM by increased risk of malformations and autosomal recessive diseases.

Previous studies by Lashen et al and Lo et al suggested BMI as a negative prognostic factor for live birth in index pregnancy[12,13]; however, Kolte et al[6] suggested no statistically significant relation. Life style factors like high intake of caffeine, tobacco and alcohol and increased stress are associated with poor obstetric outcome and therefore may also have a link in early pregnancy.

Hemminki et al in a questionnaire based study reported that risks of miscarriage varied by education and occupation[14] . Contrary to belief, miscarriage rates were higher in the middle social class rather than lower social class. Arbitrarily, it was explained by increased occupational stress levels and higher age at first pregnancy. In a more recent study by Catak et al, risk of spontaneous miscarriages were higher in women who had less than 5 years of education and also for mothers who were employed at the time of conception[15] . Further, Norsker et al in a cohort study demonstrated that women with <10 years of education had an elevated risk of spontaneous abortion when compared with women with >12 years of education (HR 1.19 (95% CI 1.05 to 1.34))[16] .

Therefore, information should be gathered about social class, educational level, occupation and daily activity levels as part of assessment and pre-pregnancy counselling.

The investigations can be performed on the:

1. Female partner
2. Male partner and/or the
3. Embryo

The clinical evaluation and strength of evidence for association with recurrent miscarriage are listed in table 1.1, table 1.2 and table 1.3.

I have collated this evidence from basic sciences, randomised controlled studies, non-randomised controlled studies, clinical reviews, observational studies, case reports and clinical guidelines.

I have classified the strength of evidence as strong, limited or unknown.

Strong – Evidence from prospective randomised or non-randomised trials, which provide a clear and compelling effect on clinical outcomes.

Limited – Evidence from uncontrolled studies and case reports which proved little or no enhancement in clinical outcomes.

Unknown – Evidence which is currently hypothetical.

Table 1.1 – Clinical Evaluation for the female partner

Risk Factors	**Strength of evidence for association with recurrent miscarriage**
Genetic	
➢ Parental karyotyping[17]	Strong
Anatomic – Uterine abnormality	
➢ Uterine septum[18]	Limited
➢ Sub mucous fibroids[19]	Limited
➢ Sub mucous polyp[20]	Limited
➢ Intrauterine adhesions[21]	Limited
➢ Hydrosalpinx[22]	Limited
➢ Cervical Insufficiency[23] – 2nd trimester	Strong
Infections[24]	
➢ Chlamydia	Limited
➢ TORCH	Limited
➢ Endometritis	Limited
Endocrinological[2]	
➢ LPD	Limited
➢ Hypothyroidism/hyperthyroidism	Limited
➢ Glucose metabolism	Limited
➢ PRL	Limited
➢ FSH, LH, E2, AMH	Limited

➢ PCOS profile	Limited
➢ Vit D	Limited
Immunological incl. autoimmune [25,26]	
➢ LA	Strong
➢ Antiphospholipid ab	Strong
➢ Beta 2 glycoprotein	Strong
➢ Auto-antibodies	Limited
➢ Anti-nuclear antibodies	Limited
Thrombophilia[26,27]	
➢ Antithrombin 3	Limited
➢ Factor V Leiden	Limited
➢ Factor 2 prothrombin	Limited
➢ MTHFR	Limited
➢ Protein C and Protein S	Limited
➢ PAI Polymorphism	Limited
➢ C4M2	Limited
Extended reproductive immunology tests[28,29,30]	
➢ Functional NK cell profile	Limited
➢ TH1:TH2 Intracellular cytokine Ratio	Limited
➢ LAD testing	Limited
➢ HLA DQ – alpha	Limited

Table 1.2 - Clinical Evaluation for the male partner

Risk Factors	Strength of evidence for association with recurrent miscarriage
Genetic	
Parental karyotyping[17]	Limited
Inherited Thrombophilia[26,27]	
➢ C4M2	Limited
➢ Factor V Leiden	Limited
Extended Reproductive immunology[28,29,30]	
➢ HLA DQ – alpha	Limited
➢ LAD	Limited
Sperm[31,32]	
➢ Sperm Aneuploidy	Limited
➢ DNA Fragmentation	Limited

Table 1.3 - Clinical Evaluation on the embryo

Risk Factors	Strength of evidence for association with recurrent miscarriage
Genetic	
➢ Fetal Karyotyping[33]	Strong

Clinical Evaluation for Recurrent Miscarriage

Genetic investigations

The incidence of chromosomal abnormalities in one partner is 2.7 % to 6% for couple suffering RM[34,35]. Due to this relatively low incidence of translocations, routine testing in form of parental karyotyping is not recommended, for cost-effective reasons. Parental karyotyping is mainly indicated if there is evidence of translocation after fetal karyotyping. However, this does not predict the risk of unbalanced translocation in the offspring[36].

Clinicians may also initiate parental karyotyping if there is history of multiple miscarriages, family history of RM or else a history of translocations in siblings of partners with RM. The most common form of translocations is balanced translocation. Other abnormalities may be inversion, deletion or duplications of parental chromosomes[37].

Uterine abnormalities

Disruption of normal development of female genital tract can result in uterine abnormalities. The prevalence of uterine abnormalities ranges between 1 to 10% in women with RM[38]. The abnormalities can be uterus with rudimentary horn, unicornuate, didelphis, septate or arcuate uterus. All uterine anomalies may result in adverse pregnancy outcomes.

Septate uterus is the most common significant uterine malformation[18]. Investigations performed are ultrasonography, hystero-salpingography, saline sonogram, hysteroscopy and/or laparoscopy. Latest studies suggests three dimensional ultrasound scan to diagnose uterine abnormalities has high sensitivity and specificity compared to hystero-laparoscopy[39].

Infections

There is no indication for screening for infections during early pregnancy unless there is evidence of acute onset or systemic evidence of infections. Infections like chlamydia, genital tuberculosis may result in fallopian tube blockage or endometrial adhesions respectively and cause subfertility rather than recurrent miscarriages[40]. Plasma cell infiltrate in endometrium as a result of repeated subclinical infections may result in chronic endometritis[41].

Mcqueen et al 2015 and Cicinelli et al suggested that incidence of endometritis can be as high as 58% in women with recurrent miscarriage[42,43]. They also suggested that prophylactic antibiotics improved live birth rates in the index pregnancy. Due to lack of randomised controlled studies routine use of antibiotics are not recommended in women with recurrent miscarriages.

Endocrine

Uncontrolled thyroid disorders, glucose metabolism and raised prolactin are related to subfertility, sporadic miscarriages and adverse obstetric outcomes[2]. The link between these conditions and recurrent miscarriage is weak. Therefore routine screening for thyroid abnormalities, HbA1C , prolactin or polycystic ovaries is not recommended. However, some clinicians perform these tests if patients present symptoms for the above aspects. As these investigations are considered to be relatively inexpensive tests, they form part of pre-pregnancy counselling in women with recurrent miscarriage. Defect in corpus luteum resulting in low progesterone levels in the mid luteal phase may result in a non-receptive endometrium and can cause recurrent miscarriages[44]. Reproductive endocrinologists consider this disorder as luteal phase defect. There are inconsistencies regarding this as some groups rely on a histological diagnosis and therefore can pose practical diagnostic difficulties in routine clinical practise. A higher prevalence for anti-thyroid antibodies is identified in women with recurrent miscarriage. A meta-analysis suggested that presence of TPO antibodies increased risk of RM by an odds ratio with 2.3[45]. However, routine testing is not advised outside research settings.

Thrombophilia

Thrombophilic factors can be acquired or inherited. Acquired thrombophilia is a well-recognised risk factor for recurrent miscarriages. Presence of lupus anticoagulant, high titres of anti-cardiolipin antibodies and/or beta 2 glycoprotein are associated with recurrent miscarriages[2].

It is postulated that thrombophilia factors can increase the risk of placental thrombosis. This results in reduced placental perfusion causing recurrent miscarriage[26]. Complement activation resulting in recruitment of inflammatory cells damages the fetus and placenta in acquired thrombophilia. Studies in mice suggest the pathway in inherited thrombophilia lack thrombomodulin protein C inhibiting placental trophoblast cells and thus impairing placental development[27]. There is conflicting evidence for the relationship between inherited thrombophilia and recurrent miscarriage. Some older studies are in favour of this relationship while more recent studies refute the evidence. Jivraj et al suggested increased risk of miscarriage in index pregnancy (after a diagnosis of recurrent miscarriage) if either the female or the male partner had evidence of inherited thrombophilia[46].

Immunological investigations

There is emerging evidence that some of the unexplained RM may be due to an abnormal maternal immune system. Recently, there is considerable interest in reproductive immunology testing, mainly the peripheral and uterine natural killer cell profile.

Peripheral and uterine NK cells differ in structure and function and there are no universally accepted normal ranges[28&30]. NK cell levels can be influenced by stress, ethnicity and age. Rai et al suggested NK cell assessment should not be routinely performed outside a research context[47]. An anti-HY antibody is a relatively new concept within reproductive immunology. Epidemiological studies conducted by Nielsen et al in Scandinavian women suggest decrease in livebirth in index pregnancy following a prior birth of a boy[48]. In women with secondary recurrent miscarriages, presence of anti-HY antibody provides negative prognostic value. Other extended immune testing involves measurements of anti-nuclear antibodies, Th1/Th2 ratio, interleukins, cytokines and anti- HLA antibodies[28, 29&30]. The value of extended reproductive immunology testing in recurrent miscarriage remains debatable. These are not routinely recommended outside a research context.

Tests for sperm DNA fragmentation and sperm aneuploidy

Suboptimal sperm results and raised DNA fragmentation are associated with subfertility. Ribas Maynou et al demonstrated that DNA fragmentation in sperm was significantly higher in male partners of women who had unexplained RM[49].85% of the partners had DNA damage compared to 33% in fertile sperm donors.

Robinson et al and Zhao et al suggested that DNA fragmentation testing should be routinely offered to couples following a diagnosis of recurrent miscarriage[50&51]. Sperm aneuploidy can also be performed by as part of investigations for male partners.

Fetal karyotyping

Fetal karyotyping is recommended for women with RM. It provides information about the current pregnancy loss. There are studies suggesting better prognosis for women in index pregnancies following an abnormal fetal karyotype. Incidence of aneuploidy may be as high as 78%[52]. Studies have used different techniques for fetal karyotyping including FISH and array CGH[53].

A fetal chromosomal abnormality may be related to a sporadic event or inherited which may warrant further investigation in the parents. Ogasawara et al suggested that the risk of aneuploidy may decrease in women who previously had higher order miscarriages[54]. Stephenson et al suggested that aneuploidy rate is independent of maternal age[17].

Treatments for Recurrent Miscarriage

The treatment options and strength of evidence for association with recurrent miscarriage are listed in table 1.4, table 1.5 and table 1.6.

Table 1.4 - Treatment options for the female partner

Risk Factors	Treatment option/s	Strength of evidence for improving outcomes in recurrent miscarriage
Genetic		
➢ Parental karyotyping[17]	PGD	Strong
	Donor gamete	Strong
Anatomic – Uterine abnormality		
➢ Uterine septum[18]	Resection	Limited
➢ Sub mucous fibroid[19]	Resection of fibroid	Strong
➢ Sub mucous polyp[20]	Resection of polyp	Strong
➢ Intrauterine adhesions[21]	Division of adhesions	Limited
➢ Hydrosalpinx[22]	Salpingostomy	Unknown
➢ Cervical Insufficiency[23] – 2nd trimester	Cervical cerclage	Strong
Infections[24]		
➢ Chlamydia	Antibiotics	Limited
➢ TORCH		
➢ Endometritis		
Endocrinological[2]		
➢ LPD	Progesterone	Limited
➢ Hypothyroidism	Thyroxine	Limited

➢ Hyperthyroidism	Anti-thyroid medications	Limited
➢ Abnormal glucose metabolism	Metformin	Limited
➢ Raised PRL	Dopamine agonists	Limited
➢ Reduced ovarian reserve	IVF	Limited
➢ Evidence of PCOS	Metformin	Limited
➢ Low Vit D	Vit D supplementation	Limited
Immunological and autoimmune disorders[25,26]		
➢ LA	Aspirin and LMWH	Strong
➢ Antiphospholipid antibodies		
➢ Beta 2 glycoprotein		
➢ Auto-antibodies	Aspirin and LMWH Glucocorticoids	Limited
➢ Anti-nuclear antibodies or evidence of systematic rheumatological issue (for e.g. Systemic lupus erythematosus		
Thrombophilia[26,27]		
➢ Antithrombin 3	Aspirin and LMWH (Folic acid in addition for cases with MTHFR mutation)	Limited
➢ Factor V Leiden		
➢ Factor 2 prothrombin		
➢ MTHFR		
➢ Protein C		

➢ Protein S ➢ PAI Polymorphism ➢ C4M2	Aspirin and LMWH	Limited
Extended reproductive immunology[28,29,30]		
➢ Functional NK cell profile ➢ TH1:TH2 Intracellular cytokine Ratio ➢ LAD testing ➢ HLA DQ – alpha	Steroids IVIL IVIG TNFα blocker G-CSF LIT	Limited

Table 1.5 - Treatment options for the male partner

Risk Factors	**Treatment option/s**	**Strength of evidence for improving outcomes in recurrent miscarriage**
Genetic		
Parental karyotyping[17]	PGD	Strong
	Donor gamete	Strong
Inherited Thrombophilia[26,27]		
➢ C4M2 ➢ Factor V Leiden	Aspirin and LMWH (Treatment is for the female partner)	Limited

Extended Reproductive immunology[28,29,30]		
➢ HLA DQ – alpha	Steroids IVIL IVIG TNFα blocker G-CSF LIT (Treatment is for the female partner)	Limited
➢ LAD		
Sperm[31,32]		
➢ Sperm Aneuploidy	PGS	Limited
➢ DNA Fragmentation	Anti-oxidants	Limited

Table 1.6 - Treatment options for embryo aneuploidy

Risk Factors	**Treatment option/s**	**Strength of evidence for improving outcomes in recurrent miscarriage**
Genetic		
➢ Fetal karyotyping[33]	PGS/PGD	Strong

Treatment for RM

There are surgical, medical, IVF with PGS/PGD and non-pharmacological treatment options for RM.

Surgical treatment options

Surgical treatment are reserved for uterine abnormalities and limited to septate uterus. Other surgical procedures such as sub-mucous myomectomy, resection of endometrial polyp and division of intrauterine adhesions in cases of Ashermans syndrome are mostly related to subfertility rather than recurrent miscarriage. Pritts et al suggested treatment of submucosal fibroids significantly improves outcomes with recurrent pregnancy losses[55].

Kowalik et al in a cochrane review suggested that there is no prospective randomised data for different treatment options[56]; however, septate uterus in a clinical setting is routinely treated by hysteroscopic metroplasty. Clinicians prefer this technique as septate uterus carries worst reproductive outcome. The poor obstetric outcomes are directly proportional to the length of the septum as measured by MRI imaging techniques or as assessed at a routine hysteroscopy

Medical treatment options

Anticoagulants:

Antiphospholipid syndrome is the only treatable cause of thrombophilia. LMWH and aspirin together showed significant improvement in LBR compared to LMWH alone in a number of studies[57,58,59&60].Even though there are some studies which suggest benefit for LMWH and aspirin for women with inherited thrombophilia, none of the trials were adequately powered[61].ALIFE 2 trial is currently in its recruitment phase and may provide further answers on efficacy of LMWH in inherited thrombophilia[62].

Skeith et al in a systematic review combined the results of 8 RCTs and found no benefit of LMWH for prevention of pregnancy loss in women with inherited thrombophilia and recurrent pregnancy loss[63]. This study suggested a RR of 0.81 ; 95% CI 0.55-1.19). Mak et al and Empson et al found a benefit with anticoagulants to improve LBR in women with RPL[64,65].

Progesterone

Progesterone preparation in the form of vaginal, oral and intramuscular preparations has been used for a long time and a number of studies have

suggested beneficial effect. Kumar et al[66] suggested a reduction of miscarriage in index pregnancy following oral dydrogestone, RR= 2.4; CI, 1.3 to 5.9. Coomarasamy et al conducted the largest multicentre trial with vaginal progesterone in women with unexplained RM and concluded lack of benefit with progesterone[67].

Immunomodulatory agents

Most of the medications in this group are new investigational medications.

IVIG

Intravenous immunoglobulin (IVIG) is a fractionated blood product used in inflammatory medical conditions. This is used as an off-license medication in treatment of recurrent miscarriage associated with abnormal immune response. Previously conducted RCTs suggested conflicting conclusions for benefit and no-benefit[58&151]. These trials had significant heterogeneity with limited number of patients and varying dose. Intravenous immunoglobulin has since been tested in a number of RCTs in unexplained RPL patients. The Cochrane review on immunotherapy in RPL by Wong et al concluded that IVIG was not beneficial in RPL[68]. Egerup at al in a systematic review and meta-analysis of IVIG in RPL suggested some benefit for women with secondary RM[69]. Due to

the heterogeneity in the study groups, IVIG is not recommended for routine use in women with RPL as it is an expensive medication with serious systemic side effects which may include transfusion reaction, anaphylactic shock and hepatitis[2].

Glucocorticoids

Prednisolone has a high affinity for glucocorticoid receptors. Uterine NK cells are different from peripheral NK cells by the presence of CD 56 and absence of CD 16 and CD 4. A number of studies suggested an increased number of uterine NK cells in endometrium of women with RM. These cells have specific glucocorticoid receptors which are bound by prednisolone. Laskin et al and Tang et al suggested an increase in LBR; however, this was not statistically significant[70,71]. There was also a high incidence of side-effects associated with administration of steroids.

TNF Alpha inhibitors

TNF alpha is a pro-inflammatory cytokine which can be blocked by monoclonal antibodies. One prospective cohort study suggested a slight reduction in first trimester miscarriages with an increased risk of birth anomalies[72] . There are no

prospective RCT for TNF Alpha in women diagnosed with RM. RCOG recommends there is no evidence to use TNF alpha blockers outside a research context[2].

G-CSF

These are hematopoietic cytokines which help with neutrophil differentiation and proliferation. Scarpellini et al suggested statistically significant increase in LBR after treatment with G-CSF[73]. A number of studies[167-181] performed in women undergoing assisted conception and those who suffered recurrent miscarriage suggested improvement in outcomes. There was also evidence with an in vitro study using GM-CSF that there might be potential benefit[166].

IVF with PGS/PGD

PGD aims at detecting single gene mutation with PCR or FISH. PGD is performed in RM where the parental karyotyping suggests chromosomal abnormality. PGS uses FISH and CGH to screen embryos for aneuploidy. Studies suggest that both PGD and PGS are beneficial for couple with combined RM and subfertility. In cases of RM, PGD may increase the LBR[74,75], whereas PGS appears to be controversial[76].

Non-pharmacological treatment

Women after suffering repeated miscarriages are understandably under considerable amount of stress and anxiety. Stray-Pederson et al and Clifford et al highlighted the importance of non-pharmacological treatment involving early pregnancy reassurance scans and counselling in improving the outcomes in women with RM[77,78].

Uncertainties in recurrent miscarriage investigations and treatment

Ideally, investigations should be performed if it can identify a risk factor or improve prognosis of a medical condition. However, RM being a diverse medical condition, the risk associations remain weak. There is no consensus amongst clinicians or professional bodies regarding the definition, investigations or managements. There are varied epidemiological and parental factors influencing the final outcome. Evidence for most of the genetic and epidemiological factors and their relation to RM are extrapolated from data from sporadic miscarriages and population registry studies.

Despite extensive investigation protocols, about half of RM cases remain unexplained. RCOG, ASRM and ESHRE have produced guidelines for investigation and management with limited consistency amongst them. Many investigations and treatment options are not advised by these professional bodies. This poses a significant clinical diagnostic dilemma for the clinician. Women with RM are vulnerable and desperate. Due to lack of NHS resources, women are often pressurised to undergo self –funded investigations that have limited or no evidence. For example, women are often labelled to have immune mediated miscarriages and are prescribed immunomodulatory medications. These treatment options for these may have harmful effects for the mother and/or the offspring. These can also cause significant financial stress for the couple with no clear benefit.

The latest addition to this group of medications was recombinant granulocyte colony stimulating factor. Evidence from a single centre study using recombinant GSF treatment for unexplained RM suggested statistically significant increase in LBR[73]. There was a need for a definitive multicentre randomised study and therefore, I proceeded with RESPONSE study to investigate benefit of recombinant human granulocyte colony stimulating factor for women with unexplained RM.

Objectives of this thesis:

1. To evaluate the efficacy of subcutaneous recombinant human granulocyte colony stimulating factor (rhG-CSF) versus placebo in pregnant women diagnosed with unexplained RM.
2. To evaluate the safety, tolerability and immunogenicity of subcutaneous rhG-CSF in pregnant women with a history of unexplained RM.

CHAPTER 2

IMMUNOLOGY OF EARLY PREGNANCY

Introduction:

Miscarriage is the most common complication in early pregnancy[79]. World Health Organisation (WHO) defines miscarriage as expulsion of fetus or embryo weighing 500 gram or less. Approximately 1 in 5 pregnancies end in a miscarriage, mainly in the first trimester. The most common reason for miscarriages is chromosomal abnormalities in the embryo[80]. In the UK, 200,000 women suffer a miscarriage each year[81].

Miscarriage causes significant emotional, physical, social and financial implications in women, their partners and their immediate families[2]. Prevention of miscarriage is one of the most challenging areas in reproductive medicine. In spite of extensive and, sometimes, exhaustive investigations for both partners, only a half of miscarriages are found to be associated with a known cause or a major risk factor[82] .

When a cause or major risk factor is not identified, it is postulated that altered immune mediated mechanisms both at the fetal and maternal compartments may play a major role.

Immuno-pathological evaluation of placenta by Kwak et al[83] in women diagnosed to have miscarriages confirmed inflammatory infiltrate, fibrin deposition and evidence of thromboembolism at the feto-maternal interface[84]. These vascular and immunological factors and the cross-interactions may form the basis of immune mediated pregnancy loss. From an obstetric perspective, these changes can result in first and second trimester miscarriages, intrauterine growth restriction and /or pre-eclampsia in the second or third trimesters and intrauterine death.

Insights for this theory are mainly from mice studies and also from failed human pregnancies[85,86]. Immunological investigations at the implantation site or placental interface in an ongoing human pregnancy are impractical and therefore, firm conclusions remain elusive. Therefore investigations, interpretation of results and treatment modalities used in reproductive immunology practice for immune mediated miscarriages remain controversial.

Immune system

Any foreign body (antigen) that enters human body initiates an immune reaction. Human immune system functions with the help of different organs, cells and molecules; the primary mechanism of immune response is to produce antibodies which attaches to the antigen[87] .

The immune system provides a host defence mechanism. This forms the basis of prevention of infection, diseases and identification and destruction of malignant tumours. To activate appropriate response to different living, non-living and tumour promoting antigens, immune system needs to generate a wide range of antibodies[88]. These antibodies should act and interact to produce effective mechanisms to eliminate any potential risk.

The two 'immune systems' termed innate immune system and adaptive immune system provide immune protection. In spite of innate and adaptive systems having different pathways, many cells have a physiological cross-linkage and complement each other. This provides an adjuvant effect resulting in homeostasis. Different components and interactions of innate and adaptive immune systems are shown in Figure 2.1.

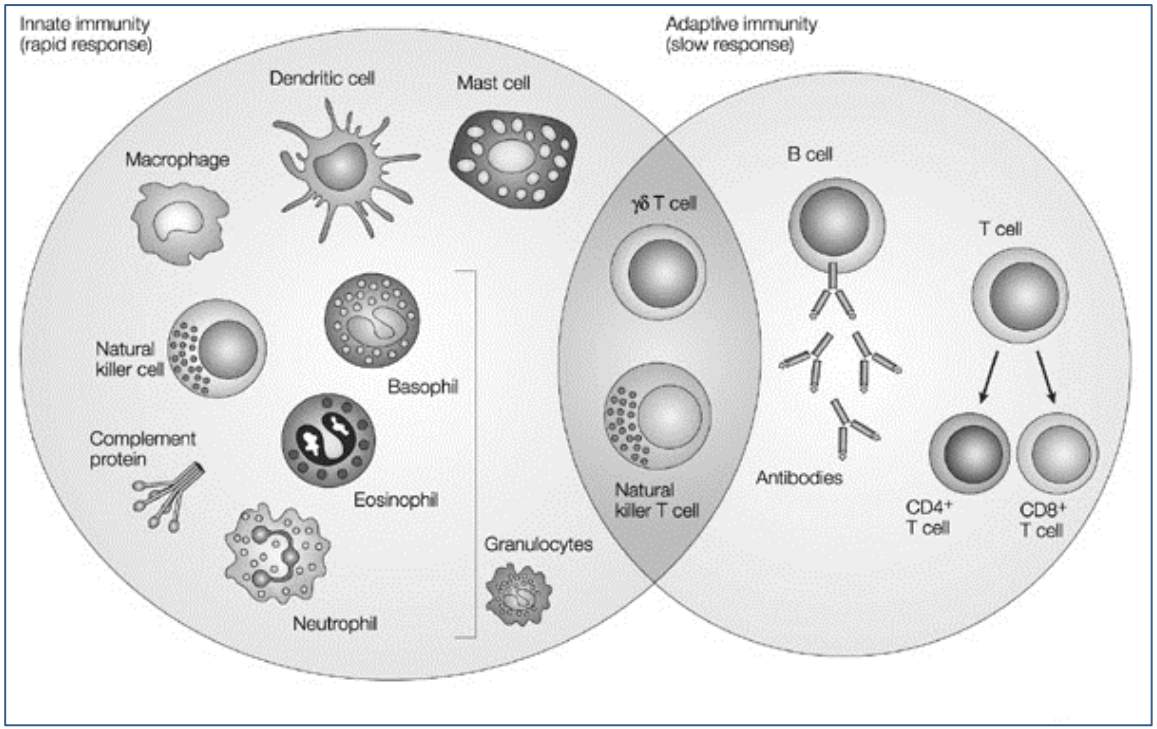


Figure 2.1 - Components of innate and adaptive immunity.
(Figure adapted from Nature reviews)[89]

Innate Immune system

This forms the initial trigger and rapid response mediated by recognition of the antigen. Antigen recognition is the hallmark of innate immunity[90]. This is effective within minutes to hours and does the function of antigen recognition and destruction sub-clinically for the final 'repair and healing' process[91].

The mechanism involves different types of epithelial barriers, cell components and soluble molecules. The components of innate compartment are mainly

(1) Dendritic cells,

(2) Macrophages and

(3) NK cells.

Antigen recognition in Innate Immunity: Key concepts

a. Antigen contains pathogen associated molecular patterns (PAMPs).
b. Mediators of innate immunity contain pattern recognition receptors (PRRs). These are identical receptors on all cells of same lineage .
c. PAMPs directly or indirectly activate the PRRs and bind with them to initiate an acute inflammatory response.
d. These inflammatory responses results in damage associated molecular patterns (DAMPs)

Adaptive Immune system

This forms a later, highly individualised immune response if the innate immunity fails to recognise and destroy the antigen. Cell discrimination (self and non-self) is the hallmark of adaptive immune system. The adaptive immune process may take up to several days or weeks. A re-infection with the same antigen elicits a highly modified and effective individual microbial recognition resulting in immunological memory. Adaptive immunity can be humoral or cell mediated. The key components of adaptive immunity are mainly lymphocytes (also a small population of B lymphocytes which provides humoral immunity) and includes

a. Cytotoxic T lymphocytes – CD8 expression,
b. Helper T lymphocytes – CD 4 expression (has both Th1 cells and Th2 cells) and
c. Regulatory T cells – CD 4 expression

Antigen recognition in adaptive immunity – Key concepts

a. Following acute inflammation, dendritic cells capture the antigen and migrate to lymph nodes or spleen. This activates specific lymphocytes for the antigen.

b. Both T cell lymphocyte and B cell lymphocyte contain highly distinctive and specific receptors. They are encoded by somatic recombinant genes. They react in a highly diverse pattern.
c. T cell lymphocyte function: Initially the microbial proteins get degraded to peptides. This binds to surface cell proteins named major histocompatibility complex (MHC) and forms the antigen presenting cell. This cell complex is identified by T cell lymphocyte receptor (TCR).
d. B cell lymphocyte function : The microbial cell wall glycoprotein contains carbohydrates. This will be recognised and bound to B cell lymphocyte receptor (BCR) in its soluble form.
e. Once activated, these lymphocytes can destroy the cell themselves (effector cells) or can initiate other cells to the inflammatory site. These cells also reach peripheral tissues and remains as memory T cells and B cells to maintain 'immunological memory'.

Maternal tolerance of fetus and immunology in a normal pregnancy

Fusion of oocyte (with maternal chromosome derived antigens) and the sperm (with paternal chromosome derived antigens) results in fertilisation[92].

Implantation of the fertilised oocyte (embryo) results in a pregnancy. Once implanted, the embryo is nurtured by the mother and a normal pregnancy results in a healthy offspring. Mammalian gestation is characterised by immune system 'deactivation' to allow the mother to accept and tolerate growth of embryo inside the uterus. This unique phenomenon is termed as viviparity[93].

Theoretically, embryo implantation process can activate the maternal immune system resulting in implantation failure. If so, a successful implantation is possible only by maternal immunosuppression. Viviparity, therefore, is important from an evolutionary perspective. It is essential that immune system adapts to the new environment and protects the mother (self) and the embryo (non-self).

A number of studies [94-98] suggested that human decidua in successful pregnancy contains a high number of immune mediators from the innate and adaptive compartment. Early pregnancy can be considered as a pro-inflammatory stage.

Further, mice and human studies confirmed depletion of inflammatory cells, mainly NK cells and dendritic cells results in placental dysfunction and early pregnancy failure[95].

Matzinger et al and Bonney et al proposed that the 'tissue damage capacity' of antigen (degree of antigenicity) is more crucial than the self and non-self theory in initiating the inflammatory response[99,100]. Innate response medicated by was discussed earlier. In a normal pregnancy there is inactivation of PAMPs –PRR response (innate immunity) and deactivation of TCR and BCR ligand production (adaptive immunity). These mechanisms result in deactivation of identification of danger signals resulting in rejection of the embryo.

Series of complex immunological changes occurring during embryo implantation, trophoblast invasion and development can be considered as a state of immunomodulation rather than immune-suppression[101,102].

Reproductive Immunology Paradox

Over the last seven decades, many theories have evolved improving the understanding of early pregnancy immunology. Following observations from transplantation immunology, Medawar et al in 1953, introduced the concept of fetus as a semi – allograft[103]. Acceptance of this semi-allogenic graft results in pregnancy success. He proposed concepts of maternal tolerance of fetus based on 3 principles:

(a) Anatomical separation of mother and fetus by a haemochorial placenta,

(b) Immaturity of the fetal antigens and,

(c) Maternal immunosuppression.

Beer at al, two decades later, identified the recognition of an early embryo by the maternal immune system and the resulting response[104,105]. This animal model study identified the role of HLA in early pregnancy maintenance. Further, different groups e.g. Billington et al[106] suggested the theory of cell mediated immunosuppression and immune-protection by different cells in placenta, and HLA sharing by parents and its implications

The major breakthrough following Medawar et al proposal was the Th1/Th2 hypothesis. Later studies identified the importance of placenta and its function in early pregnancy. The inflammatory interactions at this 'local' maternal-fetal interface and the relations to components in innate and adaptive immune system were studies in detail.

Since Wegmann et al[107] proposed the Th1:Th2 hypothesis, there has been significant changes to reproductive immunology research. Th1 cells are considered to be pro-inflammatory and Th2 cells to be anti-inflammatory. Based on this, an increased Th2 response (anti-inflammatory response) was considered beneficial for an on-going pregnancy whereas, an increased Th1 response (pro-inflammatory response) was considered harmful to a pregnancy. This opinion gradually changed when researchers focused on research on components of innate and adaptive immune system. Based on their study, Moffet et al suggested that immune adaptation during pregnancy was mediated primarily by a change in the NK cell population (innate system) rather than T lymphocytes (adaptive system)[108].

Latest Evidence

Latest research considers innate and adaptive immune system as an integral system, working in tandem to avoid the process of fetal rejection. They also support Medawar's theory of immunosuppression by focusing on the anti-inflammatory properties of T Reg cells (through cell mediated function of adaptive system). Maternal-Fetal interface favouring a normal outcome, includes maternal aspects such as (a) de-activation of adaptive immune process, (b) immune tolerance and (c) tissue re-modelling (healing and repair)[111]. Fetal and placental aspects are (a) intact structure of placenta (maintained by NK cells), (b) restricted expression of MHC antigen and (c) secretion of anti-inflammatory proteins to enhance immune-tolerance[112].

Trophoblast

Haemochorial placenta can be considered as an organ with specific immunological, endocrinological and nutritional function. Trophoblast cells are the main component at the maternal-fetal interface and can be differentiated into 3 types (a) Villous cytotrophoblast that remains in the villi, (b) syncitiotrophoblast that lines the intervillious space and (c) non –villous cytotrophoblast which migrate into decidua and the uterine myometrium.

Innate immune system in pregnancy

1. *NK cells*

NK cells are lymphocytes with two important functions, namely are cell lysis and cytokine production. Studies confirm a reduction in the production of peripheral NK cells and levels of Interferon Gamma in early pregnancy[113],[114]. These changes are consistent of an increased humoral response rather than a T cell medicated response. Peripheral NK cells express surface marker CD 56 dim 16+. Some of these cells exhibit the function of lysis, others cytokine production, but most partake in both. Uterine NK cells are activated leucocytes which produce cytokines. They have similar characteristics to peripheral NK cells; however, they express CD56 bright CD 16 -[115].

There is a significant increase in uNK cells after LH surge facilitating endometrial changes and implantation process. The major endometrial changes are remodelling of the spiral arteries, increase in production of angiogenic cytokines and VEGF. Uterine NK cells contain inhibitory receptors. These receptors bind to the MHC 1 a and 1 b on trophoblast and thus inhibit lysis. Therefore, in spite of the increased numbers of uNK cells in decidua, the non-villous cytotrophoblast is not attacked. KIR and leptin like KIRs are example of inhibitory receptors[116].

In spite of extensive studies conducted relating to NK cells and uterine NK cells, their role in human pregnancy is remains unclear[98] .

2. *Macrophages*

Macrophages are found in decidua, placental trophoblast cells and extra-placental membranes. The majority of macrophages reside in the decidua. They are mainly regulated by the hormonal changes in the uterine environment, mainly oestrogen. Their mechanism of action is somewhat related to the Th1/Th2 mentioned earlier. Activated macrophages produce 2 different types of cell, M1 and M2. M1 type is pro-inflammatory (secretes TNF and IL 12) and M2 is anti-inflammatory (secretes steroids, IL - 4, IL-10 and IL - 13)[117,118].

Macrophages provide first line of immune defence. They take part in

a. a. Antigen identification and clearance,
b. Placentation (by production of cytokines) and
c. Produce immunosuppressive prostaglandins.

Macrophages identify antigens with the help of toll-like receptors and c type lectin receptors. These receptors activate a pro-inflammatory state in cases of blood borne infection and pre-eclampsia (raised levels of TNF, cytokines and PGE_2)[119]. These events may explain the increased risk of pre-term labour associated with infections and pre-eclampsia[120].

3. *Monocytes and Granulocytes*

Pregnancy associated increase in monocytes and granulocytes results in expression of free radicals and cytokines similar to patients with sepsis. This supports the theory of innate immunity activation in pregnancy.

These changes are mediated by pregnancy related hormonal changes and placental proteins[121].

4. *Dendritic cells*

These are the most potent cell in the innate immunity and they work in close relation with T cell lymphocytes establishing a close link with adaptive immunity[87]. They are classified into 2 types: CD 83 + mature and CD 83 + immature, similar to M1 and M2 macrophages. Mature CD83+ cells are anti-inflammatory and form a major part in immune tolerance and prevention of auto-immune diseases[87]. Immature CD 83 + cells, in turn, are pro-inflammatory. However, a group of immature CD 83+ cells in presence of inflammatory cytokines can differentiate into mature CD 83 + cells. Dendritic cell cytokines like G-CSF, GM-CSF, TGF beta and IL-10 can have anti-inflammatory function by disrupting the antigen presenting pathway[88].

Adaptive immune system in pregnancy

The major component of the adaptive immune system is T lymphocyte. T lymphocytes can be divided as

a. CD 8+ Cytotoxic T cells (CTLs), which can kill cells directly and
b. CD 4+ T Helper cells (Th cells), which produce cytokines, can be sub classified into
    - Th1 cells – Pro-inflammatory (through IFN gamma and IL – 2)
    - Th17 cells – Pro-inflammatory

- Th2 cells – Anti-inflammatory (through IL-4, IL-5, IL-9, IL-10 and IL-13); and
- T reg cells – Anti-inflammatory GILL MOR

Key concepts

a. During pregnancy, there is down regulation of Th1 cells, reducing Th1/Th2 ratio[97].
b. Hormonal changes favour Th2 mediated changes by producing anti-inflammatory cytokines[106].
c. Placenta physiologically decreases pro-inflammatory cytokines[112].
d. Trophoblast cells produce indoleamine diogenase (IDO) to supress pro-inflammatory T cell activity (by reducing tryptophan)[99].

Maternal, Fetal/Trophoblast contribution during early pregnancy

An embryo in early stages is protected from inflammatory proteins in the maternal system and fallopian tube by its shell, Zona Pellucida (ZP). Once, inside the uterus the embryo hatches itself and implantation process begins. This is the most crucial phase, initiating the first breach of superficial endometrium.

An estimated 50% of embryos fail to implant; one hypothesis is that this is due to an exaggerated maternal immune response[149]. Under influence of progesterone (from an active corpus luteum), the embryo attaches and invades into the endometrium. This creates a decidual reaction in the endometrium[118]. This reaction stimulates a complex reaction at the level of uterine stroma under the influence of ovarian hormones, growth factor, cytokines, NK cells and dendritic cells[118]. These changes results in the reduction of maternal immune cells, mainly T lymphocytes and extra villous trophoblast cells enter the decidua[118]. The chorion membrane provides a physical barrier. The placenta releases fetal cells which gradually enters the maternal circulation resulting in fetal micro-chimerism. All the above changes results in immunosuppression (mediated by immune system activation).

Major Histocompatibility Complex

Major histocompatibility antigens are proteins which are expressed at the maternal–fetal interface[122]. They are also known as transplant antigens as they play a role in graft rejection. These antigens help in distinguishing self and non-self. These human leucocyte antigens are classified as:

a. HLA 1a (HLA-A, HLA-B and HLA-C) helps in immune-recognition,
b. HLA 1b (HLA-E, HLA-F, HLA-G) helps in immune-acceptance and

c. HLA 2 (HLA-DP, HLA-DQ, HLA-DR) helps in immune-recognition

Previous studies suggested trophoblasts do not express HLA 1a and HLA 2 to escape immune-recognition[123].

Trophoblasts produce HLA – 1b for immune acceptance and studies also suggested this prevents lysis of trophoblast cells by uNK cells. More recent studies confirm presence of fetal HLA in maternal serum and therefore the pregnancy equilibrium is maintained by maternal immune system 'priming' (to accept the fetus) rather than 'ignorance'[124,125].

Discussion

Advances in molecular techniques have helped in identifying new cells, their subtypes and proteins on a regular basis. This has improved the understanding of concepts and mechanisms in reproductive clinical immunology but equally, has made it more can be challenging, even to the experts[108]. Evidence suggests link to immune mediated dysfunction to unexplained sub-fertility, recurrent miscarriages, pre-eclampsia and preterm labour[126].

Any disturbances of the innate or adaptive immune system at the maternal-fetal interface may result in pregnancy failure. Theoretically, approaches to maternal immunomodulation by novel medications may improve the outcome

of immune mediated pregnancy disorders. However, a uniform understanding of immune mechanisms is needed. Until then unproven and anecdotal treatment options as part of immune suppression should be used with caution, and only within research settings.

CHAPTER 3

SYSTEMATIC NARRATIVE REVIEW OF AVAILABLE EVIDENCE OF GRANULOCYTE COLONY STIMULATING FACTORS IN REPRODUCTIVE MEDICINE

Introduction

Natural fertility potential in humans is surprisingly low. It has been estimated that about 70% of human conceptions do not reach viability of which 50% are lost before or during menstrual cycle[127].Repeated episodes of miscarriages can affect 1% of women in the childbearing age[128,129].

The most common reason for miscarriage is chromosomal abnormalities in the fetus, the risk of which increases with advancing maternal age. Other reasons include parental chromosome translocations[130], intra-uterine infections[131,132] , endocrine abnormalities[133,134] , uterine malformations[135], antiphospholipid antibody syndrome or other autoimmune conditions[136].

Investigations are reported to be normal in about half of the couples undergoing screening for recurrent miscarriages[137]. Such couples are said to have unexplained RM. This condition has generated great interest in the field of

reproductive medicine research. One proposed cause of unexplained recurrent miscarriages relates to immune dysfunction or allo-immune responses[138]. Several immune mechanisms for reproductive failure have been described. The most debated theory is the prevalence of increase in pro-inflammatory cytokines and reduction in anti-inflammatory cytokine during pregnancy which results in a high Th1:Th2 ratio. This may result in the rejection of the fetus or the 'embryonic allograft'[139].

Over the last 40 years, assisted conception techniques have gained popularity. IVF and IVF techniques with ICSI are practised for treatment of subfertility for a wide variety of reasons including RM.

HFEA statistics[140] suggests live birth rate per ET per cycle of IVF/ICSI ranging from 41.8% (for a woman under the age of 35) to 12.4% for women between the ages of 43-44.

Repeated failure of embryo implantation in assisted reproductive techniques (ART) can be defined as failure to achieve a pregnancy after a total of four good quality embryos were transferred over 3 cycles[141]. Similar to RM, there are discrepancies regarding definitions, investigations and management of recurrent implantation failure (RIF). There are many reasons for RIF including

factors relating to sperm, oocyte, embryo, uterus and fallopian tube. Majority of the factors remain unexplained. In spite of major advancement in assisted reproductive techniques, the implantation rates still remain relatively low. Embryo implantation is the rate limiting step in assisted conception treatment [142]. The final outcome is determined by a good quality embryo transferred into a receptive endometrium. A receptive endometrium supports the transformation of endometrial cells into decidua cells, blastocyst invasion and development of placenta[143]. Receptivity is facilitated by immune cells, growth factors, cytokines, and hormonal changes[144,145]. These complex immune mechanisms at the endometrial level are important and crucial in implantation process[146].

There are many immunological factors considered to be crucial for establishment and maintenance of pregnancy. Clinicians use treatment options which have limited evidence to improve the immunological milieu[147] . This is with a view to decrease RM within natural conceptions and also to try and improve outcomes with ART. There is considerable debate for the value of immunological treatment.

The commonly used immune modulation treatment includes progesterone, corticosteroids, paternal lymphocyte infusion therapy, intravenous intralipid infusion and anti – TNF drugs. Previously, clinicians used paternal leukocyte

transfusion[148], trophoblast membrane vesicle extracts[149] and seminal plasma suppositories[150]. Intravenous immunoglobulin immunonotherapy[150-152] is still used in clinical practice when intravenous intralipids are found to be ineffective.

However, all these treatments have not received general acceptance as the published results are controversial. A recent meta-analysis has shown that none of these therapies showed significant effects on patients with unexplained RM[153,154].

A recent addition to this group of medications is granulocyte colony stimulating factor. The aim of this systematic narrative review was to evaluate the effectiveness of granulocyte colony stimulating factor in reproductive medicine.

G-CSF

Granulocyte colony-stimulating factor is a cytokine, first recognized and purified in mice in 1983. Human G-CSF (hG-CSF) was cloned in 1986[155,156]. The biological activities of hG-CSF are mediated by a specific receptor on the cell surface of responding cells. This receptor (G- CSF-R) is present on myeloid

progenitor cells, myeloid leukaemia cells, mature neutrophils, platelets, monocytes, lymphoid cells and some T cells and B cells.

G-CSF is produced by bone marrow, stromal cells, endothelial cells, macrophages and monocytes[157-159]. In female reproductive tract, it is naturally found in endometrium, ovarian follicles and fallopian tube epithelial cells[160-161].

The structure and molecular characteristics of recombinant human granulocyte colony stimulating factor is shown in Figure 3.1.

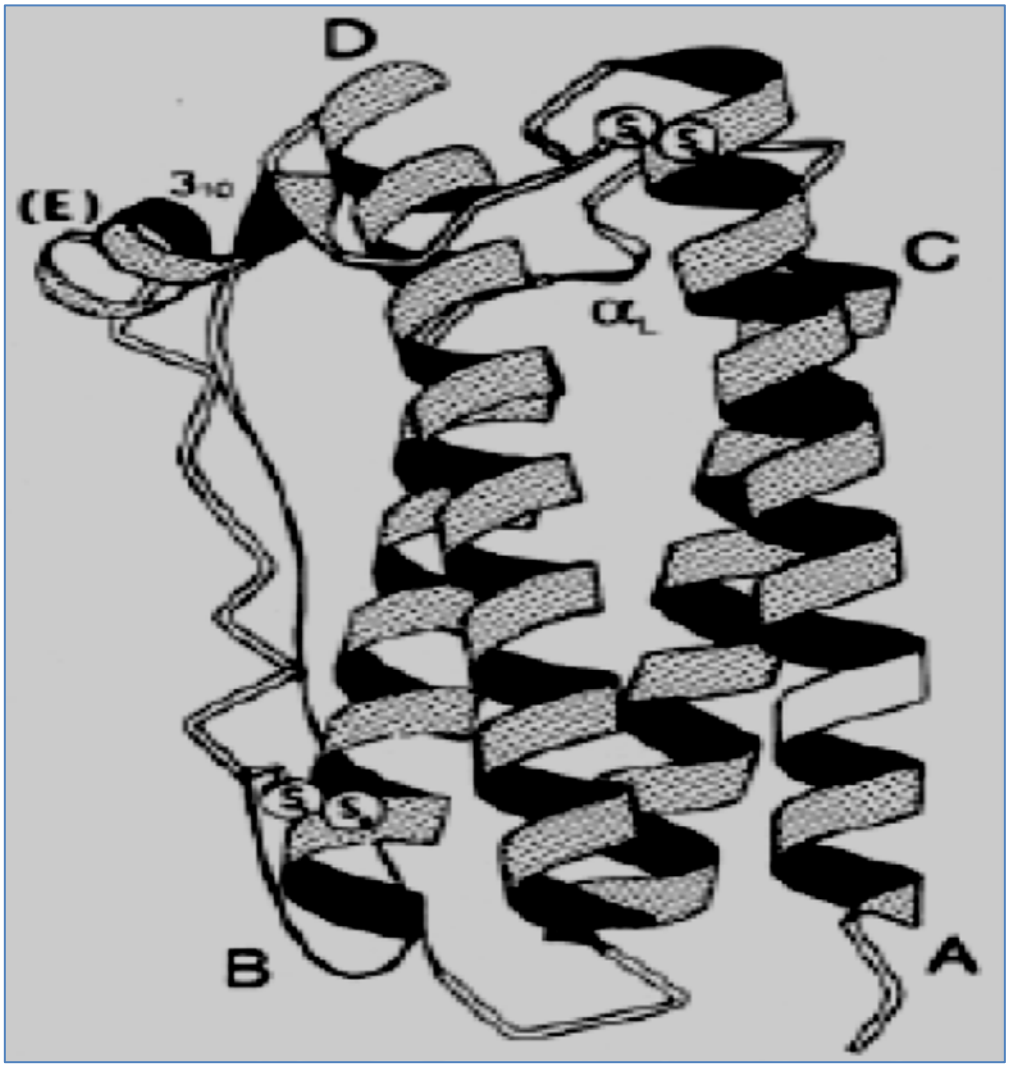


Figure 3.1 - G-CSF contains approximately 20 kD glycoprotein with 174 amino acids arranged in four anti-parallel a helices. The gene for G-CSF is found on chromosome 17 q 21-22.
(Figure courtesy: NORA Therapeutics)[162].

The main function of G-CSF is to stimulate the proliferation and differentiation of neutrophils in the bone marrow and control their release[157]. These activities are mediated by receptors on cells of haemopoitic cell lineage and in cases specific to reproduction by receptors found in lower genital tract[159,160](fallopian tube, follicular cells, placental cells and trophoblastic cells)

Studies in both humans and animals have shown that G-CSF improves endometrial thickness, ovarian follicular function and oocyte quality, which in combination enhances embryo implantation[159-161]. These actions may result in successful pregnancy outcomes by reducing pregnancy failure. In-vivo and in-vitro studies have shown low levels of G-CSF concentration in endometrial cells and follicular cells in subjects with pregnancy losses. G-CSF helps in blastocyst development and G-CSF mediated changes on decidual cells, dendritic cells, Th-2 cytokine secretion and activation of T regulatory cells may help in implantation[161].

The basis of immune mediated pregnancy loss is conventionally explained by an imbalance in T helper 1 : T helper 2 ratios, dysfunction of human T cells, N-K cells cytotoxicity and human leucocyte antigen incompatibility. The exact

mechanisms of anti-abortive actions of G-CSF in animal and human studies are not yet known.

Materials and methods

The aim of the review was to identify all studies investigating the use of G-CSF in women undergoing IVF or use of G-CSF during pregnancy for any indication. The review was conducted and reported in adherence with PRISMA statement[163]. No ethical approval was needed.

Identification of studies

The electronic databases CINAHL, Cochrane, EMBASE and Medline (from inception to April 2017) were used to perform the literature search. The following keywords and MeSH subheadings were used in the search strategy: 'G-CSF', 'granulocyte colony stimulating factor' AND 'recurrent miscarriage', 'assisted conception' OR 'IVF' OR 'reproductive medicine'. The basic search strategy was adapted to suit the database being searched. The search was restricted to humans and females. Bibliographies of relevant primary articles were also searched to identify any articles missed by the electronic searches.

Study selection and data extraction

Studies were selected following a two-step process. Firstly, the citations identified by the electronic bibliographic database searches were screened, based on their titles and abstracts. Full text papers of eligible abstracts were retrieved. Once full text papers had been located, we determined whether they fulfilled our predetermined inclusion criteria:

Population: women who received G-CSF during pregnancy or as part of ART.

Intervention: G-CSF (any route of administration)

Comparator: no treatment, another therapy

Outcome: reduction in miscarriage, increase in endometrial thickness, improvement in ART outcomes, improvement in pregnancy outcomes.

All randomised controlled trial (RCT), cohort studies and case control studies were included and case reports and reviews on the subject were excluded. Any studies where women did not administer exogenous G-CSF were excluded from the final analysis.

Two reviewers (AE and BK) independently assessed the full text papers to determine if they met the above criteria. Any disagreements regarding the eligibility of a paper were resolved by consensus. The full manuscripts of the included titles and abstracts were retrieved for further assessment. If there were

duplicate studies, the most recent and complete version were chosen. Data from the manuscripts were extracted onto a pre-designed pro-forma which included study design, sample size and population, indication for G-CSF , intervention, dose, route of administration, control and outcome. The literature search was thorough and without language restrictions.

Quality assessment of studies

The methodological quality of all manuscripts included in the study was assessed. Quality was defined as the confidence that the study design, conduct and analysis minimised bias in the estimation of effectiveness. The methodological quality of the RCTs included was assessed using the Cochrane risk bias score. The methodological quality of the non-RCT studies included was assessed using the Newcastle–Ottawa scale. A non-randomised study was considered to be of a high quality if it provided information on selection, comparability, exposure and outcome of the study participants. A maximum of one point was awarded for all items except for comparability which scored a maximum of two points. The score ranged from 0 to 7, with a score of either 0 or 1 for each item. The quality assessment is detailed in figure 3.3 and figure 3.4

Results

Figure 3.2 show the PRISMA diagram of the review process. The search of electronic databases produced 2501 citations. Two thousand four hundred and fifty four studies were excluded after examination of titles and abstracts as they did not meet the inclusion criteria.

Forty seven full manuscripts were retrieved of which 30 further studies were excluded. Five were excluded as they were duplicate publications, six studies investigated other immunomodulatory treatment in conjunction with G-CSF, 11 citations investigated effects of endogenous G-CSF and did not have any relevant data and eight were excluded as they were case reports.

Thus a total of 17 studies were included in the review.

The study characteristics of 17 studies are presented in table 3.1. The 17 studies can be divided into two groups; those which investigated efficacy of G-CSF in IVF treatment (12 studies) and those which investigated efficacy of G-CSF in RM (five studies)

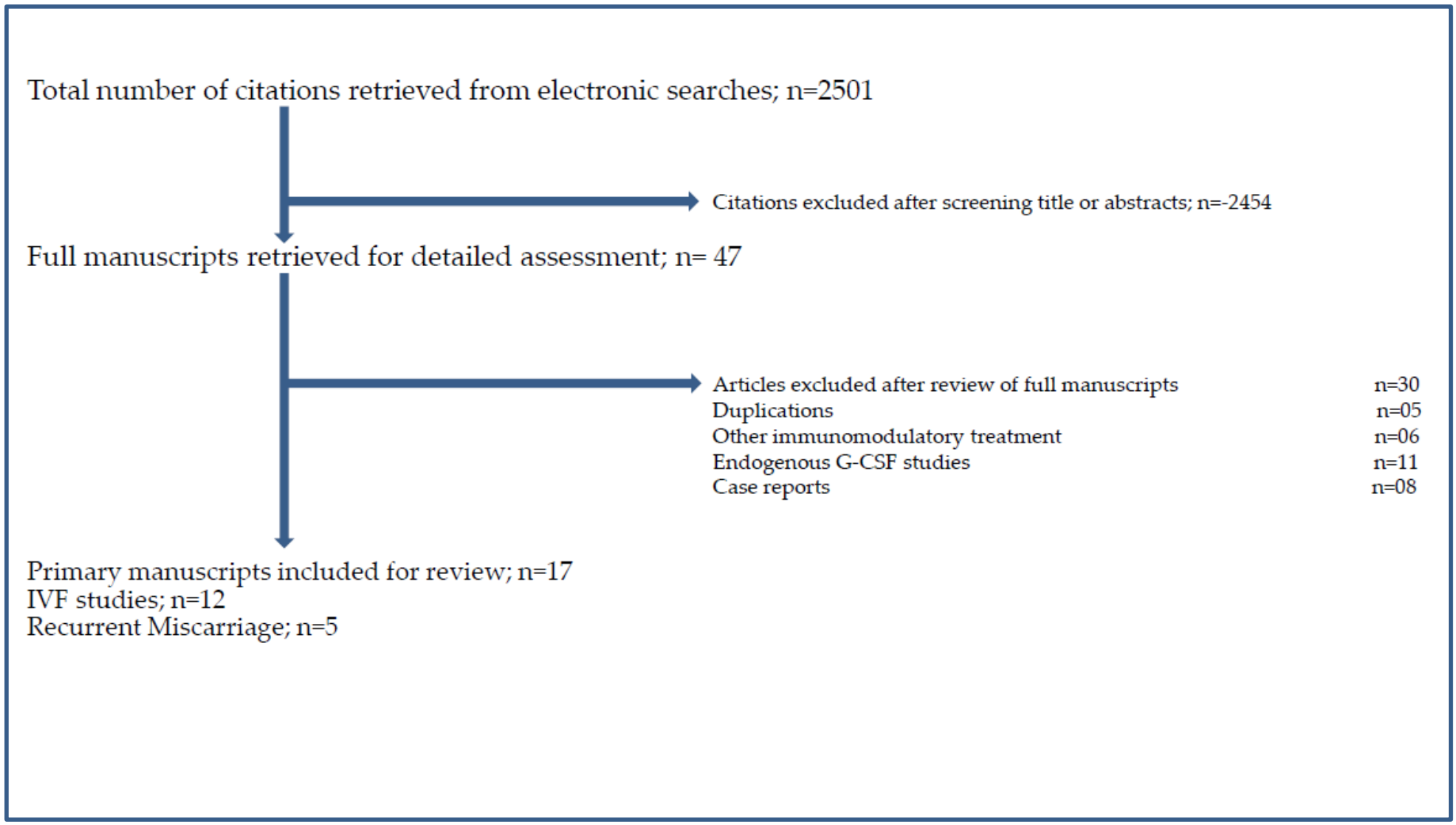


Figure 3.2 - Study selection for review of G-CSF in reproductive medicine

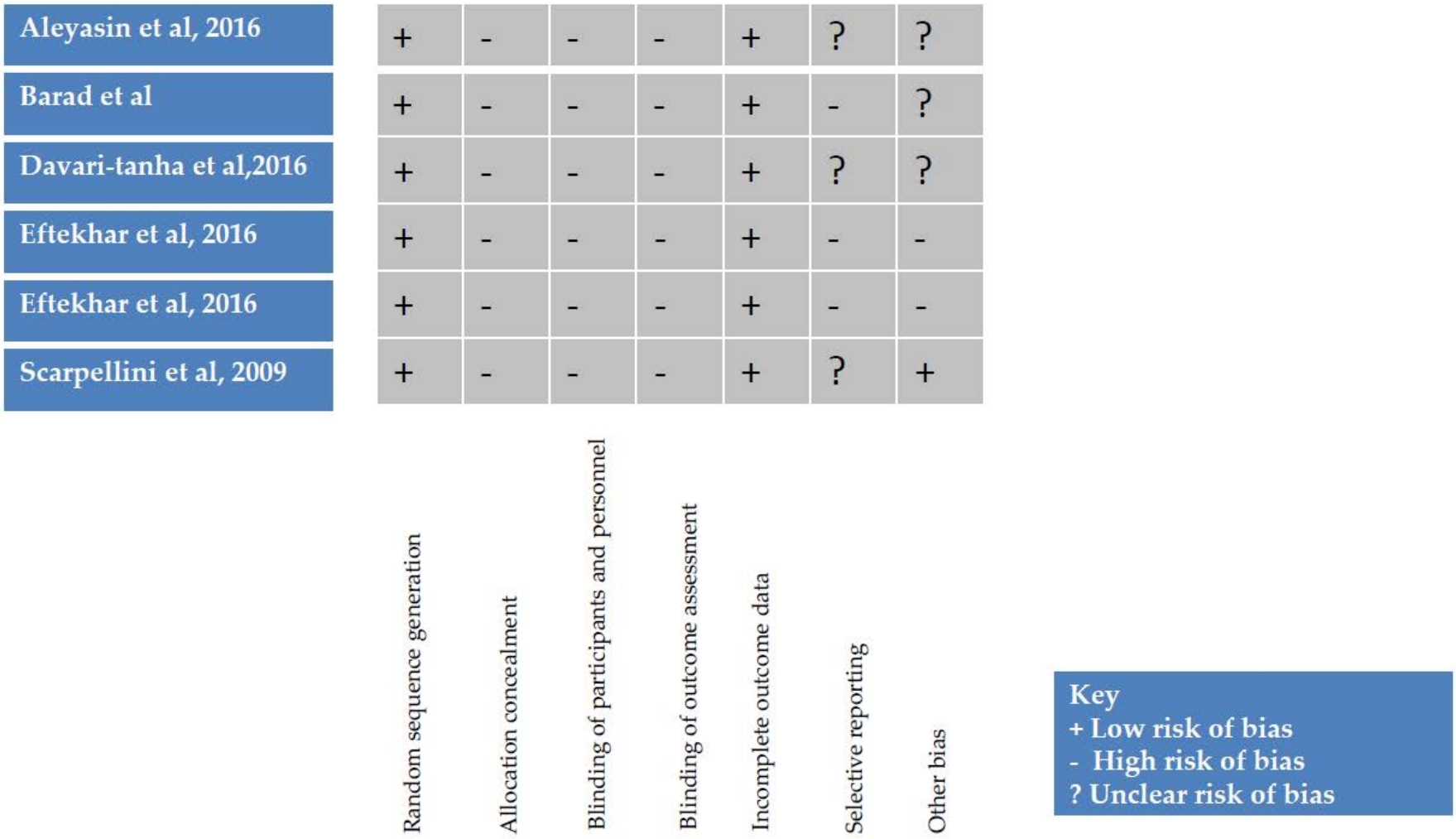


Figure 3.2a: Cochrane risk bias score for included studies

Newcastle Ottawa quality assessment for included studies in this review

Studies	Selection			Comparability	Outcome		Total score
	Representativeness of exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure		Assessment of outcome	Adequacy of completeness of follow up	
Gleicher et al, 2013	*	*	_	*	*	*	5
Kunicki et al, 2014	*	*	_	*	*	*	5
Lee et al, 2016	*	*	*	*	_	*	5
Scarpellini et al, 2012	*	*	*	*	*	*	6
Tehraninejad et al, 2015	*	*	_	*	*	*	5
Wurfel et al, 2013	*	_	_	_	_	*	2
Xu et al, 2015	*	*	*	*	*	*	6
Zeyneloglu et al, 2013	*	*	_	*	*	*	5
Boxer et al, 2015	*	_	_	_	_	*	2
Santjohanser et al, 2013	*	*	*	*	*	*	6
Zeidler et al, 2014	*	_	_	_	_	*	2

Figure 3.2b: Newcastle Ottawa quality assessment for Included studies

Table 3.1 – Characteristics of included studies of G-CSF in ART

Reference (Year)	Study design (Country)	Sample size & Population	Indication for G-CSF	Intervention & Administration route	Control	Results & Outcome
Aleyasin et al., (2016)	Randomised Trial (Iran)	112 women with repeated IVF failures	To improve IVF outcomes	300 micrograms of G-CSF instilled into uterine cavity 1 hr before ET	Controls had no additional treatment	The successful implantation (18% vs 7.2%, P=0.007), chemical pregnancy (44.6% vs 19.6%, P=0.005), and clinical pregnancy (37.5% vs 14.3%, P=0.005) rates were significantly higher in the intervention group than in the control group. Administration of G-CSF improved implantation rates, chemical pregnancy and clinical pregnancy.
Barad et al., (2014)	Randomised controlled study (USA)	141 women undergoing IVF	To improve endometrial thickness, implantation rates and clinical pregnancies	300 micrograms of G-CSF instilled into uterine cavity on day of hCG trigger	Controls had 1 ml of normal saline instilled into uterine cavity on day of hCG trigger	Endometrial thickness statistically significantly increased over the 5-day observation period for the whole group by approximately 1.36 mm. Administration of G-CSF did not result in

						statistically different improvement in endometrial thickness, implantation rates or clinical pregnancies.
Davari-tanha et al., (2016)	Randomised controlled study (Iran)	100 women with repeated IVF failures	To improve implantation rates and clinical pregnancies in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of oocyte retrieval	Controls had 1 ml of normal saline instilled into uterine cavity on day of oocyte retrieval	17 patients had a positive pregnancy test after embryo transfer [10 (25%) in G-CSF; 5 (12.5%) in saline; and 2 (10%) in placebo group]. The mean of abortion rates was 17.6% (3), two of them in G-CSF, one in saline group. The IR was 12.3% in G-CSF, 6.1% in saline and 4.7% in placebo group. Administration of G-CSF improved implantation rates, chemical pregnancy and clinical pregnancy.
Eftekhar et al., (2016)	Randomised controlled study (Iran)	100 women who had repeated IVF failures	To improve implantation rates and CPR	300 micrograms of G-CSF instilled into uterine cavity on OR day	Controls had no additional treatment	Administration of G-CSF did not results in improvement of implantation rate or CPR.

Eftekhar et al., (2016)	Randomised controlled study (Iran)	90 women who had repeated IVF failures	To improve clinical pregnancies in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of oocyte retrieval	Controls had no additional treatment	The pregnancy outcome in GCSF group was improved significantly (p=0.043). Administration of G-CSF improved implantation rates, chemical pregnancy and clinical pregnancy.
Gleicher et al., (2013)	Prospective observational study (USA)	21 women who had endometrium less than 7 mm undergoing IVF treatment	To improve endometrial thickness in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of hCG (and if needed on day of oocyte retrieval)	No controls	With 5.2 ± 1.9 days between G-CSF perfusions and embryo transfers, endometrial thickness increased from 6.4 ± 1.4 to 9.3 ± 2.1 mm ($P < 0.001$). The Δ in change was 2.9 ± 2.0 mm, and did not vary between conception and non-conception cycles. A 19.1% ongoing clinical pregnancy rate was observed, excluding one ectopic pregnancy. Administration of G-CSF improved endometrial thickness and is an

						effective treatment for chronically thin endometrium.
Kunicki et al., (2014)	Prospective observational study (USA)	37 women with endometrium less than 7 mm undergoing IVF treatment	To improve endometrial thickness in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of hCG (and if needed on day of oocyte retrieval)	No controls	Endometrium expanded significantly from 6,86 ± 1,65 to 8,80 ± 1,14 mm in the first group (who conceived) and from 6,71 ± 1,80 to 8,33 ± 1,85 mm in the second, respectively. Administration of G-CSF improved endometrial thickness after 72 hrs of instillation
Lee et al., (2016)	Retrospective observational study (Korea)	50 women with endometrium less than 8 mm on day of HCG	To improve endometrial thickness in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of hCG or on day of oocyte retrieval	No controls	The overall clinical pregnancy rate was 22.0%, the implantation rate was 15.9%, and the ongoing pregnancy rate was 20%. The clinical pregnancy rate (41.7% vs. 15.8%), the implantation rate (26.7% vs. 11.7%), and the ongoing pregnancy rate (41.7% vs.

						13.2%) were higher when G-CSF was instilled on the triggering day than when it was instilled on the retrieval day. Administration of G-CSF improved endometrial thickness.
Scarpellini et al (2012)	Prospective observational study (Italy)	109 women with RIF	To improve IVF outcomes	58 women treated with daily subcutaneous injection of 60 mg of G-CSF from ET	51 women treated with daily SC injection of saline from day of ET	Administration of G-CSF improved IVF outcomes in women with previous implantation failure.
Tehraninejad et al (2015)	Prospective observational study (Iran)	15 women undergoing IVF treatment with thin endometrium	To improve endometrial thickness in IVF	300 micrograms of G-CSF instilled into uterine cavity on day of OR or 5 days before ET	No controls	The endometrial thickness reached from 3.593±0.251 mm to 7.120 ± 0.84 mm. Administration of G-CSF may improve endometrial thickness.
Wurfel et al; (2013)	Retrospective cohort study (Germany)	301 women with RIF	To improve IVF outcomes	Varying doses	Not clear	Administration of G-CSF improved IVF outcomes in women with RIF

Xu et al., (2015)	Prospective observational study (Korea)	82 women undergoing frozen embryo transfer after IVF treatment	To improve endometrial thickness in IVF	Group A 300 mcg of G-CSF instilled into uterine cavity in follicular phase Group B G-CSF as above plus endometrial scratching	Controls had no additional treatment	Significantly higher embryo implantation and clinical pregnancy rates were observed in the G-CSF group compared with the control group (31.5% versus 13.9%; P < 0.01; 48.1% versus 25.0%; P = 0.038, respectively)Administration of G-CSF improved endometrial thickness, implantation rates and clinical pregnancy rates.

Zeynelogl u et al (2013)	Prospective observational study (Turkey)	32 women with RIF	To investigate ideal route of G-CSF administration	Group A 9 women with intrauterine administration of 48 miu/0.5 ml on day of HCG Group B 15 women had subcutaneous injection of G-CSF on a dose of 100000 iu/kg from day of oocyte retrieval for 15 days Group C 8 women had both intrauterine and subcutaneous doses as above	No controls	G-CSF is a safe medication to increase pregnancy rates in IVF treatment and dual administration seems to be best

Table 3.2 – Characteristics of included studies of G-CSF in recurrent miscarriage

Reference (Year)	Study design (Country)	Sample size & Population	Indication for G-CSF	Intervention & Administration route	Control	Results & Outcome
Boxer et al., (2015)	Retrospective observational study (USA)	107 women with 224 pregnancy events with chronic neutropenia	To treat chronic neutropenia	Varying doses ranging from daily/alternate days to twice weekly subcutaneous injections or no doses depending on severity of neutropenia	No controls	Administration of G-CSF increases WBC subsets. Decrease in abortions in G-CSF group. There were no adverse effects
Santjohanser et al., (2013)	Retrospective cohort study (Germany)	127 women with RM (2 or more miscarriages) undergoing IVF treatment	To improve outcomes in RM	Group A - Subcutaneous injection of G-CSF, 34 miu per week from embryo transfer until 12 weeks of pregnancy. Group B - Had medications which included steroids, IVIG or LMWH	Controls had no additional treatment	The LBR of patients and the subgroups differed significantly (G-CSF 32%, subgroup 1 13%, subgroup 2 14%)Administration of G-CSF resulted in statistically significant improvement in LBRs.

Scarpellini et al., (2009)	Randomised controlled study (Italy)	68 women with unexplained primary RM (4 or more consecutive miscarriages)	To prevent embryo demise	Subcutaneous injection of G-CSF, 1 microgram/kg/day from as soon as 6 days after ovulation until 9 weeks of gestation.	Controls had saline injections from as soon as 6 days after ovulation until 9 weeks of gestation.	In the group treated with G-CSF, 29 out of 35 (82.8%) women delivered a healthy baby, whereas in the placebo group, this figure was only 16 out of 33 (48.5%) (P = 0.0061, odds ratio = 5.1; 95% CI 1.5-18.4) Administration of G-CSF resulted in statistically significant decrease in miscarriages and significant improvement in LBRs
Wurfel et al; (2013)	Retrospective cohort study (Germany)	308 women with RM	To prevent miscarriages	Varying doses	Not clear	Administration of G-CSF resulted in statistically significant improvement in LBRs

Zeidler et al; (2014)	Retrospective observational study (SCNIR)	38 pregnancy events already diagnosed with chronic neutropenia	To treat chronic neutropenia	Varying doses or no doses according to severity of neutropenia	No controls	Administration of G-CSF did not result in improvements in pregnancy outcomes.

G-CSF in IVF treatment

It is hypothesised that granulocyte colony stimulating factor improves ART outcomes. The effects of G-CSF on oocyte quality, embryo development, embryo implantation and thin endometrium have been evaluated both by animal and human studies.

In early 2000, Wurfel et al conducted studies which suggested benefits of G-CSF on implantation, embryo development and increase in pregnancy rates[164]. Further studies by Frydman et al confirmed increase in implantation rates and live birth rates. Ledee et al followed up this study and suggested that oocyte grading, embryo morphology and implantation rates were better when the levels of endogenous G-CSF were higher. Surprisingly these effects were not associated with any other hormone or cytokine[165].

Improvement in embryo quality was suggested by Ziebe et al in a double blind RCT in 1149 embryo transfer events within IVF treatment[166]. This suggested a significant improvement in implantation rates and LBRs. A number of studies suggested improved outcomes in ART with use of G-CSF in women with implantation failure by improving endometrial thickness[167]. This may be by the proliferation and differentiation of endometrial cells by CAMP increase in stromal cells[167].

Aleyasin et al., performed a RCT to assess the efficacy of systemic G-CSF in 112 women who underwent IVF treatment further to having a diagnosis of recurrent implantation failure[168]. The rates for implantation (18% vs 7.2%, *P* = 0.007), chemical pregnancy (44.6% vs 19.6%, *P* = 0.005), and clinical pregnancy (37.5% vs 14.3%, *P* = 0.005) were significantly higher in the G-CSF group. Adjustments for different treatment variables were applied and the authors concluded that administration of systemic G-CSF before implantation significantly increases the IVF outcomes.

Barad et al., performed a study to assess the efficacy of intrauterine instillation of G-CSF to improve endometrial thickness and implantation rates in 141 unselected women undergoing IVF treatment[169]. There was an average increase of endometrial thickness by 1.36 mm in the G-CSF group; however, this was not statistically significant when compared to the control group. This study did not suggest improvement in IVF outcome with use of G-CSF.

In a randomised control trial of 100 women undergoing IVF treatment, Davari-tanha et al. evaluated G-CSF to improve IVF outcomes[170]. Unlike findings suggested by Barad et al., intrauterine instillation of G-CSF as a single dose on day of oocyte retrieval resulted in significant improvement in implantation rates and clinical pregnancies.

Eftekhar et al., conducted a randomised control study in 100 women undergoing IVF treatment. All women were assessed to have normal endometrial thickness prior to the study[171]. Intrauterine instillation of G-CSF did not result in a statistically significant change between the 2 groups. The authors concluded that G-CSF does not improve outcomes in women undergoing IVF with normal endometrial thickness. The same author group conducted a further RCT in 90 women undergoing IVF treatment after being diagnosed with RIF. Using the same study technique, the authors concluded that use of intrauterine instillation of G-CSF resulted in a statistically significant improvement in pregnancy outcomes ($p=0.043$).

In a prospective observational cohort study of 21 women, Gleicher et al. assessed efficacy of G-CSF to improve endometrial thickness[172]. 300 mcg of G-CSF was instilled using an intrauterine catheter on day of ovulation trigger. In this study of a highly selected participants, there was statistically significant increase in endometrial thickness, $p<0.001$. These women failed to achieve this increase with other conventional treatment techniques used within IVF treatment prior to this study.

Kunicki et al, in a prospective cohort study followed the same protocol as Gleicher et al in 37 women with an endometrial thickness less than 7 mm[143]. There was no statistically significant difference in endometrial thickness before and after the infusion. A further subgroup analysis was conducted after positive pregnancy test which suggested that women who conceived had thicker endometrium and the authors propose that the endometrium may have thickened after a delayed response to G-CSF infusion.

Lee et al. also followed a similar study technique in a retrospective observational study in women with endometrial thickness less than 8 mm in those with one previous IVF failure[173]. The mean endometrial thickness was 7.2±0.6 mm on ovulation trigger day which increased to 8.5±1.5 mm on the embryo transfer day in the G-CSF group. This was statistically significant, $p<0.001$. The G-CSF group had a higher rates for clinical pregnancy (41.7% vs. 15.8%), implantation rate (26.7% vs. 11.7%), and ongoing pregnancy rate (41.7% vs. 13.2%).

Scarpellini et al. conducted an observational study in 109 women undergoing IVF after a diagnosis of RIF[174]. 58 women were treated with a dose of 60mg/day from day of embryo transfer for 40 days. There was a statistically significant

increase in pregnancy rate in the G-CSF group (43.1%) versus the control group (21.6%), $p<0.001$.

Tehraninejad et al. observed effects of G-CSF on endometrial thickness improvement in 15 women undergoing IVF treatment[175]. These women were previously diagnosed to have thin endometrium resistant to treatment conventionally used in IVF treatment. The authors suggest that intrauterine instillation of G-CSF prior to embryo transfer improves endometrial thickness.

Wurfel et al. conducted a series of studies investigating efficacy of G-CSF in ART and further summarised the data in a retrospective analysis of 301 women who underwent IVF treatment[176]. These women were otherwise diagnosed with repeated implantation failure. Varying doses were used within the study using different preparation of G-CSF and GM-CSF and the authors suggested significant improvement in live birth rates.

Xu et al., in a prospective study divided 82 women undergoing frozen embryo transfer into 3 groups and assessed the efficacy of GCSF to improve ART outcomes in conjunction with endometrial scratching[177]. There was a slightly

higher clinical pregnancy and live birth rates in the G-CSF with endometrial scratch subgroup than the G-CSF only subgroup. The clinical pregnancy rates and live birth rates were 53.8 % and 38.5% in the G-CSF plus endometrial scratch group. The clinical pregnancy rates and live birth rates in the G-CSF only group was 42.9% and 28.6%, respectively. Compared to the control group, there were significantly higher embryo implantation rates in G-CSF group (31.5% versus 13.9%; $P < 0.01$ compared to the placebo group. The clinical pregnancy in the G-CSF only group was 48.1% versus 25.0%; $P = 0.038$ in the control group.

Zeyneloglu et al., in a prospective observational study investigated the ideal route of administration and grouped 32 women into 3 groups to receive intrauterine, subcutaneous or both respectively[178]. The pregnancy rates were 44%, 60% and 75% respectively. The authors concluded that dual administration of G-CSF may result in improvement in ART outcomes.

G-CSF in Recurrent miscarriage

Boxer at al., did a retrospective observational analysis on severe chronic neutropenia international registry (SCNIR) in 2015 and identified 107 women with 224 pregnancy events[179]. Varying doses of G-CSF started were started at different time points in pregnancy. This study suggested no improvement in

pregnancy outcome, however, confirmed that there was no increase in adverse events with use of G-CSF during pregnancy.

Wurfel et al., summarised evidence of efficacy of G-CSF in RM in a retrospective analysis in 308 women who were diagnosed with RM[176]. These women had varying doses and preparations of G-CSF within the study period. The study suggested a significant improvement in live birth rates (78.1%). Both G-CSF and GM-CSF were used in this study.

Santjohanser et al., conducted a retrospective cohort study in 127 women who had 2 or more previous miscarriages[180]. All these women had IVF treatment and were divided into two groups, group A receiving G-CSF and group B, receiving other medications to include steroids or IVIG or LMWH. The authors concluded a significant increase in LBRs in the G-CSF group.

Scarpellini et al., conducted the first randomised control study (n=68) to assess the efficacy of G-CSF in recurrent miscarriage[73]. The participants were diagnosed to have at least 4 previous miscarriages. They were randomised to G-CSF vs placebo in a 1:1 ratio. The study suggested significant reduction in miscarriages and a statistically significant increase in live birth rates. In the G-

CSF group, the live birth rate was 82.8% whereas in the placebo group, the live birth rate was 48.5%, P = 0.0061, odds ratio = 5.1; 95% CI 1.5–18.4.

Another observational analysis on SCNIR database by Zeidler et al., in 38 pregnancy events investigated G-CSF use in women with chronic neutropenia[181]. Varying doses and duration of G-CSF was noted within this study which did not confirm any improvement in pregnancy outcomes. There was 50% incidence of miscarriages in women who received G-CSF and those who didn't receive G-CSF.

Discussion

Immune mediated treatment failure in IVF and immune mediated miscarriages can cause significant dilemma and frustration to patients and clinicians alike. Many studies from early 2000s suggest and support use of G-CSF for these conditions. There is evidence to suggest increase in implantation rates for women undergoing ART and a decrease in incidence of miscarriages in women with recurrent miscarriages.

The exact mechanisms involved in improving pregnancy outcomes are unknown. Animal and human studies suggest G-CSF improves oocyte quality and maturation, endometrial thickness, endometrial receptivity and live birth

rates. Animal studies involving rats suggest anti-abortive actions of G-CSF; however, another study in rabbits did not confirm this[182,183].

G-CSF administration appears to be associated with an increase in regulatory T cells and dendritic cells and appears to influence endometrial expression of genes crucial for the implantation process. The increase in the cytokines and genes may result in endometrial vascular remodelling and immune-modulation by altering the cellular adhesion pathways[185,186].

This review incorporated a comprehensive literature search using electronic databases. All relevant citations were included and hand search of bibliography was also conducted. I also included data for pregnant women using G-CSF from population registry for other indications. (These data were not included in other available reviews till date).

The major weakness of this review was the relatively low number of controlled clinical studies. Unlike other immunomodulatory treatment options, evidence for G-CSF is relatively new and therefore considered to be a novel option. Therefore the studies available had relatively small number of participants. This makes the conclusions less reliable.

There was a high level of heterogeneity among the studies with different indications for use of G-CSF, different treatment methods and varying doses. Studies conducted by Gleicher et al[172]. , Eftekhar et al.[167] , Kunicki et al.[143] , Lee et al.[173] and Xu et al[177] had low participant number. Most of the studies used a methodology of intrauterine infusion of G-CSF as a single dose or a maximum of 2 doses.

Zeyneloglu et al. suggested dual administration of G-CSF is more effective[178]; however, there is no clear evidence for such approach. Majority of studies had no control groups and/or control groups in some studies were not clear (Wurfel et al.[184]).

Out of the 17 studies identified here, only five were randomised studies. There was evidence of selection bias in studies conducted by Scarpellini et al.[73] , Barad et al.[169], Lee et al.[173], and Xu et al[177].

A meta-analysis was not performed for the following reasons:

a. There was considerable difference in the dosage and timing of medications in each study.

b. There were no controls or else, the controls did not receive any medications in most of the studies.

c. Ideally, effect of intervention should be assessed by RCT and there was no uniformity in the RCTs identified.

d. Even though the population was matched in these studies, the interventions varied significantly.

e. With low number of participants in most of the studies, the pooled estimate would have crossed null hypothesis which would have made it difficult to make a conclusion.

f. Publication bias and lack of uniformity would have led to invalid and less meaningful interpretation of the literature.

Even though this review provides supporting evidence, there was a clear need for further conclusive studies with proper methodology to evaluate efficacy of G-CSF. This warranted the need for a large multicentre RCT trial to answer this very important clinical question.

CHAPTER 4

METHODS FOR A RANDOMISED, DOUBLE BLIND, MULTICENTRE, PLACEBO-CONTROLLED STUDY TO EVALUATE THE EFFICACY, SAFETY, AND TOLERABILITY OF RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR IN PREGNANT WOMEN WITH A HISTORY OF UNEXPLAINED RECURRENT MISCARRIAGE

Description of the study

RESPONSE study was a randomised; double blind, multi-centre, placebo-controlled study of subcutaneous Recombinant Granulocyte Colony Stimulating Factor (rhG-CSF) in pregnant women with a history of unexplained recurrent miscarriage. 150 women were randomised in a 1:1 ratio to receive subcutaneous rhG-CSF 130 mcg or placebo once daily (OD) for up to 9 weeks of treatment.

During the screening period, participants underwent eligibility evaluation. Once eligibility was established, the participant began ovulation monitoring and attempts at spontaneous conception. The participant started a daily, pregnancy test with the first morning urine sample on the 6th day after ovulation or 5 days before the start of the next expected menstrual period

(whichever came first). The test was continued until positive or until the onset of her next menstrual period.

Once the home urine pregnancy test was positive, the participant immediately scheduled a visit to the study site for a repeat urine pregnancy test (the visit took place within 3 days of the positive home pregnancy test). A repeat urine pregnancy test was performed at the study site and if positive, the participant was randomised into the study and study drug treatment was initiated.

For the purpose of the study, gestational age was calculated based on the ovulation date (Ovulation date was defined as the day after the ovulation test was reported to be positive). Weeks of gestation were calculated as ovulation date plus 2 weeks. At the time of randomisation, participants were between 3 to 5 weeks of gestation. Participants who were determined to be at greater than 5 weeks of gestation (i.e. greater than 3 weeks since the ovulation date) were not eligible for randomisation. Women who were unable to achieve a spontaneous pregnancy within 9 months of screening were discontinued from the study.

Randomised participants started daily subcutaneous injections of rhG-CSF or placebo and underwent an ultrasound examination at 6 weeks gestation for assessment of clinical pregnancy. Women who had a clinical pregnancy

underwent repeat ultrasound examinations at Weeks 8, 12 and 20 of gestation. Study drug was not continued beyond 12 weeks of gestation, as total duration of treatment was up to 9 weeks from time of randomisation. Participants who were not found to be pregnant (miscarriage, biochemical pregnancy, PUL or ectopic pregnancy) were discontinued from the study and they entered a 4-week post drug follow-up period.

The primary outcome assessment (clinical pregnancy rate at Week 20 of gestation) was assessed via ultrasound examination.

All participants were monitored for adverse events. All participants who received at least one dose of study drug were followed for safety for a minimum of 4 weeks following the last dose of study drug. Karyotype testing (where possible) was performed on products of conception as per protocol at the local recruiting site following a spontaneous pregnancy loss. Study team conducted telephone follow up assessments for women who maintained pregnancy through 20 weeks of gestation. This 8 weekly phone follow up assessed pregnancy status/outcomes and prescription medication use. One month following delivery, additional information were obtained, including pregnancy outcome, gestational age at delivery, mode of delivery, birth weight, and Apgar scores.

Rationale for study design

In addition to its granulopoietic and stem cell mobilization effects, rhG-CSF has been demonstrated to have a well-documented immune-modulatory/immune-regulatory effect in animals and humans. Multiple studies have shown that rhG-CSF can decrease T cell alloreactivity and inflammatory cytokine production[186,187]. Recombinant human granulocyte colony stimulating factor has also been demonstrated to increase regulatory T cell populations, with the net effect of induction of T cell tolerance without global immunosuppression[188]. The importance of inducing T cell tolerance in a normal pregnancy has been shown in studies conducted by Rowe at al and Samstein et al[189,190]. These experimental models support the hypothesis that rhG-CSF treatment in infertility or recurrent pregnancy loss may have beneficial effects, as an imbalanced maternal immune response has been implicated in some types of unexplained infertility, recurrent implantation failure, and recurrent pregnancy loss, for e.g. the study by Fasouliotis et al[191] .

Williams et al based on his studies on tolerance induction in pregnancy observed that this hypothesis provided biological plausibility. He proposed that administration of G-CSF may reduce the rate of miscarriage[192]. Salmassi et. al[193], McCracken et. al[194], studied links between G-CSF and pregnancy by demonstrating expression of G-CSF and G-CSF receptors in pregnancy-

associated tissues, including follicular fluid, endometrium, and placenta. Higher levels of both follicular as well as circulating endogenous G-CSF have been shown to correlate with improved pregnancy outcomes as evidenced by studies conducted by Salmassi et. al[193] and Ledee et. al[195]. A single centre placebo controlled study using recombinant G-CSF demonstrated significant improvement in live birth in women who were diagnosed with unexplained recurrent miscarriage[73]. A number of other small, single centre studies concluded the same biological and clinical effect. (Discussion of all these studies was conducted as part of a systematic narrative review ad explained in chapter 3)

Based on these previously published studies with rhG-CSF it was hypothesised that administration of rhG-CSF may have a beneficial effect in improving pregnancy outcomes in women with unexplained RPL.

A phase 1, randomised, double-blind, placebo-controlled, dose-escalation study in 48 healthy female volunteers was completed prior to the RESPONSE study design. The phase 1 study consisted of 6 single- and multiple-dose cohorts with 8 participants in each dose cohort, randomised in a 3:1 ratio to receive either rhG-CSF or placebo. rhG-CSF dose levels tested were 65, 130, and 260 mcg.

Overall, single and multiple SC doses of rhG-CSF up to 260 mcg appeared to be safe and generally well tolerated by the healthy female volunteers.

There were no deaths, serious adverse events (SAEs), or participant discontinuations due to adverse events (AEs) in the phase 1 study. No AEs met the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) v4.03 Grade 3 or higher criteria. As expected, transient neutrophilia and increases in white blood cell (WBC) counts were observed following both single and multiple doses. Vital signs and electrocardiograms remained unaffected following rhG-CSF administration. No anti-drug antibodies (ADAs) were detected in any participants. Changes in peripheral blood cell subsets were observed that are consistent with supporting a state of maternal-fetal immune tolerance. These changes included temporary induction of toleragenic cell subsets and decreased percentages of pro-inflammatory and cytotoxic cell subsets, without evidence of global immune changes or suppression. For example, overall CD4+ and CD8+ T cells were unchanged in all treatment groups. However, within the CD4 compartment, there was a selective induction of toleragenic CD4+ CD25+ positive regulatory T cells (Treg cells) and a decrease in pro-inflammatory CD4+ CXCR3+ Th1 cells. Within the CD8+ T cell compartment, there was a decrease in the cytotoxic mucosa-associated invariant T cells (MAIT cells). There were also changes within the non-T cell subsets that were consistent with induction of a toleragenic state,

including an increase in toleragenic myeloid derived suppressor cells (MDSC) and a decrease in cytotoxic natural killer (NK) cells. The CD1c+ dendritic cell (DC) subset was also decreased; these "professional" antigen cells are considered to be the most potent inducers of cytotoxic CD8+ T cells in the immune system. (NORA Therapeutics, Phase 1 study)[197]

The changes described above were observed in all multi-dose rhG-CSF groups, and were not seen in any placebo group. Furthermore, with the exception of the increase in MDSC cells, the changes were only seen in the rhG-CSF multi-dose groups and not in the single dose groups. The changes in cell subsets occurred during the period of drug administration, and the changes returned to baseline levels within 72 hours of rhG-CSF discontinuation.

A Phase 2, randomised, double-blind, placebo-controlled parallel-dose study in 156 women with a history of repeated in vitro fertilization failures was conducted at multiple investigative centres in the United States. There were no AEs of special interest as identified prospectively in the protocol and no subjects discontinued study drug due to an AE.

The pharmacokinetics of rhG-CSF were determined following SC administration in the Phase 1 study. Apparent clearance of rhG-CSF increased

between Day 1 and Day 10 were likely due to the known stimulatory effect on neutrophils and the well-established clearance of G-CSF by neutrophils. The assay for rhG-CSF measured only free drug changes in the free fraction of bioavailable dose over time due to an increased pool of neutrophils. The terminal half-life of rhG-CSF after multiple doses was approximately 8 to 11 hours. The pharmacokinetic results showed that there was no rhG-CSF accumulation with daily dosing and that exposure increased with dose, although not in an exact dose-proportional manner. These data supported daily SC injections of rhG-CSF. There was no immunogenicity to rhG-CSF found in the Phase 1 study. Treatment with rhG-CSF resulted in rapid onset of effect on neutrophils and rapid offset once dosing ceased. The rhG-CSF dose level being evaluated in this study was selected based on the results of the nonclinical and clinical studies, which support the use of 130 mcg SC daily for up to 8 weeks (±1 week).

Outcome measures

Primary outcome measure

The primary outcome measure was clinical pregnancy rate at Week 20 of gestation.

Secondary outcome measures

The secondary outcome measures were as follows:

1. Live birth

2. Live birth beyond 34 weeks of gestation

3. Clinical pregnancy at Weeks 6, 8 and 12 of gestation

4. Spontaneous pregnancy loss under 24 weeks

5. Stillbirth

6. Neonatal birth weight

7. Infants discharged alive from the hospital following delivery

8. Maternal adverse events and serious adverse events during the treatment period and within 4 weeks of the last dose of study drug

9. Changes in clinical laboratory parameters following study drug exposure

10. Major congenital anomalies

11. Diagnosis of pre-eclampsia, eclampsia, gestational hypertension, or placenta accreta.

12. Incidence of anti-drug antibody (ADA) formation

Exploratory pharmaco-dynamic outcome measure

The exploratory pharmaco-dynamic outcome measure was changes in levels of circulating white blood cell subsets.

Safety plan

Safety was monitored through the assessment of adverse events, vital signs, physical examinations, and clinical laboratory parameters throughout the treatment period and 4-week post-drug follow-up period.

In order to minimise unnecessary exposure, any participant receiving study drug who was no longer pregnant discontinued study drug. Following discontinuation, they were followed up for a minimum of 4 weeks after last dose of study drug. An external and independent Data Monitoring Committee (DMC) also followed up this study. The DMC facilitated close monitoring of safety data and serious adverse events. The DMC also reviewed all available safety data at pre-specified time points throughout the study and based on these safety reviews, the DMC made regular recommendations regarding the safe conduct of the study (including termination of dosing).

Adverse events of special interest and study stopping rules

None of the participants experienced any adverse events of special interest in this study. Therefore no participants had study discontinuation for this reason.

The adverse events of special interest were :

- 2 participants receiving active study drug with splenic rupture
- 2 participants receiving active study drug with anaphylaxis

- 2 participants receiving active study drug with acute respiratory distress syndrome (ARDS) or acute lung injury (ALI)
- 2 participants receiving active study drug with major cardiovascular event

Hyperleucocytosis

In order to avoid potential complications of hyperleucocytosis within RESPONSE study, WBC counts were monitored closely throughout the treatment period. Full blood counts (FBC) were obtained at baseline and during the treatment period. No participants had a WBC count more than 70,000/μL (which was the cut-off) for study drug treatment suspension.

Immunogenicity

Immunogenicity is a potential adverse effect of any biologic therapy, and may be of particular concern if a neutralizing antibody response occurs against recombinant forms of endogenous proteins such as G-CSF. In theory, neutralizing antibodies to rhG-CSF may cross-react with endogenous G-CSF, which may manifest clinically as induction of a neutropenic state and increased infection risk. An additional risk exists in pregnancy due to the risk of trans-placental passage of ADA and potential impact on the fetus.

Therefore, an immunogenicity monitoring plan was put in place for RESPONSE study. All participants receiving study drug had serum specimens taken at

baseline, randomisation, 6th week, 12th week, 16th week or 4 week post drug follow up as appropriate. None of the participants developed anti-drug antibody within this study.

Blinding and Minimisation of Bias

RESPONSE study was a randomised, double-blinded, and placebo-controlled to minimise potential bias in treatment assignment, participant monitoring, and endpoint evaluations.

All participants, investigative site study staff, and investigative site monitors were blinded to treatment assignment. In emergency situations such as an adverse event, the Investigator unblinded the participant's treatment assignment immediately, or as quickly as possible. The investigators had unrestricted and immediate access to unblind the participant's treatment assignment by accessing the Interactive Web Registration System (IWRS). Further, the Investigator followed-up the circumstances with the Medical Monitor. Additionally, WBC and WBC subset counts, alkaline phosphatase, uric acid, and lactate dehydrogenase (LDH) had the potential to unblind the investigators and site monitors. During the course of the study, only designated central laboratory staff, members of the DMC, and the Sponsor had access to these data. Routine pregnancy booking blood tests performed during the first

trimester typically included a WBC count as part of the full blood count (FBC). Physiological changes in WBC count as anticipated with rhG-CSF administration would have led to unnecessary confusion and study unblinding. In order to prevent these possible outcomes, participants and their general practitioners were provided with the results of the study FBC collected at the time of randomisation for use as a component of the booking blood tests. It was also requested not to include FBC as part of their booking blood tests. The DMC and sponsor were unblinded to treatment assignment and independently reviewed safety findings.

MATERIALS AND METHODS

Participants

Females aged 18-37 years with a history of unexplained recurrent pregnancy loss.

Inclusion criteria

Participants had to meet the following criteria to be eligible for randomisation into the study:

1. Pre-menopausal female 18-37 years of age at consent, trying to conceive.

2. Documented history of unexplained recurrent pregnancy loss, defined as:

- Spontaneous loss of three or more pregnancies (non-consecutive) prior to 20 completed weeks of gestation.
- At least two of the losses must have involved intrauterine clinical pregnancies prior to 20 completed weeks of gestation with normal or unknown fetal karyotype.
- Spontaneous conception, as confirmed by urine pregnancy test performed at the investigative site
- Body mass index (BMI) of 19-35 kg/m2 at consent

Exclusion criteria

Participants who had any of the following criteria were excluded from randomisation into the study:

1. Greater than 5 weeks of gestation (i.e. greater than 3 weeks from the ovulation date) when presenting for randomisation. Ovulation date was the day after the ovulation test is first positive.

2. Known karyotype abnormalities in either the participant or her current male partner.

3. Uncorrected clinically significant intrauterine abnormalities present at the time of consent (as assessed by ultrasound, hysterosonography, hysterosalpingography, or hysteroscopy within 3 years prior to consent).

4. Abnormal vaginal bleeding of unknown cause

5. Current diagnosis of infertility in either the participant or her current male partner

6. Current or past diagnosis of the following:

(a) Systemic autoimmune disease (e.g. systemic lupus erythematosus, Hashimoto's thyroiditis, Graves' disease, rheumatoid arthritis), (b) Antiphospholipid syndrome or presence of lupus anticoagulant or anti-cardiolipin antibodies, (c) Protein C or S deficiency, (d) Other thrombophilia or evidence of thrombophilia (e.g. recurrent deep vein thrombosis, pulmonary embolism), (e) Hyperprolactinemia, (f) High risk of cervical incompetence in the investigator's opinion (g) High-grade cervical dysplasia with conisation/surgery

7. Any uncontrolled clinically significant medical condition (e.g. asthma, type II diabetes, infection).

8. The following laboratory abnormalities at initial consent and within 3 months prior to randomisation

(a) Thrombocytopenia or thrombocytosis (platelet count < 75,000/μL or > 500,000/μL), (b) Neutropenia or neutrophilia (absolute neutrophil count < 1500/μL or > 10,000/μL), (c) Leucopenia or leucocytosis (white blood cell count < 3000/μL or > 15,000/μL), (d) Creatinine, hepatic

transaminases, lactate dehydrogenase (LDH), alkaline phosphatase, or uric acid ≥ 1.5x upper limit of normal (ULN).

9. Use of lithium within 1 month prior to consent.

10. Known hypersensitivity to any rhG-CSF drug product, any of its components, or any E. coli-derived proteins.

11. History of any of the following conditions:

(a) Human immunodeficiency virus (HIV) infection, (b) Malignancy within the past 5 years other than treated basal cell carcinoma or squamous cell carcinoma of the skin, (c) Splenomegaly or splenic rupture, (d) Adult respiratory distress syndrome (ARDS), acute lung injury (ALI), or pulmonary oedema, (e) Sickle cell anaemia, (f) Acute myocardial infarction, stroke, or revascularization (coronary or cerebral).

12. Previous rhG-CSF therapy for any indication.

13. In the investigator's opinion, any contraindication to the use of an investigational drug. (for e.g. anticipating poor compliance)

Method of treatment assignment

Eligible participants were randomised to receive rhG-CSF 130 mcg or placebo in a 1:1 ratio. Stratified permuted block randomisation with number of prior miscarriages (3, >3), and age (<35, 35-37) as the stratification factors, were used. An Interactive Web Response System(IWRS) was used to perform the randomisation.

Study drug

Study drug supply, storage, and preparation

Recombinant human granulocyte colony stimulating factor and placebo were supplied to the investigative site in glass 1 mL prefilled syringes. Each prefilled syringe contained 0.5 mL of a sterile, preservative-free, aqueous solution intended for single-use subcutaneous administration. The rhG-CSF 130 mcg formulation contained 260 mcg/mL of rhG-CSF in sodium acetate, D-sorbitol and polysorbate 80 at a pH of 4.0. Placebo contained sodium acetate, D-sorbitol and polysorbate 80 at a pH of 4.0. Prefilled syringes were stored at 2-8ºC (36-46ºF). Prefilled syringes were supplied in kits packaged with 8 syringes and plunger rods per kit. One kit was issued at a time via IWRS at randomisation and visits prior to the Week 6 visit (e.g. Week 4 and Week 5 visits, if applicable). Two kits were issued via IWRS at the Week 6, Week 8, and Week 10 visits. One

prefilled syringe was required for each subcutaneous injection. The investigative site personnel trained the participant on study drug administration prior to administration of the first dose according to the patient instructions. The first dose of study drug was administered at the investigative site. The participants themselves administered subsequent doses. Participants were instructed to administer this dose at approximately the same time each day (within 20 to 28 hours after the previous dose).

Route of administration

Each participant received rhG-CSF 130 mcg or placebo as a daily subcutaneous injection. The participants were instructed to rotate injections sites every day.

Dosage Modification

No dosage modification was allowed in this study.

Study Drug Suspension

There were no study suspension due to any adverse events.(Overall, there were 5 participants who had study discontinuation for adverse events ; two participant in the rhG-CSF group and three participants in the placebo group, respectively).

Missed or Delayed Doses

Each dose of study drug was administered once daily at approximately the same time each day (within 20 to 28 hours after the previous dose). If it had been longer than 28 hours after the previous dose, the dose was skipped, and the following dose was administered on the regular schedule. The skipped dose was considered and documented as a missed dose.

Labelling and study drug accountability

All prefilled syringes were labelled in kits containing eight prefilled syringes each. Study accountability was maintained for all prefilled syringes distributed to the study sites. Study kit also included an ice pack to be used in emergencies to comply with the temperature regulations.

Concomitant therapies, excluded therapies and clinical practice

Concomitant therapies

Concomitant therapy included any prescription medications or over-the-counter preparations used by a participant between randomisation through the 4-week post drug follow-up visit. A record of all concomitant medications received by participants during the treatment period and within 4 weeks of the last dose of study drug was maintained during the study for each participant. A

record of all prescription medications was maintained for participants until live birth (for women who remained pregnant) or as appropriate (for participants with other outcomes).

Participants who had well-controlled chronic diseases was allowed to continue with stables doses of medications. Participants who had adverse events were allowed to have appropriate interventions to treat the adverse events. A detailed description of the type of the drug, treatment period, dosing regimen, the route of administration and drug indication was maintained.

Excluded therapies

Excluded therapies from consent through the 4-week post drug follow-up visit, unless otherwise specified, included the following:

- Progesterone
- Low molecular weight heparin (LMWH)
- Immunomodulatory agents such as systemic corticosteroids, TNF-inhibitors, or intravenous immunoglobulin (IVIG)
- Stable doses of inhaled or intranasal corticosteroids were not excluded
- Use of lithium (beginning within 1 month prior to consent)
- Medications known to have harmful effects in pregnancy, unless medically indicated

- Any other investigational therapies.
- Use of non-standard prescription medications for "pregnancy support" of recurrent pregnancy loss will be excluded unless medically indicated for other conditions. Examples of such therapies include progesterone, intravenous immunoglobulin (IVIG), LMWH, and tumour necrosis factor (TNF) inhibitors.

Details of Study Assessments

See table 4.1. for a detailed schedule of events. The following participant flow diagram provides a summary of assessments and decision points for each participant.

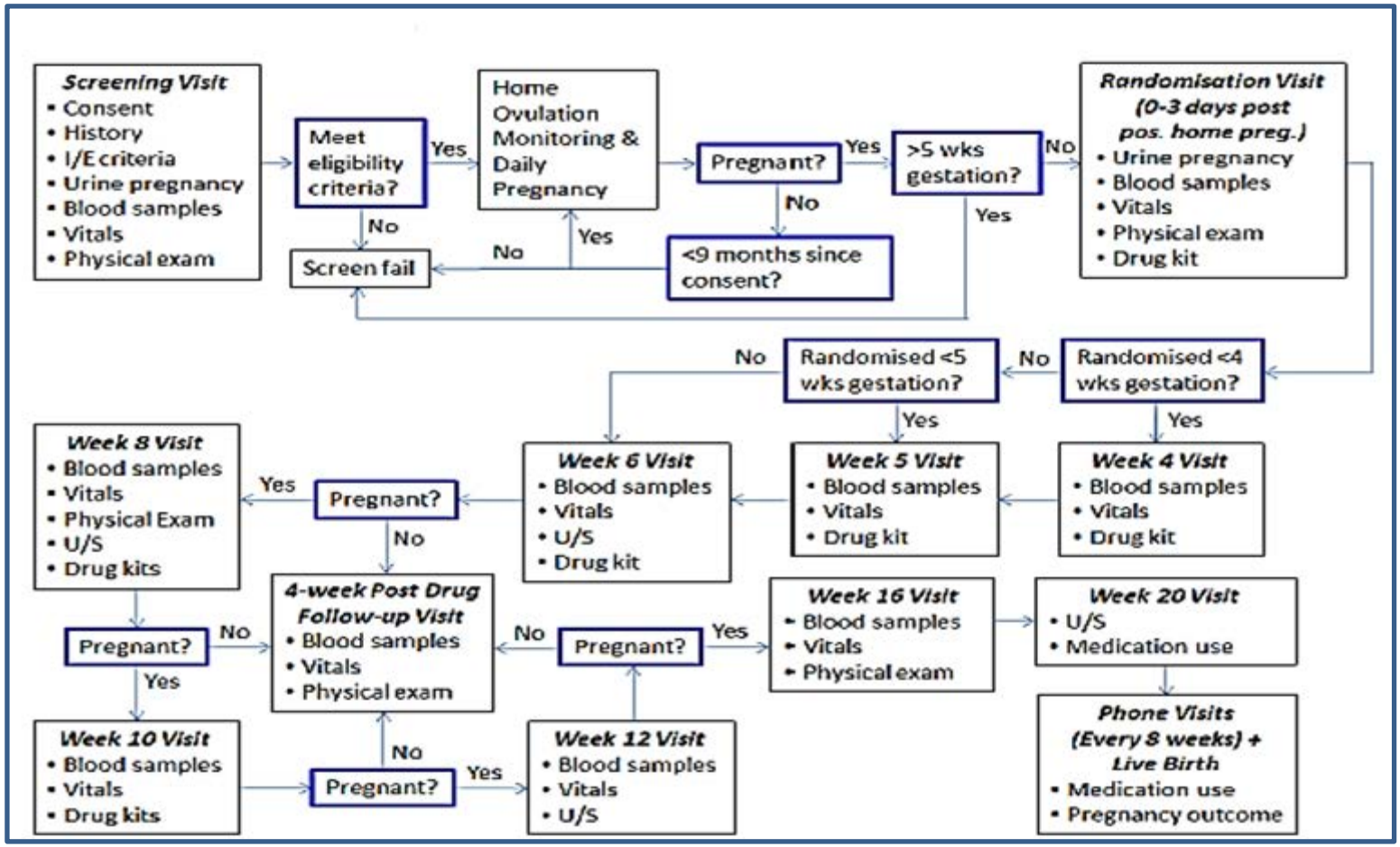


Figure 4.1 – Participant flow in the study

Table 4.1 - Schedule of Events

Study Time Points (Relevant to Gestational Age [Weeks])	Screening Visit	Random-ization Visit	Week 4 Visit[a]	Week 5 Visit[a]	Week 6 Visit	Week 8 Visit	Week 10 Visit	Week 12 Visit	Post Drug F/U[b]	Week 16 Visit	Week 20 Visit	Phone Visits	Birth Visit
		3-5	4±1d	5±1d	6±1d	8±1d	10±1d	12±1d	Variable	16±3d	20±7d	24,32,40 ±3d	~40
Informed consent	X												
Medical reproductive history	X												
Inclusion/exclusion criteria	X	X											
Study drug dispensation[(1)]		X	X	X	X	X	X						
Study drug administration			X	X	X	X	X	X					
Study site urine pregnancy test	X	X											
Home ovulation monitoring and daily urine pregnancy test	X												
Hematology	X[d]	X	X	X	X	X	X	X	X	X			
Chemistry	X[d]	X	X	X	X	X	X	X	X	X			
Screening tests[e]	X												
ADA[f]		X			X			X	X	X			
PD biomarkers		X			X	X							
Ultrasound					X[g]	X[g]		X[g]			X		
Vital signs	X	X	X	X	X	X	X	X	X	X			
Physical exam	X	X				X			X	X			
Pregnancy outcome												X	X[i]
Concomitant medications		X	X	X	X	X	X	X	X	X	X	X	X
Adverse events	X[i]	X[i]	X	X	X	X	X	X	X	X			

Abbreviations: ADA = antidrug antibodies; exam = examination; F/U = follow up; PD = pharmaco-dynamic; Tx = treatment.
Note: Randomisation visit to occur 0 to 3 days after positive home pregnancy test

a. Week 4 visit did not occur for participants randomised ≥4 weeks of gestation; Week 5 visit did not occur for participants randomised at 5 weeks of gestation.
b. The 4-week post drug Follow-up visit was only for participants who either discontinued study drug prior to Week 12 or who did not have a clinical pregnancy at Week 12.
c. After first dose, participant was observed ≥1 hour. All subsequent doses could be administered outside the clinical site. Participants were to administer study drug as close to the same time each day as possible (20 to 28 hours after previous dose).
d. During screening, haematology and chemistry samples were collected every 3 months (±1 week) in participants who have been unable to achieve pregnancy.
e. Lupus anticoagulant was not required if the participant had a negative test result within 5 years prior to consent; proteins C and S were not required if the participant had a negative test result at any time in the past.
f. Additional ADA assessments were conducted in participants who seroconvert periodically until antibody levels return to baseline through the end of the study.
g. At Week 6, 8, and 12 of gestation, the ultrasound visit window was ±3 days. Included delivery information and diagnosis of pre-eclampsia, eclampsia, gestational hypertension, or placenta accreta.
h. After informed consent, but prior to study drug administration, only SAEs caused by a protocol-mandated intervention were collected.
i. Included delivery information and diagnosis of preeclampsia, eclampsia, gestational hypertension, or placenta accreta.

Ovulation monitoring

Ovulation monitoring was performed by the participants during the screening period using a standardised commercially available ovulation monitoring kit. The date of ovulation during each menstrual cycle was recorded in the participant diary. Ovulation date was defined as the day after the ovulation test was first positive. The study staff helped participants to estimate the ovulation date if they had unreliable ovulation tests or for participants who had polycystic ovaries.

Urine pregnancy test

Participants started daily home urine pregnancy tests using a standardised commercially available home pregnancy test starting 6th day after ovulation or 5 days before the start of the next expected menstrual period (whichever came first). A urine pregnancy test at the investigative site was assessed to determine pregnancy status at the initial screening visit. Following a positive home urine pregnancy test, a repeat urine pregnancy test was performed at the time of randomisation.

Physical examinations

Physical examinations were performed at various time points. A detailed physical examination was performed at the initial screening visit, at the

randomisation visit, at 8th week of gestation, and 4 weeks after the last dose of study drug. Abnormalities identified as pre-existing before randomisation was recorded in the medical history. Clinically significant abnormalities that appeared after randomisation were recorded as adverse events. Height was recorded at screening visit and weight was recorded at all physical examinations.

Vital signs

Vital signs (including blood pressure, respiration rate, heart rate, and oral or tympanic temperature) was obtained at all study visits with the exception of follow-up phone visits and live birth visit. Blood pressure and heart rate was measured after sitting or supine for a minimum of five minutes.

Laboratory evaluations

Clinical laboratory evaluations of blood samples, including biochemistry panel and haematology, were conducted as per study schedule. Screening included evaluation of additional parameters in the collected blood samples to facilitate eligibility evaluation. All bloods were analysed at an external laboratory to maintain uniformity in laboratory assessments.

Ultrasound

Ultrasound scan was performed at weeks 6, 8, 12, and 20 of gestation to assess and confirm clinical pregnancy

Telephone Follow-up Visits

Investigative site staff contacted participants via telephone every 8 weeks (±3 days) during pregnancy, starting at Week 24 (±3 days) of gestation, to assess pregnancy outcomes and to record prescription medication use.

Live Birth Visit

One month following delivery, additional information was obtained, including pregnancy outcome, diagnosis of pre-eclampsia, eclampsia, gestational hypertension, or placenta accreta, gestational age at delivery, mode of delivery, birth weight, and Apgar scores.

Additional Visits

If any participant needed extra visits as part of assessment for AE or SAE or for any other reason (for e.g. collecting extra medications) the additional visit was logged as an extra visit linked to the nearest gestation.

Participant Discontinuation

Participants could withdraw from treatment or from participation in study visits, or withdraw consent from the study at any time. Participants who decided to withdraw from treatment and those who were not pregnant completed the 4-week post drug follow-up visit.

Early Discontinuation of Study Drug

Participants were allowed to discontinue study drug under any of the following circumstances:

- The participant wished to discontinue study drug treatment for any reason
- The presence of any medical condition that the investigator determined which may jeopardise the participant's safety if she continued with study drug treatment
- Noncompliance (e.g., missed doses, visits)
- Determination by the investigator that discontinuation was in the best interest of the participant

For participants who withdrew from study visits, every attempt was made

a. to have them complete the 4-week post-drug follow-up visit prior to withdrawal from participation, or

b. to contact them for follow-up information through their pregnancy outcome, as applicable.

If the participation was withdrawn for reasons related to an AE, every effort was made to follow the participant until resolution of the event. The reason for premature discontinuation of treatment for participants was recorded on the eCRF.

Replacement of Withdrawn Participants

Participants who discontinued study drug or discontinue from the study following randomisation were not replaced by new participants.

ASSESSMENT OF EFFICACY AND PHARMACODYNAMICS

Efficacy assessments

Clinical pregnancy was defined as evidence of at least one intrauterine fetus or gestational sac with heartbeat on obstetric ultrasound. The primary outcome of the study was clinical pregnancy rate at or beyond 20 weeks of gestation. Live birth was defined as delivery of one or more infants with any signs of life, and was reported at each participant's delivery. Live birth rate was used as a secondary efficacy assessment.

ASSESSMENT OF SAFETY

Safety assessments consisted of

- monitoring and recording protocol-defined adverse events (AEs) and serious adverse events (SAEs)
- measurement of protocol specified laboratory variables and
- measurement of protocol-specified vital signs

Safety parameters and definitions

Spontaneous pregnancy loss

Spontaneous pregnancy loss was defined as the spontaneous loss of a pregnancy from the time of positive urine pregnancy test at randomisation until 24 completed weeks of gestation. Spontaneous pregnancy losses were further categorised into

- pre-clinical losses (i.e. prior to 6-week ultrasound assessment) and
- clinical losses (i.e. following 6-week ultrasound assessment).

Stillbirth

Stillbirth was defined as death before the complete expulsion or extraction of a product of fertilization from its mother, at or after 24 completed weeks of gestation. The death was indicated by the fact that, after such separation, the

fetus does not breathe or show any other evidence of life, such as heart beat, umbilical cord pulsation, or definite movement of voluntary muscles.

Adverse event

An adverse event (AE) was defined as any event, side effect, or other untoward medical occurrence that occurs in conjunction with the use of a medicinal product in humans, whether or not it is considered to have a causal relationship to the use of the medicinal product. An AE can, therefore, be any unfavourable or unintended sign (this could include a clinically significant abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product. All AEs, whether volunteered, elicited, or noted on physical examination, were recorded from first study drug administration until the 4-week study drug follow-up visit. Pregnant participants who had a Week 16 visit instead of a 4-week study drug follow-up visit had AEs recorded from first study drug administration until the Week 16 visit.

An AE included a/an:

- exacerbation of a pre-existing illness
- increase in frequency or intensity of a pre-existing episodic event or condition
- condition detected or diagnosed even though it may have been present prior to the start of the study

Serious adverse event

A SAE was any AE occurring that results in any of the following outcomes:

- Death
- Life-threatening situation (participant is at immediate risk of death)
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant disability/incapacity
- Congenital anomaly/birth defect in the offspring of a participant who received study drug
- Significant medical event as judged by the investigator (e.g., may jeopardise the participant or may require medical/surgical intervention to prevent one of the outcomes listed above)

Clinical laboratory abnormality and other abnormal assessment as AE and SAE

Abnormal laboratory findings (e.g. clinical chemistry, haematology, urinalysis) or other abnormal assessments (e.g. vital signs) were not reported as AEs. However, abnormal findings that are deemed clinically significant (i.e. requiring medical or surgical intervention) or are associated with signs and/or symptoms were recorded as AEs (if they met the definition of an AE). Clinically significant abnormal laboratory or other abnormal findings that were present

prior to study drug administration and worsened after study drug administration were also included as AEs (and SAEs, if serious).

Major congenital anomaly

Major congenital anomalies were defined as per the Metropolitan Atlanta Congenital Defects Program (MACDP) [MACDP 2007].A major congenital anomaly was defined as a significant deviation from normal that is present at or before birth. Major anomalies were those which required medical or surgical treatment, or which had significant cosmetic, health, or developmental consequences. Investigators also reported any isolated anomalies, multiple malformation syndromes, identifiable genetic/inborn conditions or any structural anomalies. All pregnancy outcomes will be reviewed for diagnosis of major congenital anomalies – including live births , stillbirths, spontaneous pregnancy losses, and elective abortions.

Maternal obstetric events

Maternal obstetric events like pre-eclampsia, eclampsia, gestational hypertension, placenta accrete were also recorded.

Assessment of adverse event severity

AE severity (apply event-specific NCI CTCAE grading criteria) scale was defined as:

1. Mild - Transient or mild discomfort (lasting up to 48 hours); no interference with the patient's daily activities; no medical intervention/therapy required
2. Moderate - Mild to moderate interference with the patient's daily activities; no or minimal medical intervention/ therapy required
3. Severe - Considerable interference with the patient's daily activities; medical intervention/therapy required; hospitalization possible
4. Very severe, life threatening, or disabling - Extreme limitation in activity; significant medical intervention/ therapy required, hospitalization probable
5. Death related to AE

Assessment of adverse event causality/relatedness

The investigator's assessment of study drug relatedness on the AE form of the eCRF (or the SAE form if applicable) was based on the following causality:

- Likely Related: A reaction that followed a reasonable temporal sequence from administration of the study drug; that followed a known or expected response pattern to the suspected study drug; or for which other potential aetiologies were considered less likely factors than the study drug.
- Likely Unrelated: A reaction that, considering all potential aetiologies, was most likely due to factors, other than the study drug.

All investigators was requested to include a rationale for the causal relationship in the site documents which was also supported by relevant laboratory tests, histopathology evaluations, and the results of other diagnostic procedures.

Adverse event reporting period

Any AE that occurred during the study was recorded on study site source documentation. Adverse events and serious adverse events (SAEs) were recorded from the first study drug administration through the 4-week post drug follow-up visit (or the Week 16 visit for pregnant participants who have a Week 16 visit instead of a 4-week study drug follow-up visit). After informed consent (but prior to initiation of study drug administration) only SAEs caused

by a protocol-mandated intervention were collected (e.g., SAEs related to invasive procedures such as blood collection). It was the investigators duty to attempt to establish a diagnosis of the event based on the signs, symptoms, and/or other clinical information. In such cases, the diagnosis was documented as the AE (and SAE if serious) and not the individual signs/symptoms. If a clinically significant abnormal laboratory finding or other abnormal assessment meets the definition of an AE, then the AE form of the e-CRF had to be completed. The diagnosis, if known, was recorded as the AE rather than the abnormal finding or assessment.

Follow-up of AEs and SAEs

All attempts were made to follow up AE and SAE s regardless of attribution until resolved, judged stable and unlikely to resolve or until the 4-week post drug follow-up visit. Pregnant participants who had a Week 16 visit instead of a 4-week study drug follow-up visit had AEs followed up until the Week 16 visit. The investigator was responsible for ensuring that AE and SAE follow-up included any supplemental investigations as may be indicated to elucidate as completely as practical the nature and/or cause of the AE or SAE.

Reporting SAEs to Institutional Review Board (IRB), Independent Ethics Committee (IEC) and Data Monitoring committee

All investigators submitted notification of any AE or SAE s to the CRO within 24 hours. Any SAE related to study participation (e.g. procedures), which occurred before the study drug administration (but after consent), were also promptly reported to the Sponsor.

DATA ANALYSIS METHODS

Analysis was primarily descriptive in nature with the endpoints summarised by treatment group and visit. For continuous variables, the following information was presented: n, mean, standard deviation, median, minimum and maximum. These statistics were presented using the actual value at baseline and the change from baseline for later data. Baseline will be the last observation prior to treatment. For categorical variables counts and percentages were used.

Sample Size and Decision Rule

The alpha level used will be a one-sided alpha level of 10%. The target sample size was 150 randomised and treated participants. Participants was randomised in a 1:1 ratio. This sample size was selected to achieve >90% power when the clinical pregnancy rates are 60% for placebo and 80% for rhG-CSF . The sample

size calculations were performed in PASS 2008 and utilised a Chi Squared test. The primary analysis was a CMH test controlling for the stratification factors.

Handling of missing data

For summary statistics missing data were not imputed; results were reported based upon observed data except for missing or partial dates and the clinical pregnancy rate at Week 20 of gestation endpoint (primary outcome). If determination of treatment period (on treatment, pre-treatment, post treatment) was required but the corresponding date was missing or partial, then the event was considered to be within treatment (unless the portions of the date that are available indicate this is not possible). If pregnancy status at 20 weeks of gestation was not known, it was assumed that the participant was no longer pregnant (unless the subject is later found to be pregnant)

Analysis populations

Efficacy analysis were based upon an intent-to-treat philosophy. The primary efficacy population was Full Analysis Set (FAS) that included all randomised and treated participants. Participants was analysed within the treatment group to which they were randomised. The primary safety population were all treated (placebo or rhG-CSF) participants. For safety analysis, participants were classified based upon the treatment received. Participant flow was monitored

by CRO and the sponsor and accounting of all randomised participants were performed. Participants who discontinued the study drug prematurely or withdrew from the study were summarised and listed, with the description of the reason for early termination/withdrawal.

Participant characteristics

Demographic and other baseline characteristics are listed and summarised in chapter 4

Treatment Compliance and Extent of Exposure

Participant compliance with study drug dosing was accessed via a site review of the returned syringes and the compliance record maintained by the participant. From these results, summaries of treatment compliance and exposure were produced.

Efficacy Analysis

The difference in the primary efficacy outcome measure (clinical pregnancy at 20 weeks gestation) between rhG-CSF and placebo was tested using a CMH test controlling for the stratification factors (# of prior miscarriages (3, >3), age (<35, 35-37)). The hypotheses being tested were:

H0:PPlacebo ≥ PNT-100 Ha:PPlacebo < PNT-100

where *PPlacebo* is the placebo rate and *PNBT-100* is the similar rate for the rhG-CSF treatment arm. The results from this test will be compared to a 10% one-sided alpha. This alpha level provides 90% likelihood of a correct positive assessment. Descriptive statistics were used to summarise the secondary endpoints.

Safety analysis

Adverse events

The incidence of all AEs and treatment-related AEs were tabulated by treatment received. These AEs were classified by system organ class and preferred term using the Medical Dictionary for Regulatory Activities (MedDRA).If a participant reported more than one AE that was coded to the same system organ class or preferred term, the participant was counted only once for that specific system organ class or preferred term. Summaries of adverse events included event rates for each treatment arm. SAEs will be listed and summarised in a similar manner to AEs.

Clinical safety laboratory results

Clinical safety laboratory values were measured by a central laboratory. Summary statistics for actual values and for changes from baseline was

tabulated for laboratory results by scheduled visit. Graphs of laboratory values over time were produced. Shifts from baseline laboratory values was tabulated and summarised.

Vital signs

The observed data and change from baseline for each measurement day was summarised with descriptive statistics.

Interim analysis

This study included a DMC that reviewed study results to identify potential safety concerns.

ADMINISTRATIVE ASPECTS

Changes to the protocol

Protocol amendments was made only with the prior written approval of the Sponsor, investigator, and the IRB/REC. Protocol changes within RESPONSE study is listed in the appendix.

Monitoring and auditing procedures

A clinical research organisation was designated for the monitoring the conduct of this study. The study site and investigators had assessments at regular intervals as part of monitoring by the CRO.

Informed consent

The purpose of the study, the procedures to be carried out, and any potential risks of study participation was described to the participants in non-technical terms in the participant information sheet (PIS). A copy of the PIS is attached in appendix. After reviewing and understanding the PIS, participants read, signed, and dated an IRB/IEC and Sponsor approved consent form before any study-specific procedures were carried out. Participants were assured that they could withdraw from the study at any time without jeopardizing medical care related to or required as a result of study participation. The original signed consent form was maintained in the investigator site file. Copy of the signed consent form was maintained in the participant's medical records and provided to the participant.

Communication with the IRB/IEC

The protocol, informed consent form, other written participant information, and any proposed advertising material was submitted to the IRB/EC for written

approval. IRB/IEC approval of these documents and any changes was provided to the site staff through the investigator. All protocol amendments as mentioned above were also submitted to the IRB/IEC.

Records and e CRFs

All study data except central laboratory and immunogenicity data were recorded in an e CRF system. Data was entered at the site by trained site staff. All source documents from which e-CRF entries were derived was placed in the participant's medical records. E-CRF was completed for every participant screened into the study. The study monitor (CRO) reviewed all e-CRFs in detail and had access to participant medical records, laboratory data, and other source documentation to allow all e-CRF fields to be verified by source data. Data consistency and plausibility checks against data entered into the e-CRF were included in the e-CRF system. For each instance of data modification, the system required a reason for change. The system kept a full audit trail of the data values, date and time of modification, and the electronic signature of the user who performed the change. The investigator finally signed and approved the participant's e CRF following a full review of the e-CRFs. All essential documents, source data, clinical records, and laboratory data are retained by the clinical site in accordance with ICH E6 and the site's data retention policies.

CHAPTER 5

RESULTS FOR A RANDOMISED, DOUBLE BLIND, MULTICENTRE, PLACEBO-CONTROLLED STUDY TO EVALUATE THE EFFICACY, SAFETY, AND TOLERABILITY OF RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR IN PREGNANT WOMEN WITH A HISTORY OF UNEXPLAINED RECURRENT MISCARRIAGES

Patient flow through the trial

Fig 5.1 and Table 5.1 represents the flow of participants and the study disposition through the study.

A total of 340 women were screened into the study from 21 centres across the UK. Of these, 190 women were screen failures. (Reasons included those who failed to conceive within 9 months after screening, those women who had positive screening for thrombophilia or met exclusion criteria after initial screening tests or those who had change in medical status or change in personal circumstances).

150 women were randomised to RESPONSE study, of which 76 were assigned to rhG-CSF and 74 were assigned to placebo. 92/150 women (61.3%) completed

treatment and 58/150 (38.7%) discontinued early mainly due to loss of pregnancy between randomisation and 12th week of gestation (50/150 subjects [33.3%]). Overall, 140/150 women (93.3%) completed the study and 10/150 women (6.7%) discontinued the study prematurely for reasons including loss to follow up, withdrawal of consent, and other reasons (for e.g. use of any excluded therapy or a decision made by study staff to adhere to study protocol).

The participant flow in the study was broadly similar across the 2 groups. 44/76 subjects [57.9%] completed treatment in rhG-CSF group compared to 48/74 subjects [64.9%] in the placebo group. Loss of pregnancy between randomisation and Week 12 of gestation was generally similar in the 2 treatment groups (27/76 subjects [35.5%] and 23/74 subjects [31.1%] in rhG-CSF and placebo groups, respectively). All women were followed up through the study, until live birth of the offspring/s or as appropriate. All participants were included in efficacy and safety analysis.

Table 5.1 - Flow of participants within RESPONSE Study

	rhG-CSF N=76 n (%)	Placebo N=74 n (%)	Total N=150 n (%)
Received Study Drug	76 (100.0)	74 (100.0)	150 (100.0)
Completed Treatment	44 (57.9)	48 (64.9)	92 (61.3)
Early Discontinuation of Treatment	32 (42.1)	26 (35.1)	58 (38.7)
Loss Of Pregnancy Between Randomisation And Week 12 Gestation	27 (35.5)	23 (31.1)	50 (33.3)
Other Reasons	5 (6.6)	3 (4.1)	8 (5.3)
Noncompliance	1	1	2
Adverse Event	2	0	2
Investigator Decision	0	1	1
Participant Withdrew Consent	2	1	3
Completed Study	72 (94.7)	68 (91.9)	140 (93.3)
Premature study discontinuation	4 (5.3)	6 (8.1)	10 (6.7)
Lost To Follow Up	2 (2.6)	5 (6.8)	7 (4.7)
Participant Withdrew Consent	1 (1.3)	0	1 (0.7)
Other	1 (1.3)	1 (1.4)	2 (1.3)

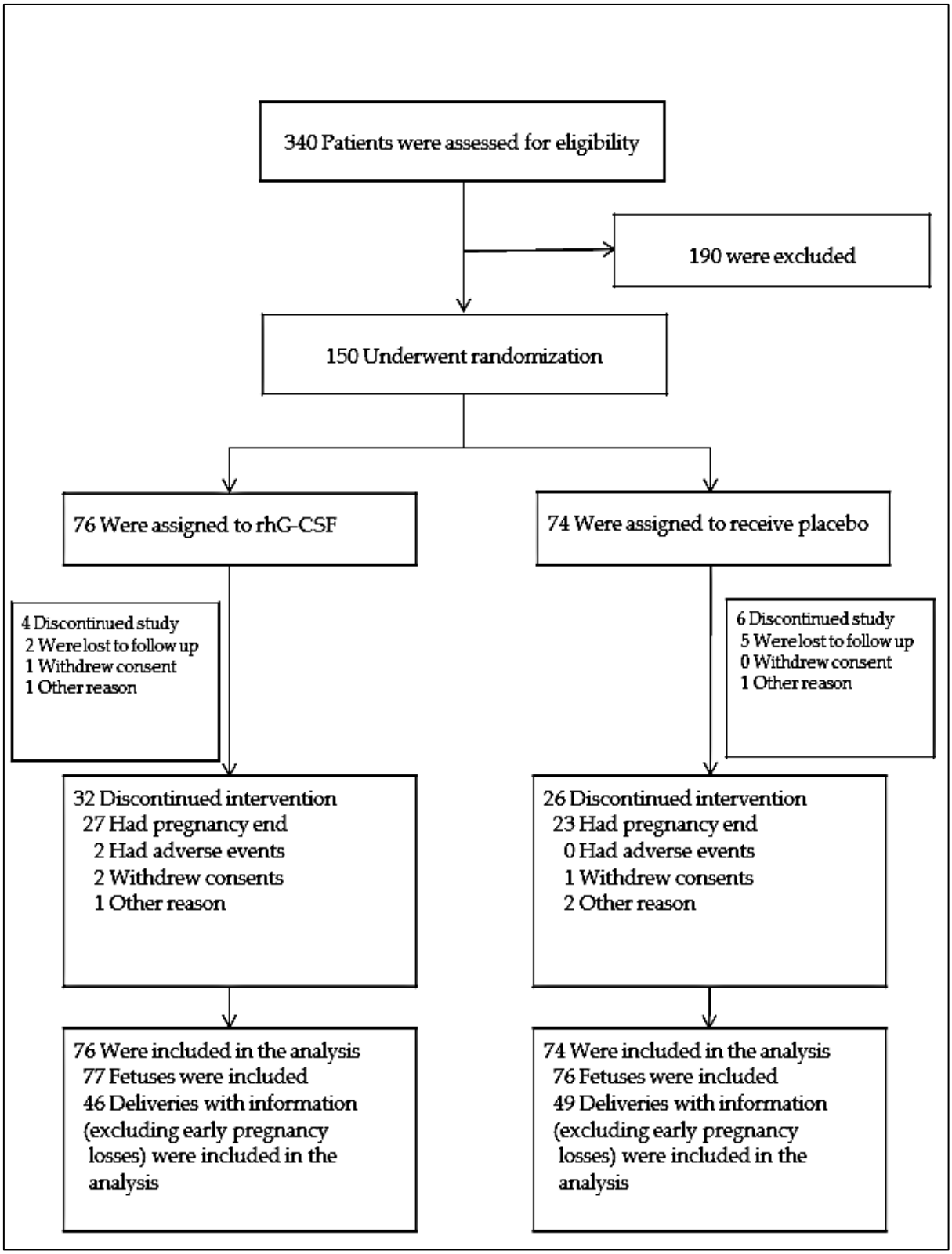


Figure 5.1 - Enrolment, Randomisation, Follow up and Analysis

Baseline characteristics

The baseline characteristics of the participants are shown in table 5.2 and table 5.3

Age

Most women (122 [81.3%]) were under the age of 35 yrs. within the study. Mean age (SD) was 31.1(3.60) in rhG-CSF group and 29.9(4.69) in the placebo group, respectively.

62 women (81.6%) were under the age of 35 and 14 women (18.4) were or over the age of 35 in the rhG-CSF group. 60 women (81.1%) were under the age of 35 and 14 women (18.9) were or over the age of 35 in the placebo group.

Ethnicity

Most women (134[89.3%]) were from the white ethnicity with the study. In the rhG-CSF group, 71(93.4%) were white, 3(3.9) were of Asian Pakistani ethnicity, 1(1.3%) from other Asian origin and 1(1.3%) from mixed race origin respectively. In the placebo group, 63(85.1%) were white, 4(5.4%) were of mixed ethnicity, 2(2.7%) were from Asian Indian ethnicity, 2(2.7%) were from Chinese ethnicity, 1(1.4%) was from Asian Pakistani origin, 1(1.4) was from black British origin and 1(1.3%) from other black ethnic origin respectively.

BMI

Most women were slightly overweight within the study. Mean BMI (SD) was 26.31(4.19) in rhG-CSF group and 25.80(4.28) in the placebo group, respectively. Both the groups were generally balanced relative to age, ethnicity and BMI.

Gestational age at start of treatment

In the rhG-CSF group, 30 women (39.5%) were randomised between three and four weeks of gestation, 42 women (55.3%) were randomised between four and five weeks of gestation and 4 women (5.3%) were randomised at 5 weeks of gestation. In the placebo group, 33 women (44.6%) were randomised between three and four weeks of gestation, 35 women (47.3%) were randomised between four and five weeks of gestation and 6 women (8.1%) were randomised at 5 weeks of gestation.

Primary or secondary miscarriage

In the rhG-CSF group, 38 women (50%) had primary RM and secondary RM respectively. In the placebo group, 37 women (50%) had primary RM and secondary RM respectively.

History of late miscarriages

Within the study a total of 23 women had previous miscarriages after 12 weeks of gestation of which 13 (17.1%) were in the rhG-CSF group and 10(13.5%) were in the placebo group.

Table 5.2 – Demographics of study population

	rhG-CSF N=76	Placebo N=74	Total N=150
Age (yrs)			
n	76	74	150
Mean (SD)	31.1 (3.60)	29.9 (4.69)	30.6 (4.20)
Median	32.0	31.0	31.0
Min, Max	22, 37	21, 37	21, 37
Age Category - n (%)			
< 35 yrs	62 (81.6)	60 (81.1)	122 (81.3)
>= 35 yrs	14 (18.4)	14 (18.9)	28 (18.7)
Race (UK) – n (%)			
Black British	0	1 (1.4)	1 (0.7)
Chinese	0	2 (2.7)	2 (1.3)
Indian	0	2 (2.7)	2 (1.3)
Mixed Race	1 (1.3)	4 (5.4)	5 (3.3)
Other Asian	1 (1.3)	0	1 (0.7)
Other Black	0	1 (1.4)	1 (0.7)
Pakistani	3 (3.9)	1 (1.4)	4 (2.7)
White	71 (93.4)	63 (85.1)	134 (89.3)

Table 5.2 – Demographics of study population

	rhG-CSF N=76	Placebo N=74	Total N=150
Baseline Height (cm)			
n	76	74	150
Mean (SD)	165.09 (7.447)	164.77 (7.374)	164.93 (7.388)
Median	165.00	165.55	165.00
Min, Max	147.5, 185.0	145.0, 178.0	145.0, 185.0
Baseline Weight (Kg)			
n	76	74	150
Mean (SD)	71.74 (14.222)	70.30 (14.044)	71.03 (14.106)
Median	70.25	67.25	69.00
Min, Max	48.9, 117.7	47.4, 101.9	47.4, 117.7
Baseline BMI (kg/m^2)			
n	76	74	150
Mean (SD)	26.31 (4.199)	25.80 (4.286)	26.06 (4.235)
Median	26.10	24.80	25.85
Min, Max	19.3, 35.1	19.4, 35.0	19.3, 35.1

Table 5.3 – Participant enrolment based on gestational age at start of treatment and previous miscarriages

	rhG-CSF N=76 n (%)	Placebo N=74 n (%)	Total N=150 n (%)
Gestational Age at Treatment Start - n (%)			
<= 3 weeks	30 (39.5)	33 (44.6)	63 (42.0)
4 weeks	42 (55.3)	35 (47.3)	77 (51.3)
>= 5 weeks	4 (5.3)	6 (8.1)	10 (6.7)
Primary or Secondary Recurrent Miscarriage			
Primary	38 (50.0)	37 (50.0)	75 (50.0)
Secondary	38 (50.0)	37 (50.0)	75 (50.0)
Prior Losses after Week 12	13 (17.1)	10(13.5)	23 (15.3)

Smoking status

Smoking status of women and their partners is presented in Table 5.4.

Overall, 27 women (18.0%) were current smokers, smoking a mean (SD) number of 6.4 cigarettes (4.50) per day. A total of 34 subjects (22.7%) had partners who smoked a mean (SD) number of 8.6 cigarettes (4.74) per day. Partners of women in the rhG-CSF group had a higher proportion of current smokers than the placebo group (19 partners [25.0%] and 15 partners [20.3%], respectively).

The number of participants who smoked was broadly balanced in both groups.

Alcohol consumption

Alcohol consumption of women is presented in table 5.5. A total of 79 women (52.7%) in the study consumed alcohol; mean (SD) units of alcohol consumed per week was 3.5 units (3.27). In the rhG-CSF Group, 40 women (52.6%) consumed alcohol; mean (SD) units of alcohol consumed per week was 3.1 units (2.31). In the placebo group, 39 women (52.7%) consumed alcohol; mean (SD) units of alcohol consumed per week was 3.9 units (34.04). Alcohol consumption (by those who consumed) was balanced in both groups.

Table 5.4 - Participant and Partner smoking status

	rhG-CSF N=76	Placebo N=74	Total N=150
Current Smoker	14 (18.4)	13 (17.6)	27 (18.0)
Cigarettes per day			
n	14	13	27
Mean (SD)	6.4 (5.30)	6.5 (3.67)	6.4 (4.50)
Median	5.0	6.0	5.0
Min, Max	1, 20	1, 10	1, 20
Partner Current Smoker	19 (25.0)	15 (20.3)	34 (22.7)
Partner's Cigarettes per day			
n	19	15	34
Mean (SD)	8.3 (5.14)	8.9 (4.32)	8.6 (4.74)
Median	8.0	10.0	10.0
Min, Max	1, 20	1, 20	1, 20

Table 5.5 Participant alcohol intake

	rhG-CSF N=76	Placebo N=74	Total N=150
Alcohol Consumption	40 (52.6)	39 (52.7)	79 (52.7)
Alcohol Consumption per week (units)			
n	40	38	78
Mean (SD)	3.1 (2.31)	3.9 (4.04)	3.5 (3.27)
Median	2.0	2.0	2.0
Min, Max	1, 10	1, 20	1, 20

Gynaecological history

Table 5.6 provides a summary of the subjects' gynaecologic history including gynaecological surgeries.

23 women (15.3%) had a documented non-surgical gynaecological history including polycystic ovarian syndrome, fibroids, endometriosis, or intrauterine abnormalities. 11 women (14.5%) had a non-surgical gynaecological condition in the rhG-CSF group. 12 women (16.2) had a non-surgical gynaecological history in the placebo group.

The gynaecological nonsurgical histories were generally balanced in both groups. The only exception was a slightly higher incidence of polycystic ovarian syndrome in the placebo group (6 women [8.1%] in the placebo group and 2 women [2.6%] in the rhG-CSF group respectively).

Gynaecological surgery

The past gynaecological surgical history for women included the following; evacuation of retained products of conception (ERPC) (78 women [52.0%]), other gynaecological surgical procedures (not otherwise specified [NOS]) (16 women [10.7%]), large loop excision of transformation zone (10 women [6.7%]), and ovarian cystectomy (7 women [4.7%]). Gynaecological surgical history was

similar in both groups except a slightly higher history of LLETZ for women in the placebo group (8 women [10.8%] in the placebo group and 2 women [2.6%] in the rhG-CSF group respectively).

Table 5.6 – Participants previous gynaecological history

	rhG-CSF n (%)	Placebo n (%)	Total n (%)
Prior Non-surgical Gynaecological History	11 (14.5)	12 (16.2)	23 (15.3)
Polycystic Ovarian Syndrome	2 (2.6)	6 (8.1)	8 (5.3)
Fibroids	5 (6.6)	3 (4.1)	8 (5.3)
Endometriosis	4 (5.3)	2 (2.7)	6 (4.0)
Intrauterine Abnormalities	2 (2.6)	5 (6.8)	7 (4.7)
Gynaecological Surgeries	46 (60.5)	44 (59.5)	90 (60.0)
Large loop excision of transformation zone	2 (2.6)	8 (10.8)	10 (6.7)
Endometriosis surgery	2 (2.6)	0 (0)	2 (1.3)
Tubal surgery	4 (5.3)	2 (2.7)	6 (4.0)
Ovarian cystectomy	3 (3.9)	4 (5.4)	7 (4.7)
ERPC	41 (53.9)	37 (50.0)	78 (52.0)
Division of intrauterine adhesions	1 (1.3)	2 (2.7)	3 (2.0)
Septum division	1 (1.3)	3 (4.1)	4 (2.7)
Other Gynaecological Surgery	6 (7.9)	10 (13.5)	16 (10.7)
Other Gynaecological Disorders	4 (5.3)	4 (5.4)	8 (5.3)

Reproductive history

Table 5.7, table 5.8 and table 5.9 presents a summary of the reproductive histories of the study population.

Women in the study had a mean (SD) of 5.1 previous pregnancies (2.21). The number of previous pregnancies ranged from three to sixteen. 37 (24.7%) had 3 previous pregnancies, 40(26.7%) had 4 previous pregnancies, 29 (19.3%) had 5 previous pregnancies and 44(29.3%) had more than 5 previous pregnancies respectively. A greater number of subjects in the rhG-CSF treatment group had a history of having had more than 5 pregnancies (25 subjects [32.9%]) compared with the placebo group (19 subjects [25.7%]).

The mean (SD) gestational ages for births were similar in the 2 treatment groups (39.5 weeks [1.76] and 39.3 weeks [1.97] in the rhG-CSF and placebo groups, respectively).

Women in the study had a mean (SD) of 4.3 previous preclinical and clinical losses (1.79). The number of previous clinical and preclinical losses ranged from three to fourteen losses. 70(46.7%) had 3 previous pregnancy losses, 36 (24.0%) had 4 previous pregnancy losses, 17(11.3%) had 5 previous pregnancy losses and 27 (18.0%) had more than 5 previous pregnancy losses respectively

More women in rhG-CSF group had a history of more than 5 previous spontaneous pregnancy losses (17 women [22.4%]) compared with the placebo group (10 subjects [13.5%]). There was a total of 647 pregnancy losses for 150 women in the study and the mean (SD) gestational age at miscarriage was 7.8 weeks (3.11) which were similar in both groups. More subjects in the rhG-CSF group had experienced preclinical losses (42 women [55.3%]) compared with the placebo group (31 women [41.9%]).

Table 5.7 - Participants previous pregnancy history

	rhG-CSF n (%)	Placebo n (%)	Total n (%)
Previous Pregnancies			
n	76	74	150
Mean (SD)	5.4 (2.51)	4.9 (1.83)	5.1 (2.21)
Median	5.0	4.0	4.0
Min, Max	3, 16	3, 10	3, 16
3 previous pregnancies – n(%)	20 (26.3)	17 (23.0)	37 (24.7)
4 previous pregnancies – n(%)	16 (21.1)	24 (32.4)	40 (26.7)
5 previous pregnancies – n(%)	15 (19.7)	14 (18.9)	29 (19.3)
>5 previous pregnancies – n(%)	25 (32.9)	19 (25.7)	44 (29.3)

Table 5.8 – Participants previous miscarriage history

	rhG-CSF	Placebo	Total
Previous spontaneous pregnancy losses (clinical and preclinical)			
n	76	74	150
Mean (SD)	4.5 (2.11)	4.1 (1.35)	4.3 (1.79)
Median	4.0	4.0	4.0
Min, max	3, 14	3, 9	3, 14
3 previous losses – n (%)	36 (47.4)	34 (45.9)	70 (46.7)
>3 previous losses – n (%)	40 (52.6)	40 (54.1)	80 (53.3)
4 previous losses – n (%)	16 (21.1)	20 (27.0)	36 (24.0)
5 previous losses – n (%)	7 (9.2)	10 (13.5)	17 (11.3)
>5 previous losses – n (%)	17 (22.4)	10 (13.5)	27 (18.0)

Table 5.9 – Participants previous pregnancy outcomes

	rhG-CSF	Placebo	Total
Prior pregnancy outcome - n (%)			
Spontaneous pregnancy loss (up to 20 weeks)	76 (100.0)	74 (100.0)	150 (100.0)
Preclinical losses	42 (55.3)	31 (41.9)	73 (48.7)
Clinical losses	76 (100.0)	74 (100.0)	150 (100.0)
Elective abortion	9 (11.8)	6 (8.1)	15 (10.0)
Still birth	0 (0)	2 (2.7)	2 (1.3)
Live birth	38 (50.0)	37 (50.0)	75 (50.0)
Other	3 (3.9)	1 (1.4)	4 (2.7)
Prior pregnancies gestational age at birth			
n	51	49	100
Mean (SD)	39.5 (1.76)	39.3 (1.97)	39.4 (1.86)
Median	40.0	40.0	40.0
Min, max	32, 42	34, 42	32, 42
Prior pregnancies gestational age at loss			
n	343	304	647
Mean (SD)	7.8 (2.69)	7.8 (3.53)	7.8 (3.11)
Median	7.0	7.0	7.0
Min, max	3, 22	4, 40	3, 40

Efficacy Results

Table 5.10 summarise the primary and secondary outcomes in the study population and by treatment group.

Primary Outcome

Primary outcome of the study was clinical pregnancy at 20 weeks and beyond. The clinical pregnancy rates at 20 weeks of gestation was 59.2% (45/76) in the rhG-CSF group, compared with 64.9% (48/74) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.2; p=0.48).

Secondary Outcomes

During the study, clinical pregnancies were confirmed by ultrasound scan at six weeks of gestation in 136 (90.7%) of the 150 randomised participants [67/76, 88.2% in the rhG-CSF group vs 69/74, 93.2% in the placebo group, RR of 0.9 (95% CI: 0.9 to 1.0; p=0.28)]. A further assessment of clinical pregnancies were conducted at 8 weeks which confirmed pregnancies in 110 randomised participants [51/76, 67.1% in the rhG-CSF group vs 59/74, 79.7%, RR of 0.8 (95% CI: 0.7to 1.0; p=0.09)]. Ongoing pregnancies were confirmed at approximately 12 weeks in 96 (64.0%) of the women [45/76, 59.2% in the rhG-CSF group vs 51/74, 68.9% in the placebo group, RR of 0.9 (95% CI: 0.7 to 1.1; p=0.224)].

The clinical pregnancy rates at 24 weeks of gestation was 59.2% (45/76) in the rhG-CSF group, compared with 64.9% (48/74) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.2; p=0.48). Six women in the placebo group had live births at less than 34 weeks of gestation confirming clinical pregnancy rates at 34 weeks of gestation at 59.2% (45/76) in the rhG-CSF group, compared with 64.9% (48/74) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.2; p=0.48). 5/45 (11.1%) women in the rhG-CSF group and 8/48 (16.7%) of women in the placebo group experienced preterm onset of labour (before 37 weeks and 0 days of gestation), giving a RR of 0.7(95% CI: 0.3 to 2.0; p=0.54). There were no stillbirths in the study.

One participant in the rhG-CSF group was diagnosed with an ectopic pregnancy. The number of spontaneous pregnancy losses up to 20 weeks was larger in subjects in the rhG-CSF group [28/76(36.8%) vs 25/74(33.8%) in the placebo group, giving a RR of 1.1 (95% CI: 0.7 to 1.7; p=0.70)]. Preclinical losses were the same in both treatment groups but there were more clinical losses in the rhG-CSF group compared with the placebo group [20/76(26.3%) vs 17/74(23.0%) in the placebo group]. Amongst the 28 pregnancies that ended in miscarriage for participants receiving rhG-CSF, the median gestation was 6

weeks (IQR 6 to 7 weeks). Amongst the 25 pregnancies that ended in miscarriage for participants receiving placebo, the median gestation was 6.5 weeks (IQR 6 to 9 weeks). The distributions of gestational age at live birth delivery for the rhG-CSF and placebo groups are given in Figure 5.2.

The median birth weight was 3420.0 gm in the rhG-CSF group vs 3300.0 gm in the placebo group. All infants, both in the rhG-CSF and the placebo group were discharged alive from the hospital (46 infants in the rhG-CSF group vs 49 infants in the placebo group). Neonatal congenital anomalies were observed in 1/46 (2.1%) of babies in the rhG-CSF group versus 1/49 (2.0%) in the placebo group (RR of 0.9; 95% CI: 0.1 to 13.4; p=0.93).

There was no incidence of antidrug antibody in the rhG-CSF group similar to findings in the phase 1 studies.

There were no pregnancy losses in the time period from primary outcome to live birth; therefore, the live birth rate was 59.2% (45/76) in the rhG-CSF group, and 64.9% (48/74) in the placebo group, giving a relative risk of 0.9 (95% CI: 0.7 to 1.2; p=0.48).

Table 5.10 – Primary and secondary outcomes in RESPONSE study

	rhG-CSF	Placebo	Relative Risk	P Value
	no./total no. (%)		(95%CI)	
Outcome				
Pregnancy outcomes				
Clinical pregnancy at 6 weeks	67/76(88.2)	69/74(93.2)	0.9 (0.9, 1.0)	0.28
Ongoing pregnancy at 8 weeks	51/76(67.1)	59/74(79.7)	0.8 (0.7, 1.0)	0.09
Ongoing pregnancy at 12 weeks	45/76(59.2)	51/74(68.9)	0.9 (0.7, 1.1)	0.22
Live birth after 20 weeks of gestation	45/76(59.2)	48/74(64.9)	0.9 (0.7, 1.2)	0.48
Live birth after 24 weeks of gestation	45/76(59.2)	48/74(64.9)	0.9 (0.7, 1.2)	0.48
Live birth after 34 weeks of gestation	45/76(59.2)	42/74 (56.8)	1.0 (0.8, 1.4)	0.76
Ectopic pregnancy	1/76(1.3)	0/74(0.0)	NA	NA
Miscarriage*	28/76(36.8)	25/74(33.8)	1.1 (0.7, 1.7)	0.70
Stillbirth	0/76(0.0)	0/76(0.0)	NA	NA
Preterm birth (before 37 weeks 0 days of gestatior	5/45(11.1)	8/48(16.7)	0.7 (0.3, 2.0)	0.54
Infant birth weight (g)				
Median	3420.0	3300.0		
Range	3005-3920	2690-3610		
Neonatal outcomes†				
Infants discharged alive from hospital	46/46(100.0)	49/49(100.0)		
Any congenital anomaly	1/46(2.2)	1/49(2.0)	0.9 (0.1, 13.4)	0.93
Adverse events§	n/N (%)	n/N(%)		
Maternal adverse events	52/76(68.4)	43/74(58.1)	1.2 (0.9, 1.5)	0.20
Serious adverse events	8/76(10.5)	6/74(8.1)	1.3 (0.5, 3.6)	0.62
Incidence of anti-drug antibody formation	0/76(0.0)	NA	NA	NA

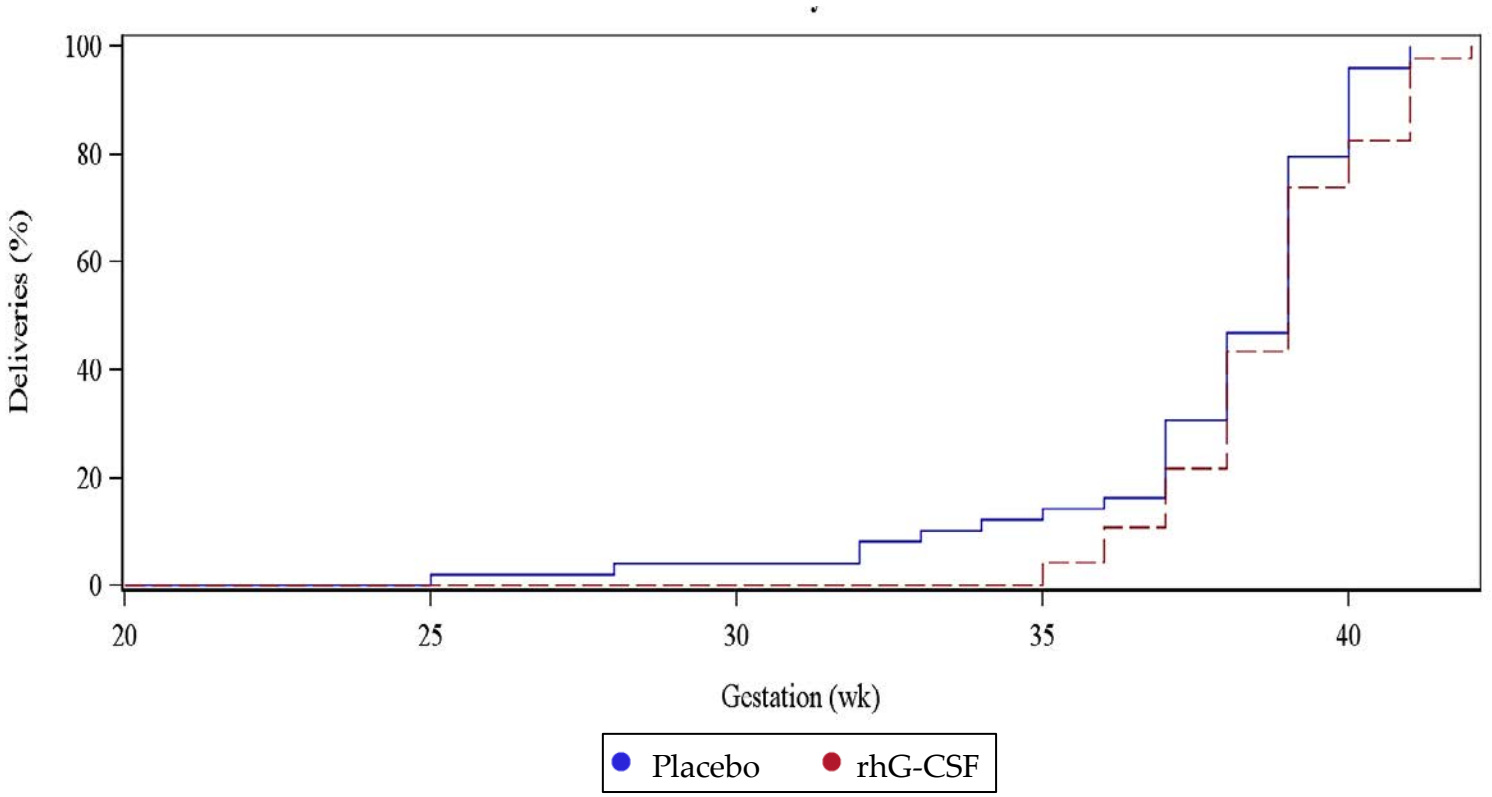


Figure 5.2 – Distribution of gestational age according to study group assignment.

Subgroup Analysis

Findings of subgroup analysis are given in table 5.11.

Maternal age at randomisation

The live birth rate for maternal age under 35 years was 58.1% (36/62) in the rhG-CSF group, compared with 66.7% (40/60) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.2; p=0.33). There was a smaller percentage of women under the age of 35 in the rhG-CSF group. The live birth rate for maternal age equal to or more than 35 years was 64.3% (9/14) in the rhG-CSF group, compared with 57.1% (8/14) in the placebo group, giving a RR of 1.1 (95% CI: 0.6 to 2.1; p=0.71). Subgroup analysis based on maternal age at randomisation did not confirm any significant effects between rhG-CSF and placebo arms.

Previous Miscarriages

The live birth rate for women who had 3 previous miscarriages were 63.9% (23/36) in the rhG-CSF group, compared with 67.6% (23/34) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.3; p=0.67). The live birth rate for women who had 4 previous miscarriages were 75% (12/16) in the rhG-CSF group, compared with 65% (13/20) in the placebo group, giving a RR of 1.3 (95% CI: 0.8 to 2.0; p=0.26). The live birth rate for women who had 5 previous miscarriages were 57.1% (4/7) in the rhG-CSF group, compared with 40% (4/10)

in the placebo group, giving a RR of 1.3 (95% CI: 0.5 to 3.6; p=0.58). The live birth rate for women who had more than 5 previous miscarriages were 35.3% (6/17) in the rhG-CSF group, compared with 80.0% (8/10) in the placebo group, giving a RR of 0.5 (95% CI: 0.2 to 1.0; p=0.06).

Subgroup analysis based on previous number of miscarriages did not confirm any significant effects between rhG-CSF and placebo arms.

Gestation at randomisation

The live birth rate for women who were randomised at less than or equal to 4 weeks of gestation was 58.3% (42/72) in the rhG-CSF group, compared with 64.70% (44/68) in the placebo group, giving a RR of 0.9 (95% CI: 0.7 to 1.2; p=0.43). Live birth rate for those randomised above 4 weeks of gestation until 5 weeks were 75.0%(3/4) in the rhG-CSF group, compared with 66.7% (4/6) in the placebo group, giving a RR of 1.1 (95% CI: 0.6 to 2.1; p=0.73).

Subgroup analysis based on gestation at randomisation did not confirm any significant effects between rhG-CSF and placebo arms.

Primary and Secondary Miscarriages

The live birth rate for women had secondary miscarriages were 63.2% (24/38) in the rhG-CSF group, compared with 62.2% (23/37) in the placebo group, giving a RR of 1.01 (95% CI:0.71to 1.44; p=0.92). Live birth rate for those with primary

miscarriages were 55.3% (21/38) in the rhG-CSF group, compared with 67.6% (25/37) in the placebo group, giving a RR of 0.81(95% CI: 0.57 to 1.17; p=0.27). Subgroup analysis based on primary and secondary miscarriages did not confirm any significant effects between rhG-CSF and placebo arms.

Previous late miscarriages

The live birth rate for women had a previous late miscarriage was 53.8% (7/13) in the rhG-CSF group, compared with 60.0% (6/10) in the placebo group, giving a RR of 0.91 (95% CI: 0.43 to 1.80; p=0.76). Live birth rate for those without late miscarriage was 60.3% (38/63) in the rhG-CSF group, compared with 65.6% (42/64) in the placebo group, giving a RR of 0.91(95% CI: 0.70 to 1.20; p=0.53). Subgroup analysis based on previous late miscarriages did not confirm any significant effects between rhG-CSF and placebo arms.

Table 5.11 – Subgroup analysis by maternal age at randomisation

Subgroup	rhG-CSF n/N(%)	Placebo n/N(%)	Relative risk (95% CI)	P value
Age*				
< 35 years	36/62(58.1)	40/60(66.7)	0.9 (0.7, 1.2)	0.33
≥ 35 years	9/14(64.3)	8/14(57.1)	1.1 (0.6, 2.1)	0.71
Previous miscarriages				
3	23/36(63.9)	23/34(67.6)	0.9 (0.7, 1.3)	0.67
4	12/16(75.0)	13/20(65.0)	1.3 (0.8, 2.0)	0.26
5	4/7(57.1)	4/10(40.0)	1.3 (0.5, 3.6)	0.58
>5	6/17(35.3)	8/10(80.0)	0.5 (0.2, 1.0)	0.06
Gestation at treatment start				
≤4 weeks	42/72(58.3)	44/68(64.70	0.9 (0.7, 1.2)	0.43
>4 weeks	3/4 (75.0)	4/6(66.7)	1.1 (0.6, 2.1)	0.73
Previous Live Birth				
Yes	24/38(63.2)	23/37(62.2)	1.01 (0.71, 1.44)	0.92
No	21/38(55.3)	25/37(67.6)	0.81 (0.57, 1.17)	0.27
Previous miscarriage after 12 weeks				
Yes	7/13(53.8)	6/10(60)	0.91 (0.43, 1.8)	0.76
No	38/63(60.3)	42/64(65.6)	0.91 (0.70, 1.20)	0.53

Adverse Events

Adverse events are listed in table 5.12.

The most common adverse event experienced was gastrointestinal disorder followed by nervous and musculoskeletal disorders. Gastrointestinal disorders were experienced by 43.4 %(33/76) in the rhG-CSF compared to 32.4 %(24/74) in the placebo group, giving a RR of 1.3(95% CI: 0.9 to 2.0; p=0.17). Nervous system disorders were experienced by 27.6 %(21/76) in the rhG-CSF compared to 18.9% (14/74) in the placebo group, giving a RR of 1.5(95% CI: 0.8 to 2.6;p=0.22). Musculoskeletal disorders were experienced by 26.3%(20/76) in the rhG-CSF compared to 8.1%(6/74) in the placebo group, giving a RR of 3.2(95% CI: 1.4 to 7.5;p=0.01).There were 10.5 %(8/76) serious adverse events in the rhG-CSF group vs 8.1% (6/74) in the placebo group, giving a RR of 1.3(95% CI: 0.5 to 3.6; p= 0.62). All SAEs in both treatment groups were considered to be likely unrelated. The occurrence of SAEs was broadly balanced in the 2 treatment groups, and no trend in their occurrence was obvious. The majority of SAEs reported during the study could be considered to be pregnancy related, and no SAE was considered to be related to study drug. The rhG-CSF group reported fewer AEs leading to discontinuation than the placebo group (2 women [2.6%] compared with 3 subjects [4.1%], respectively). Women in rhG-CSF group experienced more AEs related to study drug than the placebo treatment group. AEs that occurred during this study were generally mild or moderate in

severity and balanced in severity between the 2 treatment groups. The occurrence of AEs considered to be related to study therapy was also similar in the 2 treatment groups. No AEs of special interest were reported during this study. Among these, serious adverse events in the rhG-CSF group comprised of 2 occurrences of gastrointestinal disorders, a diagnosis of cholecystitis, a diagnosis of lower respiratory tract infection and 1 occurrence of severe headache, whereas serious adverse events in the placebo group comprised of a diagnosis of pneumonia and a diagnosis of endometritis.

Table 5.12 – Adverse events in RESPONSE study

Adverse event	rhG-CSF n(n/N)	Placebo n(n/N)	Relative Risk (95% CI)	P value
Number of participants	76	74		
Blood and lymphatic system disorders	4 (5.3)	1(1.4)	3.9 (0.4, 34.2)	0.22
Cardiac disorders	1(1.3)	NA	NA	
Gastrointestinal disorders	33(43.4)	24(32.4)	1.3 (0.9, 2.0)	0.17
General disorders	10(13.2)	13(17.6)	0.7 (0.4, 1.6)	0.44
Hepatobiliary disorders	1(1.3)	NA	NA	
Immune system disorders	2(2.6)	NA	NA	
Infections and infestations	16(21.1)	10 (13.5)	1.6 (0.8, 3.2)	0.23
Injury, poisoning and procedural complications	2(2.6)	3(4.1)	0.6 (0.1, 3.7)	0.63
Significantly deranged serum parameters	8(10.5)	5(6.8)	1.6 (0.5, 4.6)	0.42
Musculoskeletal and connective tissue disorders	20(26.3)	6(8.1)	3.2 (1.4, 7.5)	0.01
Nervous system disorders	21(27.6)	14(18.9)	1.5 (0.8, 2.6)	0.22
Pregnancy, puerperium and perinatal conditions	8(10.5)	4(5.4)	2.0 (0.6, 6.1)	0.25
Psychiatric disorders	1(1.3)	NA	NA	
Renal and urinary disorders	1(1.3)	1(1.4)	1.0 (0.1, 15.5)	0.99
Reproductive system and breast disorders	19(25.0)	1(14.9)	1.7 (0.9, 3.3)	0.13
Respiratory, thoracic and mediastinal disorders	4(5.3)	2(2.7)	1.9 (0.4, 10.3)	0.44
Skin and subcutaneous tissue disorders	8(10.5)	2(2.7)	3.9 (0.9, 17.8)	0.08
Vascular disorders	1(1.3)	1(1.4)	1.0 (0.1, 15.2)	0.98

Haematology Variables

In the rhG-CSF group, a general trend was noted in shifts from normal values to higher individual WBC values and higher neutrophil counts, which shifted back to normal at 4 weeks post treatment. An additional trend was noted in shifts from baseline values in lymphocytes to low values on treatment.

Figure 5.3 to figure 5.7 demonstrates the changes from baseline in haemoglobin, WBCs, neutrophils, and lymphocytes. While haemoglobin values over time were generally similar in both the active and placebo treatment groups, values in rhG-CSF groups were strikingly different from placebo across time points and served to demonstrate that the treatment was active in producing the intended biological effect.

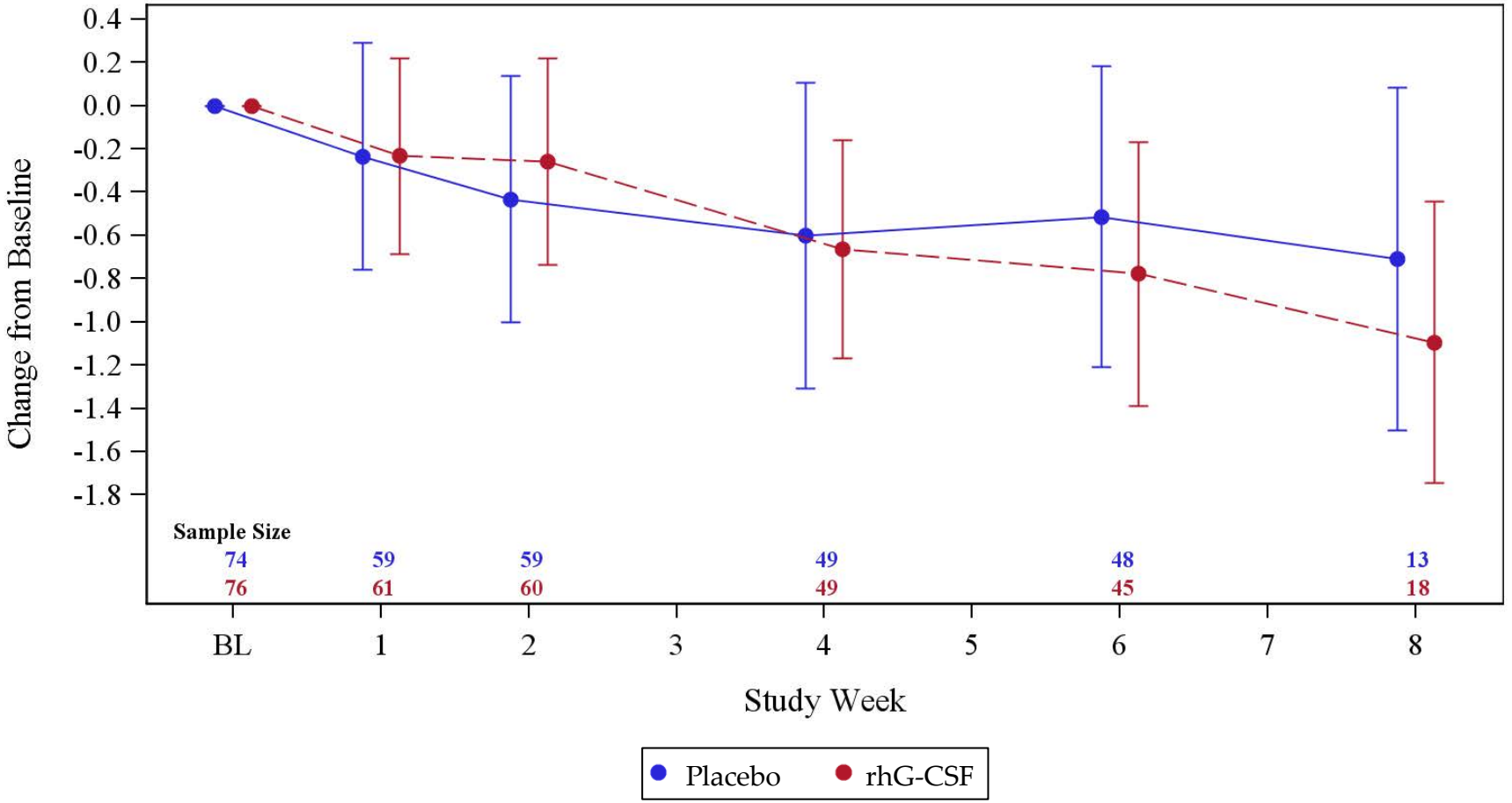


Figure 5.3 – Mean change from baseline (+/- SD) in Haemoglobin

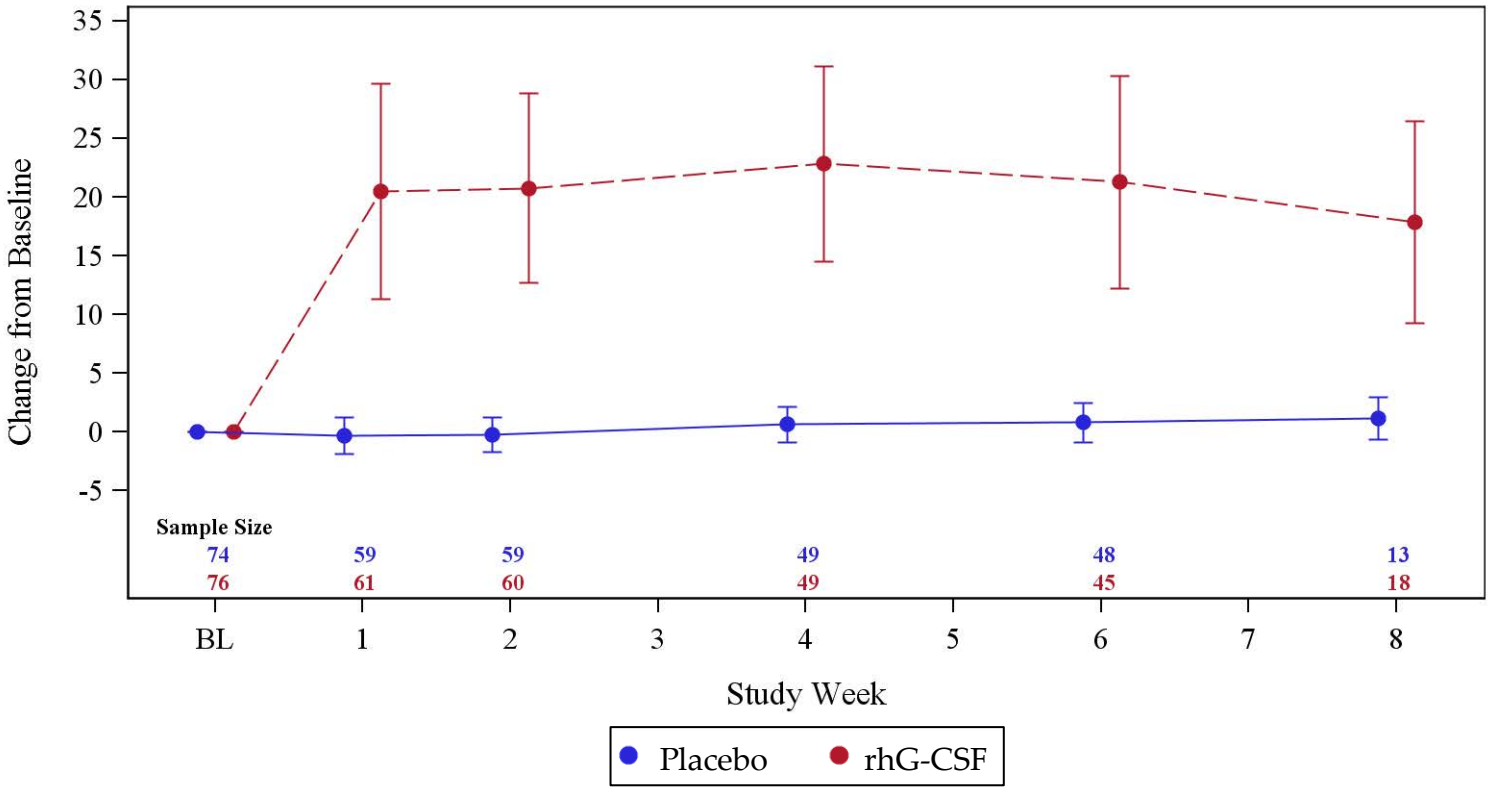


Figure 5.4 – Mean change from baseline (+/- SD) in White Blood Cells

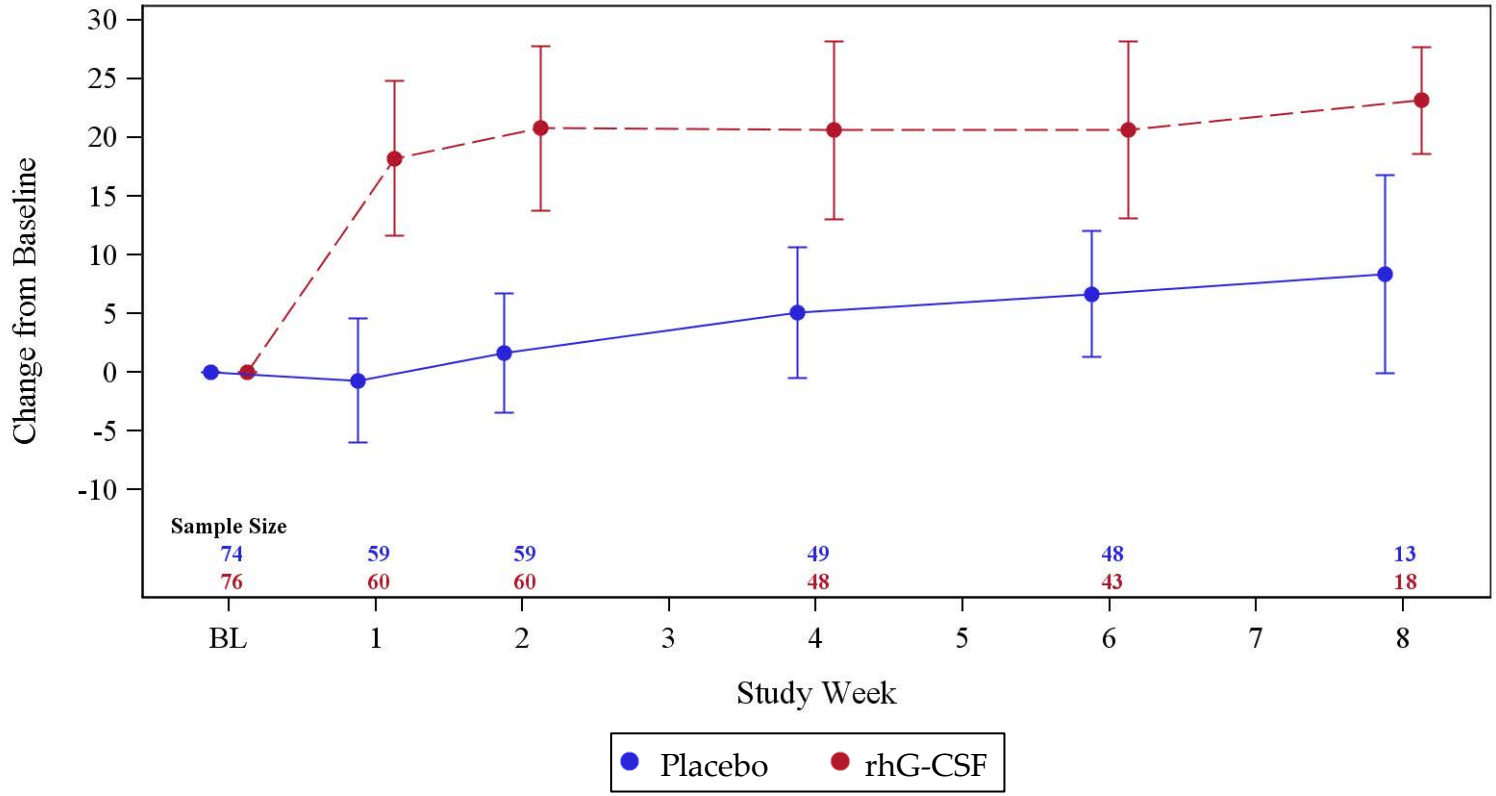


Figure 5.5 – Mean change from baseline (+/- SD) in Neutrophils.

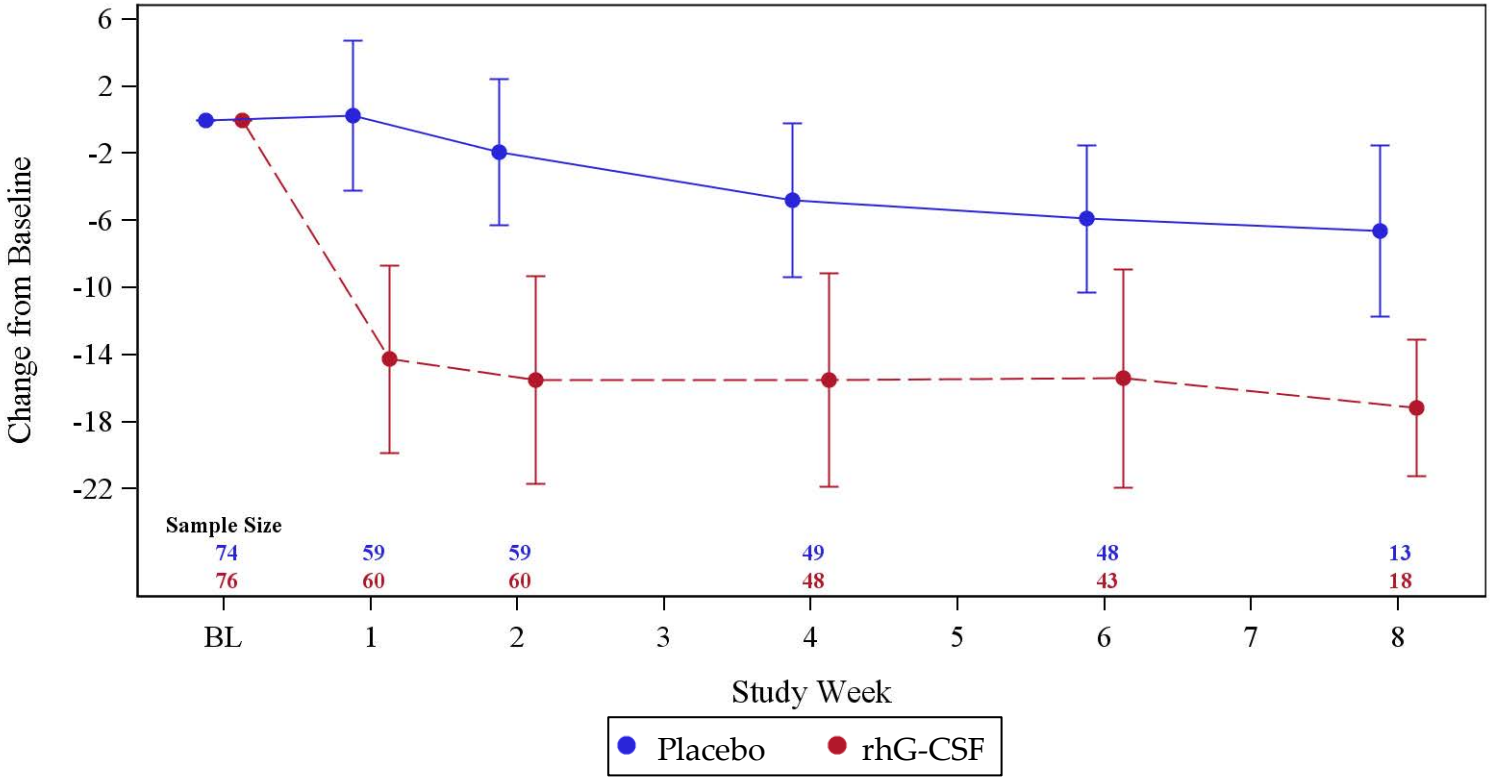


Figure 5.6 – Mean change from baseline (+/- SD) in Lymphocytes.

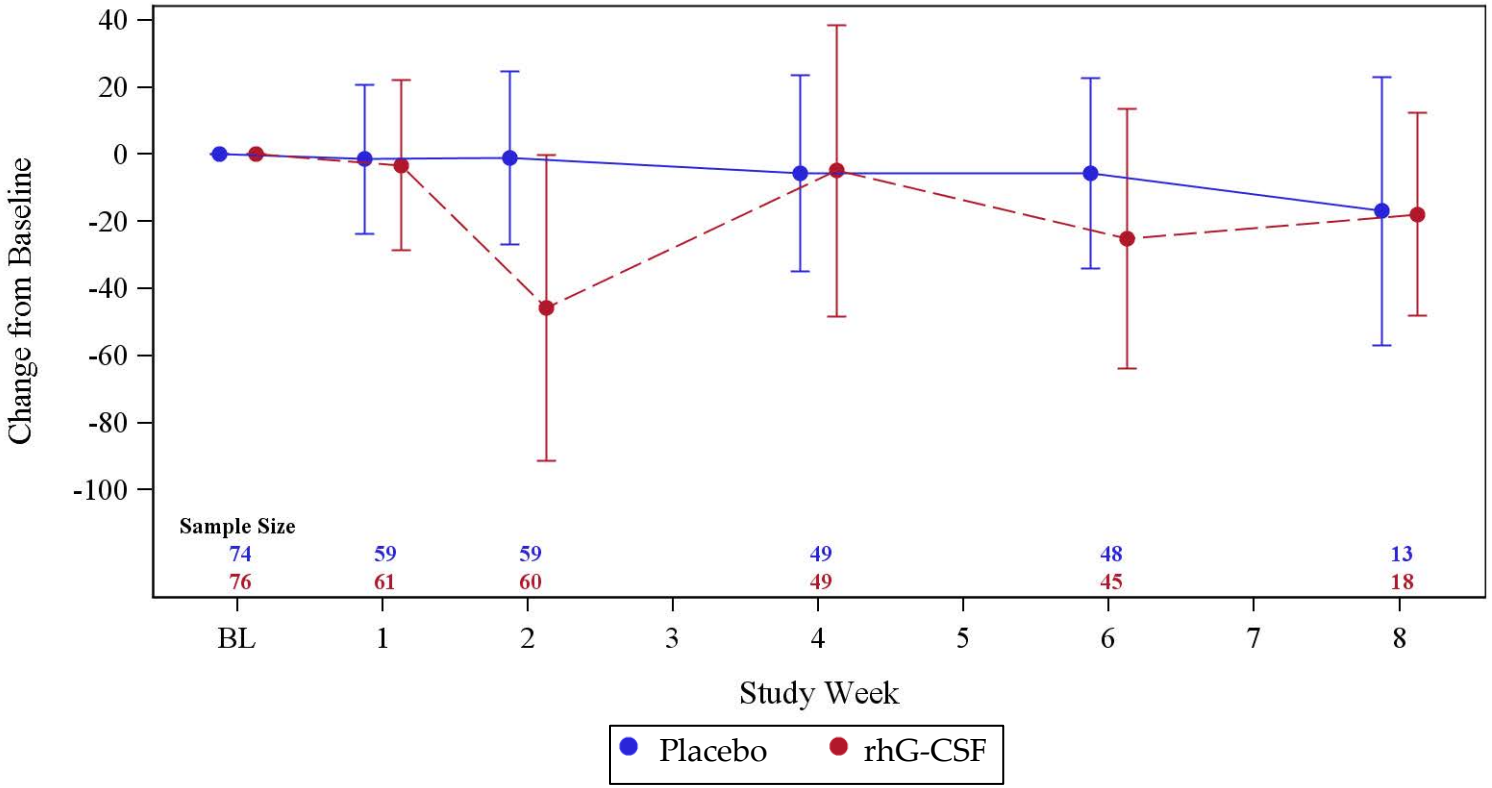


Figure 5.7 – Mean change from baseline (+/- SD) in Platelets.

Biochemistry Variables

The values in the biochemistry variables for the 2 treatment groups were generally balanced at baseline.

The treatment groups had similar alkaline phosphatase values at baseline, but following study treatment at the study Week-1 time point, the subjects in rhG-CSF group had consistently higher mean alkaline phosphatase values compared with the placebo group at all time points while on treatment. At 4 weeks after study treatment, values for ALP returned to being again similar in the 2 treatment groups.

Mean alanine aminotransferase values were generally similar at baseline; on-treatment values were modestly higher in rhG-CSF group at all on-treatment time points. Mean LDH was broadly comparable at baseline, but increased steeply at on-treatment time points in rhG-CSF group. By the 4 weeks post-treatment visit, values across the 2 treatment groups were nearly the same for LDH.

Mean values in gamma glutamyl-transferase were very slightly higher in the placebo treatment group at baseline but by on-treatment study Week 1 became higher in rhG-CSF group. The higher values in rhG-CSF group for GGT continued to be higher in the rhG-CSF group at all on-treatment time points,

and dropped at the 4 weeks post treatment time point to be slightly lower than gamma glutamyl-transferase values in the placebo group.

Figure 5.8 to 5.12 graphically shows changes from baseline across study time points in alkaline phosphatase, ALT, AST, LDH, and GGT.

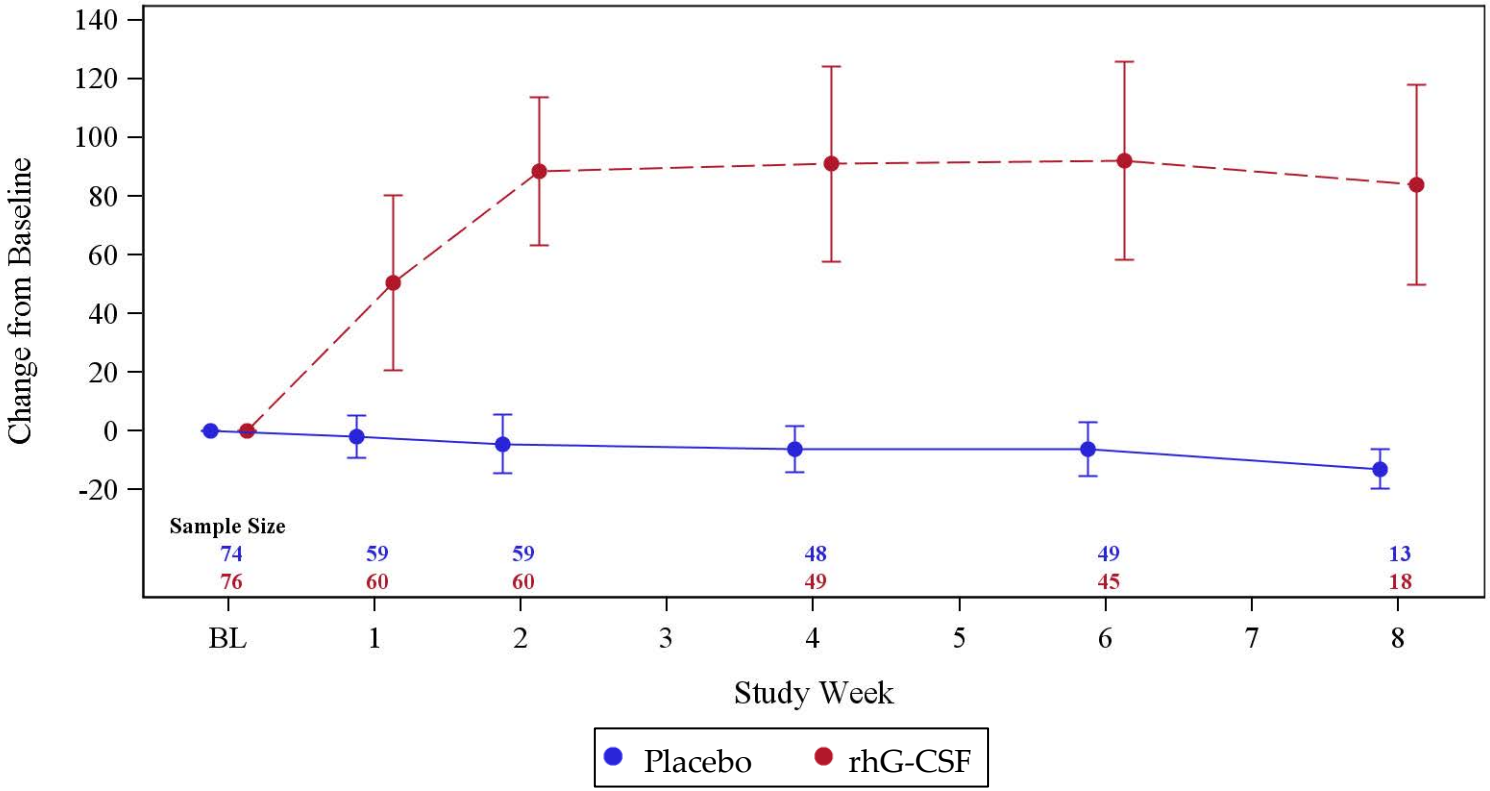


Figure 5.8 – Mean change from baseline (+/- SD) in Alkaline Phosphatase.

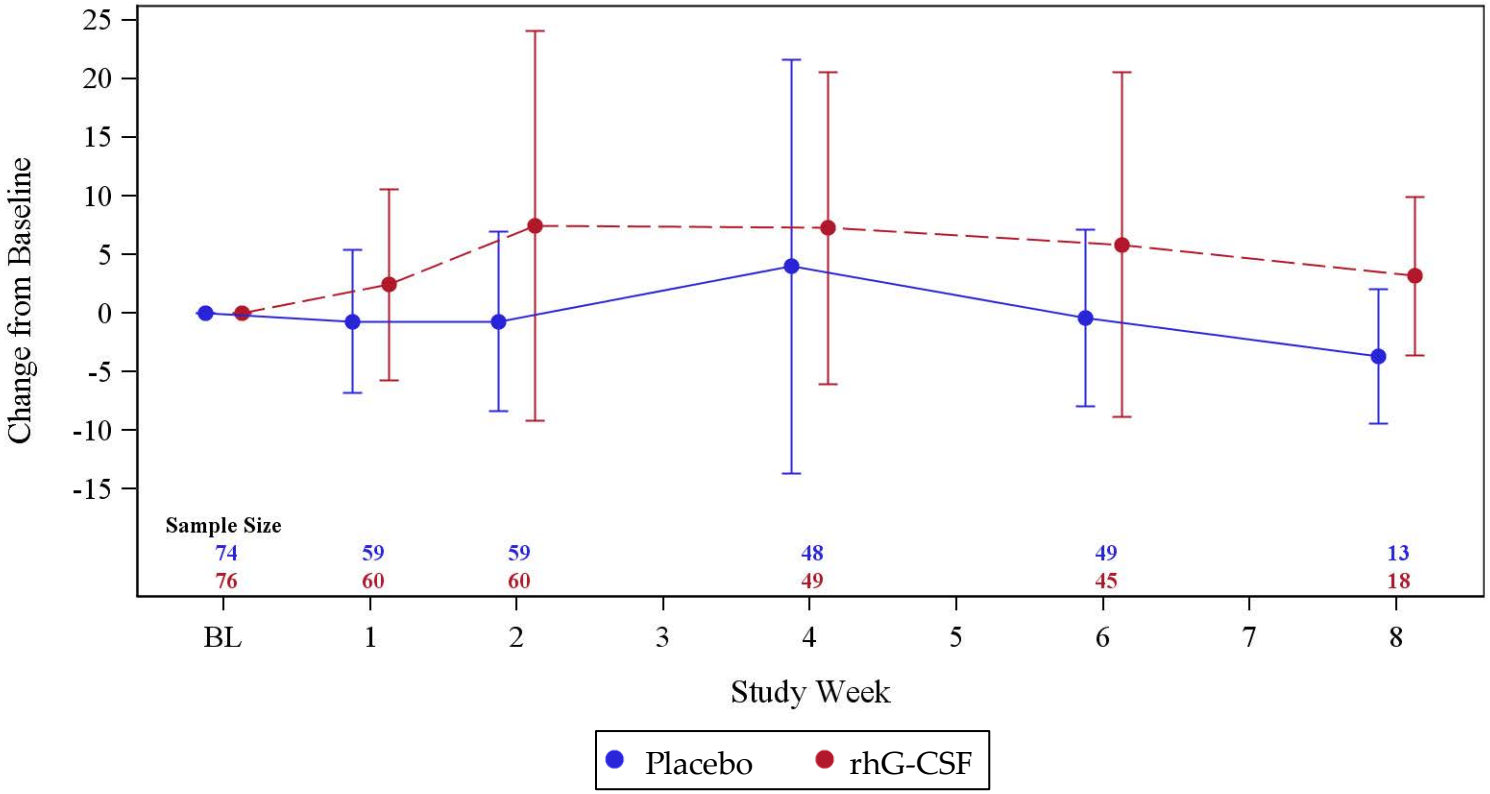


Figure 5.9 – Mean change from baseline (+/- SD) in Alanine Transaminase.

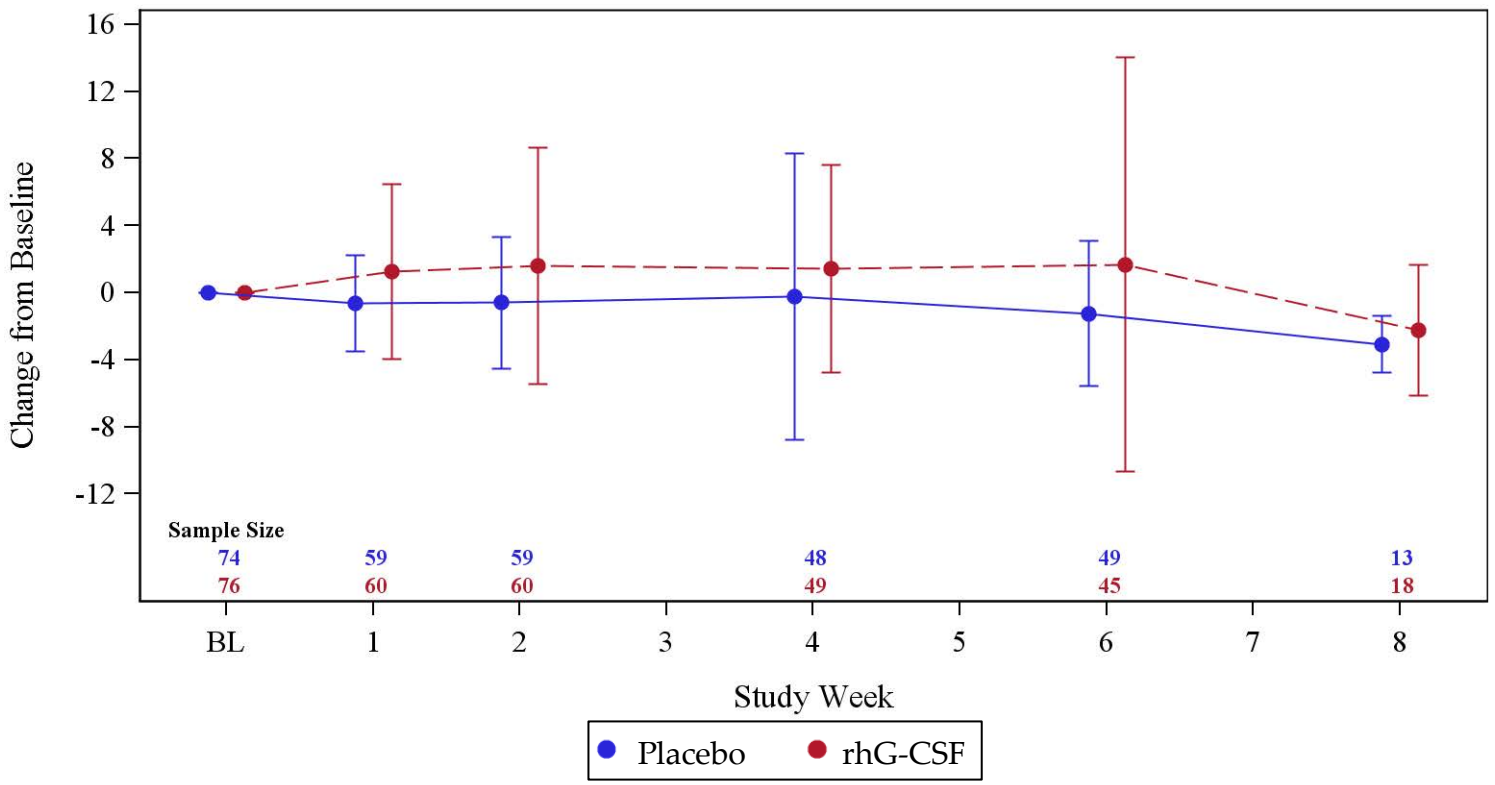


Figure 5.10 – Mean change from baseline (+/- SD) in Aspartate Amino Transferase.

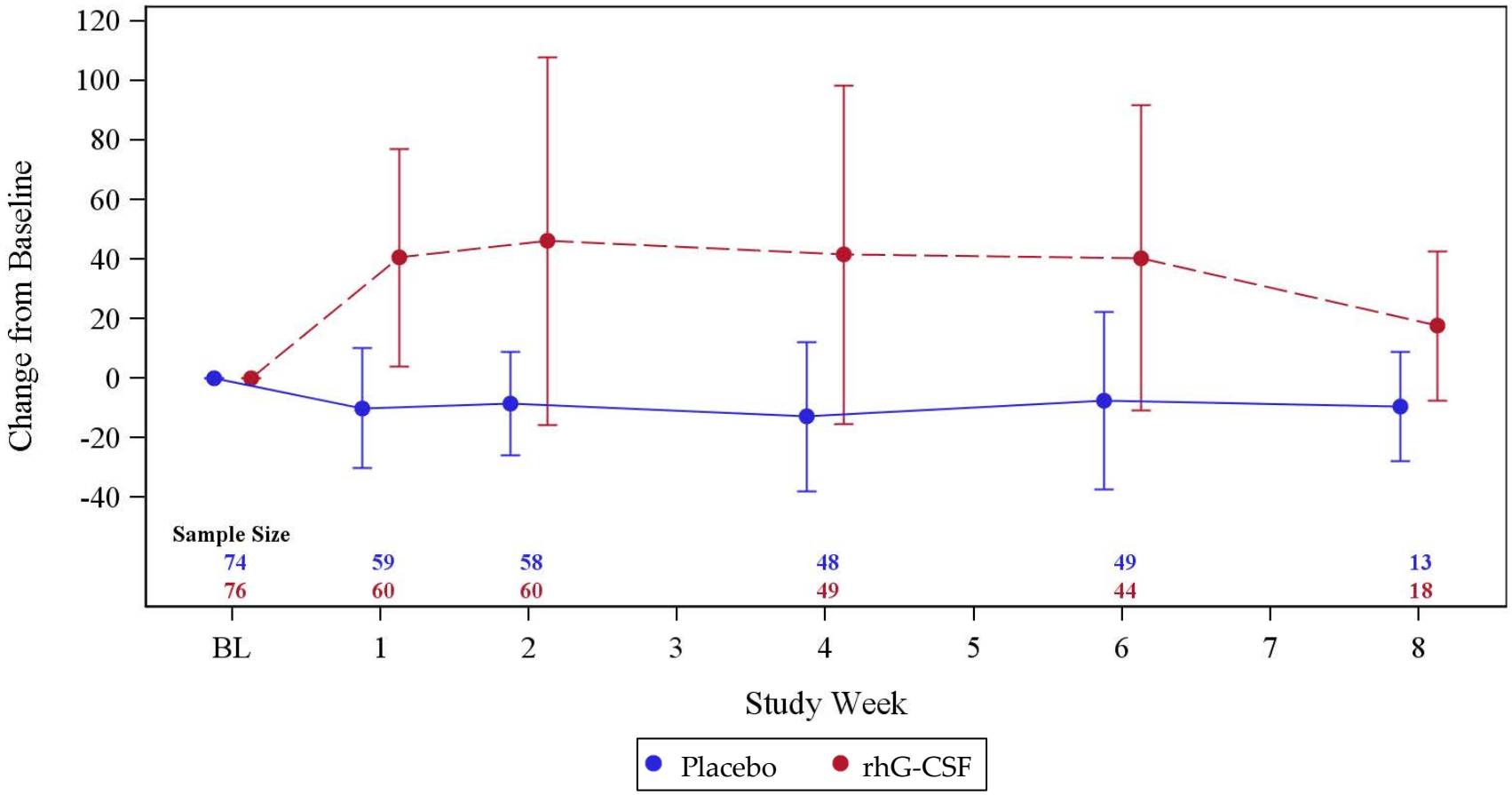


Figure 5.11 – Mean change from baseline (+/- SD) in Lactate Dehydrogenase.

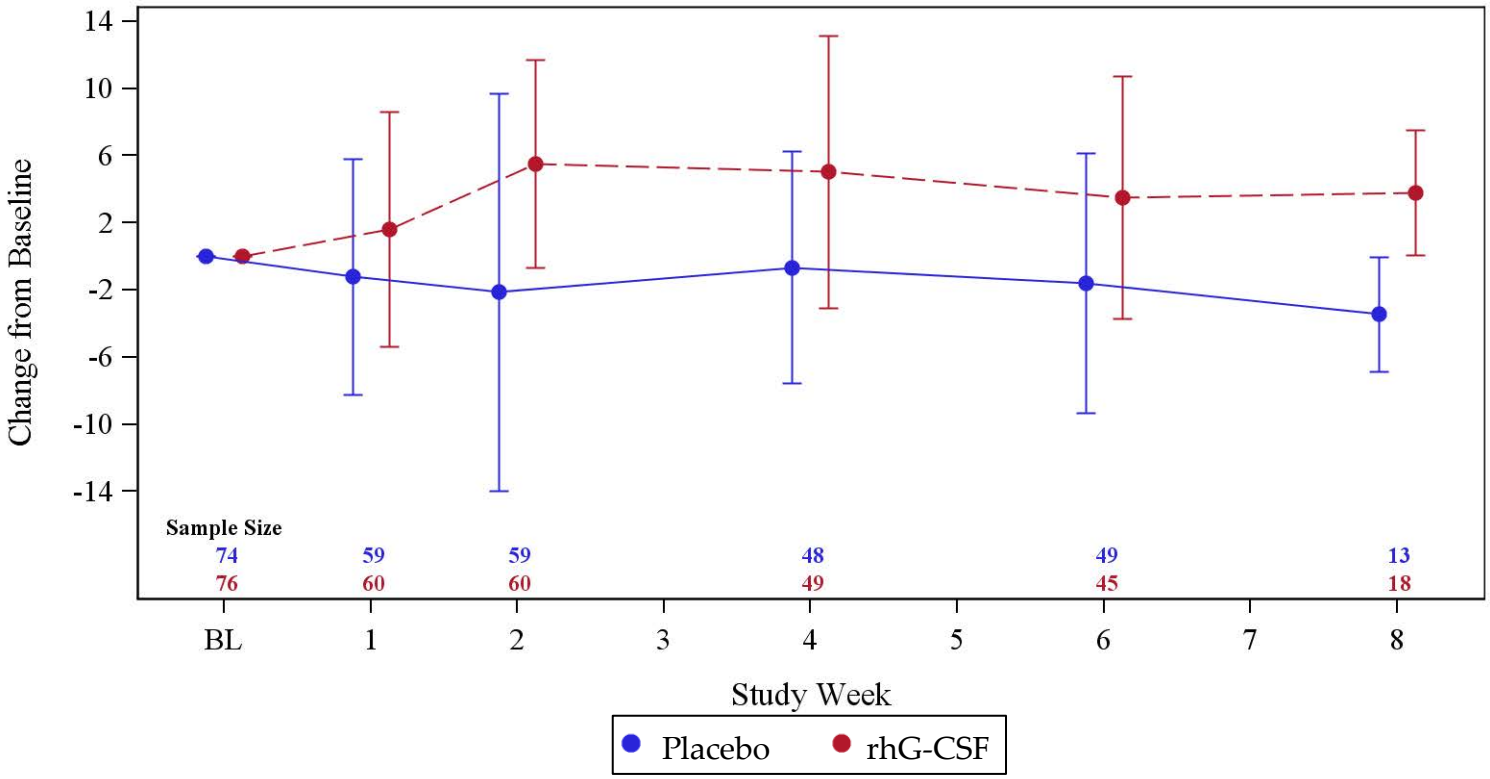


Figure 5.12 – Mean change from baseline (+/- SD) in Gamma –glutamyl Transferase.

Summary of Results

Efficacy Profile

Recombinant human granulocyte colony stimulating factor did not demonstrate efficacy in this study compared to placebo, in either the primary outcome measure of clinical pregnancy rate at Week 20 of gestation, or the secondary efficacy outcome measures. There were no statistically significant differences in clinical pregnancy rates and live birth rate between rhG-CSF and placebo. Three subjects in rhG-CSF group and 1 subject in the placebo group had an ectopic pregnancy. Interestingly, all women who maintained clinical pregnancy through Week 20 had outcomes of live birth (i.e, there were no late-term losses or stillbirths). All infants were discharged alive from the hospital.

Safety profile

Recombinant human granulocyte colony stimulating factor appeared to be generally safe and well tolerated in this study when administered as daily SC injections of 130 mcg for up to 9 weeks. The vast majority of reported TEAEs were of mild to moderate severity, and there were no statistically significant differences between the rhG-CSF and placebo groups in rates or types of AEs leading to discontinuation, AEs related to study drug, or SAEs. One Grade-4

event was reported in a rhG-CSF treated subject (ectopic pregnancy), but this was considered to be likely unrelated to study drug.

Laboratory changes, including elevations in WBC and neutrophil counts, were transient and consistent with the known effects of rhG-CSF treatment. There were no clinically significant differences in vital sign parameters between the rhG-CSF and placebo groups. No evidence of immunogenicity was observed in this study.

Headache was reported by 27.6% of s rhG-CSF subjects and by 13.5% of placebo subjects. Injection site pain was not reported by any in the rhG-CSF group but by 4.1% of placebo subjects. Vomiting was seen at a higher rate in rhG-CSF group (14.5%) compared to placebo subjects (6.8%). There were no statistically significant differences in neonatal outcomes between rhG-CSF and placebo groups. All women who had a clinical pregnancy at 20 weeks of gestation progressed to live birth, and all infants were discharged alive from the hospital. Birth weights and 1- and 5-minute Apgar scores were comparable. There were only two major congenital anomalies, one urethral cyst in rhG-CSF group and one heart defect in the placebo group respectively.

CHAPTER 6

DISCUSSION AND CONCLUSIONS OF A RANDOMISED, DOUBLE BLIND, MULTICENTRE, PLACEBO-CONTROLLED STUDY TO EVALUATE THE EFFICACY, SAFETY, AND TOLERABILITY OF RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR IN PREGNANT WOMEN WITH A HISTORY OF UNEXPLAINED RECURRENT MISCARRIAGES

Discussion

Immune mediated mechanisms are thought to contribute to recurrent pregnancy losses. Wide ranges of treatment options aimed at modifying maternal immune response are used and the latest addition to this group of medication is G-CSF.

This large multicentre, randomised, placebo-controlled trial investigated the efficacy and safety of recombinant human granulocyte colony stimulating factor in women with a history of unexplained recurrent miscarriages. The study showed that rhG-CSF therapy used throughout the first trimester of pregnancy

did not result in a significant increase in clinical pregnancies at 20 weeks or live births among women with a history of unexplained recurrent pregnancy losses.

Participant flow within the study and the baseline characteristics were similar between the study groups. There were no statistically significant differences in the primary and secondary outcomes in the study. Out of the 340 women screened, 150 women were randomised in a 1:1 ratio and all women in the study were followed up as appropriate. Subgroup analysis based on maternal age at randomisation also did not show any differences between the two treatment arms.

As anticipated, there were significant increases in both haematology and biochemistry parameters in the active treatment group. These changes returned to baseline values after discontinuation of the medications. The study did not show any significant increase in adverse events between the two groups.

This study does not support the findings of the only previous randomised control study evaluating G-CSF in recurrent pregnancy losses[73]. In this previous, smaller, single center study of 68 patients, Scarpellini et al., suggested

statistically significant improvement in live birth rates between the rhG-CSF group and in the placebo group (82.8% versus 48.5%).

A systematic narrative review of previous studies suggested significant clinical effect of G-CSF in improving outcomes for women having assisted reproductive techniques and also for those who had G-CSF as a treatment option for recurrent miscarriages. Out of the 17 studies, 12 investigated efficacy of G-CSF in assisted conception techniques and five investigated the efficacy of G-CSF in recurrent miscarriages.

12 studies within the ART population included three randomised controlled studies and nine observational studies. Three RCT conducted by Aleyasan et al., Davari-Tanha et al., and Eftekhar et al. confirmed benefits with G-CSF[168,170&171]. The observational studies also suggested benefit. One RCT[169] by Barad et al., and an observational study[167] by Eftekhar et al., did not confirm any significant improvement in outcomes. Most of the above studies used G-CSF regimen based on body weight dependant target dose. The active medication was administered as a single dose or multiple dose. The route of administration was subcutaneous or as an intrauterine infusion. An observational study by Zeyneloglu et al., used a dual administration technique[178].

Five studies investigating the effect of G-CSF in women with recurrent miscarriage included one RCT and 4 observational studies. RCT by Scarpellini et al[73] and 3 observational studies confirming benefit with G-CSF whereas the observational study based on population registry on SCNIR by Zeidler suggested no benefit with G-CSF[181].

However, all the above studies were of poor quality.

Zhao et al. published a systematic review and meta-analysis investigating the effects of G-CSF in ART suggested improvement in all outcomes in ART, if G-CSF was administered as a subcutaneous injection[198]. A small cohort study by Zeyneloglu et al., also suggested that dual administration, in form of subcutaneous and intrauterine infusion, of G-CSF significantly improved live birth rates[178].

The above studies both in the ART population and RM population had significant heterogeneity due to the variable patient population, indication for G-CSF, dose and routes of administration. Therefore, researchers called for larger well-designed studies.

RESPONSE study utilised the biological effect of G-CSF to rapidly increase the peripheral blood cell subsets, mainly the neutrophil population. This effect was hypothesised to result in changes consistent with supporting a state of maternal-fetal immune tolerance by temporary induction of toleragenic cell subsets and decreased percentages of pro-inflammatory and cytotoxic cell subsets.

Rise in WBC subsets was confirmed by the observed changes in haematology parameters confirming biological effect of rhG-CSF, as anticipated.

These biological effects, however, did not translate into any meaningful increase in clinical outcomes within this study. Therefore, it may be postulated that the clinical effects were overstated in previous studies.

This study on efficacy of G-CSF in unexplained RM also had some limitations. First of all, the tests performed as part of screening did not include any specific immune tests to identify immune dysfunction as the reason for pregnancy losses. This was mainly because of lack of a universally accepted test(s) for immune dysfunction in reproductive immunology. However, addition of

immune testing specific to implantation G-CSF deficiency syndrome may have provided more information on both immune physiology and treatment outcomes. Herrler et al and Makrigiannakis et al in their studies investigating maternal fetal interactions suggested that killer immunoglobulin like receptor along with uterine NK cells had a major role in implantation and development[199,200]. These are controlled by activation and inhibition genes. Lack of suppression of inhibitory genes as suggested by Varla-Leftherioti et al can derange the implantation process[201]. Hiby et al suggested the same outcome if there was lack of activator genes[202,203]. Benefits of G-CSF administration for women lacking these genes were demonstrated by Wurfel et al and Santjohanser et al[196,204] The concept of implantation specific G-CSF deficiency syndrome was introduced by these study groups.

Secondly, this study did not routinely perform analysis for fetal karyotyping for women who suffered miscarriages. Wurfel et al suggested majority of miscarriage events in G-CSF group in their study were due to fetal chromosomal abnormalities[130].

The strengths of this study include the multicentre study design involving 21 hospitals spread across the United Kingdom. After comprehensive

investigations 340 women with unexplained recurrent pregnancy losses were screened into this study. Thus, this study represents the largest placebo-controlled randomised control study for rhG-CSF in women with unexplained recurrent pregnancy losses. Of these 150 women were randomised. These were women from different ethnic backgrounds. Participant compliance rate was high and all participants were followed up until completion of study endpoints, as appropriate. We initiated optimum dose of rhG-CSF as soon as the pregnancy was confirmed, which started as early as 7 days after ovulation.

This study with strong methods provides further evidence for clinicians and researchers in reproductive medicine regarding efficacy and safety of rhG-CSF. They can translate this information to clinical practice to counsel women considering treatment for immune mediated miscarriages. This hopefully will prevent women from administering treatment options with no clinical effect which is expensive and which may add potential risk to the mother and/or the baby.

This study has opened up a few research questions for the future. We are now considering a retrospective analysis of prospectively collected date for biomarker bloods to assess immune mechanisms. There is also possibility of

developing an investigation panel for different up regulator and down regulator proteins or genes which control embryo implantation process.

Conclusions

In conclusion, this trial showed no significant increase in clinical pregnancy or live births with the use of rhG-CSF in the first trimester of pregnancy among women with recurrent miscarriages. There was no increased risk of congenital anomalies among offspring of women treated with rhG-CSF, although the study was not powered for such rare outcomes.

References

1. Silver RM, Branch DW, Goldenberg R, Iams JD, Klebanoff MA. Nomenclature for pregnancy outcomes: time for a change. Obstet Gynecol. 2011;118(6):1402-8.

2. RCOG. The Investigation and Treatment of Couples with Recurrent First-trimester and Second trimester Miscarriage. Green top Guideline 17. 2011.

3. Larsen EC CO, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. BMC Med. 2013;Jun 26(11):154.

4. ASRM . Evaluation and treatment of recurrent pregnancy loss: a committee opinion. 2012.

5. Jaslow CR, Kutteh WH. Effect of prior birth and miscarriage frequency on the prevalence of acquired and congenital uterine anomalies in women with recurrent miscarriage: a cross-sectional study. Fertil Steril. 2013;99(7):1916-22.

6. Kolte AM, Quenby S, Farquharson RG, Stephenson M, Goddijn M, Christiansen OB;. Non-visualized pregnancy losses are prognostically important for unexplained recurrent miscarriage. ESHRE Special Interest Group Early PregnancyHum Reprod 2014;29(5):931-7.

7. Kolte AM OL, Mikkelsen EM, Christiansen OB, Nielsen HS. Depression and emotional stress is highly prevalent among women with recurrent pregnancy loss. Hum Reprod 2015;Apr;30(4):777-82. doi: 10.1093/humrep/dev014.

8. Rowsell R LC, Donoghue J. Atypical antipsychotics in the treatment of schizophrenia. Validity of dropout rates as proxy measure of tolerability is unknown. BMJ. 2001; 14;322(7291):925.

9. Kagami M MT, Koizumi T, Miyazaki K, Nishikawa-Uchida S, Oda H, Uchida H, Fujisawa D, Ozawa N, Schmidt L, Yoshimura Y. Psychological adjustment and psychosocial stress among Japanese couples with a history of recurrent pregnancy loss. Hum Reprod. 2012;Mar;27(3):787-94. doi: 10.1093/humrep/der441.

10. Elzaan C van Niekerk IS, Theunis Frans Kruger. An evidence-based approach to recurrent pregnancy loss. SAJOG. 2013;Vol 19, No 3.

11. Brigham SA, Conlon C, Farquharson RG. A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. Hum Reprod. 1999;14(11):2868-71.

12. Lashen H FK, Sturdee DW. Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. Hum Reprod. 2004;Jul;19(7):1644-6.

13. Lo W, Rai R, Hameed A, Brailsford SR, Al-Ghamdi AA, Regan L. The effect of body mass index on the outcome of pregnancy in women with recurrent miscarriage. J Family Community Med. 2012;19(3):167-71.

14. Hemminki E , Forssas. Epidemiology of miscarriage and its relation to other reproductive events in Finland. Am J Obstet Gynecol. 1999 Aug;181(2):396-401.

15. Catak B, Oner C, Sutlu S, Kilinc S. Effect of socio-cultural factors on spontaneous abortion in Burdur, Turkey: A population based case-control study.Pak J Med Sci. 2016 Sep-Oct;32(5):1257-1262.

16. Norsker FN, Espenhain L, A Rogvi S, Morgen CS, Andersen PK, Nybo Andersen AM. Socioeconomic position and the risk of spontaneous abortion: a study within the Danish National Birth Cohort. BMJ Open. 2012 Jun 25;2(3). pii: e001077. doi: 10.1136/bmjopen-2012-001077.

17. Mary D. Stephenson, Sony Sierra. Karyotype - Reproductive outcomes in recurrent pregnancy loss associated with a parental carrier of a structural chromosome rearrangement. Human Reproduction, Volume 21, Issue 4, 1 April 2006, Pages 1076–1082,https://doi.org/10.1093/humrep/dei417 Published:05 January 2006

18. Tullio Ghi1, Francesca De Musso1, Elisa Maroni, Aly Youssef, Luca Savelli, Antonio Farina, Paolo Casadio, Marco Filicori, Gianluigi Pilu, and Nicola Rizzo1. The pregnancy outcome in women with incidental diagnosis of septate uterus at first trimester scan. Human Reproduction,

Vol.27, No.9 pp. 2671–2675, 2012 Advanced publication on June 29, 2012 doi:10.1093/humrep/215

19. Sotirios H. Saravelos, Junhao Yan, Hassan Rehmani, Tin-Chiu Li. The prevalence and impact of fibroids and their treatment on the outcome of pregnancy in women with recurrent miscarriage. Human Reproduction, 2011

20. Beth W. Rackow, Endometrial polyps affect uterine receptivity. Fertil Steril. 2011 June 30; 95(8): 2690–2692. doi:10.1016/j.fertnstert.2010.12.034

21. I-Ferne TanAustralas. The role of imaging in the investigation of Asherman's syndrome. J Ultrasound Med. 2011 Aug; 14(3): 15–18. Published online 2015 Dec 31. doi: 10.1002/j.2205-0140.2011.tb00118.xPMCID: PMC5024900.

22. H Harb, Coomarasamy. The effect of presence and management of hydrosalpinx on miscarriage in IVF. Fertil & Stertil. September 2014,Volume 102, Issue 3, Supplement, Page 298

23. T.C.Li, M.Makris , M.Tomsu , E.Tuckerman and S.Laird. Recurrent miscarriage: aetiology, management and prognosis. Human Reproduction Update, Vol.8, No.5 pp. 463±481, 2002

24. Sevi Giakoumelou, Nick Wheelhouse, Kate Cuschieri, Gary Entrican, Sarah E.M. Howie, and Andrew W. Horne. The role of infection in miscarriage. Human Reproduction Update, Vol.22, No.1 pp. 116–133, 2016 Advanced Access publication on September 19, 2015 doi:10.1093/humupd/dmv041.

25. Song Y, Wang HY, Qiao J, Liu P, Chi HB.Antiphospholipid Antibody Titers and Clinical Outcomes in Patients with Recurrent Miscarriage and Antiphospholipid Antibody Syndrome: A Prospective Study. Chin Med J (Engl). 2017 Feb 5;130(3):267-272. doi: 10.4103/0366-6999.198934. PMID: 28139508

26. Karadağ C, Yoldemir T, Karadağ SD, İnan C, Dolgun ZN, Aslanova L.Obstetric outcomes of recurrent pregnancy loss patients diagnosed wıth inherited thrombophilia. Ir J Med Sci. 2017 Feb 7. doi: 10.1007/s11845-017-1569-0.

27. Fishel S, Patel R, Lytollis A, Robinson J, Smedley M, Smith P, Cameron C, Thornton S, Dowell K, Atkinson G, Shaker A, Lowe P, Kazem R, Brett S, Fox A. Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype. Reprod Biomed Online. 2014 Jul;29(1):80-7. doi: 10.1016/j.rbmo.2014.03.019.

28. Persson G, Melsted WN, Nilsson LL, Hviid TVF.HLA class Ib in pregnancy and pregnancy-related disorders. Immunogenetics. 2017 Aug;69(8-9):581-595. doi: 10.1007/s00251-017-0988-4. Epub 2017 Jul 11.

29. Colucci F. The role of KIR and HLA interactions in pregnancy complications.Immunogenetics. 2017 Aug;69(8-9):557-565. doi: 10.1007/s00251-017-1003-9. Epub 2017 Jul 10. Review.

30. Morin SJ, Treff NR, Tao X, Scott RT 3rd, Franasiak JM, Juneau CR, Maguire M, Scott RT Combination of uterine natural killer cell immunoglobulin receptor haplotype and trophoblastic HLA-C ligand influences the risk of pregnancy loss: a retrospective cohort analysis of direct embryo genotyping data from euploid transfers. Fertil Steril. 2017 Mar;107(3):677-683.e2. doi: 10.1016/j.fertnstert.2016.12.004. Epub 2017 Jan 6. PMID: 28069185

31. Caseiro AL, Regalo A, Pereira E, Esteves T, Fernandes F, Carvalho J. Implication of sperm chromosomal abnormalities in recurrent abortion and multiple implantation failure. Reprod Biomed Online. 2015 Oct;31(4):481-5. doi: 10.1016/j.rbmo.2015.07.001. Epub 2015 Jul 8. Review.

32. Zidi-Jrah I, Hajlaoui A, Mougou-Zerelli S, Kammoun M, Meniaoui I, Sallem A, Brahem S, Fekih M, Bibi M, Saad A, Ibala-Romdhane S. Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss. Fertil Steril. 2016 Jan;105(1):58-64. doi: 10.1016/j.fertnstert.2015.09.041. Epub 2015 Oct 19. PMID: 26493117

33. Garrido-Gimenez C, Alijotas-Reig J. Recurrent miscarriage: causes, evaluation and management. Postgrad Med J 2015;91:151–162.

34. Tharapel AT, Tharapel SA, Bannerman RM. Recurrent pregnancy losses and parental chromosome abnormalities: a review. Br J Obstet Gynaecol. 1985 Sep;92(9):899-914.

35. Abuelo DN B-BG. Prognosis for couples who have experienced repeated pregnancy loss. Fertil Steril Dec;40(6):844-5. 1983.

36. Zhang S, Gao L, Liu Y, Tan J, Wang Y, Zhang R, Liu Y, Chen H, Zhang J.Reproductive outcome and fetal karyotype of couples with recurrent miscarriages. Clin Exp Obstet Gynecol. 2014;41(3):249-53.

37. Fred Kavalier, Investigation of recurrent miscarriages - A successful pregnancy is the most likely outcome. BMJ. 2005 Jul 16; 331(7509): 121–122. doi: 10.1136/bmj.331.7509.121 PMCID: PMC558686

38. Pedro Acie´n and Maribel I. Acie´n. The history of female genital tract malformation classifications and proposal of an updated system† Human Reproduction Update, Vol.17, No.5 pp. 693–705, 2011 doi:10.1093/humupd/dmr021

39. Graupera, B., Pascual, M. A., Hereter, L., Browne, J. L., Úbeda, B., Rodríguez, I. and Pedrero, C. (2015), Accuracy of three-dimensional ultrasound compared with magnetic resonance imaging in diagnosis of Müllerian duct anomalies using ESHRE–ESGE consensus on the classification of congenital anomalies of the female genital tract. Ultrasound Obstet Gynecol, 46: 616–622. doi:10.1002/uog.14825

40. Gorwitz RJ, Wiesenfeld HC, Chen PL, Hammond KR, Sereday KA, Haggerty CL, Johnson RE, Papp JR, Kissin DM, Henning TC, Hook EW , Steinkampf MP, Markowitz LE, Geisler WM. Population-attributable fraction of tubal factor infertility associated with chlamydia. Am J Obstet Gynecol. 2017 Sep;217(3):336.e1-336.e16. doi: 10.1016/j.ajog.2017.05.026. Epub 2017 .

41. Hyun Jong Park, You Shin Kim, Tae Ki Yoon, Woo Sik Lee. Chronic endometritis and infertility. Clin Exp Reprod Med. 2016 Dec; 43(4): 185–192. Published online 2016 Dec 26. doi: 10.5653/cerm.2016.43.4.185 PMCID: PMC5234283

42. Mcqueen DB, Perfetto CO, Hazard FK, Lathi RB. Pregnancy outcomes in women with chronic endometritis and recurrent pregnancy loss. Fertil Steril. 2015;104(4):927-31.

43. Cicinelli E MM, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, De Ziegler D, Resta L. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. Reprod Sci 2013;May;21(5):640-7. doi: 10.1177/1933719113508817.

44. Kaur R, Gupta K Endocrine dysfunction and recurrent spontaneous abortion: An overview. . Int J Appl Basic Med Res. 2016 Apr-Jun;6(2):79-83. doi: 10.4103/2229-516X.179024. PMID: 27127734

45. Thangaratinam S TA, Knox E, Kilby MD, Franklyn J, Coomarasamy A. Association between thyroid autoantibodies and miscarriage and preterm birth: meta-analysis of evidence. BMJ. 2011;May 9;342:d2616. doi: 10.1136/bmj.d2616.

46. Jivraj S RR, Underwood J, Regan L. Genetic thrombophilic mutations among couples with recurrent miscarriage. Hum Reprod. 2006;May;21(5):1161-5. Epub 2006 Jan 23.

47. Rai R, Sacks G, Trew G. Natural killer cells and reproductive failure--theory, practice and prejudice. Hum Reprod. 2005;20(5):1123-6.

48. Nielsen HS AA, Kolte AM, Christiansen OB. A firstborn boy is suggestive of a strong prognostic factor in secondary recurrent miscarriage: a confirmatory study. Fertil Steril. 2008;Apr;89(4):907-11. doi: 10.1016/j.fertnstert.2007.04.029. Epub 2008 Jan 28.

49. Ribas-Maynou J, Garcia-Peiro A, Fernandez-Encinas A, Amengual MJ, Prada E, Cortes P, et al. Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. PLoS One. 2012;7(9):e44679.

50. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17.

51. Zhao J ZQ, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril. 2014;Oct;102(4):998-1005.e8. doi:10.1016/j.fertnstert.2014.06.033. Epub 2014 Sep 1.

52. Marquard K, Westphal LM, Milki AA, Lathi RB. Etiology of recurrent pregnancy loss in women over the age of 35 years. Fertil Steril. 2010;94(4):1473-7.

53. Mathur N, Triplett L, Stephenson MD. Miscarriage chromosome testing: utility of comparative genomic hybridization with reflex microsatellite analysis in preserved miscarriage tissue. Fertil Steril. 2014;101(5):1349-52.

54. Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril. 2000;73(2):300-4.

55. Pritts EA, Parker WH, Olive DL. Fibroids and infertility: an updated systematic review of the evidence. Fertil Steril. 2009;91(4):1215-23.

56. Kowalik CR, Goddijn M, Emanuel MH, Bongers MY, Spinder T, de Kruif JH, et al. Metroplasty versus expectant management for women with recurrent miscarriage and a septate uterus. Cochrane Database Syst Rev. 2011(6):CD008576.

57. Rai RS,Regan L,Clifford K, Pickering W, Dave M, Mackie I, et al.Antiphospholipid antibodies and beta-2-glycoprotein-I in 500 women with recurrent miscarriage:results of a comprehensive screening approach.Hum Reprod 1995;10:2001–5.

58. Triolo G, Ferrante A,Ciccia F,Accardo-Palumbo A, Perino A, Castelli A, et al.Randomized study of subcutaneous low molecular weight heparin plus aspirin versus intravenous immunoglobulin in the treatment of recurrent fetal loss associated with antiphospholipid antibodies.Arthritis Rheum 2003;48:728–31.

59. Rai RS,Clifford K,Cohen H,Regan L. High prospective fetal loss rate in untreated pregnancies of women with recurrent miscarriage and antiphospholipid antibodies.Hum Reprod 1995;10:3301–4.

60. Elisabeth Pasquier, Luc de Saint Martin, Caroline Bohec, Céline Chauleur, Florence Bretelle, Gisèle Marhic,Grégoire Le Gal, Véronique Debarge, Frédéric Lecomte, Christine Denoual-Ziad, Véronique Lejeune-Saada,Serge Douvier, Michel Heisert, and Dominique Mottier. Enoxaparin for prevention of unexplained recurrent miscarriage: a multicenter randomized double-blind placebo-controlled trial. Blood. 2015 Apr 2; 125(14): 2200–2205.Prepublished online 2015 Jan 30. doi: 10.1182/blood-2014-11-610857PMCID: PMC4432556

61. Laura Ormesher, Louise Simcox, Clare Tower and Ian A Greer. Management of inherited thrombophilia in pregnancy. Womens Health (Lond). 2016 Jul; 12(4): 433–441.Published online 2016 doi: 10.1177/1745505716653702PMCID: PMC5373275

62. Paulien G de Jong et al. ALIFE2 study: low-molecular-weight heparin for women with recurrent miscarriage and inherited thrombophilia - study protocol for a randomized controlled trial. Trials. 2015; 16: 208.Published online 2015 May 7. doi: 10.1186/s13063-015-0719-9PMCID: PMC4453290

63. Skeith L, Carrier M, Kaaja R, Martinelli I, Petroff D, Schleussner E, et al. A meta-analysis of low-molecular-weight heparin to prevent pregnancy loss in women with inherited thrombophilia. Blood. 2016;127(13):1650-5.

64. Mak A CM, Cheak AA, Ho RC. Combination of heparin and aspirin is superior to aspirin alone in enhancing live births in patients with recurrent pregnancy loss and positive anti-phospholipid antibodies: a meta-analysis of randomized controlled trials and meta-regression.Rheumatology (Oxford). 2010 Feb;49(2):281-8. doi: 10.1093/rheumatology/kep373. Epub 2009 Dec 4. .

65. Empson M LM, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant.. Cochrane Database Syst Rev. 2005;Apr 18;(2):CD002859. Review.

66. Kumar A, Begum N, Prasad S, Aggarwal S, Sharma S. Oral dydrogesterone treatment during early pregnancy to prevent recurrent pregnancy loss and its role in modulation of cytokine production: a double-blind, randomized, parallel, placebo-controlled trial. Fertil Steril. 2014;102(5):1357-63 e3.

67. Coomarasamy A, Williams H, Truchanowicz E, Seed PT, Small R, Quenby S, et al. A Randomized Trial of Progesterone in Women with Recurrent Miscarriages. N Engl J Med. 2015;373(22):2141-8.

68. Wong KS, Connan K, Rowlands S, Kornman LH, Savoia HF. Antenatal immunoglobulin for fetal red blood cell alloimmunization. Cochrane Database Syst Rev. 2013(5):CD008267.

69. Egerup P LJ, Gluud C, Christiansen OB. The effects of immunotherapy with intravenous immunoglobulins versus no intervention, placebo, or usual care in patients with recurrent miscarriages: a protocol for a systematic review with meta-analyses, trial sequential analyses, and individual patient data meta-analyses of randomised clinical trials. Syst Rev. 2014;Aug 15;3:89. doi: 10.1186/2046-4053-3-89.

70. Laskin CA, Bombardier C, Hannah ME, Mandel FP, Ritchie JW, Farewell V, et al. Prednisone and aspirin in women with autoantibodies and unexplained recurrent fetal loss. N Engl J Med. 1997;337(3):148-53.

71. Tang AW, Alfirevic Z, Turner MA, Drury JA, Small R, Quenby S. A feasibility trial of screening women with idiopathic recurrent miscarriage for high uterine natural killer cell density and randomizing to prednisolone or placebo when pregnant. Hum Reprod. 2013;28(7):1743-52.

72. Corinna Weber-Schoendorfer. Pregnancy outcome after TNF-α inhibitor therapy during the first trimester: a prospective multicentre cohort study. Br J Clin Pharmacol. 2015 Oct; 80(4): 727–739. Published online 2015 May 28. doi: 10.1111/bcp.12642PMCID: PMC4594709

73. Scarpellini F SM. Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: a randomised controlled trial. Hum Reprod 2009;Nov;24(11):2703-8. doi: 10.1093/humrep/dep240. Epub 2009 Jul 17.

74. Ogilvie CM, Braude P, Scriven PN. Successful pregnancy outcomes after preimplantation genetic diagnosis (PGD) for carriers of chromosome translocations.Hum Fertil (Camb) 2001;4:168–71.

75. Munné S,Chen S, Fischer J,Colls P,Zheng X, Stevens J, et al. Preimplantation genetic diagnosis reduces pregnancy loss in women

aged 35 years and older with a history of recurrent miscarriages. Fertil Steril 2005;84:331–5.

76. Raoul Orvieto and Norbert Gleicher. J Assist Reprod Genet. 2016 Nov; 33(11): 1445–1448.Published online 2016 Sep 15. doi: 10.1007/s10815-016-0801-6PMCID: PMC5125154 Should preimplantation genetic screening (PGS) be implemented to routine IVF practice?

77. Stray-Pedersen B, Stray-Pedersen S. Etiologic factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. Am J Obstet Gynecol. 1984;148(2):140-6.

78. Clifford K, Rai R, Regan L. Future pregnancy outcome in unexplained recurrent first trimester miscarriage. Hum Reprod. 1997;12(2):387-9.

79. Diagnosis and management of first trimester miscarriage BMJ 2013;346:f3676 doi: 10.1136/bmj.f3676 (Published 19 June 2013)

80. Merel M.J. van den Berg a. Genetics of early miscarriage. Biochimica et Biophysica Acta 1822 (2012) 1951–1959

81. Miscarriage Association, UK. www.miscarriageassociation.org.uk

82. Chan-Wei Jia, Li Wang, Yong-Lian Lan, Rui Song, Li-Yin Zhou, Lan Yu, Yang Yang, Yu Liang, Ying Li, Yan-Min Ma, andShu-Yu Wang. Aneuploidy in Early Miscarriage and its Related Factors. Chin Med J (Engl). 2015 Oct 20; 128(20): 2772–2776. doi: 10.4103/0366-6999.167352 PMCID: PMC4736891

83. Kwak JY, Beer AE, Kim SH, Mantouvalos HP. Immunopathology of the implantation site utilizing monoclonal antibodies to natural killer cells in women with recurrent pregnancy losses. Am J Reprod Immunol. 1999;41(1):91-8.

84. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med. 1986;163(3):740-5.

85. Levi M, Dorffler-Melly J, Reitsma P, Buller H, Florquin S, van der Poll T, et al. Aggravation of endotoxin-induced disseminated intravascular coagulation and cytokine activation in heterozygous protein-C-deficient mice. Blood. 2003;101(12):4823-7.

86. Levi M, Keller TT, van Gorp E, ten Cate H. Infection and inflammation and the coagulation system. Cardiovasc Res. 2003;60(1):26-39.

87. Janeway CA Jr, Travers P, Walport M, et al. The Immune System in Health and Disease. Immunobiology: 5th edition. New York: Garland Science; 2001.

88. Lindsay B. Nicholson. The immune system. Essays Biochem. 2016 Oct 31; 60(3): 275–301.Published online 2016 Oct 26. doi: 10.1042/EBC20160017. PMCID: PMC5091071Nature Reviews. Immune System. 2001

89. Costantino Iadecola & Josef Anrather Cells of the innate immune system. The immunology of stroke: from mechanisms to translation. Nature Medicine 17,796–808 (2011) doi:10.1038/nm.2399

90. G Leoni, P-A Neumann, R Sumagin TL Denning, and A Nusrat. Wound repair: role of immune–epithelial interactions. Mucosal Immunol. 2015 Sep; 8(5): 959–968.Published online 2015 Jul 15. doi: 10.1038/mi.2015.63. PMCID: PMC4916915

91. Robertson SA, Mau VJ, Hudson SN, Tremellen KP 1997 Cytokine-leukocyte networks and the establishment of pregnancy. Am J Reprod Immunol 37:438—442.

92. Medawar PB 1953 Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp Soc Exp Biol 7:320-328.

93. Billingham RE, Head JR. Recipient treatment to overcome the allograft reaction, with special reference to nature's own solution. Prog Clin Biol Res. 1986;224:159-85.

94. Hansel W, Hickey GJ. Early pregnancy signals in domestic animals. Ann N Y Acad Sci. 1988;541:472-84.

95. HM. Weitlauf. Embryonic signaling at implantation in the mouse. Prog Clin Biol Res 1989;294:359-76.

96. Soares MJ. The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. Reprod Biol Endocrinol. 2004;2:51.

97. Moffett A, Loke YW. The immunological paradox of pregnancy: a reappraisal. Placenta. 2004;25(1):1-8.

98. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991-1045

99. Bonney EA MP. Much IDO about pregnancy. Nat Med 1998;Oct;4(10):1128-9.

100. Cardenas I, Means RE, Aldo P, Koga K, Lang SM, Booth CJ, et al. Viral infection of the placenta leads to fetal inflammation and sensitization to bacterial products predisposing to preterm labor. J Immunol. 2010;185(2):1248-57.

101. Mor G, Cardenas I. The immune system in pregnancy: a unique complexity. Am J Reprod Immunol. 2010;63(6):425-33.

102. Medawar PB. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. . Sym SocExp Biol 1953. 1953;7:320–38.

103. Beer AE, Quebbeman JF. Immunological mechanisms of survival and "rejection" of the fetal allograft. Mead Johnson Symp Perinat Dev Med. 1986(24):20-6.

104. Beer AE, Semprini AE, Zhu XY, Quebbeman JF. Pregnancy outcome in human couples with recurrent spontaneous abortions: HLA antigen profiles; HLA antigen sharing; female serum MLR blocking factors; and paternal leukocyte immunization. Exp Clin Immunogenet. 1985;2(3):137-53

105. Billington WD. The immunological problem of pregnancy: 50 years with the hope of progress. A tribute to Peter Medawar. J Reprod Immunol. 2003;60(1):1-11.

106. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today. 1993;14(7):353-6.

107. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol. 2002;2(9):656-63.

108. Edmonds DK, Lindsay KS, Miller JF, Williamson E, Wood PJ. Early embryonic mortality in women. Fertil Steril. 1982;38(4):447-53.

109. Alberman E. Antenatal and perinatal causes of handicap: epidemiology and causative factors. Baillieres Clin Obstet Gynaecol. 1988;2(1):9-19.

110. Erlebacher A, Vencato D, Price KA, Zhang D, Glimcher LH. Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. J Clin Invest (2007) 117(5):1399–411. doi:10.1172/JCI28214

111. Joan S Hunt, Daudi K Langat, Ramsey H McIntire, Pedro J Morales. The role of HLA-G in human pregnancy. Reprod Biol Endocrinol. 2006; 4(Suppl 1): S10. doi: 10.1186/1477-7827-4-S1-S10 PMCID: PMC177506

112. Shawn P. Murphy. Interferon Gamma in Successful Pregnancies Biol Reprod. 2009 May; 80(5): 848–859.Prepublished online 2009 Jan

113. Bulmer JN, Lash GE. Human uterine natural killer cells: a reappraisal. Mol Immunol 21. doi: 10.1095/biolreprod. PMCID: PMC2849832

114. Marianne J van den Heuvel, A Review of Trafficking and Activation of Uterine Natural Killer Cells. Am J Reprod Immunol. 2005 Dec; 54(6): 322–331. doi: 10.1111/j.1600- 897.2005.00336.xPMCID: PMC2967519.

115. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. CD56^{bright}natural killer (NK) cells: an important NK cell subset. Immunology. 2009;126(4):458-465. doi:10.1111/j.1365-2567.2008.03027.x.

116. Varla-Leftherioti M[1]. Role of a KIR/HLA-C allorecognition system in pregnancy.J Reprod Immunol. 2004 Jun;62(1-2):19-27.

117. Kammerer, U., Eggert, A.O., Kapp, M., McLellan, A.D., Geijtenbeek, T.B., Dietl, J., van Kooyk, Y., Kampgen, E., 2003. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. Am. J. Pathol. 162, 887–896.

118. Svensson, J., Jenmalm, M.C., Matussek, A., Geffers, R., Berg, G., Ernerudh, J., 2011. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. J. Immunol. 187, 3671–3682.

119. Houser, B.L., Tilburgs, T., Hill, J., Nicotra, M.L., Strominger, J.L., 2011. Two unique human decidual macrophage populations. J. Immunol. 186, 2633–2642.

120. Staff, A.C., Johnsen, G.M., Dechend, R., Redman, C.W., 2014. Preeclampsia and uteroplacental acute atherosis: immune and inflammatory factors. J. Reprod. Immunol. 101–102, 120–126.

121. Lampe, R., Kover, A., Szucs, S., Pal, L., Arnyas, E., Adany, R., Poka, R., 2015. Phagocytic index of neutrophil granulocytes and monocytes in healthy and preeclamptic pregnancy. J. Reprod. Immunol. 107, 26–30.

122. Jens Kieckbusch, MHC-dependent inhibition of uterine NK cells impedes fetal growth and decidual vascular remodelling. Nat Commun. 2014; 5: 3359. doi: 10.1038/ncomms4359. PMCID: PMC3948146

123. Ashley Moffett· Uterine NK cells: active regulators at the maternal-fetal interface. J Clin Invest. 2014 May 1; 124(5): 1872–1879. Published online 2014 May 1. doi: 10.1172/JCI68107 PMCID: PMC4001528

124. L. Mellor IMMUNOLOGY AT THE MATERNAL-FETALINTERFACE: Lessons for T Cell Tolerance and Suppression Rev. Immunol. 2000. 18:367–391 Annual Reviews. 0732–0582/00/0410–036714.00 367A

125. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25, 677–686

126. Ana Sofia Figueiredo. The T helper type 17/regulatory T cell paradigm in pregnancy. Immunology. 2016 May; 148(1): 13–21. Published online 2016 Apr 4. doi: 10.1111/imm.12595

127. Edmonds DK, Lindsay KS, Miller JF, Williamson E, Wood PJ. Early embryonic mortality in women. Fertil Steril. 1982;38(4):447-53.

128. Alberman E. Antenatal and perinatal causes of handicap: epidemiology and causative factors. Baillieres Clin Obstet Gynaecol. 1988;2(1):9-19.

129. Stirrat GM. Recurrent miscarriage. Lancet. 1990;336(8716):673-5.

130. Portnoi MF, Joye N, van den Akker J, Morlier G, Taillemite JL. Karyotypes of 1142 couples with recurrent abortion. Obstet Gynecol. 1988;72(1):31-4.

131. Rae R, Smith IW, Liston WA, Kilpatrick DC. Chlamydial serologic studies and recurrent spontaneous abortion. Am J Obstet Gynecol. 1994;170(3):782-5.

132. Summers PR. Microbiology relevant to recurrent miscarriage. Clin Obstet Gynecol. 1994;37(3):722-9.

133. Craig LB, Ke RW, Kutteh WH. Increased prevalence of insulin resistance in women with a history of recurrent pregnancy loss. Fertil Steril. 2002;78(3):487-90.

134. Arredondo F, Noble LS. Endocrinology of recurrent pregnancy loss. Semin Reprod Med. 2006;24(1):33-9.

135. Guimaraes Filho HA MR, Pires CR, Araujo Junior E, Moron AF, Nardozza LM. . Prevalence of uterine defects in habitual abortion patients attended on at a university health service in Brazil. Arch Gynecol Obstet. 2006;274:345–8.

136. Vitagliano A, Noventa M, Gizzo S. Autoimmunity, systemic inflammation, and their correlation with repeated implantation failure and recurrent miscarriage: Is chronic endometritis the missing piece of the jigsaw? Am J Reprod Immunol. 2017 Jan;77(1). doi: 10.1111/aji.12597. Epub 2016 Nov 8

137. Toth B, Jeschke U, Rogenhofer N, Scholz C, Wurfel W, Thaler CJ, et al. Recurrent miscarriage: current concepts in diagnosis and treatment. J Reprod Immunol. 2010;85(1):25-32.

138. Allison JL, Schust DJ. Recurrent first trimester pregnancy loss: revised definitions and novel causes. Curr Opin Endocrinol Diabetes Obes. 2009;16(6):446-50.

139. Michimata T SM, Miyazaki S, Ogasawara MS, Suzumori K, Aoki K, Nagata K, Saito S. . Decrease of T-helper 2 and T-cytotoxic 2 cells at implantation sites occurs in unexplained recurrent spontaneous abortion with normal chromosomal content. . Hum Reprod 2003;18:1523–1528.

140. HFEA. National data for fertility treatment in 2013.Trends and figures. Published 17th Dec 2014.

141. Coughlan C, Ledger W, Wang Q, Liu F, Demirol A, Gurgan T, et al. Recurrent implantation failure: definition and management. Reprod Biomed Online. 2014;28(1):14-38.

142. Singh N TV, Kumar S, Vanamail P, Madhu M. . Does endometrial injury enhances implantation in recurrent in-vitro fertilization failures? A prospective randomized control study from tertiary care center. . J Hum Reprod Sci 2015; Oct-Dec;8(4):218-23. doi: 10.4103/0974-1208.170401.

143. Kunicki M, Lukaszuk K, Woclawek-Potocka I, Liss J, Kulwikowska P, Szczyptanska J. Evaluation of granulocyte colony-stimulating factor effects on treatment-resistant thin endometrium in women undergoing in vitro fertilization. Biomed Res Int. 2014;2014:913235.

144. Psychoyos A. Uterine receptivity for nidation. Ann N Y Acad Sci. 1986;476:36-42.

145. Sharkey A. Cytokines and implantation. Rev Reprod. 1998;3(1):52-61.

146. Taylor C, Faulk WP. Prevention of recurrent abortion with leucocyte transfusions. Lancet. 1981;2(8237):68-70.

147. Kwak-Kim J, Han AR, Gilman-Sachs A, Fishel S, Leong M, Shoham Z. Current trends of reproductive immunology practices in in vitro fertilization (IVF) - a first world survey using IVF-worldwide.com. Am J Reprod Immunol 2013; 69: 12–20

148. Johnson PM, Chia KV, Hart CA, Griffith HB, Francis WJ. Trophoblast membrane infusion for unexplained recurrent miscarriage. Br J Obstet Gynaecol. 1988;95(4):342-7.

149. Stern JJ, Coulam CB. Current status of immunologic recurrent pregnancy loss. Curr Opin Obstet Gynecol. 1993;5(2):252-9.

150. Mueller-Eckhardt G, Heine O, Neppert J, Kunzel W, Mueller-Eckhardt C. Prevention of recurrent spontaneous abortion by intravenous immunoglobulin. Vox Sang. 1989;56(3):151-4.

151. Christiansen OB, Pedersen B, Rosgaard A, Husth M. A randomized, double-blind, placebo-controlled trial of intravenous immunoglobulin in the prevention of recurrent miscarriage: evidence for a therapeutic effect in women with secondary recurrent miscarriage. Hum Reprod. 2002;17(3):809-16.

152. Yamada H, Morikawa M, Furuta I, Kato EH, Shimada S, Iwabuchi K, et al. Intravenous immunoglobulin treatment in women with recurrent abortions: increased cytokine levels and reduced Th1/Th2 lymphocyte ratio in peripheral blood. Am J Reprod Immunol. 2003;49(2):84-9.

153. Scott JR. Immunotherapy for recurrent miscarriage. Cochrane Database Syst Rev. 2003(1):CD000112.

154. Porter TF, LaCoursiere Y, Scott JR. Immunotherapy for recurrent miscarriage. Cochrane Database Syst Rev. 2006(2):CD000112.

155. Metcalf D. The granulocyte-macrophage colony-stimulating factors. Science. 1985;229(4708):16-22.

156. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, et al. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature. 1986;319(6052):415-8.

157. Thomas J, Liu F, Link DC. Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr Opin Hematol 2002; 9: 183-189.

158. Carton E, Bellesoeur A, Mir O. Colony-stimulating factors for febrile neutropenia. N Engl J Med. 2013 Jul 18;369(3):285

159. Williams Z. Inducing tolerance to pregnancy. N Engl J Med. 2012 Sep 20;367(12):1159-61.

160. Root RK, Dale DC. Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor: Comparisons and Potential for Use in the Treatment of Infections in Nonneutropenic Patients. J Infect Dis 1999; 179: S342-S352.

161. Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F, Tobita M, et al. Identification and characterization of receptors for granulocyte colony stimulating factor on human placenta and trophoblastic cells. Proc Natl Acad Sci USA 1989; 86: 9323-9326.

162. NORA Therapeutics Inc. California

163. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ. 2009;339:b2535.

164. Würfel W. Approaches to better implantation. J Assist ReprodGenet. 2000;17, 473.

165. Osipova A, Piccinni, M.P., Taieb, J., Gallot, V., Lombardelli, L., Petitbarat, M.,Fanchin, R., Chaouat, G., Frydman, N., Frydman, R., Lédée, N. The value of follicular fluid G-CSF as a biomarker of embryo implantationpotential in monofollicular IVF cycle. Mol. Human Reprod 2009;24 (Suppl.1), 48.

166. Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M, et al. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. Fertil Steril. 2013;99(6):1600-9.

167. Eftekhar M, Sayadi M, Arabjahvani F. Transvaginal perfusion of G-CSF for infertile women with thin endometrium in frozen ET program: A non-randomized clinical trial. Iran J Reprod Med. 2014;12(10):661-6.

168. Aleyasin A, Abediasl Z, Nazari A, Sheikh M. Granulocyte colony-stimulating factor in repeated IVF failure, a randomized trial. Reproduction. 2016;151(6):637-42.

169. Barad DH, Yu Y, Kushnir VA, Shohat-Tal A, Lazzaroni E, Lee HJ, et al. A randomized clinical trial of endometrial perfusion with granulocyte colony-stimulating factor in in vitro fertilization cycles: impact on endometrial thickness and clinical pregnancy rates. Fertil Steril. 2014;101(3):710-5.

170. Davari-Tanha F, Shahrokh Tehraninejad E, Ghazi M, Shahraki Z. The role of G-CSF in recurrent implantation failure: A randomized double blind placebo control trial. Int J Reprod Biomed (Yazd). 2016;14(12):737-42.

171. Eftekhar M, Miraj S, Farid Mojtahedi M, Neghab N. Efficacy of Intrauterine infusion of granulocyte colony stimulating factor on patients with history of implantation failure: A randomized control trial. Int J Reprod Biomed (Yazd). 2016;14(11):687-90.

172. Gleicher N, Kim A, Michaeli T, Lee HJ, Shohat-Tal A, Lazzaroni E, et al. A pilot cohort study of granulocyte colony-stimulating factor in the treatment of unresponsive thin endometrium resistant to standard therapies. Hum Reprod. 2013;28(1):172-7.

173. Lee D, Jo JD, Kim SK, Jee BC, Kim SH. The efficacy of intrauterine instillation of granulocyte colony-stimulating factor in infertile women with a thin endometrium: A pilot study. Clin Exp Reprod Med. 2016;43(4):240-6.

174. Scarpellini F, Sbracia, M. . G-CSF treatment improves IVF outcome in women with recurrent implantation failure in IVF. J Reprod Immunol94, 103. 2012.

175. Tehraninejad E, Davari Tanha F, Asadi E, Kamali K, Aziminikoo E, Rezayof E. G-CSF Intrauterine for Thin Endometrium, and Pregnancy Outcome. J Family Reprod Health. 2015;9(3):107-12.

176. Würfel JW. G-CSF treatment of patients with recurrent implantation failures (RIF) and recurrent spontaneous abortions (RSA). . JReprod Immunol 2013;101/102 (15), S25.

177. Xu B, Zhang Q, Hao J, Xu D, Li Y. Two protocols to treat thin endometrium with granulocyte colony-stimulating factor during frozen embryo transfer cycles. Reprod Biomed Online. 2015;30(4):349-58.

178. Zeyneloglu HB, Onalan, G., Durak, T., Alyazici, I., Unal, E. Granulocyte macrophage colony stimulating factor (G-CSF) administrationfor art patients with repeated implantation failure (RIF): which routeis best? Fertil Steril. 2013; 100, S291–S292.

179. Boxer LA, Boyard, A.A., Marrero, T.T., Alter, B.P., Bonilla, M.A., Link, D.,Newburger, P.E., Rosenberg, P.S., Shimamura, A., Dale, D.C. 490 outcomes of pregnancies for women with severe chronic neutropenia with and without G-CSF treatment. Hematology In: 52nd ASH AnnualMeeting, Abstract 4786. 2010.

180. Santjohanser C, Knieper, C., Franz, C., Hirv, K., Meri, O., Schleyer, M.,Würfel, W., Toth, B. Granulocyte-colony stimulating factor as treatment option in patients with recurrent miscarriage. . ArchImmunol Ther Exp (Warsz). 2013;61, 159–164.

181. Zeidler C, Grote UA, Nickel A, Brand B, Carlsson G, Cortesao E, et al. Outcome and management of pregnancies in severe chronic neutropenia patients by the European Branch of the Severe Chronic Neutropenia International Registry. Haematologica. 2014;99(8):1395-402.

182. Litwin S LM, Barrientos G, Roux ME, Margni R, Miranda S. Comparative immune-histochemical study of M-CSF and G-CSF in feto-maternal interface in a multiparity mouse model. Am J Reprod Immunol 2005;54: 311-320.

183. Kato Y KT, Itoh T, Hiura M, Hatori A, Shigematsu A, et al. . A possible relationship between abortions and placental embolism in pregnant rabbits given human granulocyte colony-stimulating factor. J Toxicol Sci. 2001;26: 39-50.

184. Würfel W SC, Hirv K, Bühl M, Meri O, Laubert I, et al. . High pregnancy rates with administration of granulocyte colony-stimulating factor in ART-patients with repetitive implantation failure and lacking

killer-cell immunoglobulin-like receptors. Hum Reprod 2010;25: 2151-2152.

185. Rahmati M, Petitbarat M, Dubanchet S, Bensussan A, Chaouat G, Ledee N. Granulocyte-Colony Stimulating Factor related pathways tested on an endometrial ex-vivo model. PLoS One. 2014;9(9):e102286.

186. Roberts AW. G-CSF: a key regulator of neutrophil production, but that's not all! Growth Factors 2005;Mar;23(1):33-41.

187. Hartung T, Docke WD, Gantner F, Krieger G, Sauer A, Stevens P, et al. Effect of granulocyte colony-stimulating factor treatment on ex vivo blood cytokine response in human volunteers. Blood. 1995;85(9):2482-9.

188. Toh HC, Sun L, Soe Y, Wu Y, Phoon YP, Chia WK, et al. G-CSF induces a potentially tolerant gene and immunophenotype profile in T cells in vivo. Clin Immunol. 2009;132(1):83-92.

189. Rowe JH EJ, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature 2012;Oct 4;490(7418):102-6. doi: 10.1038/nature11462.

190. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. Cell. 2012;150(1):29-38.

191. Fasouliotis SJ, Spandorfer SD, Witkin SS, Schattman G, Liu HC, Roberts JE, et al. Maternal serum levels of interferon-gamma and interleukin-2 soluble receptor-alpha predict the outcome of early IVF pregnancies. Hum Reprod. 2004;19(6):1357-63.

192. Williams Z. Inducing tolerance to pregnancy. N Engl J Med. 2012;367(12):1159-61.

193. Salmassi A, Schmutzler AG, Huang L, Hedderich J, Jonat W, Mettler L. Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells. Fertil Steril. 2004;81 Suppl 1:786-91.

194. McCracken SA, Grant KE, MacKenzie IZ, Redman CW, Mardon HJ. Gestational regulation of granulocyte-colony stimulating factor receptor expression in the human placenta. Biol Reprod. 1999;60(4):790-6.

195. Lédée N, Lombroso, R., Lombardelli, L., Selva, J., Dubanchet, S., Chaouat, G., Frankenne, F., Fodart, J.M., Maggi, E., Romagnani, S., Ville, Y., Piccini, M.P. . Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. . Hum Reprod. 2008; 23, 2001–2009

196. Santjohanser C, Franz, C., Wuerfel, W., Meri, O., Fiedler, K., Krüsmann, G.,Krüsmann, J., Hirv, K., Toth, B. . 564 cycles with G-CSF applicationin patients with fertility disorders. . J Reprod Immunol 2011;90 (159), S51.

197. NORA Therapeutics, Phase 1 study. NORA Therapeutics Inc.

198. J. Zhao, B. Xu, S. Xie, Q. Zhang and Y. P. Li Whether G-CSF administration has beneficial effect on the outcome after assisted reproductive technology? A systematic review and meta-analysis. Zhao et al. Reproductive Biology and Endocrinology (2016) 14:62. DOI 10.1186/s12958-016-0197-2

199. Herrler A, von Rango, U., Beier, H.M. Embryo-maternal signalling:how the embryo starts talking to its mother to accomplish implantantion. Reprod BioMed 2003;Online 6, 244–256.

200. Makrigiannakis A, Karamouti, M., Drakakis, P., Loutradis, D., Antsaklis, A. Fetomaternal immunotolerance. Am J Reprod Immunol. 2008;60,482–496.

201. Varla-Leftherioti M, Spyropoulou-Vlachou, M., Keramitsoglou, T.,Papadimitropolous, M., Tsekoura, C., Graphou, O., Papadopoulou, C.,Gerondi, M., Stavropoulos-Giokas, C. Lack of the appropriate natural killer cell inhibitory receptors in women with spontaneous Hum Immunol 2004;66, 65–71.

202. Hiby SE, Regan L, Lo W, Farrell L, Carrington M, Moffett A. Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. Hum Reprod. 2008;23(4):972-6.

203. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. J Clin Invest. 2010;120(11):4102-10.

204. Wurfel W. Treatment with granulocyte colony-stimulating factor in patients with repetitive implantation failures and/or recurrent spontaneous abortions. J Reprod Immunol. 2015;108:123-35.

Appendices

Appendix 1. Protocol Amendments

The RESPONSE protocol was effective on 05 Feb 2014. There were 2 amendments that were prepared to document changes in the conduct of the study.

Version 2 (first amendment to the effective protocol), dated 21 Mar 2014, made the following changes:

- Clarified that participants were between 3 to 5 weeks of gestation at the time of randomisation and did not continue study drug administration beyond the Week 12 of gestation assessment. Therefore, the total duration of treatment was up to 9 weeks. Also clarified that visit timing was based on weeks of gestation.

- Added language that the investigator had unrestricted and immediate access to unblind the participant's treatment assignment by accessing the IWRS.

- Removed "G-CSF levels" from the list of potentially unblinding laboratory values.

- Removed Factor V Leiden mutation, prothrombin (Factor II) 20210 G>A mutation, prolactin, and anti-thyroid peroxidase antibodies from the list of screening tests.

- Added a statement that daily home urine pregnancy tests had to start 5 days before the start of the next period if the sixth day after ovulation had not yet occurred.

- Removed the sentence "Subjects who are determined to be greater than 5 weeks of gestation will not eligible for randomisation."

Version 3 (second amendment to the effective protocol, dated 15 Aug 2014, made the following changes:

- Revised the wording in the background section that states the estimated prevalence of recurrent loss of a clinical pregnancy, including a new reference to a 2012 American Society for Reproductive Medicine Practice Committee opinion.
- Added a new exploratory pharmacodynamics outcome measure: changes in levels of circulating WBC subsets.

- Clarified in the Immunogenicity section that participants who developed an ADA response and were pregnant were cared for by an appropriate specialist.

- Added language to the section on Blinding and Minimisation of Bias to standardize the process for informing participants and their general practitioners of pre randomisation FBC results and requesting that routine booking blood tests during pregnancy not include an FBC.

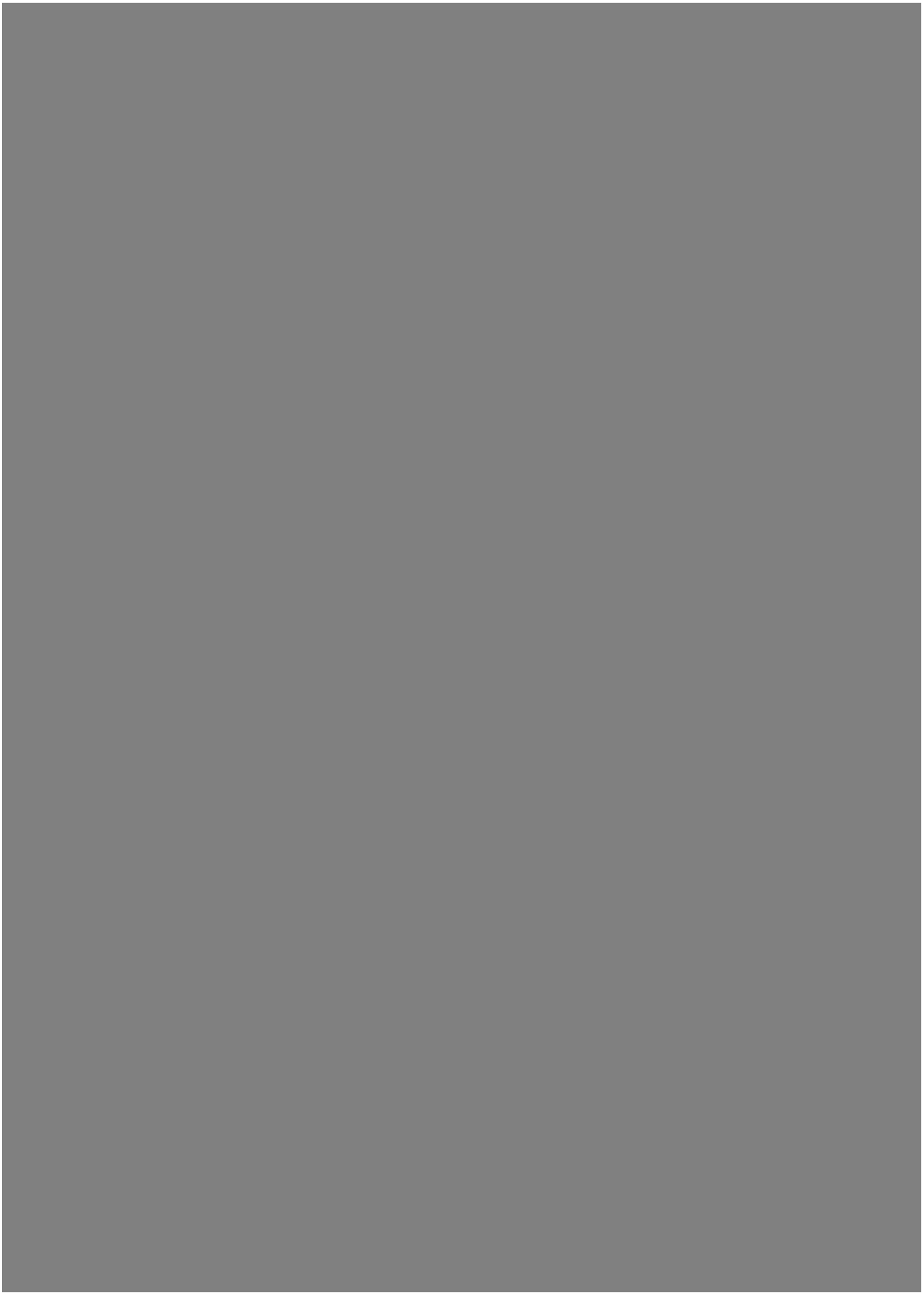
- Clarified the wording in inclusion criterion #2 used to define documented history of unexplained RPL.

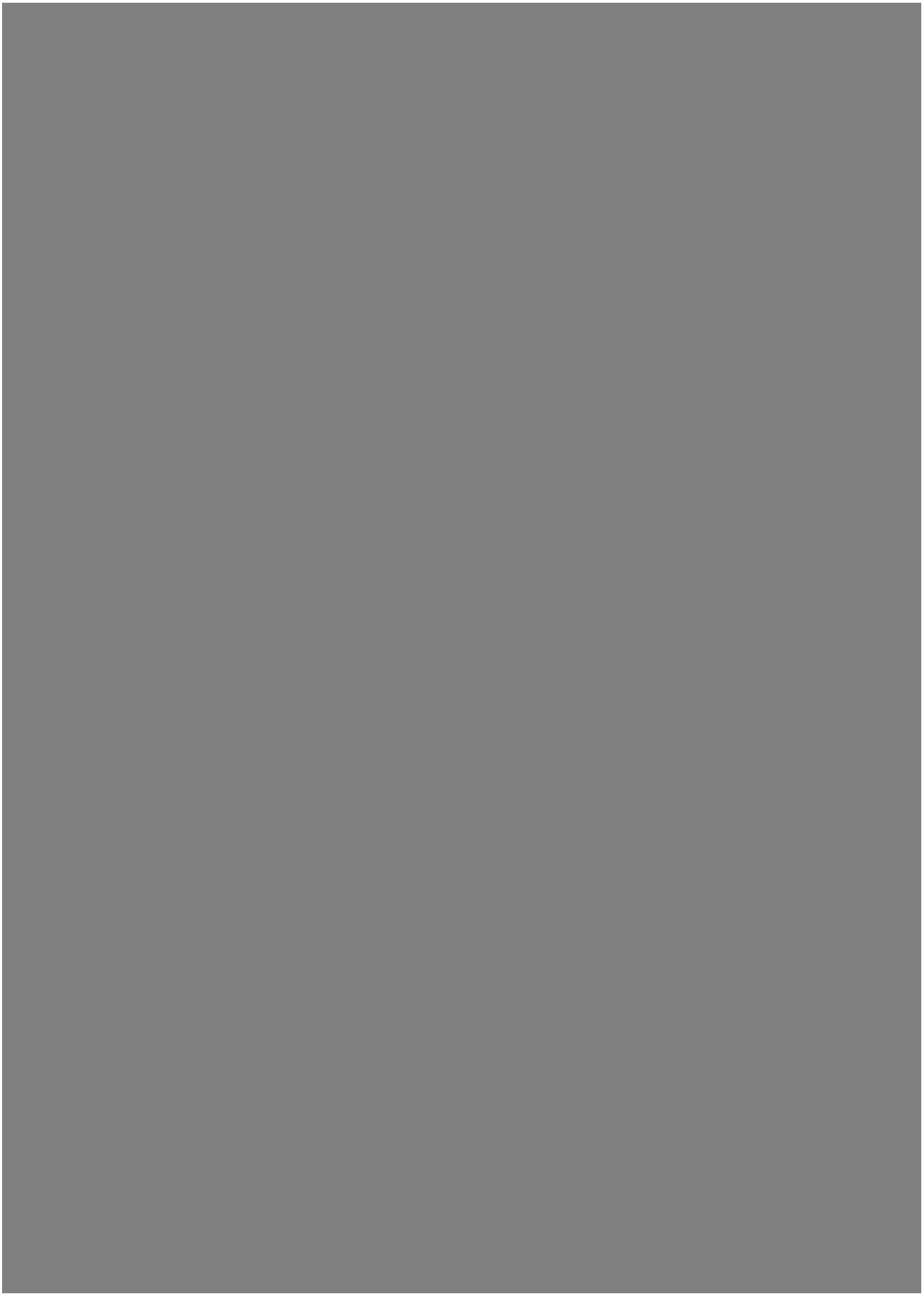
- Clarified wording of exclusion criterion #6f, current or past diagnosis of cervical incompetence

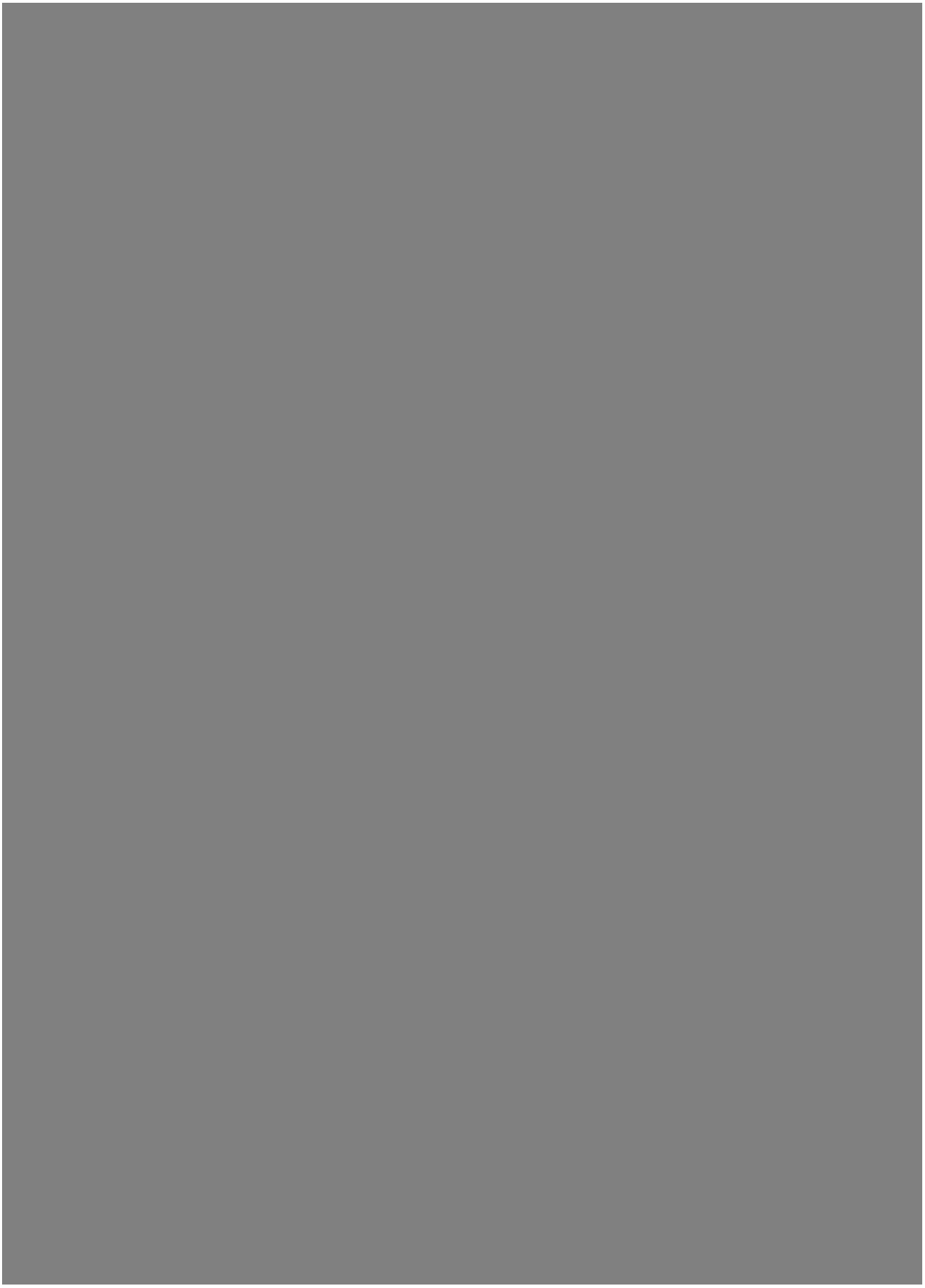
- Added a statement to the Excluded Therapies section that aspirin use was not excluded

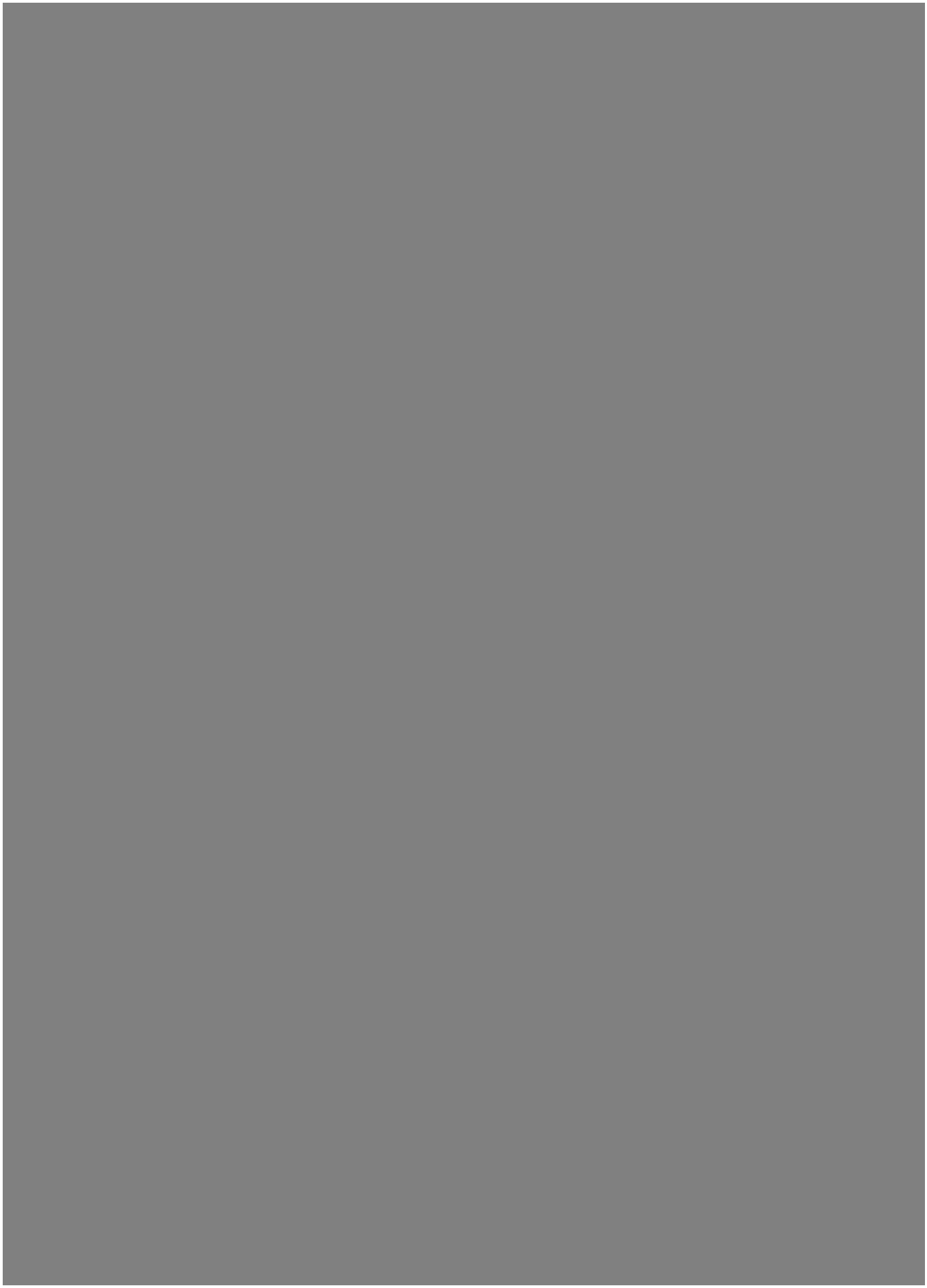
- Increased the time window of the Week 20 of Gestation Visit from ±3 days to ±1 week.

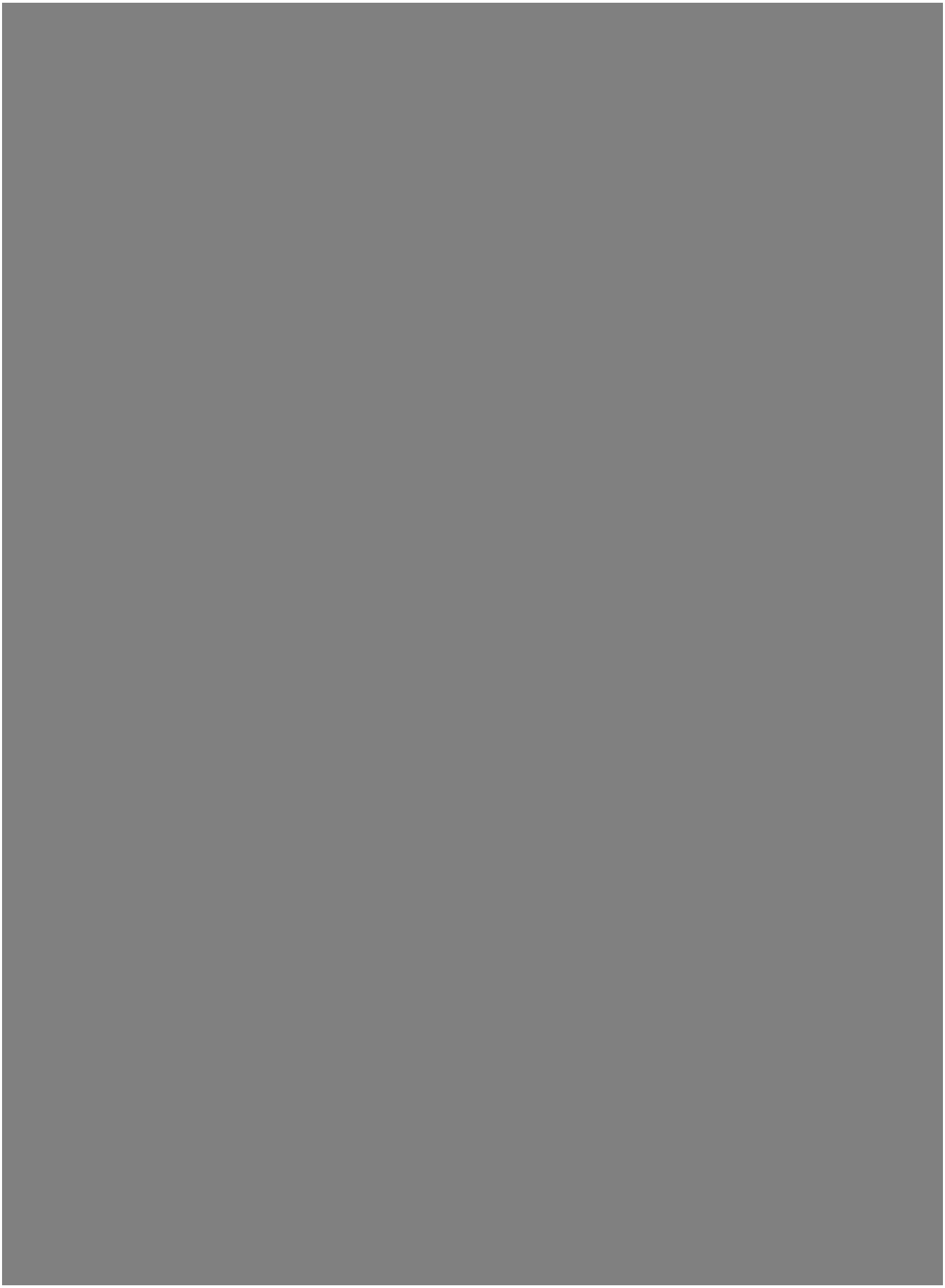
- Revised the title of Section 5 to "Assessment of Efficacy and Pharmacodynamics". Added Section 5.2, a Pharmaco-dynamic assessment that describes the set of exploratory research biomarkers that were examined to better understand the effects of pregnancy and rhG-CSF on immune cells. Added confirmation that genetic testing was not performed on any samples.

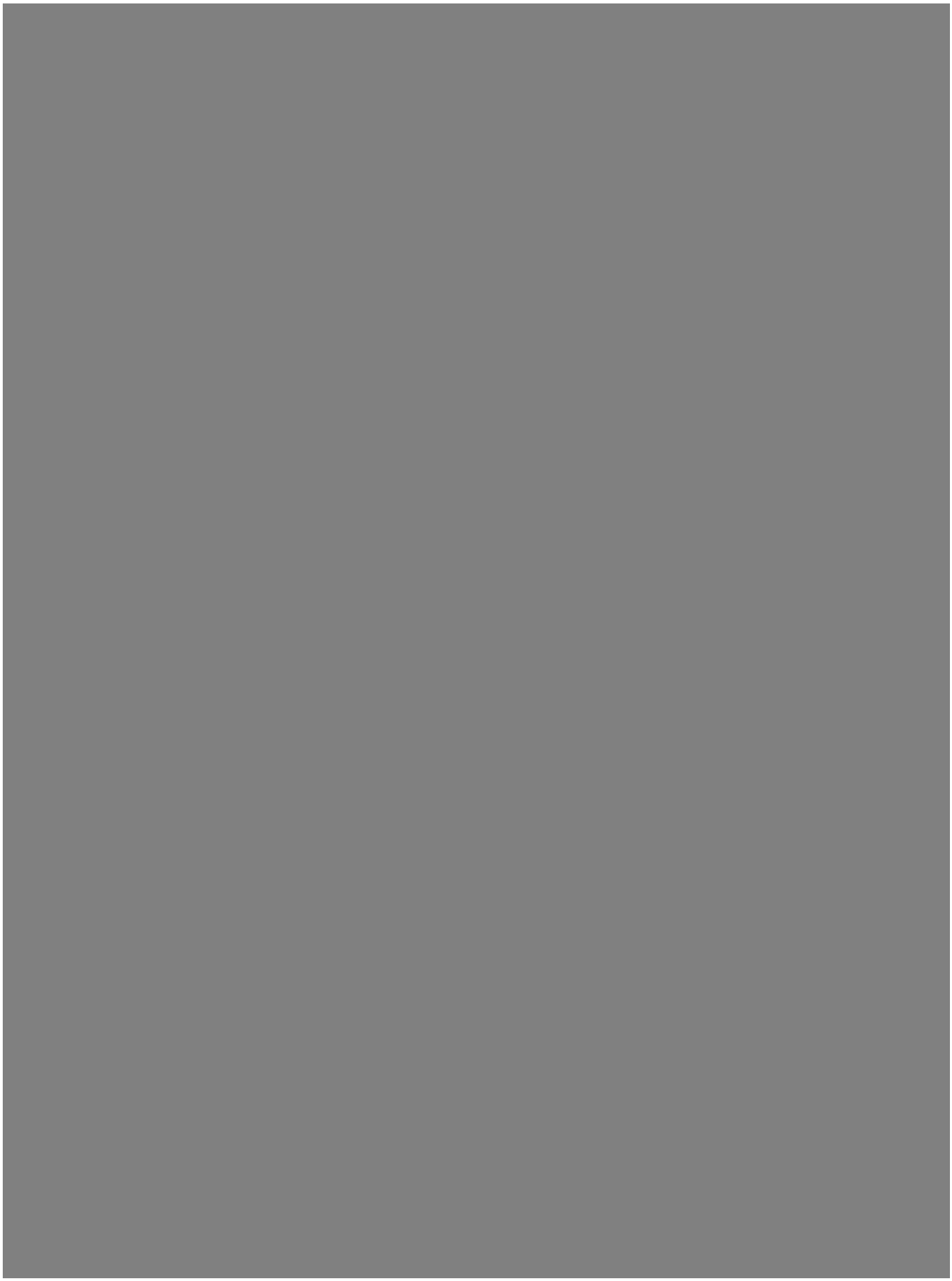


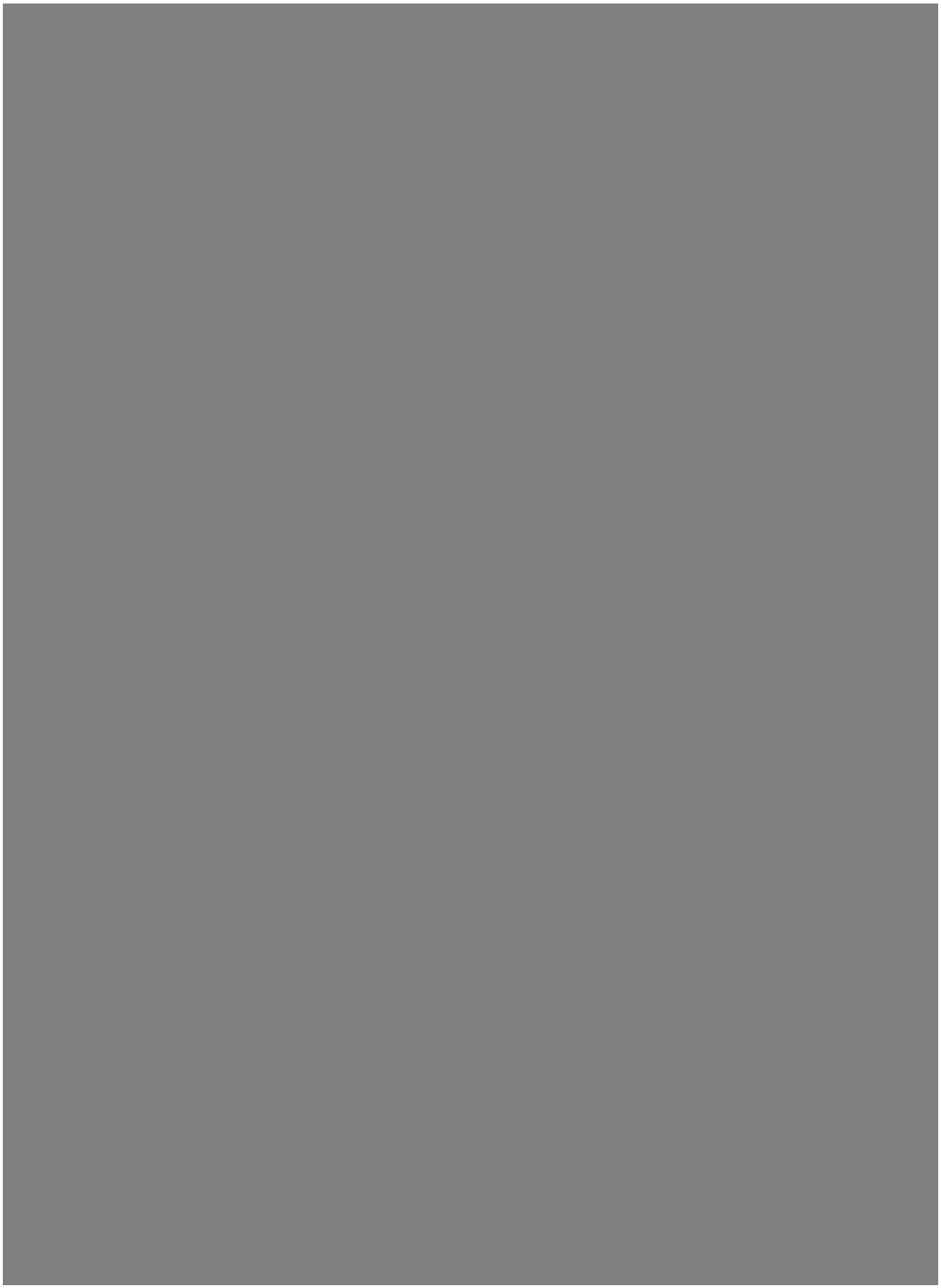


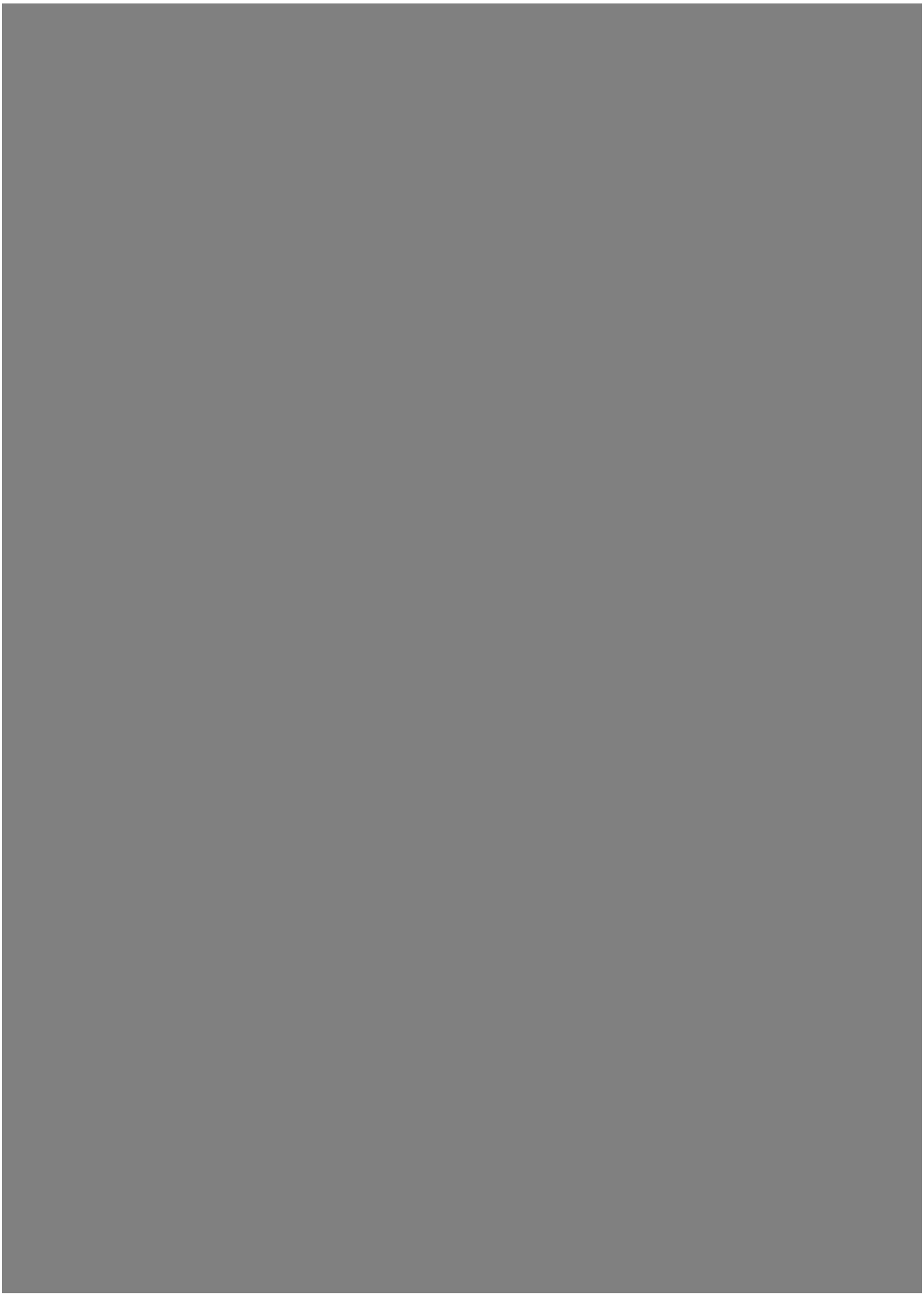


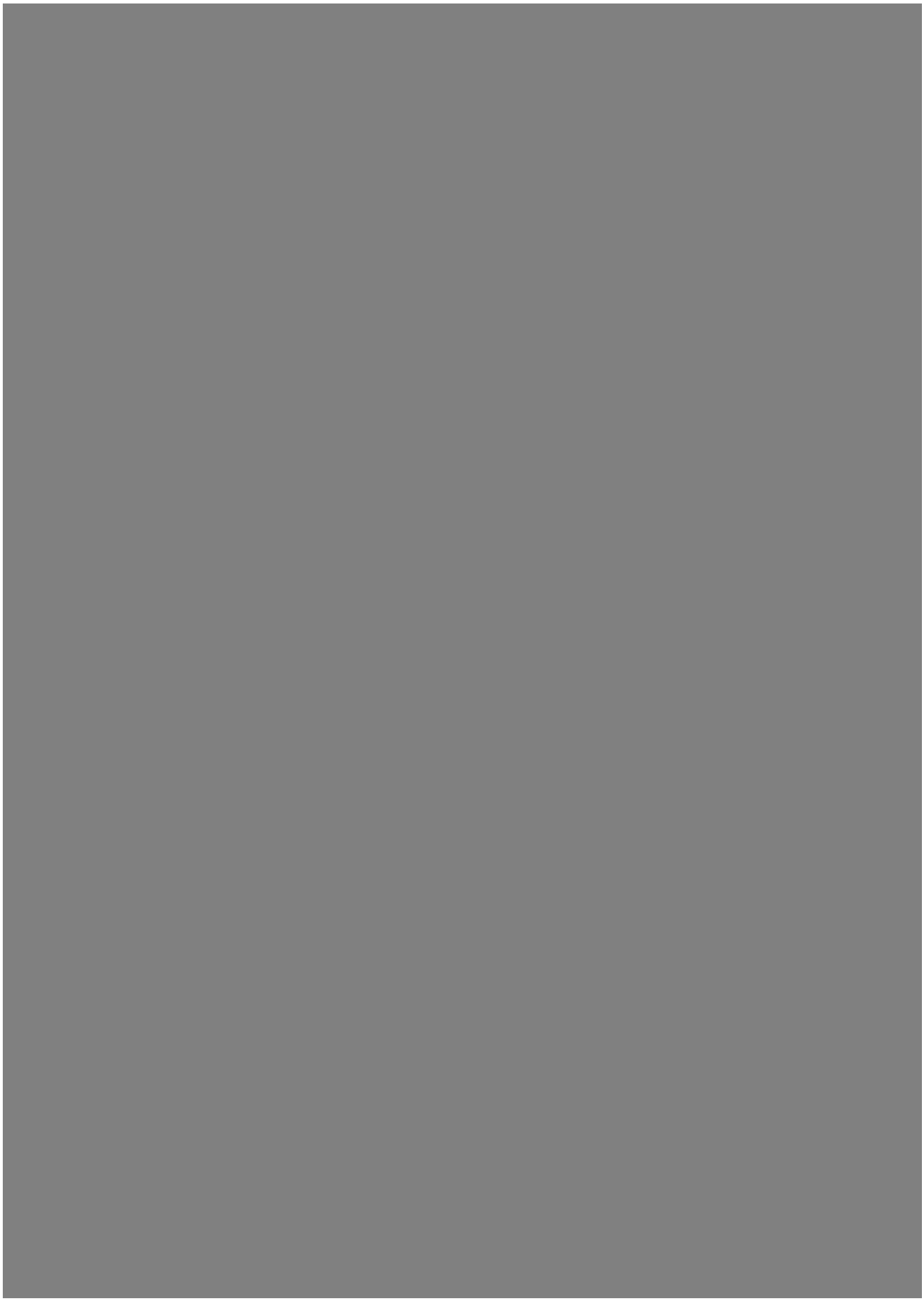


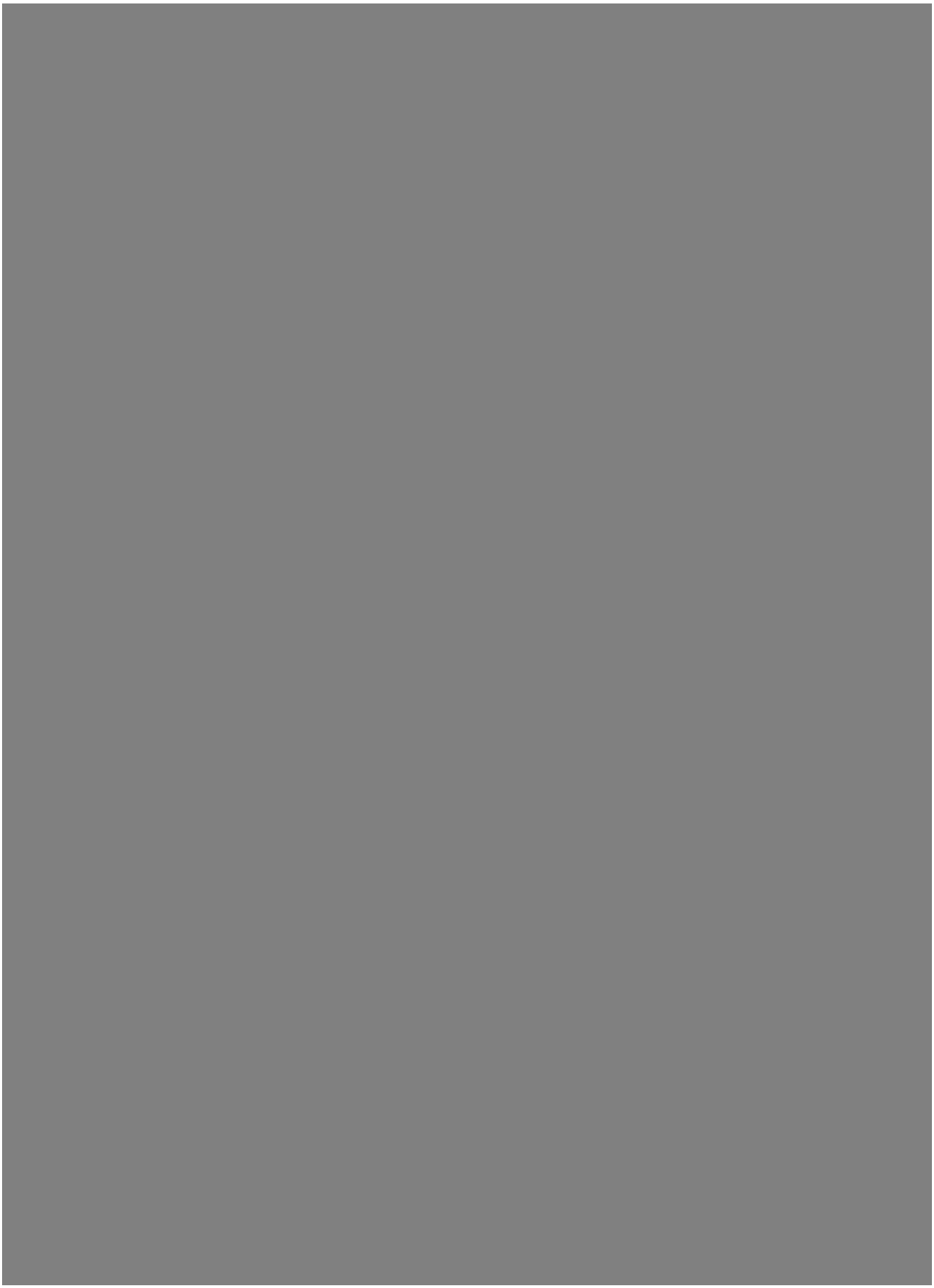


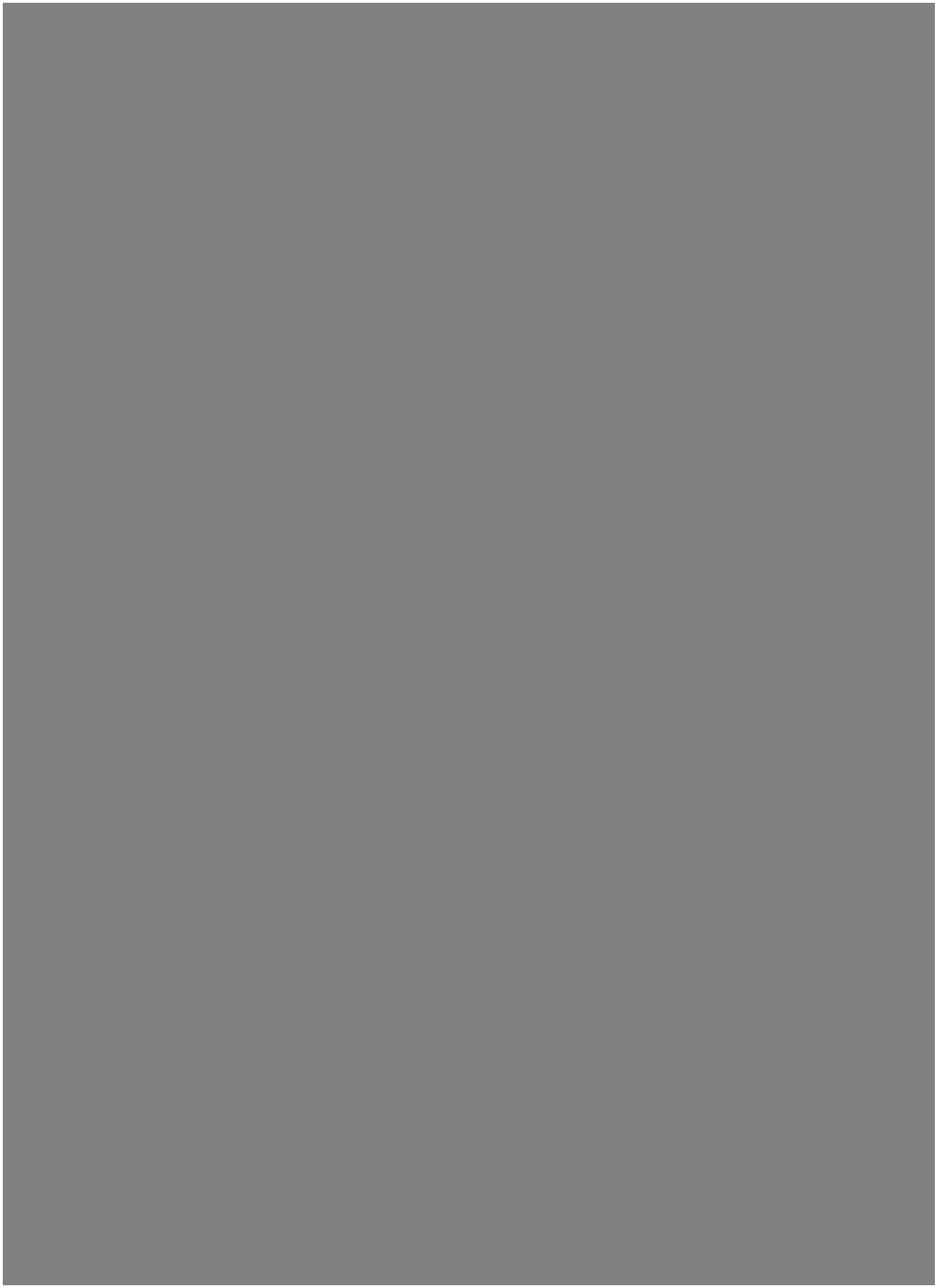


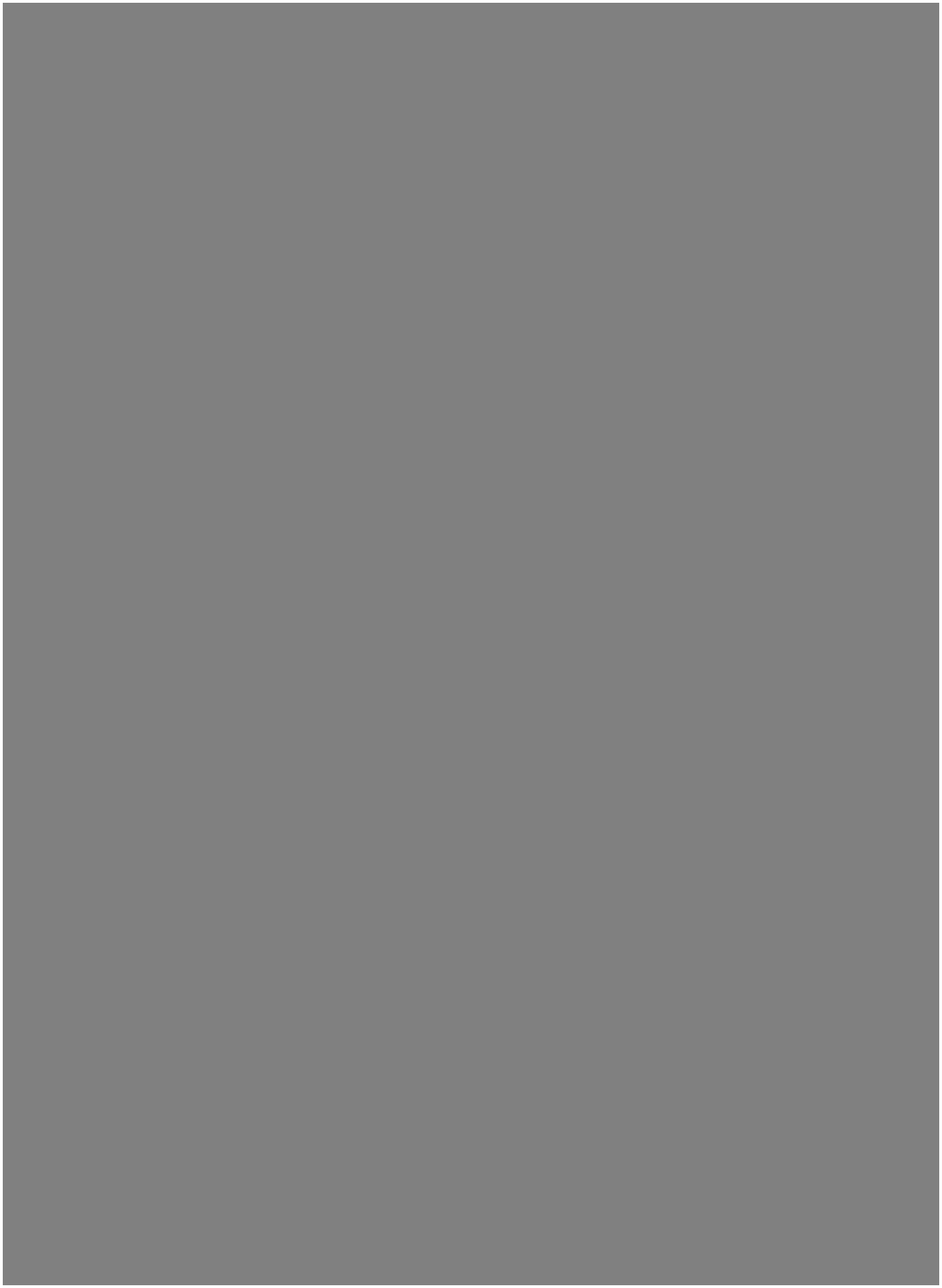


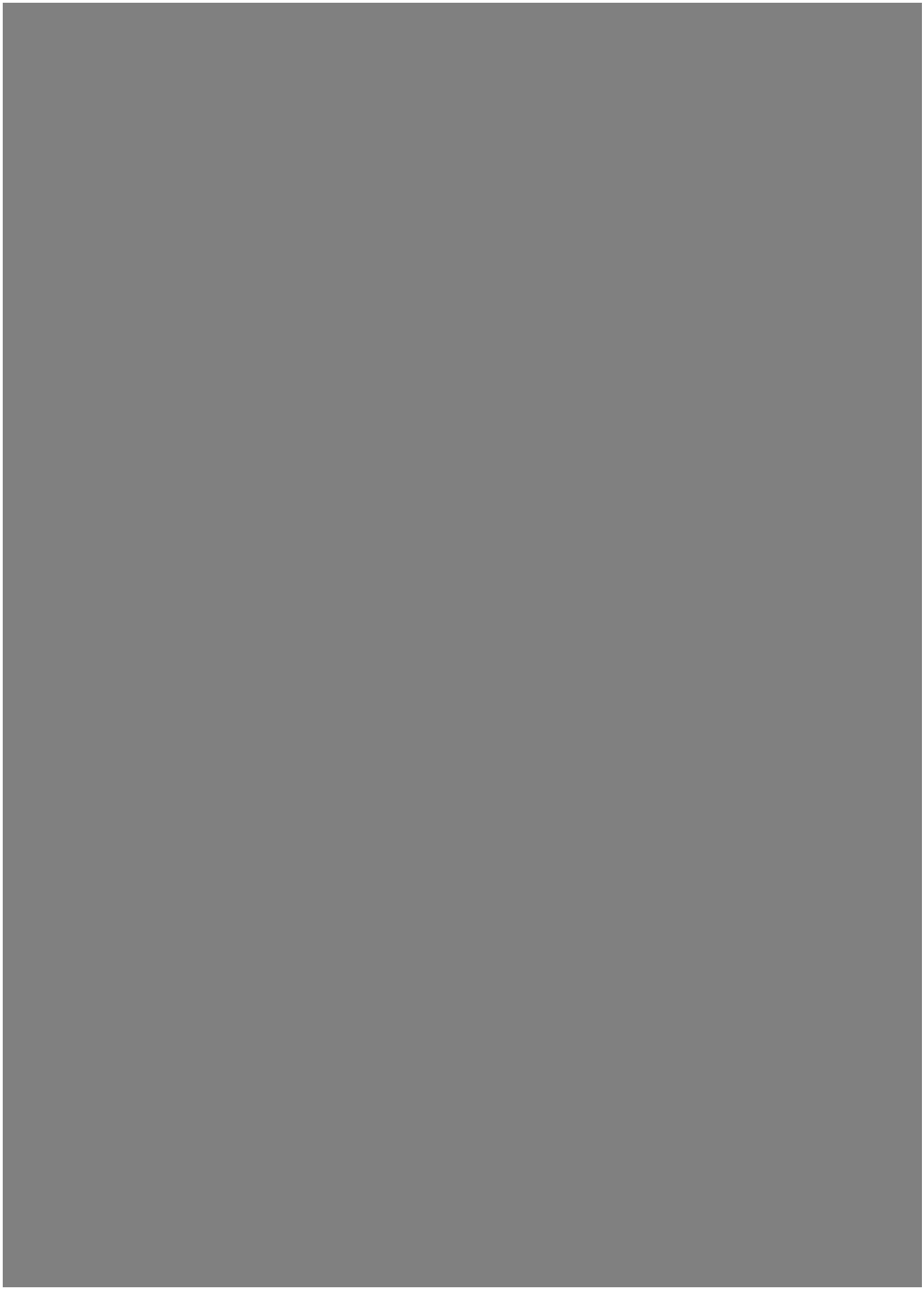


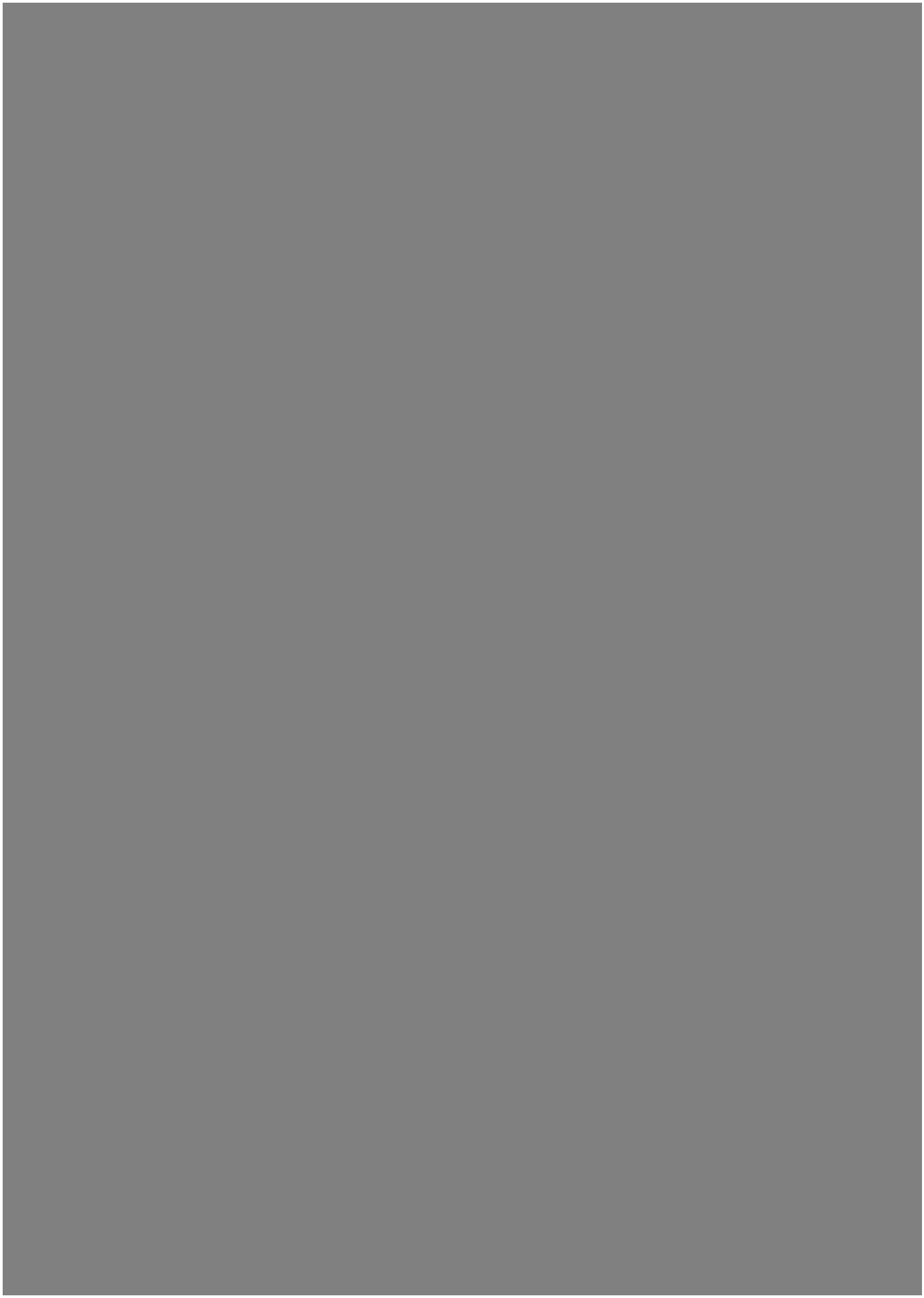


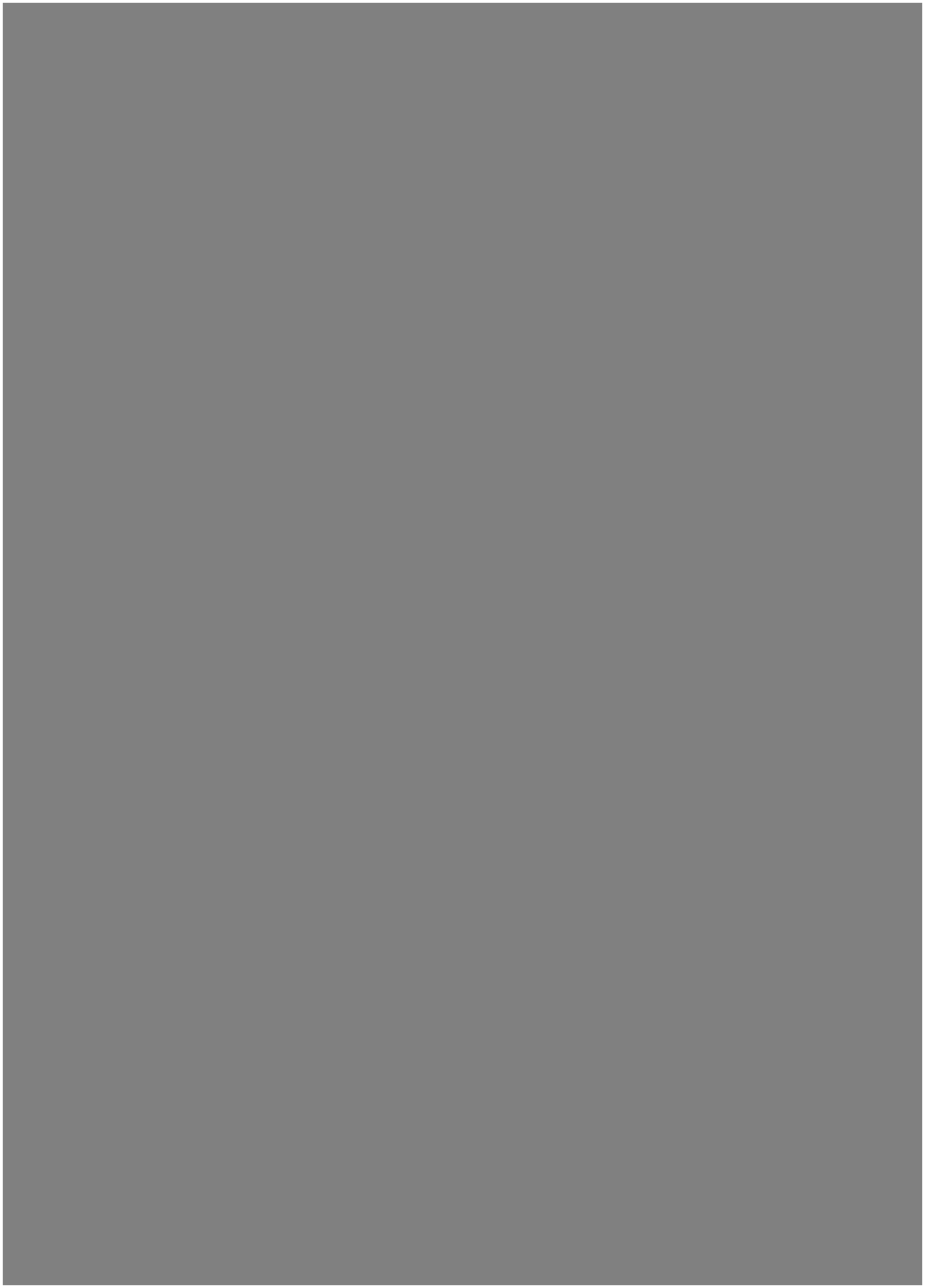


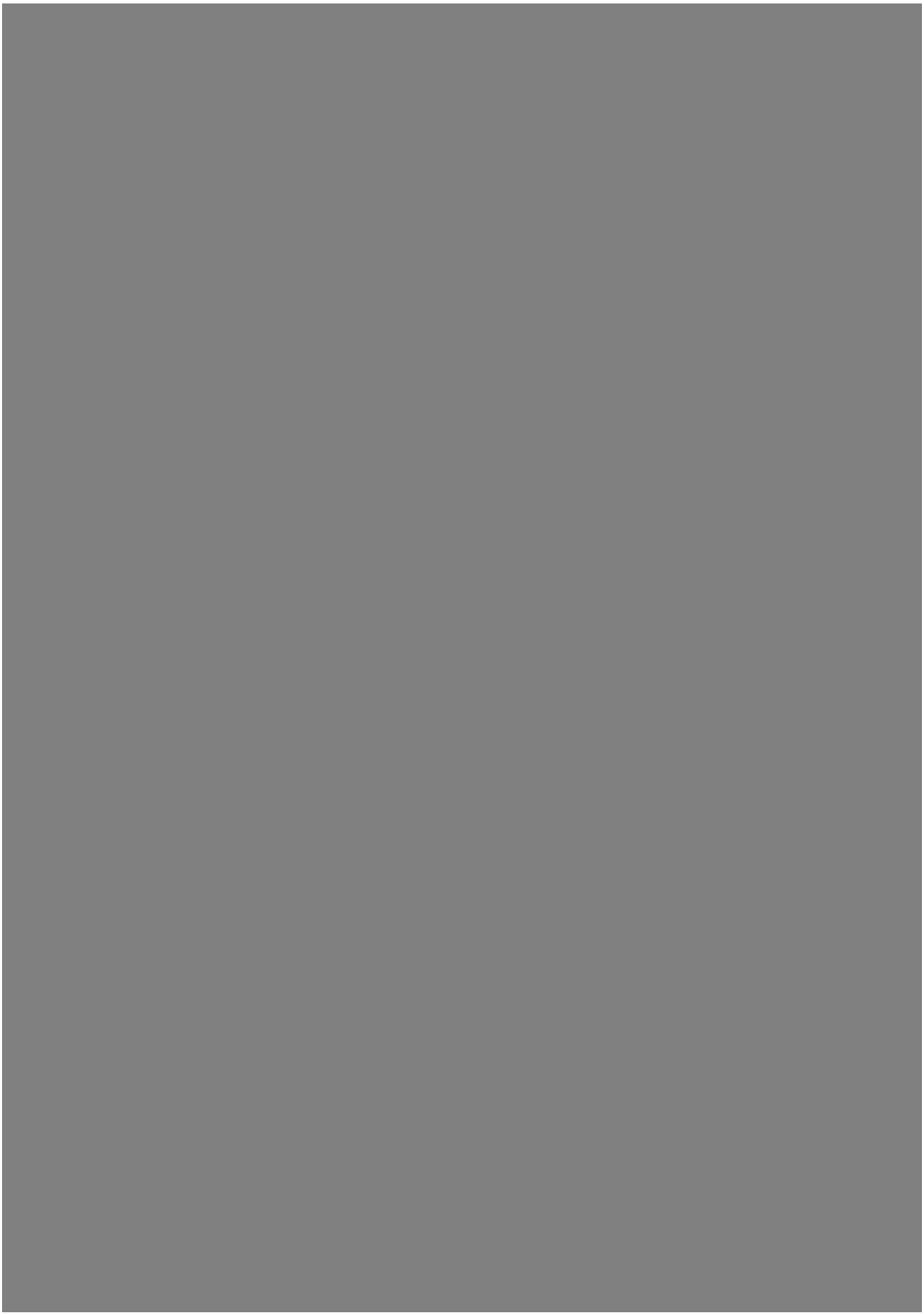


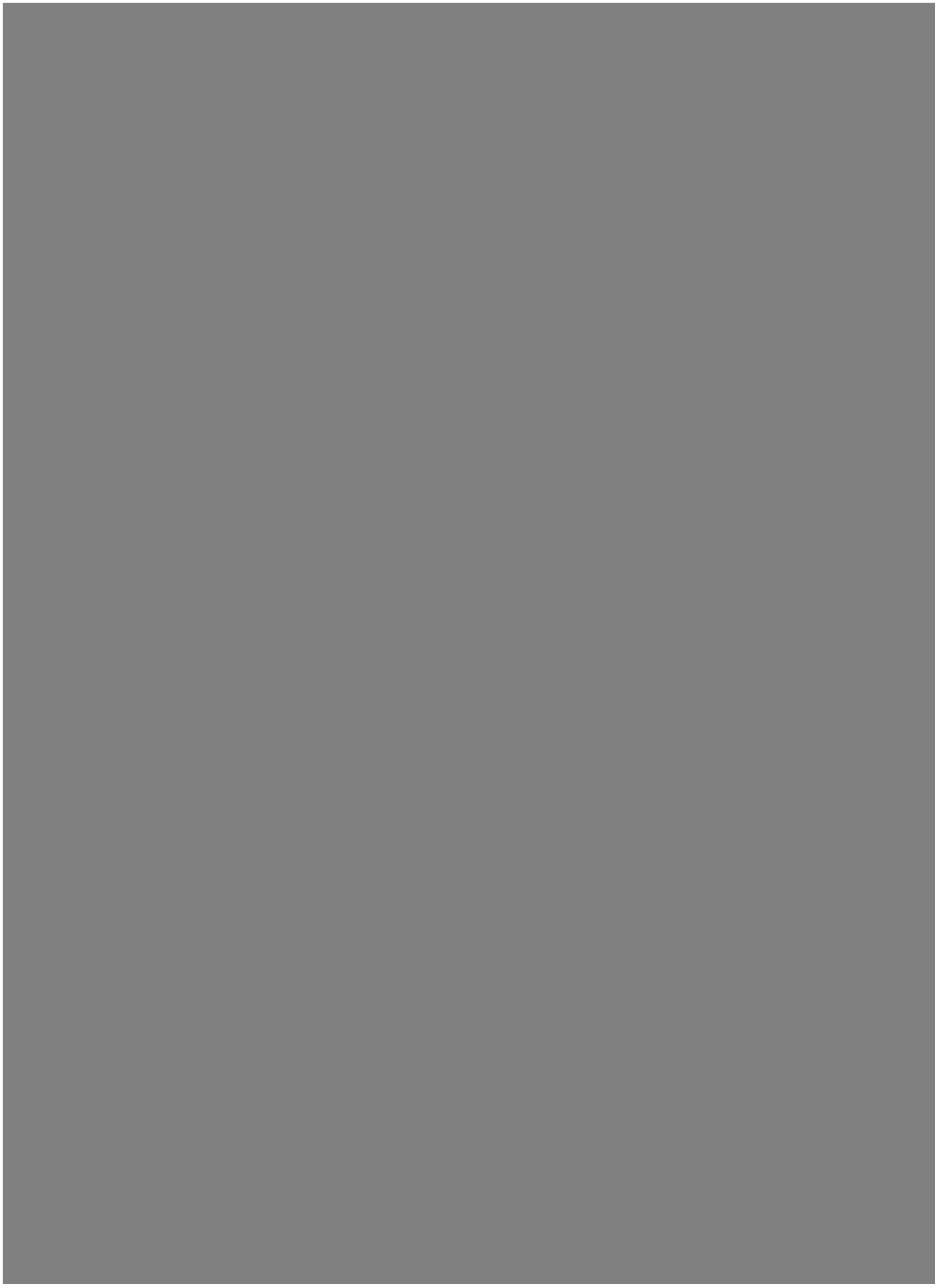


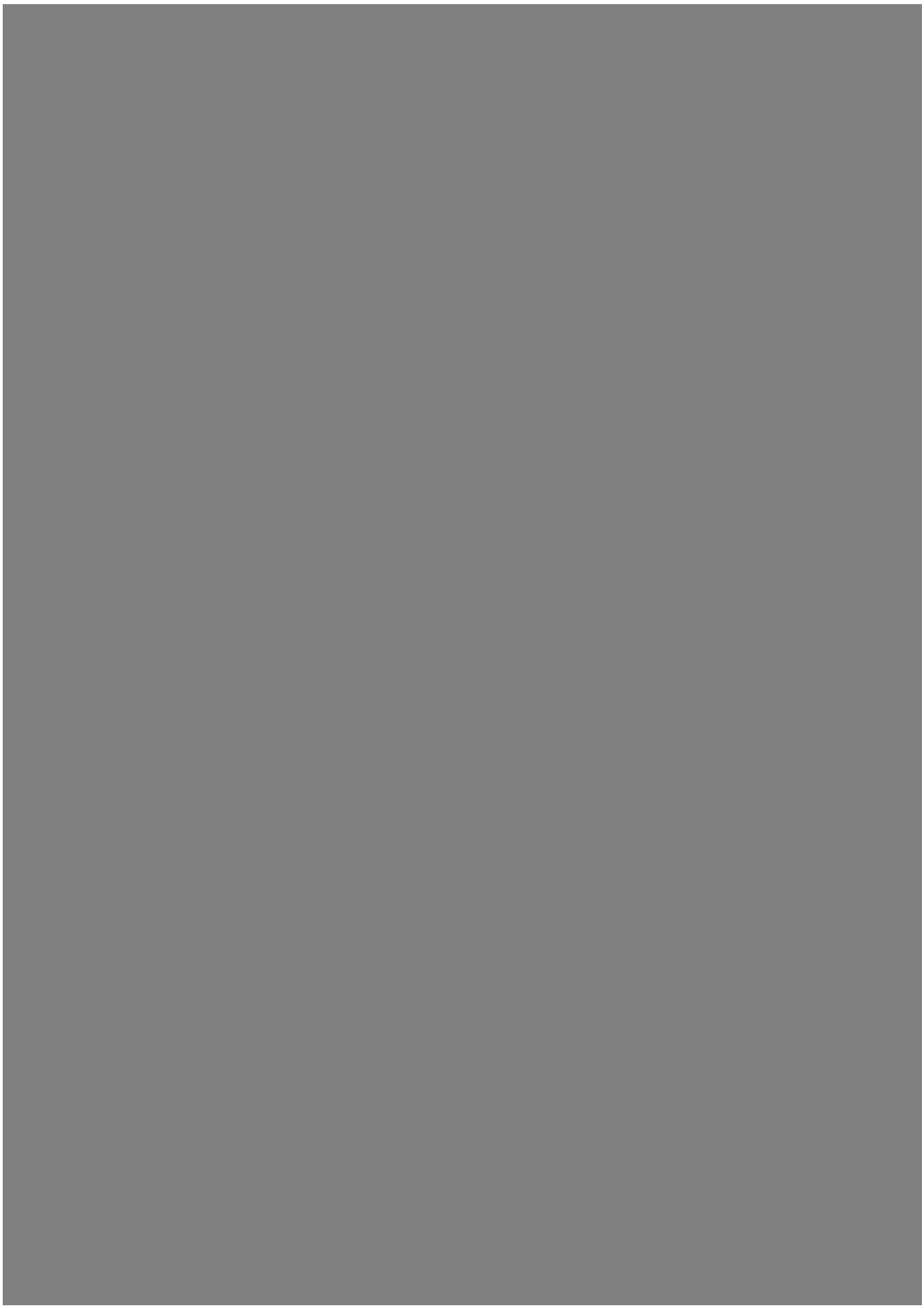


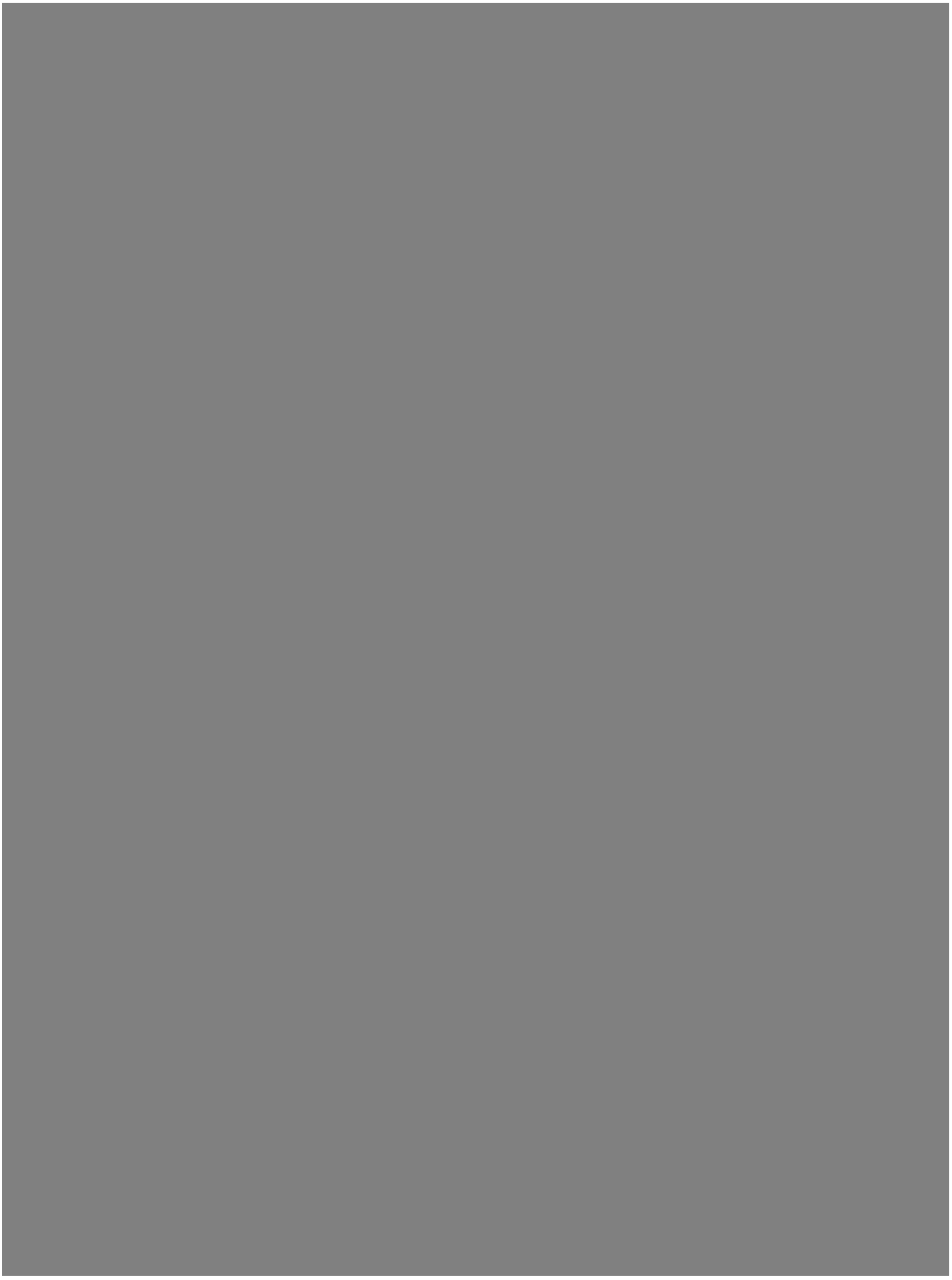


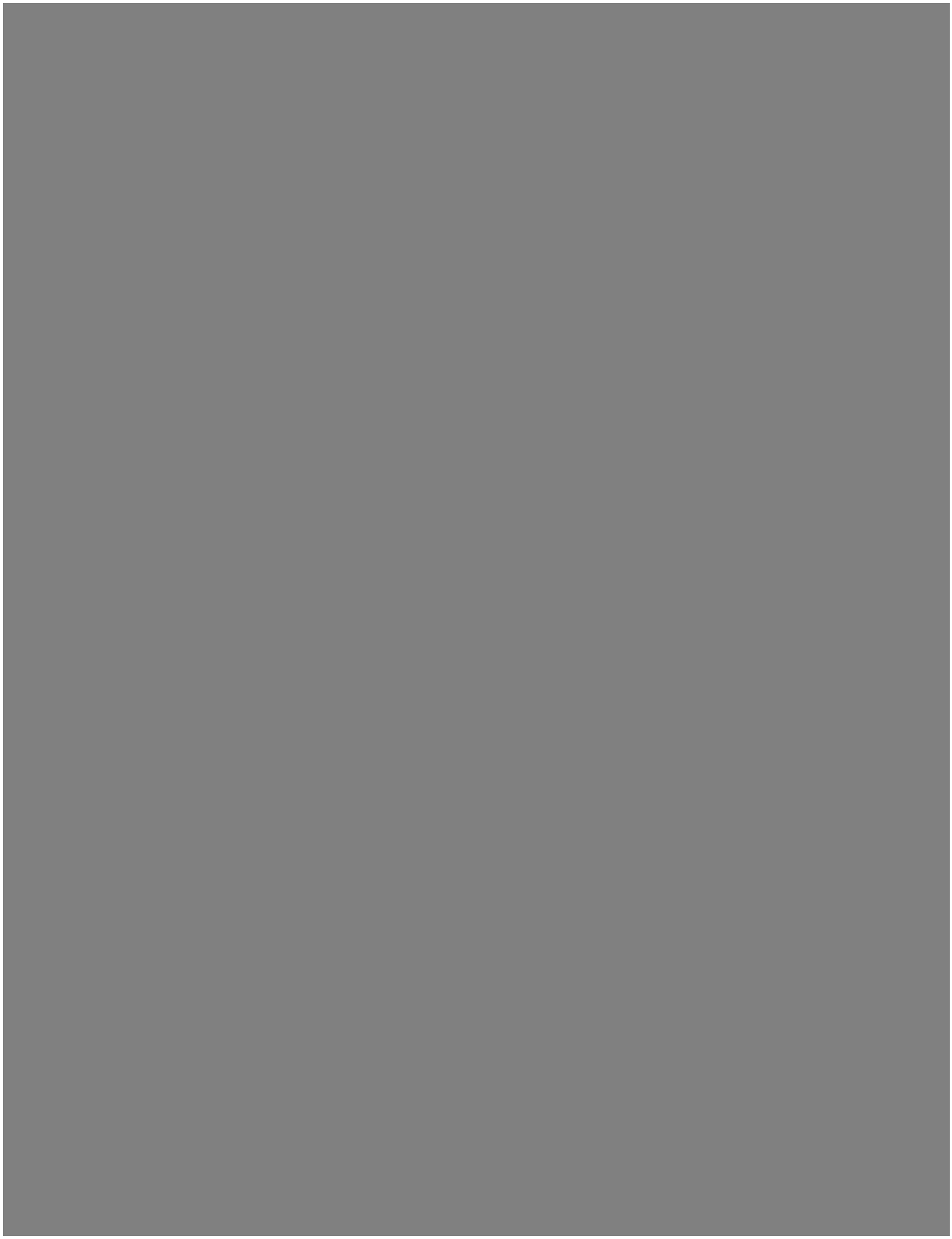


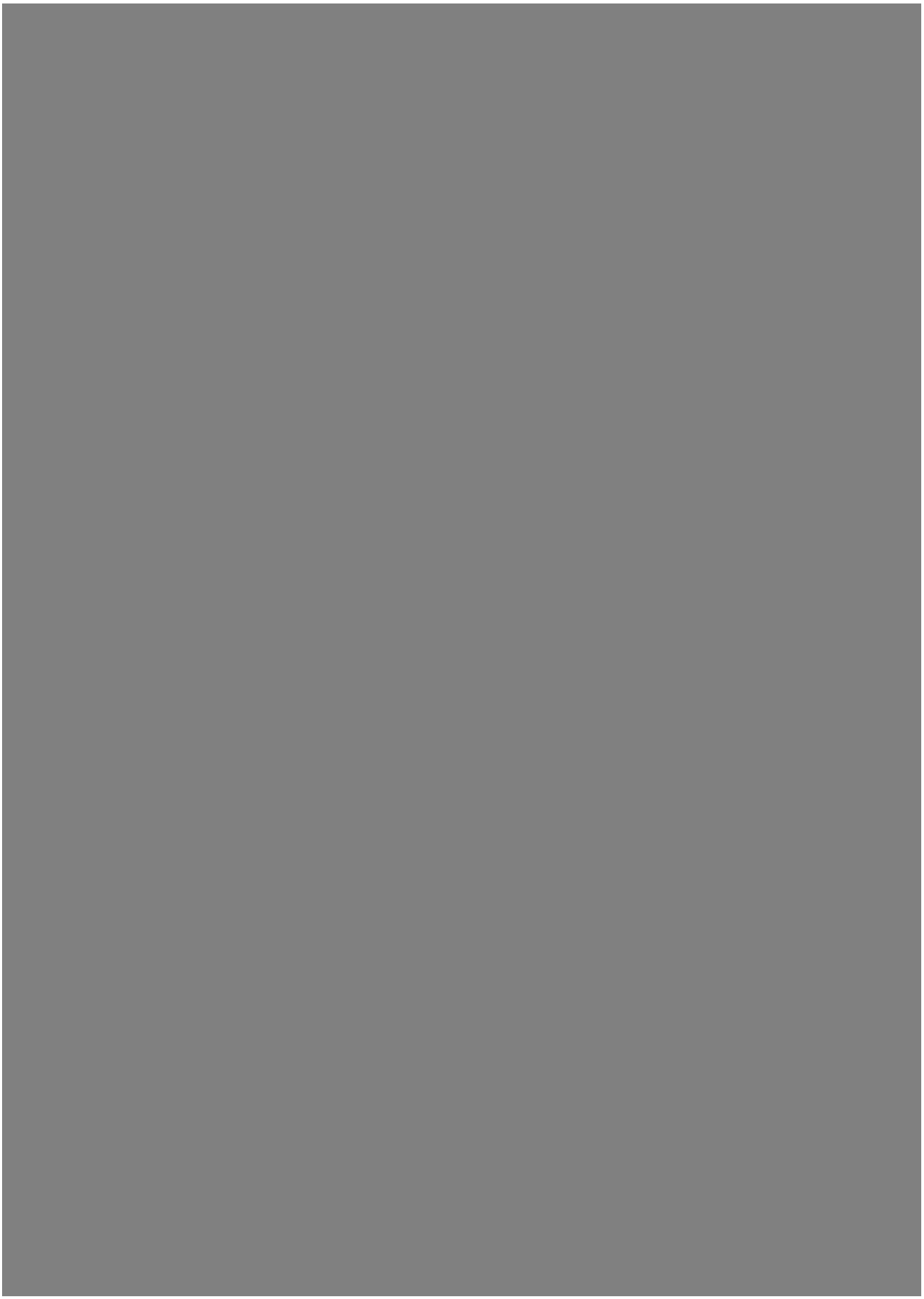












Appendix 8: Study Acknowledgements

I wish to thank all the women who participated in this study; all the principal investigators for supervising recruitment and randomization at the study centres; all the RESPONSE research nurses who assisted in study visits and collection of data; study pharmacists; the data and safety monitoring committee; Premier research, clinical research organisation; The Miscarriage Association; and all those not otherwise mentioned above who have contributed to the RESPONSE study.