THE UNIVERSITY OF ADELAIDE

Doctor of Philosophy Thesis

The ASK TEAMS approach: A systematic way to investigate couples with recurrent miscarriages

Catherine Dolores McCormack

Principal Supervisor: Claire Roberts

Co-Supervisor: Gustaaf Dekker

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Discipline of Obstetrics and Gynaecology

Adelaide Medical School

January 2020

Table of Contents

Abstract

The ASK TEAMS approach: A systematic way to investigate couples with recurrent miscarriages

C D McCormack

Introduction

Miscarriages are one of the commonest reasons that women in their reproductive years present to an Emergency Department, and they account for a large proportion of admissions, procedures, and morbidity of such patients. While a single miscarriage occurring in 15-20% of all clinically recognized pregnancies may be an isolated event for most women, recurrent pregnancy losses (RPL) account for 1-4% of all losses and are a separate clinical entity. Most recurrent miscarriage clinics now divide miscarriages according to gestational age, with those less than or equal to ten weeks termed embryonic losses, and those from ten to twenty weeks as fetal losses, instead of the original first or second trimester losses.

It is a frustrating situation both for the Couples suffering the losses, and for their Physicians, given the lack of good quality evidence regarding the management of these couples.

Recurrent pregnancy loss is seldom due to a single pathogenic factor, and most have a multifactorial background, involving multiple genetic and environmental risk factors.

In a dedicated recurrent miscarriage clinic, the aim was to develop an evidence-based diagnostic pathway for this group of women presenting with recurrent miscarriages.

Methods

A detailed history was obtained, and the diagnostic testing eventually led to the “ASK TEAMS” approach to identify potential issues that could lead to patient specific, safe and effective interventions.

The “ASK TEAMS” approach is as follows:

A **A**ge: Patients were offered an Anti Müllerian Hormone test as part of the work up;

S **S**tructure: Patients were offered a luteal phase 3D ultrasound for assessment of the pelvis and uterus;

K **K**aryotype: Miscarriage products were sent for karyotyping. Parental Karyotyping was restricted to those in whom a translocation had been detected in the products of conception;

T **T**hrombophilia: Screening for hereditary thrombophilias was restricted to those with a strong personal or family history of venous thromboembolism;

E **E**ndocrine: All patients were offered thyroid function tests, a 75g oral glucose tolerance test (OGTT) including insulin levels;

A **A**utoimmune: Antibody tests included thyroid peroxidase antibodies (TPO), anti-thyroid receptor antibodies (ATA), antinuclear antibody (ANA) titres. The antiphospholipid syndrome antibodies were also included;

M **M**etabolic: OGTT and Insulin studies, and patients also had fasting circulating homocysteine concentration quantified;

S **S**pecific: Vitamin D, B12 and folate studies were performed. Male partners were also tested for factors thought to contribute to sperm DNA damage, which may contribute to early miscarriages.

Results

While many of the findings were consistent with those of recurrent miscarriage clinics world-wide, some novel observations were made:

a) AMH levels were found to be significantly lower than in a normal population;

b) Women with raised fasting or two-hour insulin levels had an increased risk of gestational diabetes in a subsequent pregnancy;

c) Low vitamin D levels were significantly associated with hyperinsulinism in miscarriage patients.

Conclusions

The ASK TEAMS approach to investigations in an RPL Clinic facilitates a consistent work-up. Tests are adjusted as evidence accumulates regarding miscarriage causes/associations. Live birth rates are excellent following this approach.

Declaration of Authorship

I certify that this work does not contain any material that has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, does not contain any material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed: _____

Dated: 20/1/2020

It always seems impossible until it's done.

Nelson Mandela

Publications arising from this thesis

1) **Catherine D McCormack**, Shalem Y Leemaqz, Denise L Furness, Gustaaf A Dekker, Claire T Roberts. Anti Müllerian Hormone levels in recurrent embryonic miscarriage patients are frequently abnormal, and may affect pregnancy outcomes. Journal of Obstetrics and Gynaecology 2018.

https://doi.org/10.1080/01443615.2018.1552669.

2) **Catherine D McCormack**, Denise L Furness, Gustaaf A Dekker, Karen Shand, Claire T Roberts. 3D Ultrasound findings in women attending a South Australian recurrent miscarriage clinic. Australian Journal of Ultrasound in Medicine November 2016; 19(4):142-146.

3) **Catherine McCormack**, Shalem Leemaqz, Denise Furness, Gustaaf Dekker, Claire Roberts. Association between Vitamin D status and Hyperinsulinism. Journal of Maternal-Fetal and Neonatal Medicine 2018;

https://doi.org/10.1080/14767058.2018.148030.

4) **Catherine D McCormack**, Shalem Y Leemaqz, Denise L Furness, Gustaaf A Dekker, Claire T Roberts. Do raised two-hour pre-pregnancy insulin levels confer the same risks of developing GDM, as raised fasting levels, in recurrent miscarriage patients? Journal of Obstetrics and Gynaecology 2019;

https://doi.org/10.1080/01443615.2019.1672139.

5) **Catherine McCormack**, Shalem Leemaqz, Denise Furness, Gustaaf Dekker, Claire Roberts. The ‘ASK TEAMS’ approach to investigations for women with recurrent miscarriages. (To be submitted)

Acknowledgements

This work has been a phenomenal journey of discovery and hope that would not have been possible without so many people who have been a part of it since the beginning.

My Mentor, Keith Gunston, whose pragmatic support and encouragement was unwavering as I began my journey into Obstetrics and Gynaecology.

My Fellow researchers in the PAPO group, who recruited patients and enthusiastically collected the vital information.

My Scientific Supervisor, Professor Claire Roberts, with her incredible knowledge, who tirelessly directed me towards the scientific method, with great humour and endless patience.

My Co-Supervisor Professor Gustaaf Dekker whose boundless enthusiasm, optimism and knowledge was the reason I registered for a PhD at the beginning of the journey.

Shalem Leemaqz, whose statistical brilliance solved many of my questions and provided insights into the relevance of the work.

My Parents who never allowed me to think that there was anything I could not achieve, and who always supported me unconditionally.

My Family who have always been there for me, especially my husband whose support has been my inspiration.

And finally, my patients, who, despite their grief and anguish, selflessly joined the Research group in the hopes of achieving their dreams, and who were willing to help others even if their dreams were impossible. Being a part of their journey and ultimate joy, has been a profound privilege.

List of Figures

List of Tables

CHAPTER 1: Literature Review

1.1 Introduction

Recurrent Pregnancy Loss (RPL) is a life changing event for couples planning parenthood, and frequently a perplexing and challenging issue for their physicians. Despite the prevalence, research activity on recurrent miscarriages as measured by the the frequency of publications in relevant medical journals, or presentations at medical conferences, is extremely sparse, compared to research into assisted reproductive technology. There is also little or no consensus on the timing, gestational age or even the number of losses that comprise 'recurrent' losses. This vulnerable group of patients certainly comprise a significant proportion of patients who complain about the standard of care at an Institution, citing the lack of compassion for their situation, and the dearth of information about possible causes or interventions. Given the relatively minor medical impact of a miscarriage in comparison to major pregnancy complications and emergencies, it is obvious why such situations arise. However, if Institutions do not set up Early Pregnancy Units or Miscarriage Clinics, these patients are left with few explanations and minimal information, and may descend into depression and anxiety, or access some non-evidence based, potentially harmful, interventions.

1.2 Background

Pregnancy losses are the most common complication of pregnancy, occurring in up to 50 - 75% of all couples who are planning parenthood. Many of these losses were previously unrecognized, and occured before or with the next period, or slightly later, at which time they would have been presumed to be just a late period (RCOG, 2003).

However, with the advent of increasingly sensitive home pregnancy tests, more of the previously named ‘biochemical pregnancies’ are being identified by patients, and then counted as losses. A pregnancy that is confirmed by serum or urine Beta-HCG (β-HCG) levels, including non-visualized pregnancy losses after 6 weeks, (post heavy bleeding but no confirmatory ultrasound), and treated pregnancies of unknown location, are included. Ectopic pregnancies, molar pregnancies and implantation failures are not included. However, miscarriages after ART treatments should be included (Kolte et al., 2015). These pregnancies are further divided into Recurrent Early Pregnancy Losses, (REPL) or Embryonic Losses, before ten weeks of gestation. Losses from 10 weeks until the unit viability definition, would be Fetal or Recurrent Late Pregnancy losses (Atik et al., 2018).

Approximately 5% of couples planning a pregnancy will have two losses, and approximately 1% of couples will suffer three losses during their reproductive lives. As it has been estimated that the probability of three consecutive losses is about 0.34%, this is unlikely to be due purely to random chance (RCOG, 2003). Of recognized losses, 15 – 20% are ectopic pregnancies or spontaneous miscarriages that are diagnosed after clinical recognition of pregnancy. Unfortunately, the risk for a subsequent pregnancy loss increases with each successive loss. After one loss, the couple has approximately the normal population baseline risk of having another one, which is approximately 15% (Nybo Andersen et al., 2000). However, should they suffer two losses the subsequent risk increases to approximately 25%, depending on the combined age of the couple. Several studies have estimated that the risk of pregnancy loss after three successive losses is 30 – 45%, and up to 75% if the combined age of both partners is greater than 75 years (Petrozza et al., 2004; de la Rochebochard et al., 2003).

Recurrent Pregnancy Loss (RPL) is an independent risk factor for other adverse pregnancy outcomes including preterm labour, preeclampsia and gestational diabetes (Yang et al., 2006). Therefore, investigation of these patients could identify novel risk factors for such outcomes, and possibly inform early opportunities for intervention and treatment. Current trends in research in fetal medicine focus on the prediction (at 12 weeks of gestation) of patients at risk of adverse outcomes such as, preeclampsia, *abruptio placenta,* intrauterine growth restriction and stillbirth, but this is too late for women with embryonic loss. Given that first trimester placental development is implicated in a wide range of pregnancy complications (Roberts, 2010), identification of women at risk at 12 weeks' may be too late to reverse the aberrant placental developmental trajectory. By investigating the recurrent miscarriage patients following their loss, blood and other tests can be performed, potentially guiding substantial improvements to lifestyle, medication and/or supplementation. Identification of these patients prior to pregnancy would be far more cost effective than finding and treating them in the first trimester. Test results would presumably reflect the internal environment that may contribute to the losses, before women have taken steps to improve them, as the latter can confound future investigations and interventions. The prevailing belief has been that about 50% of recurrent pregnancy losses may have a risk factor detected (Bashiri et al., 2016, Li et al., 2002), which may or may not be the actual cause. Very few cases of RPL are caused by a single factor, the majority being an interaction between multiple genetic and environmental risk factors (Bashiri et al., 2016). However, in 2018 Popescu et al., investigated the addition of a 24-chromosome microarray of miscarriage tissue, along with routine investigations offered at their institution, at the time of a second or

later loss, and detected a definate or probable cause in 95% of the patients investigated (Popescu et al., 2018).

The heterogeneity and complexity make RPL difficult to research. To identify causal factors and test therapeutic interventions, large patient and control populations in multi-centre trials are needed to obtain level 1 evidence. As RPL is a far rarer condition than infertility, and given that there are very few dedicated RPL clinics, current evidence is limited to small trials.

Christiansen et al., (2014) suggested that there were two possible scenarios. Chromosomal errors resulting in losses with the rate of such losses being explained by chance and increased risk. The other, that maternal environmental issues such as endocrine, thrombophilic, structural or immunological abnormalities, could increase the losses of euploid conceptions, which, if detected, could be treatable. Such treatments/interventions would need to be evaluated in randomized controlled trials (Christiansen et al., 2014).

Identifying this complex group should be the role of recurrent miscarriage clinics, leading to evidence-based interventions. Genetic factors detected in families with RPL predisposition, may also increase risk for adverse pregnancy outcomes such as intrauterine growth restriction, preeclampsia and perinatal complications (Christiansen et al., 2014). A Maternal predisposition to endothelial cell inflammatory injury may also be part of the problem, as a factor that negatively affects placental growth, thus increasing the association between adverse obstetric and neonatal outcomes in pregnancies in patients with RM (Christiansen et al., 2014).

1.3 Causes

The pathogenesis of miscarriage involves a complex interaction of multiple factors, including environmental and life style factors and genetic factors as well (Zetterberg et al., 2004). There is also controversy regarding the number of miscarriages that constitute ‘recurrent’ pregnancy loss, and thus confusion as to when to classify a patient as ‘recurrent’ and in need of a full RPL evaluation. The Royal College of Obstetricians and Gynaecologists (RCOG) Green-top guidelines state that “recurrent miscarriage, defined as the loss of three or more consecutive pregnancies, affects 1% of couples trying to conceive” (RCOG 2011). The American Society for Reproductive Medicine (ASRM) in 2012, defined RPL as “a distinct disorder defined by two or more failed clinical pregnancies” (ACOG 2008). However, Kolte et al., (2014) showed that even non-visualized pregnancy losses are prognostically important in unexplained recurrent miscarriages. Specifically, each additional non-visualized loss conferred a similar risk for not achieving a live birth as the risks of each clinical miscarriage. Jaslow et al., (2010), showed that the findings in recurrent miscarriage patients after two, three or more losses did not differ significantly among the women with these losses and suggested that investigations should be performed following two or more consecutive losses. The latest European Society of Human Reproduction (ESHRE) Guidelines on RPL has accepted ‘recurrent pregnancy loss’ as two or more spontaneous losses before the fetus reaches viability. This would therefore include all pregnancies from conception until 24 weeks (Atik et al., 2018). However, many countries, including Australia, do not include losses over 20 weeks, and such losses are subsequently included in the Perinatal Morbidity and Mortality statistics for each Institution. Such losses would then be investigated as extreme preterm labour and stillbirth, despite the non-viability status. Each Institution may also

have different cut-offs for determining viability and deciding when intervention may be offered (Bashiri et al., 2016).

Consensus among clinicians working with RPL patients has not yet been achieved regarding the optimal work up, classification and interventions that should be offered. Clinicians working in the area should strive to consolidate existing knowledge, develop protocols and guidelines for management, and hopefully support Multicentered Randomized Controlled Trials, that could advance evidence-based treatment.

The presumed causes are varied and often controversial, with more than one ‘etiological factor’ being present. More than 50% of miscarriages are currently considered ‘unexplained’. The loss of aneuploid pregnancies is expected, and there is a good prognosis following these losses, with a lower risk of a repeated miscarriage. It is the loss of euploid pregnancies that are of most concern, especially as many studies have suggested that the recurrence risk increases with the number of previous losses (Christiansen et al., 2014; Jaslow et al., 2010).

Prognosis should therefore be estimated by monitoring the number of live births per unit time after a diagnosis of recurrent miscarriages is determined (Jaslow et al., 2010). In a Danish study using complete national birth registries, over a period of 22 years, it was found that in women identified as ‘recurrent miscarriage patients’, 66.7% had achieved a live birth after 5 years and 71.1% after 15 years (Jaslow et al., 2010). Among women who had experienced 6 or more miscarriages, 50.2% had achieved a live birth after 5 years (Jaslow et al., 2010).

Primary RPL is defined as pregnancy loss without any previous live births, and secondary RPL is when the couple has had at least one live birth, and experience

pregnancy losses as well. Controversy remains as to whether there are differences in findings between these two groups, regarding the live birth rates (Christiansen et al., 2007; Bashiri et al., 2012).

Defining the gestational age of RPL's for the purposes of counselling and possible investigations is very important. Couples experiencing extremely early losses are more likely to have aneuploidy than those experiencing later losses in the second trimester. In the Second trimester losses are more likely to have structural abnormalities, cervical insufficiency and thrombophilias than first trimester losses, but no presumed etiology is restricted to a certain gestational age (Ohno et al., 1991; Drakely et al., 1998; Fritz et al., 2011; Bashiri et al., 2016).

Every couple experiencing RPL should have access to a complete work up and possible evidence-based interventions. How each clinic defines RPL and determines their investigations depends on their population and the availability of the selected tests for presumed causes.

The most common presumed 'causes of' or 'associations with' RPL's are as follows:

Genetic, structural, autoimmune, thrombophilic, endocrine, parental age, metabolic and lifestyle (Bashiri et al., 2016).

1.3.1 Age

Maternal age is one of the commonest independent risk factors associated with pregnancy loss, and the risk of loss increases with advancing maternal age. Nybo Andersen et al., (2000) showed that the risk of miscarriage at age 20-24 years was approximately 8.9%, increasing to 74.4% in women aged 45 years or more. The ESHRE guidelines suggest that women ought to be informed that the risk of

pregnancy loss is lowest in women aged 20-35 years, and should have a discussion regarding the age of the couple, and the fact that the risk of pregnancy loss increases significantly after the age of 40 years (Atik et al., 2018).

The term that is used to describe a woman's reproductive potential, as it relates to the quality of her oocytes and her own individual follicular depletion, is ovarian reserve. Serum Anti-Müllerian Hormone (AMH) levels are an ideal marker of a woman's ovarian reserve, as AMH is highly correlated with the number of antral follicles remaining. (Visser et al., 2006; Barad et al., 2009). AMH is a member of the transforming growth factor β family of growth and differentiation factors. AMH has an inhibitory effect on primordial follicle recruitment in the ovary, as well as on the responsiveness of the growing follicles to follicle stimulating hormone (FSH) (Barad et al., 2009).

The ovary-specific expression pattern in granulosa cells of the growing non-selected follicles thus makes AMH an excellent marker for the size of the remaining ovarian follicle pool. The size of this follicle pool is established very early intrauterine life. Germ cells populate the ovary and become surrounded by somatic cells, forming the so-called primordial follicles (Gruitjers et al., 2003). At birth, about one million oocytes are present. This number decreases pre-pubertally, resulting in a primordial follicle pool of 300 000 to 500 000 follicles at menarche (Gruitjers et al., 2003). Throughout a woman's reproductive life, follicles leave the primordial follicle pool, and enter the growing antral follicle pool. Most of these growing follicles will be lost as a result of atresia, unless they are rescued by FSH (Baird et al., 2005). This rescue begins after puberty when the pituitary-gonadal endocrine axis has been activated. Only one follicle is selected to become the dominant follicle each menstrual cycle, ovulating under the influence of luteinizing hormone (LH) (McGee et al., 2005). This process

continues until the primordial pool is exhausted, resulting in menopause (Seifer and Naftolin, 1998). Fertility declines in the years preceding menopause, and the menstrual cycle becomes irregular, ultimately ceasing. There is significant individual variation in the age of menopause and, subsequently, also in the age of 'subfertility' (te Velde et al., 1998). Chronological age is thus a poor indicator of individual reproductive ageing and also of actual ovarian reserve (Faddy et al., 1998).

Traditionally the 'perimenopause' was described as the time when ovarian function began to decline to the extent that clinical symptoms such as irregular menstruation started (Faddy et al., 1992). In recent years, assisted reproductive technology (ART) has given us a better understanding of the events that happen before the menopause (Dolleman et al., 2013). A significant decline in ovarian function begins much earlier than previously thought, most likely in the mid-thirties, (Volarcik et al., 1998) and possibly earlier in some women (Faddy et al., 1998). As a result of compromised endocrine, paracrine and autocrine signals due to the effects of ageing, there is altered communication between granulosa cells and the oocytes, which results in abnormal nuclear and cytoplasmic maturation within the oocyte. An accelerated loss of follicles then occurs, as well as qualitative changes in those remaining (Nikolau et al., 2002), (Bopp et al.,1998). The clinical result is an increase in the incidence of miscarriage and a decrease in fertility rates, both spontaneous conceptions and with ART. This frequently occurs without obvious clinical symptoms of endocrine deficiency, during the peri-menopausal period (Baird et al., 2005).

Lyttle Schumaker et al., (2018) showed a marked increase in miscarriages in spontaneously conceived pregnancies of women with markedly decreased AMH levels. Their rate was twice that of women with normal levels, suggesting that AMH levels are inversely associated with miscarriage risk (Lyttle Schumaker et al., 2018).

1.3.2 Structural and Pelvic abnormalities

Congenital uterine abnormalities (CUA's) have been thought to be a cause of pregnancy loss and adverse pregnancy outcomes (Christiansen et al., 2005). The reported prevalence of these abnormalities in women suffering recurrent pregnancy loss varies from 6 to 38% (Salim et al., 2003). CUA's are thought to result from abnormal formation, fusion or resorption of the Müllerian ducts during fetal life (Jaslow et al., 2013). The prevalence of major CUA's is thought to be at least 3-fold higher in women with a history of recurrent miscarriages in both embryonic (gestational age of ≤10 weeks from the last menstrual period) and fetal (gestational age of >10 weeks) loss, compared with the low risk population. Therefore, CUA's may indeed be responsible for pregnancy loss in a significant proportion of women with recurrent miscarriages (Saravelos et al., 2005).

Polycystic ovarian syndrome is a complex endocrine disorder characterized by anovulation and oligomenorrhoea, infertility, hyperandrogenism, and metabolic dysfunction (Trikudanathan et al., 2015). Polycystic ovaries may be detected on 3D ultrasound, as large volume ovaries containing greater than 25 peripherally distributed follicles, each measuring from 2 to 9mm in diameter. However, the detection of 'Polycystic Ovaries' does not establish a diagnosis of the Polycystic Ovarian Syndrome (PCOS), unless other features are present such as hyperandrogenism and chronic anovulation (Zhu et al., 2016).

One study suggested that the prevalence of fibroids in women experiencing recurrent pregnancy losses was about 8.2% while that of the infertile population was 1 - 2.4% (Saravelos et al., 2011). Prevalence in the general population is thought to be from 3

-10%, however, it is difficult to estimate (Laughlin et al., 2009). Furthermore, fibroids are thought to be associated with mid-trimester losses amongst women with recurrent miscarriages (Saravelos et al., 2007). Pregnancy loss in women with submucosal fibroids is thought to be primarily due to decreased implantation rates. Resection of fibroids that distort the uterine cavity can eliminate the mid-trimester losses and result in a doubling of the live birth rate (Saravelos et al., 2007). In their group, the live birth rate increased from 23.3 to 52.0% ($P<0.05$) post myomectomy.

Adenomyosis is a non-neoplastic condition, affecting many women, characterized by a benign invasion of the myometrium by ectopic endometrium, that is accompanied by hyperplasia of the adjacent smooth muscle (Martines-Conejero et al., 2011). Such invasion may be detected during a 3D ultrasound but there are insufficient studies available to be able to assign causation in relation to RPL. Endometriomas and endometriosis may be detected by 3D ultrasound. There is conflicting evidence regarding surgical removal of endometriomas, as such removal could damage the ovarian reserve, and compromise future fertility (Ahmadi et al., 2014).

Asherman's syndrome is described as the presence of intrauterine adhesions composed of fibrotic tissue and is a pathological condition associated with pregnancy loss. It is thought to arise following trauma to the basal layer of the endometrium, usually following a curettage, infection or inflammation, with a high level of recurrence, even after treatment (Ahmadi et al., 2014).

1.3.3 Karyotype/Chromosomal

Carp et al., (2001) noted, in a series of 167 cases, chromosomal abnormalities in 36 (21.6%) of them, the commonest of which was trisomy, found in 24 (66.6%) of the 36

cases; 5 out of the 24 were trisomy 21, which was the commonest aberration, followed by trisomies 16 and 18, triploidy, monosomy X, and unbalanced translocations. Warburton et al., (1987) reported on 273 women who had two losses karyotyped and concluded there is no increased risk of another trisomy after a previous trisomic miscarriage, resulting in a very favourable prognosis for these women in subsequent pregnancies (Warburton et al., 1987).

It is thought that 33% of all malformed fetuses approximately and 66% of malformed embryos have underlying chromosomal abnormalities. 3% of cytogenetically abnormal concepti also have structural rearrangements. In the familial translocations, 66% are maternally derived and the remainder are of paternal origin. However, approximately 50% of all unbalanced translocations arise *de novo* during gametogenesis (Jacobs et al., 2013). Balanced parental rearrangements are found in 3-6% of RPL's and the most commonly encountered parental rearrangements include balanced translocations and inversions. The risk of future pregnancy losses in these cases is influenced by both the size and the genetic makeup of these rearranged chromosomal segments (Jacobs et al., 2013).

While routine karyotyping of the couple was previously recommended, this has changed in recent years, particularly after it was shown by Franssen et al., (2006) that couples with at least two previous miscarriages who carried translocations or inversions, had the same chance of having a healthy child as non-carrier couples with at least two miscarriages, 83% and 84%, respectively. More importantly, these couples also had a low chance (0.8%) of carrying pregnancies with an unbalanced karyotype beyond the end of the first trimester (Warburton et al., 1987). Detection of an unbalanced translocation in the fetus or embryo should be followed by parental

studies. The latest ESHRE guidelines also suggest that parental studies should only be offered after a translocation is detected in the previous loss (Atik et al., 2018).

Genome damage may be associated with adverse pregnancy outcomes, including recurrent miscarriages. Individual diets as a key factor in determining genomic stability is more relevant than previously thought, because it is now known that diet impacts on all relevant pathways, i.e. exposure to dietary carcinogens, activation/detoxification of carcinogens, DNA repair, DNA synthesis and apoptosis (Fenech et al., 2001).

In 2018 Popescu et al., included a 24-chromosome microarray analysis on the products of miscarriages, as well as the recommended American Society for Reproductive Medicine (ASRM) standard work up, to establish if this would explain most losses. They discovered that a definite or probable cause was then identified in over 90% of RPL patients at the time of a second or subsequent loss (Popescu et al., 2018.

1.3.4 Thrombophilias

In the 1980's, it was suggested that antiphospholipid antibodies (aPL's) could have a causal association with recurrent miscarriages, following the detection of placental clots in patients with positive serum aPL's and pregnancy loss (Kutteh et al.1996). In the 1990's it was noted that treatment of these patients with aspirin and Heparin increased the live birth rate (Kutteh et al., 1996). The assumption was that clotting issues were the most likely cause,and assumed that the inherited thrombophilia's such as factor V Leiden, (FVL 1691→A) prothrombin gene mutation, (20210G PGM), methylene tetrahydrofolate reductase (MTHFR) deficiency and antithrombin III (ATIII)

deficiency, could also cause pregnancy losses due to their direct or indirect ability to induce hypercoagulable states. MTHFR polymorphisms are thought to be associated with clotting due to the association with mild to moderate homocystinaemia, which is an independent risk factor for arterial thrombosis (Kutteh et al., 1996). These thrombophilias induce a hypercoagulable state via indirect or direct augmentation of prothrombin to thrombin, its active clot-inducing form. It was thought that these thrombophilias may also cause other adverse pregnancy outcomes such as intrauterine growth restriction (IUGR), placental abruption, preeclampsia and stillbirth (Kist et al., 2008). Despite numerous studies, results are conflicting, and level one evidence is lacking. Most of this inconsistency is due to including the incorrect patient groups, i.e. including embryonic losses when evaluating the effects of, for instance factor V, instead of just fetal losses. Kist et al., (2008) showed a higher incidence of FVL in women with recurrent fetal loss in the 2nd and 3rd trimester than in the 1st. Given the absence of a functional intervillous space up to 9-10 weeks of gestation (embryonic losses) it is unlikely that placental thrombosis would be an issue at this early stage (Kist et al., 2008).

Activated factor V is a clotting protein that acts in conjunction with activated factor X to directly convert prothrombin to thrombin. A single gene defect (F5c.1691G>A and p.Arg506Gin) results in a protein that is resistant to inactivation, increasing levels of factor V, increased thrombin and therefore an increased risk of venous thromboembolism (VTE) (Lockwood et al., 2011). The heterozygous form of this mutation is the commonest inherited thrombophilia in the general population. However, the prevalence is low and less than 0.3% of women will have a VTE in pregnancy (Lockwood et al., 2011).

Two reviews concluded that female carriers of FVL G1691A have an increased relative risk for RPL (odds ratio [OR] 1.52, 95% confidence interval [CI] 1.06-2.19 and OR 2.02., 95% CI 1.60-2.55) (Bradley et al., 2012; Roger et al., 2010).

However, the maternal-fetal medicine (MFM) network also emphasized a very low absolute risk (4.2%) of actual pregnancy loss in the women who carry the FVL gene defect (Roger et al., 2010). Atik et al., (2018), in the ESHRE guidelines on recurrent miscarriage, suggest that screening should not be performed unless it is in the context of research or in women with additional risk factors for thrombophilia. It should also be noted that such losses would be fetal and not embryonic (Atik et al., 2018).

PGM G20210 substitution mutation (F2 c20210G>A) is the second most common inherited thrombophilia, and causes a decrease in thrombin, with a resulting increase in concentration of prothrombin in the plasma of carriers. VTE incidence is low in heterozygotes at an absolute risk of 0.5% while homozygotes have a risk of 2-3%. Although Bradley et al., (2012) suggested an increased risk of RPL in F2 c20210G>A carriers, the MFM network did not find any association (Silver et al., 2010).

Given that FVL and PGM have a low prevalence in the general population, and that women with these variants can have a normal pregnancy, it is felt that they have a relatively small impact on the absolute risk of a pregnancy loss, and high-quality evidence is lacking. The balance of benefit versus harm regarding these treatments has not been studied in any major trials. Bradley et al., 2012, in a large meta-analysis, suggested that evidence is moderate that anticoagulation of women with recurrent pregnancy loss and FVL/PGM variants could lead lead to harm (Bradley et al., 2012). Each RPL case should be evaluated separately and the history of each

patient with regard to documented gestational age of previous losses taken into account. Fetal losses after establishment of a placenta may require a different approach with regard to anticoagulation, compared to embryonic losses.

The efficacy and safety of aspirin and heparin in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia have a limited number of studies (De Jong et al., 2014). A Cochrane review found no beneficial effect of anticoagulants in studies at low risk of bias, and thus the review does not support the use of anticoagulants in women with unexplained recurrent miscarriage. Further randomized controlled trials are needed; at present there is no evidence of a beneficial effect (De Jong et al., 2014).

1.3.4.1. MTHFR

One-carbon metabolism during embryonic development has been studied predominantly with regard to the development of the nervous system in the fetus. Pregnant women who are folate deficient have a greatly increased risk of having babies with neural tube defects (NTD) and peri conceptional folic acid supplementation protects against this effect. The molecular events that result in NTD due to folate deficiency are unknown but may include dysfunctional methylation of crucial metabolites in the developing embryo and/or abnormalities in neural cell proliferation, differentiation and apoptosis, which may be due to DNA nucleotide misincorporation that resulting from folate deficiency in rapidly proliferating cells (Govindaiah et al., 2009). It is thus possible that these critical events also might lead to decreased fetal viability and possibly pregnancy loss as well. A population-based case control study showed an increased risk of early miscarriages in pregnant women with low plasma folate levels and/or Vitamin B12 deficiency during

pregnancy, that results in elevated homocysteine concentrations in the embryo and increases the incidence of NTD (Govindaiah et al., 2009; Van Der Put et al., 1997; Steen et al., 1998). Three other studies suggested a direct embryotoxic effect of homocysteine (Rosenquist et al., 1997; Greene et al., 2003; Zetterberg et al., 2004). Recently there has been more emphasis on the likely role of epigenetic processes and DNA methylation that occurs throughout gestation. B6/B12 deficiencies also affect DNA methylation (Kos et al., 2018).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that exists in the cytoplasm of cells and irreversibly reduces 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate (Zetterberg et al., 2004). This is used to convert homocysteine to methionine by the enzyme methionine synthase. MTHFR is involved in regulating the circulatory levels of homocysteine. Folic acid, vitamin B6 and B12 are also important cofactors in this metabolic pathway. Two single nucleotide polymorphisms (SNPs) are currently investigated in the MTHFR genotype of couples with recurrent miscarriages, C677T and A1298T. The normal 'wild type' allele at position 677 is C and the normal wild type allele at position 1298 is A. If substitutions occur at these loci, the patient then becomes 'heterozygous or homozygous' for the common thermolabile polymorphism (Zetterberg et al., 2004).

Mutations in the MTHFR gene causes the enzyme to be thermolabile with mild loss of activity. Homozygosity for this gene variant is present in at least 11% of the Caucasian population and can lead to hyperhomocysteinaemia when there is a coexisting nutritional folate deficiency. Deficiencies of Vitamin B12 (cobalamin) and folic acid can also result in elevated plasma homocysteine levels (Zetterberg et al., 2004). As the fetus inherits two of these alleles from the Mother and two from the

Father, couples with 2 or more polymorphisms in total, have the chance of transmitting at least 2 of these alleles to the fetus.

In women, hyperhomocysteinaemia is associated with increased risk of having a child with a neural tube defect or a fetal loss. (Kos et al. 2018). However, folic acid supplementation during pregnancy lowers homocysteine concentration and reduces the risk of these adverse pregnancy outcomes. The association of MTHFR gene mutation with RPL is controversial, with conflicting reports in the literature. Nelen et al., (2000), noted that hyperhomocysteinaemia was a risk factor for recurrent pregnancy loss. Other authors found that women carrying the mutant allele of the MTHFR C677T polymorphism had elevated serum levels of homocysteine and idiopathic recurrent miscarriage, in a Middle-European White population (Unfried et al., 2002; Couto et al., 2005). The presence of an anti-cardiolipin antibody together with the MTHFR C677T mutation, is associated with recurrent abortions (Kumar et al., 2003), while an increased risk of spontaneous abortions with maternal MTHFR C677T genotype has been demonstrated (Lissak et al., 1999). However other reports indicate that MTHFR C677T homozygosity is not associated with recurrent pregnancy loss (Foka et al., 2000; Holmes et al., 1999).

The biochemical consequences of these polymorphisms can be modified by increasing folate and Vitamin B12 supplementation in women. Whether the supplementation should be routine ‘standard’ doses or increased doses needs to be investigated. The only evidence to date regarding ideal doses is with reference to decreasing homocysteine concentrations, in non-pregnant patients (Holmes et al., 1999). Studies have demonstrated that daily doses of folic acid at 5-10 mg resulted in higher serum folate levels, lower total plasma homocysteine levels, and improved endothelial function (De Vecchi et al., 2003). However, ACOG and the MFM network

does not recommend screening women for the MTHFR mutation with either adverse fetal outcomes or a history of a prior VTE (Lockwood et al., 2011; Bezemer et al., 2007).

1.3.4.2. Protein C and Protein S Deficiency.

Congenital protein C or S deficiency is an inherited disorder that causes abnormal blood clotting. Heterozygous protein C deficiency occurs in approximately 1 in 300 adults (Clouse et al., 1986). Protein S deficiency occurs in about 1 in 20,000 people (Clouse et al., 1986).

ESHRE guidelines suggested that hereditary thrombophilia should not be screened for except unless patrt of research studies, or in women likely to have additional risk factors for thrombophilia, such as a personal history of thrombosis or a strong family history of thrombosis (Atik et al., 2018). It must be stressed, however, that they are referring to all recurrent pregnancy losses, and not just embryonic.

1.3.4.3 Antiphospholipid syndrome

The Antiphospholipid syndrome (APS) was recognized over three decades ago to have a causal association with recurrent pregnancy loss and in 1996, Kutteh et al., demonstrated that treatment of pregnant women with antiphospholipid antibodies (APL) syndrome with aspirin and heparin increased the live birth rate to 80% (Kutteh et al., 1996).

Antiphospholipid antibodies are a heterogeneous group of autoantibodies, that are detected in the sera of patients with both autoimmune and various non-autoimmune diseases. However, they may also be detected in subjects with no overt underlying disease – the primary antiphospholipid syndrome. (PAPS) (Kutteh et al., 2006). High titres of APL are associated with arterial and venous thrombosis, recurrent fetal loss, and thrombocytopenia, (Leaf et al., 2017, Roubey et al 1996), as well as stillbirth, preterm birth, preeclampsia and intrauterine growth restriction (Branch et al., 1989, Ticani et al., 2002).

The clinically relevant APL include the Lupus anticoagulant – Kaolin clotting time, (KCT) Dilute Russell Viper Venom test (DRVVT), anti-β_2 glycoprotein 1 antibodies and anticardiolipin antibodies. In the general population the prevalence is 1-7%, (Lockwood et al., 1989), while in patients with RPL it is found in up to 20% (Lockwood et al., 1989, Jaslow et al.,2010).

Criteria for the classification of patients with definite APS (Sapporo criteria) developed in 1998, (Lockshin et al., 2000) provide a basis for including patients with the syndrome in research protocols rather than a guide to diagnosing the syndrome in individual patients. In order to fulfil these “Sapporo criteria”, patients must have had either vascular thrombosis or fetal loss and demonstrate evidence of APL either by the detection of anticardiolipin antibodies or a positive lupus anticoagulant. Furthermore, autoantibodies must be detected on at least 2 occasions, 12 weeks apart, in order to distinguish persistent autoimmune antibody responses from transient responses caused by infection or drug exposures (Lockshin et al., 2000). Although these criteria suggested three or more pregnancy losses, evidence by van den Boogaard et al., (2013) has shown no difference in the sequence of pregnancies, the total number of pregnancy losses or even in maternal age, between women with RPL and APS and women with unexplained RPL. Therefore, it is now considered justifiable to test for APS in women attending a RPL clinic who have a history of two or more pregnancy losses, whether consecutive or not (Van der Boogaard et al., 2013; Atik et al., 2018; Miyakis et al., 2006).

A diagnostic workup for APL’s should be considered in all patients with venous or arterial thrombosis and/or fetal/embryonic losses for which there is no alternative

explanation, especially if the losses are recurrent (Miyakis et al., 2006). Likewise, unexplained thrombocytopenia, haemolytic anaemia and prolongation of any phospholipid coagulation tests should lead to determination of APL status (Oshiro et al.,1996, Bouvier et al., 2014).

Although the risk of pregnancy loss in women with APL was initially thought to be the greatest from the 10th week of gestation onward, (fetal period) (Lockshin et al., 1985. Lima et al., 1996), it is now known that APL are also associated with early pregnancy loss, due to placental damage, including inadequate trophoblast invasion, which is the most common histopathologic finding (Kutteh et al., 2014). There is also evidence that women with APL have an increased risk of adverse pregnancy outcomes such as giving birth to a premature infant because of pregnancy-associated hypertension and uteroplacental insufficiency (Kutteh et al., 2014).

Tong et al., (2014) in a systematic review concluded that although the underlying mechanisms by which APL cause adverse pregnancy outcomes are unknown, they reiterated the effects on trophoblast invasion, including reducing trophoblast viability, syncytialization and invasion *in vitro*. There is some corroborating evidence of these effects *in vivo* (Sebire et al., 2002; Bose et al., 2006) which could contribute to impaired placental development and dysfunction in APL affected pregnancies.

Several studies also reported that APL may affect the production of cytokines, hormones and other signalling molecules by trophoblasts *in vitro* (Van Horn et al., 2004; Stone et al., 2006). There is also some evidence that APL may affect placental coagulation and complement activation. However, thrombosis and complement activation are not likely to be the main mechanisms for the problems experienced in

women with APL, particularly regarding embryonic losses (Sebire et al., 2002, Salafia and Cowchock 1991).

Determining the presence of APL's could allow therapeutic intervention, and hopefully, prevent not only miscarriages, but also the associated adverse later pregnancy outcomes (Kutteh et al., 2014). Indeed, the recent ESHRE guidelines have recommended screening for APS in women with recurrent pregnancy loss as it can allow treatment that may not only prevent pregnancy loss but also decrease subsequent pregnancy complications (Atik et al., 2018).

1.3.5 Endocrine

Thyroid disorders have been considered as one of the commonest causes/associations with RPL. Hypothyroidism is thought to be one of the most common endocrinopathies detected among young fertile women, and thyroid disorders have been found to be associated with several adverse pregnancy outcomes, such as infertility, early pregnancy loss, preeclampsia, preterm labour and delivery and stillbirths (Negro et al., 2011; Vissenberg et al., 2015). Thyroid antibodies are also associated with several adverse pregnancy outcomes such as recurrent pregnancy losses and preterm deliveries even in women who are apparently euthyroid (Negro et al., 2011). Thyroid antibodies have been associated with increased rates of preterm deliveries in pregnant cohorts, when compared with women who were thyroid antibody negative (Glinoer et al.,1994; Ghafoor et al., 2006). However, this association is not consistently demonstrated in other pregnancy cohorts (Tierney et al., 2009; Stagnaro-Green et al., 2005). Another study found an increase in perinatal infant death among patients who were antibody positive (Mannisto et al., 2009).

The definition of ‘normal’ thyroid stimulating hormone (TSH) during pregnancy is controversial, with some evidence suggesting that women with levels below 2.5 mlU/L had significantly fewer pregnancy losses than women with higher levels (Negro et al., 2010). Subclinical hypothyroidism (SCH) in pregnancy has recently been defined as a TSH level above the pregnancy-related reference range with a normal serum thyroxine concentration (Stagnaro-Green et al., 2011). The majority of SCH is caused by autoimmune thyroiditis but may be due to iodine deficiency in some populations. (Lazarus et al., 2014; Bernadi et al., 2013).

1.3.6 Autoimmune

Women with autoimmune diseases such as systemic lupus erythematosus (SLE), have a higher risk of embryonic and fetal losses than women without such autoimmunity. (Christiansen et al., 2008). Some patients may have the Lupus anticoagulants (Kaolin clotting time KCT, or the dilute russell viper venom test DRVVT) positivity, concomitantly, and these are known to be associated with RPL. Antinuclear antibodies (ANA) have been studied in relation to losses, but the evidence is unconvincing regarding a direct link. It is more likely that they indicate an interruption in the women’s immune tolerance, and may be a marker of other autoimmune problems (Christiansen 1996).

Antiphospholipid antibodies could be included in the autoimmune section, however, they have been discussed above for historical reasons, as many institutions still believe that they are associated with clotting. This is unlikely in the embryonic group due to the trophoblast structure. However, thrombophilias are most likely to be an issue in fetal losses.

Thyroid antibodies associated with thyroid autoimmunity is the commonest cause of thyroid dysfunction, and the presence of thyroglobulin autoantibodies (Tg-Abs) or thyroid peroxidase autoantibodies (TPO-antibodies) induces a chronic lymphocytic thyroiditis that can destroy the thyroid gland (Crawford et al., 2016). These antibodies are fairly common in women of reproductive age, and although they can lead to thyroid problems, most women with them are euthyroid (Bülow-Pedersen et al., 2009). They are probably the manifestation of gene environment interactions. The genetic predisposition is most likely polygenic, involving several low-penetrance, low risk alleles. Environmental factors such as maternal low birth weight, selenium deficiency, iodine excess, and reproductive life span may also play a role in the development of antibodies (Prummel et al., 2004). Although the fetus does not make thyroxine until the second trimester, it does influence the maternal supply of thyroxine to itself. Thus, the conceptus, through the production of placental human chorionic gonadotrophin (hCG), indirectly increases the demand for thyroxine (T4), and also directly stimulates the maternal thyroid to produce T4 (Glinoer et al., 1990). In women with normal thyroid function, thyroxine equilibrium is maintained. Women with abnormal thyroid function need to increase their T4 levels in order to compensate for the demands of pregnancy and to maintain normal TSH levels (Alexander et al., 2004).

A meta-analysis of 22 studies showed a clear association between thyroid autoimmunity and miscarriage with a pooled odds ratio (OR) of 2.5 in eight case-control studies and a pooled relative risk (RR) of 2.3 in 14 cohort studies (Chen et al., 2011). Another meta-analysis of 31 studies evaluated linkage between anti-thyroid antibodies and miscarriage, with 28 studies showing a positive association (Thangaratinam et al., 2011). After dividing the meta-analysis into cohort and case-

control studies, the data in the cohort showed an OR of 3.9 for miscarriage with the presence of thyroid autoantibodies. The OR of miscarriage for women with RPL with positive thyroid antibodies was 4.22. For case-control studies the OR for miscarriage was 1.8, and slightly higher in women with RPL (OR 1.86, *p*=0.008). (Thangaratinam et al., 2011). Clinical practice guidelines cosponsored by the American Association of Clinical Endocrinologists and the American Thyroid Association, state that anti-TPO measurement should be considered when evaluating patients with RPL, regardless of infertility (Garber et al., 2012). A recent systematic review and meta-analysis confirmed the benefits of levothyroxine (LT4) supplementation on both naturally conceived and ART pregnancies in women with SCH. Supplementation was associated with a decrease in both pregnancy loss and pre-term delivery (Rao et al., 2019).

The ESHRE guidelines suggest that thyroid screening (TSH and Thyroid peroxidase antibodies -TPO) is recommended in women with RPL, and that abnormal TSH and TPO-antibody levels should be followed up by thyroxine (T4) testing in these women. (Atik et al., 2018).

1.3.7 Metabolic

1.3.7.1 Diabetes

Women with uncontrolled pregestational diabetes, appear to have higher rates of pregnancy loss. Monitoring the haemoglobin A1c (HbA1c) levels is an excellent indicator of blood glucose control, and women with HbA1c levels higher than 8% have higher rates of pregnancy loss. Diabetic women who manage to control blood glucose levels within the normal range, have similar rates of pregnancy loss to women who are not diabetic (Mills et al., 1988).

1.3.7.2 Hyperinsulinism

Insulin resistance (hyperinsulinism) is a condition in which the sub-optimal efficacy of insulin in promoting the uptake and utilization of available glucose by tissues, organs and cells, is lower than in non-insulin resistant individuals. This often occurs in the context of completely normal glucose levels but glucose levels may be raised (Wang et al., 2011).

Insulin resistance/Hyperinsulinism (IR/HI) has been thought to be associated with pregnancy loss, particularly in patients with the polycystic ovarian syndrome (PCOS). Wang et al., (2011), considered the fasting, one, two and three-hour insulin levels, of recurrent miscarriage patients in the first trimester of pregnancy. They found a significant difference in both the insulin and glucose levels at one, two and three hours of testing, even when they excluded patients with PCOS. They concluded that there is an increased incidence of insulin resistance in recurrent miscarriage patients, in the first trimester of pregnancy, and suggested that only testing for fasting insulin and glucose in recurrent miscarriage patients, would be inadequate to determine insulin resistance (Wang et al., 2011). Maryam et al., (2012) also found insulin

resistance in 24% of women with RPL, compared to only 8% of controls, in a case-control study.

The mechanism underlying the association between HI and the risk for miscarriage is unknown. A few possible mechanisms have been suggested by studies involving patients with PCOS. Jakubowicz et al., (2004) found that HI led to reduced serum concentrations of insulin-like growth factor binding protein-1 (IGFBP-1) and glycodelin in early pregnancy, which would be expected to increase the chance of miscarriages.

Glycodelin is an immunomodulatory protein involved in implantation, and Glycodelin - A is found in abundant levels in the decidua in early pregnancy (Lee et al., 2016). It plays an important role in placental development and feto-maternal defence. Abnormal levels are associated with unexplained infertility and recurrent pregnancy loss (Lee et al., 2016). Insulin can regulate the concentrations of glycodelin and IGFBP-1 negatively, increasing the risk of miscarriages. HI may also increase the level of plasminogen activator inhibitor-1 and induce villous thrombosis, thereby reducing placental perfusion and villous hypoplasia, resulting in miscarriage (Glueck et al., 2006). Another hypothesis is that HI may cause an uncontrolled diabetic-like state in the fetal environment resulting in increased first trimester loss (Glueck et al., 2006). High insulin levels have been shown *in vitro* to increase the transport of glucose by first trimester cytotrophoblasts independent of glucose level (probably by upregulation of the GLUT1 glucose transporter system) (Glueck et al., 2006).

A recent study by Vega et al., 2019, found that elevated insulin levels were directly toxic to cultured first trimester trophoblasts, resulting in apoptosis of these cultured cells, increased damage to their DNA, and decreased cell survival. These effects

were prevented by the addition of Metformin (Vega et al., 2019). Insulin is typically considered as an antiapoptotic and progrowth hormone, however, exposure of human primary trophoblast cells to insulin in the absence of raised glucose levels resulted in significant damage (Vega et al., 2019). Despite the above reports, the current ESHRE guidelines have suggested that assessing for PCOS, fasting insulin and fasting glucose is not recommended in women with RPL as a method of improving subsequent pregnancy prognosis. It needs to be emphasized that this statement is mainly based on insufficient evidence (Atik et al., 2018).

1.3.8 Specific

1.3.8.1 Immunological causes

The feto-placental unit is equivalent to an allograft and is similar to a heart or kidney transplant (Van Kampen et al., 2001). The maternal immune system needs to adapt to the different paternal alloantigens present and such ‘immune recognition’ is essential for the survival of the pregnancy (Christiansen et al., 2008). Antipaternal human leukocyte antigen (HLA) antibodies have been observed in 10-30% of all normal pregnancies (Van Kampen et al., 2001; Lashley et al., 2013). Immunological biomarkers associated with RPL in the blood have been extensively researched as potential causes/associations regarding recurrent miscarriages. These include: natural killer (NK) cells in the blood or decidual tissue, cytokines in blood or decidua, and investigations of classical or nonclassical HLA polymorphisms in patients with RPL, as well as studies of HLA protein expression on trophoblast (Laird et al., 2011).

HLA incompatibility was previously thought to be an important cause of RPL but a large case-control study did not support a link between RPL and anti-HLA antibody

formation, and thus discounted the diagnostic value of alloantibodies detected as part of an RPL work-up (Bartel et al., 2011).

ESHRE guidelines have suggested that there is in inadequate research into an association between pregnancy outcomes and HLA polymorphisms in women suffering RPL, and that the investigation of HLA-DR or other classical HLA genes should only be undertaken in the context of research (Atik et al., 2018).

Class II HLA in women with secondary RPL after the birth of a male baby (HLA-DRB1*15:01 and HLA-DRQB1*05:01/05:2) has been used as part of a work -up in Scandinavian women, with secondary loss after the birth of a male and in a research context (Atik et al., 2018). However, HLA-C has also recently been shown to be associated with recurrent miscarriages (Meuleman et al., 2017). HLA-C is the only classical HLA-I antigen expressed on trophoblast. It has been shown that HLA-C incompatibility between couples is significantly associated with unexplained recurrent miscarriage (Meuleman et al., 2017).

1.3.8.2 Anti-HY antibodies

Anti-HY antibodies are those directed against male-specific minor histocompatibility (HY) antigens that are expressed on most or all nucleated cells from males, (Nielsen et al., 2010) and hence could be expected in pregnancies bearing a male fetus. The evidence for testing these antibodies is sparse so warrants further investigation. The ESHRE guidelines do not recommend testing for them in clinical practice (Atik et al., 2018).

1.3.8.3 Natural Killer Cells

NK cells are part of the innate immune system and can recognize and react against target antigens, without prior sensitization (Christiansen et al., 2008). This reaction can result in the destruction of cells or the secretion of an array of cytokines (Christiansen et al., 2008). Decidua and endometrium have a unique composition of NK cells called uterine NK (uNK) cells, that are thought to play a key role in the establishment of successful pregnancy by facilitating immunologic adaptation of the semiallogenic developing embryo (Lachapelle et al., 1996).

More than 90% of lymphocytes in the luteal phase endometrium or decidual tissue in early pregnancy are low cytotoxicity, high cytokine producing NK cells, which carry a high density of the CD56 surface marker ($CD56^{bright}$) assessed by flow cytometry but are negative for the CD16 marker (Laird et al., 2011). In contrast, 90% of the NK cells in peripheral blood carry the $CD56^{DIM}CD16$ markers, which are associated with high cytotoxicity and low cytokine production. Although many studies have suggested that the levels of CD56 cells in peripheral blood taken prior to pregnancy is significantly higher in RPL women than in controls, (King et al., 2010; Lee et al., 2013) other studies have not found this association (Emmer et al., 1999). Several studies investigated the impact of NK cytotoxicity on the subsequent pregnancy outcome in RPL patients. Aoki et al., (1995) reported that RPL patients with high peripheral blood NK cytotoxicity before pregnancy had a significantly higher rate of pregnancy loss (71%) in the next pregnancy, than patients with a lower NK cytotoxicity (20%). Yamada et al., (2003) found a significantly higher NK cytotoxicity in patients with a subsequent euploid miscarriage compared with those with a live birth while Morikawa et al. (2001) found a non-significant tendency for the same. In contrast, another study found no difference. (Liang et al. 2012). Katano et al. (2013), in a large prospective

study however, provided the strongest argument against a significant role for the quantification of NK cytotoxicity in RPL patients. In a logistic regression analysis adjusting for recognized risk factors for miscarriage, high NK cytotoxicity before pregnancy had no impact on subsequent pregnancy loss rate.

The composition of endometrial lymphocytes fluctuates significantly during the menstrual cycle with a six to ten-fold increase in the late luteal phase in comparison to the follicular phase, and similarly in the peripheral blood samples and endometrium (Russell et al., 2013). Thus, it has been questioned whether the endometrial NK cell subsets can accurately reflect those in the peripheral blood.

Templer and Sacks 2016, highlighted the complexity of this issue regarding NK cells and reproductive failure. While NK cell activity may not favour successful reproduction, such NK cell activity may provide alternative protection against disease. Hence, the presumed benefits of the use of potent immunosuppressive agents needs to be assessed aganst the potential harms of such agents (Templer and Sacks, 2016)

The ESHRE guidelines have stated that there is insufficient evidence in women suffering RPL, to recommend testing of either peripheral blood NK cells or endometrial NK cells (Atik et al., 2018).

1.3.8.4. KIR and HLA-C

Extravillous trophoblast (EVT) of fetal origin is responsible for the remodelling of the maternal spiral arteries via the destruction of the media, essential for normal placentation. Specific uterine natural killer cells (uNK) are present in the decidua during the placental formation and interact with the invading trophoblastic cells (Moffett et al., 2016). The EVT expresses high levels of HLA-C, of maternal and

paternal origin, that is recognized by uterine NK cells (uNK) killer immunoglobulin-like cell surface receptors (KIRs) (Alesandru et al., 2017). KIRs are a complex family that includes both activator an inhibitory receptors, and the activation profile is genetically determined in each individual. In uNKs, KIRs are the most variable receptors and there is diversity in gene numbers between individuals and allelic diversity at individual KIR loci (Alesandru et al., 2017). KIRs bind to ligands on trophoblastic cells and dysfunction has been associated with reproductive failure (Díaz-Peña et al., 2019).

This leads to diverse levels of functionality for NK and T cells once they actively engage with specific HLA class 1 molecules (Díaz-Peña et al., 2019). Combinations of KIR and HLA genes are associated with pregnancy complications such as recurrent pregnancy loss and preeclampsia. The maternal KIR genes and their fetal ligands are highly variable, so different KIR/HLA-C genetic combinations occur in each pregnancy. KIR genes can be inhibitory or activating, and inhibitory KIRs are found more often in women who suffer pre-eclampsia, recurrent miscarriages or significant fetal growth restriction (Moffett et al., 2016).

Faridi et al., (2010) showed that KIR and HLA-C allorecognition patterns implicative of dominant activation of natural killer cells contributed to recurrent miscarriages in their cohort. Dambaeva et al., (2016) showed that RPL in women with the KIR2DS1 genotype was associated with an increased HLA-C2 allelic frequency, and suggested that KIR and HLA-C genotyping could be useful for predicting immune related problems in women with RPL. Díaz-Peña et al., (2019) suggested that demographic genetic studies need to be performed to better understand the successful KIR/HLA-C allotype combinations of a succcessful pregnancy outcome (Díaz-Peña et al., 2019).

1.3.8.5 Vitamin D deficiency

There is increasing, but not always consistent, evidence that a deficiency of Vitamin D increases the risk of many adverse pregnancy outcomes, including miscarriages, gestational diabetes, intrauterine growth restriction, and preeclampsia (Aghakjafari et al., 2013). Vitamin D is thought to affect placental implantation, angiogenesis, oxidative stress, immune function, glucose homeostasis and endothelial function (Wei et al., 2014). The biological activity of Vitamin D occurs via two pathways – a slow genomic response, and a rapid, non-genomic response (Mizwiki and Norman, 2009). The target organs for the non-classical actions of vitamin D include the adaptive and innate immune systems, heart, cardiovascular system, the pancreatic-β-cells, brain and reproductive system (Christakos et al., 2007). Vitamin D is associated with multiple tissue responses, including regulation of hormone secretion, modulation of immune responses, and a control of cellular proliferation and differentiation (Mizwiki et al., 2009). Vitamin D has been reported to inhibit the proliferation of T helper 1 (Th1) cells and limit their production of cytokines, such as interferon gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) (Mizwiki et al., 2009). Conversely, vitamin D induces T helper 2 (Th2) cytokines, such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Adams et al., 2008). Vitamin D has also been thought to be a modifiable environmental factor for Th 1-mediated autoimmune diseases and may be important for susceptibility to and severity of the disease (Chen et al., 2007). It regulates B cell immunity by down-regulating the proliferation and differentiation of B lymphocytes and inhibits IgG production (Ota et al., 2014). Some investigators have shown that vitamin D influences local inflammatory responses and induces decidualization for successful pregnancy. It may thus be an immune modulator during implantation (Diaz et al., 2009; Barrera et

al., 2008). A dominant Th2 response is important to maintain maternal-fetal relationships for a successful pregnancy outcome. Vitamin D is thought to skew the T cell compartment from a Th1 to a Th 2 type (Ota et al., 2014). Women with low vitamin D levels have significantly increased odds for autoimmune abnormalities, a risk factor for reproductive failure (Ota et al., 2014).

A meta-analysis revealed that maternal vitamin D deficiency is associated with increased risk of gestational diabetes during pregnancy (Lu et al. 2016). Several studies have shown that vitamin D is required for the normal insulin production and secretion by the pancreas (Zitterman et al., 2006, Pittas et al., 2012). Verburg et al. (2016), showed a seasonal variation for GDM in a population-based study. Several lifestyle and psychosocial factors could have been influential in the pathophysiological mechanisms of the development of GDM in this study. Several other factors such as ambient temperature, nutrient intake, physical activity and vitamin D levels could affect maternal physiology, and fetal and placental development at a cellular level and thus contribute to the development of GDM. High serum vitamin D at 15 +/- 1 weeks' gestation has been shown to protect against the development of gestational diabetes (Wilson et al., 2018).

Vitamin D has anti-inflammatory effects, and vitamin D sufficiency has beneficial effects on improving islet-cell functions, insulin release and decreasing insulin resistance (Wimalawansa et al., 2016). These are likely due to signaling actions of vitamin D receptors present on pancreatic β-cells (Wimalawansa et al., 2016). Interestingly one study showed that there is an increased prevalence of insulin resistance in women with a history of recurrent pregnancy loss, compared with matched controls (Craig et al., 2002).

1.3.8.6 Sperm DNA damage

The male partner confers 50% of the genomic material to the conceptus, and contributes not only to placental development, but also to that of the embryo (Carlini et al., 2017). Therefore, genetic and epigenetic alterations of sperm may have significant consequences, contributing to early pregnancy loss. Minor DNA damage in spermatozoa as a result of endogenous or exogenous insults are repaired by pre- and post-replication repair mechanisms but major damage cannot be repaired. This results in poor-quality sperm. Thus, the male partner may contribute to pregnancy loss, despite morphologically normal sperm, by harboring sperm with DNA damage. DNA damage causes alterations in sperm quality and function, sperm-oocyte interaction, implantation and early placental and embryonic development, resulting in pregnancy loss (Robinson et al., 2012; Aitken et al., 2016). Paternal factors, therfore, may including age, obesity, smoking and exposure to environmental contaminants (Aitken et al., 2016; Carlini et al., 2017). These factors involved in poor sperm quality cause oxidative stress, and are also associated with pregnancy problems such as small for gestational age babies and preeclampsia (McCowan et al., 2011; Raad et al., 2017).

The ESHRE guidelines suggest that as there is moderate evidence indicating associations between RPL and poor-quality sperm, and that there is evidence that lifestyle contributes, clinicians should alert couples to this, and possibly offer sperm DNA fragmentation tests if available (Atik et al., 2018).

1.3.8.7 Progesterone/Luteal phase defects

Some studies suggest that the use of progesterone supplements in RM patients decreases miscarriages (El-Zibdeh et al., 2005). In a Cochrane database review in

2013, a subgroup analysis of women with RPL (defined as 3 or more consecutive losses) treated with progesterone demonstrated a statistically significant decrease in miscarriage rate compared to placebo or no treatment. (OR 0.39;95% CI 0.21-0.72) (Carp et al., 2015). However, the PROMISE trial in 2015 found that the administration of progesterone in the first trimester did not result in a significantly higher rate of live births among women with a history of unexplained recurrent miscarriages (Coomarasamy et al., 2015). However, the trial did not perform genetic analyses of the products of conception in the losses, and may have missed a significant contributor to losses, that could have altered the findings. Furthermore, the trial may have had insufficient statistical power to detect a difference. Recently Coomarasamy et al., (2019) in a randomized controlled trial of progesterone in women with bleeding in early pregnancy, showed that the administration of progesterone did not result in a higher incidence of live births. However, in a subgroup analysis of women with three or more miscarriages and bleeding in early pregnancy, there was a suggestion of benefit from progesterone (Coomarasamy et al., 2019). This requires validation.

1.3.8.8 Domestic violence

Intimate partner violence and domestic violence have been associated with an increased risk of both induced and spontaneous miscarriages (Fanslow et al., 2008; Silverman et al, 2007). Complex social situations, especially among poorly educated and financially disadvantaged women, are prevalent and healthcare systems and physicians need to be cognizant of the link between violence and miscarriages.

1.3.8.9 Smoking and alcohol

Maternal smoking is known to be associated with adverse pregnancy and neonatal outcomes, including ectopic pregnancies, congenital abnormalities, preterm birth,

small for gestational age babies, placenta praevia and stillbirth (Leung and Davies 2015; Zhang et al., 2010). There have also been reports of sudden infant death syndrome, as well as other problems during childhood (Leung and Davies, 2015). However, evidence regarding pregnancy loss is lacking but, given the adverse outcomes, couples may best be advised to stop (Atik et al., 2018).

1.3.8.10 Obesity

Maternal obesity is a risk factor for RPL and has a significant impact on female reproductive health overall (Pandey et al., 2010; Boots and Stephenson, 2011). A body mass index greater than 30kg/m^2 has been evaluated as a risk factor for RPL and a higher prevalence of RPL was reported in obese women with such a BMI, compared to women with a normal BMI. (0.4% versus 0.1%; OR 3.51; 95% CI 1.03 – 12.01) (Lashen et al., 2004; Boots and Stephenson, 2011). An increase in euploid miscarriages among obese women compared to women of a normal weight (58% versus 37%, relative risk RR 1.63; 95% CI 1.08-2.47) has also been found (Boots et al., 2014). Lo et al., (2012) demonstrated that BMI, female age, number of previous losses and ethnicity were significantly associated with pregnancy outcomes, and maternal obesity, as demonstrated by a BMI ≥30 kg/m^2, was significantly associated with an increased risk of miscarriages, in couples with unexplained RPL (OR 1.73;95%CI 1.06-2.83). The possible pathways for the association between obesity and pregnancy failure include an adverse impact on the development of the endometrium or an adverse effect on ovaries affecting the quality of oocytes and thus embryo viability/quality or a combination of both (Boots and Stephenson 2011).

Cavalcante et al., (2019) found a positive correlation between obesity and RPL. The actual mechanisms are unknown, and interventions such as weight control have not

been adequately investigated in RCT's. Obesity is associated with chronic inflammation associated with numerous inflammatory markers such as high levels of C-reactive protein and interlukein-6 (IL-6) (Grimstad and Krieg 2016). Other possible mechanisms are abnormalities in the hypothalamic-pituitary-gonadal hormonal axis (Calvacante et al., 2019). Given that maternal obesity is associated with RPL, women should be advised to strive for a healthy weight before embarking on a pregnancy, both for the prevention of RPL and to decrease the known pregnancy complications associated with obesity, such a gestational diabetes mellitus, and its complications for both mother and fetus (Atik et al. 2018).

1.4 Conclusions

Miscarriage is a complex and heterogeneous condition, and is a significant negative life event that has a major emotional impact on couples. When the losses become repetitive, the impact is intensified, and feelings of grief, anxiety and personal failure affect both partners. The psychosocial needs of couples affected by RPL need to be considered when planning care, and these couples need access to expertise, investigations and individualized treatment plans that will reduce their losses. These need to be evidence based and ethical (Atik et al., 2018).

References Chapter 1

Adams JS, Hewisen M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. Nat Clin Pract Endocrinol Metab. 2008;4:80-90.

Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O'Beirne M, Rabi DM. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: a systematic review and meta-analysis of observational studies. BMJ 2013;346:f1169.

Ahmadi F, Javam M. Role of 3D sonohysterography in the investigation of uterine synechiae/asherman's syndrome: Pictorial assay. J Med Imaging and Rad Oncol. 58:2014;199-202.

Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatazoa. Reproduction, Fertility and Development. 2016;28:1-10. http://dx.doi.org/10.1071/RD15325.

Alarcon-Segovia D, Sanchez-Guerrero J. Primary antiphospholipid syndrome. J Rheumatol 1989;16:482-8.

Alarcon-Segovia D, Deleze M, Oria CV, Fernandez L, DeLeon SP. Antiphospholipid antibodies and the antiphospholipid syndrome in systemic lupus erythematosus. A prospective analysis of 500 consecutive patients. Medicine (Baltimore) 1989;68:353-65.

Alecsandru D, Garcia-Velasco JA. Why natural killer cells are not enough: a further understanding of killer immunoglobulin-like receptor and human leukocyte antigen. Fertil. Steril 2017;107(6):1273-1278. https//doi.org/101016/jfertnstert.2017.04.018

Alexander EK, Marqusee E, Lawrence J, Jarolim P, Fischer GA, Larsen PR. Timing and magnitude of increases in levothyroxine requirements during pregnancy in women with hypothroidism. N Eng J Med 2004;351(3):241-249.

Alfirevic Z, Roberts D, Martlew V. How strong is the association between maternal thrombophilia and adverse pregnancy outcome? A systematic review. Eur J Obstet Gynecol Reprod Biol. 2002;101(1):6-14.

American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss. ACOG Pract Bull 2001;24:1-12.

Aoki K, Kajiura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y et al. Preconceptional natural-killer-cell actvity as a predictor of miscarriage. Lancet.1995;345:1340-2.

Asherson RA, Khanashta MA, Hughes GR. Antiphospholipid antibodies, lupus-like disease and the "primary" antiphospholipid syndrome. Clin Rheumatol 1989;8:115-7.

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.

Baird DT, Collins J, Egozcue J, et al. Fertility and ageing. Human reproduction update 2005;11:261-76.

Barad DH, Weghofer A, Gleicher N. Comparing anti-Mullerian hormone (AMH) and follicle-stimulating hormone (FSH) as predictors of ovarian function. Fertil Steril 2009;91:1553.

Barrera D, Avila E, Hernandez G, Halhali A, Larrea F, Morales A et al. Calcitrol affects hCGgene transcription in cultured human syncytiotrophoblasts. Reprod Biol Endocrinol 2008;6:3.

Bartel G, Walch K, Wahrmann M, Pils S, Kussel L, Polterauer S, et al. Prevalence and qualitative properties of circulating anti-human leukocyte antigen alloantibodies after pregnancy: no association with unexplained recurrent miscarriage. Hum Immunol 2011;72:187-192.

Bashiri A, Harlev A, Argawal A. (Editors) Recurrent Pregnancy Loss. (Book) Springer International Publishing Switzerland 2016.

Bashiri A, Shapira E, Ratzon R, Shoham-Vardi I, Sejienko R, Mazor M. Primary vs secondary recurrent pregnancy loss- epidemiological characteristics, etiology, and the next pregnancy outcome. J Perinat Med. 2012;40:389-96.

Bernadi LA, Cohen RN, Stephensen MD. Impact of subclinical hypothyroidism in women with recurrent early pregnancy loss. Fertil Steril 2013;100:1326-1331.

Bezemer ID, et al. No association between the common MTHFR 677C→T polymorphism and venous thrombosis: results from the MEGA study. Arch Intern Med. 2007;167(5):497-501.

Bodnar LM, Catov JM, Simhan HN. Maternal vitamin D deficiency increases the risk of preeclampsia. J Clin Endocrinol Metab 2007;92:3517-3522.

Bopp BL, Seifer DB. Oocyte loss and the perimenopause. Clinical obstetrics and gynecology 1998;41:898-911.

Bouvier S, Cochery-Nouvellon E, Lavigne-Lassalde G, Mercier E, Marchetti T, Balducchi JP et al. Comparative incidence of pregnancy outcomes in treated obstetric antiphospholipid syndrome; the NOH-APS observational study. Blood 2014;123:404-413.

Boots C, Stephenson MD. Does obesity increase the risk of miscarriage in spontaneous conception: a systematic review. Semin Reprod Med 2011;29:507-513.

Boots CE, Bernardi LA, Stephenson MD. Frequency of euploid miscarriage is increased in women with recurrent early pregnancy loss. Fertil Steril 2014;102:455-459.

Bose P, Kadyrov M, Goldin R, Hahn S, Backos M Regan L, Huppertz B. Abberations of early trophoblast differentiation predispose to pregnancy failure: lessons from the anti-phospholipid syndrome. Placenta 2006;27:869-875.

Bradley LA, Palomaki GE, Bienstock J, et al. Can Factor V Leiden and prothrombin G20210A testing in women with recurrent pregnancy loss result in improved pregnancy outcomes?: results from a tergeted evidence-based review. Genet Med 2012;14(1):39-50.

Branch DW, Silver RM. Blackwell, J.L et al. Outcome of treated pregnancies in women with antiphospholipid syndrome: an update of the Utah experience. Obstet Gynecol. 1992:614-20.

Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Thromb Haemost 1995;74:1185-90.

Bülow Pedersen I, Laurberg P, Knudsen N et al. Lack of association between thyroid autoantibodies and parity in a population study argues against microchimerism as a trigger of thyroid autoimmunity. Eu J Endocrinol;2009;154(1):39-45.

Carlini T, Paoli D, Pelloni M, Faja F, Dal Lago A, Lombardo F et al., Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss. Reprod Biomed Online 2017;85: 446-450.

Carp HJA, Toder V, Aviram A, Daniely M, Mashiach S, Barkai G. Karyotype of the abortus in recurrent miscarriage. Fertil Steril. 2001;5:678–82.

Carp H. Karyotype of the abortus in recurrent miscarriage. Fertil Steril. 2001;75(4):678-82. Doi:10.1016/S0015-0282(00)01801-X.

Carp H. A systematic review of dydrogesterone for the treatment of recurrent miscarriage. Gynecol Endocrinol.2015;1-9.

Chen L, Hu R. Thyroid autoimmunity and miscarriage: a meta-analysis. Clin Endocrinol (Oxf) 2011;74(4):513-9.

Chen TC, Chimeh F, Lu Z, Mathieu J, Person KS, Kohn N et al. Factors that influence the cutaneous synthesis and dietary sources of Vitamin D. Arch Biochem Biophys 2007b;460:213-217.

Christakos S, Dhawen P, Benn B, Porta A, Hediger M, Oh GT, et al. Vitamin D: molecular mechanism of action. Ann N Y Acad Sci 2007;1116:340-348.

Christiansen OB. A fresh look at causes and treatments of recurrent miscarriage, especially its immunological aspects. Hum Reprod Update 1996;2: 271-293.

Christiansen OB. Epidemiology of recurrent pregnancy loss. In: Carp H, editor. Recurrent pregnancy loss: causes, controversies and treatment. 1st ed UK: Informa healthcare; 2007.p1-14.

Christiansen OB. Recurrent miscarriage is a useful and valid clinical concept. Acta Obstet Gynecol Scand. 2014;93:852–7.

Christiansen OB, Steffersen R, Nielsen HS, Varming K. Multifactorial etiology of recurrent miscarriage and its scientific and clinical implications. Gynecol Obstet Invest. 2008;66:257-67.

Cines DB, McCrae KR. The antiphospholipid -protein syndrome. J Clin Immunol;1995;15:86S-100S.

Clouse LH, Com PC. The regulation of hemostasis: the protein C system. N Engl J Med, 1986;314:1298-304.

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;373:2141-8. DOI:10.1056/NEJMoa1504927.

Coomarasamy A, Devall AJ, Cheed H, Harb LJ, Middleton ID, Gallos H et al., A randomized trial of progesterone in women with bleeding in early pregnancy. NEJM 2019;3801815-24. DOI:10.1056/NEJMMoa1813730.

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

Crawford NM, Steiner AZ. Thyroid autoimmunity and Reproductive function. Semin Reprod Med 2016;34:343-350.

Cuoto EB, Zaccharia R. Association of anticardiolipin antibody and C677T in methylenetetrahydrofolate reductase mutation in women with recurrent spontaneous abortions: a new path to thrombophillia? Sao Paulo Med J 2005;123-128.

Dale, T. Tanbo, E. Haug, et al. The impact of insulin resistance on the outcome of ovulation induction with low-dose follicle stimulating hormone in women with polycystic ovary syndrome Hum Reprod, 13 (1998), pp. 567-570.

Dambaeva SV, Lee DH, Sung N, Chen C, Bao S, Gilman-Sachs et al. Recurrent Pregnancy loss in Women with Killer Cell Immunoglobulin-Like Receptor KIR2DS1 is Associated with an Increased HLA-C2 Allelic Frequency.Am J Reprod Immunol 2016;75:94-103

de Jong PG, Kaandorp S, Di Nisio M, Goddijn M, Middeldorp S. Aspirin and/or heparin for women with unexplained recurrent miscarriage with or without inherited thrombophilia. Cochrane Database of Systematic Reviews 2014, Issue 7. Art. No.: CD004734. DOI: 10.1002/14651858.CD004734.pub4.

de la Rochebrochard E, Thonneau P. Paternal age and maternal age are risk factors for miscarriage: Results of a Multicentre European Trial. Human Reproduction 2002;17:1649-56.

Diaz L, Noyola-Martinez N, Barrera D, Hernandez G, Avila E, Halhali A et al. Calcitrol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. J Reprod Immunol 2009;81:17-24.

Díaz-Peñ a R, de los Santos MJ, Lucia A and Castro-Santos P. Understanding the role of killer cell immunoglobulin-like receptors in pregnancy complications. J Assis Reprod and Genetics, 2019;36:827-835.Dolleman M, Depmann M, Eijkemans MJ, et al. Anti-Mullerian hormone is a more accurate predictor of individual time to menopause than mother's age at menopause. Human Reproduction 2014;29:584-91.

Drakely A, Quenby S, Farquharsen R. Mid-trimester loss-appraisal of a screening protocol. Human Reprod. 1998;13(7):1975-80.

El-Zibdeh MY. Dydrogesterone in the reduction of recurrent spontaneous abortion. J Steroid Biochem Mol Biol. 2005;97(5):431-4.

Emmer PM, Veerhoek M, Nelen WLDM. Steegers EAP, Joosten I. Natural killer cell reactivity and HLA-G in recurrent spontaneous abortion. Transplant Proc. 1999;31:1838-40.

Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson FJ. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Human Reprod,(10)1992;13342-1346.

Fanslow J, Silva M, Whitehead A, Robinson E. Pregnancy outcomes and intimate partner violence in New Zealand. Aust N Z J Obstet Gynecol. 2008; 48(4):391-7.

Faridi RM, Agrawal S. Killer immunoglobulin-like receptors (KIR's) and HLA-C allorecognition patterns implicative of dominant activation of natural killer cells contribute to recurrent miscarriages. Human Reprod (26) 2011; 2:491-497.Fenech M.

Recommended dietary allowances (RDAs) for genomic stability. Mutation Research 2001 480-481 51-4.

Foka ZJ, Lambropoulous AF, Saravelos H, Karas GB, Karavidas a, Agorastas T et al. Factor V Leiden and Prothrombin G20210A mutations, but not methylenetetrahydrofolate reductase C677T, are associated with recurrent miscarriages. Human Reproduction 2000.;15:458-62.

Franssen MT, Korevaar JC, van der Veen F, Leschot NJ, Bossuyt PM, Goddijn M. Reproductive outcome after chromosomal analysis in couples with two or more miscarriages: index [corrected]-control study. BMJ.2006;332(7544):759-63.

Fritz M. Recurrent early losses. In: Fritz M, Speroff L, editors. Clinical gynecologic endocrinology and infertility. 8th ed. Philadelphia, PA: Lippincott

Williams & Wilkins; 2011. p. 1191–220.

Galli M, Beretta G, Daldossi Bevers EM, BarbuiT. Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. Thromb Haemost, 1997;77:486-91.

Garber JR, Cobin RH, Gharib H, Hennessey JV, Klein I, Mechanick JI *et al*. Clinical guidelines for hypothyroidism in adults: cosponsored by the American Association of Clinical Endocrinologists and the American Thyroid Association. Thyroid. 2012;22(12):1200-35.

Ghafoor F, Mansoor M, Malik T, Malik MS, Khan AU, Edwards R, Akhtar W. Role of thyroid peroxidase antibodies in the outcome of pregnancy. J Coll Physicians Surg Pak. 2006; 16:468-471.

Gladson CL, Scharrer I, Hach, V. Beck KH, Griffin JH. The frequency of type I heterozygous protein S and protein C deficiency in 141 unrelated young patients with venous thrombosis. Thromb Haemost, 1988; 59:18-22.

Glinoer D, de Nayer P, BourdouxP et al. Regulation of maternal thyroid during pregnancy. J Clin Endocrinol Metab, 1990;71(2):276-287.

Glinoer D, Raihi M, Grün JP, Kinthaert J. Risk of subclinical hypothyroidism in pregnant women with asymptomatic thyroid disorders. J Clin Endocrin Metabol. 1994;79:197-204.

Glueck CJ, Phillips H, Cameron D, et al. Continuing metformin throughout pregnancy in women with polycystic ovary syndrome appears to safely reduce first-trimester spontaneous abortion: a pilot study Fertil Steril, 75 (2001), pp. 46-52.

Glueck CJ, Sieve L, Zhu B, et al: Plasminogen activator inhibitor activity, 4G5G polymorphism of the plasminogen activator inhibitor- 1 gene and first-trimester miscarriage in women with polycystic ovary syndrome. Metabolism 2006; 55: 345–352.

Glueck CJ,, P. Wang P, Goldenberg N, et al. Pregnancy loss, polycystic ovary syndrome, thrombophilia, hypofibrinolysis, enoxaparin, metformin Clin Appl Thromb Hemost, 10 2004, pp. 323-334

Gold JE, Haubenstock A, Zalusky R. Lupus anticoagulant and AIDS. N Engl J Med 1986;314:1252-3.

Govindaiah V, Naushad SM, Prabhakara K, Krishna PC, Radha R, Devi A. Association of parental hyperhomocysteinemia and C677T Methylene tetrahydrofolate reductase (MTHFR) polymorphism with recurrent pregnancy loss. Clin Biochem 2009;42:380-6.

Greene ND, Dunlevy L.P. Copp, A.J. Homocysteine is embryotoxic, but does not cause neural tube defects in mouse embryos. . Anat and Embry 2003; 206:185-91.

Grimstad F, Krieg S. Immunogenetic contributions to recurrent pregnancy loss. J Ass Reprod Genet 2016;33:833-847.

Gruijters MJ, Visser JA, Durlinger AL, Themmen AP. Anti-Mullerian hormone and its role in ovarian function. Mol Cell Endocrinol 2003;211:85-90.

Haas DM, Ramsey PS. Progesterone for preventing miscarriage. Cochrane Database Syst Rev 2013;10, CD003511.

Holmes ZR, Regan L, Chilcott I, Cohen H. . The C677T MTHFR gene mutation is not predictive of risk for recurrent fetal loss. Br J Haematol 1999;105:98-101.

Hunt JE, McNeil JE, HP Morgan GJ, Crameri RM, Krilis SA. A phospholipid-beta 2-glycoprotein I complex is an antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection. Lupus.1992;1:75-81.

Jacobs PA. Retrospective and prospective epidemiological studies of 1,500 karyotyped spontaneous human abortions. Birth Defects Res A Clin Mol Teratool. 2013;97(7):487-8doi10.1002/bdra.2323145.

Jakubowicz DJ, Essah PA, Seppala M, et al: Reduced serum glycodelin and insulin-like growth factor-binding protein-1 in women with polycystic ovary syndrome during first trimester of pregnancy. J Clin Endocrinol Metab 2004; 89: 833–839.

Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril 2010;93:1234-1243.

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. Fertility Sterility 2013;99:1916-1922.

Josso N, Cate RL, Piccard JY et al. Anti-Müllerian hormone: the Jost factor Recent progress in Hormone research 1993; 48(1):1-59.

Josso N, Racine C, di Clemente N, Rey R, Xavier F. The role of anti-Mullerian hormone in gonadal development. Mol Cell Endocrinol 1998;145:3-7.

Katano K, Suzuki S, Ozaki Y, Suzumori N, Kitaori T, Sugiura-Ogasawara M. Peripheral natural killer cellactivity as a predictor of recurrent pregnancy loss: a cohort study. Fertil Steril. 2013;100:1629-34.

King K, Smith S, Chapman M, Sacks G. Detailed analysis of peripheral blood natural killer cells (NK) in women with recurrent miscarriage. Hum Reprod.2010;25:52-8.

Kist WJ, Janssen NG, Kalk JJ, Hague WM, Dekker GA, de Vries JIP. Thrombophilias and adverse pregnancy outcome- A confounded problem! Thromb Haemost 2008;99:77-85.

Kolte AM, van Oppenraaij RH, Quenby S, Farquharson RG, Stephenson M, Goddijn M, Christiansen OB, on behalf of the ESHRE Special Interest Group Early

Pregnancy. Non-visualized pregnancy losses are prognostically important for unexplained recurrent miscarriage. Human Reprod, 2014;29, (5):931-937.

Krilis SA, (editor). Immunology of antiphospholipid antibodies. Immunology of connective tissue diseases.1993: 279-304.

Kos BJP, Leemaqz SY, McCormack CD, Andraweera PH, , Furness DL, Roberts CT & Dekker GA.(2018): The association of parental methylenetetrahydrofolate reductase polymorphisms (MTHFR 677C > T and 1298A > C) and fetal loss: a case–control study in South Australia, The Journal of Maternal-Fetal & Neonatal Medicine, DOI: 10.1080/14767058.2018.1500546

Kumar KS, Naushad, SE. Plasma homocysteine levels correlated to interactions between folate status and methylene tetrahydrofolate reductase gene mutation in women with unexplained recurrent pregnancy loss. J Obstet Gynaecol 2003;23:55-8.

Kutteh WH, Triplett DA. Thrombophilias and recurrent pregnancy loss. Semin Reprod Med 2006;24:54-66.

Kutteh WH, Hinote CD. Antiphospholipid syndrome. Obstet Gynecol Clin N Am 2014;41:113-132.

Kutteh WH. Antiphospholipid antibody-associated recurrent pregnancy loss: treatment with heparin and low-dose aspirin is superior to low-dose aspirin alone. Am J Obstet Gynecol. 1996;174(5):1584-9.

Lachapelle MH, Miron P, Hemmings R, Roy DC. Endometrial T, B and natural killer cells in patients with recurrent spontaneous abortion. Aletred profile and pregnancy outcome. J Immunol 1996;156:4027-34.

Laird S, Mariee N, Wei L, Li TC. Measurements of CD56+ cells in peripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ. Hum Reprod. 2011;26:1331-7.

Laughlin SK, Baird DD, Savitz DA, Herring AH, Hartman KE. Prevalence of uterine leiomyomas in the first trimester of pregnancy: an ultrasound-screening study. Obstet Gynecol.2009;113:630-635.

Lashen LW, Fear K, Sturdee DW. Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. Hum Reprod 2004;19:1644-1646.

Lazarus J, Brown RS, Daumerie C, Negro R, Vaidya B. 2014 European thyroid association guidelines for the management of subclinical hypothyroidism in pregnancy and in children. European Thyroid Journal;2014;3(2):76-94.

Leaf RK, Connors JM. The role of anticoagulants in the prevention of pregnancy complications. Clin and App Thromb/Hemo. 2017:23(2);116-23.

Lee KL, Na BJ, Kim JY, Hur SE, Lee M, Gilman-Sachs A, Kwak-Kim J. Determination of clinical cellularimmune markers in women with recurrent pregnancy loss. Am J Reprod Immunol. 2013;70:398-411.

Lee CL, Lam KKW, Vijayan M, Koistinen H, Seppala M, Ng EHY, Yeung WSB, Chiu PCN. The pleiotropic effect of glycodelin-A in early pregnancy. Am J Reprod Immunol;2016;75:290-297.

Lee MM, Donaghue PK. Mullerian inhibiting substance: a gonadal hormone with multiple functions. Endocrine Reviews 1993;14:152-64.

Li TC, Makris M, Tomsu M, Tuckerman E, Laird S. Recurrent Miscarriage: aetiology, management and prognosis, Hum Reprod Update, 2002;8:463-481.

Li T-C, Iqbal T, Antie B, Gillham J, Amer S, Wood K, et al. An analysis of the pattern of pregnancy loss in women with recurrent miscarriage. Fertil Steril. 2002;78:1100–6.

Liang P, Mo M, Li G-G, Yin B, Cai J, Wu T.et al. Comprehensive analysis of peripheral blood lymphocytes in 76 women with recurrent miscarriage before and after lymphocyte immunotherapy. Am J Reprod Immunol. 2012;68:164-74.

Lima F, Khamashta MA, Buchanan NMM, Hunt BJ, Hughes GRV. A study of sixty pregnancies in patients with the antiphospholipid syndrome. Clin Exp Rheumatol 1996;14:131-6.

Lissak A, Sharon A, Fruchter, O, Kassal A, Sanderovitz J, Abramovici H. Polymorphism for mutation of cytosine to thiamine at location 677 in the

methylenetetrahydrofolate reductase gene is associated with recurrent early fetal loss. Am J Obstet Gynecol .1999;181:126-30.

Lo W, Rai R, Brailsford SR, Al-Ghamdi AA, Regan L. The effect of body mass index on the outcome pregnancy in women with recurrent miscarriage. J Family Community Med 2012;19:167-171.

Lockwood CJ, Romero R, Feinberg RF, Clyne LP, Coster B Hobbins JC. The prevalence and biologic significance of lupus anticoagulant and anticardiolipin antibodies in a general obstetric population. Am J Obstet Gynecol 1989;161:369-373.

Lockwood C, Wendel G. Practice bulletin no 124: inherited thrombophilias in pregnancy. Obstet Gynaecol. 2011;118(3):730-40.

Lockshin MD, Sammaritano L, Schwartzman S. Validation of the Sapporo criteria for antiphospholipid syndrome. Arthritis Rheum 2000;43:440-3.

Lockshin MD, Druzin ML, Goei S,Jovanovic L, Ferenc M. Antibody to cardiolipin as a predictor of fetal distress or death in pregnant patients with systemic lupus erythematosus. N Engl J Med. 1985;313:152-6.

Lu M, Xu Y, Lv L, Zhang M. Association between vitamin D status and the risk of gestational diabetes mellitus: a meta-analysis. 2016;293:959-966.

Lyttle Schumaker BM, Jukic AMZ, Steiner AZ. Antimüllerian hormone as a risk factor for miscarriage in naturally conceived pregnancies. Fertil steril 2018;109:1065-1071.

Mackworth-Young CG, Loizou S, Walport MJ. Primary antiphospholipid syndrome: features of patients with raised anticardiolipin antibodies and no other disorder. Annals of Rheu dis 1989;48(5):362-367.

Männisto T, Vääräsmäki M, Pouta A, Hartikainen AL, Roukonen A, Surcel HM et al. Perinatal outcome of children born to mothers with thyroid dysfunction or antibodies: a prospective population-based cohort study. J Clin Endocrinol Metab 2009;94:772-779.

Martinez-Conejero JA, Morgan M, Montesinos M Fortuno S, Meseguer M, Simon C et al. Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. Fertil Steril 2011;96:943-950.

Maryam K, Bouzari Z, Basirat Z, Kashifard M, Zadeh MZ. The comparison of insulin resistance frequency in patients with recurrent early pregnancy loss to normal individuals. BMC Res Notes 2012;5:133.

Mateo J, Oliver A, Borrell M, Sala N, Fontcuberta J. Laboratory evaluation and clinical characteristics of 2,132 consecutive unselected patients with venous thromboembolism--results of the Spanish multicentric study on thrombophilia (EMET-Study) Thrombosis and Haemostasis 1997:77(3);444-451.

McCowan LM, North RA, Kho EM, Black MA, Chan EH, Dekker GA, Poston L, Taylor RS and Roberts CT (2011) Paternal contribution to small for gestational age babies: a multicenter prospective study. Obesity 19:1035-9. doi: 10.1038/oby.2010.279.

McCullough, LE, Miller EE, Calerwood LE, Hebert JR, Hoyo C. Maternal inflammatory diet and adverse pregnancy outcomes: Circulating cytokines and genomic imprinting as potential regulators? Epigenetics 2017;12:688-697.

McGee EA, Hseueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocrine Reviews 2005;21:200-14.McNeil HP Krilis SA . Antiphospholipid antibodies. *ANZJOG*;1991:21(4);20316-20322.

Meuleman T, Haasnoot GW, van Lith JMM, Verduijn W, Bloemenkamp KWM, Claas FHJ. Paternal HLA-C is a risk factor in unexplained recurrent miscarriage Am J Reprod Immunol. 2018 Feb;79(2). doi: 10.1111/aji.12797.

Miletich J,Sherman L. Broze, G. Jr. Absence of thrombosis in subjects with heterozygous protein C deficiency. N Engl J Med 1987;317:991-6.

Mills JL, Simpson JL, Driscoll SG, Jovanovic-Peterson L, Van Allen M, Aarons JH et al. Incidence of spontaneous abortion among normal women and insulin-dependent diabetic women whose pregnancies were identified within 21 days of conception. N Engl J Med. 1988;319(25):1617-23.

Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295-306.

Mizwiki A, Norman AW. The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. Sci Signal 2009;2:re4.

Moffett A, Chazara O, Colucci F and Johnson H. Variation of maternal KIR and fetal HLA-C genes in reproductive failure: too early for clinical intervention. Reproduuctive BioMedicine online 2016;33(6): 763-769. https://doi.org?10.1016/j.rbmo.08.019

Moore JE. Biologically false positive serologic tests for syphilis. JAMA;1952;150:467-73.

Moore KL, Persaud TVN, Torchia MG. The Urogenital System. Before we are born: Essentials of Embryology and Birth Defects, 7th edn, Philadelphia: Saunders/Elsevier, 2008, 162-189.

Morikawa M, Yamada H, Kato EH, Shimada S, Ebina Y, Yamada T et al. NK cell activity and and subsets in women with a history of spontaneous abortion. Gynecol Obstst Invest. 2001; 52:163-7.

Moore KL, Persaud TVN, Torchia MG. The Urogenital System. Before we are born: Essentials of Embryology and Birth Defects, 7th edn, Philadelphia: Saunders/Elsevier, 2008, 162-189.

Myones BL McCurdy D. The antiphospholipid syndrome: immunologic and clinical aspects. Clinical spectrum and treatment. J Rheumatol 2000;58(Suppl 27):20-8.

Nahass GT. Antiphospholipid antibodies and the antiphospholipid antibody syndrome.Journ Amer Acad Derm;2000:36(2)149-68.

Negro R, Schwartz A, Gismondi R, Tinelli A, Mangieri T, Stagnaro-Green A. Increased Pregnancy Loss Rate in Thyroid Antibody Negative Women with TSH Levels between 2.5 and 5.0 in the First Trimester of Pregnancy. J Clin Endocrinol Metab. 2010;95(9):E44-E48.

Negro R, Schwartz A, Gismondi R, Tinelli A, Mangieri T, Stagnaro-Green A. Thyroid Antibody Positivity in the First Trimester of Prgenancy Is Associated with Negative Pregnancy Outcomes. J Clin Endocrinol Metabol, 2011:96(6):E920-E924.

Nelen WLDN, Blom HJ. Steegers EAP, den Heijer M, Eskes TKAB. Hyperhomocystinaemia and recurrent pregnancy loss: (A meta-analysis.). FertilSteril, 2000; 74:1196-9.

Nielsen HS, Wu F, Aghai Z, Steffensen R, van Halteren AG, Spierings et al. H-Y antibody titers are increased in unexplained secondary recurrent miscarriage patients and associated with low male:female ratio in subsequent live births. Hum Reprod 2010b;25:2745-2752.

Nikolaou D, Lavery S, Turner C, Margara R, Trew G. Is there a link between an extremely poor response to ovarian hyperstimulation and early ovarian failure? Human Reproduction 2002;17:1106-11.

Nybo Andersen AN, Wohlfahrt J, Christens P, Olsen J Melbye M. Maternal age and fetal loss:population based register linkage study. BMJ 2000;320:1708-12.

Ohno M, Maaeda t, Matsunobu A. A cytogenetic study of spontaneous abortions with direct analysis of chorionic villi. Obstet Gynecol. 1991;77(3):394-8.Oshiro BT, Silver R, Scott, JR, Yu H, Branch DW. Antiphospholipid antibodies and fetal death. *Obstet Gynecol*;1996;87:489-93.

Ota K, Dambaeva S, Han A, Beaman K, Gilman-Sachs A, Kwak-Kim J. Vitamin D deficiency my be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity. Human Reprod. 2014;29(2):208-219.

Palermo GD, Neri QV, Cozzubbo T, Rosenwaks z. Perspectives on the assessment of human sperm chromatin integrity. Fertil Steril. 2014;102(6):1508-17.

Pandey S, Pandey S, Maheshwari A, Bhattacharya S. The impact of female obesity on the outcome of fertility treatment. J Hum Reprod Sci 2010;3:62-67.

Petrozza J.C. Obrien B. Early pregnancy loss. wwwemedicinecom/med/topic3241 2004.

Pittas AG, Nelson J, Mitri W, Hillman C, Garganta DM, Nathan et al. Plasma 25-hydroxyvitamin D and progression to diabetes in patients at risk for diabetes: an ancillary analysis in the Diabetes Prevention Program. Diab Care. 2012;35:(3) ;565-573.

Popescu F, Jaslow CR, Kutteh WH. Recurrent pregnancy loss evaluation combined with 24-chromosome microarray of miscarriage tissue provides a probable or definate cause of pregnancy loss in over 90% of patients. Human Reprod 2018;33:579-587.

Prummel MF, Streider T, Wiersinga WM. The environment and autoimmune thyroid diseases diseases. Eu J Endocrinol 2004; 150(5):605-618.

Raad G, Hazzouri M, Bottini S, Trabucchi M, Azoury J and Grandjean V (2017) Paternal obesity: how bad is it for sperm quality and progeny health? Basic Clin Androl 27:20. doi: 10.1186/s12610-017-0064-9.

Rao M, Zeng Z, Zhou F, Wang H, Liu J, Wang R et al. Hum Reprod Update 2019;3:344-361.

RCOG. The investigation of Recurrent Miscarriage: College; 2003 June 2003.

Roberts CT (2010) IFPA Award in Placentology Lecture: complicated interactions between genes and the environment in placentation, pregnancy outcome and long term health. Placenta 31:S47-S53.

Robinson L, Gallos ID, Connor SJ, Rajkhower 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;27:2908-2917.

Rodger MA, Betancourt MT, Clark P et al. The association of factor V Leiden and prothrombin gene mutation and placenta-mediated pregnancy complications: a systematic review and meta-analysis of prospective cohort studies. PLoS Med 2010;7(6):e1000292.

Roubey RAS. Autoantibodies to phospholipid-binding plasma proteins: a new view of lupus anticoagulants and other “antiphospholipid” autoantibodies. Blood 1994; 84:2854-67.

Royal College of Obstetricians and Gynaecologists. The investigation and treatment of couples with recurrent first-trimester and second trimester miscarriage. In: Green-top guidelines No17. London: Royal College of Obstetricians and Gynaecologists; 2011.

Russell P, Sachs G, Tremelen K, Gee A. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. III: further observations and reference ranges. Pathology. 2013;45:393-401.

Salim R, Regan L, Woelfer B, Backos M, Jurkovic D. A comparative study of the morphology of congenital uterine anomalies in women with and without a history of recurrent first trimester miscarriage. Hum Reprod 2003;18:162-6.

Saravelos SH, Yan J, Rehmani H, Li TC. The prevalence and impact of fibroids and their treatment on the outcome of pregnancy in women with recurrent miscarriage. Hum Reprod 2011;26(12):3274-3279.

Saravelos SH, Cocksedge KA, Li TC. Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal. Hum Reprod Update 2008;14:415-29.

Sebire NJ, Fox H, Backos M, Rai R, Paterson C, Regan L. Defective endovascular trophoblast invasion in primary antiphospholipid syndrome- associated early pregnancy failure. Hum Reprod 2002;17:1067-1071.

Seifer DB, Naftolin F. Moving toward an earlier and better understanding of perimenopause. Fertility and sterility 1998;69:387-8.

Shapiro SS. The lupus anticoagulant/antiphospholipid syndrome. Annu Rev Med 1996;47:533-53.

Silver RM, ZhaoY, Spong CY, et al. Prothrombin gene G20210A mutation and obstetric complications. Obstet Gynecol 2010;115(1):14-20.

Silverman JG, Gupta J, Decker MR, Kapur N, Raj A. Intimate partner violence and unwanted pregnancy, miscarriage, induced abortion and stillbirth among a antional sample of Bangladeshi women.BJOG. 2007;114(10):1246-52.

Stagnaro-Green A, Chen X, Bogden JD, Davies TF, Scholl TO. The thyroid and pregnancy: a novel risk factor for very preterm delivery. Thyroid 2005;15:351-357.

Steen MT, Boddie A.M, Fisher AJ, Dembure PP, Elsas LJ. Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid**.** Prenat Diagn 1998;18:545-55.

Tan EM Cohen AS, Fries JF, Talal N, Winchester RJ. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-7.

te Velde ER, Scheffer GJ, Dorland M. et al Developmental and endocrine aspects of normal ovarian ageing. Molecular and Cellular Endocrinology 1998 145:67- 72.

Templer S, Sacks G. A blessing and a curse: is high NK cell activity good for health and bad for reproduction? Human Fert 2016.http://dx.doi.org/10.1080/14647273.2016 .121072.

Thangaratinam S, Tan A, Knox E, Kilby MD, Franklyn J, Coomarasamy A. Association between thyroid autoantibodies and miscarriage and preterm birth: meta-analysis of evidence. *BMJ*. 2011;342:d2616.

Thomas PK, Hoffbrand A.V, Smith IS. Neurological involvement in hereditary transcobalamin II deficiency. J Neurol Neurosurg Psychiatry, 1982; 45:74-7.

Tincani A, Lojacona A, Taglietti M, Motta M, Biasini C, Decca L, et al., Pregnancy and neonatal outcome in primary antiphospholipid syndrome. Lupus 2002;11:649.

Tierney K, Delpachitra P, Grossman M, Onwude J, Sikaris K, Wallace EM, Hamilton EJ, Tong S. Thyroid function and autoantibody status among women who spontaneously deliver under 35 weeks of gestation. *Clin Endocrin* (Oxf) 2009;71:892-895.

Tong M, Viall CA, Chamley LW. Antiphospholipid antibodies and the placenta: a systematic review of their in vitro effects and modulation by treatment. Hum Reprod update 2015;21:97-118.

Torodova MBM. Some recent insights into the prothrombogenic mechanisms antiphospholipid antibodies. (Review) Current Medicinal Chemistry 2007;13:811 - 26.

9788) 9990) Vaarala OP, Kleemola M. Anticardiolipin response in acute infections. *Clin Immunol Immunopathol*. 1986;41:8-15.

Trikudanathan S. Polycystic Ovarian Syndrome. Med Clin N Amer 99; (2015) 221-

225.

Tripodi A, Asti D, Chantarangkul V, Biguzzi E, Mannucci PM. . Interference of factor V Leiden on protein S activity: evaluation of a new prothrombin time-based assay. *Blood Coagul Fibrinolysis* 2007;18:543-6.

Unfried G, Griesmacher A, Weismüller W, Huber JC, Tempfer CB. The C677T Polymorphism of the Methylenetetrahydrofolate Reductase Gene and Idiopathic Recurrent Miscarriage. Obstetrics & Gynecology 2002;99:614-9.

Vaarala OP, Kleemola M. Anticardiolipin response in acute infections. *Clin Immunol Immunopathol*. 1986;41:8-15.

Van den Boogaard E, Cohn DM, Korevaar JC, Dawood F, Vissenberg R, Middledorp S et al. Fertil Steril 2013;99:188-192.

Van Der Put NMJ, Thomas CMG, Eskes, TKAB, Smeitink JAM, Blom H. Altered folate and vitamin B12 metabolism in families with spina bifida offspring**.** Qjm 1997;90:505-10.

Van Horn JT, Craven C, Ward K, Branch DW, Silver RM. Histologic features of placentas and abortion specimens from women with antiphospholipid syndrome and antiphospholipid-like syndromes. Placenta 2004;25:642-648.

Van Kampen CA, Versteeg-van der Voort Maarschaalk MF, Langerak-Langerak J van BE, Roelen BL, Claas FH. Pregnancy can induce long-persisting primed CTLs specific for inherited paternal HLA antigens. *Hum Immunol.* 2001;62:201-7.

Verburg PE, Tucker G, Scheil W, Erwich JJHM, Dekker GA, Roberts CT. Seasonality of gestational diabetes mellitus: a South Australian population study. BMJ Open Diabetes Research and Care 2016;4:e000286. Doi:10.1136/bmjdrc-2016-000286.

Vidogner M, Shrivastava V, Gutstein LE, Schneider J, Nieves J, Goldstein M et al. Localization and identification of sumoylated proteins in human sperm: excessive sumolyation is a marker of defective spermatozoa. Hum Reprod. 2013;28(1):210-23.

Vissenberg R, Manders VD, Mastenbroek S, Fliers E, Afink GB, Ris-Stalpers C et al. Pathophysiological aspects of thyroid hormone disorders/thyroid peroxidase autoantibodies and reproduction. Hum Reprod Update 2015;21:378-387.

Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 2006;131:1-9.

Volarcik K, Sheean L, Goldfarb J, Woods L, Abdul-Karim FW, Hunt P. The meiotic competence of in-vitro matured human oocytes is influenced by donor age: evidence that folliculogenesis is compromised in the reproductively aged ovary. Human Reproduction 1998;13:154-60.

Wang Y, Zhao H, Li Y, Zhang J, Tan J, Liu Y. Relationship between Recurrent Miscarriage and Insulin Resistance. *Gynecol Obstet Invest* 2011;72:245-51.

Warburton D, Kline J, Stein Z, Hutzler M, Chin A, Hassold T. Does karyotype of a spontaneous abortion predict the karyotype of a subsequent abortion? Evidence from 273 women with two karyotyped spontaneous abortions. Am J Hum Genet. 1987;41(3):465-83.

Wei SQ. Vitamin D and pregnancy outcomes. Curr Opin Obstet Gynecol. 2014;26(6):438-447.

Wimalawansa SJ. Associations of vitamin D with insulin resistance, obesity, type 2 diabetes, and metabolic syndrome. J Steroid Biochem. Mol Biol. 2016. http://dx.doi.org/10.1016/j.jsbmb.09.017.

Wilson RL, Leviton AJ, Leemaqz SY, Anderson PH, Grieger JA, Grzeskowiak LE, Verburg PE, McCowan L, Dekker GA, Bianco-Miotto T and Roberts CT (2018) Vitamin D levels in an Australian and New Zealand cohort and the association with pregnancy outcome. BMC Pregnancy and Childbirth 18:251.

Wilson WA, Ghavri AE, Koike, T, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum* 1999;42:1309-11.

Van Kampen CA, Versteeg-van der Voort Maarschaalk MF, Langerak-Langerak J van BE, Roelen BL, Claas FH. Pregnancy can induce long-persisting primed CTLs specific for inherited paternal HLA antigens. *Hum Immunol.* 2001;62:201-7.

Yamada H, Morikawa M, Kato EH, Shimada S, Kobashi G, Minikami H. Pre-conceptional natural kiler cell activity and percentage as predictors of biochemical

pregnancy and spontaneous abortion with normal chromosome karyotype. Am J Reprod Immunol. 2003;50:351-4.

Yang CJ, Stone P, Stewart AW. The epidemiology of recurrent miscarriage: a descriptive study of 1214 prepregnant women with recurrent miscarriage. Aust N Z J Obstet Gynaecol 2006;46:316-22.

Zarrabi MH, Zucker S, Miller F,Harnett JA, Varma AO. Immunologic and coagulation disorders in chlorpromazine-treated patients. *AnnIntern Med* 1979;91:194-9.

Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol and Endocrin 2004:2-7.

Zhu R-Y, Wong Y-C, Yong E-L. Sonographic evaluation of polycystic ovaries. Best practice & research. Clinbical obstetrics and gynaecology. 2016 http://dx.doi.org/10.1016/j.bpobgyn.2016.02.005

Zitterman A. Vitamin D and disease prevention with special reference to cardioascular disease. Prog Biophys Mol Biol. 2006 (1):39-48.

CHAPTER 2: The Recurrent Miscarriage Clinic

2.1 Background

Traditionally, women with recurrent miscarriage have been offered various tests in order to identify the cause and to guide management. These have been in various clinical settings including primary care, fertility and obstetric services. This dedicated clinic was established to better serve the needs of women who have suffered recurrent miscarriage.

This cohort of patients was recruited from the Recurrent Miscarriage Clinic at the Women's and Children's Hospital in Adelaide, South Australia, from 2009 until 2017.

This study formed part of a Clinical Trial named the PAPO (**P**rediction of **A**dverse **P**regnancy **O**utcomes) study, (Clinical trial number ACTRN12609000254291). The study was approved by the Women's and Children's Hospital Human Research Ethics Committee in North Adelaide South Australia, REC1481/6/09. The patients were informed about the research and written informed consent was obtained. They had an average of four visits during the work up, and many were followed in the subsequent pregnancy, in a single clinic.

The purpose of the study was to offer as full an evidence based recurrent miscarriage work up as possible, given the paucity of research in the area, evaluate the findings, on an individual basis, and advise on the best method of treatment for the presumed causes/associations, so that the patients could achieve their objective of a live birth.

There were 1532 patients referred to the Recurrent Miscarriage Clinic, of whom 480 fitted the criteria of recurrent embryonic miscarriages, (losses at 10 weeks or less,

post the last menstrual period), as described before. The reasons for the exclusions included non-consecutive losses, proven genetic abnormalities, or unproven pregnancies based on recall alone. Patients who suffered both embryonic and fetal losses were only included if they had experienced 2 or 3 consecutive embryonic losses prior to their index visit. The recent European Society of Human Reproduction and Embryology (ESHRE) guidelines, however, have suggested that non-consecutive losses could be included, and this would have increased the eligible numbers considerably (Atik et al., 2018).

There were 283 women aged less than or equal to 35 years (≤35), and 197 greater than 35 years of age (>35). They were referred to the Recurrent miscarriage clinic if they had suffered two or more consecutive pregnancy losses, with the same partner.

2.2 Work-up

The patients were referred to the clinic approximately six weeks after the miscarriage. A full history was taken including past medical, surgical, obstetric and gynaecological history, as well as family history, social status and work environment, dietary habits, smoking habits, drug use and exercise. Weight and height measurements were also performed, and BMI calculated. A family history of losses, congenital abnormalities, and any major illnesses was included. Details of the previous losses included the dating of previous losses in order to ascertain if they were embryonic or fetal, and any history relevant to this, history of medical or surgical management of the losses. If they had not had an abdominal CT scan, MRI or previous laparoscopy, dye studies or Hysterosalpingo-contrast-sonography, (HyCoSy), they were offered a 3D Ultrasound with pelvic studies.

Partners were also asked for their relevant surgical and Medical History, as well as relevant family history. If the previous miscarriage products had been sent for genetic testing, and the result was an aneuploid loss, they were not included in the assessment unless they had previously had more than two consecutive genetically normal/unknown losses.

2.3 Demographics

2.3.1 Ethnicities

	≤ 35 years N (%)	> 35 years N (%)
Total	283 (59)	197 (41)
Ethnicities		
Caucasian	224 (79.2)	166 (84.3)
Indian	23 (8.1)	9 (4.6)
Other Asian	15 (5.3)	9 (4.6)
Middle Eastern	18 (6.4)	7 (3.6)
African	3 (1.1)	7 (3.6)
BMI		
Underweight < 20	0	0
Healthy 20.1 – 24.9	148 (52.3)	116 (58.9)
High > 25	135 (47.7)	81 (41.1)

Body Mass index: BMI was calculated and recorded.

Table 2.3.1: The Ethnic structure and BMI of the patients in the RPL clinic.

Patients were divided into two age groups, ≤35 years of age or > 35 years of age at their first visit. The reason for assessing these patients in the different age groups was due to the well documented effect of maternal age on fetal loss, as described by Nybo Anderson et al., (2000). They reported a steep increase in fetal loss after the age of 40 years, which was already increased in the 30's, but particularly after 35 years of age (Nybo Andersen et al., 2000). Women attending the clinic are heterogeneous in their journeys and experience of multiple miscarriages. It soon became obvious that a systematic approach to their care was required. Therefore, the ASK TEAMS approach was developed and is the subject of this thesis.

This approach was used to ensure that all patients had the same tests, as opposed to random or incomplete work ups. Given the large number of tests, a simple acronym was helpful in reminding physicians to arrange the Ultrasounds, fasting and non-fasting bloods. Many Centres still offer a work-up based on outdated recommendations, and even the acronym will need to change as more evidence becomes available.

The acronym is “ASK TEAMS” and is as follows:

A **A**ge: A realistic discussion regarding the effects of age on reproductive potential was held with all couples, particularly those aged over 40. Patients were also offered an Anti Müllerian Hormone test as part of the work up, if they were prepared to arrange this separately, as it is not covered by the National Insurance (MEDICARE).

S **S**tructure: If they had not previously undergone a diagnostic laparoscopy, hysteroscopy, hysterosalpingogram, HyCoSy, CT scan of the abdomen/pelvis or MRI of the pelvis, they were offered a luteal phase 3D ultrasound for assessment of the uterus, to detect congenital uterine abnormalities, such as a unicornuate, bicornuate, septate or arcuate uterus. Acquired abnormalities such as fibroids, Ashermans syndrome, adenomyosis or endometriosis, as well as ovarian morphology were also investigated.

K **K**aryotype: All patients were asked to consent to send the products of conception for karyotyping, particularly if they had more than one loss. This was not always possible. If an aneuploidy was detected, the miscarriage was not included in patient selection. Parental karyotyping was initially performed on all those attending the clinic; however, the yield was so low, that it was finally restricted to those in whom a translocation was detected in a previous miscarriage, or in those in whom no other possible cause had been found.

T **T**hrombophilia: Initially all patients were offered a full thrombophilic screen, for both genetic and acquired thrombophilias. Recent evidence has suggested that screening for hereditary thrombophilias has a very low yield, given their low

prevalence in population, and so the screening was restricted to those with a strong family history of venous thromboembolism, (VTE) or those who had experienced any thromboembolic events prior to presentation (Davenport et al., 2014). While evidence is conflicting, clinicians in most countries now agree that thrombophilias are unlikely to be associated with embryonic losses, especially as maternal blood flow to the intervillous space of the placenta is only initiated after ten weeks when gestation enters the Fetal period (Jauniaux et al. 2000). The 2018 ESHRE guidelines do not recommend screening for hereditary thrombophilias unless the screening is performed in the context of a research project, or in women who have had thrombotic events. The clinical utility of the testing was judged as minimal and the harms of testing thought to outweigh the benefits (Atik et al., 2018).

Thyroid issues such as subclinical hypothyroidism and the presence of thyroid antibodies could replace the Thrombophilia heading.

E **E**ndocrine: All patients were offered thyroid function tests, a 75g oral glucose tolerance test (OGTT), including insulin and prolactin levels. Reproductive hormone levels were not routinely tested, unless the patient presented with cycle irregularity, or features of the polycystic ovarian syndrome (PCOS). If PCOS was suspected from history or from the 3D ultrasound ovarian morphology, a full androgenic work-up was undertaken including sex hormone binding globulin levels, testosterone levels and the free androgen index.

A **A**utoimmune**:** Antibody tests included thyroid peroxidase antibodies (TPO), anti-thyroid receptor antibodies (ATA), antinuclear antibody (ANA) titres. Extractable nuclear antibody tests were only performed if the ANA titres were high. Antiphospholipid antibodies were tested in order to detect the Antiphospholipid

syndrome. They comprised: Anticardiolipin antibodies IgG and IgM, Beta-2 Glycoprotein 1 (B2GP1) antibodies and the lupus anticoagulant antibodies; the dilute russell viper venom test (DRVVT) and Kaolin clotting time (KCT). (Lockwood et al., 2013).

M **M**etabolic**:** Insulin levels were tested as part of the endocrine work up, as were the glucose levels, detecting elevated fasting glucose, impaired glucose tolerance or overt diabetes. Fasting homocysteine levels were also tested.

S **S**pecific**:** Vitamin D, B12 and serum folate studies were performed. Infectious screens were performed if there was a positive history of vaginal infections, such as chlamydia or bacterial vaginosis. A detailed nutritional history was obtained, and coeliac studies performed for a history of dietary intolerances. Body mass index was calculated at the initial visit and recorded. A history of depression and anxiety was also sought, and relevant referrals made for a mental health plan, if they were deemed to have a significant problem in need of additional psychological support.

Partners were included in the work up, as long as they had been the father of at least the previous two losses. Their work up included a detailed history, and a fasting blood work up. This included fasting homocysteine, glucose, insulin, Vitamin D, folate, and B12 levels. Karyotype was initially performed on all partners but was eventually limited to those in whom the previous pregnancy loss had yielded a translocation on karyotyping.

Female	
Non-Fasting bloods	Complete blood picture
	AMH (Privately funded test)
	+/- Karyotype - History dependent
	Thyroid function tests - TSH T4
	Thyroid antibodies
	Anti-Cardiolipin antibodies
	Beta 2 GP1 antibodies
	Lupus anticoagulant –DRVVT, Kaolin clotting time
	Antinuclear antibodies
Fasting bloods	Glucose tolerance test, 75g, with insulin studies
	Homocysteine levels
	Folate, Vitamin B12 and Vitamin D studies
Structural tests	3 D Ultrasound/ HyCoSy/ MRI/ laparoscopy/ hysteroscopy
Male	
Fasting bloods	+/- Karyotype – History dependent
	Glucose and insulin levels
	Homocysteine levels
	Vitamin D, B12 and Folate levels

Table 2.3.2 Investigations in the RPL Clinic,

offered to both partners.

References Chapter 2

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.

Davenport WB, Kutteh WH. Inherited Thrombophilias and Adverse Pregnancy Outcomes. A Review of Screening Patterns and Recommendations. Obstet Gynecol Clin N Amer. 2014;(41)133-144.

Egerup P, Kolte AM, Larsen EC, Krog M, Nielsen HS, Christiansen OB. Recurrent pregnancy loss: what is the impact of consecutive versus non-consecutive losses? Hum Reprod 2016;31: 2428-2434.

Farquharson RG, Jauniaux E, Exalto N. Updated and revised nomenclature for description of early pregnancy events. Hum Rep 2005;20:(11)3008-11.

Lockwood C, Wendel G, Silverman N. ACOG Practice Bulletin 138. Inherited Thrombophilias in Pregnancy. Obstet Gynecol 2013:122(3);706-16.

Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN and Burton GJ (2000) Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 2000:157;111-22.

Nybo Anderson AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population-based register linkage study. BMJ 2000; 320:1708-12.

Petrozza JC. Recurrent Early Pregnancy Loss. 2012.

Practice Committee of the American Society of the American Society for Reproductive Medicine. Definition of infertility and recurrent pregnancy loss: Fertil Steril 2013;99(1):63.

RCOG. The Investigation and Treatment of Couples with Recurrent First-trimester and second-trimester miscarriage. 2011.

CHAPTER 3: Age

The term that is used to describe a woman's reproductive potential, as it relates to the quality of her oocytes and her own individual follicular depletion, is ovarian reserve. Serum Anti-Müllerian Hormone (AMH) levels are an ideal marker of a woman's ovarian reserve, as AMH is highly correlated with the number of antral follicles remaining. (Visser et al., 2006; Barad et al., 2009). Fertility declines in the years preceding menopause, and the menstrual cycle becomes irregular, ultimately ceasing. There is significant individual variation in the age of menopause and, subsequently, also in the age of 'subfertility' (te Velde et al., 1998). Chronological age is thus a poor indicator of individual reproductive ageing and also of actual ovarian reserve (Faddy et al., 1998).

Anti Müllerian Hormone levels are an indirect reflection of ovarian reserve, and levels known to correlate with the remaining number of antral follicles. (Gruitjers et al., 2003; Kelsey et al., 2012).

Severe endometriosis, pelvic inflammatory disease, ovarian surgery, various systemic illnesses, chemotherapy and possibly smoking are all known factors affecting ovarian reserve. (Faddy et al.,1997; Sharara et al.,1998). It is possible that such 'sub fertility' could play a role in miscarriages, and AMH levels could predict the live birth chances of patients attending the clinic (Lukaszuk et al., 2009). Therefore, quantification of circulating AMH was offered in the clinic.

References Chapter 3

Barad DH, Weghofer A, Gleicher N. Comparing anti-Mullerian hormone (AMH) and follicle-stimulating hormone (FSH) as predictors of ovarian function. Fertil Steril. 2009;91(4 Suppl):1553-5.

Faddy M, Gosden R, Gougeon A, Richardson S, Nelson J. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Human Reproduction 1997;7 1342-6.Gruijters MJ, Visser JA, Durlinger AL, Themmen AP. Anti-Müllerian hormone and its role in ovarian function. Mol Cell Endocrinol. 2003;211(1-2):85-90.

Kelsey TW, Anderson RA, Wright P, Nelson SM, Wallace WH. Data-driven assessment of the human ovarian reserve. Mol Hum Reprod 2012;**18**:79-87.

Lukaszuk K, Kunicki M, Liss J, Bednarowska A, Jakiel G. 2014. Probability of live birth in women with extremely low anti-müllerian hormone concentrations. Reproductive Biomedicine Online 28:64–69.

Sharara FI, Seifer DB, Flaws JA. Environmental toxicants and female reproduction. Fertility and Sterility. 1998;70(4):613-22.

te Velde ERS, G.J. Dorland, M. et al. Developmental and endocrine aspects of normal ovarian ageing. Molecular and Cellular Endocrinology 1998 145:67-73.

Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-Mullerian hormone: a new marker for ovarian function. Reproduction. 2006;131(1):1-9.

Statement of Authorship (SOA) AMH paper

Published Paper: AMH

Statement of Authorship

Title of Paper	Anti Müllerian Hormone levels in recurrent embryonic miscarriage patients are frequently abnormal and may affect pregnancy outcomes.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Published: Journal of Obstetrics and Gynaecology. https://doi.org/10.1080/01443615.2018.1552669

Principal Author

Name of Principal Author (Candidate)	Dr C D McCormack
Contribution to the Paper	Planning of project, selection of patients, written text
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11/6/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr S Leemaqz
Contribution to the Paper	Statistics, contribution to written text
Signature	Date 15/9/18

Name of Co-Author	Dr D L Furness
Contribution to the Paper	Selection of patients, contribution to written text
Signature	Date 15/9/2018

Name of Co-Author	Professor G A Dekker		
Contribution to the Paper	Project development, contribution to written text		
Signature		Date	17-10-18

Name of Co-Author	Professor C T Roberts		
Contribution to the Paper	Contribution to written paper		
Signature		Date	18.10.18

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

The Journal of Obstetrics and Gynaecology

Taylor & Francis
Taylor & Francis Group

Journal of Obstetrics and Gynaecology

ISSN: 0144-3615 (Print) 1364-6893 (Online) Journal homepage: https://www.tandfonline.com/loi/ijog20

Anti-Müllerian hormone levels in recurrent embryonic miscarriage patients are frequently abnormal, and may affect pregnancy outcomes

Catherine D. McCormack, Shalem Y. Leemaqz, Denise L. Furness, Gustaaf A. Dekker & Claire T. Roberts

To cite this article: Catherine D. McCormack, Shalem Y. Leemaqz, Denise L. Furness, Gustaaf A. Dekker & Claire T. Roberts (2019): Anti-Müllerian hormone levels in recurrent embryonic miscarriage patients are frequently abnormal, and may affect pregnancy outcomes, Journal of Obstetrics and Gynaecology, DOI: 10.1080/01443615.2018.1552669

To link to this article: https://doi.org/10.1080/01443615.2018.1552669

Published online: 27 Mar 2019.

JOURNAL OF OBSTETRICS AND GYNAECOLOGY
https://doi.org/10.1080/01443615.2018.1552669

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Anti-Müllerian hormone levels in recurrent embryonic miscarriage patients are frequently abnormal, and may affect pregnancy outcomes

Catherine D. McCormack[a,b], Shalem Y. Leemaqz[a], Denise L. Furness[a], Gustaaf A. Dekker[a] and Claire T. Roberts[a]

[a]Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide, Australia; [b]Department of Obstetrics, Women's and Children's Hospital, North Adelaide, Australia

ABSTRACT

This prospective cohort study measured anti-Müllerian hormone (AMH) levels in recurrent miscarriage (RM) patients, compared them to a normal population, and assessed the pregnancy outcomes. The RM patients demonstrated AMH levels that were significantly lower than the normal population, both in women aged ≤35 years, and those aged >35 years. AMH percentiles were found to be significantly lower in the study group of RM patients ≤35 years ($p < .004$) in the 5th and 50th percentiles, and in all percentiles in women >35 years ($p < .03$), were compared to women from a normal population. Serum AMH levels may reflect quality, and quantity of the remaining oocytes in these patients, and RM patients may have a low ovarian reserve, and a potentially poor oocyte quality, as shown by low circulating AMH. The evaluation of AMH levels in a RM work up may allow realistic counselling and possible ART referral in RM patients.

KEYWORDS

Anti-Müllerian hormone; recurrent miscarriages; ovarian reserve; subfertility; embryonic losses; foetal losses

IMPACT STATEMENT

- **What is already known on this subject**? There is some evidence to show that low AMH levels are associated with recurrent miscarriages and this is thought to be due to a decreased oocyte quality. The AMH levels are lower in the patients with endometriosis, and are often significantly higher in the patients with polycystic ovarian syndrome. Both conditions are independently associated with miscarriages.
- **What the results of this study add?** Anti-Müllerian hormone (AMH) levels were found to be significantly lower in recurrent miscarriage patients, compared to a normal population. This may be another factor contributing to miscarriages. The spontaneous pregnancy rates in the miscarriage group significantly improved with increasing AMH levels. This may confirm that patients with low AMH levels have poorer quality oocytes, and thus may be considered 'sub-fertile'. It was also found that the utilisation of assisted reproductive technologies (ART) to achieve a pregnancy was significantly reduced in the groups with a higher serum AMH.
- **What the implications are of these findings for clinical practice and/or further research?** Serum AMH levels should be offered to all patients as part of a recurrent miscarriage work up. Detecting the low AMH levels and counselling the patients on these findings may allow them the option of accessing ART. ART may have the ability to expedite conception rates, and with pre-implantation genetic analyses, could possibly select the embryos with the greatest chance of survival. Further research is needed to establish how the decreased AMH levels contribute to recurrent miscarriages.

Introduction

Ovarian reserve is the terminology used to describe the existing number of follicles within the ovaries. Although serum AMH levels are an indirect reflection of ovarian reserve, they have already been shown to be highly correlated with the remaining number of antral follicles, another indirect measure of ovarian reserve (Gruijters et al. 2003; Visser et al. 2006; Barad et al. 2009; Hansen et al. 2011; Kelsey et al. 2012). Early pregnancy losses are the most common complication of human gestation, occurring in 50–75% of all women trying to conceive (Petrozza 2006). Many of these losses occur before or with the next expected menses. With the advent of sensitive pregnancy detection kits available to the general public, previously unrecognised losses are frequently detected. With the losses that occur after the first missed menstrual cycle, approximately 15–20% are spontaneous miscarriages or ectopic pregnancies (Petrozza 2006). Approximately, 5% of couples trying to conceive will have two consecutive losses, and approximately 1% of couples will suffer three consecutive losses during their reproductive lives which is unlikely to be due merely to chance since it has been estimated that the probability of three consecutive losses is 0.34% (Bopp and Seifer 1998; RCOG 2011; Petrozza 2012). Christiansen stated that a risk factor could be detected in approximately 50% of couples experiencing pregnancy loss, there was very seldom a single factor, and that the majority of losses have a multifactorial background involving

CONTACT Catherine D. McCormack Dee.mccormack@sa.gov.au Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide, South Australia 5005, Australia.

the interaction of multiple genetic and environmental risk factors (Christiansen 2016). It is possible that some of these patients may have low ovarian reserves, and poorer quality oocytes. The purpose of this study was to assess the AMH levels in patients attending a recurrent miscarriage (RM) clinic, compare the results with those of a normal population, and to document the subsequent pregnancies.

Materials and methods

Recurrent embryonic miscarriage patients attending a RM clinic between June 2008 and May 2014 were included. They were offered serum anti-Müllerian hormone (AMH) testing, as well as the routine investigations that are typically offered in a RM clinic, which includes a 3D ultrasound, blood tests for endocrine (thyroid, prolactin levels) metabolic (GTT and insulin studies, homocysteine levels) autoimmune (thyroid antibodies, antinuclear antibodies), obstetric antiphospholipid syndrome, nutritional vitamins D and B, and folate, and a karyotype of both partners. Women with at least two consecutive embryonic losses (less than 10 weeks from the last menstrual period) were included (Kolte et al. 2015). An informed consent was obtained from all of the patients.

The women were divided into two groups, those aged 35 years or younger, and those aged over 35, because of the well-known effects of advanced maternal age (Nybo Andersen et al. 2000).

AMH assay

The serum AMH was quantified by immunoassay at Clinpath Laboratories using a DSL AMH generation II method (Beckman-Coulter Anti-Müllerian immunoassay).

The AMH levels were recorded and all of the pregnancy outcomes documented, including those in each group who did not achieve a pregnancy over a period of two years after the test was done, and those who only had further pregnancy losses. These outcomes were documented for all AMH levels (low, normal and high) in the two age groups. The women were followed up in the clinic for two years to ascertain whether they had achieved their pregnancy spontaneously or by ART and whether they had a live birth (LB).

Ethics approval

This study formed part of a Clinical Trial named the PAPO (Prediction of Adverse Pregnancy Outcomes) study (Clinical Trial Number ACTRN12609000254291). The study was approved by the Human Research Ethics Committee REC1481/6/09.

Statistics

The serum AMH concentrations in these women were compared with those from a healthy cohort using data obtained with permission from Shebl et al. (2011). The 5th, 50th and 90th percentiles in the age groups $\leq$35 and >35 years were compared, and data were analysed using the Chi-square test. The pregnancy outcomes in the women from the RM clinic who achieved an ongoing pregnancy, spontaneously or via ART, with low (<10 pmol/L), medium (10.1–30 pmol/L) or high (>30 pmol/L) serum AMH were compared by ANOVA (SPSS Version 19, SPSS Inc., Chicago, IL).

Results

During the study period, 202 patients were offered AMH testing, 182 women accepted, and 20 declined testing. Serum AMH results in the miscarriage group were compared with 1105 women in a presumably healthy sub group. The 5th, 50th and 90th percentiles were compared in the two groups – women aged $\leq$35 years, and women aged >35 years. The ethnicities of the patients were: Caucasian 88%, African 3.4%, Vietnamese/Chinese 3.4%, Middle Eastern 3.4% and Indian 1.7%. The numbers of losses ranged from 2 to 11. AMH centiles were found to be significantly lower in the study group of RM patients $\leq$35 years ($p<$ .004) and in those aged greater than 35 years ($p<$ .03), compared to women from a healthy population (Figures 1 and 2).

The percentages of women with low, normal and high serum AMH concentrations were assessed. Overall, 53.3% of all patients tested had low serum AMH, 34.6% were in the normal range and 12.1% were in the high range. In the group of women aged $\leq$35 years, 41.2% had serum levels of AMH considered to be low, 36.8% had normal levels and

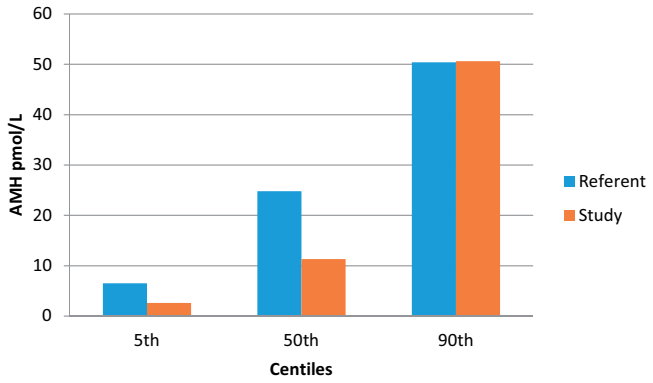


Figure 1. Anti-Müllerian hormone (AMH) levels in women aged $\leq$35 years, compared to a normal (referent) population (data derived from Shebl et al. 2011, with permission). The 5th, 50th and 90th percentiles were considered. The normal population showed significantly higher AMH levels in the 5th and 50th centiles ($p < 0.004$).

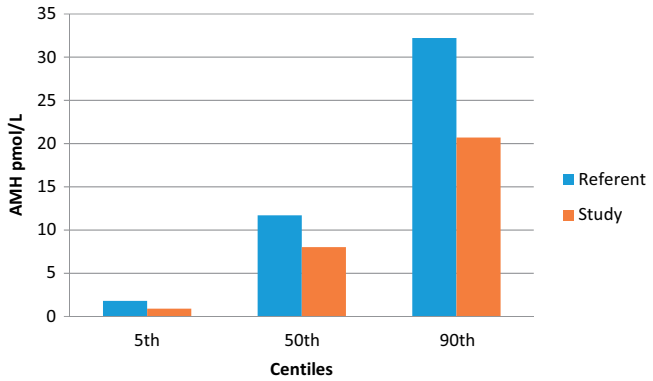


Figure 2. AMH levels in women aged >35 years compared to a normal (referent) population. The 5th, 50th and 90th centiles were compared. The normal population showed significantly higher levels in all centiles ($p < 0.03$).

Table 1. Outcomes regarding infertility, pregnancies, pregnancy losses and deliveries in women aged ≤35 years, in each of the AMH groups.

≤35 years; 37.4%; 68	≤10 pmol/L	10.1–30 pmol/L	>30 pmol/L
Total % patients	41.2% (28)	38.8% (25)	22% (15)
No pregnancies achieved	21.4% (6)	8% (2)	13% (2)
Pregnancies	78.57% (22)	92% (23)	87% (12)
Spontaneous	50% (11)	78.3% (18)	100% (12)
ART	50% (11)	21.7% (5)	0% (0)
Miscarriages	27.3% (6)	26.1% (6)	25% (3)
Spontaneous	66.7% (4)	66.7% (4)	100% (3)
ART	33.3% (2)	33.3% (2)	60% (0)
Deliveries	57.1% (16)	68% (17)	75% (9)
Spontaneous	43.7% (7)	82.35% (14)	100% (9)
ART	56.3% (9)	17.65% (3)	0% (0)

Low, <10 pmol/L; normal, 10.0–30 pmol/L; high, >30 pmol/L.

22% had high levels. PCOS had been diagnosed in 84% of those with an AMH > 30 pmol/L.

The women aged >35 years had lower AMH levels compared to women 35 years or less ($p<$ .000) as would be expected with 60.5% with low AMH, 33.3% with normal levels, and 6% had high levels. PCOS had been diagnosed in 50% of those with AMH levels >30 pmol/L.

Pregnancy outcomes

Patient follow up showed that among women aged ≤35 years with low AMH levels, 21.4% were designated infertile; in that they did not achieve a desired pregnancy over a two-year period. Precisely, 27.3% only had miscarriages, resulting in an overall LB in women with low AMH levels of 57.1%. Of the latter, 43.7% were spontaneously conceived pregnancies, and 56.3% utilised assisted reproductive technologies (ART). In the women ≤35 years with normal AMH levels, 8% were infertile, 26.1% had only miscarriages, and the overall LB rate was 68%; 82.4% of which were spontaneous pregnancies, and 17.6% were the result of ART (Table 1).

The higher rate of ART between the women with low and those with normal AMH levels was statistically significant ($p<$ .002). The women with high AMH levels had a 13% infertility rate, 25% pregnancy loss rate and an LB rate of 60%, all following spontaneous conceptions (Figure 3).

In the women aged >35 years, with low AMH levels, 36.2% were infertile, and 52.3% of them experienced only miscarriages. There was an LB rate of 30.45%, of which 47.6% were spontaneous conceptions, and 52.4% were the result of ART. Those women with normal AMH levels had infertility rates of 28.9%, and 'losses only' of 55.6%. The overall LB rate was 44.4%, of which 91.7% were conceived spontaneously and 8.3% were the result of ART. The higher rate of ART in women with low versus normal AMH levels was statistically significant ($p<$ .007) (Figure 4).

In the women with high AMH levels, there were no infertile patients, 'losses only' 57.1%, and the LB rate was 42.85%, and all were conceived spontaneously (Table 2). The LB rate was reduced in all three AMH groups in women aged >35 years, as would be expected, with the greatest losses occurring in the women >35 with high AMH levels.

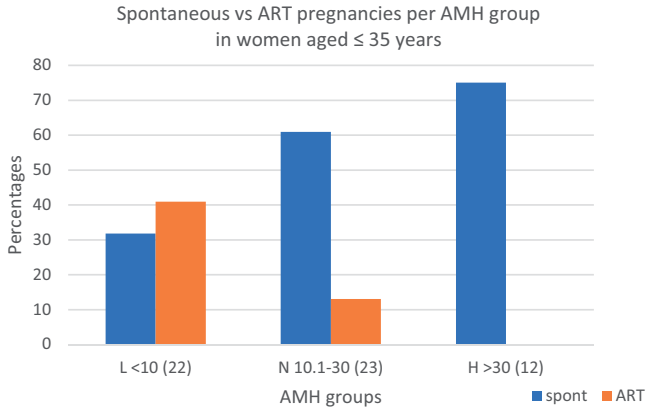


Figure 3. Spontaneous pregnancies versus assisted reproductive technology (ART) pregnancies in low, normal and high AMH groups, in women aged ≤35 years, in each of the AMH groups. The need for ART decreased in women in normal and high serum AMH levels ($p < 0.002$). Spont: spontaneous pregnancies; ART: assisted reproductive technology; L: low; N: normal; H: high AMH levels.

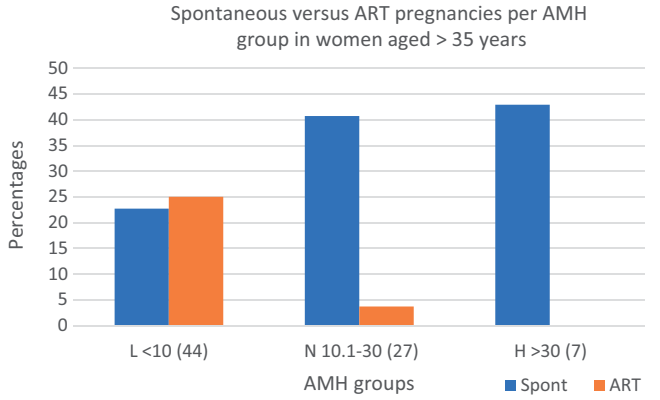


Figure 4. Spontaneous pregnancies versus ART pregnancies in low, normal and high AMH groups in women aged >35 years. The need for ART decreased in the normal and high AMH groups ($p < 0.007$). Spont: spontaneous pregnancies; ART: assisted reproductive technology; L: low; N: normal; H: high AMH levels.

Discussion

Fertility trends in the twenty-first century are different from previous centuries, with many women choosing to delay the birth of their first child until their mid-thirties, due to career choices, divorces and re-marriages. Traditionally the 'perimenopause' is described as the time when ovarian function declines, resulting in clinical symptoms such as menstrual irregularities. It is

Table 2. Outcomes regarding infertility, pregnancies, pregnancy losses and deliveries in women aged >35 years, in each of the AMH groups.

>35; 62.5% (114)	≤10 pmol/L	10.1–30 pmol/L	>30 pmol/L
Total patients	60.5% (69)	33.3% (38)	6.2% (7)
Infertility	36.23% (25)	28.95% (11)	0
Pregnancies	63.77% (44)	71.05% (27)	100% (7)
Spontaneous	50% (22)	70.37% (19)	85.71% (6)
ART	50% (22)	29.63% (8)	14.29% (1)
Miscarriages	52.27% (23)	55.56% (15)	57.14% (4)
Deliveries	30.44% (21)	31.58% (12)	42.85% (3)
Spontaneous	47.62% (10)	91.67% (11)	100% (3)
ART	52.38% (11)	8.33% (1)	0

Low, ≤10 pmol/L; normal, 10.1–30 pmol/L; high, >30 pmol/L.

thought to be due to the continuous loss of oocytes with ageing; however, in recent years, ART have identified the events that precede menopause (Seifer and Naftolin 1998; Dólleman et al. 2014). An accelerated deterioration of ovarian function begins much earlier than previously thought, probably in the mid-thirties (Bopp and Seifer 1998), and possibly earlier in some women, often without obvious symptoms. While it is well established that ovarian reserve declines with age (Nikolaou et al. 2002; Baird et al. 2005), the rate of this decline seems to vary among individuals and depends on the family history, medical history, as well as on various environmental and genetic factors. Severe endometriosis, pelvic inflammatory disease, ovarian surgery, various systemic illnesses, chemotherapy and possibly smoking are all known factors affecting ovarian reserve (Faddy et al. 1992; Sharara et al. 1998). It is possible that such 'sub fertility' could play a role in miscarriages, and AMH levels could predict the LB chances of patients attending the clinic (Lukaszuk et al. 2014). It is not ethically possible to directly measure ovarian reserve as this would involve an invasive and potentially ovarian damaging biopsy. Studies have shown highly significant positive correlations between AMH levels and the ovarian reserve assessed by manual stereological counts of non-growing follicles in ovarian tissue samples (Hansen et al. 2011). Whether low AMH levels reflect the quantity and quality of the remaining oocyte pool or not, has been widely discussed. Lehmann et al. (2014) described AMH as a 'valuable biomarker of oocyte quality in IVF'. Irez et al. (2011) also showed that AMH levels may predict the oocyte quality. Conversely, high levels reflect the polycystic ovarian syndrome, and thus the metabolic factors associated, such as hyperinsulinism, dyslipidaemia and hypertension, may be independent risk factors for pregnancy losses (Cocksedge et al. 2009).

Pils et al. (2016) and Atasever et al. (2016) both demonstrated that AMH levels were significantly lower in the RM groups compared to controls. Our findings suggest that a low ovarian reserve may be a factor in RM patients, and as such, should possibly be considered when offering assessments of such couples, as the detection of low levels, particularly in a younger patient, may be significant for their pregnancy planning. Kedem et al. 2013, showed that it is unnecessary to distinguish between low and extremely low AMH levels, when counselling patients or referring them for ART. They showed that both the low and extremely low AMH group had similar pregnancy rates in IVF cycles (Kedem et al. 2013).

The spontaneous pregnancy rates improved with increasing AMH levels, this may suggest that patients with low AMH levels have poorer quality oocytes and may thus be considered as 'sub-fertile' (Gnoth et al. 2005; Ebner et al. 2006). Greenwood et al. 2017 found that patients with rigorously defined infertility had AMH levels and antral follicle counts (AFC) no different to community-based controls. Their findings challenge the assumption that ovarian reserve is an indicator of fertility. AMH correlates well with AFC in normo-ovulatory IVF populations and is also strongly associated with a poor ovulatory response to stimulation when low, and has the ability to predict an excessive response to ovarian stimulation when high (Greenwood et al. 2017). However, RM patients may be an entirely different cohort with additional factors contributing to decreased AMH levels, as well as adverse pregnancy outcomes. Detecting the low AMH levels and counselling the patients about these findings may have increased the use of ART in this group. ART may have the ability to expedite conception rates, and with pre-implantation genetic analyses, ART could possibly select embryos with the greatest chance of survival (Brezina et al. 2013). In support of this concept, the need for ART was significantly reduced in the groups with higher serum AMH. Detecting high levels could alert the clinician to the possibility of PCOS and its consequences.

The LB rate was reduced in all three AMH groups in the women aged greater than 35 years, as would be expected; with the greatest losses occurring in the women aged over 35 with high AMH levels. High levels at this age are more likely to reflect the contribution of PCOS, and the consequent metabolic disturbances that may be embryotoxic (Kdous et al. 2009).

Strengths and limitations

This is a small study; larger studies are needed to confirm these findings. AMH levels are not thought to be influenced by the stage of the menstrual cycle, and as such AMH is an excellent marker of ovarian reserve, allowing the clinician to assess where the patient is in her reproductive lifespan. This may be critical in patients experiencing miscarriages.

Conclusions

The patients attending a RM clinic may have a low ovarian reserve, and potentially a poor oocyte quality, as shown by low circulating AMH. Establishing the ovarian reserve early in the work up allows the clinician to concentrate on possible interventions that may expedite the patient's journey towards the goal of achieving a successful pregnancy. Women should still undergo a full miscarriage work up, and any potential contributing factors should be treated as expeditiously as possible. Early detection allows a realistic counselling, reassurance and relevant interventions in this traumatised group of patients.

Acknowledgements

We thank the patients for participating in this study. We thank Fertility and Sterility (Elsevier) for permission to use AMH data from a healthy cohort (Shebl et al. 2011).

Disclosure statement

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

C.T. Roberts was supported by a National Health and Medical Research Council of Australia (NHMRC) Senior Research Fellowship (GNT1020749) (http://www.nhmrc.gov.au).

ORCID

Catherine D. McCormack http://orcid.org/0000-0002-6209-6904

References

Atasever M, Soyman Z, Demirel E, Gencdal S, Kelekci S. 2016. Diminished ovarian reserve: is it a neglected cause in the assessment of recurrent miscarriage? A cohort study. Fertility and Sterility 105:1236–1240.

Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, et al. 2005. Fertility and ageing. Human Reproduction Update 11:261–276.

Barad DH, Weghofer A, Gleicher N. 2009. Comparing anti-Müllerian hormone (AMH) and follicle-stimulating hormone (FSH) as predictors of ovarian function. Fertility and Sterility 91:1553–1555.

Bopp BL, Seifer DB. 1998. Oocyte loss and the perimenopause. Clinical Obstetrics and Gynecology 41:898–911.

Brezina PR, Ke RW, Kutteh WH. 2013. Preimplantation genetic screening: a practical guide. Clinical Medicine Insights. Reproductive Health 7: 37–42.

Christiansen OB. 2016. Foreward in: recurrent pregnancy loss. In: Bashiri A, Harlev A, Argarwal A, editors. Evidence-based evaluation, diagnosis and treatment. Switzerland: Springer International Publishing.

Cocksedge KA, Saravelos SH, Metwally M, Li TC. 2009. How common is polycystic ovary syndrome in recurrent miscarriage? Reproductive BioMedicine Online 19:572–576.

Dólleman M, Depmann M, Eijkemans MJC, Heimensem J, Broer SL, van der Stroom EM, et al. 2014. Anti-Mullerian hormone is a more accurate predictor of individual time to menopause than mother's age at menopause . Human Reproduction (Oxford, England) 29:584–591.

Ebner T, Sommergruber M, Moser M, Shebl O, Schreier-Lechner E, Tews G. 2006. Basal level of anti-Müllerian hormone is associated with oocyte quality in stimulated cycles. Human Reproduction (Oxford, England) 21:2022–2026.

Faddy M, Gosden R, Gougeon A, Richardson S, Nelson J. 1992. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Human Reproduction 7:1342–1346.

Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges J, Freundel G. 2005. Definition and prevalence of subfertility and infertility. Human Reproduction (Oxford, England) 20:1144–1147.

Greenwood EA, Cedars MI, Santoro N, Eisenberg E, Kao C, Haisenleder DJ. 2017. Antimüllerian hormone levels and antral follicle counts are not reduced compared with community controls in patients with rigorously defined unexplained fertility. Fertility and Sterility 108: 1070–1077.

Gruijters MJ, Visser JA, Durlinger AL, Themmen AP. 2003. Anti-Müllerian hormone and its role in ovarian function. Molecular and Cellular Endocrinology 211:85–90.

Hansen KR, Hodnett GM, Knowlton N, Craig B. 2011. Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertility and Sterility 95:170–175.

Irez T, Ocal P, Guralp O, Cetin M, Aydogan B, Sahmay S. 2011. Different serum anti-Müllerian hormone concentrations are associated with oocyte quality, embryo development parameters and IVF-ICSI outcomes. Archives of Gynecology and Obstetrics 284:1295–1301.

Kdous M, Chaker A, Bouyahia M, Zhioua F, Zhioua A. 2009. Increased risk of early pregnancy loss and lower live birth rate with GNRH antagonist vs. long GNRH agonist protocol in PCOS women undergoing controlled ovarian hyperstimulation. La Tunisie Medicale 87:834–842.

Kedem A, Haas J, Lerner Geva L, Yerushalmi G, Kanety H, Hourvitz A. 2013. Ongoing pregnancy rates in women with low and extremely low AMH levels. A multivariate analysis of 769 cycles. Fertility and Sterility 100:S324.

Kelsey TW, Anderson RA, Wright P, Nelson SM, Wallace WH. 2012. Data-driven assessment of the human ovarian reserve. Molecular Human Reproduction 18:79–87.

Kolte AM, Bernardi LA, Christiansen OB, Quenby S, Farquharson RG, Goddijn M, Stephenson MD. 2015. ESHRE Special Interest Group Early Pregnancy. 2015. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Human Reproduction (Oxford, England) 30:495–498.

Lehmann P, Vélez MP, Saumet J, Lapensée L, Jamal W, Bissonnette F, et al. 2014. Anti-Müllerian hormone (AMH): a reliable biomarker of oocyte quality in IVF. Journal of Assisted Reproduction and Genetics 31:493–498.

Lukaszuk K, Kunicki M, Liss J, Bednarowska A, Jakiel G. 2014. Probability of live birth in women with extremely low anti-Müllerian hormone concentrations. Reproductive Biomedicine Online 28:64–69.

Nikolaou D, Lavery S, Turner C, Margara R, Trew G. 2002. Is there a link between an extremely poor response to ovarian hyperstimulation and early ovarian failure? Human Reproduction 17:1106–1111.

Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. 2000. Maternal age and fetal loss: population based register linkage study. BMJ (Clinical Research Ed) 320:1708–1712. [PMC][10864550][Mismatch]

Petrozza JC. 2006. Early pregnancy loss. eMedicine WebMD. Available from: http://wwwemedicinecom/med/topic3241htm (Topic 3241).

Petrozza JC, Berin I, Cowan BD, Smith CV, Gaup FB. 2012. Recurrent early pregnancy loss. eMedicine. Medscape, [cited 2010 Jan 22]. Available from: http://emedicine.medscape.com/article/260495.overview.

Pils S, Promberger R, Springer S, Joura E, Ott J. 2016. Decreased ovarian reserve predicts inexplicability of recurrent miscarriage? A retrospective analysis. PLoS ONE 11:e0161606.

RCOG. 2011. The investigation and treatment of couples with recurrent first-trimester and second-trimester miscarriage. RCOG. Greentop Guideline No. 17.

Seifer DB, Naftolin F. 1998. Moving toward an earlier and better understanding of perimenopause. Fertility and Sterility 69:387–388.

Sharara FI, Seifer DB, Flaws JA. 1998. Environmental toxicants and female reproduction. Fertility and Sterility 70:613–622.

Shebl O, Ebner T, Sir A, Schreier-Lechner E, Mayer RB, Tews G, Sommergruber M. 2011. Age-related distribution of basal serum AMH level in women of reproductive age and a presumably healthy cohort. Fertility and Sterility 95:832–834.

Visser JA, de Jong FH, Laven JS, Themmen AP. 2006. Anti-Müllerian hormone: a new marker for ovarian function. Reproduction (Cambridge, England) 131:1–9.

CHAPTER 4: Structure

Statement of Authorship AJUM

Published paper. Structural AJUM

Statement of Authorship

Title of Paper	3D Ultrasound findings in a South Australian recurrent miscarriage clinic.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Australasian Journal of Ultrasound in Medicine – AJUM. November 2016 19(4) 142-147. doi: 10.1002/ajum.12032

Principal Author

Name of Principal Author (Candidate)	Dr C D McCormack
Contribution to the Paper	Patient selection, data gathering, document written
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 7/7/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr D L Furness
Contribution to the Paper	Patient selection, contribution to written text
Signature	Date 15/9/2018

Name of Co-Author	Professor G A Dekker
Contribution to the Paper	Project design, contribution to written text
Signature	Date 17/10/2018

Name of Co-Author	Dr K Shand		
Contribution to the Paper	Performance of 3D Ultrasounds, contribution to written text		
Signature		Date	18/10/18

Name of Co-Author	Professor C T Roberts		
Contribution to the Paper	Project design, contribution to written text.		
Signature		Date	18/10/18

3D ultrasound findings in women attending a South Australian recurrent miscarriage clinic

Catherine D. McCormack, MBChB, FCOG (SA), FRANZCOG[1,2], **Denise L. Furness**, BSc, PhD, RNut.[1], **Gustaaf A. Dekker**, MD, PhD, FRANZCOG[1], **Karen Shand**, BMBS, FRANZCOG, COGU[2] and **Claire T. Roberts**, BA, BSc (Hons), PhD[1]

[1]Robinson Research Institute, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia, Australia
[2]Department of Obstetrics, Women's and Children's Hospital, North Adelaide, South Australia, Australia

Abstract

Background: Women who suffer recurrent miscarriage are a heterogeneous group. Known causes include genetic and endocrine abnormalities, anti-phospholipid syndrome and autoimmune disease. Congenital uterine abnormalities (CUAs) such as bicornuate, unicornuate, septate and arcuate uterine abnormalities are known to negatively impact on pregnancy rates, and to increase the miscarriage rates of genetically normal pregnancies. In some countries, such as Britain, 3D ultrasound of the pelvis is offered routinely to women with recurrent miscarriages.

Aim: To determine the prevalence of CUAs and other pelvic pathology, in women attending a South Australian recurrent miscarriage clinic.

Materials and methods: 3D transvaginal ultrasounds performed during the luteal phase of the menstrual cycle were offered to all patients attending the recurrent miscarriage clinic, who had not previously had a hysteroscopy, laparoscopy, HyCoSy or MRI study of their pelvis. A Philips IUI 8 MHz transvaginal probe for freehand sweep, and dedicated 3D transvaginal probe was used. 3D scans provide a coronal view of the uterus, ideal for detecting abnormalities which may be missed during routine conventional 2D scanning.

Results: A total of 210 women were recruited, 200 results were available, and 29% were found to have a CUA. 15% had polycystic ovaries detected, 15% were found to have fibroids, 12% adenomyosis and 1.5% Asherman's syndrome.

Conclusions: 3D ultrasound evaluation of patients attending a recurrent miscarriage clinic detects CUAs, and has a high detection rate of other pelvic abnormalities that may contribute to recurrent miscarriages.

Keywords: 3D ultrasounds, bicornuate uterus, congenital uterine abnormalities, recurrent miscarriages, septate uterus.

Introduction

3D ultrasound investigations may be used in conjunction with 2D ultrasounds for a full evaluation of the pelvis, so that structural abnormalities as well as ovarian, tubal, myometrial, endometrial and cervical areas can be evaluated in a single study period. 3D ultrasound is a non-invasive method of investigation that allows the uterine dimensions to be measured, which helps in the diagnosis of congenital uterine abnormalities (CUAs).[1]

Congenital uterine abnormalities have been thought to be a cause of pregnancy loss and adverse pregnancy outcomes, and the reported prevalence of these abnormalities in women suffering recurrent pregnancy loss varies from 6% to 38%.[2] CUAs are thought to result from abnormal formation, fusion or resorption of the Müllerian ducts during fetal life.[1] Although miscarriages are frequently caused by a genetic problem such as an abnormal karyotype in the embryo or fetus, the parental causes most likely to impact on a pregnancy are chromosomal translocations, autoimmune diseases, endocrine or metabolic disorders, uterine anomalies and age-related issues.[3] The prevalence of major CUAs is thought to be at least threefold higher in women with a history of recurrent miscarriages in both embryonic (gestational age of 10 weeks or less from the last menstrual period) and fetal (gestational age of greater than 10 weeks), compared with the low-risk population, and thus CUAs may indeed be responsible for pregnancy loss in a significant proportion of women with recurrent miscarriages

Correspondence to email dee.mccormack@sa.gov.au
doi: 10.1002/ajum.12032

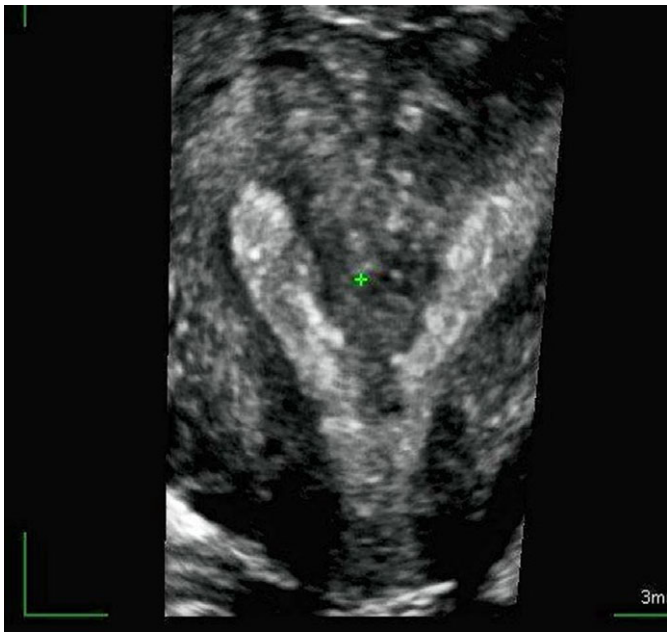


Figure 1: A septate uterus on 3D ultrasound.

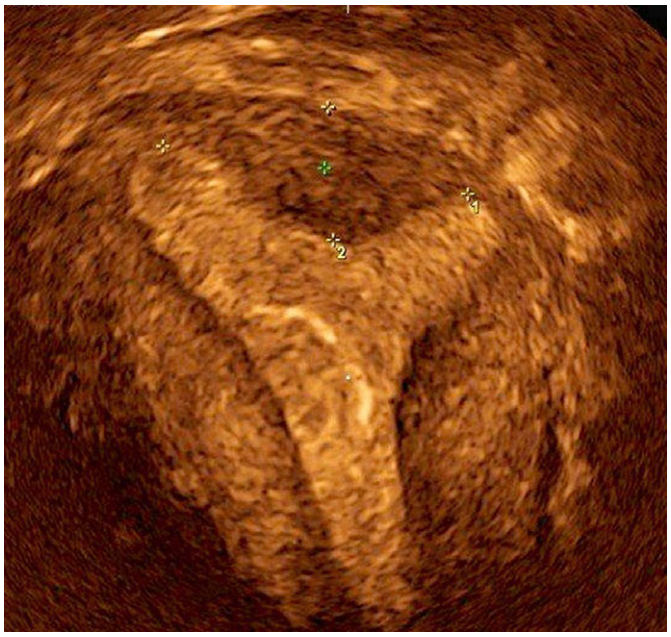


Figure 2: An arcuate uterus on 3D ultrasound.

(Figures 1 and 2).[4] 3D ultrasound was offered as part of the work up in the RM clinic to ascertain if it could contribute towards the assessment of these patients.

Materials and methods

This prospective cohort observational study formed part of a Clinical Trial named PAPO (Prediction of Adverse Pregnancy Outcomes) study (Clinical trial number ACTRN12609000254291). The study was approved by the Women's and Children's Hospital Human Research Ethics Committee in North Adelaide South Australia, REC1481/6/09. Informed and written consent was obtained from all participants. CUAs were classified in accordance with the modified American Fertility Society Classification (Table 1).[5]

Table 1: American Fertility Society (AFS) classification of Müllerian duct anomalies.

Class I	Hypoplasia and agenesis. (a) vaginal, (b) cervical, (c) fundal, (d) tubal, (e) combined
Class II	Unicornuate. (a) communicating, (b) non-communicating, (c) no cavity, (d) no horn
Class III	Didelphys
Class IV	Bicornuate. (a) partial, (b) complete
Class V	Septate. (a) partial, (b) complete
Class VI	Arcuate
Class VI	Diethylstilbestrol (DES) drug related

Women with a history of at least two consecutive miscarriages had 3D ultrasounds performed in the Women's Ultrasound department in a tertiary referral hospital in Adelaide. The scans were performed in the luteal phase of the menstrual cycle using a Philips IU22 C8-4V freehand sweep +/− Philips 3D 9-3V with dedicated 3D vaginal probes, or GE Voluson E8, RIC 5-9-D vaginal probe. 3D scans provide a coronal view of the uterus, ideal for detecting the abnormalities that may be been missed during routine conventional 2D scanning. Polycystic ovaries may be detected on 3D ultrasound, as large volume ovaries containing greater than 12 follicles each, measuring from 2 to 9 mm in width.[6] Adenomyosis is a non-neoplastic condition characterised on ultrasound by the presence of myometrial cysts or heterogeneous areas, diffuse vascularity, asymmetry of the myometrial walls, and a globular or bulky uterine configuration.[7] HyCoSy was offered to the patients if the uterine findings were unclear on the 3D scan alone. The sonographers are all employees at a Public Hospital offering Tertiary Obstetric Ultrasounds, and all were reported by a Senior COGU Specialist. The women also had the full routine miscarriage investigations offered by the clinic, which include thrombophilic, endocrine, autoimmune, metabolic and genetic investigations (Table 2). The reason for dividing the patients into two groups, ≤35 years and >35 years, is due to the independent effect of maternal age on the risk of spontaneous abortion.[8]

Results

210 patients were offered a 3D ultrasound, 200 were available for assessment and 10 were lost to follow-up. The ages of these women ranged from 22 to 44 years of age, and they had had from 2 to 15 losses prior to presentation. There were 108

Table 2: Recurrent miscarriage workup.

Work up	Tests
AGE	AMH levels
Structure	3D Ultrasound
Genetics	Karyotype products of conception
Thrombophilia (TP)	APS. Protein C, Protein S, ATIII levels (Only test for genetic thrombophilias if the patient has had a prior thrombotic event)
Endocrine	Thyroid, prolactin
Autoimmune	ANA, ENA, thyroid antibodies
Metabolic	GTT and insulin studies, homocysteine
Infectious	Vaginal swabs

patients aged 35 years or less, mean 31 years, and 92 patients aged 36 years or more, mean age 39 years. Primary miscarriage was defined as 'two or more consecutive miscarriages with the same partner, without a live birth'. Secondary miscarriage was defined as 'two or more consecutive losses with the same partner, after at least one live birth'.[2] There were 92 primary patients and 108 secondary patients. The majority of the losses were embryonic losses prior to 10 weeks from the last menstrual period, 10 had both embryonic and fetal losses, and one had two fetal losses. Preterm labours (>20 weeks) were not included.

Overall, 29% of the women presenting to the clinic had a CUA. In women aged 35 years or less (≤35 years), 35.2% had structural abnormalities detected. 7.9% had a bicornuate uterus, 2.6% had a unicornuate uterus, 57.9% had a septum (Figure 3) and 31.6% were found to have an arcuate-shaped uterus. In the women aged >35 years, 21.7% had structural abnormalities. 10% comprised a bicornuate uterus and there were no unicornuate uteri detected, 65% had a septum and 25% had an arcuate shape detected.

In women ≤35 years, 35.2% had a CUA detected as described, and when these patients were evaluated according to age and previous pregnancy status, 68.4% of them (primary patients) had a CUA detected. In patients in this age group who had a past live birth (secondary patients), 31.6% had a CUA detected. There was a statistically significant difference in the incidence of CUAs between the primary and secondary patients, P = 0.04.

In women >35 years, 21.7% had a CUA detected, 35% of these were in the primary group, and 65% were in the secondary group. In primary patients aged ≤35 years, the estimated odds ratio for a CUA was 3.92 (95% CI 1.12–14.96) compared to women aged >35 years.

Other findings included polycystic ovaries, fibroids, adenomyosis and Asherman's syndrome, polycystic ovaries (greater than 12 follicles per ovary, measuring 2–9 mm) were noted in 30 (15%) of the patients, 23 (21.3%) in women ≤35 years and 7 (7.6%) in women aged >35. An ultrasound detection of polycystic ovarian morphology was correlated with endocrine findings, before a diagnosis of polycystic ovarian syndrome was made. Fibroids were detected in 30 (15%) of the patients, 8 (26.7%) in the age group ≤35 years and 22 (73.3%) in the women aged >35, as would be expected. One patient with fibroids also had an endometrioma. Adenomyosis was diagnosed in 23 (12%) patients presenting to the clinic, 13 (12%) in women ≤35 years and 10 (10.9%) in women >35. Asherman's syndrome was diagnosed in three patients in the clinic (Figure 4).

Discussion

3D ultrasound is a non-invasive method of investigation that allows the uterine dimensions to be measured, which helps in the diagnosis of CUAs.[1] 3D ultrasound is used in conjunction with 2D ultrasound for a full evaluation of the pelvis, so that structural abnormalities as well as ovarian, tubal, myometrial, endometrial and cervical areas can be evaluated in a single study period. The true population prevalence of CUAs is difficult to assess as the diagnostic tests are rarely applied to a low risk population. A systematic review by Chan *et al.* showed a

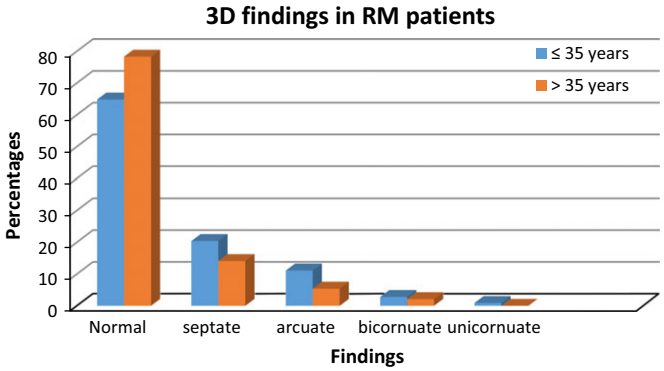


Figure 3: 3D congenital uterine abnormalities (CUAs) detected in patients aged ≤35 years or >35 years.

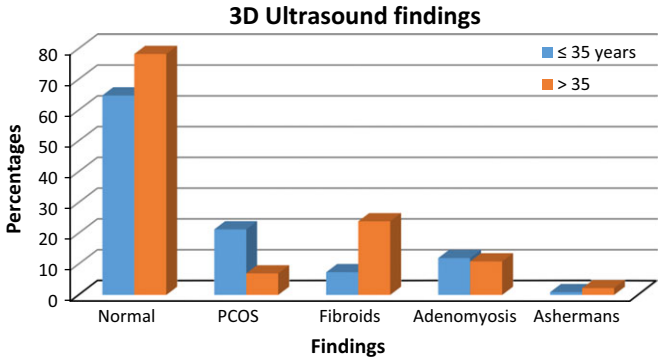


Figure 4: Other pathology detected at the time of the 3D ultrasound, in women aged ≤35 years compared with those aged >35 years.

prevalence of 5.5% in an unselected population, 8% in an infertile population and 13.3% in women with miscarriages, and 24.5% in women with infertility and miscarriages.[4] The limitation of this study is the relatively small size, however, it has demonstrated the usefulness of 3D ultrasound in the evaluation of these patients.

In total, 72% of the patients who had a 3D ultrasound were found to have uterine and/or pelvic abnormalities that could have contributed to their pregnancy losses. In this study, 29% of the patients presenting to the clinic who underwent a 3D ultrasound were found to have a CUA. A systematic review, evaluating the impact of congenital uterine abnormalities on reproductive outcomes, showed that women with canalisation defects, such as septate and subseptate uteri, had the poorest reproductive performance.[9] In addition to having a reduced conception rate, they are at an increased risk of first trimester miscarriage, preterm birth and fetal malpresentation at delivery.

Previous studies of women suffering recurrent early pregnancy losses have shown a variable prevalence of CUAs. Salim *et al.*[2] detected a prevalence of 23.8% of all abnormalities, and 6.9% major abnormalities in 509 women with recurrent miscarriages. Clifford *et al.*[10] found 1.8% major abnormalities in 500 cases. The prevalence in our study is broadly in agreement with the findings of Salim *et al.*[2] The lower finding by Clifford *et al.*[10] could have been the use of 2D ultrasound, which is known to be less sensitive for the detection of these abnormalities than 3D ultrasound.

Some studies have suggested that the pathophysiology of early pregnancy loss in those patients with septate uteri may be explained by the presence within the uterus, of a relatively avascular septum, and thus the inability of this septum to provide an adequate blood supply to the implanted and developing embryo.[11] Histological evaluation of some septae that showed significantly reduced vascular supply in relation to the rest of the uterus, supported this view.[12] However, other studies have suggested that there is an increased blood supply that interferes with the implantation of the conceptus.[13,14] Other causes may be increased uterine contractions or reduced uterine capacity. Salim *et al.*[2] tried to quantify the degree of uterine distortion by calculating the ratio between the fundal distortion and the length of the cavity on hysterosalpinography. They found no association between the severity of the uterine abnormality and the number of previous miscarriages. In contrast, the results of this study showed that the degree of distortion of the uterine cavity in septate uteri was higher in women with recurrent miscarriages, compared to low-risk women, mainly due to a reduced length of the unaffected cavity.

Some studies have reported an increase in adverse pregnancy outcomes in women with an arcuate uterus, mainly second trimester/fetal losses.[4] The pathophysiology of miscarriage in these women is unknown. However, other studies did not find this association. The prevalence of an arcuate uterus in women suffering recurrent miscarriages was 8.5% in our study, 6% in the 35 and under age group, and 2.5% in those aged over 35. Interestingly, these women were offered a hysteroscopy as part of their assessment, and a small septum was detected in 4 of 12 cases that had not been detected even on 3D ultrasound. This septum may have accounted for the losses.

Polycystic ovarian syndrome is a heterogeneous endocrine disorder characterised by anovulation, hyperandrogenism, infertility and metabolic dysfunction.[6] Polycystic ovaries may be detected on 3D ultrasound, as large volume ovaries containing greater than 12 follicles each, measuring from 2 to 9 mm in width. However, the detection of 'polycystic ovaries' does not establish a diagnosis of the polycystic ovarian syndrome, unless other features are present, such as hyperandrogenism and chronic anovulation. Recently, Dewailly *et al.*[15] suggested that PCOS should be defined as >25 follicles per ovary or ovarian volume greater than or equal to 10 mL, depending on the available technology. However, for this study, performed prior to this definition, the '12 follicles' definition was used.

We detected 'polycystic ovaries' in 14 patients, but only eight were finally confirmed as having the polycystic ovarian syndrome.

A recent study suggested that the prevalence of fibroids in women experiencing recurrent pregnancy losses is about 8.2%. This study showed that fibroids are associated with mid-trimester losses among women with recurrent miscarriages. Resection of fibroids associated with distortion of the uterine cavity can eliminate the mid-trimester losses and result in a doubling of the live birth rate.[16] A prevalence of 15% was detected in the whole group. Many of the fibroids were small and did not distort the uterine cavity. These patients were offered a referral to a gynaecologist for a surgical discussion regarding the feasibility of removal of the fibroids.

Adenomyosis is a non-neoplastic condition, affecting many women, characterised by a benign invasion of the myometrium by ectopic endometrium, that is accompanied by hyperplasia of the adjacent smooth muscle.[7] Such invasion may be detected during a 3D ultrasound; however, there are insufficient studies available to be able to assign causation. Endometriomas and endometriosis may be detected by 3D ultrasound, however there is conflicting evidence regarding surgical removal of endometriomas, as such removal could damage the ovarian reserve.[17]

Asherman's syndrome is described as the presence of intrauterine adhesions composed of fibrotic tissue, and is a pathological condition that is thought to arise following trauma to the basal layer of the endometrium, usually following a curettage, infection or inflammation. Only three cases of Asherman's syndrome were detected, with significant synechiae. This was lower than expected considering that many of the patients presenting to a recurrent miscarriage clinic have had multiple dilatations and curettage procedures, a known risk factor in the development of this syndrome.[18] Knopman *et al.* showed that

3D ultrasound was able to demonstrate the presence of Asherman's syndrome, and identify the severity of the disease, regarding the percentage of the uterine cavity that was obstructed.[19]

Conclusion

This study demonstrates the usefulness of 3D ultrasound scans in evaluating women suffering recurrent pregnancy losses. Larger studies are needed to confirm this. The information gathered may be used to refer the patients to gynaecologists for the hysteroscopic or laparoscopic evaluation of the uterus and pelvis, and possible removal of septae, synechiae or fibroids. The ultrasound study should be performed during the luteal phase following a miscarriage, in conjunction with the serological workup, so that valuable time is not wasted in this vulnerable group of patients.

References

1 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: 1916–22.

2 Salim R, Regan L, Woelfer B, Backos M, Jurkovic D. A comparative study of the morphology of congenital uterine anomalies in women with and without a history of recurrent first trimester miscarriage. *Hum Reprod* 2003; 18: 162–6.

3 Moore KL, Persaud TVN, Torchia MG. The urogenital system. In: Before we are born: Essentials of Embryology and Birth Defects, 7th edn. Philadelphia, PA: Saunders/Elsevier; 2008. 162–89.

4 Saravelos SH, Cocksedge KA, Li TC. Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal. *Hum Reprod Update* 2008; 14: 415–29.

5 AFS. The American fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, Müllerian anomalies and intrauterine adhesions. *Fertil Steril* 1988; 49: 944–55.

6 Trikudanathan S.Polycystic ovarian syndrome. *Med Clin N Am* 2015; 99: 221–35

7 Martinez-Conejero JA, Morgan M, Montesinos M, *et al.* Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. *Fertil Steril* 2011; 96: 943–50.

8 Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. *BMJ* 2000; 320: 1708–12

9 Chan YY, Jayaprakasan K, Tan A, Thornton JG, Coomarasamy A, Raine-Fenning NJ. Reproductive outcomes in women with congenital uterine anomalies: a systematic review. *Ultrasound Obstet Gynecol* 2011; 38: 371–82.

10 Clifford K, Rai R, Watson H, Regan L. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. *Hum Reprod* 1994; 9: 1328–32.

11 Burchell RC, Creed F, Rasoulpour M, Whitcomb M. Vascular anatomy of the human uterus and pregnancy wastage. *Br J Obstet Gynaecol* 1978; 85: 698–706.

12 Dabirashafi H, Bahadori M, Mohammed K, *et al.* Septate uterus: new idea on the histological features of the septum in this abnormal uterus. *Am J Obstet Gynecol* 1995; 171: 105–7.

13 Dabirashrafi H, Mohammad K, Nikbin B, Tabrizi NM, Azari A. Histocompatibility leukocyte antigens in Rokitansky-Kuster-Hauser syndrome. *Am J Obstet Gynecol* 1995; 172: 1504–5.

14 Kupesic S, Kurjak A, Skenderovic S, Bjelos D. Screening for uterine abnormalities by three-dimensional ultrasound improves perinatal outcome. *J Perinat Med* 2002; 30: 9–17.

15 Dewailly D, Lujan ME, Carmina E, *et al.* Definition and significance of polycystic ovarian morphology: a task force report from the Androgen Excess and Polycystic Ovary Syndrome Society. *Hum Reprod Update* 2014; 20(3): 334–52.

16 Saravelos SH, Yan J, Rehmani H, Li TC. The prevalence and impact of fibroids and their treatment on the outcome of pregnancy in women with recurrent miscarriage. *Hum Reprod* 2011; 26: 3274–9.

17 Koch J, Rowan K, Rombaults L, Yazdani A, Chapman M, Johnsom N. Endometriosis and Infertility – a consensus statement from ACCEPT (Australasian CREI Consensus Expert Panel on Trial evidence). *Aust N Z J Obstet Gynaecol* 2012; 52: 513–22.

18 Ahmadi F, Javam M. Role of 3D sonohysterography in the investigation of uterine synechiae/asherman's syndrome: pictorial assay. *J Med Imag Rad Oncol* 2014; 58: 199–202.

19 Knopman J, Copperman AB. Value of 3D ultrasound in the management of suspected Asherman's Syndrome. *J Reprod Med* 2007: 52; 1016–22.

CHAPTER 5: Karyotype

5.1 Introduction

When the clinic initially opened, routine karyotyping of the couple was offered. However, the RCOG Guidelines in 2011 suggested that only couples in whom a translocation was identified in the products of conception should be evaluated, as the costs were excessive, especially given the prevalence in 1-5% of recurrent pregnancy losses (RCOG GT guidelines 2011). However, the American Society for Reproductive Medicine (ASRM) in 2012 recommended performing genetic karyotypes on both partners. Popescu et al., (2018) proposed a new algorithm for the evaluation and treatment of recurrent pregnancy loss (RPL) based on the results of genetic testing obtained from the products of conception (POC) at the time of the next pregnancy loss. The algorithm suggests that couples with a single miscarriage should not have a work up performed, while those with two or more losses should have genetic testing of the POC performed. If the POC are found to be aneuploid, no further work up is required. If an unbalanced translocation is detected, both parents should undergo genetic testing. If euploid, a full recurrent miscarriage work up should be performed (Popescu et al., 2018).

The ESHRE Guidelines on Recurrent Pregnancy Loss (RPL) 2018, state that parental karyotyping is not routinely recommended in couples with RPL (Atik et al., 2018).

Currently our institution offers Karyotyping of the products of conception, and Chromosomal microarrays are not available.

5.2 Findings

In the women aged ≤35 years, 254 had a karyotype performed. 11 women were found to have a translocation (4.3%). 164 men had a karyotype performed and 2 had translocations (1.2%). In the women aged > 35 years, 168 were karyotyped and 3 translocations were found (1.8%). 110 of the partners were karyotyped and 1 carried a translocation (0.9%).

Detection of an abnormal karyotype in either parent warrants a referral for genetic counsellors who have the ability to discuss pregnancy options with the couple, which could include assisted reproductive technologies with genetic testing of the embryos. Future pregnancy losses should be karyotyped, and if found to be euploid, other causes/associations should be investigated.

References Chapter 5

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.

Popescu F, Jaslow CR, Kutteh WH. Recurrent pregnancy loss evaluation combined 24-chromosome microarray of miscarriage tissue provides a probable or definate cause of pregnancy loss in over 90% of patients.Hum Reprod 2018;(33):4:579-587.

Practice Committee of the American Society of the American Society for Reproductive Medicine. Definition of infertility and recurrent pregnancy loss: Fertil Steril 2013;99(1):63.

RCOG Green-top Guideline No 17. Royal College of Obstetricians and Gynaecologists. April 2011.

CHAPTER 6: Thrombophilia

6.1 Introduction

When the recurrent miscarriage clinic first opened, a thrombophilic work up was routinely performed. As evidence accumulated suggesting that thrombophilias were unlikely to be a cause of early pregnancy loss in the embryonic group, we stopped routinely performing both the Factor V Leiden (FVL) and Prothrombin Gene Mutation genotype studies, unless there was a strong personal or family history of thromboembolism. MTHFR genpotyping was continued, along with a fasting homocysteine level until the Public Health system decided that MTHFR was not a significant thrombophilia, and ceased funding the test. We no longer perform the genotypes, even if the fasting homocysteine levels are high, as the treatment is the same regardless of the polymorphisms. However, MTHFR and fasting homocysteine results were available for 316 of the clinic patients.

The American College of Obstetrics and Gynecology (ACOG) recommends testing for the inherited thrombophilias (Factor V Leiden, Prothrombin gene mutation, Protein C, Protein S and antithrombin III deficiencies) when a patient has a personal history of venous thromboembolism (VTE) in the setting of a non-recurrent risk factor (such as surgery) or has a first-degree relative with a known or suspected high-risk thrombophilia (The Practice Committee ASRM, 2012). The test result would then be used for potential treatment of the pregnancy woman during pregnancy and not for the prevention of adverse pregnancy outcomes.

6.2 Methods

Patients presenting to the RPL clinic were offered the genetic thrombophilia testing until this was discontinued by SA Health. (All patients are however, offered testing for

the Obstetric Antiphospholipid Syndrome, initially thought to be an acquired thrombophilia, however this is now believed to be a placental damage issue, not a clotting issue, in patients with embryonic losses).

6.3 Results

The prevalence of Factor V Leiden heterozygosity (FVLh) in our women was 4.6%, and homozygosity (FVL H) 0.2%. Said et al., found prevalences of 5.3% and 0.05%, respectively, in an Australian antenatal population (Said et al., 2008).

Prothrombin gene mutation (PGM G20210 substitution mutation F2 c20210G>A) prevalence was 2.4% in the clinic, and in Said et al. it was also 2.4%.

MTHFR homozygosity for the 677 polymorphism was 15.49% in our group, and 11.62% in the antenatal population (Said's group.) ($p = 0.046$). However, MTHFR homozygosity for the 1298 polymorphism was 9.78% in our cohort and 9.98% in the general antenatal population (Said et al., 2008). Compound heterozygosity prevalence was 18.75% in our cohort and 20.34 in Said's group (Non significant)

Table 6.1

Genetic thrombophilias	RM total N	RM N (%)	Aus cohort N (Said et al.)	N %	P
FVL Homozygous	419	1 (0.2)	2019	1 (0.1)	NS
FVL Heterozygous	419	19 (4.5)	2019	107 (5.3)	NS
PGM Homozygous	419	0	2018	0	
PGM Heterozygous	419	10 (2.4)	2018	49 (2.4)	NS
MTHFR 677 Hetero	368	151 (41)	2014	870 (43.2)	NS
MTHFR 677 Homo	368	57 (15.5)	2014	23 (11.6)	**0.046**
MTHFR 1298 Hetero	368	151 (41)	2014	84 (41.9)	NS
MTHFR 1298 Homo	368	36 (9.8)	2014	201 (9.9)	NS
Compound Hetero	368	69 (18.8)	2014	404 (20.1)	NS-

NS =not significant

Table 6.1 Comparison of polymorphisms for thrombophilia between the Recurrent Miscarriage clinic and a general Australian antenatal population*

*Data from Said et al., 2008 with permission.

6.4 Discussion

The finding that MTHFR 677 homozygosity was higher in our population than in the antenatal population was reflected in the homocysteine levels in this group. MTHFR homozygous patients were more likely to have higher levels of homocysteine than patients without the polymorphisms. However, Atik et al., (2018), have concluded that the evidence associating raised homocysteine (Hcy) levels and RPL is inconsistent, and have thus suggested that it should not be routinely tested for in a RPL work-up.

References Chapter 6

De Jong PG, Goddijn M, Middledorp S. Testing for inherited thrombophilia in recurrent miscarriage.Sem Rep Med 2011;29(6):540-547.

Kos BJP, Leemaqz SY, McCormack CD, Andraweera PH, , Furness DL, Roberts CT & Dekker GA.(2018): The association of parental methylenetetrahydrofolate reductase polymorphisms (*MTHFR* 677C > T and 1298A > C) and fetal loss: a case–

control study in South Australia. The Journal of Maternal-Fetal & Neonatal Medicine, DOI: 10.1080/14767058.2018.1500546.

Kutteh WH, Triplett DA. Thrombophilias and Recurrent Pregnancy Loss. Sem Rep Med 2006;54-65.

Nelen WLDN, Blom HJ. Steegers EAP, den Heijer M, Eskes TKAB. Hyperhomocystinaemia and recurrent pregnancy loss:(A meta-analysis.). FertilSteril, 2000; 74:1196-9.

Practice Committee of the American Society of the American Society for Reproductive Medicine. Fertil Steril 2012;98(5):1103-10.

Said JM, Brennecke SP, Moses EK, Walker SP, Monagle PT, Campbell J et al. The prevalence of inherited thrombophilic polymorphisms in an asymptomatic Australian antenatal population. Aust N Z J Obstet Gynaecol 2008;48:536-541.

Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol and Endocrin 2004:2-7.

CHAPTER 7: Endocrine

7.1 Thyroid disease

All patients had thyroid stimulating hormone (TSH) levels checked as well as free thyroxine (FT4) levels. In the age group ≤35 years, 9.25% were found to have clinical or sub clinical hypothyroidism. We considered TSH levels above 2.5mlU/L as subclinical hypothyroidism. In the age group >35 years, 11.22% had thyroid disease. This is higher than normally described in the literature, but was due to the inclusion of the subclinical hypothyroidism group. Subclinical hypothyroidism in pregnancy has recently been defined as a TSH level above the pregnancy-related reference range with a normal serum thyroxine concentration. The majority of subclinical hypothyroidism is caused by autoimmune thyroiditis, but may be due to iodine deficiency in some areas (Lazarus et al., 2014).

7.2 Hyperinsulinism

We offered a 75g OGTT with insulin studies to all patients. 245 patients accepted this. There were 5 patients with impaired fasting glycaemia or impaired glucose tolerance. With respect to insulin, 123 patients were normal, and 122 were found to have raised fasting, two hour (2h) or both fasting and 2h levels. 6.5% had raised fasting levels, 26.3% had both raised fasting and 2h levels. 67.2% had only raised 2h insulins. This has not been previously described in the literature, as most studies focus on the fasting levels. We later found that such hyperinsulinism significantly raised the risk of the patients developing gestational diabetes in a subsequent pregnancy.

References Chapter 7

Lazarus J, Brown RS, Daumerie C, Negro R, Vaidya B. 2014 European thyroid association guidelines for the management of subclinical hypothyroidism in pregnancy and in children. *European Thyroid Journal*;2014;3(2):76-94.

Rao VR, Lakshmi A, Sadhnani MD. Prevalence of hypothyroidism in recurrent pregnancy loss in first trimester. Indian J Med Sci 2008;62: 357-361.

Statement of Authorship GDM HI

GDM HI paper insert

Statement of Authorship

Title of Paper	Do raised two-hour pre-pregnancy insulin levels confer the same risks of developing GDM, as raised fasting levels, in recurrent miscarriage patients?
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Dr C D McCormack
Contribution to the Paper	Patient selection, data gathering, document written
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 13/6/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr S Leemaqz
Contribution to the Paper	Statistics, contribution to written document
Signature	Date 15/05/2019

Name of Co-Author	Dr D L Furness
Contribution to the Paper	Patient selection, contribution to written paper
Signature	Date 15/9/2018

Name of Co-Author	Professor G A Dekker		
Contribution to the Paper	Project design, contribution to written text		
Signature		Date	17-10-18

Name of Co-Author	Professor C T Roberts		
Contribution to the Paper	Contribution to written text		
Signature		Date	18.10.18

The Journal of Obstetrics and Gynaecology

Taylor & Francis
Taylor & Francis Group

Journal of Obstetrics and Gynaecology

ISSN: 0144-3615 (Print) 1364-6893 (Online) Journal homepage: https://www.tandfonline.com/loi/ijog20

Do raised two-hour pre-pregnancy insulin levels confer the same risks of developing GDM, as raised fasting levels, in recurrent miscarriage patients?

Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker & Claire T. Roberts

To cite this article: Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker & Claire T. Roberts (2019): Do raised two-hour pre-pregnancy insulin levels confer the same risks of developing GDM, as raised fasting levels, in recurrent miscarriage patients?, Journal of Obstetrics and Gynaecology, DOI: 10.1080/01443615.2019.1672139

To link to this article: https://doi.org/10.1080/01443615.2019.1672139

Published online: 02 Dec 2019.

JOURNAL OF OBSTETRICS AND GYNAECOLOGY
https://doi.org/10.1080/01443615.2019.1672139

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Do raised two-hour pre-pregnancy insulin levels confer the same risks of developing GDM, as raised fasting levels, in recurrent miscarriage patients?

Catherine McCormack[a,b], Shalem Leemaqz[a], Denise Furness[c], Gustaaf Dekker[c] and Claire T. Roberts[a]

[a]Robinson Research Institute, University of Adelaide, Adelaide, South Australia, Australia; [b]Department of Obstetrics, Women's and Children's, North Adelaide, South Australia, Australia; [c]Adelaide Medical School, University of Adelaide, Adelaide, South Australia, Australia

ABSTRACT

This study questioned whether raised pre-pregnancy two-hour (2 h) insulin levels, measured in recurrent embryonic miscarriage (RM) patients via a 75 g Oral Glucose Tolerance Test (OGTT), are associated with an increased risk of gestational diabetes mellitus (GDM) in a subsequent pregnancy. Patients had a 75 g OGTT and insulin levels evaluated ($n = 170$). 54.1% had normal glucose and insulin levels, 45.9% had levels indicating hyperinsulinism (HI). In the 98 patients who achieved a pregnancy, the prevalence of GDM was 3.7% in those without HI, and 35.7% in the patients who only had raised 2 h insulin levels. While HI has been described as a risk factor for miscarriages only in relation to raised fasting (basal) insulin levels, this study demonstrated that raised 2 h insulin levels predict an increased risk of GDM in a subsequent pregnancy. Thus raised 2 h insulin levels likely confer a similar risk to raised fasting insulin levels in RM patients.

IMPACT STATEMENT

- **What is already known on this subject?** Fasting hyperinsulinism is known to be associated with an increased risk of gestational diabetes mellitus (GDM) in pregnancy. Hyperinsulinism, as reflected by the fasting (basal) insulin levels >20mU/L, has been recognized as a risk factor for recurrent miscarriages, particularly in patients with polycystic ovarian syndrome (PCOS), in the World literature. Raised two-hour insulin levels have not been considered as a risk factor in the literature before.
- **What do the results of the study add?** We have demonstrated a 10-fold increase in the development of GDM in patients with fasting insulin resistance, and/or raised 2h insulin levels, and an almost 10-fold increase in patients with only raised 2h levels. 58.8% of the patients who subsequently developed GDM only had raised 2h levels and would have been missed with routine testing.
- **What are the implications of these findings for clinical practice and/or further research?** Our study has demonstrated that GDM was three times more prevalent in the patients with only raised 2h levels, than in those only with raised fasting levels, reflecting insulin resistance/hyperinsulinism. Insulin studies including 2h insulin levels are therefore an important factor to consider when working up these patients. Insulin studies pre-pregnancy may be useful in identifying women at risk of suffering miscarriages or of developing GDM in a subsequent pregnancy.

KEYWORDS

Recurrent miscarriages; hyperinsulinism; insulin resistance; gestational diabetes mellitus

Introduction

Fasting hyperinsulinism is known to be associated with an increased risk of gestational diabetes mellitus (GDM) in pregnancy. Hyperinsulinism, as reflected by the fasting (basal) insulin levels >20mU/L, has been recognized as a risk factor for recurrent miscarriages, particularly in patients with polycystic ovarian syndrome (PCOS) (Boomsma et al. 2008; Glueck et al. 2008; Wang et al. 2011). Some investigators have shown an increased prevalence of insulin resistance in women who suffer recurrent pregnancy losses (Craig et al. 2002). Gestational diabetes mellitus (GDM) and maternal obesity have been shown to be independently associated with adverse neonatal and maternal outcomes, and both share metabolic characteristics such as hyperglycaemia, increased insulin resistance and hyperinsulinaemia (Catalano et al. 2012).

First we found that a significant proportion of our patients undergoing investigations for recurrent miscarriages had markedly raised two-hour (2 h) insulin levels, with normal fasting insulin and normal glucose levels. Given that fasting hyperinsulinism is known to be associated with an increased risk of gestational diabetes mellitus (GDM) in pregnancy, we followed these patients in a subsequent pregnancy to establish if raised 2 h levels did in fact confer as similar a risk of developing GDM as fasting hyperinsulinism. If so, it may be possible that raised two-hour levels are also a risk factor for miscarriages and should be routinely tested in a recurrent miscarriage work-up.

CONTACT Catherine McCormack deemccormack@hotmail.com Adelaide Medical School, Obstetrics and Gynaecology, Robinson Research Institution, Adelaide 5001, Australia; Adelaide Women's and Babies Division, Women's and Children's Hospital, North Adelaide 5006, Australia

Methods

Study population

We conducted a prospective cohort observational study as part of the PAPO study. (Prediction of Adverse Pregnancy Outcomes – ACTRN126090002542910).

Patients referred to the recurrent miscarriage clinic from July 2010 until January 2013 were evaluated for possible causes of recurrent miscarriages, including genetic, structural, autoimmune, endocrinologic, thrombophilic, metabolic and lifestyle factors. Patients were included if they had two or more documented (positive bloods tests/ultrasound examinations) losses with the same partner. Written informed consent was obtained from each patient. They were then followed up in a subsequent pregnancy. The study was approved by the Hospital Ethics Committee. (REC1481/6/09).

Data collection

All patients had their tests performed at the same laboratory and were included if all requested tests were performed. A 75 g oral glucose tolerance test (OGTT) was performed after a 10 h fast, and fasting and two-hour glucose and insulin levels were recorded. Demographic information was collected during the face-to-face visits with the physician, and BMI calculated. Patients' body mass index (BMI) was determined as weight divided by the height in metres squared. A BMI of 18–24.9 is regarded as normal, 25–29.9 as overweight and $\geq$30 as obese.

Insulin was measured using the Advia Centaur analytical system. This insulin assay detects recombinant insulin analogues in addition to endogenous insulin (Clinical and Laboratory Standards Institute (CLSI) 2006). The fasting insulin levels accepted as normal by our laboratory are 0–12mU/L. We accepted the glucose:insulin ratio $>$4.5 and insulin levels $<$20 mU/L as normal, and a glucose:insulin ratio $<$4.5 and insulin levels $\geq$20 mU/L as fasting hyperinsulinism. Normal two-hour levels in our laboratory are 10–40 mU/L. In our normal group the mean two-hour insulin levels were 25.88, and so we used three standard deviations from this mean as our cut-off for the diagnosis of two-hour hyperinsulinism 52.6 mU/L, as there are no guidelines in the literature.

GDM was defined as a fasting glucose greater than 5.5 mmol/L, and/or a 2 h level $>$8.0 mmol/L, following a 75 g GTT, at 28 weeks of gestation.

Statistical analysis

Univariate analyses were performed on maternal demographics including age, BMI, previous miscarriages and ethnicity, comparing between women with and without hyperinsulinism. The association between GDM and fasting and two-hour insulin levels was examined using Fishers exact test, with further analysis using Logistic regression adjusted for age, BMI and ethnicity to estimate the odds ratios and corresponding 95% confidence intervals. R version 3.3.2 was used to perform the analyses and results are considered statistically significant at 5% significance level ($p < .05$).

Given that the GDM prevalence in South Australia was 5.8% at the beginning of the study, we calculated that a sample size of 90 patients would be needed to achieve 90% power to detect a doubling of the GDM rate with alpha 0.05. We had 98 patients who went on to achieve pregnancy and live birth.

Results

Overall 182 patients were recruited. Twelve patients did not complete the study, had incomplete results, or were already pregnant at the initial test, and were not included. 170 undertook the required 75 g GTT and completed the study; 44 (25%) of patients did not achieve a pregnancy during the data collection period; 28 (22.2%) had miscarriages, and 98 achieved a live birth (77.8%).

Women were predominantly Caucasian Australians (Caucasian 81.8%, Indian 3.5%, Asian 4.7%, African 1.8% and Middle Eastern 8.2%). The ages ranged from 19 to 45 years, and the numbers of previous losses from 2 to 10 (Table 1).

92 (54.1%) patients were found to have normal glucose and insulin levels. 78 (45.9%) were found to have raised fasting levels and/or 2 h levels and 3 (3.8%) had impaired GTT's. In the group with increased insulin levels (hyperinsulinism/insulin resistance; HI/IR), 8.9% had raised fasting insulin, 23.1% had raised fasting and 2 h levels, and 67.9% had raised 2 h insulin levels only, with normal glucose levels. The normal fasting insulin levels ranged from 0.5 to 19 mU/L, and the raised (hyperinsulinism) levels ranged from 20 to 78 mU/L in the hyperinsulinism group (Figure 1). The 2 h insulin levels ranged from 2.8 to 49 mU/L in the normal group and from 60 to 370 mU/L in the group with hyperinsulinism (Figure 2).

Ninety-eight patients achieved an ongoing pregnancy following the testing. In these pregnancies, in the group of women with raised 2 h insulin levels who did not develop GDM, the insulin levels ranged from 60 to 120 mU/L, and in the group who did develop it, insulin levels ranged from 62 to 170 mU/L (Figure 3). The association between 2 h HI and GDM was significant ($p = .0013$).

There was a clear association between Ethnicity and HI (overall $p = .0026$). Although the numbers in some of the groups were small, nearly 79% of the women of Middle Eastern origin had HI.

Table 1. Demographics of patients attending Recurrent Pregnancy Loss clinic.

RM Clinic	No HI	HI	*p* value
N (%)	92 (54)	78 (46)	–
AGE (years)	22–43	21–45	ns
Mean	33.8	33.7	ns
Miscarriages			
Range	2–10	2–8	ns
Mean	3.20	3.22	ns
BMI (kg/m^2)			
20–24.9	48 (52)	29 (37)	ns
$\geq$25	44 (48)	49 (63)	ns
Ethnicity			0.0026
Caucasian	83 (90)	56 (72)	
Asian	5 (5)	3 (4)	
Middle Eastern	3 (3)	11 (14)	
Indian	1 (1)	5 (6)	
African	0 (0)	3 (4)	

Hi: Hyperinsulinism; ns: non-significant.

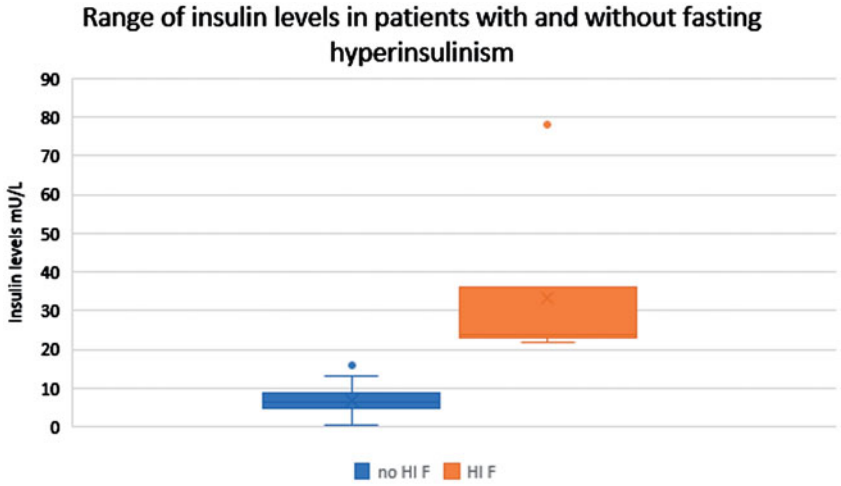


Figure 1. Pre-pregnancy fasting insulin levels in patients with and without hyperinsulinism. Hyperinsulinism was defined as insulin levels >20mU/L or a fasting glucose to insulin ratio <4.5.

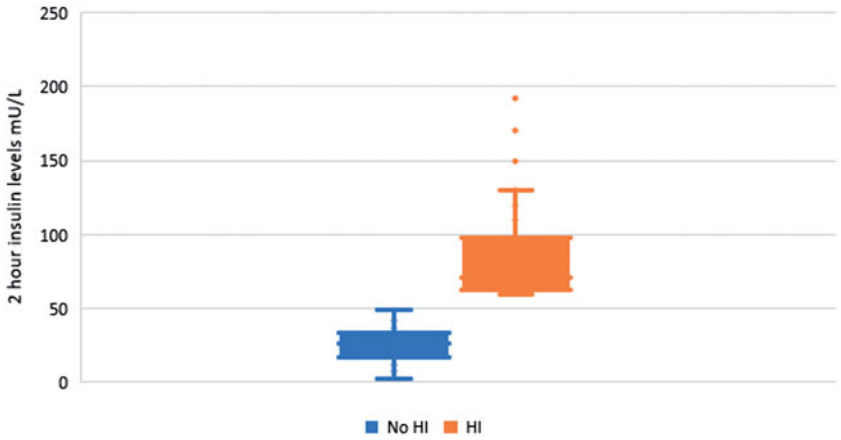


Figure 2. Pre-pregnancy 2-hour insulin ranges in patients with and without hyperinsulinism. Hyperinsulinism was defined in this study as a level >60mU/L.

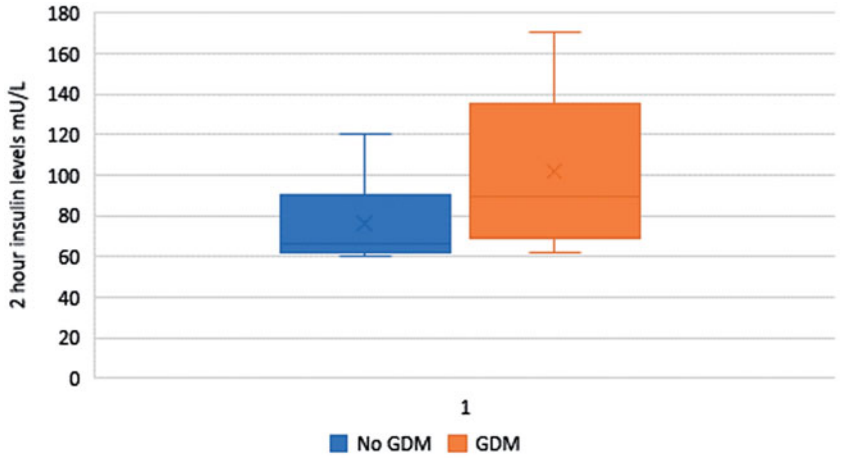


Figure 3. Pre-pregnancy raised 2-hour insulin levels (hyperinsulinism). Women who developed Gestational Diabetes Mellitus in a subsequent pregnancy, versus those with hyperinsulinism who did not.

Table 2 shows the prevalence of GDM in each group. Overall, 38.6% in those with raised fasting and/or two-hour levels developed GDM in a subsequent pregnancy. Of the patients who developed GDM in the subsequent pregnancy after the detection of abnormal insulin levels, 17.7% were the group who only had raised fasting insulin levels, and 58.8% were those who only had raised 2 h levels; 23.5% had both high fasting and 2 h insulin levels. After correcting for age, ethnicity and BMI, the risk of developing GDM with raised fasting or 2 h insulin levels was increased by between 10 and 28-fold (Table 2).

Although we used a conservative three standard deviations from the mean in our normal group to identify a cut-off for our high two-hour insulin group, this could be

Table 2. Associations between hyperinsulinism and Gestational Diabetes Mellitus, in the Recurrent Miscarriage clinic.

Insulin levels	No GDM	GDM	Total	% GDM	% Group	OR (95% CI)	*p*
Normal fasting – normal 2-hour	52	2	54	3.7%	2%	1	–
Normal fasting – high 2-hour	18	10	28	35.7%	10.2%	10.21 (1.81–5.64)	.0085
High fasting – normal 2-hour	2	3	5	60%	7.1%	28.22 (2.55–312.79)	.0065
High fasting – high 2-hour	7	4	11	36.4%	4.1%	11.32 (1.40–91.58)	.0229
TOTAL	79	19	98				

reduced to two standard deviations, which would probably increase the detection rate of HI in women with subsequent GDM.

Discussion

Women who suffer recurrent miscarriage form a heterogeneous group. However, previous reports link insulin resistance with recurrent miscarriage in a sub-set of patients (Wang et al. 2011). We have demonstrated a 10-fold increase in the development of GDM in patients with fasting insulin resistance, and/or raised 2 h insulin levels, and an almost 10-fold increase in patients with only raised 2 h levels. 58.8% of the patients who subsequently developed GDM only had raised 2 h levels and would have been missed with routine testing.

Most of the available literature considers hyperinsulinism to be reflected only by the fasting (basal) levels. Wang et al. (2011) considered the fasting, one, two and three-hour levels, however the patients were in the first trimester of pregnancy. They found a significant difference in both the insulin and glucose levels at one, two and three hours of testing, even when they excluded patients with polycystic ovarian syndrome (PCOS). They concluded that there is an increased incidence of insulin resistance in recurrent miscarriage patients, in the first trimester of pregnancy, and suggested that only testing for fasting insulin and glucose in recurrent miscarriage patients, would be inadequate to demonstrate insulin resistance (Wang et al. 2011).

Jakubowicz et al. (2004) found that hyperinsulinaemia led to reduced concentrations of insulin-like growth factor binding protein-1 (IGFBP-1) and glycodelin in early pregnancy, thereby increasing the chance of miscarriages. Glycodelin is an immunomodulatory protein involved in implantation, and Glycodelin -A is found in abundant levels in the decidua in early pregnancy. It plays an important role both in placental development and fetomaternal defence. Abnormal levels are associated with unexplained infertility and recurrent pregnancy loss (Glueck et al. 2008). Interestingly, insulin can negatively regulate the concentrations of glycodelin and IGFBP-1, and this may be the mechanism by which it increases the risk of miscarriages.

Hyperinsulinemia may also increase the level of plasminogen activator inhibitor-1 and induce villous thrombosis, thereby reducing the blood supply to the placenta and leading to trophoblastic hypoplasia, resulting in miscarriage (Gordon et al. 1995).

Lee et al. (2016) hypothesised that HI could cause an uncontrolled diabetic-like state in the fetal environment resulting in increased first trimester losses. High insulin levels have been shown *in vitro* to increase the transport of glucose by first trimester cytotrophoblasts independent of glucose level probably by upregulation of the GLUT1 glucose transporter system (Lee et al. 2016).

In conclusion, our study has demonstrated GDM was three times more prevalent in recurrent miscarriage patients with raised 2 h insulin levels than in those with raised fasting levels, reflecting insulin resistance/hyperinsulinism. Insulin studies including 2 h levels are an important factor to consider when working up these patients. This finding needs to be replicated in other populations. A full work-up should be performed on all patients, and all presumed 'causes/associations' of miscarriages addressed. Insulin studies pre-pregnancy may be useful in identifying women at risk of having miscarriages or developing GDM in a subsequent pregnancy and therefore identify women who may benefit from early intervention, such as diet and life style interventions.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

CTR was supported by a National Health and Medical Research Council of Senior Research Fellowship ([GNT1020749).]. (http://www.nhmrc.gov.au).

ORCID

Catherine McCormack http://orcid.org/0000-0002-6209-6904

References

Boomsma CM, Fauser B, Macklon NS. 2008. Pregnancy complications in women with polycystic ovary syndrome. Seminars in Reproductive Medicine 26:072–084.

Catalano PM, McIntyre DH, Cruickshank JK, McCance DR, Dyer AR, Metzger BE, et al. 2012. The hyperglycemia and adverse pregnancy outcome study. Diabetes Care 35:780–786.

Clinical and Laboratory Standards Institute (CLSI). Clinical Laboratory Technical Procedure Manuals; Approved Guideline, GP02-A5, 2006.

Craig LB, Ke RW, Kutteh WH. 2002. Increased prevalence of insulin resistance in women with a history of recurrent pregnancy loss. Fertility and Sterility 78:487–490.

Glueck JC, Pranikoff J, Aregawi D, Wang P. 2008. Prevention of gestational diabetes by metformin plus diet in patients with polycystic ovary syndrome. Fertility and Sterility 86:625–634.

Gordon MC, Zimmerman PD, Landon MB, Gabbe SG, Kniss DA. 1995. Insulin and glucose modulate glucose messenger ribonucleic acid expression and glucose uptake in trophoblasts isolated from

first-trimester chorionic villi. American Journal of Obstetrics and Gynecology 173:189–197.

Jakubowicz DJ, Essah PA, Seppälä M, Jakubowicz S, Baillargeon J-P, Koistinen R, et al. 2004. Reduced serum glycodelin and insulin-like growth factor-binding protein-1 in women with polycystic ovary syndrome during first trimester of pregnancy. The Journal of Clinical Endocrinology and Metabolism 89:833–839.

Lee C-L, Lam KKW, Vijayan M, Koistinen H, Seppala M, Ng EHY, et al. 2016. The pleiotropic effect of glycodelin-A in early pregnancy. American Journal of Reproductive Immunology 75:290–297.

Wang Y, Zhao H, Li Y, Zhang J, Tan J, Liu Y. 2011. Relationship between recurrent miscarriage and insulin resistance. Gynecologic and Obstetric Investigation 72:245–251.

CHAPTER 8: Autoimmune

8.1 Thyroid autoantibodies

All patients had their thyroid autoantibodies tested. In the ≤35 year age group, only 1 patient did not choose to have the test. 247 (87.6%) were normal and 35 (12.4%) were positive for thyroid autoantibodies. These patients also had thyroid function tests, and in the antibody positive group, 19 (54.3%) had thyroid disease, versus 7 (2.8%) in the antibody negative group.

In the women aged >35 years only 2 patients did not test, 177 (90.8%) were normal and 18 (9.2%) had thyroid antibodies. 77.8% of the women with thyroid antibodies had overt thyroid disease, as opposed to 8 (4.5%) of those without antibodies.

8.2 Antiphospholipid Syndrome

Obstetric Antiphospholipid (antibody) syndrome (APS) refers to a specific situation in pregnancy that comprises recurrent early pregnancy loss or sporadic late loss with positive antiphospholipid antibodies (APL's) - lupus anticoagulant, anti-β2-glycoprotein 1, and anticardiolipin antibodies – without concurrent VTE (Leaf et al., 2017). It has been shown that circulating APL's are one of the main risk factors in 7-25% of early pregnancy losses and the prevalence in the population is 1-5% (Kutteh et al., 2014). It is now thought that early losses are mainly due to defective endovascular decidual trophoblast invasion. APS incidence has been found to be 15-20% in couples with RPL, in comparison to only 5% in women without a history of adverse pregnancy outcomes (Kutteh et al., 2014). Abnormal early trophoblast

invasion and placental development comprise the most likely pathogenic mechanisms in obstetric APS. Studies have shown that APL's can directly impede trophoblast invasiveness, diminish human chorionic gonadotrophin secretion and impair trophoblast differentiation and maturation (Kutteh 2016). Molecular studies have shown that APL's inhibit the trophoblast cell adhesion molecules ([alpha]1 and [alpha]5 integrins, as well as E and VE cadherins) (Je and Giradi, 2004). APL's activate complement on the trophoblast surface inducing an inflammatory response (Kutteh et al., 2014). Thus the most frequently observed histological abnormality in APS associated pregnancy loss, is defective decidual endovascular trophoblast invasion, rather than intervillous thrombosis (Ornoy et al., 2003).

ESHRE guidelines on recurrent pregnancy loss recommend screening for APS after two pregnancy losses (Atik et al., 2018).

8.2.1 Findings

In the women in the ≤35 year age group, 2 did not test, 212 (75.4%) did not have antibodies, and 69 (24.6%) were positive for APL's on at least two occasions, post pregnancy loss and 6-12 weeks after the initial positive test. In the women aged > 35 years, 2 did not test, 149 were normal (77.4%) and 46 (23.6%) were positive for APL's. These prevalences are in keeping with other recurrent miscarriage clinics.

8.3 Discussion

Atik et al., (2018) in the ESHRE guidelines strongly recommend that APS be tested in women who are diagnosed as RPL patients. The current recommendations are that women with 2 or more losses before ten weeks of gestation (embryonic losses) or 1 loss after ten week, should be tested. These tests should be repeated after 6-12 weeks, and if a diagnosis of APL is made, treatment should be offered.

References Chapter 8

American College of Obstetricians and Gynecologists Committee on Practice Bulletins-Obstetrics. ACOG Practice Bulletin No. 118: antiphospholipid syndrome. Obstet Gynecol 2011;117(1):192-199

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.
Je S, Girardi G. The role of complement in the antiphospholipid syndrome. Curr Dir Autoimmun 2004;7:33-48

Kutteh WH, Hinote CD. Antiphospholipid Antibody Syndrome. Obstet Gynecol Clin N Am.2014:41;113-132.

Kutteh WH. Novel Strategies for the Management of Recurrent Pregnancy Loss. Seminars in Reproductive Medicine. Volume 33(3), May 2015, p 161–168

Leaf RK, Connors JM. The role of anticoagulants in the prevention of pregnancy complications. Clin and App Thromb/Hemo 2017:23(2);116-23.

Ornoy A, Yacobi S, Matalon ST et al. The effects of antiphospholipid antibodies obtained from women with SLE/APS and associated pregnancy loss on rat embryos and placental explants in culture. LUPUS 2003;2:573-8.

Tong M, Viall CA, Chamley LW. Antiphospholipid antibodies and the placenta: a systematic review of their in vitro effects and modulation by treatment. Hum Reprod update 2015;21:97-118.

CHAPTER 9: Metabolic

9.1 Homocysteine

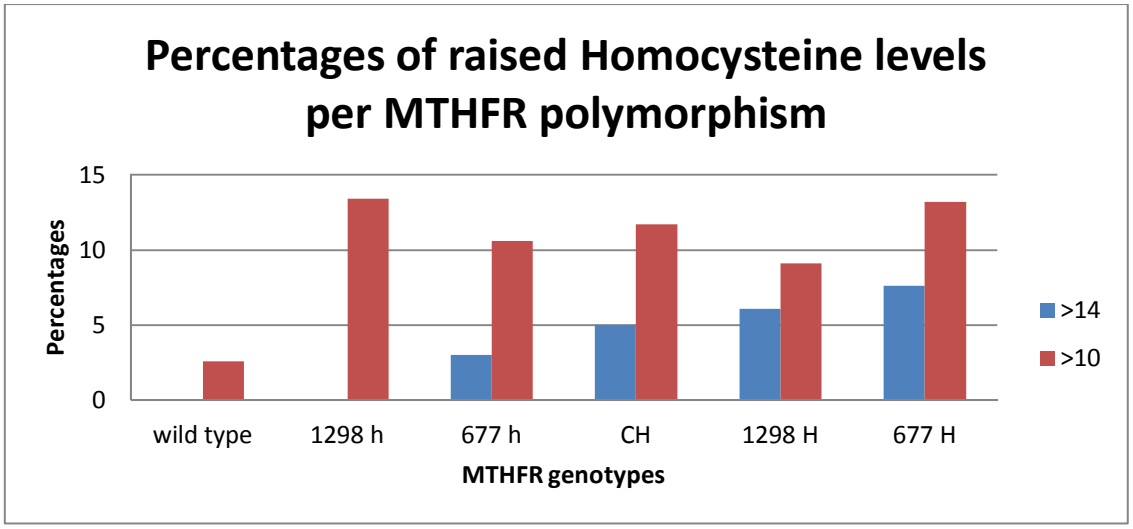
The confirmed association between increased circulating homocysteine concentration and neural tube defects (NTD), (Zetterberg, 2004) has led to the hypothesis that high concentrations of homocysteine might be toxic to the embryo, and lead to decreased fetal viability, in that environment. Recently there has been more emphasis on the likely role of epigenetic processes and DNA methylation that occur throughout gestation. The molecular events that result in NTD due to folate deficiency are unknown but may include insufficient methylation of crucial metabolites in the developing embryo and/or abnormalities in neural cell proliferation, differentiation and apoptosis, which may be due to DNA nucleotide misincorporation that resulting from folate deficiency in rapidly proliferating cells (Govindaiah et al., 2009).

9.1.1 Findings

Fasting Homocysteine was measured in women presenting to the clinic.

Homocysteine	>10 µmol/L N (%)	>14 µmol/L N (%)
Wild type	1 of 38 (2.6)	0 (0)
677 heterozygous	7 of 66 (10.6)	2 of 66 (3.0)
677 Homozygous	7 of 53 (13.2)	4 of 53 (7.6)
1298 Heterozygous	9 of 67 (13.4)	0 (0)
1298 Homozygous	3 of 33 (9.1)	2 of 33 (6.1)
Compound Heterozygote	7 of 60 (11.7)	3 of 60 (5)

Table 9.1 Frequency of hyperhomocysteinaemia per MTHFR polymorphism (> 10 or >14µmol/L).



Footnote: Homocysteine >10 µmol/L but < 14 µmol/L, or > 14 µmol/L

Figure 9.1. Percentages of increased homocysteine levels in each MTHFR polymorphism group

While a small percentage of patients without the MTHFR polymorphisms (wildtype) had raised fasting homocysteine levels, greater than 10µmol/L, none exceeded 14 µmol/L. There was a tendency for homocysteine to increase as the polymorphisms increased, with women having both polymorphisms having the highest circulating homocysteine levels. We do test for it, and we also check the serum folate and B12 levels. Supplementation is suggested, as well as lifestyle changes.

References Chapter 9

Govindaiah V, Naushad SM, Prabhakara K, Krishna PC, Radha R, Devi A. Association of parental hyperhomocysteinemia and C677T Methylene tetrahydrofolate reductase (MTHFR) polymorphism with recurrent pregnancy loss. Clin Biochem 2009;42:380-6.

Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol and Endocrin 2004:2-7.

CHAPTER 10: Specific

The ASK TEAMS approach allows for additional tests to be performed according to the individual histories of the patients. This could include a toxic or infectious screen, should such a history be elicited. Drug screens could also be offered, and every patient should be questioned about diet, psychosocial situations and domestic violence issues. We encouraged patients to attend with their partners if possible, given the profound effect of miscarriages on both partners. 274 partners (57%) of the 480 women attended and did the tests an answered lifestyle questions.

10.1 Smoking

Cigarette smoke contains a significant number of toxic substances, including polycyclic aromatic hydrocarbons, heavy metals, alkaloids, nitrosamines and others. These componds may damage the embryo, impair endometrial maturation, implantation and possibly even placentation (Dechanet et al., 2011). Any of these mechanisms could cause the increase in miscarriages noted by Xu et al., (2014). These associations are further increased according to the number of cigarettes smoked per day (Xu et al., 2011). Atik et al., (2018) suggest that couples with RPL should be informed of the possible negative impact on their pregnancy plans. Obtaining a smoking history is important, as well as having a discussion about cessation. All couples were asked about their smoking history, and were strongly advised to stop, even if only one was a smoker. 10.2% (28) of the men admitted to smoking and only 2.8% (25) of the women.

10.2 Nutrition

Nutrition is also important, and many vitamin deficiencies such as folate and B12 deficiencies, known to increase the risk of hyperhomocystinaemia and neural tube

defects in pregnancies, (Zetterberg, 2004), were detected, as well as vitamin D deficiency or insufficiency, during the work up. Australia fortifies bread making flour with folic acid, and good quality pre-natal vitamins are available. Many patients accessed the social media networks and started self treating with micronutrients long before presentation to the RM clinic. Many had visited Naturopaths and Homeopathy practitioners, prior to their appointments. The couples were advised not to plan a pregnancy until all tests had been completed and possible contributory deficiencies had been identified, and dietary issues addressed. Some were referred to their GP's for longer follow up and referral to nutritionists or dietitions if deficiencies were were deemed important.

10.3 Vitamins

There is increasing evidence that a deficiency of Vitamin D increases the risk of all adverse pregnancy outcomes, including miscarriages, gestational diabetes, intrauterine growth restriction, and pre-eclampsia (Wei et al., 2014). Vitamin D is thought to affect embryo implantation, placental angiogenesis, oxidative stress, immune function, glucose homeostasis and endothelial function (Wei et al., 2014). A recent study showed that Vitamin D has a role in regulating T regulatory cells (Treg) and T helper 17 cells (Th17) (Ji et al., 2019).

Fasting vitamin D ($25OHD_3$) was routinely measured in both partners. Supplementation was advised if the levels were below those recommended. Vitamin D levels were defined as: Deficient below 50nmol/L, Insufficient as 50 – 74.9 nmol/L and Sufficient as ≥75 nmol/L (Kramer et al., 2014).

Vitamin D has been shown to also affect male fertility via positive effects on sperm motility, and direct actions on spermatozoa and activation of the molecular pathways

involved in the acrosomal reaction, sperm capacitation and motility (De Angelis et al., 2017).

10.4 Male factor

The ‘male factor’ in RPL has been investigated in numerous studies, particularly in relation to sperm aneuploidy (Gil-Villa et al., 2010, Collodel et al., 2009, Zidi-Jrah et al., 2016). When sperm from couples experiencing RPL was compared with sperm from couples without pregnancy loss, a lower percentage of normal sperm morphology, concentration and progressive motility was found in the pregnancy loss group compared to those without loss. Obviously, sperm quality may affect embryonic development by genetic, as well as epigenetic, mechanisms (Zidi-Jrah et al., 2016. Aitken et al.,2016). These sperm-borne epigenetic marks are, in turn, affected by a variety of paternal factors, including genotype, age, obesity, smoking and exposure to environmental contaminants (Aitken et al., 2016). Raised circulating homocysteine in men has also been shown to possibly be toxic to sperm and may negatively affect sperm parameters (Gil-Villa et al., 2010).

The ‘Lifestyle’ questions that the men were asked included caffeine intake, drug habits, alcohol intake, smoking, exercise patterns. 251 patients had their BMI recorded and 165 of these were overweight or obese.

Two hundred and seventy-four partners attended the clinic. Once their blood tests were available, a discussion was held regarding possible interventions, such as folate, vitamins B and D supplementation. Of the 155 men who were tested for fasting homocysteine, 68.4% had raised levels. They were advised to improve lifestyles, including factors that affect sperm quality, such as decreasing caffeine,

alcohol, smoking, and increasing vitamin supplementation if needed, all factors also know to increase homocysteine levels.

Only 1.8% admitted to illicit drug intake among the men.

Interestingly, the discussion regarding lifestyles and the possible impact on pregnancy outcomes by the male partner was accepted with profound relief by the women, predominantly because the overwhelming belief in many societies that the woman is responsible for pregnancy loss. Whether this factor decreased their stress levels and was thus part of their success later on, remains to be investigated. Supplementing vitamin deficiencies and lowering homocysteine levels via supplementation with homocysteine lowering agents, as well as changes to diet and habits, was offered, and a repeat blood check after a month. Once the levels were back to the accepted normal levels, they were encouraged to attempt the next pregnancy.

References for Chapter 10

Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatazoa. Reproduction, Fertility and Development. 2016;28:1-10. http://dx.doi.org/10.1071/RD15325.

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018

.

Collodel G, Giannerini V, Pascarelli AN, Federico MG, Comodo F, Moretti E. TEM and FISH studies in sperm from men of couples with recurrent pregnancy loss. Andrologica 2009;41:352-60.

de Angelis, C., Galdiero, M., Pivonello, C. et al. Rev Endocr Metab Disord (2017) 18: 285. https://doi.org/10.1007/s11154-017-9425-0

Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S et al., Effects of cigarette smoking on reproduction. Hum Reprod Update 2011;17:76-95.

Gil-Villa AM, Cardona-Maya W, Argawal A, Sharma R, Cadavid A. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. Fertil Steril 2010;94:1465-72.

Kramer CK, Swaminathan B, Hanley AJ, Connelly PW, Sermer M, Zinman B, Retnakaran R. Vitamin D and Parathyroid Hormone Status in Pregnancy: Effect on insulin Sensitivity, β-cell Function, and Gestational Diabetes Mellitus. 2014; J Clin Endocrinol Metab 99:4506-4513.

Wei SQ. Vitamin D and pregnancy outcomes. Curr Opin Obstet Gynecol. 2014;26(6):438-447.

Xu GL, Wu YM, Yang LM, Yuan L, Guo HF, Zhang FQ et al., Risk factors for early miscarriage among chinese: A hospital-based case-control study. Fertility and Sterility 2014; 101:1663-1670.

Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol and Endocrin 2004:2-7.

Zidi-Jrah I, Hajlaoui A, Moogou-Zerelli S, Kammoun M, Meniaoui I, Sallem A et al. Relationship between sperm anueploidy, sperm DNA integrity, chromatin packaging, tyraditiuonal semen parameters, and recurrent pregnancy loss. Fertil Steril 2016;105:1:58-64.

Statement of Authorship Vit D HI:

Statement of Authorship

Title of Paper	Association between vitamin D and hyperinsulinism.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	The Journal of Maternal-Fetal & Neonatal Medicine. June 2018 p1-4. DOI: 10.1080/14767058.2018.1481030

Principal Author

Name of Principal Author (Candidate)	Dr Catherine McCormack
Contribution to the Paper	Project development, selection of patients, gathering of data, written text
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11/6/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr S Leemaqz
Contribution to the Paper	Statistics, contribution to written document
Signature	Date

Name of Co-Author	Dr D L Furness
Contribution to the Paper	Patient selection, contribution to written paper
Signature	Date 15/9/2018

Name of Co-Author	Professor G A Dekker		
Contribution to the Paper	Project design, contribution to written text		
Signature		Date	17-10-18

Name of Co-Author	Professor C T Roberts		
Contribution to the Paper	Contribution to written text		
Signature		Date	18.10.18

The Journal of Maternal-Fetal & Neonatal Medicine

ISSN: 1476-7058 (Print) 1476-4954 (Online) Journal homepage: https://www.tandfonline.com/loi/ijmf20

Association between vitamin D status and hyperinsulinism

Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker & Claire Roberts

To cite this article: Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker & Claire Roberts (2018): Association between vitamin D status and hyperinsulinism, The Journal of Maternal-Fetal & Neonatal Medicine, DOI: 10.1080/14767058.2018.1481030

To link to this article: https://doi.org/10.1080/14767058.2018.1481030

Published online: 08 Jun 2018.

THE JOURNAL OF MATERNAL-FETAL & NEONATAL MEDICINE
https://doi.org/10.1080/14767058.2018.1481030

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Association between vitamin D status and hyperinsulinism

Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker and Claire Roberts

Paediatrics and Reproductive Health, University of Adelaide Robinson Institute, North Terrace, Adelaide, Australia

ABSTRACT

Aims: Some studies have suggested that vitamin D deficiency is associated with an increased risk of first trimester miscarriages, others have suggested that it is associated with an increased risk of hyperinsulinism/insulin resistance and the development of gestational diabetes. Hyperinsulinism is also thought to increase miscarriages. We investigated the association between vitamin D levels and hyperinsulinism in a cohort of recurrent miscarriage patients.

Methods: Patients undergoing miscarriage investigations had insulin and vitamin D levels tested. Vitamin D levels were classified as: sufficient ($\geq$75 nmol/L), insufficient (50–74.9 nmol/L) or deficient ($<$50 nmol/L). Hyperinsulinism was assessed *via* a 75 g oral glucose tolerance test (OGTT) with insulin studies.

Results: One hundred and fifty-five patients underwent the testing. Hyperinsulinism was detected in 58.3% of the vitamin D deficient group, 38.7% of the insufficient group, and 33.3% of the sufficient group (chi-square $p = .034$). There were no significant associations between BMI and vitamin D levels, or BMI and hyperinsulinism. Caucasians comprised 82% of the clinic, and 67% of these women had vitamin D insufficiency/deficiency. Noncaucasians comprised 18% of the clinic but 89% of these patients had vitamin D insufficiency/deficiency.

Discussion: We found that insufficient or deficient vitamin D levels were significantly associated with hyperinsulinism in these patients. Vitamin D deficiency is also thought to contribute to an increased risk of adverse pregnancy outcomes including preeclampsia, preterm birth, small-for-gestational-age gestational diabetes mellitus, and miscarriages. Larger level one trials are needed to establish if increasing serum vitamin D levels prior to conception or in early pregnancy improves adverse pregnancy outcomes.

ARTICLE HISTORY
Received 10 January 2018
Revised 8 May 2018
Accepted 22 May 2018

KEYWORDS
Hyperinsulinism; insufficiency and deficiency; vitamin D sufficiency; recurrent miscarriages

Introduction

Vitamin D has been implicated as a modifiable risk factor for miscarriages, due to its function as an immune modulator and because it may modify maternal immune tolerance, without which the semiallogeneic human fetus cannot survive. Andersen et al., found the adjusted hazard of first-trimester miscarriage was lower with higher vitamin D concentrations [1–5]. Vitamin D is classically involved in the regulation of calcium homeostasis, directly *via* affecting intestinal calcium and bone absorption, and indirectly by suppression of parathyroid hormone (PTH) secretion. It also has several well established "nonclassical" roles in the immune system and in functional regulation of a wide variety of cell types [6]. Accumulating evidence has linked vitamin D deficiency with abnormal glucose metabolism, thought to mainly occur *via* direct effects of vitamin D, either on pancreatic β-cell function or on insulin sensitivity [7–9]. Studies have shown lower vitamin D levels in diabetic subjects compared to nondiabetics [10–14]. Lu et al. in a meta-analysis of observational studies involving 16,515 patients from 20 studies revealed that low vitamin D levels were associated with a 45% increased risk of the development of Gestational Diabetes Mellitus (GDM) [8]. Insulin resistance and the resultant hyperinsulinism is known to increase the risk of gestational diabetes and pregnancy loss, particularly in patients with polycystic ovarian syndrome.

Subjects

Patients referred to a Recurrent Miscarriage Clinic in a Tertiary Public Hospital. They were evaluated if they had had two or three clinically recognized, consecutive miscarriages, with the same partner.

CONTACT Catherine McCormack deemccormack@hotmail.com Paediatrics and Reproductive Health, University of Adelaide Robinson Institute, North Terrace, Adelaide, Australia

Materials and methods

Vitamin D testing was offered to all patients presenting to the recurrent miscarriage clinic, with the routine blood work up. Vitamin D (25-hydroxy vitamin D) assessment was performed using the Thermo Scientific™ LC-MS system by SA Pathology. 155 patients also had a 75 g Oral Glucose Tolerance test (OGTT) together with fasting and 2 h insulin levels measured as well. Insulin was measured using the Advia Centaur analytical system. This insulin assay detects recombinant insulin analogs in addition to endogenous insulin. The fasting insulin levels accepted as normal by our laboratory are 0–12 mU/L, we used the glucose: insulin ratio <4.5 and insulin levels ≥ 20 mU/L to diagnose fasting hyperinsulinism. Normal 2-h insulin concentrations in our laboratory are 10–40 mU/L. Using 10–40 mU/L gave a mean of 25.88 in our group of normal patients. We used three standard deviations from this mean as our cutoff for the diagnosis of 2-h hyperinsulinism, 52.6 mU/L, as there are no guidelines in the literature.

BMI was calculated as weight in kilograms divided by the height in meters squared, and 20–24.9 as normal BMI, 25–29.9 as overweight and ≥ 30 obese. Vitamin D levels were defined as deficient below 50 nmol/L, insufficient as 50–74.9 nmol/L and sufficient as ≥ 75 nmol/L [9].

Statistics

The association between hyperinsulinism, vitamin D levels, BMI categories, and ethnicity were examined using chi-squared test, or Fisher's exact test where appropriate. A Breslow-Day test was also performed to assess the homogeneity of odds ratios between hyperinsulinism and vitamin D levels across different BMI categories. Statistical analyses were performed in R 3.4.1 [15].

Results

The vitamin D results for 294 patients were available for assessment and 155 of these had 75 g OGTT's. Eighty-two% of the patients were Caucasian, and only 18% non-Caucasian, comprising Indian (5%), African (1.9%), Middle Eastern (6.5%), and Asian (4.6%).

Vitamin D levels in recurrent miscarriage patients were sufficient in 33% of Caucasians, and just 11% of non-Caucasians (Figure 1). Hyperinsulinism was significantly more common in recurrent miscarriage (RM) patients who had vitamin D deficiency or insufficiency ($p = .034$) (Figure 2) than in patients who had sufficient

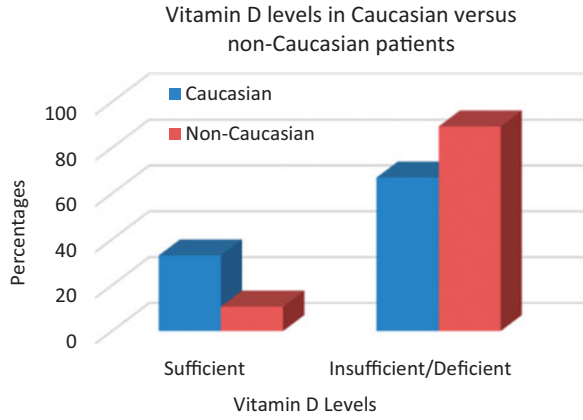


Figure 1. Vitamin D levels in Caucasian and non-Caucasian patients in the sufficient and insufficient/deficient groups.

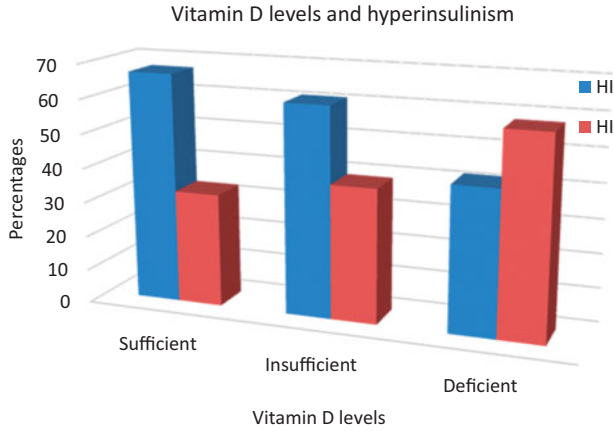


Figure 2. Vitamin D levels and hyperinsulinism. The risk of hyperinsulinism increased with decreasing vitamin D levels. S: sufficient; I: Insufficient; D: deficient vitamin D; HI: hyperinsulinism.

levels, and this was independent of BMI ($p = .41$). Vitamin D levels were also independent of BMI ($p = .09$).

Discussion

We have demonstrated that a low serum concentration of vitamin D is associated with hyperinsulinism. We have previously shown a significant increase in GDM in RM patients with prepregnancy raised fasting or 2-h insulin levels, in a subsequent pregnancy (submitted). The coexistence of vitamin D deficiency/insufficiency with hyperinsulinism in RM patients strengthens the link between insulin resistance and poor vitamin D status. Furthermore, it may indicate that improving vitamin D status and metabolic health could have important benefits for women planning pregnancy. Recurrent miscarriage (RM) affects from

1 to 4% of a population of reproductive age couples and is multifactorial in origin [16]. Women who suffer recurrent miscarriages are a heterogeneous group. Known causes include genetic and endocrine abnormalities, antiphospholipid syndrome, autoimmune disease and uterine structural abnormalities [16]. While genetic factors are an important cause of losses, lifestyle factors, many of which are modifiable, are significant. Increased maternal age and increased prepregnancy BMI are two such modifiable risk factors [15–17]. Vitamin D insufficiency and deficiency in pregnant women is common worldwide, particularly in the winter months, and vitamin D supplementation is thought to be a public health strategy that could improve both maternal and fetal outcomes [18].

Vitamin D is synthesized in the skin following sun exposure and to a lesser extent is present in the diet [18]. Dark skin pigmentation, cold climates with little sun exposure, clothing that limits sun exposure, sun screen, as well as inadequate diets, all lead to vitamin D deficiency. This is not necessarily predictable. People with darker skins are known to make less cutaneous vitamin D than those with little pigmentation [18]. However, in countries such as Australia, with extremely high incidence of malignant melanoma many people tend to limit sun exposure. Hence, hypovitaminosis D is increasing, and is suspected to be a public health problem in many parts of the world [18].

Vitamin D deficiency is associated with type 2 diabetes mellitus in adults [8] while in pregnancy it has been associated with an increased risk of preeclampsia, GDM, preterm birth, small for gestational age babies (SGA), as well as problems in infancy such as rickets [8]. Alternative actions for vitamin D in pregnancy loss have been reported. Ota et al. [19] showed that a high proportion of women with recurrent pregnancy loss had vitamin D deficiency citing its association with increased cellular and autoimmunity. They found that women with low vitamin D status had significantly increased antithyroid antibodies (ATA) antinuclear antibodies (ANA) and antiphospholipid antibodies (APA) [17,18]. APA's are known to cause pregnancy loss [19], whereas ANA's and ATA's are associated with losses [20].

We have demonstrated an association between decreased vitamin D levels and hyperinsulinism, which may suggest that vitamin D is a modifiable risk factor for adverse pregnancy outcomes. Large, well designed multicentre randomized controlled trials are required to actually determine whether vitamin D supplementation in women with low vitamin D status reduces the risk of adverse pregnancy outcomes.

Acknowledgements

This study formed part of a Clinical Trial named the PAPO (Prediction of Adverse Pregnancy Outcomes) study, (Clinical trial number ACTRN12609000254291). The study was approved by the Women's and Children's Hospital Human Research Ethics Committee in North Adelaide South Australia, REC1481/6/09. All patients gave written informed consent.

Disclosure statement

The authors declare that there are no conflicts of interest associated with this manuscript.

Funding

C.T. Roberts was supported by a National Health and Medical Research Council of Australia (NHMRC) Senior research fellowship [GNT1020749] (http://www.nhmrc.gov.au).

ORCID

Catherine McCormack http://orcid.org/0000-0002-6209-6904

References

[1] Feodor NS, Andersen P, Strandberg LK, et al. Risk factors for miscarriage from a prevention perspective: a nationwide follow-up study. BJOG: Int J Obstet Gy. 2014;121:1439.
[2] Hewison M. Vitamin D and innate immunity. Curr Opin Investig Drugs. 2008;9:485–490.
[3] Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. Nat Clin Pract Endocrinol Metab. 2008;4:80–90.
[4] Lagishetty V, Liu NQ, Hewison M. Vitamin D metabolism and innate immunity. Mol Cell Endocrinol. 2011;347:97–105.
[5] Andersen LB, Jørgensen JS, Jensen TK, et al. Vitamin D insufficiency is associated with increased risk of first-trimester miscarriage in the Odense Child Cohort. Am J Clin Nutr. 2015;102:633–638.
[6] Clifton-Bligh RJ, McElduff P, McElduff A. Maternal vitamin D deficiency, ethnicity and gestational diabetes. Diabet Med. 2008;25:678–684.
[7] Scragg R, Holdaway I, Singh V, et al. Serum 25-hydroxyvitamin D3 levels decreased in impaired glucose tolerance and diabetes mellitus. Diabetes Res Clin Pract. 1995;27:181–188.
[8] Lu M, Xu Y, Lv L, et al. Association between vitamin D status and the risk of gestational diabetes mellitus: a meta-analysis. Arch Gynecol Obstet. 2016;293:959–966.
[9] De Pergola G, Nitti A, Bartolomeo N, et al. Possible role of hyperinsulinemia and insulin resistance in lower vitamin D levels in overweight and obese patients. BioMed Res Int. 2013;2013:921348.
[10] Kramer CK, Swaminathan B, Hanley AJ, et al. Vitamin D and parathyroid hormone status in pregnancy:

effect on insulin sensitivity, β-cell function, and gestational diabetes mellitus. J Clin Endocrinol Metab. 2014;99:4506–4513.

[11] Chiu KC, Chu A, Go VL, et al. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. Am J Clin Nutr. 2004;79:820–825.

[12] Forouhi NG, Luan J, Cooper A, et al. Baseline serum 25-hydroxy vitamin D is predictive of future glycemic status and insulin resistance: the Medical Research Council Ely prospective study 1990–2000. Diabetes. 2008;57:2619–2625.

[13] Mattila C, Knekt P, Männistö S, et al. Serum 25-hydroxyvitamin D concentration and subsequent risk of type 2 diabetes. Diabetes Care. 2007;30:2569–2570.

[14] Zhang C, Qiu C, Hu FB, et al. Maternal plasma 25-hydroxyvitamin D concentrations and the risk for gestational diabetes mellitus. PLoS One. 2008;3:e3753.

[15] Team RDC. R:A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2011.

[16] Petrozza JC. Early pregnancy loss. emedicine; 2006. Available from: http://wwwemedicinecom/med/topic3241.htm.

[17] Nybo Andersen AM, Wohlfahrt J, Christens P, et al. Maternal age and fetal loss: population based register linkage study. BMJ. 2000;320:1708–1712.

[18] WHO. Guidelines: Vitamin D Supplementation in Pregnant Women. Geneva: World Health Organization; 2012.

[19] Ota K, Dambaeva S, Han AR, et al. Vitamin D deficiency may be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity. Hum Reprod. 2014;29:208–219.

[20] Kutteh WH, Rote NS, Silver R. Antiphospholipid antibodies and reproduction: the antiphospholipid antibody syndrome. Am J Reprod Immunol. 1999;41:133–152.

Paper Published: Vit D and HI paper

CHAPTER 11: Conclusions

11.1 General

The ASK TEAMS Acronym is useful for clinicians who deal with RM patients, as it not only reminds them of the extensive tests needed, but also allows additional tests to be added for individual patients and will allow other investigations to be added as they are discovered and found to be useful. It is also a reminder that a ‘Team’ approach to these patients, such as additional psychological support, may be of benefit. RPL patients are not a static group, but one of the most focussed and desperate groups of patients that one can encounter. They are psychologically damaged and are looking for help, and have often been traumatized by the frequent lack of compassion afforded their plight in busy Emergency departments facing more serious issues. They are also some of the most loyal patients ever likely to attend clinics and follow all advice given. Many are desperate to try for pregnancy as soon as possible, frequently before much of the work-up is completed. Thus, every couple needs to be treated as individually as possible, while trying to keep within the bounds of a predetermined but potentially flexible work-up.

Constant auditing of findings, investigations, outcomes and the latest evidence is essential, and offering these couples the best investigations of the current available evidence is a duty of all physicians attending these couples.

The European Society of Human Reproduction and Embryology (ESHRE) develops guidelines for clinical practice in Europe, to provide clinical recommendations in order to improve the quality of healthcare delivery within the field of human reproduction and embryology. These guidelines were produced after careful consideration of the scientific evidence available at the time of preparation of the document and are the most recent guidelines on this difficult but ubiquitous problem. However, the authors are quick to point out that adherence to them cannot guarantee a successful outcome, nor do they establish a standard of care for institutions. The ESHRE guidelines do not replace the healthcare professional's clinical judgement, nor clinical decisions, which need to be made on an individual basis using their clinical knowledge and expertise, while considering the circumstances, wishes and conditions of each individual couple (Atik et al. 2018).

Thus, running RM clinics and dealing with such couples is time consuming and requires dedication, especially with the vast amount of information, not necessarily accurate or safe, available via social media forums, blogs etc. The acronym approach has allowed a consistent approach to the potentially massive work up, and is modified regularly, especially if individual investigations are eventually deemed non-contributory, such as the thrombophilias. While the ESHRE guidelines no longer suggest investigating fasting glucose and insulin levels, our findings of a significantly increased risk of gestational diabetes in patients with hyperinsulinism has encouraged us to continue these investigations, and to strongly suggest to patients that they modify their lifestyle before embarking on their next pregnancy, if possible, which may or may not be directly linked to miscarriages, but which may have a profound impact on the morbidity of both mother and fetus and their future health.

Recurrent miscarriages are difficult to study in a research setting, especially as they are common but heterogeneous. While there has been a vast improvement in the last three years with dedicated recurrent miscarriage conferences, they remain in the ‘too hard box’ for large level one evidence randomized controlled trials. However, we believe that consensus reached by a large number of clinicians is of use in the interim, until such trials can be undertaken.

AGE:

One of the most important facts regarding the age of the patients is that miscarriages increase with increasing maternal age. It is essential that this discussion occurs early in the work up, so that patients are aware of the facts regarding their pregnancy outcomes, and can hopefully make informed choices, prior to embarking on difficult and costly journeys with little chance of success.

Pregnancy at an advanced maternal age is increasing worldwide in both developed and developing countries, particularly via advances in assisted reproductive technology (ART) (Kahveci et al., 2018). Unfortunately concomitant co-morbidities such as hypertension and glucose intolerance also increase with age, and may contribute to miscarriages or adverse pregnancy outcomes. However, not all patients can conceive when they plan to do so. Some women may need an assessment of their ovarian reserve, as low reserves may not only contribute to poorer egg quality, but also to miscarriages. Lyttle Schumaker et al., (2018) showed a marked increase in miscarriages in spontaneously conceived pregnancies of women with markedly decreased AMH levels. Their rate was twice that of women with normal levels, suggesting that AMH levels are inversely associated with miscarriage risk (Lyttle Schumaker et al., 2018).

Serum Anti-Müllerian Hormone (AMH) levels are an ideal marker of a woman's ovarian reserve, as AMH is highly correlated with the number of antral follicles remaining (Visser et al., 2006; Barad et al., 2009.

Offering this test to patients with a view to referring them to ART in a timely fashion was useful, especially when we showed that their levels were lower than in a normal population.

STRUCTURE

The use of the 3 D ultrasound proved beneficial in detecting abnormalities that could potentially be corrected surgically.

KARYOTYPE

Genetics is advancing at a rapid rate, and new techniques are becoming available every year. Each institution needs to evaluate what they can offer in a cost effective manner that will yield the most benefit to these patients. Our Institution does not offer chromosomal microarrays yet, as they believe that the Karyotype currently offers more than the arrays could. Ongoing audit is currently in place.

THROMBOPHILIA

When the clinic was first established, thrombophilias were thought to be a cause of miscarriages. However, this line of thinking has changed significantly since then. It was assumed that clotting in the placenta could lead to adverse pregnancy outcomes, therefore these women were treated with anticoagulants.

A systematic review by Skeith et al., in 2016, reported no benefit of low molecular weight heparin (LMWH) for prevention of pregnancy loss in women with hereditary thrombophilia and prior late (≥ 10 weeks) pregnancy loss (LBR LMWH versus no LMWH: RR 0.81; 95% CI 0.38-1.72; 5 RCTs; n=308) or recurrent early (< 10 weeks) pregnancy loss (LBR LMWH versus no LMWH: RR 0.97; 95% CI 0.80-1.19; 2 RCTs; n=66) (Skeith et al., 2016).

A Cochrane review on anticoagulant treatment for women with RPL with or without hereditary thrombophilia combined nine RCTs including 1228 women with predominantly early losses. The reviewers reported no significant effect of treatment (aspirin, LMWH, LMWH + aspirin) compared to placebo. The risk ratio for live birth was 0.94 (95% CI 0.80-1.11; n=256) in the comparison of aspirin versus placebo, 1.23 (95% CI 0.84-1.81; n=453; studies at high risk of bias included) for LMWH versus no treatment, and 1.01 (95% CI 0.87-1.16; n=322) for LMWH and aspirin compared to no treatment. In the comparison of LMWH versus aspirin the risk ratio for live birth was 1.08 (95% CI 0.93-1.26; n=239), in the comparison of LMWH and aspirin versus aspirin alone it was 1.11 (95% CI 0.94-1.30; n=327) (de Jong et al., 2014).

Given placentation and clotting issues, thrombophilias can only be associated with fetal losses, not embryonic ones (≤ 10 weeks from LMP), thus a good history is essential if treatment is to be considered.

We changed from treating all women with genetic thrombophilias to only those who had had thrombotic events outside of pregnancy, or those with a fetal loss or unexplained stillbirth and a genetic thrombophilia. More research is required to clarify causality.

ENDOCRINE

Our findings of significantly raised insulin levels in a large number of patients independent of their BMI, and the subsequent increase in GDM in a subsequent pregnancy, has lead us to conclude that hyperinsulinism is an issue in these patients. Thyroid disorders were also common and were treated. Elevated prolactin was not routinely treated unless clinical symptoms were detected.

AUTOIMMUNE

Autoantibodies were tested, particularly the antiphospholipid syndrome. Thyroid antibodies were also tested, but only sub-clinical or clinical hypothyroidism was treated. Hyperthyroidism was also treated.

METABOLIC

A fasting 75g OGTT is offered to all patients, and detection of abnormal levels warrants a visit to their GP's for long term lifestyle advice, and future health.

SPECIFIC

This area allows additional tests to be added as new evidence becomes available. Patients referred to recurrent miscarriage clinics are psychologically fragile and vulnerable. Pregnancy loss is classified as a significant life event, and ESHRE now suggests that that there is a need to study the emotional impact on men, as well as the women (Atik et al., 2018). Considerable time needs to be spent dealing with RM couples, reassuring them that it was highly unlikely that either of them was to blame for the losses, explaining the relative dearth of level one evidence regarding treatments, and explaining the possible tests that could be offered, as well as lifestyle changes that should be instituted if issues are detected.

While many of the findings were consistent with those of recurrent miscarriage clinics world-wide, some unique observations were made:

a) AMH levels were found to be significantly lower than in a normal population. Thus many of these women would be compromised if they were to waste significant time prior to planning the next pregnancy, as AMH is a marker of ovarian reserve.

b) Fasting and two-hour insulin levels were tested, and when compared to patients with normal insulin levels, women with raised two-hour insulin levels had a 10-fold increased risk of gestational diabetes in a subsequent pregnancy, whereas women with raised fasting levels had a 4-fold increase. Lifestyle interventions such as increasing exercise and decreasing carbohydrate intake may improve insulin levels.

c) Low vitamin D levels were significantly associated with hyperinsulinism in miscarriage patients and Vitamin D needs to be supplemented in many patients.

The raised two-hour insulin levels and the subsequent increased risk of gestational diabetes in these women has not been described before. If this factor is taken into consideration, it would suggest that instead of the quoted figure of 50% of recurrent miscarriage patients not having a 'cause/association' detected during a work-up, the actual number may be considerably less. Recent work by Vega et al., (2019) showing that elevated insulin levels were directly toxic, *in vitro*, to trophoblasts from first trimester, resulting in apoptosis, increased DNA damage and overall reduced cell survival, is interesting. It suggests that increased insulin may be associated with placental damage. They also showed that this effect was reduced by the addition of Metformin *in vitro* (Vega et al., 2019).

While it is difficult to calculate the exact number, the women aged 35 years or less in whom no obvious cause/association could be found numbered 8 (2.8%), when APS,

CUA, Vitamin D levels, homocysteine levels and hyperinsulinism were all considered. In the women aged over 35 years, this number was 5 (2.5%).

11.2 Findings

Investigations	**Test N**	≤ 35 - **283**	>35 - **197**
		N (%)	N (%)
Age -low AMH	182	28 of 68 (41.2)	69 of 114 (60.5)
Structure congenital uterine abnorms	331	48 (24.4)	25 (18.7)
Karyotype	422	11 (4.3)	3 (3.3)
Thrombophilia FVL heterozygous	399	11 (4.6)	8 (4.5)
Thrombophilia PGM heterozygous	399	4 (1.7)	6 (3.4)
Endocrine –thyroid	424	26 (9.2)	22 (11.2)
Endocrine fasting hyperinsulinism	245	3 (4.1)	5 (10.6)
Endocrine 2-hour hyperinsulinism	245	48 (64.9)	33 (70.2)
Endocrine fasting and 2-hour HI	245	23 (31.1)	9 (19.2)
Autoimmune- Thyroid antibodies	480	35 (12.4)	18 (9.2)
Autoimmune – APS	476	69 (25)	46 (24)
Metabolic-vitamin D insufficiency	363	72 (33)	61 (42)
Metabolic- Vitamin D deficiency	363	61 (28.1)	39 (26.7)
Metabolic ↑Homocysteine	423	95 (37)	59 (35.1)
Specific MTHFR 677 Homozygous*	368	28 (13.7)	29 (17.8)
Specific MTHFR 1298 Homozygous*	368	20 (9.8)	16 (9.8)

* While MTHFR was originally viewed as a thrombophilia, it is no longer, and testing has ceased.

Table 11.1. The final test results in the two age groups in the women attending the RM Clinic.

11.3 Pregnancy outcomes

In the women aged ≤35 years, 12.7% did not achieve a pregnancy within two years of attending the clinic, 12.5% had only losses and 87% achieved a live birth (LB) (76.3% of all attending the clinic). In the >35 years age group, 24.9% did not achieve a pregnancy, 25% had losses only and 75% achieved a LB.

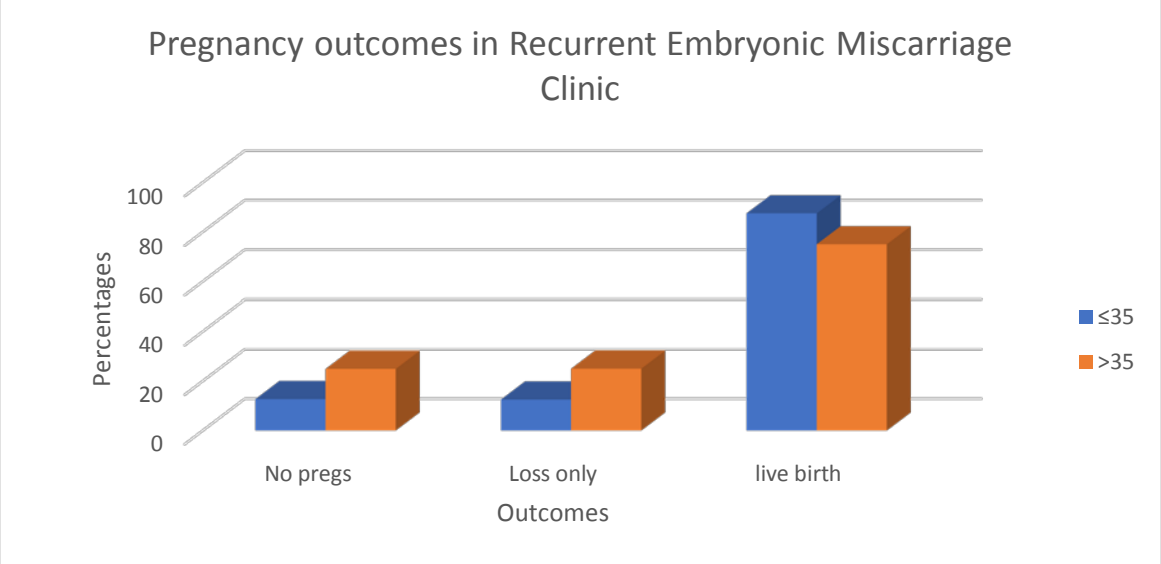


Figure 11.1 The pregnancy outcomes in the women attending the clinic.

Research has shown that women who suffer recurrent miscarriages are at an increased risk of future cardiovascular disease of total coronary heart disease after adjusting for traditional cardiovascular risk factors (Oliver-Williams et al., 2013). The risk appears to be independent of BMI, hypertension, waist-to-hip ratio and white cell count (Parker et al.,2014). Thus, miscarriages may reflect a bigger picture of overall cardiovascular health, and as such, could be a primary health care issue with possible preventative interventions for this group.

11.4 Summary

Every institution dealing with RM patients should decide on their baseline tests, and these should be consistently applied to all patients. The ASK TEAMS approach suits a service in which there may be a large change-over of staff on a regular basis, especially if such a service does not offer pre-printed investigation forms or pre determined blood sets per diagnosis. A clear distinction between recurrent miscarriages and infertility is also essential, especially if the institution does not offer ART. Referring a patient to an infertility specialist may allow for male factors to be identified that may be contributing to miscarriages or delayed pregnancies. The World Conference on Recurrent Pregnancy losses is now in its 5th year, and the bringing together of physicians with an interest in this complex issue has opened the area for the exchange of ideas, and possible collaborations in the future.

Recurrent miscarriage patients are a heterogeneous group, and research in this area is difficult, especially as many patients do not want to wait for the necessary large multicentered randomized and placebo-controlled trials that are so vital to being able to provide evidence-based investigations and interventions.It is essential, however, that these trials be performed on an international platform, so that, ultimately, we may all achieve the safest and best outcomes for these unfortunate patients.

References Chapter 11

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.

de Jong PG, Kaandorp S, Di Nisio M, Goddijn M, Middeldorp S. Aspirin and/or heparin for women with unexplained recurrent miscarriage with or without inherited thrombophilia. Cochrane Database Syst Rev 2014;7: Cd004734.

Kahveci B, Melecoglu R, Evruke IC, and Cetin C. The effect of advanced maternal age on perinatal outcomes in nulliparous singleton pregnancies. BMC Pregnancy and Childbirth 2018;18:343. https://doi.org/10.1186/s12884-018-1984-x.

Lyttle Schumaker BM, Jukic AMZ, Steiner AZ. Antimüllerian hormone as a risk factor for miscarriage in naturally conceived pregnancies. Fertil steril 2018;109:1065-1071.

Oliver-Williams CT, Heydon EE, Smith GCS, Wood AM. Miscarriage and future maternal cardiovascular disease: a systematic review and meta-analysis.Heart 2013;99:1636-1644.

Parker DR, Lu B, Sands-Lincoln M, Kroenke CH, Lee CC, O'Sullivan M et al. Risk of Cardiovascular Disease Among Postmenopausal Women with Prior Pregnancy Loss: The Women's Health Initiative. Ann Fam Med 2014;302-309. doi: 10.1370/afm.1668.

Skeith L, Carrier M, Kaaja R, Martinelli I, Petroff D, Schleussner E, Laskin CA, Rodger MA. A meta-analysis of low-molecular-weight heparin to prevent pregnancy loss in women with inherited thrombophilia. Blood 2016;127: 1650-1655.

Vega M, Mauro MM, Williams Z. Direct toxicity of insulin on the human placenta and protection by metformin. Fertil Steril 2019;111:3:489-496.

Statement of Authorship: ASK TEAMS

Paper to be submitted: ASK TEAMS

The ‘ASK TEAMS’ approach to investigations for women with recurrent miscarriages.

Running title: ASK TEAMS approach to recurrent miscarriages.

Catherine McCormack, Shalem Leemaqz, Denise Furness, Gustaaf Dekker, Claire Roberts.

Keywords: Recurrent miscarriages, miscarriage investigations, embryonic miscarriages, spontaneous pregnancy losses, recurrent abortions.

ABSTRACT:

Background: Recurrent embryonic miscarriage (RM) patients who presented to a recurrent miscarriage clinic were offered a structured work up.

Aims:

To identify presumed causes/associations of RM's and streamline investigations.

Methods:

Women were included if they had had two or three embryonic losses (Gestational age of ≤10 weeks).

Results:

The testing that was developed and subsequent acronym, was as follows:

Age: Antimüllerian Hormone testing was offered.

Structure: Patients were offered a luteal phase 3D ultrasound of their pelvis.

Karyotype: Although this was routinely performed initially, later evidence suggested that it should be restricted to the miscarriage products. Parental karyotyping was then undertaken if translocations were detected.

Thrombophilia: Initially all patients were tested. However, as thrombophilias are no longer considered as causal in embryonic losses, testing was ceased.

Endocrine: Thyroid function tests and prolactin levels were tested.

Autoimmune: Thyroid antibodies and antiphospholipid antibodies were tested.

Metabolic: Fasting Homocysteine levels, and a 75 g oral glucose tolerance test with insulin studies was offered, as well as vitamin D, Folate and B12 levels.

Specific: Male partners were tested for factors in their lifestyles that could contribute to sperm DNA damage and possibly contribute to pregnancy losses. Tests included fasting glucose, insulin, homocysteine, vitamin D, Folate and B12 levels. Other tests were initiated according to the history of the patient, such as an infectious screen.

Outcomes: After risk assessment, counselling and patient specific interventions, the subsequent live birth rates were 87% in those ≤35, and 75% in >35 years.

Conclusions: The 'ASK TEAMS' approach facilitates a consistent work-up. Tests are adjusted as evidence accumulates regarding miscarriages causes/associations. Live birth rates are excellent following this approach.

Background:

Recurrent pregnancy losses are possibly one of the most frustrating areas of medicine, not only for the couples who suffer them, but also for the physicians

attempting to alleviate the situation. Despite considerable research, this heterogenous condition remains one of the commonest reasons for women presenting to Emergency Departments, and the causes are most likely the result of multiple interactions of genetic and environmental factors, many of which have yet to be elucidated.[1]

Miscarriage is the term applied to the loss of a pre-viable baby and most institutions will consider miscarriage as a loss up to 20 weeks of gestation. Losses from the diagnosis of pregnancy until an estimated 10 weeks from the last menstrual period are termed embryonic losses, and those from 10-20 weeks are defined as fetal losses. Recurrent pregnancy loss is a heterogenous condition, with considerable confusion as to the numbers of consecutive losses required for the actual definition. The American Society for Reproductive Medicine (ASRM) has accepted 2 or more consecutive pregnancy losses as their definition[2], whereas the European Society for Human Reproduction and Embryology (ESHRE) had previously used 3 or more pregnancy losses in their definition but they have changed this to 2 in the latest guidelines.[3]

Pregnancy losses are the most common complication of humanreproduction , occurring in 50 - 75% of all couples planning a pregnancy.[4] Many of these losses occur before or with the next expected menstrual period. With the advent of sensitive pregnancy detection kits available to the public, previously unrecognized losses are frequently detected. With the losses that occur after the first missed menstrual cycle, approximately 15-20% are spontaneous miscarriages or ectopic pregnancies.[5] Approximately 5% of couples trying to conceive will have two consecutive losses, and approximately 1% of couples will suffer three consecutive losses during their reproductive lives which is highly unlikely to be due purely to chance since it has

been estimated mathematically that that the probability of two consecutive losses should be in the region of 2.25% and that of three consecutive losses about 0.34%.[6,7]

Aims: Recurrent miscarriages are associated with a plethora of presumed 'causes' and/or 'associations,' many of which are not evidence based. The aim of this study was to develop a structured approach to investigations in a dedicated clinic, to ensure consistency for these couples, and to facilitate comparisons with other miscarriage clinics for research purposes. Frequent literature reviews are necessary to update investigations and implement 'evidence-based' interventions for these couples in their endeavour to become parents.

Materials and methods:

Ethics approval:

This study formed part of a Clinical Trial named the PAPO (Prediction of Adverse Pregnancy Outcomes) study, (Clinical trial number ACTRN12609000254291). The study was approved by the Women's and Children's Ethics Committee, REC1481/6/09.

Patients were divided into two age groups, ≤35 years of age or > 35 years of age at their first visit. The reason for assessing these patients in the different age groups was due to the well documented effect of maternal age on fetal loss, as described by Nybo Anderson et al.[6] They showed a steep increase in fetal loss after the age of 40 years, which was already increased in the 30's, but particularly after 35 years of age.[6]

It became apparent that an acronym could be used to systematise the large number of tests these patients underwent, and that the results could also be assessed via the acronym.

Women attending the clinic are heterogeneous in their journeys, needing pathways, tests, and a systematic method, so the ASK TEAMS approach was developed.

The acronym is “ASK TEAMS” and is as follows:

A **A**ge: Patients were offered an Anti Müllerian Hormone (AMH) test as part of the work up, if they were prepared to arrange this separately, as it is not covered by the National Insurance (MEDICARE). AMH Assay: Serum AMH was quantified by immunoassay at Clinpath laboratories using a DSL AMH generation II method (Beckman-Coulter Anti-Müllerian immunoassay). Serum AMH concentrations in women attending the Recurrent Miscarriage Clinic were compared with those from a healthy cohort using data obtained with permission from Shebl et al.[8]

S **S**tructure: If they had not previously undergone a diagnostic laparoscopy, hysteroscopy, hysterosalpingogram, HyCoSy, CT scan of the abdomen/pelvis or MRI of the pelvis, women were offered a luteal phase 3D ultrasound for assessment of the uterus, to detect congenital uterine abnormalities, such as a unicornuate, bicornuate, septate or arcuate uterus. Acquired abnormalities such as fibroids, Asherman’s syndrome, adenomyosis or endometriosis, as well as ovarian morphology were also investigated.

K **K**aryotype: All patients were asked to send the products of conception for karyotyping, particularly if they had more than one loss. This was not always possible. If an aneuploidy was detected, the miscarriage was not included in patient selection. Parental Karyotyping was initially performed on all those attending the clinic but the yield was so low, that it was finally restricted to those in whom a translocation was detected in a previous miscarriage, or in those in whom no other possible cause had been found.

T **T**hrombophilia: Initially all patients were offered a full thrombophilic screen, for both genetic and acquired thrombophilias. Recent evidence has suggested that screening for hereditary thrombophilias has a very low yield, given their low prevalence in the population, and so screening was restricted to those with a strong family history of venous thromboembolism (VTE) or those who had experienced any thromboembolic events prior to presentation.[9] While evidence is conflicting, most Countries now agree that thrombophilias are unlikely to be associated with embryonic losses, especially as maternal blood flow to the intervillous space is initiated at ten weeks (Fetal period). The 2018 ESHRE guidelines do not recommend screening for hereditary thrombophilias unless the screening is performed in the context of a research project, or in women who have had thrombotic events. The clinical utility of the testing was judged as minimal and the harms of testing thought to outweigh the benefits.[3] Acquired thrombophilias, e.g. antiphospholipid antibodies, were grouped under auto-immune conditions.

E **E**ndocrine: All patients were offered thyroid function tests, a 75 g oral glucose tolerance test (OGTT) including insulin and prolactin levels. Reproductive hormone levels were not routinely tested, unless the patient presented with cycle irregularity or features of the polycystic ovarian syndrome (PCOS). If PCOS was suspected from history or from the 3D ultrasound ovarian morphology, a full androgenic work-up was undertaken including sex hormone binding globulin levels, testosterone levels and the free androgen index.

A **A**utoimmune**:** Antibody tests included thyroid peroxidase antibodies, anti-thyroid receptor antibodies (ATA), antinuclear antibody (ANA) titres. Extractable nuclear antibody tests were only performed if the ANA titres were high. Antiphospholipid antibodies were tested in order to detect the Antiphospholipid

syndrome. They comprised: Anticardiolipin antibodies IgG and IgM, Beta-2 Glycoprotein 1 (B2GP1) antibodies and the lupus anticoagulant antibodies; the dilute russell viper venom test (DRVVT) and Kaolin clotting time (KCT).[10]

M **M**etabolic**:** Insulin levels were tested as part of the endocrine work up, as were the glucose levels, detecting impaired fasting glucose levels, impaired glucose tolerance or overt diabetes. Fasting homocysteine levels were tested as well due to the potential association with thrombophilias.

S **S**pecific**:** Vitamin D, B12 and folate studies were performed. Infectious screens were performed if there was a positive history of vaginal infections, such as chlamydia or bacterial vaginosis. A detailed nutritional history was obtained, and coeliac studies performed for a history of dietary intolerances. Body mass index was calculated at the initial visit and recorded. A history of depression and anxiety was also sought, and relevant referrals made for a mental health plan, if they were deemed to have a significant problem in need of additional psychological support.

Partners were included in the work up, if they had been the father of at least the previous two losses. Their work up included a detailed history, and a fasting blood work up. This included fasting homocysteine, glucose, insulin, Vitamin D, Serum Folate, and B12 levels. Vitamin deficiencies may reflect poor lifestyle choices, and have been associated with sperm DNA damage, thought to contribute to losses. Karyotype was initially performed on all partners but was eventually limited to those in whom the previous pregnancy loss had yielded a translocation on Karyotyping.[11]

There were 1532 patients referred to the Recurrent Miscarriage Clinic, 480 of whom fitted the criteria of recurrent embryonic miscarriages, (losses at ≤10 weeks, post the last menstrual period). The reasons for the exclusions included non-consecutive

losses, proven genetic abnormalities, or unproven pregnancies based on recall alone. Patients who suffered both embryonic and fetal losses were only included if they had experienced 2 or 3 consecutive embryonic losses prior to their index visit. The recent European Society of Human Reproduction and Embryology (ESHRE) guidelines in 2018, however, have suggested that non-consecutive losses could be included, and this would have increased the eligible numbers considerably.[3,12] There were 283 women aged less than or equal to 35 years (≤35), and 197 greater than 35 years of age.

The Clinic accepted referrals of patients/couples deemed to have had recurrent miscarriages. A detailed history was obtained. The patients were asked to have two sets of tests performed, one fasting, and the other non-fasting. If they had not had a 3-Dimensional ultrasound (3D), laparoscopy, hysteroscopy, HyCoSy, MRI or CAT scan of the pelvis, they were offered a luteal phase 3D ultrasound scan. BMI was calculated, and Ethnicity recorded.

Most patients required 2 or 3 visits. The first visit was used to obtain a detailed history and to plan the tests. The second visit was used for a review of the results, and to give the patient and partner a detailed plan of intervention, if needed, depending on the findings.

Investigations – Table 1

Table 1 The current investigations offered to couples presenting to the RM clinic.

FEMALE:	
Non-Fasting bloods	Complete blood picture
	AMH (Private test – incurred fees)
	TSH, T4
	Thyroid Autoantibodies
	Anti-cardiolipin IgM and IgG antibodies (+ on 2 occasions)
	Beta 2 GP1 antibodies (+ on 2 occasions)
	Lupus Anticoagulant – DRVVT and Kaolin clotting time (+ x 2)
	Karyotype (if POC* abnormal)
Fasting Bloods	75 g Oral Glucose Tolerance Test with insulin studies
	Fasting Homocysteine levels
	Serum Folate, vitamin B12 and vitamin D levels
3D Pelvic Ultrasound	
MALE:	Karyotype (if POC* abnormal)
	Fasting Glucose and Insulin levels
	Fasting Homocysteine levels
	Serum Folate, vitamin B12 and vitamin D levels.

POC – Products of Conception

Results:

Age: - Serum Anti Müllerian Hormone levels were performed. 41.2% of women ≤35 years had low levels (concentration??), 36.7% were normal (concentration range??) and 22.1% had high (concentration) levels. In women aged >35 years, 60.8% had low levels, 33.3% were normal and 6.1% had high levels.

Structure: - 24.2% of women aged ≤35 years had congenital uterine abnormalities detected by 3D ultrasound as did 18.7% of those > 35 years.

Karyotype: - 4.3% of the women aged ≤35 years had abnormal karyotypes, as did 1.8% of women >35 years.

Thrombophilias: - Testing was initially routinely performed but it was later discontinued.

Endocrine: - 54.8% of women aged ≤35 years had insulin levels tested and 47.7% of those had hyperinsulinism detected. The fasting insulin levels accepted as normal by our laboratory are 0-12mU/L. We accepted the glucose:insulin ratio >4.5 and insulin levels <20mU/L as normal, and a glucose:insulin ratio <4.5 and insulin levels ≥20mU/L as fasting hyperinsulinism. Normal 2 h levels in our laboratory are 10-40mU/L. In our normal group the mean 2 h insulin levels were 25.88mU/L, and so we used three standard deviations from this mean as our cut-off for the diagnosis of 2 h hyperinsulinism (52.6mU/L), as there are no guidelines in the literature. 45.7% aged >35 were tested and 52.2% of those had increased insulin levels.

Autoimmune: - 12.4% of women tested aged ≤35 years had positive thyroid autoantibodies and 54.3% of these women had thyroid problems. 9.2% of women > 35 years had thyroid autoantibodies and 77.8% had thyroid problems. The obstetric antiphospholipid syndrome was detected in 22% of women aged ≤35 years and 19% of those > 35 years.

Metabolic: - Vitamin D levels, as 25(OH)D3, were tested in 75% of women; 35.8% were sufficient (>75nmol/L), 36.6% were insufficient (>50nmol/L-75nmol/L), and 27.6% were deficient (>50nmol/L).

Specific: - Infectious screen if relevant, other tests according to history.

Sub-clinical hypothyroidism was treated with low dose thyroxine, Vitamin D, B12 or Folate deficiency with supplementation. Raised Homocysteine levels were treated with dietary advice such as decreasing caffeine, alcohol and smoking, as well as supplementation with folic acid and vitamin B12 if they were found to be low. If positive antiphospholipid antibodies were detected, a second test was required, with a minimum time of 6-12 weeks for the repeat test, before the diagnosis could be established. Once the obstetric antiphospholipid syndrome (OAPS) was diagnosed, the patients were advised to start low dose aspirin until they were pregnant, and once pregnancy was confirmed, Heparin Sodium was added. Raised insulin or glucose levels were discussed and patients were frequently referred to dietitians for advice. Patients found to have abnormal Karyotypes were referred to Geneticists, and those with abnormal uterine findings, to gynaecologists, if surgical intervention was considered.

The subsequent live birth rates were 87% in those ≤35, and 75% in >35 years.

Table 2 Results of the investigations of couples who presented to the RM clinic.

The final test results in the two age groups in the women attending the RPL Clinic.

Investigations	**Test N**	≤ 35 - **283**	>35 – **197**
		N (%)	N (%)
Age -low AMH	182	28 of 68 (41.2)	69 of 114 (60.5)
Structure congenital uterine abnorms	331	48 (24.4)	25 (18.7)
Karyotype	422	11 (4.3)	3 (3.3)
Thrombophilia FVL heterozygous	399	11 (4.6)	8 (4.5)
Thrombophilia PGM heterozygous	399	4 (1.7)	6 (3.4)
Endocrine –thyroid	424	26 (9.2)	22 (11.2)
Endocrine fasting hyperinsulinism	245	3 (4.1)	5 (10.6)
Endocrine 2-hour hyperinsulinism	245	48 (64.9)	33 (70.2)
Endocrine fasting and 2-hour HI	245	23 (31.1)	9 (19.2)
Autoimmune- Thyroid antibodies	480	35 (12.4)	18 (9.2)
Autoimmune – APS	476	69 (25)	46 (24)
Metabolic-vitamin D insufficiency	363	72 (33)	61 (42)
Metabolic- Vitamin D deficiency	363	61 (28.1)	39 (26.7)
Metabolic ↑Homocysteine	423	95 (37)	59 (35.1)
Specific MTHFR 677 Homozygous*	368	28 (13.7)	29 (17.8)
Specific MTHFR 1298 Homozygous*	368	20 (9.8)	16 (9.8)

* Footnote: While MTHFR was originally viewed as a thrombophilia, it is no longer, and testing has ceased.

Discussion:

The Royal Australian and New Zealand College of Obstetrics and Gynaecology utilizes the Royal College of Obstetrics and Gynaecology Green Top Guidelines regarding recurrent miscarriage investigations, dated 2011. They need updating. The European Society of Human Reproduction and Embryology (ESHRE) develops guidelines for clinical practice in Europe, to provide clinical recommendations in order to improve the quality of healthcare delivery within the field of human reproduction and embryology. These guidelines are produced after careful consideration of the scientific evidence available at the time of preparation and are the most recent guidelines on this difficult but prevalent problem. However, the authors are quick to

point out that even strict following of them does not guarantee a successful outcome, nor do they establish a standard of care that supersedes individual institutions. They do not override the healthcare professional's clinical judgement, or clinical decisions, which need to be made on a case-by-case basis using their clinical knowledge and expertise, while considering the circumstances, wishes and conditions of each individual couple.[3] Thus, running these clinics and dealing with such couples is time consuming and requires dedication, especially with the vast amount of information, not necessarily accurate or safe, that is available via social media forums, blogs etc. The acronym approach allows a consistent and systematic approach to the potentially massive work up, and is modified regularly, especially if individual investigations are eventually deemed non-contributory, such as the thrombophilias. While the ESHRE guidelines no longer suggest investigating fasting glucose and insulin levels, our findings of a significantly increased risk of gestational diabetes in patients with hyperinsulinism has encouraged us to continue these investigations. We strongly suggest to patients that they modify their lifestyle before embarking on their next pregnancy, if possible, which may not be directly linked to miscarriages, but which may have a profound impact on the morbidity of both mother and fetus. Recurrent miscarriages are difficult to study in a research setting, especially as they are common but heterogeneous. While there has been a vast improvement in the last three years with dedicated 'World Recurrent Miscarriage Conferences', they remain in the 'too hard box' for large level-one evidence randomized controlled trials. However, we believe that consensus reached by a large number of clinicians is of use in the interim, until such trials can be undertaken.

The ASK TEAMS approach has systematised the work up for RM patients and made it consistent between patients. It facilitates appropriate and timely interventions and

counselling for these women and has resulted in more than acceptable live birth rates.

Acknowledgements:

We thank the patients for participating in this study. We thank Fertility and Sterility (Elsevier) for permission to use AMH data from a healthy cohort (Shebl et al. 2011).

References:

1) Christiansen OB. 2016. Foreword in: Recurrent Pregnancy Loss. Evidence-Based Evaluation, Diagnosis and Treatment Bashiri A, Harlev A, Argarwal A editors. Springer. DOI 10.1007/978-3-319-27452-2.
2) Practice committee for the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss; A committee opinion. *Fert Steril* 2012;99:63.
3) Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middledorp S, et al. ESHRE guideline: recurrent pregnancy loss. Human Reprod open 2018.
4) Petrozza JC. Early Pregnancy Loss. eMedicine. 2006. http://wwwemedicinecom/med/topic3241htm
5) Petrozza JC. Recurrent early pregnancy loss. January 2011. http://www.emedicine.medscape.com.
6) Nybo Andersen AN, Wohlfahrt J, Christens P, Olsen J Melbye M.Maternal age and fetal loss:population based register linkage study. BMJ 2000;320:1708-12.
7) Royal College of Obstetricians and Gynaecologists. The investigation and treatment of couples with recurrent first-trimester and second trimester miscarriage. In: Green-top guidelines No17. London: Royal College of Obstetricians and Gynaecologists; 2011.

8) Shebl O, Ebner T, Sir A, Schreier-Lechner E, Mayer RB, Tews G, et al. Age-related distribution of basal serum AMH level in women of reproductive age and a presumably healthy cohort. Fertility and sterility. 2011;95(2):832-4.

9) Davenport WB, Kutteh WH. Inherited Thrombophilias and Adverse Pregnancy Outcomes. A Review of Screening Patterns and Recommendations. Obstet Gynecol Clin N Amer. 2014;(41)133-144.

10) Lockwood C, Wendel G, Silverman N. ACOG Practice Bulletin 138. Inherited Thrombophilias in Pregnancy. Obstet Gynecol 2013:122(3);706-16.

11) Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J, Coomarasamy A. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Human Reproduction, Volume 27, Issue 10, 1 October 2012, Pages 2908–2917, https://doi.org/10.1093/humrep/des261

12) Egerup P, Kolte AM, Larsen EC, Krog M, Nielsen HS, Christiansen OB. Recurrent pregnancy loss: what is the impact of consecutive versus non-consecutive losses? *Hum Reprod* 2016;31: 2428-2434.