# The Effect of Dietary Folic Acid Supplementation on Female Germ Cell Aneuploidy

By

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# Abstract

Aneuploidy refers to cells with an abnormal number of chromosomes. In female germ cells, aneuploidy originates primarily during the first meiotic division of oogenesis as a result of chromosome segregation errors. Aneuploidy can lead to pregnancy loss and genetic defects in newborns. The incidence of aneuploidy in oocytes increases with advanced maternal age. There is evidence suggesting that dietary intervention can mitigate the effect of maternal age on oocyte aneuploidy. Folic acid, an essential B vitamin, is required for DNA synthesis, chromosome stability, methylation reactions, and proper gene expression; these processes are fundamental for female reproductive physiology. The major hypothesis is that folic acid supplementation will reduce the incidence of oocyte aneuploidy associated with advanced maternal aging. Here, I examined the effect of dietary folic acid supplementation on maternal age-induced oocyte aneuploidy using Bub1-heterozygous mice, a transgenic mouse model of aging-associated oocyte aneuploidy. Results showed that old female mice (24 weeks on a diet) fed a folic acid supplemented diet (8 mg/kg) had significantly more (2-fold) normal oocytes compared to the control group (2 mg/kg). Second, I examined the effect of dietary folic acid deficiency, adequacy and supplementation on colchicine-induced oocyte aneuploidy, as a model for studying age-induced oocyte aneuploidy. Results showed that colchicine treatment arrested a high proportion of oocytes (68%) in meiosis I leaving only 32% of meiosis II oocytes for analysis. No differences among the diet groups were observed. These preliminary results indicate that a larger sample size of mice would be required to determine whether diet has an effect on colchicne-induced oocyte aneuploidy and/or the use of a lower colchcine dose or mouse strain may prove more efficient. My data suggest that folic acid supplementation might mitigate the effect of aging on oocyte aneuploidy and that folate may play a causal role in the development of oocyte aneuploidies. These

data, if replicated, suggest that women, especially older women, may benefit from consuming a folic acid supplement in the pre-conception period to avoid aneuploidy-affected pregnancies such as miscarriage and trisomy syndromes.

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# Thesis Overview

This thesis is organized by chapters according to the objectives of the project. Chapter 1 is written as a review and includes a detailed overview of the potential molecular mechanisms associated with increased chromosome segregation errors during meiosis and oocyte aneuploidy with advanced maternal age.

Chapter 2 discuss the association between folic acid status and aneuploidy in which I reviewed folate metabolism and the relationship of FA and folate status with human health, including female fertility and aneuploidy.

Chapter 3 addresses the first experimental objective of the project which is examining the role of folic acid supplementation on mitigating oocyte aneuploidy associated with maternal age using Bub1 transgenic mice. This chapter has its own introduction, materials and methods, results and discussion.

Chapter 4 addresses the second objective which is examining the effect of dietary folic acid on chemically-induced oocyte aneuploidy. This chapter has its own introduction, materials and methods, results and discussion.

Chapter 5, general discussion, consists of a summary of the findings, implications for female reproductive health, future directions and conclusions.

# Statement of Contribution

**Chapter 1: Mechanisms of Oocyte Aneuploidy Associated with Maternal Age**

- **Writing of Chapter:** Myy Mikwar

**Chapter 2: Association between Folic Acid and Oocyte Aneuploidy**

- **Writing of Chapter:** Myy Mikwar
- **Chapter 3: Investigating the Effect of Dietary Folic Acid Supplementation on Oocyte Aneuploidy Associated with Advanced Maternal Age in Bub1 Heterozygous Female Mice**
- **Study Design:** Dr. Amanda MacFarlane & Dr. Francesco Marchetti
- **Sample Collection:** Myy Mikwar, Nathalie Behan, Fernando Matias
- **Breeding and Colony Maintenance:** Cina Aghazadeh, Michelle Lalande, Myy Mikwar
- **Genotyping:** Myy Mikwar & Nathalie Behan
- **Cytogenetic Analysis:** Myy Mikwar
- **Plasma & RBC Folate:** Myy Mikwar & Raisa Rahman
- **Homocysteine Analysis:** Penny Jee
- **Hormone Analysis:** Dominique Patry
- **Statistical Analysis:** Myy Mikwar
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**Chapter 4: Investigating the Effect of Dietary Folic Acid Intake on Chemically Induced Aneuploidy in Wild Type Female Mice**

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**Chapter 5: General Discussion**

- **Writing of Discussion:** Myy Mikwar

# Chapter 1: Mechanisms of Oocyte Aneuploidy Associated with Maternal Age

**Mechanisms of Oocyte Aneuploidy Associated with Maternal Age**

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# Chapter 1
# Mechanisms of Oocyte Aneuploidy Associated with Maternal Age


## Abstract

It is known that maternal age is associated with a rapid decline in the production of healthy and high-quality oocytes that result in reduced fertility in women older than 35 years of age. In particular, chromosome segregation errors during meiotic divisions are increasingly common and lead to the production of oocytes with an incorrect number of chromosomes, a condition known as aneuploidy. When an aneuploid oocyte is fertilized by a sperm it gives rise to an aneuploid embryo that, except in rare situations, will result in a spontaneous abortion. As females advance in age, they are at higher risk of infertility, miscarriage, or having a pregnancy affected by congenital birth defects such as Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), and Turner syndrome (monosomy X). Here, we review the potential molecular mechanisms associated with increased chromosome segregation errors during meiosis as a function of maternal aging. Our review shows that multiple exogenous and endogenous factors contribute to the age-related increase in oocyte aneuploidy. Specifically, the weight of evidence indicates that recombination failure, cohesin deterioration, spindle assembly checkpoint (SAC) failure, and mitochondrial dysfunction are the leading causes of oocyte aneuploidy associated with maternal aging.

## 1.1 Introduction

### 1.1.1 Significance of Aneuploidy

Aneuploidy is the presence of an incorrect number of chromosomes in a cell. Chromosome segregation errors during either mitosis or meiosis lead to aneuploid cells containing a number of chromosomes that differs from the exact haploid number (Tsutsumi et al., 2014). Aneuploidy in germ cells is a major cause of reproductive complications in humans. It is one of the most common causes of infertility, pregnancy failure, and serious genetic disorders in the offspring. In humans, aneuploidy is present in approximately 0.3% of newborns (with the most common abnormalities being trisomy 21 and sex chromosome trisomies), 30-60% of embryos, 30-70% of oocytes and 35% of spontaneous abortions (Hassold & Hunt, 2001; Nagaoka et al., 2012). Studies in humans showed that most aneuploid embryos survive until the blastocyst stage and are then lost during implantation (Fragouli et al., 2013). Most monosomic embryos undergo embryonic death and are spontaneously aborted in the early stages of development due to severe developmental defects. Therefore, most studies on aneuploidy have focused on trisomic conditions, specifically those that are compatible with life, such as trisomy 13, 18, and 21, and sex chromosome aneuploidies (Hassold et al., 2007).

Trisomy 21, known commonly as Down syndrome, is one of the few viable autosomal aneuploidies with fetuses able to survive to term, although as many as 80% of Down syndrome embryos do not make it to term (Hassold et al., 2007). The association between advanced maternal age and the incidence of trisomy 21 was documented in the early 1930s (Penrose, 2009). The risk of a trisomy 21 birth rises from about 3% for younger women in their 20s to 30% in older women in their 40s (Hassold & Hunt, 2009). A case-control study on trisomy 21 in the Atlanta area, Georgia, included 1215 families with an infant with trisomy 21 (cases) and 1375 families with an infant without trisomy 21 (controls) reported important observations: 1) the vast majority of

trisomy 21 cases were of maternal origin; and, 2) the chromosome segregation error occurred mostly during meiosis I (MI). The vast majority of trisomy 21 cases were of maternal origin and derived from an error at MI.

These findings are not unique to trisomy 21 as most aneuploidies, independently of the chromosome involved, are due to meiotic errors in the female germline. An analysis of nearly 1,200 trisomic pregnancies indicated that the extra chromosome originated during maternal meiosis in ~84% of cases, while paternal meiosis and post-zygotic errors contributed to ~11% and ~5% of cases, respectively (Hassold & Hunt, 2001). Furthermore, the same analysis indicated that that majority of meiotic errors in female germ cells occur during the first meiotic division. For example, in the case of trisomy 16, which is not compatible with life, 100% of aneuploidies are due to MI errors. Similarly, about 65% of trisomy 21, 76% of trisomy 15, and 94 % of trisomy 22 cases are due to MI errors. However, ~60% of trisomy 18 cases are associated with errors during meiosis II (MII) (Hassold & Hunt, 2001).

### 1.1.2 Meiotic Divisions and Oogenesis

Meiosis is essential for sexual reproduction and producing mature gametes (sperm and oocytes). Haploid gametes are generated through two specific cell divisions known as MI and MII with no intermediate S-phase. In female mammals, MI is initiated with a prophase stage in the fetal ovary after mitotic divisions of the primordial germ cells. A series of events happen during prophase I, including homologous chromosome pairing, synapsis and crossing over (recombination) to exchange genetic material between maternal and paternal pairs (El Yakoubi & Wassmann, 2017). Crossover generates a physical link between the homologous chromosomes, called chiasmata, that secures the homologous pair and maintains their appropriate orientation to the opposite poles of the spindle during the metaphase stage (Duro & Marston, 2015). At birth, oocytes arrest at diplotene or dictyotene stage of prophase I (germinal vesicle arrest) and remain

arrested for decades until ovulation (Clift & Schuh, 2013). During this meiotic arrest, oocytes are enclosed by a single layer of flat somatic cells called follicular cells and this structure is known as the primordial follicle. Follicular cells transfer nutrients to the oocyte via gap junctions; as a result, the oocyte grows and its volume increases from 15-20 μm to 80-100 μm in diameter depending on the species, as it develops into the mature egg (Buccione et al., 1990).

In every menstrual cycle in a sexually mature female, a surge of luteinizing hormone is released from the anterior pituitary gland, which causes oocytes to resume MI (Buccione et al., 1990). At the cellular level, the germinal vesicle breaks down, the spindle assembles, chromosomes align at the equator of the spindle at metaphase I, and then the oocyte extrudes half of the homologous chromosomes into the first polar body. At the molecular level, the meiosis-specific cohesion subunit Rec8 residing on chromosome arms is cleaved by separase before anaphase I; however, centromere cohesion is protected from cleavage by shugoshin protein (Sgo) (Watanabe, 2012). Together, this allows homologous chromosomes to segregate to opposite poles, while sister chromatids migrate to the same pole.

After segregation of homologous chromosomes, oocytes are ovulated and enter MII where they remain arrested at metaphase II until fertilization. Upon fertilization, oocytes resume MII. At the cellular level, the second meiotic spindle assembles, sister chromatids are bi-oriented and attach to microtubules from opposite spindle poles, and the second polar body is extruded with half of the sister chromatids. At the molecular level, the cohesion subunit Rec8 is cleaved from the centromeric region of sister chromatids allowing their migration to the same pole (Marston & Amon, 2004; Watanabe, 2012). Chromosomes from the sperm and oocyte form separate pronuclei in the zygote and remain physically and spatially separated until the first mitotic division of the embryo (Clift & Schuh, 2013).

Different meiotic chromosome segregation pathways are suggested to contribute to the genesis of oocyte aneuploidy. Nondisjunction occurs when homologus chromosomes or sister chromatids fail to segregate during MI or MII, respectively, leading to gain or loss of chromosomes (Hassold et al., 2007). Alternatively, several studies reported that aneuploidy induced during MI results from premature sister chromatid separation (PSCS) and random segregation of single chromatids (Angell, 1997; Pellestor et al., 2003). Analyses of polar bodies suggest that meiotic segregation errors caused by PSCS are more frequent than those caused by NDJ (Gabriel et al., 2011; Handyside et al., 2012). It should be noted that PSCS does not always lead to aneuploidy and some oocytes will still have the right number of chromosomes at the end of MII. Those oocytes are thought to have completed meiosis by reverse segregation as suggested by the analysis of polar body-oocyte and polar body-embryo trios (Ottolini et al., 2015). In cases of reverse segregation, sister chromatids separate at MI instead of MII, resulting in a euploid egg with one sister chromatid from each homologus chromosome (having different parental origin) instead of both chromatids from a single homologous chromosome (Webster & Schuh, 2017).

### 1.1.3 Maternal Age Effect on Oocyte Aneuploidy

Aging affects female fertility resulting in reduced oocyte quality and implantation rates, and increased spontaneous abortions (Ge et al., 2015). The occurrence of fetal miscarriage gradually increases from 5.3% in women aged ≤ 30 years to 7.6% in women aged 31-34 years, 12.8% in women aged 35-39 years and 22.2% in women aged ≥ 40 years (Spandorfer et al., 2004). Also, the incidence of pregnancies affected by aneuploidy is lower in woman aged < 40 years (65%) compared with women aged > 40 years (82%) (Spandorfer et al., 2004). Incidence of trisomy cases occur at a low frequency ~2% among women under the age of 25 years after which the frequency increases to about 35% at ages > 40 years (Hassold & Hunt, 2001). Maternal age is

therefore a significant inducer of female germ cell aneuploidy but the etiology is not well understood (Nagaoka et al., 2012).

Women with advanced maternal age (≥ 35 years) have an increased risk of chromosome segregation errors during meiosis. Errors that affect chromosome segregation can occur at any stage during oocyte development: (1) in the fetal ovary during mitotic proliferation of primordial germ cells to generate oogonia; (2) during meiotic divisions of the oocyte; and (3) during the mitotic divisions of the early embryo following fertilization (Hassold & Hunt, 2009; Jones & Lane, 2013). Within these stages of oocyte development, three processes may be particularly vulnerable: (1) meiotic recombination and synapsis in the fetal ovary; (2) follicle formation during the second trimester of fetal development; and (3) oocyte growth in the adult ovary, including completion of MI and metaphase II oocyte arrest (Hunt & Hassold, 2008).

Several mechanisms have been proposed to explain the higher incidence of oocyte aneuploidy in older females including: (1) meiotic recombination failure; (2) deterioration of chromosome cohesion; (3) defects of spindle assembly checkpoint mechanism; and (4) mitochondrial dysfunction. In the next few sections, we will discuss the evidence that supports the involvement of these mechanisms in the maternal age effect.

## 1.2 Molecular Mechanisms of Oocyte Aneuploidy Associated with Maternal Aging

### 1.2.1 Meiotic recombination failure

Meiotic recombination is the exchange of genetic material between homologous chromosomes during MI prophase, initiated by programmed DNA double strand breaks (Herbert et al., 2015). During crossover, a physical linkage known as chiasmata maintains the connection between the homologous chromosomes before segregation at anaphase of MI. Studies of human trisomies suggest that the number and the location of recombination events affect chromosome segregation and can lead to aneuploidy. A reduction in recombination events has been observed

in different types of maternally derived trisomies including trisomy 15, 16, 18, 21 and sex chromosome (Bugge et al., 1998; Hassold et al., 1995; Robinson et al., 1998; Thomas et al., 2001). Studies of recombination positions show that different recombination patterns are associated with NDJ. For example, trisomy 21 is associated with several patterns of homologous chromosome recombination established in the fetal ovary that were associated with increased risk for NDJ (Oliver et al., 2012). These include the absence of any exchange, or exchanges occurring near a centromere or telomere. Failure to crossover between homologous chromosomes (no chiasmata formed) leads to an increased risk of an MI error because unconnected homologous chromosomes cannot achieve a stable bipolar orientation on the MI spindle. A single exchange that forms close to the telomere (distal crossover) can result in reduced amounts of cohesion and less efficient promotion of bi-orientation, increasing the risk of a MI error. Crossover sites formed near the centromere (proximal crossover) may compromise sister chromatid cohesion or prevent the removal of cohesion increasing the risk of a MII error and NDJ (Herbert et al., 2015; Hunter, 2015; Sherman et al., 2006).

Studies in mice support the important role of meiotic recombination in assuring normal chromosome segregation. Mice deficient for the synaptonemal complex protein SCP3 had reduced recombination, and resulted in a high incidence of oocyte aneuploidy (Yuan et al., 2002). Inter-specific crossing between two inbred mouse strains (B6 x SPRET) with an estimated sequence divergence of ~1% was used to develop a model of reduced recombination. Aneuploidy incidence in oocytes increased from less than 1% in control mice to 10% in 4 week-old and 20% in 8-11 week old F1 mice (Koehler et al., 2006). From these human and mouse observations, we can infer that recombination frequency or position can affect the recombination process and lead to aneuploidy.

Advanced maternal age has been linked to recombination failure in several human trisomies including trisomy 16, 18, and 21 (Hassold et al., 1995 ; Fisher et al., 1995; Lamb et al., 2005; Oliver et al., 2008). Analysis of 400 trisomy 21 cases of maternal origin showed that approximately 50% of maternal MI errors in both young and old women were due to failed recombination (no crossover). In addition, an exchange near the telomere is a major contributor to NDJ among young women (younger than 29 years), whereas it is less of a contributor among older women (35 years or older). However, an exchange near the centromere is more common in older women. The susceptible recombination (near the telomere or centromere) occurs more frequently during maternal aging because older oocytes have a limited ability to resolve recombination errors (Lamb et al., 2005; Sherman et al., 2006). Thus, reduced recombination and altered recombination patterns are risk factors for NDJ that increase with advanced maternal age.

### 2.2.2 Deterioration of Chromosome Cohesion with Age

Sister chromatid cohesion is a process that requires a ring-like structure generated by the cohesin complex; it is established during DNA replication in both mitosis and meiosis to hold sister chromatids together and promote the recombination procedure in meiosis to achieve faithful chromosome segregation (Watanabe, 2012). The cohesion complex comprises four subunits: a core V-shaped heterodimer of two Structural Maintenance of Chromosome (SMC) family proteins subunits; a kleisin subunit that stabilizes the ring-like structure; and Stromalin Antigen (SA) or STAG subunit that associates with kleisin (MacLennan et al., 2015). In mitosis, cells have two SMC subunits termed Smc1 (Smc1α) and Smc3; the kleisin subunit is Rad21, also known as Scc1 or Med1 in other species, and the SA or STAG subunit is either SA1 or SA2 (also known as Scc3). In meiotic cells, additional SMC and SA subunits named Smc1β and STAG3, and additional kleisin subunits named Rec8 and RAD21L are expressed (Jessberger, 2012).

Sister chromatid cohesion is required for two reasons: (1) to maintain the proper alignment of sister chromatids on the spindle during metaphase and physical attachment of homologous chromosomes by chiasmata; and (2) to generate tension around the centromere during the bipolar attachment of the chromosomes (Vogt et al., 2008). In meiosis, the loss of sister chromatid cohesion from chromosome arms at anaphase I and from centromeres at anaphase II is required to achieve faithful chromosome segregation. At the onset of anaphase, the APC system promotes the degradation of securin, which activates the enzyme separase to cleave the cohesion subunit Rec8 along the chromosome arms. Then, chiasmata are resolved and the cohesion ring opens allowing homologous chromosomes to separate. The chromosomes segregate to opposite poles by the pulling forces generated by the spindle to allow for appropriate chromosome separation during MI (Kurahashi et al., 2012). However, centromeric cohesion remains to avoid PSCS and promote sufficient chromosome attachment at metaphase II (Vogt et al., 2008). Two main subunits contribute to protection of the centromeric cohesion during MI: (1) the meiosis-specific subunit Spo13 has a crucial role to prevent the degradation of Rec8; and (2) the shugoshin, kinetochore proteins, Sgo1 and Sgo2, that protect centromeric Rec8 (Watanabe, 2004). The loss of arm and centromeric cohesion may promote NDJ and PSCS, respectively (Kurahashi et al., 2012). Indeed, PSCS is the major cause of MI and II errors for all chromosomes (most frequently aneuploidies for chromosomes 16, 22, 21, 19, 11, and 15) (Handyside et al., 2012).

Mice deficient in Smc1β or Rec8 cohesion subunits provide evidence that cohesion is important to avoid aneuploidy in mammalian oocytes. Oocytes that have a reduced level of *Smc1β* exhibit a high incidence of univalent and single chromatids associated with chiasmata loss (Hodges et al., 2005). Moreover, Rec8 deficiency induces germ cell failure, disruption of meiosis at

prophase I, loss of synaptic association between homologous chromosomes, PSCS, a high incidence of embryonic lethality and sterility (Bannister et al., 2004; Xu et al., 2005).

In different mammalian species, two major defects that affect chromosomes structure are associated with advanced maternal age: (1) reduction in cohesion subunit levels; (2) and an increase in the inter-kinetochore (iKT) distance between sister chromatids (associated with the incorrect attachment of the kinetochore to the spindle fiber). Table 1.1 shows a summary of the effect of maternal aging on cohesion function.

Several mammalian species demonstrate lower levels of cohesion subunits with maternal aging. Levels of the meiotic cohesion subunits REC8 and SMC1β were lower in older women (40 years old) compared to younger women (20 years old) (Tsutsumi et al., 2014). Similarly in mice, oocytes from older females had lower levels of Rec8 and Smc1β compared to younger females (Chiang et al., 2010; Chiang et al., 2011; Lister et al., 2010; Liu & Keefe, 2008; Tsutsumi et al., 2014). However, findings are not always consistent; for example Smc1 β and Rec8 protein levels were higher in activated oocytes of old mice in comparison to younger mice (Shimoi et al., 2019). Different results have been reported in human and mouse showing that the transcriptome level of *SMC1 β* did not differ between young and old female (Fu et al., 2014; Garcia-Cruz et al., 2010). Also, Sgo2 was reduced in aged mice, which was associated with PSCS and lengthening of the iKT distance (Lister et al., 2010; Yun et al., 2014). These findings suggest that age-associated depletion of meiotic cohesin subunits leads to cohesion loss and causes precocious chromosome separation and aneuploidy.

The iKT distance between sister chromatids is another factor affected by maternal aging. Observations in human oocytes reported a difference in iKT distance between young and old women up to 25.5% (Duncan et al., 2012; Patel et al., 2015). In addition, the fraction of split

kinetochores was increased with advanced maternal age. Around 5% of sister kinetochore pairs in young women are separated by gaps or completely separated into single univalent chromosomes and this proportion increased to around 30% in older women (Zielinska et al., 2015). Mouse studies have shown results consistent with those observed in humans. The difference in iKT distance between young and old female mice can increase between 25% and 54% (Chiang et al., 2010; Merriman et al., 2012; Yun et al., 2014). An increase in iKT distances, which means that sister kinetochores are not fused together and do not act as a single unit, indicates that changes in kinetochore structure are associated with advanced maternal age.

Loss of sister chromatid cohesion and larger distances between sister kinetochores changes the geometry of kinetochore-microtubule attachment, which leads to incorrect attachment, missegregation and aneuploidy. The number of merotelic kinetochore-microtubule attachments, when a kinetochore attaches to microtubules originating from both spindle poles, increases with maternal aging. The proportion of merotelical kinetochore-microtubule interactions increase significantly from 7% in oocytes of younger women to 21% in oocytes of older women (Zielinska et al., 2015). They increase similarly in mouse oocytes from 4% in younger mice to 10% in older mice (Shomper et al., 2014).

In summary, cohesion deterioration is a major factor that contributes to oocyte aneuploidy related to maternal aging. Studies in human and mouse oocytes show that cohesion subunits are established in the fetal ovary during DNA replication and the level of cohesion subunits is degraded gradually and eventually falls below the threshold required to hold homologous chromosomes together and prevent segregation errors. Evidence from mouse studies suggests that Rec8-containing cohesion complexes are present during DNA replication during the early stages of meiosis and there is no replenishment during the long dictyate arrest (months in mouse to

decades in humans) and oocyte growth until after fertilization (Burkhardt et al., 2016). In addition, the level of cohesion is lower in both older women and mice (Chiang et al., 2010; Chiang et al., 2011; Lister et al., 2010; Liu & Keefe, 2008; Tsutsumi et al., 2014). Moreover, the distance between sister kinetochores in oocytes of older female increases (Chiang et al., 2010; Duncan et al., 2012; Merriman et al., 2012; Patel et al., 2015; Yun et al., 2014; Zielinska et al., 2015). These factors alone or together may diminish cohesion strength leading to increased meiotic errors, chromosome missegregation and aneuploidy (Ge et al., 2015; Nagaoka et al., 2012) with advancing maternal age.

**Table 1. 1.** Effect of Maternal Age on Cohesin Function

| End point | Species (strain) | Type of study | Yong female age (n=number of oocytes analysed) | Old female age (n=number of oocytes analysed) | Methodology | Effect of maternal age* | P-Value | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Cohesin Level** | *Human* | *in vivo* | ≤29 year (n=127 MI) | ≥40 year (n=105 MI) | Immunofluorescence | 24% lower REC8 protein level | $p<0.01$ | (Tsutsumi et al., 2014) |
| | | | ≤29 year (n=138 MI) | ≥40 year (n=96 MI) | | 38% lower SMC1β protein level | $p<0.01$ | |
| | *Human* | *in vivo* | 19-23 year (n=22 GV) | > 35 year (n=17 GV) | RT-PCR | No change in *SMC1β* transcript level | NS | (Garcia-Cruz et al., 2010) |
| | *Mouse (SAM)(AKR/J)* | *in vivo* | 2-3 months (n=23 MI) | 10-14 months (n=22 MI) | Immunofluorescence | > 80% lower Rec8 protein level in SAM mice | NI | (Liu & Keefe, 2008) |
| | | | | | | > 70% lower Smc1β protein level in SAM mice | NI | |
| | *Mouse Hybrid BCC3F1(C57BL/6 x C3H)* | | 2-3 months (n=20) | 10-14 months (n=20) | | Lower Rec8 & Smc1β protein level in hybrid F1 mice | NI | |
| | *Mouse (B6D2F1/J)* | *in vivo* | 3 months (n=21 MI) | 17 months (n=3 MI) | Immunocytochemistry | > 90% lower Rec8 protein level | NI | (Chiang et al., 2010) |
| | *Mouse (C57BL/6/Icrfat)* | *in vivo* | 2 months (n=8) | 14 months (n=7) | Immunofluorescence | 13% lower Rec8 protein level | $p<0.001$ | (Lister et al., 2010) |
| | *Mouse (B6D2F1/J)* | *in vivo* | 6-14 weeks (n=17) | 12 months (n=13) | Immunocytochemistry | 50% lower Rec8 protein level | $P=0.007$ | (Chiang et al., 2011) |
| | *Mouse (Swiss CD1)* | *in vivo* | 6 weeks (n= 200 GV) | 9 months (n= 200 GV) | RT-PCR | No change in *Smc1β, Smc3, Stag3* transcript level | NS | (Fu et al., 2014) |
| | *Mouse (C57BL/6NCR)* | *in vivo* | 2 months (n=95 MI) | 10 months (n=83 MI) | Immunofluorescence | 50% lower Rec8 protein level | $p<0.01$ | (Tsutsumi et al., 2014) |
| | | | 2 months (n=82 MI) | 10 months (n=67 MI) | | 3% lower Smc1β protein level | $p<0.01$ | |
| | *Mouse (ICR)* | *in vivo* | 0 h of oocyte collection | 12 h of oocyte collection | Western blot | 3.6-fold higher Smc1β protein level | $P<0.05$ | (Shimoi et al., 2019) |
| | | | | | | ND in Smc3 protein level | NS | |
| | | | | | | 2.2-fold higher Rec8 protein level | $P<0.05$ | |
| **Distance Between Sister Kinetochores** | *Human* | *in vivo* | 16.4 year (n=18) | 37.3 year (n=18) | In situ chromosome spreading | 25.5% increase in iKT distance | $p<0.001$ | (Duncan et al., 2012) |
| | *Human* | *in vivo* | < 30 year (NI) | > 35 year (NI) | Immunofluorescence | 25% increase in iKT distance | $p<0.0001$ | (Zielinska et al., 2015) |
| | *Human* | *in vivo* | < 33 year (n= 214 kinetochore pairs from 7 oocytes) | > 38 year (n= 216 kinetochore pairs from 7 oocytes) | Immunofluorescence | ~18% increase in iKT distance | $P<0.0001$ | (Patel et al., 2015) |
| | *Mouse (C57BL6/Icrfat)* | *in vivo* | 6-14 weeks (n=480 kinetochore pairs from 24 oocytes) | 16-19 weeks (n=340 kinetochore pairs from 17 oocytes) | Immunocytochemistry | ~34% increase in iKT distance | $p<0.05$ | (Chiang et al., 2010) |

| End point | Species (strain) | Type of study | Yong female age (n=number of oocytes analysed) | Old female age (n=number of oocytes analysed) | Methodology | Effect of maternal age* | P-Value | Ref. |
|---|---|---|---|---|---|---|---|---|
| | *Mouse (Swiss CD1)* | *in vivo* | 1 month (n= 303 kinetochore pairs) | 15 months (n= 345 kinetochore pairs) | Immunofluorescence | 54% increase in iKT distance | $p<0.01$ | (Merriman et al., 2012) |
| | *Mouse (Swiss CD1)* | *in vivo* | 1 month (n = 597 kinetochore pairs) | >12 months (n= 257 kinetochore pairs) | Immunofluorescence | 25% increase in iKT distance | $p<0.0001$ | (Yun et al., 2014) |

**Footnotes:** (NI) Not Indicated; (NS) Not Significant; (iKT) inter-kinetochore; (SAM) Senescence-accelerated Mice; (REC8) Recombinant8; (SMC1 β) Structural Maintenance of Chromosomes 1 β; (STAG3) Stromal Antigen 3.
*Values presented in this table are calculated relative to young females

### 1.2.3 Spindle Assembly Checkpoint (SAC) Dysfunction

#### 1.2.3.1 Overview of SAC

The spindle assembly checkpoint (SAC) is a cell cycle surveillance mechanism that maintains genome stability by controlling both the time and order of cell cycle events in both mitotic and meiotic cells (Musacchio, 2015). SAC monitors proper kinetochore attachment to the spindle and prevents chromosome missegregation by delaying anaphase until accurate attachment to the spindle apparatus occurs (Duro & Marston, 2015). The SAC senses unattached kinetochores on spindle microtubules and prevents the cell from progressing to anaphase until full attachment is achieved. Unattached kinetochores activate the SAC mechanism, whereas full attachment of all kinetochores silences SAC, permitting anaphase to proceed (Mailhes, 2008).

The SAC is composed of at least 14 evolutionarily conserved proteins observed in almost all eukaryotes. They were first identified in yeast and have been characterized in eukaryotic somatic cells (Jones & Lane, 2013). SAC proteins include members of the mitotic–arrest deficient protein family (Mad1, Mad2 and BubR1 or Mad3), members of the budding uninhibited by benzimidazole family (Bub1 and Bub3), and other proteins including monopolar spindle I (Mps1) (Vogt et al., 2008). SAC function is controlled by two factors: the kinetochore–microtubule attachment, which is regulated by factors such as Mad1 and Mad2; and, the kinetochore tension, which is monitored by factors such as Bub1 and BubR1 (Lara-Gonzalez et al., 2012).

The primary target for SAC in both mitosis and MI is the anaphase promoting complex/cyclosome (APC/C). The APC/C is an E3 ubiquitin ligase that targets several proteins for proteolytic degradation and whose activity is dependent on the activator protein cell division $cycle_{20}$ ($Cdc_{20}$). To block the metaphase-anaphase transition in meiosis and mitosis, SAC prevents APC/C-$Cdc_{20}$ from targeting two key proteins, securin and cyclin B. Securin is an inhibitor of the protease separase that is required to cleave the cohesion complex that holds chromosomes together.

In MI, separase is required in anaphase to cleave the meiotic cohesion complex that holds the homologous chromosomes together. The cohesion located near sister centromeres is protected to keep sister chromatids together. In contrast, in mitosis and MII, separase cleaves the cohesion subunit between sister chromatids (Touati & Wassmann, 2016; Watanabe, 2005). Cyclin B is the activator of the master metaphase kinase cyclin-dependent kinase 1 (Cdk1). The degradation of cyclin B during anaphase of MI allows for cytokinesis of the cytoplasm in preparation for MII. When sister kinetochores of homologous chromosomes are either co-oriented to the same poles (loss of tension) or not fully attached to spindle microtubules, SAC is activated and inhibits APC/C activity. In this situation, kinetochores accumulate a high concentration of checkpoint signaling proteins leading to the assembly of the APC/C inhibitor, the mitotic checkpoint complex (MCC) (Vogt et al., 2008).

The MCC is a signaling system comprised of Mad2, BubR1, Bub3, and $Cdc_{20}$. The sub-complexes Mad2-$Cdc_{20}$ and BubR1-Bub3 form during the first stage of the MCC inhibitor complex assembly. Mad2 inhibits anaphase by directly binding $Cdc_{20}$ and preventing it from activating the APC/C (Lara-Gonzalez et al., 2012). The other sub-complex, Bubr1-Bub3, exists throughout the cell cycle. The two sub-complexes interact in the presence of unattached kinetochores, causing $Cdc_{20}$ sequestration making unable to activate APC/C, which in turn prevents anaphase by inhibiting cyclin B and securin degradation by the ubiquitin/protesome system (Homer et al., 2009; Musacchio, 2015; Vogt et al., 2008).

SAC signals are turned off when bi-orientation of every chromosome is achieved. The two sister kinetochores must be attached to the microtubules of the spindle poles. Therefore, tension between the sister kinetochores must be generated at the metaphase plate in mitosis and MI. In this case, APC is activated and binds to $Cdc_{20}$ to form $APC^{cdc}{}_{20}$ complex. Degradation of cyclin

B and securin leads to inactivation of Cdk1 and activation of separase and allows anaphase to proceed (Figure 1.1). Consequentially, Mad2, Bub3 and BubR1 are removed from the kinetochores that are stably attached to microtubules (Jones & Lane, 2013).

Other SAC components, such as Bub1, BubR1 and Mps1 are required to generate effective signals to promote the recruitment of SAC proteins to kinetochores (Musacchio & Salmon, 2007). Bub1 is a protein kinase that localizes to the kinetochores of unaligned chromosomes early during prophase in mitosis and meiosis (Sun & Kim, 2012). It is required to inhibit the activity of $Cdc_{20}$ via binding to Mad2 through the phosphorylation of Mad1 leading to disassociation of the Mad1-Mad2 complex and release of free Mad2 which binds and inhibits $Cdc_{20}$. Furthermore, Bub1 plays an important role in the inhibition of APC activity through the activation of BubR1-Bub3-Mad2 complexes in kinetochores in response to spindle damage (Marchetti & Venkatachalam, 2010). In meiosis, Bub1 recruits shugoshin proteins to kinetochores and maintains Rec8 at sister kinetochores until anaphase II onset (Mailhes, 2008). BubR1 is required for stable attachment of homologous chromosomes to the spindle in oocytes (Baker et al., 2013), regulates chromosome alignment (Homer et al., 2009) and the establishment of kinetochore-microtubule attachments in a meiosis-specific manner (Wei et al., 2010). Mps1 is required for meiotic progression and chromosome segregation in mouse oocytes (Hached et al., 2011).

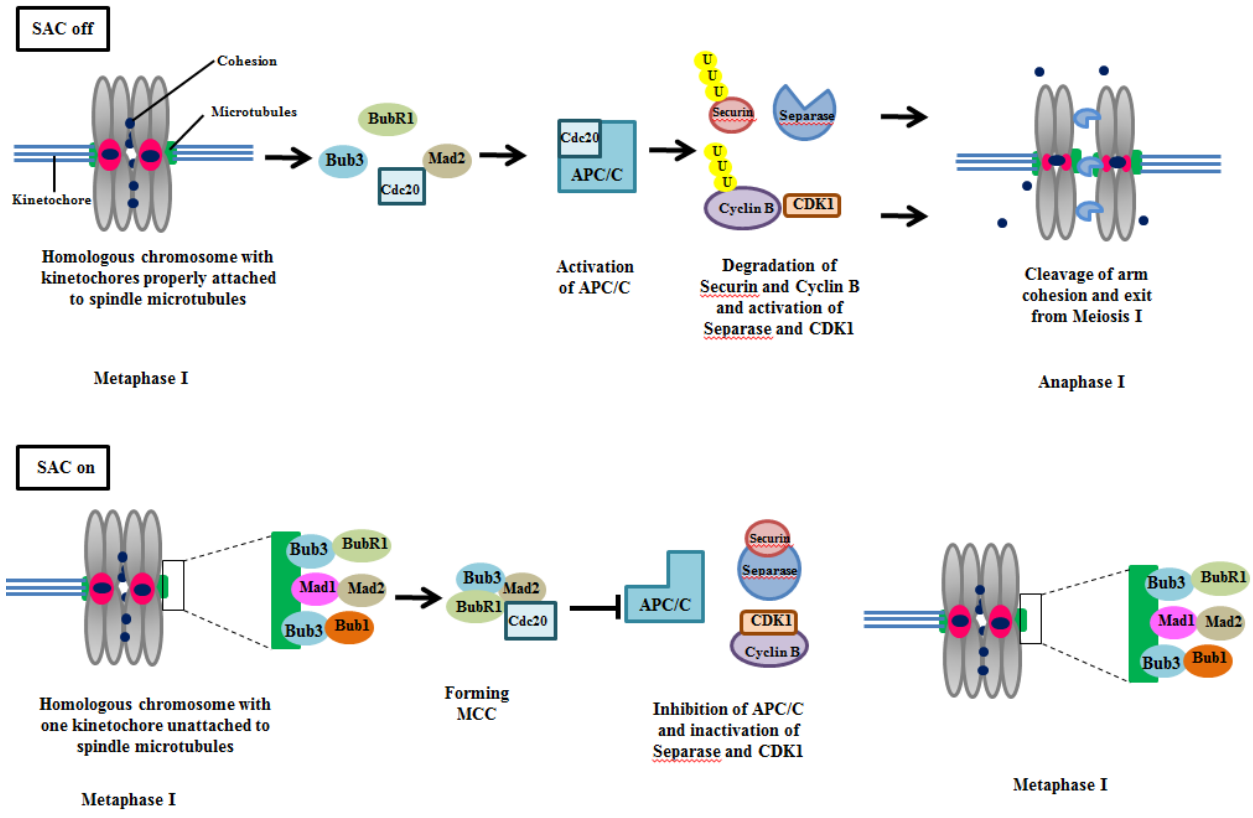



**Figure 1. 1. Signaling pathway of spindle assembly checkpoint (SAC) mechanism.** At anaphase I, correct and full attachment of kinetochores to spindle microtubules lead to disassembly of the mitotic checkpoint complex (MCC) from kinetochores (SAC off). Cdc20 is free to activate the anaphase promoting complex/Cyclosome (APC/C), then Securin and cyclin B are targeted for degradation. Separase is activated and cleaves the cohesion subunit Rec8 from chromosomes arms allowing homologous chromosomes to separate and metaphase I-anaphase I transition. Unattached kinetochores activate SAC (SAC on). SAC proteins localize to unattached kinetochores and form MCC, and sequester Cdc20 preventing it from activation of APC/C. Securin and Cyclin B levels remain high, separase is not activated therefore cohesion is not removed and metaphase I-anapahse I transition is delayed in oocytes.

Despite many similarities, it is thought that SAC operates differently in meiosis than mitosis (Hunt & Hassold, 2002). A single unattached kinetochore in mitosis is efficient to activate SAC and delay anaphase. In contrast, the ability of SAC to detect an unattached kinetochore is limited in meiosis. Evidence shows that the control of the cell cycle by SAC in oocytes is potentially less stringent and could be satisfied when most but not all chromosomes are attached to the bipolar spindle (Nagaoka et al., 2011), and oocytes can progress through meiosis despite misalignment of chromosomes (Touati & Wassmann, 2016). Live cell tracking of oocyte progression to anaphase revealed that human oocytes with no chromosome alignment defect and those with moderate and severe misalignment progressed to anaphase (Zielinska et al., 2015). A study in XO female mice, which have one X chromosome only and thus cannot form a bivalent (a MI structure of homologous chromosomes), demonstrated that SAC is weakened in mammalian oocytes; most oocytes initiated anaphase and completed chromosome segregation despite the misalignment of the X chromosome at the metaphase plate (LeMaire-Adkins et al., 1997). In addition, oocytes lacking MLH1, required for recombination and formation of chiasmata, exhibited multiple univalent chromosomes that escaped SAC detection and initiated anaphase and completed homologous segregation even though not all chromosomes were aligned at metaphase (Nagaoka et al., 2011). Mice deficient in Sycp3, a component of the synaptonemal complex that maintains the homologous chromosomes together, demonstrate the failure of the SAC response in oocytes. The Sycp3 deficient oocytes entered MI with no detected delay to anaphase despite the presence of univalent chromosomes (Kouznetsova et al., 2007). In addition, SAC might not be responsive to small errors in kinetochore-microtubule attachments in oocytes. Multiple studies have established that APC becomes active and anaphase starts even though chromosome alignment and kinetochore attachment is not achieved properly (Gui & Homer, 2012; Kolano et al., 2012;

Lane et al., 2012; Sebestova et al., 2012). Together, these findings indicate that SAC in oocytes is not sensitive enough to detect errors and may ignore the misaligned chromosomes and/or misattached kinetochores, thus initiating anaphase, which leads to segregation errors at meiosis and oocyte aneuploidy.

#### 1.2.3.2 Alteration of the SAC mechanism

SAC dysfunction is a potential contributing factor to oocyte aneuploidy. Various approaches have been used to study the consequences of the loss of core SAC proteins (including: Mad1, Mad2, Bub1, Bub3, BubR1, and Mps1) in oocytes of different species (Table 1.2).

A number of studies established the importance of checkpoint control for meiosis by modifying the expression of core SAC components. Disruption of Mad2 expression using RNAi or morpholino injection led to elimination of SAC control and premature activation of APC/C, acceleration of MI, spindle abnormalities, and increase aneuploidy incidence in mouse oocytes (Homer et al., 2005a; Homer et al., 2005d; Marangos et al., 2015; Wang et al., 2007). Additionally, mutant Mad2 expressed in mouse oocytes leads to abnormal metaphase (Wassmann et al., 2003a; Wassmann et al., 2003b), chromosome missegregation (Dobles et al., 2000), generation of aneuploidy at MI, reduced fertility (Niault et al., 2007), and increased embryonic apoptosis (Dobles et al., 2000).

Similarly, specific depletion of Bub1 in mouse oocytes results in precocious anaphase and polar body extrusion (Yin et al., 2006), strong acceleration of MI and massive chromosome missegregation leading to high levels of PSCS and aneuploidy (McGuinness et al., 2009). Moreover, expression of a dominant negative mutant of the *Bub1* gene in mice resulted in a shortened duration of MI (Tsurumi et al., 2004), high proportion of oocyte aneuploidy and PSCS, and loss of fertility associated with increasing maternal age (Leland et al., 2009).

Inhibition of BubR1 and Bub3 function either by morpholino injection, depletion, or knock out causes a failure of prophase I arrest, reduction in Cdh1 level (Homer et al., 2009; Wei et al., 2010), chromosome misalignment and missegregation, infertility, and birth defects (Baker et al., 2013; Baker et al., 2004; Li et al., 2009; Touati et al., 2015). Finally, accelerated meiosis, chromosome missegregation and increased incidence aneuploidy with loss of Mps1function in mouse oocytes (Hached et al., 2011; Lane & Jones, 2014; Marangos et al., 2015; Touati et al., 2015).

The expression (gene and/or protein) of core SAC components is affected by aging, which is evident in different mammalian species (Table 1.3). MI oocytes from older women showed a decline in the mRNA expression of *MAD2* and *BUB1* (Steuerwald et al., 2001). *In vitro* cultured porcine oocytes exhibited a reduction in the protein content of Mad2 with aging, which was associated with PSCS (Ma et al., 2005). Similarly, postovulatory aging of mouse oocytes showed a time-dependent decrease in *Mad2* mRNA expression. The decline of *Mad2* was associated with a significant increase in PSCS (Steuerwald et al., 2005). Comparison of global patterns of gene expression between oocytes from young (6-12 weeks old) and old (66 weeks old) mice showed that the transcripts of several SAC components, including *Bub1*, decreased with aging and were associated with increases in aneuploid oocytes (Pan et al., 2008). Induction of DNA damage in aged mouse oocytes (> 50 weeks) elicited a 15% and 35% decline in Bub1 and BubR1 accumulation at kinetochores, respectively, with respect to younger oocytes. In addition, those aged oocytes progressed to MI and MII despite the presence of DNA damage (Marangos et al., 2015). In conclusion, the disturbance of the expression and/or function of core SAC proteins in oocytes, as observed in aging, could contribute to the increased incidence of aneuploidy.

**Table 1. 2.** Loss of SAC function in oocytes in different species

| SAC component | Species (strain) | Approach used | Effect observed | Ref. |
|---|---|---|---|---|
| **Bub1** | *Mouse (FVB)* | (Dominant negative mutant) Truncated form of Bub1 contains (1-331 aa), missing the kinase domain | Acceleration of MI<br>Not required for CSF arrest | (Tsurumi et al., 2004) |
| | *Mouse (kunming white mice)* | (Blocking antibody) Bub1 function is depleted by microinjection of anti-Bub1 antibody to oocyte cytoplasm | Misaligned chromosome at metaphase plate<br>Precocious anaphase | (Yin et al., 2006) |
| | *Mouse (C75BL/6 x 129SV/E)* | Hypomorphic allele | No fertility defects | (Jeganathan et al., 2007) |
| | *Mouse (C75BL/6 x 129/SV)* | (Conditional deletion) Fluxed bub1 allele and a Zp3-Cre recombinase transgene | Misaligned chromosome at metaphase plate<br>Acceleration of MI<br>Oocyte aneuploidy<br>PSCS | (McGuinness et al., 2009) |
| | *Mouse (B6C3F1)* | (Heterozygous mutation) Gene-trap leading to generate truncated form of BUB1 contains (1-269 aa),missing kinase domain, has kinetochore binding domain | PSCS; Female-specific germ cell aneuploidy<br>Aneuploidy inherited to zygotes | (Leland et al., 2009) |
| | *Mouse (MF1)* | (Dominant negative mutant) Dominant negative form of Bub1 prepared from the pEFT7MCS-DnBub1 plasmid | SAC inactivation (60% reduction in BubR1 level) | (Marangos et al., 2015) |
| | *Drosophila* | (Null mutants)lK06109 and lK03113 P-element insertions | Miotic abnormality includes: acceleration from MI; Chromosome missegregation; Chromosome fragmentation leads to high level of apoptosis | (Basu et al., 1999) |
| **Bub3** | *Mouse (ICR)* | (RNAi) Bub3 siRNA transfection (Down regulation of *Mad2*) | Misaligned chromosome at metaphase plate<br>60% incidence of abnormal polar body<br>80% of oocyte aneuploidy incidence | (Li et al., 2009) |
| **BubR1** | *Mouse (NI)* | (Hypomorphic allele)Truncated form of BubR1 after 187 aa using Cre-mediated recombination | Misaligned chromosome at metaphase plate<br>Chromosome missegregation<br>PSCS<br>Infertility associated with birth defect | (Baker et al., 2004) |
| | *Mouse (FVB)* | (Dominant negative mutant) Mutated form pfBubR1express (351-700 aa) | Not required for CSF arrest | (Tsurumi et al., 2004) |
| | *Mouse (MF1)* | (Morpholino) Depletion of 80% of endogenousBubR1 by morphilino antisense oligos | MI arrest; Misaligned chromosome at metaphase plate; Chromosome missegregation; Impaired kinetochore-microtubule attachment<br>60% reduction in Cdh1 level | (Homer et al., 2009) |
| | *Mouse (ICR)* | (Morpholino)Deplition of BubR1 by morphilino antisense oligos | Acceleration of MI; Misaligned chromosome at metaphase plate; Disruption of spindle microtubules | (Wei et al., 2010) |
| | *Mouse (C57BL/6/Sv129 )* | (knockout) BubR1 allele is flanked by LoxPsequences26 to generate a conditional knockout | Acceleration of MI<br>Misaligned chromosome at metaphase plate<br>85% of oocyte aneuploidy incidence<br>Loos of spindle stability<br>Not required for CSF arrest | (Touati et al., 2015) |
| | *Mouse (ICR)* | (Acetylation-mimetic mutant) Substitution of lysine (K) with a glutamine (Q) mimics an acetylated amino acid state | Kinetochore-microtubule missattachment<br>Chromosome congressing failure<br>Spindle malformation | (Qiu et al., 2018) |
| | *Drosophila* | (Hypomorphic allele) lK03113 P-element insertions | Early developmental arrest<br>Abnormal syncytial nuclear division<br>Defects in chromosome congression<br>PSCS<br>Irregular chromosome distribution | (Perez-Mongiovi et al., 2005) |
| **Mad1** | *Mouse (Kunming white mice)* | (Blocking antibody) Mad1 function is depleted by microinjection of anti-Mad1 antibody to oocyte cytoplasm | Misaligned chromosome at metaphase plate | (Zhang et al., 2005) |

| SAC component | Species (strain) | Approach used | Effect observed | Ref. |
|---|---|---|---|---|
| **Mad2** | *Hct-116 human colon carcinoma cell line; Mouse (embryonic fibroblasts)* | (Deletion) Replacing the 5kb genomic fragment encoding *Mad2* with PGK-neomycine resistance cassette | PSCS<br>Chromosome missegregation | (Michel et al., 2001) |
| | *Human (HeLa and 293T cells)* | (Dominant negative mutant) Phosphorylation on multiple serine residues of C-terminus in somatic cell | Disruption of interaction with Mad1 and APC/C<br>Interfering with loading the endogenous Mad2 to APC/C<br>Inhibition of the checkpoint response | (Wassmann et al., 2003a) |
| | *Mouse (C75BL/6 x BL/6) (mitotic cells, embryos)* | (Deletion)Replacing the 5kb genomic fragment encoding *Mad2* with PGK-neomycine resistance cassette | Unable to arrest in response to spindle disruption<br>Chromosome missegregation<br>Apoptosis in embryos | (Dobles et al., 2000) |
| | *Mouse (OF1)* | Dominant negative mutant (injection of dn Flag-Mad2 to oocyte) Mutant protein carries 3 serine to aspartic acid substitution | 85% of oocytes have abnormal metaphase | (Wassmann et al., 2003b) |
| | *Mouse (FVB)* | (Dominant negative mutant) Mutated form pf Mad2 lacks 10 aa at the C-terminus | Not required for CSF arrest | (Tsurumi et al., 2004) |
| | *Mouse (MF1)* | (Morphilino) Depletion of Mad2 by morphilino antisense | Acceleration of MI<br>32% incidence of oocyte aneuploidy<br>Destabilisation of securin and APC/C | (Homer et al., 2005b, 2005c) |
| | *Mouse (C75BL/6 x 129SV/E)* | (Heterozygous mutation) Deletion of one allele of *Mad2* by replacing the 5 kb genome encoding *Mad2* with a PGK-neomycin resistance cassette | Acceleration of MI<br>Elevated level of oocyte aneuploidy at MI<br>Decline in female fertility (42.4% loss of litter size) | (Niault et al., 2007) |
| | *Mouse (Kunming white mice)* | (RNAi) Mad2 siRNA transfection (Down regulation of *Mad2*) | Acceleration of MI<br>Spindle abnormality | (Wang et al., 2007) |
| | *Mouse (MF1)* | (Morphilino) Deplition of Mad2 by morphilino antisense | SAC inactivation (Dissociation of Mad2 from kinetochore)<br>Acceleration of MI | (Marangos et al., 2015) |
| | *Yeast* | Homozygous mutant | MI nondisjunction | (Shonn et al., 2003) |
| **Mps1** | *Mouse (C57BL/6/Sv129 )* | (Loss of function mutation) Mutated protein harbors a 107 aa deletion with kinetochore localization domain | Acceleration of MI<br>Misaligned chromosome at metaphase plate<br>70% of oocyte aneuploidy incidence<br>Disruption of Aurora B/C localization | (Hached et al., 2011) |
| | *Mouse (C57BL/6/CBA)* | (MPS1 inhibitor) Inhibition of Mps1 kinase activity | Acceleration of MI<br>Misaligned chromosome at metaphase plate<br>PSCS<br>30% of oocyte aneuploidy incidence | (Lane & Jones, 2014) |
| | *Mouse (MF1)* | (Inhibitor (Mps1i)) Mps1 by the use of the specific inhibitor AZ3146 (Mps1i); inhibition of Mps1 kinase activity | Spindle disruption<br>Misaligned chromosome at metaphase plate | (Marangos et al., 2015) |
| | *Mouse (C57BL/6/Sv129 )* | (Knockout) Inhibition of Mps1 kinase activity | Acceleration of MI<br>Misaligned chromosome at metaphase plate<br>> 80% of oocyte aneuploidy incidence<br>Microtubule depolymerisation | (Touati et al., 2015) |
| | *Zebrafish* | (Hypomorphic allele) Negative block of mps1 | Chromosome missegregation<br>Aneuploidy<br>Developmental defects | (Poss et al., 2004) |
| | *Drosophila* | (Hypomorphic allele) P(GS:13084) insertion | Acceleration of MI<br>Chromosome segregation defects | (Gilliland et al., 2005, 2007) |

**Footnote:** (APC/C) Anaphase Promoting Complex/Cyclosome; (SAC) Spindle Assembly Checkpoint; (CSF) Cytostatic Factor; (MI) Meiosis I; (PSCS) Premature Sister Chromatid Separation

**Table 1. 3.** Effect of Maternal Age on Core SAC Components

| SAC component | Species (strain) | Type of study | Yong female age (n=number of oocytes analysed) | Old female age (n=number of oocytes analysed) | Methodology | Effect of maternal age on SAC component* | P-Value | Additional phenotypes | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **Bub1** | *Human* | *in vivo* | < 36 year (n=~8 GV) | > 36 year (n=~8 GV) | RT-PCR | 56% lower mRNA level | *P*=0.044 | | (Steuerwald et al., 2001) |
| | | | < 36 year (n=~8 MI) | > 36 year (n=~8 MI) | | 92% lower mRNA level | *P*<0.001 | | |
| | | | < 36 year (n=~22 MII) | > 36 year (n=~22 MII) | | 11% lower mRNA level | *P*=0.017 | | |
| | *Human* | *in vivo* | < 32 year (n=10) | > 40 year (n=10) | Microarray | 1.42-fold lower mRNA level | NI | | (Steuerwald et al., 2007) |
| | *Human* | *in vivo* | 31 year (n= single MII) | 39 year (n= single MII) | NanoString nCounter assay | 1.5 lower mRNA level | 0.006 | | (Riris et al., 2014) |
| | *Mouse (B6SJL F1)* | *in vivo* | 6 week (n=25) | 66 week (n=25) | Microarray | 1.28 decline in mRNA level | NI | Hyperploidy | (Pan et al., 2008) |
| | *Mouse (MF1)* | *in vivo* | 6-8 week (n= 243 kinetochores) | >50 week (n= 249 kinetochores) | Immunofluorescence | 15% lower protein level in kinetochore accumulation | *P*<0.0001 | | (Marangos et al., 2015) |
| **Bub3** | *Human* | *in vivo* | < 32 year (n=10) | > 40 year (n=10) | Microarray | 1.58-fold lower mRNA level | NI | | (Steuerwald et al., 2007) |
| **BubR1** | *Human* | *in vivo* | < 32 year (n= 10) | >37 year (n= 10) | Immunoblot | ~1.5-fold lower protein level | NI | | (Riris et al., 2014) |
| | *Mouse (MF1)* | *in vivo* | 6-8 week n= (238 kinetochores) | >50 week (n=168 kinetochores) | Immunofluorescence | 35% reduction of protein level in kinetochore accumulation | *P*<0.0001 | | (Marangos et al., 2015) |
| **Mad2** | *Human* | *in vivo* | < 36 year (n=~8 GV) | > 36 year (n=~8 GV) | RT-PCR | 24% lower mRNA level | *P*=0.043 | | (Steuerwald et al., 2001) |
| | | | < 36 year (n=~8 MI) | > 36 year (n=~8 MI) | | 72% lower mRNA level | *P*=0.005 | | |
| | | | < 36 year (n=~22 MII) | > 36 year (n=~22 MII) | | No change in mRNA level | NS | | |
| | *Mouse (ICR)* | *in vivo* | 14 h post ovulation (n=20 MII) | 24 h post ovulation (n=25 MII) | RT-PCR | 58% lower mRNA | *p*<0.05 | High incidence of PSCS | (Steuerwald et al., 2005) |
| | *Mouse (MF1)* | *in vivo* | 6-8 week (n=112 kinetochores) | >50 week (n= 77kinetochores) | Immunofluorescence | 10% lower protein level in kinetochore accumulation | NS | | (Marangos et al., 2015) |
| | *Mouse (ICR)* | *in vitro* | 0 h of oocyte collection | 6-12 h of oocyte collection | Western blot | No change in protein level | NS | 40% of oocyte aneuploidy | (Shimoi et al., 2019) |
| | *Bovine* | *in vitro* | Ave=27.8±0.3 months, 0 h of culture (n=60) | Ave=152.3±2.6 months, 21h of culture (n=60) | RT-PCR | No change in mRNA level | NS | 45.6% higher abnormal fertilization rate* | (Iwata et al., 2011) |

| SAC component | Species (strain) | Type of study | Yong female age (n=number of oocytes analysed) | Old female age (n=number of oocytes analysed) | Methodology | Effect of maternal age on SAC component* | P-Value | Additional phenotypes | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| | *Porcine* | *in vitro* | 40 h of culture (n= 42) | 60, 72 h of culture (n= 52, 42) | Immunofluorescence | 45%, 65% lower protein level | $p<0.001$ | | (Ma et al., 2005) |

**Footnotes:** (GV) Germinal Vesicle; (MI) Metaphase I; (MII) Metaphase II;(NI) Not indicated; (NS) Not significant;(PSCS) Premature Sister Chromatid Separation
*Values presented in this table are calculated relative to young females
*Abnormal fertilization is defined as when an oocyte has more or less than 2 polar bodies and 2 pronuclei.

### 1.2.4 Mitochondrial Dysfunction

The oocyte is the largest cell in the organism and has the largest number of mitochondria (Eichenlaub-Ritter et al., 2004). In fact, mitochondria are the most abundant organelle in oocytes (Eichenlaub-Ritter et al., 2011). Oocyte mitochondrion have a unique morphology with round shape, few cristae, thick membrane and generally one or two mitochondrial DNA (mtDNA) genomes (Eichenlaub-Ritter et al., 2011), although up to fifteen copies have been reported (Zhang et al., 2017). The mtDNA is a circular double-stranded DNA of 16,569 base pairs, has no introns, histones, DNA repair enzymes or antioxidant mechanism, and is attached to the inner membrane of the mitochondrion (Meldrum et al., 2016). The mtDNA contains 37 genes, of which 13 encode for proteins that are part of the electron transfer chain, 22 encode for transfer RNA, and the remaining 2 encode for ribosomal RNA subunits (Meldrum et al., 2016; Zhang et al., 2017).

In mammals, mtDNA copy number increases during oocyte maturation. In humans, the primary oocyte has 500 copies of mtDNA, whereas a mature MII oocyte has 150,000-700,000 copies of mtDNA (Grindler & Moley, 2013; Reynier et al., 2001; Wai et al., 2010). mtDNA in the zygote is inherited almost exclusively from the oocyte, and the quality of the embryo depends on the quality of the inherited mitochondria (Demain et al., 2017).

Oocytes require energy to undergo essential processes, including fertilization, transcription and translation during maturation, intracellular signaling, spindle formation, chromosome segregation, and polar body extrusion (Eichenlaub-Ritter, 2012). Mitochondria produce energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation to phosphorylate adenosine diphosphate (Bentov et al., 2011). Proper oocyte maturation, fertilization and embryo development are dependent on the amount of mtDNA molecules, membrane potential, and ATP production (Grindler & Moley, 2013). In assisted reproductive technologies, mitochondrial function is a key indicator for oocyte quality and successful fertilization.

Mitochondrial dysfunction negatively affects oocytes and increases with advanced maternal age. Mitochondria are the major source of reactive oxygen species (ROS) that cause oxidative stress, which in turn may contribute to the decline in mitochondrial function associated with the aging (Richter, 1995). With aging, the activity and number of mitochondria falls and impairs oocyte maturation processes including spindle activity and chromosome segregation. The drop in mitochondria number and activity also increases mtDNA mutation by reducing ATP production via impairment of the follicular oxidative phosphorylation pathway (Eichenlaub-Ritter et al., 2011). Age-related mitochondrial dysfunction increases mitochondrial damage, which reduces mitochondrial membrane potential, impairs the ability of mitochondria to accumulate and produce ATP, and alters mitochondrial gene expression (Lord & Aitken, 2013). This can lead to meiotic spindle damage, chromosome misalignment, aneuploidy and, in most cases, oocyte death (Eichenlaub-Ritter et al., 2004).

Advanced maternal age negatively affects mitochondria morphology, ATP production, mtDNA copy number and increases mtDNA mutation rates. Studies that examined the effect of maternal aging on mitochondrial function are summarized in Table 1.4. The mitochondria in old oocytes from many mammalian species, including, mouse, hamster, and pig, appear to be more differentiated, have numerous crista, have an elongated shape instead of a round shape, are smaller in size, and aggregate to the center of the oocyte compared with younger oocytes (Babayev et al., 2016; Kushnir et al., 2012; Ma et al., 2005; Simsek-Duran et al., 2013). In addition, oocytes from aged female mice had an approximately 15% reduction in mitochondrial membrane potential compared with younger oocytes (Igarashi et al., 2016).

Aging contributes to increased mtDNA mutations and deletions. In oocytes from women undergoing IVF, 93% of oocytes from patients aged ≥ 38 years harbored the common mtDNA

4977 bp deletion compared with only 28% of oocytes from younger women (Keefe et al., 1995). Similarly, older women (≥ 35 years) had a significantly higher incidence of the 4977 bp deletion compared to young women(Chan et al., 2005). Moreover, older women exhibited a mtDNA point mutation (T414G) in 39.5% of oocytes compared to 4.4% in younger women, which may affect the regulation of mtDNA transcription and replication during oocyte and post-embryonic development (Barritt et al., 2000).

In several species, aging affected mtDNA copy number and ATP levels. In humans, old oocytes have decreased mtDNA copy number in comparison to younger oocytes (Chan et al., 2005; Murakoshi et al., 2013). Additionally, Fragouli et al reported a decrease in mtDNA copy number in the blastomeres of old women compared to younger women (Fragouli et al., 2015). In cows, advanced maternal age is correlated with a lower mtDNA copy number and ATP content (Iwata et al., 2011). Similarly, aged mice and hamsters have lower oocyte ATP levels, which is correlated with decreased mtDNA (Igarashi et al., 2005; Simsek-Duran et al., 2013). Also, older mice had significantly less mtDNA in contrast to younger mice (Babayev et al., 2016; Kushnir et al., 2012). In mares, mtDNA copy number was lower in old oocytes subjected to *in vitro* maturation compared to younger oocytes (Rambags et al., 2014). In contrast to studies with human, mouse and bovine oocytes, it should be noted that Cree et al reported a negative correlation between advanced maternal age in bovine and mtDNA copy number (Cree et al., 2015).

Transcriptomic analysis of mature oocytes and embryos, performed using Next Generation Sequencing, revealed that mRNA expression for genes associated with mitochondrial dysfunction and ROS levels tend to increase in aged cows (Takeo et al., 2013b). Similarly, microarray analysis reported that genes involved in mitochondrial function and oxidative damage were downregulated in older oocytes in humans (Steuerwald et al., 2007) and mice (Hamatani et al., 2004).

Additionally, RNA-Seq performed on individual growing follicles from young and old mice revealed that genes involved in mitochondrial function and meiotic chromosome segregation change with aging (Duncan et al., 2017).

Mitochondria deterioration affects normal fertilization and cleavage ability and is associated with aging. Aged female bovine oocytes had abnormal fertilization rates (up to 56%), deterioration of oocyte cleavage, and a decline in developmental competence (i.e.; blastocysts with low cell numbers) (Iwata et al., 2011; Takeo et al., 2013b). Additionally, older mice had significantly prolonged time to conception and decreased fertility outcomes (Kushnir et al., 2012).

In summary, there is clear evidence that advanced maternal age negatively affects mitochondria function. Mitochondrial damage, including morphological change, lower mtDNA number and ATP content, increased mutation rate and elevated levels of ROS, are aspects of female reproductive aging common among different mammalian species. Deterioration of mitochondrial function affects oocyte quality, fertilization ability, embryo development and overall female fertility since mtDNA is inherited from the mother.

**Table 1. 4.** Effect of Maternal Age on Mitochondrial Function

| Endpoint | *Species (strain)* | Type of study | Yong female age (n= number of oocytes analysed) | Old female age (n= number of oocytes analysed) | Methodology | Effect of maternal age* | P-Value | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Mitochondria Morphology; Number; Distribution** | *Mouse (C57BL/6)* | *in vivo* | ~3 months (n=31) | ~ 10 months (n=64) | TEM assay | Change in mitochondria morphology from spherical to elongated with numerous cristae and more differentiated | *P<0.001* | (Kushnir et al., 2012) |
| | *Mouse (B6CBAF1)* | *in vivo* | 2-4 months (n=11) | 10-12 months (n=11) | TEM assay | 25.8% decrease in mitochondria number<br>50.9% of vacuolated mitochondria | NS<br>*p*<0.0005 | (Simsek-Duran et al., 2013) |
| | *Mouse (C57BL/6)* | *in vivo* | 9 weeks (n=26) | 12 months (n=27) | TEM assay | 43% decrease in mitochondria coverage<br>Smaller mitochondria | *P<0.05*<br>*P<0.05* | (Babayev et al., 2016) |
| | *Mouse (B6C3F1)* | *in vitro* | 14 h post HCG (n=20) | 20-24 h post HCG (n=20) | JC-1 staining | 15% reduction in mitochondrial membrane potential | *P<0.05* | (Igarashi et al., 2016) |
| | *Hamster (golden Syrian)* | *in vivo* | 2-4 months (n=10) | 10-12 months (n=10) | TEM assay | 15% decrease in mitochondria number<br>38.9% less matrix density Change in mitochondria and cytoplasmic lamellae structure | *P<0.05*<br>*P<0.001* | (Simsek-Duran et al., 2013) |
| | *Porcine* | *in vitro* | 40, 48 h in culture (n= 64, 72) | 60, 72 h in culture (n= 69, 75) | Immunofluorescence | 65 -70% of oocytes have mitochondria aggregates to the centre | *P*<0.001 | (Ma et al., 2005) |
| | *Porcine* | *in vitro* | 44, 56 h in culture | 68, 80 h in culture | TEM assay | Change in mitochondria morphology from spherical to elongated | NI | (Hao et al., 2009) |
| | *Equine (Equus caballus)* | *in vitro* | < 12 year (n=5) | ≥ 12 year (n=5) | TEM assay | Grossly swollen mitochondria/ loss of cristae/ low matrix density | NI | (Rambags et al., 2014) |
| **mtDNA Mutation** | *Human* | *in vivo* | < 38 year (n=13) | ≥ 38 year (n=33) | Nested PCR | 232% of oocytes with common mtDNA deletion (4977 bp) | *P*<0.0001 | (Keefe et al., 1995) |
| | *Human* | *in vivo* | 26-36 year (n= 23) | 37-42 year (n= 43) | DNA sequence analysis | 797% of oocytes with mtDNA point mutation (T414G) rate | *P*<0.01 | (Barritt et al., 2000) |
| | *Human* | *in vivo* | < 35 year (n=97) | ≥ 35 year (n=58) | Nested PCR | 107%of oocytes with common mtDNA deletion (4977 bp) | *P=0.018* | (Chan et al., 2005) |
| **mtDNA Copy Number** | *Human* | *in vivo* | < 35 year (n=97) | ≥ 35 year (n=58) | RT-QPCR | 16% less mtDNA copy number | *P=0.037* | (Chan et al., 2005) |
| | *Human* | *in vivo* | < 40 year (n=15) | ≥ 40 year (n=14) | RT-PCR | 23% less mtDNA copy number | *P<0.05* | (Murakoshi et al., 2013) |

| Endpoint | *Species (strain)* | Type of study | Yong female age (n= number of oocytes analysed) | Old female age (n= number of oocytes analysed) | Methodology | Effect of maternal age* | P-Value | Ref. |
|---|---|---|---|---|---|---|---|---|
| | *Human* | *in vitro* | Ave=33.7 year; range 29-37 year (n=19 blastomeres)<br>Ave=34.8 year; range 26-37 year (n=109 blastocysts) | Ave=39.2 year; range 38-42 year (n=20 blastomeres)<br>Ave=39.8 year; range 38-42 year (n=94 blastocysts) | RT-QPCR | 26% less mtDNA copy number<br>19% higher mtDNA copy number | *P*=0.003<br>*P*=0.01 | (Fragouli et al., 2015) |
| | *Mouse (C57BL/6)* | *in vivo* | ~ 3 months (n=31) | ~ 10 months (n=64) | RT-QPCR | 2.7 fold less mtDNA copy number | *P<0.001* | (Kushnir et al., 2012) |
| | *Mouse (B6CBAF1)* | *in vivo* | 2-4 months (n=80) | 10-12 months (n=74) | RT-QPCR | 44% less mtDNA copy number | *P=0.039* | (Simsek-Duran et al., 2013) |
| | *Mouse (CD-1)* | *in vivo* | 6-9 weeks (n=~85 blastocysts) | 13.5 months (n=~85 blastocysts) | NGS | 101% higher mtDNA level | *P*<0.0001 | (Tao et al., 2017) |
| | *Mouse (C57BL/6)* | *in vivo* | 9 weeks (n=52) | 12 months (n=33) | RT-QPCR | 64% less mtDNA copy number | *P<0.01* | (Babayev et al., 2016) |
| | *Hamster (golden Syrian)* | *in vivo* | 2-4 months (n=63) | 10-12 months (n=55) | RT-QPCR | 51.8% less mtDNA copy number | *P=0.0015* | (Simsek-Duran et al., 2013) |
| | *Bovine* | *in vitro* | 21-89 months of 43 female (n=10) | > 89 months of 22 female (n=10) | RT-QPCR | mtDNA copy number decline gradually when female reaches 90 months (7% less mtDNA) | *P*=0.041 | (Iwata et al., 2011) |
| | *Bovine* | *in vitro* | 25-45 months of 18 female (n=10) | > 180 months of 7 female (n=10) | RT-QPCR | Reduction in mtDNA copy number in embryos derived from oocytes of >180 months female | NS | (Takeo et al., 2013a) |
| | *Bovine* | *in vivo* | 3 year (n=7) | 10 year (n=5) | RT-QPCR | ND | NS | (Cree et al., 2015) |
| | *Equine (Equus caballus)* | *in vitro* | < 12 year (n=79) | ≥ 12 year (n=92) | RT-QPCR | 4% less mtDNA copy number | *P*<0.001 | (Rambags et al., 2014) |
| **ATP Level** | *Mouse (B6C3F1)* | *in vitro* | 12.5 h post HCG (n=30) | 18.5 h post HCG (n=30) | Luciferin-luciferase assay | 18% lower ATP level | *P<0.05* | (Igarashi et al., 2005) |
| | *Mouse (B6CBAF1)* | *in vivo* | 2-4 months (n=56) | 10-12 months (n=61) | Bioluminescent assay | 38.4% lower ATP level | *P<0.0001* | (Simsek-Duran et al., 2013) |
| | *Hamster (golden Syrian)* | *in vivo* | 4 months (n=90) | 12 months (n=73) | Bioluminescent assay | 35.4% lower ATP level | *P=0.002* | (Simsek-Duran et al., 2013) |
| | *Porcine* | *in vitro* | 44 h in culture (n=NI) | 68, 80 h in culture (n=NI) | Luciferin-luciferase bioluminescent assay | 44%, 55% lower ATP level | *p*<0.05 | (Hao et al., 2009) |
| | *Bovine* | *in vitro* | 20-89 months (n=63 MII) | ≥ 90 months (n=113 MII) | Luciferin-luciferase bioluminescent assay | Positive correlation of ATP content and maternal aging | *P*=0.013 | (Iwata et al., 2011) |

| Endpoint | *Species (strain)* | Type of study | Yong female age (n= number of oocytes analysed) | Old female age (n= number of oocytes analysed) | Methodology | Effect of maternal age* | P-Value | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Stress Response Expression** | *Mouse (C57BL/6)* | *in vivo* | 9 weeks (n=26) | 12 months (n=27) | RT-QPCR | 2-fold increase in *Hspd1* transcript level | *P<0.05* | (Babayev et al., 2016) |
| | *Mouse (C57BL/6)* | *in vivo* | 9 week (n=40 GV, 32 MII) | 12 months (n=39 GV, 35 MII) | Fluorescence intensity of Carboxy-H2DCFDA | 42%, 33% higher ROS level GV and MII oocytes | *P<0.05* | (Babayev et al., 2016) |
| | *Bovine* | *in vitro* | Ave= 34.2±3.7 months of 8 females (n=30) | Ave= 168.9±9.7 months of 9 females (n=30) | Immunostaining | 1.8 -fold increase of ROS level<br>1.4-fold increase of SIRT1 level | NI<br>NI | (Takeo et al., 2013b) |
| | *Bovine* | *in vivo* | 20-30 months MII oocytes (n=40) | ≥ 120 months MII oocytes (n=55) | NGS | Higher expression of genes associated with oxidative phosphorylation and mitochondrial dysfunction in MII oocytes | NI | (Takeo et al., 2013b) |
| **Fertility Outcomes** | *Mouse (B6C3F1)* | *in vitro* | 14 h post HCG (n=15) | 20-24 h post HCG (n=15) | Microscopic analysis | ND in fertilization rate and embryonic development | NS | (Igarashi et al., 2016) |
| | *Bovine* | *in vitro* | Ave=29.8 ±1.0 months of 60 females (n=152) | Ave= 156.3±2.7 months of 60 females (n=154) | Microscopic analysis | 56% of abnormal fertilization rate**<br>18% lower cells in blastocyst stage | *p*<0.05<br>*p*<0.01 | (Takeo et al., 2013b) |
| | *Bovine* | *in vitro* | 20-89 months (n=43) | ≥ 90 months (n=22) | Microscopic analysis | 14% of abnormal fertilization rate**<br>5% failure to extrude second polar body | *p<0.05*<br>*p<0.01* | (Iwata et al., 2011) |

**Footnotes:** (ATP) Adenosine Triphosphate; (*Hspd1*) heat shock 60 KDa protein 1; (ND) No Difference; (NGS) Next Generation Sequencing; (NS) Not Significant; mitochondrial DNA; (ROS) Reactive Oxygen Species; (RT-QPCR) Real-Time Polymerase Chain Reaction; (SIRT1) Sirtuin1; (TEM) Transmission Electron Microscopy

*Values presented in this table are calculated relative to young females

**Abnormal fertilization is defined as when an oocyte has more or less than 2 polar bodies and 2 pronuclei.

### 1.3 Concluding remarks

Female fertility decreases with advanced maternal age. Aged oocytes experience chromosome segregation errors during meiosis that can result in aneuploidy and poor oocyte quality. Several molecular pathways likely contribute to age-associated chromosome segregation errors in oocytes. With aging, chromosomes undergo structural changes, which promote incorrect attachment of kinetochores to spindle microtubules. Cohesin subunits are gradually degraded, with little to no turnover. The loss of cohesion directly affects chromosome segregation by destabilizing chiasmata, reducing kinetochore orientation, and increasing the distance between sister kinetochores. The SAC mechanism is less sensitive and exhibits a reduced expression of core SAC components in older oocytes. Finally, mitochondrial dysfunction including reduced ATP content, mtDNA copy number, and increased mtDNA mutation rate contribute to reduce oocyte quality. Missegregation of chromosomes in aged oocytes could result from one or more of these impaired molecular pathways and contribute to the observed increase in oocyte aneuploidy with aging (Figure 2.2).

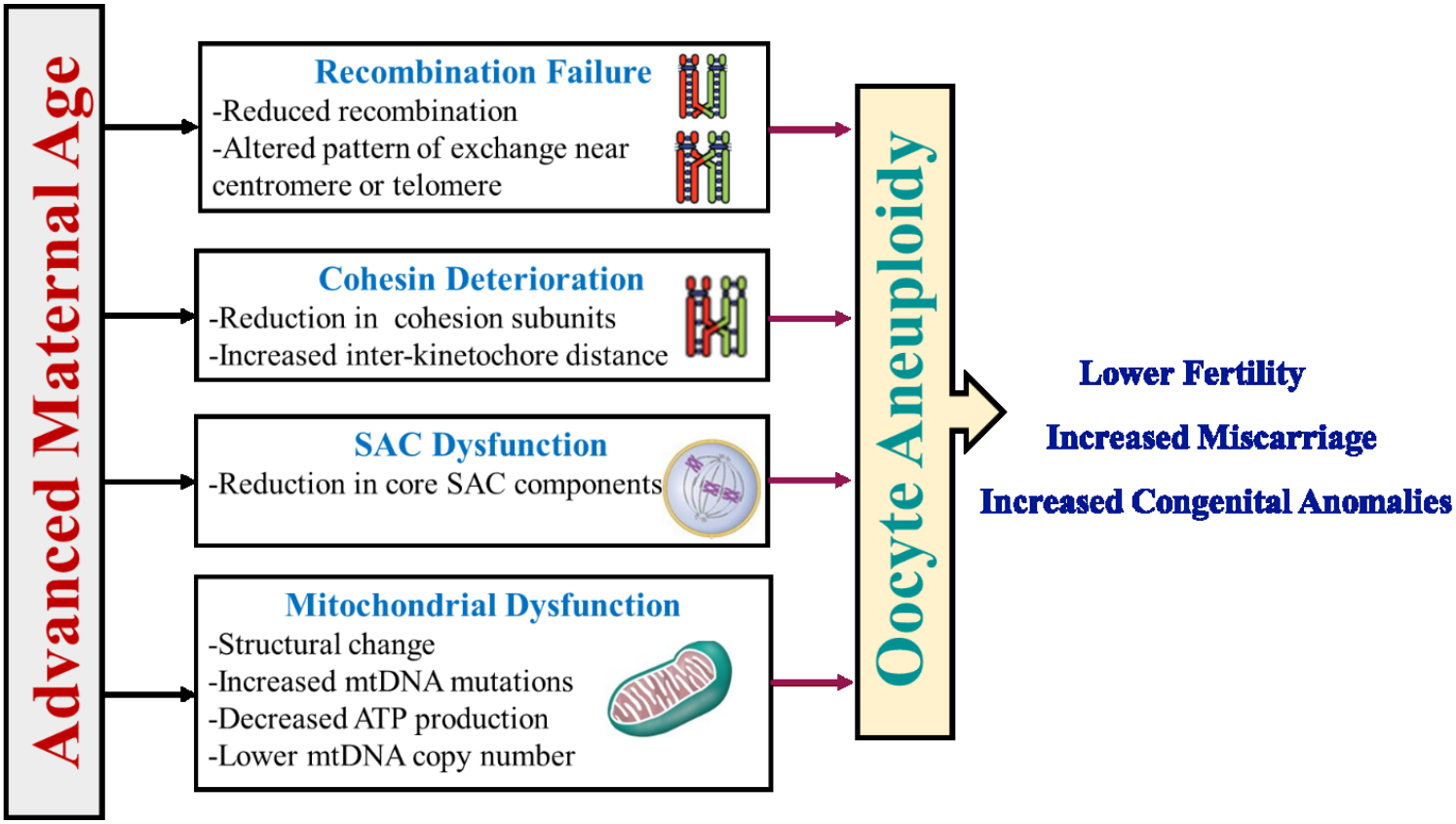



**Figure 1. 2. Summary figure shows the effect of advanced maternal age on oocyte aneuploidy and the potential molecular mechanisms affected by aging**. Maternal aging can alter recombination and the function of cohesin, SAC and mitochondria. Alteration of theses mechanisms leads to aneuploidy occurance in oocyte and further lower fertility rate and increased miscarriage and congenetal anomalis in offspring

# Chapter 2: Association between Folic Acid and Oocyte Aneuploidy

# Chapter 2
# Association between Folic Acid and Oocyte Aneuploidy

## 2.1 Overview of Folate

Folate, otherwise known as vitamin B9, is an essential water-soluble vitamin that is required for *de novo* nucleotide and methionine synthesis. Structurally, folate is a pteroylglutamate comprised of a pteridine ring and a *p*-aminobenzoic acid attached by a methylene group (Figure 2.1). Because folate cannot be synthesized endogenously, mammals rely on dietary sources such as fresh fruit and vegetables, dark green leafy vegetables, beans, whole grains, liver and milk to maintain folate adequacy. Cooking decreases folate content in food leading to irreversible degradation of folate to two products, pteridine and *p*-aminobenzoylglutamate. As such, fortified foods, such as enriched white flour, and vitamin supplements generally include the synthetic, stable, oxidized, and monoglutaminated form of folate called folic acid (FA) (Stover, 2004).

The majority of naturally occurring folate from food is in the form of 5-methyl-tetrahydrofolate (5-methyl-THF) with a polyglutamate tail length ranging from 2 to 9 residues depending on the food. In the gastrointestinal tract, these folate polyglutamates must be converted to the monoglutamate form by γ-glutamyl hydrolyase before intestinal absorption can take place. Uptake of folate in the intestine is mediated by the proton-coupled folate transporter (PCFT) located predominantly in the proximal jejunum and duodenum (Zhao et al., 2009). FA, which is not biologically active, is transported across the intestinal epithelium via PCFT and must be sequentially reduced to dihydrofolate (DHF) and then to THF by dihydrofolate reductase (DHFR) before it can contribute to one-carbon metabolism. 5-methyl-THF is circulated and transported into cells through the reduced folate carrier (RFC) or receptor-mediated endocytosis of folate receptors (FR) (Stover, 2009; Zhao & Goldman, 2013). Intracellular THF is processed into

functional metabolic cofactors through the addition of the polyglutamate peptide comprised of up to 9 glutamate residues linked by γ-peptide linkages. The glutamate polypeptide is essential to retain the vitamin within cells and increases its affinity for folate-dependent enzymes.

Intracellular THF cofactors function as a family of metabolic cofactors that chemically activate and either accept or donate single carbons for metabolic pathway known as one-carbon metabolism. There are three one-carbon substituted forms of THF cofactors. The one-carbon moiety is attached at the N5 and/or N10 position at the oxidation level of formate (e.g. 10-formyl-THF), formaldehyde (5,10-methylene-THF) or methanol (5-methyl-THF) (Bailey et al., 2015). THF carries and activates one-carbon units for three main biosynthetic pathways: *de novo* synthesis of purines and thymidylate, and the synthesis of methionine from the remethylation of homocysteine (Fox & Stover, 2008; Stover, 2009). As such, folate is required for nucleotide synthesis, DNA repair, and cellular methylation.

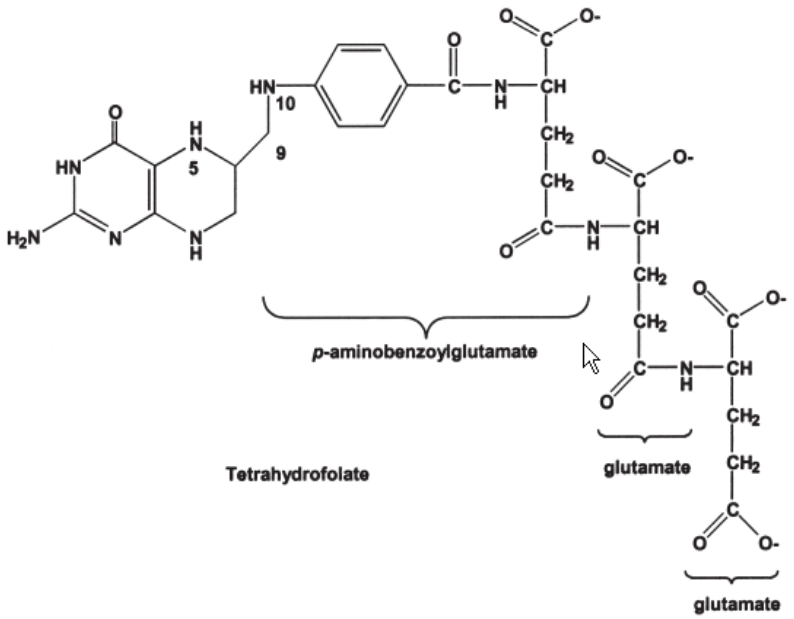



**Figure 2. 1.** Structure of tetrahydrofolate triglutamate

## 2.2 Folate Mediated one-carbon Metabolism

In mammalian cells, folate metabolism occurs in the nucleus, mitochondria, and cytoplasm (Figure 2.2). In the nucleus, one-carbon metabolism is required for synthesis of thymidylate at the replication fork. In the mitochondria, one-carbon metabolism has three primary functions: the generation of one-carbon units in the form of formate; the generation of the amino acid glycine; and the synthesis of formylmethionyl-transfer RNA. In the cytoplasm, one-carbon metabolism is a network of biosynthetic pathways that catalyze the *de novo* synthesis of purine nucleotides and remethylation of homocysteine to methionine. These pathways compete for a limiting pool of folate cofactors. Folate cofactors compete for 5,10-methylene-THF for *de novo* dTMP biosynthesis and remethylation of homocysteine (Fox & Stover, 2008; Scotti et al., 2013).

Folate is transported into mitochondria in the form of THF monoglutamate via the mitochondrial folate transporter SLC25A32 (McCarthy et al., 2004). One-carbon metabolism in mitochondria has three main functions: the generation of one-carbon units in the form of formate from the catabolism of serine, glycine, dimethylglycine and sarcosine for cytoplasmic reactions; the generation of the amino acid glycine from serine; and the synthesis of N-formylmethionyl-transfer RNA using 10-formyl-THF as a cofactor for mitochondrial protein synthesis (Bailey et al., 2015). The hydroxymethyl group of serine is the primary source of one-carbon units in the mitochondria (Tibbetts & Appling, 2010). The catabolism of the amino acids serine, glycine, dimethylglycine and sarcosine, generates 5, 10-methylene-THF which is then oxidized to 5,10-methenyl-THF and to 10-formylTHF catalyzed by the bi-functional enzymes methyl-THF dehydrogenase 2 (MTHFD2) and (MTHFD2L). 10-formyl-THF is hydrolyzesd to formate which

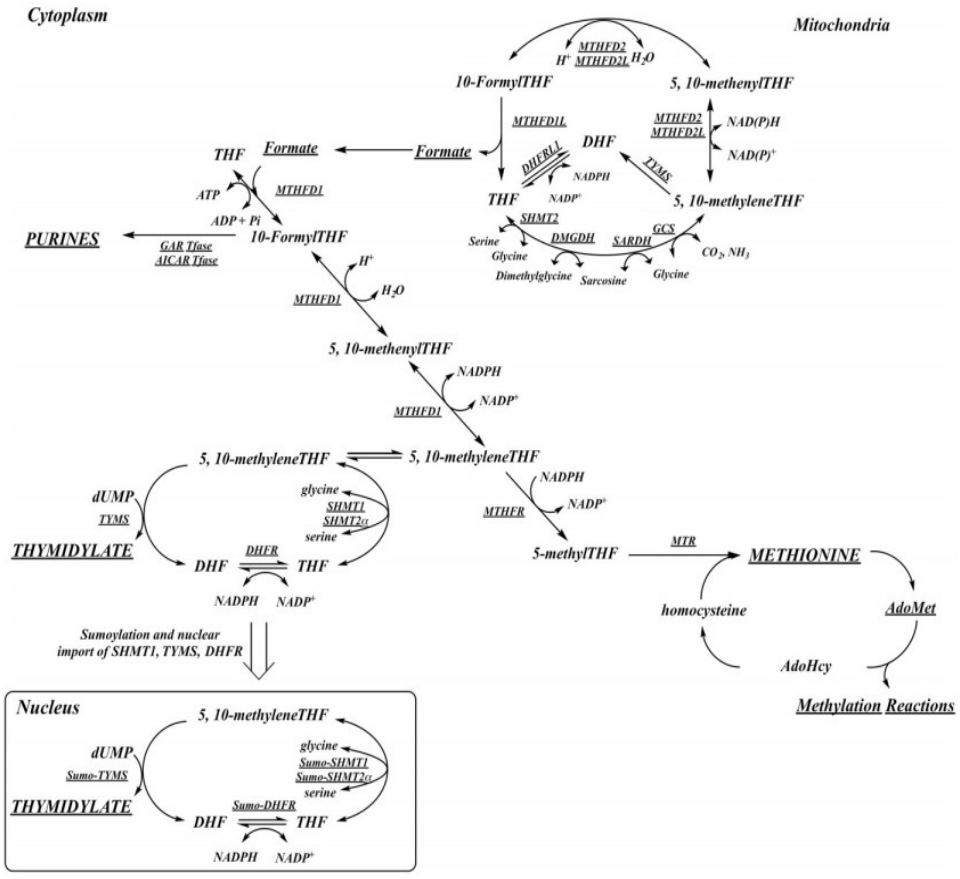



**Figure 2. 2. Folate mediated one-carbon metabolism in the nucleus, mitochondria and cytoplasm.** One-carbon metabolism in the cytoplasm is required for purine, thymidylate and methionine synthesis. One-carbon metabolism in the mitochondria is required to generate formate for one-carbon metabolism in the cytoplasm. One-carbon metabolism in the nucleus is required for thymidylate synthesis. Enzymes are indicated with *italics*. Abbreviations: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; *DHFR*, dihydrofolate reductase; dUMP, deoxyuridine monophosphate; GCS, glycine cleavage system; *MTHFD*, methylenetetrahydrofolate dehydrogenase; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; SHMT1, cytoplasmic serine hydroxymethyltransferase; SARDH, sarcosine dehydrogenase; Sumo, small ubiquitin-like molecule; THF, tetrahydrofolate; *TYMS*, thymidylate synthase. **Diagram from Bailey et al., 2016 with permission (Appendix I)**.

is catalyzed by MTHFD1L in an ATP dependent reaction (Scotti et al., 2013). Formate enters the cytoplasm and provides the majority of one-carbon units required for cytoplasmic and nuclear one-carbon metabolism. Approximately 40% of total cellular folate polyglutamates are present in mitochondria. The communication between mitochondria and cytoplasm folate metabolism occurs via the exchange of one carbon donors including serine, glycine and formate (Stover & Field, 2011; Tibbetts & Appling, 2010).

In the cytoplasm, which accounts for 50% of cellular folate metabolism, a series of metabolic processes take place. Formate from mitochondrial one-carbon metabolism is the primary source of one-carbon units for cytoplasmic reactions. Other sources of one-carbon units include the catabolism of histidine, purines and serine. Formate condenses with THF to form 10-formyl-THF which is required for *de novo* purine synthesis. This reaction is catalyzed by 10-formyl-THF synthetase enzyme in an ATP-dependent reaction (Stover & Field, 2011).

*De novo* purine biosynthesis involves a pathway of ten chemical reactions. These reactions are catalyzed by the multi-enzyme complex known as purinosome, which is responsible for *de novo* purine biosynthesis in the cytoplasm when the salvage pathway is not active (Chan et al., 2015). The purinosome consists of six enzymes whose association and dissociation can be regulated dynamically with changing purine concentrations or exogenous factors. These enzymes include: 1) phosphoribosylpyrophosphate amidotransferase; 2) a trifunctional enzyme which is composed of glycinamide ribonucleotide synthetase, glycinamide ribonucleotide formyltransferase and aminoimidazole ribonucleotide synthetase; 3) formylglycinamidine ribonucleotide synthase; 4) a bifunctional enzyme which is composed of carboxyaminoimidazole ribonucleotide synthase and succinoaminoimidazolecarboxamide ribonucleotide synthetase; 5) adenylosuccinate lyase; and 6) a bifunctional enzyme which is composed of

aminoimidazolecarboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase (An et al., 2008; Zhao & Goldman, 2013). The reactions catalyzed by glycinamide ribonucleotide formyltransferase and aminoimidazolecarboxamide ribonucleotide transformylase are dependent on the folate cofactor 10-formyl-THF, which is required for providing the number 2 and 8 carbons in the formation of the purine ring (Scotti et al., 2013).

Alternatively, 10-formyl-THF can be converted to 5,10-methenyl-THF by methenyl-THF cyclohydrolase and further reduced to 5,10-methylene-THF catalyzed by methylene-THF dehydrogenase 1 in an NADPH dependent conversion. 5,10-methylene-THF can be used for thymidylate biosynthesis through the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP).

The *de novo* synthesis of dTMP from dUMP requires several enzymatic activities: thymidylate synthase (TYMS); serine hydroxymethyltransferase (SHMT); methylene-THF dehydrogenase 1(MTHFD1); and dihydrofolate reductase (DHFR). 5,10-Methylene-THF is the one-carbon donor for the conversion of dUMP to dTMP and DHF catalyzed by TYMS. In this reaction, the folate cofactor serves as a one-carbon donor and source of 2 electrons through the oxidation of THF to DHF. DHF is converted to THF in a NADPH-dependent reaction catalyzed by DHFR. The pathway is completed by the generation of 5,10-methylene-THF from THF in a reaction catalyzed by SHMT1 and SHMT2α. Alternatively, 5,10-methylene-THF can be generated from formate, ATP, NADPH and THF in a reaction catalyzed by MTHFD1 or from serine through vitamin B6 dependent activity of SHMT that catalyzes the transfer of the hydroxymethyl group of serine to THF to generate glycine and 5, 10-methylene-THF (Scotti et al., 2013; Stover & Field, 2011; Tibbetts & Appling, 2010).

Approximately 10% of cellular folate is present in the nucleus. The enzymes that constitute the thymidylate synthesis pathway undergo UBC9-mediated modification with the small ubiquitin-like modifier (SUMO)-dependent translocation of SHMT1, TYMS, and DHFR during S-phase of the cell cycle (Anderson et al., 2007; Woeller et al., 2007). Impairment of folate-dependent *de novo* thymidylate biosynthesis results in dUMP accumulation and uracil incorporation into DNA instead of thymidine during DNA synthesis leading to DNA double strand breaks (Chon et al., 2017).

In the cytoplasm, 5,10-methylene-THF can be irreversibly reduced to 5-methyl-THF by methylene-THF reductase (MTHFR) in a NADPH-dependent reaction. 5-methyl-THF donates a methyl group to homocysteine to generate THF and methionine, a reaction catalyzed by the enzyme methionine synthase (MTR). Methionine can be converted to *S*-adenosylmethionine (SAM) by methionine adenosyltransferase. SAM is the universal methyl donor used for cellular methylation reactions, such as the methylation of DNA, RNA, histones and synthesis of the neurotransmitters (Fox & Stover, 2008; Stover, 2009). There are several biomarkers for impaired homocysteine remethylation and reduced cellular methylation potential, including decreased plasma SAM concentrations and increased plasma homocysteine and s-adenosylhomocysteine (SAH) concentrations. Reduced cellular methylation potential can lead to changes in DNA methylation patterns which affect genome stability and gene expression. Also, the accumulation of 5-methyl-THF in cells due to lower MTR activity can result in a methyl trap, leading to impaired purine and thymidylate biosynthesis (Bailey et al., 2015).

**2.3 Folate and Human Health**

Impairment in folate metabolism can result from folate deficiency due to poor dietary folate intake or genetic polymorphisms in folate dependent enzymes. Impaired folate metabolism has been linked to multiple pathologies in humans including megaloblastic anemia, cardiovascular

disease, several types of cancer, cognitive dysfunction and neural tube defects (Bailey et al., 2015). Insufficient folate intake affects DNA synthesis and cellular methylation capacity that can lead to altered genome stability and gene expression which represent possible mechanisms behind those pathologies.

### 2.3.1 Folic Acid and Neural Tube Defects

Development of the neural tube occurs during early embryogenesis in the first trimester of pregnancy between days 21-28 post fertilization. At this stage, most women are unaware that they are pregnant. NTDs develop when the neural tube fails to completely close (Greene & Copp, 2014). The recent global assessment of NTD prevalence from 75 countries indicates that the prevalence ranges from 0.3 to 199.4 NTDs per 10,000 births (Zaganjor et al., 2016). NTDs are serious congenital birth defects of the central nervous system including the brain and spine; NTDs cause serious morbidity and mortality. Anencephaly and spina bifida (including meningocele and meningomyelocele) are the most commonly observed NTDs in humans, accounting for 1/3 and 2/3 of NTDs respectively. Anencephaly, when the cranial neural tube fails to close, results in a defect in which the brain neural fold remains open. It causes fetal death either before or shortly after birth. Spina bifida involves a defect in the caudal end of the neural tube and results in spinal cord damage in the lumbosacral region. Spina bifida is not lethal but the severity of co-morbidities depends on the degree and location of the NTDs (Copp & Greene, 2013; Pitkin, 2007).

An association between maternal folate status and the occurrence of NTDs was proposed in the early sixties. Hibbard first observed that low folate status was associated with NTD occurrence by investigating the folate status of 1,484 pregnant patients through the analysis of the formiminoglutamic acid (FIGLU) excretion in the urine. Patients with abnormal (higher than 20 μg/ml) FIGLU, an indicator of folate deficiency, were twice as likely to have a baby with congenital birth defects (3.0%) compared to patients with a normal FIGLU excretion (1.5%)

(Hibbard, 1964). Emery and colleagues reported that low maternal folate status, determined by measuring serum folate, was associated with the occurrence of spina bifida and anencephaly (Emery et al., 1969).

Following these observational studies, a series of case/control studies reported the relation between maternal FA supplementation and NTDs in the offspring. In these studies, the cases (infant with NTDs) were compared to control (infant with no NTDs). A study in the UK of nearly 5500 mothers with a history of NTD pregnancies reported a reduced recurrence rate of NTDs from 4.3% in mothers who consumed a multivitamin containing 0.36 mg/day FA compared to 0.5% in non-supplemented mothers (Smithells et al., 1981). Other case/control studies performed in the US, Australia and Canada also reported decreases in NTD occurrence ranging between 35% to 75% associated with consumption of multivitamins containing either 0.4 or 0.8 mg/day of FA before conception (Bower & Stanley, 1989; Mulinare et al., 1988; Shaw et al., 1995; Werler et al., 1993).

Three large-scale clinical trials investigated the effect of periconceptional supplementation of FA on NTD incidence. In 1991, the Medical Research Council of the UK (MRC Vitamin Study Research Group) launched a large-scale, international, multicenter, double-blind randomised prevention trial involving seven countries (UK, Hungary, Israel, Australia, Canada, France and USSR) to investigate the effect of periconceptional vitamin supplementation with or without FA in mothers at high risk of NTD pregnancies (mothers who had a history of NTD affected infant). The study included 1817 mothers given either a periconceptional supplement containing 4.0 mg/day FA, a multivitamin containing vitamins A, B1, B2, B6, C, D and nicotinamide, both or neither. The study showed that periconceptional supplementation of FA had a 72% protective effect against NTD recurrence in high risk women (MRC Vitamin Study Research Group, 1991).

Another large-scale trial initiated in Hungary aimed to investigate the effect of FA supplementation on the prevention of a first occurrence NTD. In this study, women who were planning a pregnancy were given either a vitamin supplement containing 0.8 mg/day FA or a trace elements supplement with no FA from one month before conception until the third month of pregnancy. The FA treated group had 0% NTD incidence compared with 0.3% NTD incidence in the control group (Czeizel & Dudás, 1992). A large intervention study performed in China demonstrated that periconceptional supplementation with 0.4 mg/day FA reduced the risk of NTD incidence in high (5 to 6 per 1000 birth) and lower (~1per 1000 birth) rates of NTD areas in China. The NTD incidence declined from 4.8 to 1.0 and from 1.0 to 0.6 per 1000 births in high and lower NTD prevalence areas, respectively (Berry et al., 1999).

Based on these observational studies and clinical trials, the periconceptional consumption of FA clearly reduces the incidence of NTD. There are three potential approaches to deliver FA to the general population including: improvement of dietary habits, food fortification with FA and supplement use. In 1991, the CDC recommended the use of 4 mg/day FA for women at high risk of NTD and 0.4 mg/day FA for all other women of childbearing age (CDC Recommendations for Folic Acid Supplementation, 1991). In 1992, the US Public Health Service (PHS) recommended to use 0.4 mg/day FA for all women including high risk women to reduce the NTD incidence (Recommendations for the use of folic acid, 1992). In 1998, food fortification of white flour, enriched pasta and cornmeal products with FA became mandatory in the United States and Canada to increase the average consumption of FA for women of childbearing age. Food fortification led to a 46% reduction of the prevalence of NTD in Canada and reduced the prevalence of folate deficiency from 30% to less than 1% in the Canadian population (Colapinto et al., 2011; De Wals et al., 2007). In Canada, women of childbearing age or their male partners with no known NTD

risk factors are recommended to take a daily multivitamin containing 0.4 mg/day FA in addition to consuming folate enriched foods, three months prior to pregnancy, and throughout pregnancy and lactation. It is recommended that women with moderate (women or their partners have a family history of NTD in a first or second degree relative) or high risk (women or their partners with a personal NTD history or a previous NTD pregnancy) NTD to take 1.0 and 4.0 mg/day FA (daily multivitamin), respectively, in addition to consuming folate enriched food (Wilson et al., 2015).

### 2.3.2 Folic Acid and Female Fertility

Folic acid is required for nucleotide synthesis, chromosome stability and methylation reactions. These processes are fundamental for female reproductive physiology including ovulation, fertilization, and fetal development and growth. DNA synthesis also plays an important role in germ cell development, and therefore, folate is important for reproduction (Laanpere et al., 2010).

The use of assisted reproductive technologies (ART) including in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT) and intracytoplasmic sperm injection (ICSI) has increased dramatically in recent years. It has increased from approximately 50,000 cycles in 1995 to 256,000 cycles in 2017 in North America (Assisted Reproductive Technology in the United States and Canada, 1995). Infertility is defined as the inability of a couple to achieve pregnancy after 12 months of regular sexual activity. It affects approximately 15-24% of couples in Western Countries (Slama et al., 2012; Thoma et al., 2013).

Interest in the association between nutrition and female fertility has been an expanding area of research in the last decades; however, little is known about the effect of folate or FA on oocyte development and maturation. Several studies have investigated the effect of FA supplementation prior to conception in women undergoing ART cycles. A randomised Polish trial including 40

women found that women who received FA supplementation prior to IVF treatment had lower HCY in follicular fluid (FF) (FF is the microenvironment of the oocyte) and serum, better oocyte quality and higher degree of oocyte maturity compared to women who did not receive FA (Szymanski & Kazdepka-Zieminska, 2003). In addition, a study on patients with polycystic ovary syndrome undergoing IVF treatment demonstrated that higher HCY concentrations, a functional marker of folate status, in FF was associated with significantly a lower fertilization rate, and oocyte and embryo quality (Berker et al., 2009). Together these data indicate that FA supplementation impacts the folate environment of the oocyte and may alter oocyte quality.

Improved fertility and live birth rates in ART patients may require FA doses higher than recommended FA for NTD prevention (> 0.4 mg/day). In US, a cohort of women undergoing IVF treatment reported that women who were in the highest quartile for supplemental FA intake (more than 0.8 mg/day) had a 20% higher live birth rate compared to women in the lowest quartile (less than 0.4 mg/day). Highest supplemental FA consumption was also associated with significantly higher fertilization rates and lower cycle failure rates before embryo transfer (Gaskins et al., 2014a). A follow up study by the same group reported that women in the highest quartiles of serum folate and vitamin B12 concentrations before ART treatment had 1.62 and 2.04 times the live birth rate compared to women in the lowest quartiles (Gaskins et al., 2015).

Several studies have shown that the use of FA before or during pregnancies is associated with reduced risk of miscarriage. A cohort study performed by Byrne showed that supplemental FA intake three months before and during early pregnancy (during first trimester) was associated with a 35% and 60% reduction in miscarriage rate respectively (Byrne, 2011). Another cohort study of 15,950 pregnancies has shown that women in the high category of supplemental folate

intake (>730 μg/day) had a 20% lower miscarriage rate compared to women who did not consume supplemental folate (0 μg/day) (Gaskins et al., 2014b).

Consumption of multivitamins including FA was shown to affect pregnancy rate. A randomized controlled trial on 5,502 women with confirmed pregnancy showed that the pregnancy rate was significantly higher in women who consumed multivitamins containing 800 μg/day FA at least a month before pregnancy and during pregnancy in comparison with the control group of women who consumed a trace element (Czeizel et al., 1994).

A cohort study of Danish women planning a pregnancy suggested that periconceptional FA supplementation was associated with higher fecundability, a higher probability of becoming pregnant in a single menstrual cycle, and reduced odds of short cycle length (Cueto et al., 2016). In addition, regular use of multivitamin supplements that include FA at least three times a week was reported to decrease the risk of ovulatory infertility (Chavarro et al., 2008). Recently, HCY concentrations among healthy women with adequate folate status were suggested to impact the ovulatory cycle, which has implications for fertility. Higher plasma HCY concentrations were associated with a 33% increased risk of sporadic anovulation and the failure to produce a mature egg which might be indicative of impaired ovulatory function (Michels et al., 2017).

A recent pilot study that included 100 women who were undergoing IVF/ICSI reported that women who received multinutrient supplement including 800 μg FA, selenium, vitamin E, catechins, and omega-3 for a maximum of 56 days prior to ovulation had better embryo quality in comparison to women who received FA (400 μg/day) alone (Nouri et al., 2017).

In short, maternal nutrition, and specifically FA intake, during the periconceptional period may positively affect the reproductive performance including oocyte quality and maturity,

fertilization, implantation, placentation, fetal growth and organ development, by supporting one carbon metabolism (Ebisch et al., 2007).

The majority of studies investigated the effect of FA supplementation on several aspects of female fertility; studies that have investigated the effect of FA deficiency on female fertility in humans or animal models are more limited. Maternal folate deficiency prior to or/and during gestation affects female fertility and embryo viability. An animal study investigated the effect of folate deficiency showed that mouse dams fed a deficient folate diet (75 nmol folate/kg of chow) for 8 weeks prior to and during gestation had a significant reduction in pregnancy rate, number of implanted embryos, number of live pups and reduction in body weight of offspring compared to dams fed a sufficient folate diet (1200 nmol folate/kg of chow) (Xiao et al., 2005).

### 2.3.3 Folate Deficiency and Mutagenic potential

Folate deficiency can occur as a result of poor dietary intake of micronutrients that are necessary for folate metabolism (primarily folate, secondarily other B-vitamins such as vitamin B12 and B6), and genetic variation in folate-dependent enzymes (Stover, 2004).

Low folate status can lead to nucleotide imbalances by affecting dTMP biosynthesis, such that the dTMP synthesis rate decreases, and dUMP accumulates in the cell. The frequency of uracil misincorporation into DNA is dependent on the dUTP:dTTP ratio; accumulation of dUMP leads to uracil nucleotide misincorporation into DNA. The increased level of dUTP leads to increased uracil incorporation into DNA (Stover, 2009). The removal of uracil from DNA via uracil-DNA glycosylase and apyrimidinic endonuclease requires the initiation of the base excision repair pathway. The base excision repair generates a single strand DNA breaks (nicks). If single strand DNA breaks occur on opposing sides of a double stranded DNA (two opposing nicks), double strand brakes (DSBs) are generated. This has been shown to occur in cells of chronic folate deficiency (Blount et al., 1997; Fenech, 2001). DNA DSBs can cause chromosome deletion,

translocation, duplication or in the worst case chromosome breakage (Blount et al., 1997). DSBs can also lead to micronucleus formation; micronuclei are small nuclei containing chromosome fragments that did not migrate to the polar ends of the cell during anaphase of cell division (Blount et al., 1997; Fox & Stover, 2008). Impaired *de novo* thymidylate synthesis leads to increased DSBs which leads to DNA instability.

Low folate status can also cause nucleotide imbalances and DNA mutations through impairment of *de novo* purine synthesis; however, an understanding of these pathways is limited. A study done in Chinese hamster ovary cells showed that purine starved cells had abnormal DNA repair, decreased DNA replication and increased lethality and mutagenicity of DNA (Collins et al., 1988).

Folate deficiency has been associated with decreased global DNA methylation which is critical for the regulation of gene and maintenance of genome stability. DNA methylation occurs in cytosine residues within CpG sites of genomic DNA when a methyl group from SAM is transferred to the 5 position of cytosine to generate 5-methylcytosine. Methylation can regulate gene expression/repression, control the localization of transcription factors, and alter chromatin structure (Crider et al., 2012). In cases of folate deficiency, SAM concentrations (the major methyl donor) is decreased resulting in diminished DNA methylation capacity thus altering gene expression and DNA stability (Fox & Stover, 2008). An insufficient rate of remethylation of HCY results in elevated plasma HCY concentrations, a decrease in SAM production, and an increase in SAH concentrations leading to decrease SAM:SAH ratio and decreased level of 5-methylcytosine in DNA and hypomethylation (Stover, 2009). SAH is also a negative regulator of many methyltransferase enzymes, thus further inhibiting methylation reactions. Different types of human cancers and tumors are associated with global DNA hypomethylation (Linhart et al., 2009).

Deficiency of vitamin B12 can also impair folate mediated one-carbon metabolism. MTR catalyzes the vitamin B12-dependent remethylation of HCY using 5-methyl-THF which produces methionine and THF (Stover, 2004). Vitamin B12 deficiency diminishes MTR activity and results in accumulation of 5-methyl-THF. The accumulation of 5-methyl-THF depletes the relative concentrations of other forms of folate such as THF and inhibits nucleotide synthesis. The accumulation of 5-methyl-THF due to reduced MTR activity is known as the "methyl trap" since the production of 5-methyl-THF is irreversible, and only MTR can regenerate THF from 5-methyl-THF which is required for *de novo* purine and thymidylate synthesis (Stover, 2009).

Several variants have been identified in genes involved in folate mediated one-carbon metabolism. A common 677C>T variant of the *MTHFR* gene has been extensively investigated (Laanpere et al., 2011). The *MTHFR* 677C>T variant results in an amino acid alanine-to-valine substitution in the protein. The substitution leads to reduced enzyme activity which leads to reduced 5-methylTHF production resulting in an accumulation of HCY and impairment of cellular methylation reactions (Laanpere et al., 2010). Increased concentrations of HCY (hyperhomocysteinaemia) have been linked to different human pathologies including pregnancy complications (Forges et al., 2007).

**2.4 Folic acid and Aneuploidy Incidence**

Deficiency of FA has been associated with chromosome segregation errors and aneuploidy incidence. Multiple studies have indicated that FA deficiency is associated with chromosome aneuploidy in somatic cells. An *in vivo* metabolic study in humans implicated that folate deficiency is a risk factor for abnormal chromosome segregation and cell division. The study revealed that lymphocytes from women (aged 49-63 years) who were on a baseline folate intake of 195 µg/day followed by five weeks depletion of folate intake (56 µg/day) had a significant increase in the frequency of kinetochore micronuclei, and that transition from a depletion to a

repletion folate intake (~516 µg/day) for seven weeks was associated with a significant decline in kinetochore micronuclei, a strong marker for abnormal chromosome segregation (Titenko-Holland et al., 1998). An *in vitro* study showed that cultured human lymphocytes in a FA deficient medium (12 nmol/L) for nine days is associated with a significant increase of 26 and 35% in aneuploidy frequency of chromosomes 17 and 21 respectively (Wang et al., 2004). Additionally, another *in vitro* study in which human lymphocytes were cultured in a FA deficient medium (20 nmol/L) for 9 days reported a 31.8 and 26% increase in frequency of aneuploidy of chromosomes 8 and 17 respectively (Ni et al., 2010). Also, culture of human WIL2-NS lymphoblastoid cell line for 10 days in low FA concentrations (0.2 and 2 nmol/L) was associated with a significant increase in chromosome 21 aneuploidy (Beetstra et al., 2005). A recent study by Guo et al. showed for the first time a linkage among folate intake, SAC dysfunction and chromosome instability in somatic cells. In that study, human NCM460 colon mucosal cells were cultured in a folate free medium for 21 days. Folate deficiency induced SAC dysfunction by dysregulating the expression of core SAC components including *BUB1, BUBR1, MAD1, MAD2* and *MPS1*. The dysregulation of SAC components contributed to significant induction of chromosome instability, and mitotic aberration presented by higher frequency of chromosome misalignment and missegregation, and spindle abnormality (Guo et al., 2017). This study indicates that folate could have a direct effect on SAC function. In addition to human data, male C57BL/6 mice fed a FA deficient diet (0 mg/kg) for seven weeks had a significant increase of micronuclei in erythrocytes (McDorman et al., 2002).

In male germ cells, a study of healthy human males showed that males who had higher folate intake (1150 µg/day) had a 19% lower frequency of sperm aneuploidy compared with men with moderate folate intake (349 µg/day), and 20% lower aneuploidy compared with men with low intake (115 µg/day ) (Young et al., 2008).

To date, no study has directly examined the effect of FA intake on oocyte aneuploidy in humans and there have been only a limited number of studies that examined the effect of FA on oocyte quality and maturation in female germ cells in animal models. An *in vivo* study reported that three-week old Cr1:CD1 female mice fed a FA deficient diet (0 mg/kg) for ~two months did not affect oocyte meiosis and did not increase the frequency of oocyte abnormalities as indicated by the percentage of abnormal spindle formation compared with females fed a control diet (2.4 mg/kg FA) (Tsuji et al., 2016). *In vitro* cultured porcine oocytes exposed to FA supplemented medium (10 ng/ml) for 44 hours were shown to have improved oocyte maturation and embryonic development after IVF, as indicated by a higher number of blastocyst cells compared with oocytes cultured in a control medium (1 ng/ml) (Kim et al., 2009).

**2.5 Maternal Folate Status and the Incidence of Down Syndrome**

Down Syndrome (DS) or trisomy 21 is a complex genetic disorder that occurs in 1:600-700 newborns. It is characterised by the presence of three copies of chromosome 21 as a result of chromosome non disjunction (NDJ) during oogenesis. The extra chromosome in 95% of DS cases is maternally derived and the incidence increases with advanced maternal aging. DS is associated with multiple comorbidities including: intellectual disabilities, congenital heart defects, metabolic disorder, Alzheimer disease, and specific phenotype features (Mazurek & Wyka, 2015). The presence of genetic variants in folate related genes and lack of maternal folic acid supplementation in the periconceptional period have been linked to meiotic error and chromosome 21 NDJ. Several studies have shown an association between maternal single nucleotide polymorphisms in genes encoding enzymes that are involved in the folate metabolic pathway and increased risk for having a DS birth. The first case-control study to suggest that polymorphisms in folate related genes were a maternal risk factor for a DS birth was conducted almost 19 years ago by James et al. in North America which examined the frequency of the *MTHFR* 677C>T variant in 57 mothers of children

with DS (< 40 years) (cases) and 50 aged-matched control mothers (children with no DS). *MTHFR* catalyzes the reduction of 5,10-methylene-THF to 5-methyl-THF that is required for remethylation of homocysteine to methionine (Figure 2.2). The study found that mothers that were homo/and heterozygous for the variant *MTHFR* gene were 2.6-fold more likely to have a birth with DS. Also, plasma HCY concentrations were significantly higher in the mothers of children with DS. The authors hypothesised that the *MTHFR* 677C>T variant might reduce enzyme activity, folate metabolism and methylation reactions leading to meiotic NDJ, which makes the variant a risk factor for DS (James et al., 1999). A subsequent study by Hobbs et al analysed the maternal polymorphisms in two genes in the folate pathway *MTHFR* 677C>T and *MTRR* 66A>G in a larger population of 157 case and 144 control mothers in North America. The study confirmed that a polymorphism in the *MTHFR* variant is associated with higher risk (1.91-fold increase) of having a child with DS and further indicated that the homozygous variant of *MTRR* confers a significant increased risk (2.44 fold) of having a child with DS. *MTRR* maintains the methionine synthase enzyme in an active form for remethylation of homocysteine to methionine. Also, the study showed that the presence of both variants increases the risk (4.08-fold) of having a DS child (Hobbs et al., 2000). It was hypothesised that polymorphisms in metabolic genes involved in folate metabolism alter folate metabolism and lead to modified methylation of the pericentromeric region of chromosome 21 and, as a consequence, results in abnormal segregation during maternal meiosis and trisomy 21.

A recent meta-analysis of case-control studies revealed that among several polymorphisms, there are at least three maternal folate polymorphisms namely: *MTHFR* 677C>T, *MTRR* 66A>G, and *RFC1* 80G>A, that might be maternal risk factors for having a DS birth (Coppede, 2015). Since the original observations, more than 50 case-control studies from around the world (about

15 countries) have been conducted to confirm the association between polymorphisms in folate related genes and a birth of a child with DS. However, the findings are not always consistent with some studies finding no link between DS birth and folate gene variants and others reporting a positive association between variant of several folate genes and DS birth [for review, see (Coppede, 2015)].

Maternal diet containing folate prior to conception could provide a protective effect against chromosome 21 NDJ. Data analysed from a population case-control study of the National Down Syndrome Project compared the use of FA containing supplements before conception among 702 mothers of infants with DS (cases) and 983 mothers of infants with no DS (control). This study suggested that lack of FA supplementation during the periconceptional period might be a risk factor for chromosome 21 NDJ error (Hollis et al., 2013).

### 2.6 Models for Studying Oocyte Aneuploidy Associated with Advanced maternal age *in vivo*

To study the potential effect of FA supplementation in mitigating the increase in aneuploidy incidence in oocytes caused by advanced maternal age, three mouse models have been proposed employed. The first model is the naturally aged mouse model. This mouse model allows oocytes to age *in vivo* which mimics reproductive aging in humans. The C57BL/6 NCrl (C57BL/6) mouse strain was used as a naturally aged mouse model. Females weaned, at three weeks of age, on either control or supplemented FA diets for ~50 weeks. The effect of FA supplementation on oocyte aneuploidy was assessed at ~12 months of age as aneuploidy incidence at this age was reported to significantly increase by up to 37% in wild type CD1 mice (Merriman et al., 2012).

The second model is the transgenic Bub1 ($Bub1^{+/m}$) mouse model. This mouse model represents a dysfunction of the SAC mechanism, which is one of the proposed mechanisms negatively affected by maternal aging. In this mouse model, female mice harbor a heterozygous mutation in the Bub1 gene that leads to the generation of oocytes with a high incidence of

aneuploidy at a young age. This mouse model provides the opportunity to study aneuploidy at an earlier age which cannot be achieved using the naturally aged mouse model. The transgenic Bub1$^{+/m}$ females were generated by intercrossing C57BL/6 NCrl (C57BL/6) females and Bub1$^{+/m}$ males. Females weaned, at three weeks of age, on either control or supplemented FA diets for 8, 16, and 24 weeks. The effect of FA supplementation on oocyte aneuploidy was assessed in young (11-week), middle-aged (19-week), and old (27-week) females.

The third model is a model of chemically-induced aneuploidy in mouse oocytes. We used the meiotic inhibitor colchicine to induce aneuploidy in oocytes. In this mouse model, aneuploidy can be induced at any age including in relatively young mice which allowed us to study aneuploidy incidence in 10-week old mice. Also, this model provides a time savings compared to the *in vivo* aging model which can take up to 12 months for the assessment of oocyte aneuploidy and risks reduced numbers of ovulated oocytes for analysis. The C57BL/6 NCrl (C57BL/6) mouse strain was used for the colchicine-induced oocyte aneuploidy study to align with the other studies. Females weaned, at four weeks of age, on either deficient, control or supplemented FA diets for six weeks. The effect of FA deficiency, adequacy, and supplementation on oocyte aneuploidy was assessed in 10-week old mice.

The main reasons we used the C57BL/6 NCrl (C57BL/6) mouse strain were: (1) the background of the Bub1$^{+/m}$ females is C57BL/6; (2) a dietary intervention was shown to reduce oocyte aneuploidy in this mouse strain, namely caloric restriction (Selesniemi et al., 2011); and (3) to utilize a consistent mouse strain throughout the three studies to allow comparison of results.

Ethically, oocyte aneuploidy studies are challenging since human oocyte studies are often performed in oocytes isolated from women having fertility issues. Also, as a result of this limitation the oocytes included in these studies are those that are not suitable for fertilization which

can bias observations. Because of these issues, the use of mouse models to study aneuploidy incidence in oocytes is an important and suitable substitution for human oocytes. Mice demonstrate a similar age effect as that observed in humans, and large numbers of oocytes are available for analysis which allow for a better understanding of reproductive aging in women (Chiang et al., 2011; Merriman et al., 2012).

### 2.7 Effect of Dietary Intervention on Oocyte Aneuploidy Associated with Advanced Maternal Age

There is accumulating evidence that dietary factors may play a role in the reduction or prevention of different aspects of maternal aging on oocyte health and function. Chronic administration of oral antioxidants (mixture of vitamin C and E) was shown to reduce the negative effect of aging on oocyte quantity and quality in female mice (Tarin et al., 2002). Supplementation of the culture medium with antioxidants (mixture of α-lipoic acid, α-tocopherol, hypotaurine, N-acetylcysteine and sirtuin) resulted in improved oocyte quality, mitochondrial function (higher membrane potential and ATP production) and embryo development (more blastocysts developed) in oocytes and embryos derived from aged female mice (Silva et al., 2015). In addition, supplementation of culture medium with the antioxidant melatonin prevented several aspects of postovulatory aging (*in vitro*) in mouse and pig oocytes, including maintenance of normal oocyte morphology, alleviating oxidative stress, decreasing apoptosis and improving embryonic development (Lord et al., 2013; Wang et al., 2017).

Caloric restriction has been implicated in oocyte aging. Moderate caloric restriction initiated during mouse adulthood extended the reproductive lifespan in aged females by improving the maintenance of the ovarian reserve and increasing the primordial follicle number. Caloric restriction also improved both litter size and offspring survival rates (Selesniemi et al., 2008). A follow-up study by the same group reported that maintenance of aged female mice on caloric

restriction diet prevented age-associated increases in oocyte aneuploidy, spindle abnormalities, chromosome misalignment, and mitochondrial dysfunction (Selesniemi et al., 2011).

Other nutrients or dietary components have also been implicated in oocyte health and function. Lifelong consumption of a diet rich in omega-3 fatty acids improved oocyte quality (evaluated by the structure of the spindle apparatus and mitochondrial dynamics) and delayed ovarian aging in aged female mice (Nehra et al., 2012). Supplementation of mice with putrescine, an amino acid metabolite, improved oocyte quality and reproductive outcomes through the reduction of the embryo resorption rate and an increase in the number of blastocysts and live pups derived from aged females (Tao et al., 2015). Recently, caffeine was shown to delay oocyte aging by maintaining normal meiotic spindle morphology and thus oocyte quality (Zhang et al., 2017). In summary, dietary interventions may mitigate the effect of maternal aging on oocyte quality and quantity, oocyte aneuploidy, thus improving fertility outcomes.

## 2.8 Thesis Rationale, Hypothesis and Objectives

### 2.8.1 Rationale

Chromosome segregation errors during meiosis lead to the production of aneuploid oocytes with an incorrect number of chromosomes. It is well known that the incidence of oocyte aneuploidy increases with advanced maternal age. The extra chromosome in Trisomy 21 cases is 95% maternally derived and the incidence increases with advanced maternal aging (Hassold & Hunt, 2001). Folic acid, an essential B vitamin, is required for DNA synthesis, chromosome stability and methylation reactions. The presence of genetic variants in folate related genes such as *MTHFR* and *MTRR* has been associated with meiotic error and nondisjunction of chromosome 21 (James et al., 1999 ; O'Leary et al., 2002). It has been reported that lack of FA supplementation during the periconceptional period has been associated with Trisomy 21 birth (Hollis et al., 2013). It was reported that FA supplementation is associated with better quality and maturation of oocytes, higher fertilization, pregnancy and live birth rates and overall female fertility (Berker et al., 2009; Gaskins et al., 2014a; Szymanski & Kazdepka-Zieminska, 2003; Westphal et al., 2006).

In the mouse models that were used in our studies (the naturally aged, transgenic Bub1, and the chemically induced aneuploidy mouse models), the FA intervention was initiated from weaning when oocytes were meiotically arrested at prophase I. This arrest takes years in humans and months in mice which represents a long window during which oocyte quality could be affected by a number of exogenous and endogenous factors (Clift & Schuh, 2013). For example, during this long arrest, cohesion subunits that hold chromosomes together are degraded, key SAC proteins are downregulated, and mitochondrial function is affected as reported in human and mouse studies (Simsek-Duran et al., 2013; Steuerwald et al., 2001; Tsutsumi et al., 2014). We proposed that FA supplementation during mouse adult life might mitigate the effect of maternal aging on oocyte aneuploidy by maintaining or preventing the degradation of the above mechanisms that are

negatively affected by maternal aging (Figure 2.3). Since adequate folate intake is required for proper nucleotide synthesis and cellular methylation reactions (Stover, 2009), we hypothesized that FA supplementation during the adult life of female mice would reduce the adverse effect of maternal aging on specific mechanisms that lead to a high incidence of segregation errors and oocyte aneuploidy.

### 2.8.2 Major Hypothesis

Dietary FA supplementation during adult life will decrease the high incidence of aneuploidy in female germ cells associated with advanced maternal age.

### 2.8.3 Objectives

1- Examine the effect of FA supplementation on oocyte aneuploidy associated with advanced maternal age in two mouse models: the naturally aged mouse model and the transgenic Bub1 heterozygous mouse as a model of premature maternal age induced oocyte aneuploidy.

2- Examine the effect of dietary FA intake on chemically-induced oocyte aneuploidy as a model for studying an age-induced oocyte aneuploidy.

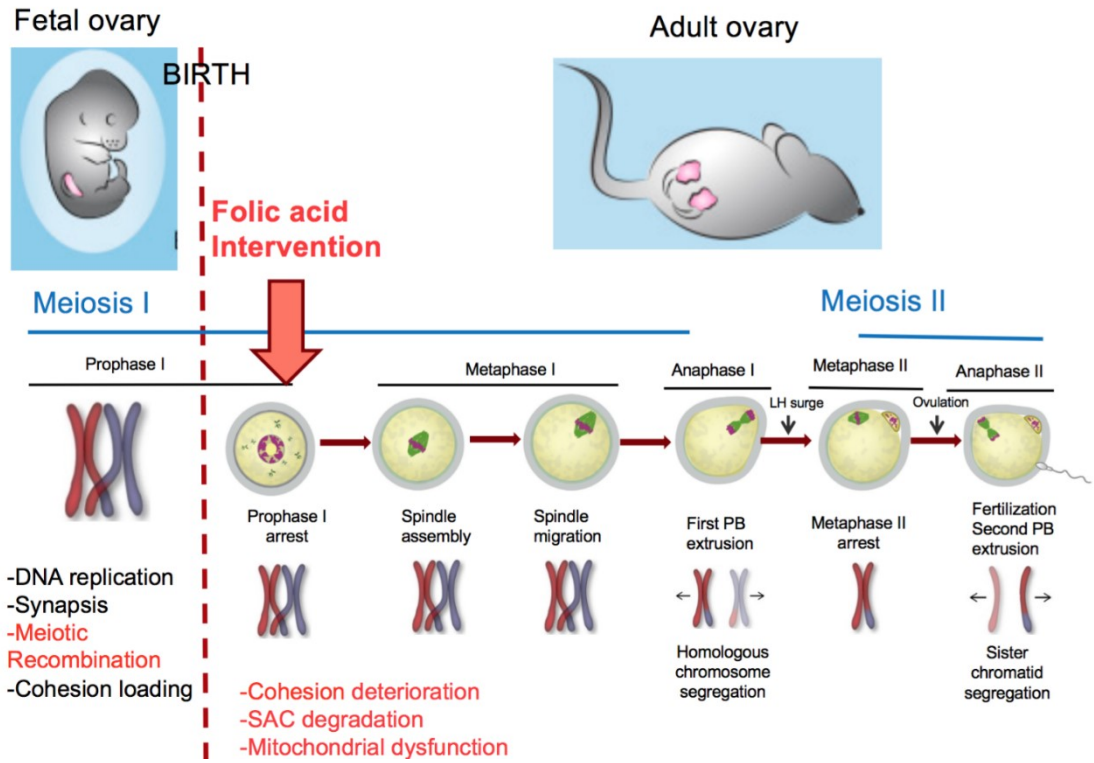



**Figure 2.3. Proposed molecular mechanisms that could be affected by folic acid intervention during the adult life of female mice.** In females, meiosis is initiated in the fetal ovary after mitotic divisions of the primordial germ cells. Oocytes enter prophase during which homologous recombination takes place. Upon completion of the meiotic recombination, oocytes progress to the diplotene stage of prophase I and after which they are arrested until after birth. During the time of arrest, oocytes become surrounded by a single layer of somatic cells, forming the primordial follicle. Primordial follicles remain arrested for years in humans and months in mice until they are recruited for growth. During this long arrest, cohesion subunits that hold chromosomes could degrade, the expression of key SAC proteins could be dysregulated, and mitochondrial function might be affected. In a sexually mature female, primordial follicles are recruited to grow under the effect of follicular stimulating hormone (FSH). The growing follicles (called antral follicles) resume MI in response to the luteinizing hormone (LH) surge and oocytes extrude half of the homologous chromosomes to the first polar body. After MI, oocytes are ovulated, a second meiotic spindle forms and the fully-grown oocytes remain arrested at metaphase II until fertilization. Fertilization triggers the completion of MII and oocytes segregate half of their sister chromatids to the second polar body. After the division, chromosomes from the oocyte and the sperm form separate pronuclei in the zygote which then fuse for the mitotic divisions of the embryo. Intervention with FA during the adult life of female mice might help reduce the adverse effect of maternal aging on specific mechanisms that could lead to high incidence of segregation errors and aneuploidy

# Chapter 3: Investigating the Effect of Dietary Folic Acid Supplementation on Oocyte Aneuploidy Associated with Advanced Maternal Age in Bub1 Heterozygous Female Mice

# Chapter 3

## Investigating the Effect of Dietary Folic Acid Supplementation on Oocyte Aneuploidy Associated with Advanced Maternal Age in Bub1 Heterozygous Female Mice


### Abstract

Chromosome segregation errors during female meiotic divisions lead to aneuploidy in the oocyte and the resulting embryo. Aneuploidy in female germ cells is the leading genetic cause of spontaneous abortions and developmental disorders in humans. The incidence of aneuploidy in oocytes rises significantly with advanced maternal age. Evidence suggests that dietary intervention can mitigate the effect of maternal age on oocyte aneuploidy. Folic acid, an essential B vitamin, is required for DNA synthesis, chromosome stability, methylation reactions, and proper gene expression; these processes are fundamental for female reproductive physiology. In this chapter, we examined the effect of FA supplementation (8 mg/kg) on young (11 weeks old), middle-aged (19 weeks old) and old (27 weeks old) Bub1-heterozygous female mice, a transgenic mouse model that prematurely produces oocytes with high incidence of aneuploidy, and which increases with advanced maternal age. We reported that old female mice fed a FA supplemented diet for 24 weeks had significantly more (2-fold) normal oocytes, having the exact chromosome number (20 chromosomes in mice), compared to females fed a FA control diet (2 mg/kg). This phenotype was not detected in either young or middle-aged females. Thus, FA supplementation throughout adult life may maintain chromosome stability and proper segregation during MI which increases the proportion of normal oocytes in old females.

## 3.1 Hypothesis

Dietary FA supplementation will reduce the incidence of oocyte aneuploidy associated with advanced maternal age in Bub1 heterozygous females.

## 3.2 Study Rationale

The incidence of oocyte aneuploidy increases with advanced maternal age (Hassold & Hunt, 2001). Maternal aging has been associated with dysfunction of the SAC mechanism which increases the risk of aneuploidy incidence in oocytes. It has been reported in mice that heterozygosity for a mutation in a core SAC component, Bub1, affecting the function of the Bub1 protein kinase, and leads to an increased frequency of aneuploidy oocytes that further increases with advanced maternal age (Leland et al., 2009). A recent study by Guo et al. 2017 showed for the first time in cultured human colonic cells that folate deficiency induced SAC dysfunction by dysregulating the expression of core SAC components including Bub1 and that it contributed to a significant induction of chromosome misalignment and missegregation, and spindle abnormality (Guo et al., 2017). This study indicates that folate status could have a direct effect on SAC function. In contrast, FA supplementation has been shown to improve oocyte quality and maturation (Kim et al., 2009; Westphal et al., 2006). As such we proposed that FA supplementation might mitigate the effect of oocyte aneuploidy associated with advanced maternal age in Bub1 heterozygous females.

## 3.3 Introduction

### 3.3.1 The Importance of Bub1 Component in the Spindle Assembly Checkpoint Mechanism

The spindle assembly checkpoint (SAC) is a cell cycle surveillance mechanism that monitors proper kinetochore attachment to the spindle and prevents chromosome missegregation by delaying anaphase until accurate attachment to the spindle apparatus occurs (Duro & Marston, 2015). The SAC senses unattached kinetochores on spindle microtubules and prevents the cell

from progressing to anaphase until full attachment is achieved. Unattached kinetochores activate the SAC mechanism, whereas full attachment of all kinetochores silences SAC, permitting anaphase to proceed (Mailhes, 2008). The SAC is composed of at least fourteen evolutionarily conserved proteins including members of the Mad and Bub families (Vogt et al., 2008). The primary target for SAC is the anaphase promoting complex/cyclosome (APC/C) that targets several proteins for proteolytic degradation including the activator protein cell division cycle 20 ($Cdc_{20}$).

The budding uninhibited by benzimidazole 1 (Bub1) protein is a kinase that localizes to the kinetochores of unaligned chromosomes early during prophase and is required for the recruitment of other SAC components (Mad2, BubR1, Cenp-E) to the kinetochore and for monitoring microtubule attachment to kinetochores (Taylor & McKeon, 1997). In response to spindle damage, Bub1 inhibits the activity of $Cdc_{20}$, the APC activator, through the phosphorylation of Mad1 leading to disassociation of the Mad1-Mad2 complex and release of free Mad2 which binds and inhibits $Cdc_{20}$. In addition, Bub1 inhibits APC activity through the activation of BubR1-Bub3-Mad2 complexes in kinetochores in response to spindle damage (Marchetti & Venkatachalam, 2010). In both mitosis and meiosis, Bub1 monitors centromeric cohesion either through regulating the localization of shugoshin, which protects centromeric cohesion Rec8 from separase cleavage, (Kitajima et al., 2005; Kitajima et al., 2004) or through the activation of SAC in response to an unattached kinetochore (Perera et al., 2007).

Bub1 is an essential component of SAC that controls many aspects of the mitotic and meiotic processes including the timing of division, chromosome congression and segregation (Taylor & McKeon, 1997; Yin et al., 2006). Studies using transgenic animal models demonstrated that the depletion of Bub1 leads to premature anaphase, and an increased incidence of abnormal

chromosome alignment and separation in mitosis (Meraldi & Sorger, 2005; Perera et al., 2007). Multiple mouse models have been developed to study the effect of losing Bub1 function in female meiosis. Results from these studies demonstrate that the depletion of Bub1 leads to similar phenotypes in both meiosis and mitosis with the acceleration of MI, misaligned chromosomes at the metaphase plate, the presence of premature sister chromatid separation (PSCS) and the occurrence of oocyte aneuploidy (Leland et al., 2009; McGuinness et al., 2009; Tsurumi et al., 2004; Yin et al., 2006).

Maternal aging is associated with the disturbance of core SAC components which could be a contributing factor to the increased incidence of oocyte aneuploidy. Several studies have reported that maternal aging affects the expression (gene and/or protein) of Bub1. In humans (Steuerwald et al., 2001) and mice (Pan et al., 2008; Marangos et al., 2015), a decline in the mRNA level of Bub1 has been reported in oocytes of older females with respect to younger females. Together these data indicate the importance not only of the SAC mechanism, but also the role of Bub1 in oocyte health.

## 3.4 Materials and Methods

### 3.4.1 Generation of Bub1 Heterozygous Mice

All mice were cared in accordance with the Guidelines of the Canadian Council on Animal Care (CACC), as described in the CACC Guide to the Care and Use of Experimental Animals (Olfert et al., 1993). The study was approved by the Health Canada Ottawa Animal Care Committee. Female mice were pair-housed in plastic, HEPA-filtered cages and maintained under a 12 hour light/dark photoperiod at a room temperature of 22 ± 2 ºC and standard humidity of 40% - 60%. Mice had *ad libitum* access to food and water.

Bub1 heterozygous mice ($Bub1^{+/m}$) were previously generated using the Baygenomics gene-trap embryonic stem cell resource as described in (Leland et al., 2009). Briefly, the mutation

was generated using a gene-trap cassette consisting of a splice acceptor-βgeo cassette (β-galactosidase-neomycin fusion gene). At the gene level, the gene trap was integrated within intron 8 of the *Bub1* gene. The insertion of the gene trap leads to loss of expression of the 17 downstream exons. At the protein level, the gene-trap leads to the generation of a putative truncated Bub1-βgeo fusion protein containing the first 269 aa of the 1,057 aa long wild type (WT) protein. The transgenic Bub1 protein lacks the kinase domain but retains the Mad3-Bub1 domain and the kinetochore binding domain fused to the βgeo reporter protein and is therefore able to bind to the kinetochore. The transgenic protein presumably interferes with the recruitment and binding of WT Bub1 to other SAC components (Figure 3.1) (Leland et al., 2009).

For this study, $Bub1^{+/m}$ female mice (total of 156 mice) were generated by mating $Bub1^{+/m}$ males from the Health Canada in-house colony and WT females (C57BL/6). The C57BL/6 females were purchased from Charles River Laboratories (Quebec, Canada).

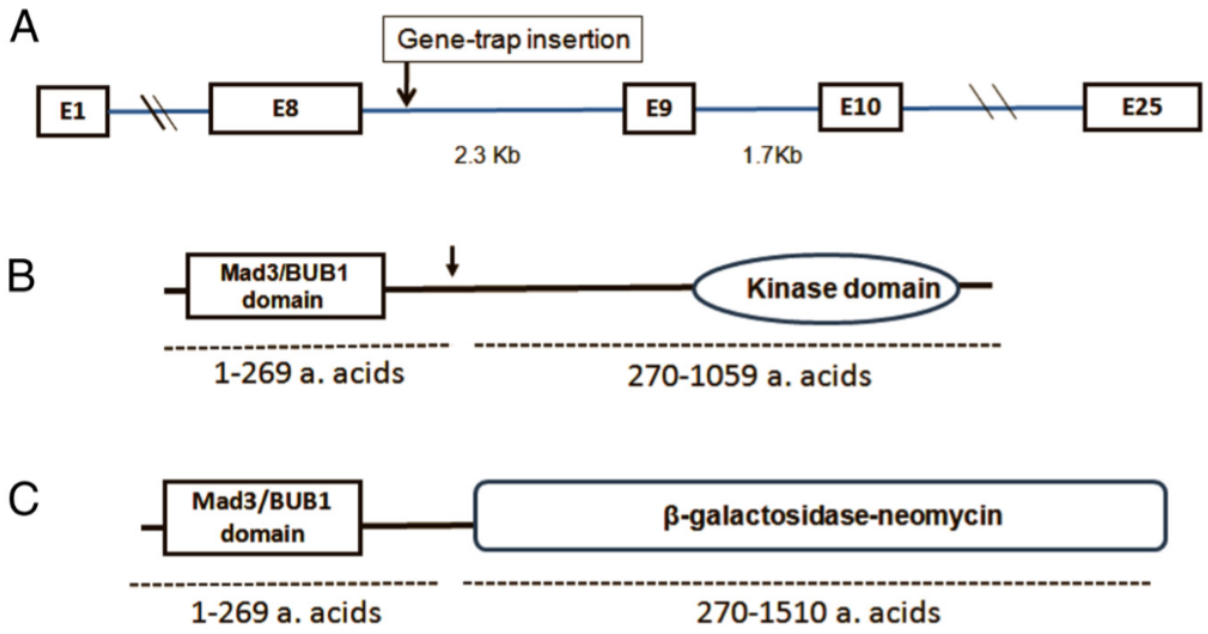



**Figure 3. 1 Schematic model of Bub1 gene disruption**. **(A)** Gene structure of the WT Bub1 gene; arrow indicates the gene-trap insertion site. **(B)** Protein organization of the WT Bub1 showing the two functional domains: the kinetochore binding domain and the kinase domain; arrow indicates the relative trap insertion site. **(C)** Organization of the transgenic Bub1 protein (Bub1-βgeo) showing the replacement of the kinase domain by βgeo reporter peptide. **Figure from (Leland et al., 2009) with permission (Appendix II).**

### 3.4.2 Genotyping of the Generated Mice

Genotyping for Bub1$^{+/m}$ mice was performed as described by Leland et al., 2009. Genotyping was done using tail snips collected at fourteen days of age. Tail snips (approximately 3 mm) were collected in sterile nuclease free 1.5 ml microcentrifuge tubes. Tail snips were either used immediately to extract the DNA or frozen at -20°C until later use. DNA was extracted from tail snips using either Chelex 100 Resin from BioRad (Cat. # 1422822) or the High Pure PCR Template Preparation Kit from Roche (Cat. # 11796828001). For DNA extraction using Chelex, a cross sectioned tail snip was transferred to a mixture of 30 µl of (50:50 dissolved Chelex in $H_2O$) and 70 µl of RNase-free $H_2O$ in 5 ml falcon tube. The mixture was boiled at 95°C for 10 minutes then cooled down in ice for 30 minutes.

For the DNA extraction using the High Pure PCR Template Preparation Kit, the "Isolation of Nucleic Acids from Mouse Tail" protocol included with the kit was followed according to the manufacturer's instructions. Briefly, the extracted DNA was eluted using pre-warmed Elution Buffer. The eluted DNA was either used directly or stored at -20°C for later analysis.

The concentration of total DNA was verified using the NanoDrop spectrophotometer (NanoDrop-2000, Technologies, Inc.). PCR was performed using the illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Life Science, Cat. # 27-9557-02) in a final volume of 25 µl. Each PCR reaction contained 150 ng of DNA, 3 µl of 10 µM optimized primer mix which are: (Bub1_GS1 5`-AAG AAG CAT GAG CAG TGG GTT-3`; Bub1_GS2 5`-AGG CAC ACT CCA AAC ATG TCA-3`; and Bub1_TR2 5`ACC TGG CTC CTA TGG GAT AG-3`), and Nuclease-Free Water to a total of 25 µl. The PCR cycling conditions were as following: 2 minutes at 95 °C, 35 cycles of: 45 seconds at 95°C, 35 seconds at 61°C, 1:30 minutes at 72°C and a final extension for 7 minutes at 72°C, then last step holding at 4°C. The PCR product was separated on 2% agarose gel stained with ethidium bromide (E-Gel double comb 2% agarose gel (Invitrogen, Cat #

Y141217). The separated PCR products were visualized using the ChemiDoc$^{TM}$ Imaging System (Bio-Rad, Life Science Research.Inc). When amplified by Bub1_GS1 and Bub1_GS2 primer sets, a single upper band approximately 1000 bp long indicates a WT female. Double bands (with the second band appearing approximately at 800 bp) amplified by the Bub1_GS1 and Bub1_TR2 primer sets indicate Bub1$^{+/m}$ female. The E-Gel Low Range Quantitative DNA Ladder was used (Invitrogen, Cat # 12373-031) (Figure 3.2).

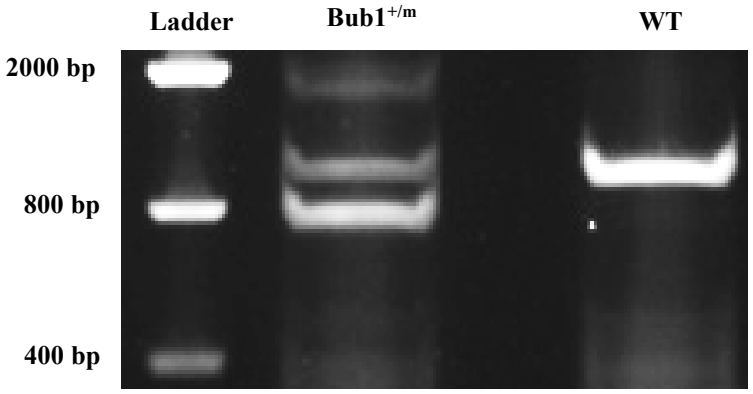



**Figure 3. 2 Genotyping analysis of the generated Bub1$^{+/m}$ female mice**. Genotyping was done using tail snips collected from 14-day old mice. PCR-based genotyping analysis is shown for WT and transgenic Bub1 females. A single 1000 bp band (right lane) indicates a WT female, whereas double bands at 1000 and 800 bp (left lane) indicates a Bub1$^{+/m}$ female.

### 3.4.3 Experimental Design and Diets

The experiment was designed to examine the effect of FA supplementation on age-associated aneuploidy (Figure 3.3). Bub1$^{+/m}$ female mice (total of 156 mice) were produced by breeding Bub1$^{+/m}$ males and WT females (C57BL/6). At weaning age (21±1 days), female mice were fed one of two FA-defined diets based on the AIN-93G formula (n = 78 per diet). The diets contained 2 mg FA/kg (control) or 8 mg FA/kg (supplemented) (Dyets, Inc.; Bethlehem, PA). The control diet is analogous to the recommended dietary allowance 0.4 mg FA/ day for women who could become pregnant. It is representative of an adequate dietary FA intake for rodents as recommended by the American Institute of Nutrition (Reeves et al., 1993). The supplemented diet is 4-fold the control diet and approximates the combined FA intake 1.6 mg FA/day for adults, representing a maximum dose of FA intake which can be achieved from folate intake from the natural sources, FA from fortified foods and prenatal FA supplements available in the Canadian market. Females were fed the diets for 8 (n = 40, 20/diet group), 16 (n = 40, 20/diet group) or 24 (n = 76, 38/diet group) weeks. The rationale for using a minimum of 20 females was to generate a sufficient number of oocytes for the analysis of the presence of aneuploidy. The target was to analyse ~200 MII oocytes for the presence of aneuploidy. If it is assumed that each female ovulates ~25 oocytes, 20 females per group would ovulate a total of 500 oocytes. Considering that oocytes are lost during the preparation and a number of oocytes will not be analysed because of chromosome over-spreading and clumping, we assumed that we will collect 200 oocytes per group for aneuploidy analysis. Taking into consideration that oocyte number decreases with aging, and 6-month old CD1 female mice have ~50% fewer ovulated oocytes compared to 3-month old female mice (Merriman et al., 2012), an almost doubling of female (n = 37) was used for the 24 week collection. Each female was weighed weekly from weaning until the day of necropsy for 8 ± 1, 16 ± 1 and 24 ± 1 weeks. Females were defined as follows: young female (8 weeks on diet/11

weeks of age), middle-aged female (16 weeks on diet/19 weeks of age) and old female (24 weeks on diet/27 weeks of age). According to the aneuploidy incidence in the $Bub1^{+/m}$ model reported in Leland et al., 2009, and the graphs of human and mouse oocyte aneuploidy incidence reported in Jones & Lane, 2013 and Merriman et al., 2013, the classification of the ages used in our mouse study approximated the following human ages: the young $Bub1^{+/m}$ female mice is equivalent to women in her 20s (< 30 years); the middle-aged $Bub1^{+/m}$ female is equivalent to ~35 years in women; and the old $Bub1^{+/m}$ female is equivalent to > 45 years in women (Jones & Lane, 2013; Leland et al., 2009; Merriman et al., 2012).

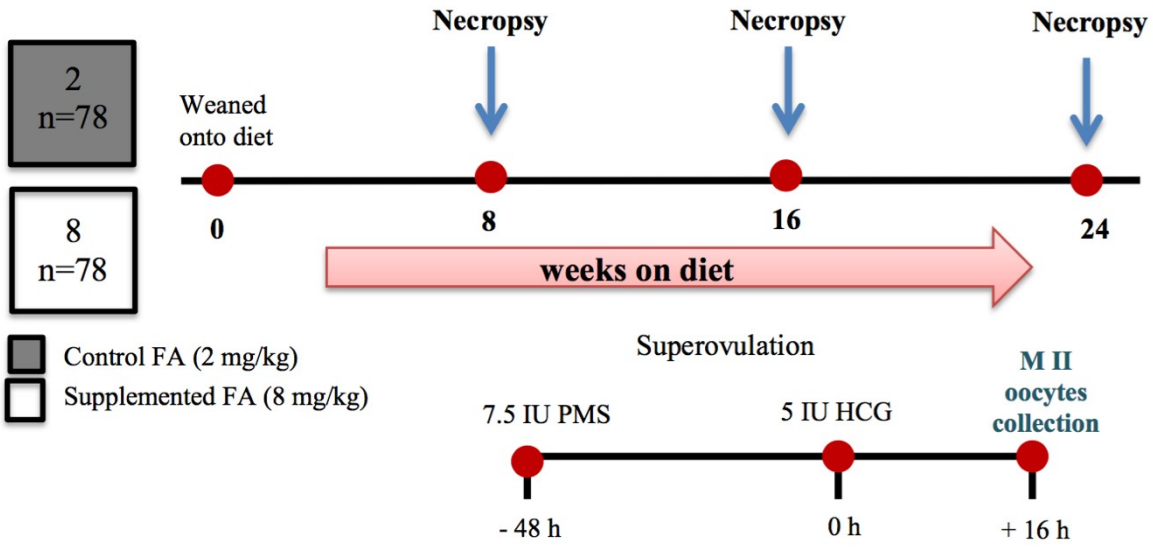



**Figure 3. 3 Study design and superovulation method.** Bub1$^{+/m}$ female mice were weaned to either a FA control or supplemented diet for 8, 16 or 24 weeks. Females were superovulated by i.p. injection with 7.5 IU of PMS followed 48 h later by 5 IU of HCG to stimulate ovulation. Oocytes were harvested 16 h after HCG administration.

### 3.4.4 Superovulation Method

Females were superovulated by intraperitoneal injection (i.p.) with 7.5 IU pregnant mare's serum (PMS; Sigma-Aldrich) followed 48 h later by an i.p. injection of 5.0 IU human chorionic gonadotrophin (HCG; Sigma-Aldrich) to stimulate ovulation as described by Mailhes & Yuan, 1987a. Females were anesthetized under isoflurane 16 h after the administration of HCG (Figure 3.3) and euthanized by cardiac puncture followed by cervical dislocation.

### 3.4.5 Tissues Collected at Necropsy

Oocytes were collected and processed for the metaphase II chromosome analysis as described below in section 3.5.6. Blood was collected by cardiac puncture into EDTA coated Vacutainer® tubes. Red blood cells (RBCs) and plasma were separated by centrifugation at 1000× *g* at 4ºC. Three plasma aliquots were made for the analysis of circulating folate, hormones and homocysteine. Plasma and the RBC pellet were stored at -80°C for further analysis.

### 3.4.6 Cytogenetic Analysis of MII Oocytes

At each necropsy, a group of five to eight females were euthanized and their oocytes were pooled for analysis. Oocytes were processed for the metaphase II chromosome analysis to assess the presence of aneuploidy and PSCS as described previously (Mailhes & Yuan, 1987a). Briefly, the entire reproductive tract including ovaries, oviduct and part of the uterine horn were isolated from both sides of a female and placed in warm Hank's Balanced Salt Solution (37ºC) (HBSS; Sigma-Aldrich). Using a dissecting microscope, the oviduct was isolated from the ovary and the uterine horn, then oocytes were harvested from oviducts and treated with 200 units/ml hyaluronidase solution (Sigma-Aldrich) for 20 minutes at 37ºC to remove the cumulus cells surrounding the oocytes. Oocytes (pooled from 7 to 12 females) were rinsed with HBSS and then treated with hypotonic solution (sodium citrate 0.3%; Fluka) for 30 minutes at 20ºC. Oocytes were then fixed with two sequential dilutions of (methanol:acetic acid vol:vol; 3:1 and 1:1) with four

steps for a total of 25 minutes. Fixed oocytes were dropped onto a wet microscope slide and stained with ProLong™ Diamond Antifade Mountant with DAPI (Cat# P36962, Life Technologies) and covered with a cover slip. Chromosomes were scored for aneuploidy and PSCS using a fluorescence microscope (Zeiss Axiophot Fluorescent Microscope with Zeiss Axio Cam) at 10× then 100× NeoFluor objectives. The number of chromosomes and chromatids was counted at 100× magnification with oil immersion. An oocyte was considered aneuploid if it contained a chromosome number that was different than 20 when both dyads and single chromatids were counted (the haploid number in mice is 20). For instance, if the oocyte contained 19 dyads and 2 chromatids (total of 20) it was considered normal, whereas an oocyte with 19 dyads and 4 chromatids (total of 21) was considered aneuploid. Oocytes with chromosome clumping or over-spreading were excluded from the analysis. The same observer performed the preparation and scoring for all slides in a blinded fashion. For the young and old females, ~70% and 50% respectively of the targeted number of MII oocytes (200) to analyse for the presence of aneuploidy were achieved. However, the middle-aged females had some technical issues such as losing oocytes during the fixation process which led to a loss of ~80% of the targeted oocyte number.

### 3.4.7 Folate Extraction from RBCs

Folate was extracted from RBCs as described by Molloy & Scott, 1997 as follows: 75 µl of the RBC pellet (n = 15 samples/diet/time point) were mixed with 200 µl of extraction buffer (2% (w/v) sodium ascorbate (Sigma-Aldrich, St. Louis, MO); 0.2 M beta-ME; 0.05 M HEPES pH 7.85 (Sigma,); and 0.05 M CHES pH 7.85 (Sigma)). Samples were centrifuged at 1500× *g* for 5 minutes at 4°C then placed on ice. 20 µl of the supernatant was removed from each sample and stored at -80°C for later use for the Lowry assay as described below (section 3.5.9). The remaining 255 µl were boiled at 100°C for 10 minutes then placed on ice for 10 minutes. Samples were

centrifuged at 21,000× *g* for 5 minutes at 4°C. Supernatant was collected into a 1.5 ml tube, and the exact volume was recorded for each sample. A 0.25× volume of dialyzed Rat Conjugase Serum was added to each sample, then samples were incubated in a water bath at 37°C for 3 hours. Samples were boiled for 5 minutes at 100°C then placed on ice for 5 minutes. Samples were centrifuged at 21,000× *g* for 5 minutes at 4°C. The supernatant was collected into a 1.5 ml tube on ice and the exact volume was recorded. Samples were stored at -80°C until further use to run the folate assay as described below (section 3.5.8).

### 3.4.8 Plasma and RBC Folate Measurement

Plasma and extracted RBC folate (section 3.5.7) were measured in a subset of samples (n = 15 samples/diet/time point) using the *Lactobacillus casei* (*L. casei*) microbiological assay as described by (Molloy & Scott, 1997). In this assay, folate is extracted from the tissue of interest and plated with *L. casei*. *L. casei* are dependent on folate for growth, therefore their growth can be correlated with total folate content in a sample. The *L. casei* are only able to metabolize monoglutamate forms of folate, so folates in a sample have to be converted to the monoglutamate form by adding of rat serum conjugase (Shane, 2011). To measure folate from plasma and RBCs, plasma samples were diluted 40× in 0.5% (w/v) sodium ascorbate, and the extracted RBC folate was diluted 100× in sterile water. In both cases, master mix containing extraction buffer (2% sodium ascorbate, 0.2 M beta-ME, 0.05 M HEPES, and 0.05bM CHES), working buffer (1 M potassium phosphate, sodium ascorbate, and Milli-Q $H_2O$), *L. casei* media and sterile filtered water was prepared according to the number of samples being analysed. Each well contained 1 µl extraction buffer, 8 µl working buffer, 150 µl *L. casei* media, and 111 µl sterile filtered water. *L. casei* media (Difco™ Folic Acid Casei Media, Becton Dickinson and Company, Sparks, MD, USA) was prepared according to manufacturer's instructions. *L. casei* bacteria was diluted 100×

in sterile filtered 0.9% NaCl. In a sterile clear 96 well plate, 270 µl of master mix, 20 µl of 0.9% NaCl and 10 µl of sterile water were added to blank wells. 6.22 ml of diluted *L. casei* was added to the remaining master mix, then 290 µl of this master mix *L. casei* was added to sample and standard wells. Seven dilutions of standard ranging from 2-100 foml/µl folate was prepared. 10 µl of sterile water was added to the 0 points of the standard curve. 10 µl of either standard or samples was added to all remaining wells. Outside wells (top and bottom rows, first and last columns) were not used for standards or samples; instead, 200 µl of sterile water was added to them. All plates were covered and incubated overnight for approximately 16 hours at 37°C in a 5% $CO_2$ incubator. Absorbance was read at 595 nm single wave length.

### 3.4.9 RBC Protein Content

Since folate in tissues is protein bound, RBC folate concentrations were normalized to total protein content. The modified Lowry assay was used to quantify the protein content in a given sample as described in (Bensadoun & Weinstein, 1976). RBC samples were diluted in water at a ratio of 1:30. In a 1.5 ml tube, 5 µl of the diluted samples were added to 670 µl of water. Five dilutions of standard ranging from 2-30 ug/µl was prepared from 5 mg/ml BSA stock. To each standard/sample tube, 5 µl of 2% deoxycholic acid and 75 µl of 100% trichloroacetic acid (TCA) were added. Samples were incubated on ice for 15 minutes then centrifuged at max speed at 4°C for 15 minutes and the supernatant was discarded from pellets. 1 ml of fresh Lowry C (50:1 mix of Lowry A with Lowry B) was added to each pellet and vortexed well until dissolved. Lowry A contains 10 g $Na_2CO_3$, 2 g NaOH, 0.1 g Potassium sodium tartrate tetrahydrate, and 500 ml Milli-Q $H_2O$; Lowry B contains 2.5 g $CuSO_4$-5$H_2O$in 500 ml Milli-Q $H_2O$. Samples were incubated at room temperature for 10 minutes. Folin-Ciocalteu's Phenol (F-C) was diluted 1:1 with Milli-Q water, and then 100 µl of diluted F-C was added to each sample and incubated 40 minutes in the

dark at room temperature. 200 µl of sample was added to each well in a sterile clear 96 well plate and the absorbance was immediately read at 740 nm.

### 3.4.10 Homocysteine (HCY) Analysis

plasma HCY is a biomarker for functional folate status, as it indicates the ability of cells to remethylate homocysteine to form methionine (Kopp et al., 2017). Plasma HCY was analyzed using the Advia Centaur XP immunoassay (Siemens Canada), as described by the manufacturer. The HCY assay is an immunoassay using chemiluminescent technology based on reducing different forms of HCY in the sample to free HCY. Free HCY is then converted to S-adenosylhomocysteine (SAH) and coupled to paramagnetic particles with anti-SAH. The report for total HCY represents the relative amount of light units detected by the system as measured by Advia Centaur system. The steps were performed as follows: 20 µl of plasma was added to a cuvette; 50 µl of reducing reagent was added to the cuvette; sample was incubated for 3 minutes at 37ºC; 50 µl of enzyme reagent was added to the cuvette; the sample was incubated for 2.5 minutes at 37ºC; 250 µl of solid face reagent was added and the sample incubated for 2.5 minutes at 37ºC; 100 µl of lite reagent was added and the sample incubated for 2.5 minutes at 37ºC; supernatant was aspirated, and the cuvettes were washed with reagent water, 300 µl each of acid reagent and base reagent were added to the sample to initiate the chemiluminescent reaction.

### 3.4.11 Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) Analysis

High levels of FSH and LH contribute to the incidence of oocyte aneuploidy in humans and mice (Roberts et al., 2005; Xu et al., 2011) and elevated levels of FSH and LH are considered to be endocrine biomarkers for aging in humans and mice (Ahmed Ebbiary et al., 1994; MacNaughton et al., 1992 ; Parkening et al., 1980), making them of interest for this study. Plasma FSH and LH concentrations were measured using the Milliplex® MAP Mouse Pituitary Magnetic

Bead Panel-Endocrine Multiplex assay (Cat # MPTMAG-49K; EMD Millipore, Billerica, MA, USA) following the manufacturer's instructions. The multiplex assay is a type of immunoassay that uses fluorescent magnetic beads pre-coated with analyte-specific capture antibodies known as MagPlex®-C-microspheres to simultaneously measure multiple analytes in a single sample. Briefly, the sample is added to a mixture of color-coded beads with two fluorescent dyes pre-coated with specific capture antibodies. After the analyte from the sample is captured by the bead, it binds to the biotinylated detection antibody. The reaction mixture then incubated with the reporter molecule (Streptavidin-PE conjugate) to complete the reaction on the surface of each bead. Beads are then read on a high-speed digital-single detection instrument to identify each bead and quantify the result based on the fluorescent reporter signals. A customised panel for FSH and LH only was used in this analysis. Each sample was run in duplicate in a 96-well plate. 200 µl of assay buffer was added to each well of the plate and shacked for 10 minutes at room temperature (RT). Assay buffer was decanted, 25 µl of standard and 10 µl of plasma sample were added to the appropriate wells followed by 25 µl of matrix solution. 25 µl of the mixed beads was added to the wells, plate was sealed and incubated on a shaker overnight at 4ºC. Then well contents were removed, and the plate washed 2× with 200 µl wash buffer. 50 µl of detection antibodies was added to wells, plate incubated on a shaker for one hour at RT. 50 µl of streptavidin-phycoerythrin was added to each well, incubated for 30 minutes at RT, then washed 2× with 200 µl wash buffer. 100 µl of sheath fluid was added to wells and shacked for 5 minutes. Finally, plate was read on Luminex 200™ Instrument and the median fluorescent intensity was used to calculate the analytes concentrations in a sample.

### 3.5 Statistical Analysis

Statistical analyses were performed using SigmaPlot 12.5 software for Windows (Systat Software, Inc.). Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance. Data were transformed if they did not meet the parametric assumption. Statistical significance was tested using Two-Way analysis of variance (ANOVA), the two variables are diet and age, followed by Tukey multiple comparison test to evaluate the differences in body weight, plasma and RBC folate, plasma HCY, plasma FSH and LH and number of ovulated oocytes between the two diet groups at three time points. Chi-square test was used to compare the euploid oocyte frequency, the frequency of premature sister chromatid separation (PSCS) and premature anaphase (PA) between the two diet groups at each time point. Euploidy, PSCS and PA frequencies were calculated relative to the total number of MII oocytes analysed. Statistical differences were considered significant if $p < 0.05$. Data are presented as mean±standard error of the mean for body weight, plasma and RBC folate, plasma HCY, plasma FSH and LH concentrations, and number of ovulated oocytes; and as a percentage for euploidy, PSCS and PA frequencies.

### 3.6 Results

#### 3.6.1 Body Weight

Body weight did not differ between females fed control and supplemented FA diets at any time point. Not unexpectedly, body weight increased significantly over time ($p < 0.001$). Total weight gain from weaning did not differ between females fed control and supplemented FA diets ($p = 0.406$). Total weight gain was greater as the females aged ($p < 0.001$) (Figure 3.4).

#### 3.6.2 Plasma Folate

Females fed a FA supplemented diet had higher plasma folate concentration compared to females fed a control diet at all three time points ($p < 0.001$). Also, a time effect was observed,

such that plasma folate concentration was lower as the females aged, regardless of whether they were fed the control or supplemented diet ($p < 0.001$) (Figure 3.5).

### 3.6.3 RBC Folate

Old females fed the FA supplemented diet had higher RBC folate concentration compared to females fed a control diet ($p < 0.001$). FA supplemented diet had no effect on RBC concentrations in young ($p = 0.157$) and middle-aged ($p = 0.411$) females. No time effect ($p = 0.569$) or interaction between diet and time ($p = 0.070$) on RBC folate concentration was detected (Figure 3.6).

### 3.6.4 Plasma Homocysteine

Plasma HCY concentration did not differ between the females fed control and supplemented FA diets at all three time points ($p = 0.199$). However, a time effect was observed such that plasma HCY was lower in young ($p = 0.017$) and old ($p = 0.006$) females compared to middle-aged females respectively (Figure 3.7).

### 3.6.5 Plasma FSH and LH

FSH and LH concentrations did not differ between females fed the FA supplemented diet and control diets at all three time points ($p = 0.416$ for FSH; $p = 0.194$ for LH). Also, there was no time effect on FSH and LH concentrations ($p = 0.479$ for FSH; $p = 0.261$ for LH). An interaction between diet and time was not detected for either hormones ($p = 0.052$ for FSH; $p = 0.312$ for LH) (Figure 3.8. A, B).

### 3.6.6 Number of Superovulated Oocytes

The number of oocytes ovulated by each female did not differ significantly between females fed a FA supplemented diet or a control diet at any time point ($p = 0.340$). As expected, the oocyte number was significantly lower as females aged, $p = 0.002$ when comparing young to

middle-aged females; $p = 0.010$ when comparing middle-aged to old females; $p < 0.001$ when comparing young to old females (Table 3.1) (Figure 3.9)

### 3.6.7 Euploidy in MII Oocytes

The number of normal (euploid) oocytes (oocytes that have the haploid number of chromosomes = 20) for the two diet groups at three time points was analysed. The number of normal oocytes did not differ between the females fed control and supplemented FA diets in young ($p = 0.989$) and middle-aged females ($p = 0.993$). However, old females fed a FA supplemented diet had significantly 2-fold higher normal oocytes (25%) compared to the control group (9%) ($p = 0.005$) (Table 3.1) (Figure 3.10). The frequency of euploid oocytes did not change over time ($p = 0.356$).

### 3.6.8 Premature Sister Chromatid Separation (PSCS) Frequency in MII Oocytes

The PSCS frequency did not differ between the FA control and supplemented diets in the young ($p = 0.697$), middle-aged ($p = 0.183$), or old females ($p = 0.189$) (Table 3.1) (Figure 3.11). The PSCS frequency in normal oocytes did not differ between old females fed either the FA control or supplemented diet ($p = 1.000$) (Table 3.2). The PSCS frequency decreased significantly over the time ($p = 0.012$).

### 3.6.9 Premature Anaphase II (PA) Frequency in MII Oocytes

The PA frequency did not differ between the FA control and supplemented diets in the young ($p = 0.567$), middle-aged ($p = 0.675$) or old females ($p = 0.426$) (Table 3.1) (Figure 3.12). The PA frequency decreased significantly over the time ($p = <0.001$).

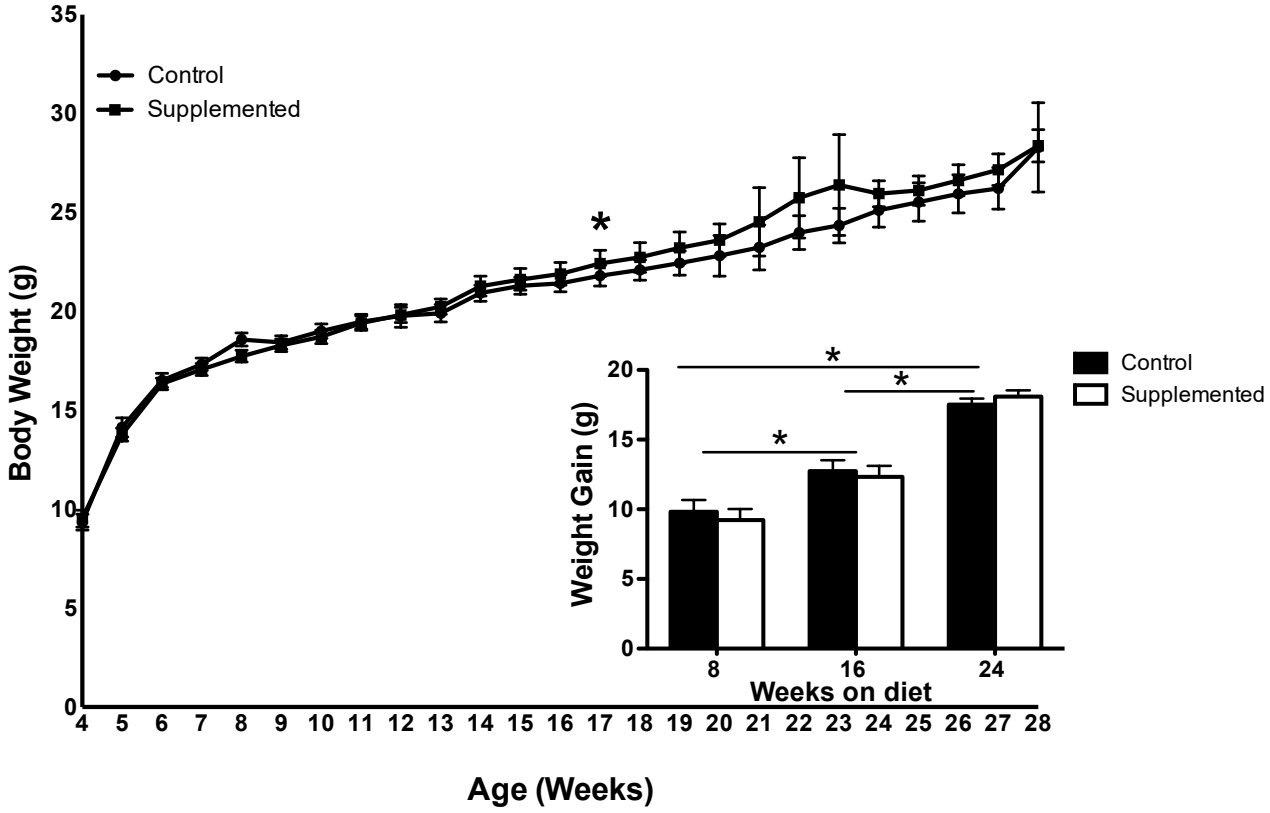



**Figure 3. 4 Body weight at each time point and overall weight gain of Bub1$^{+/m}$ female mice fed a folic acid control or supplemented diet.** For body weight, circle, control diet; square supplemented diet. *Inset*, total weight gain from meaning. Black bars, control diet; white bars, supplemented diet. Data are presented as mean ± SEM. Two-way ANOVA followed by Tukey multiple comparison tests was used to assess the differences between the two diet groups at each time point. Asterisk indicates a significant effect of time ($p < 0.001$) for body weight and overall weight gain.

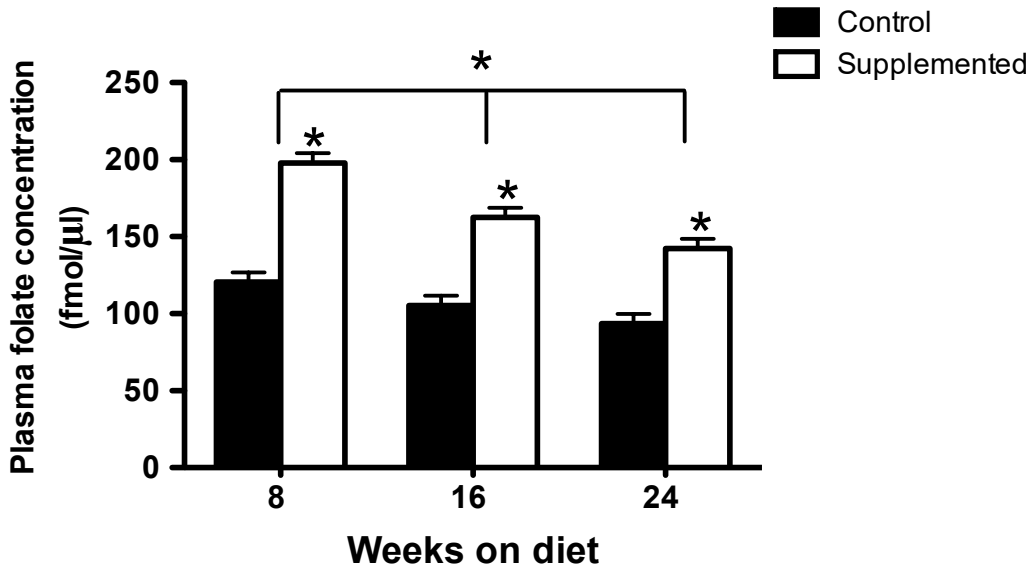



**Figure 3. 5 Effect of dietary folic acid intake on plasma folate in female mice fed a folic acid control or supplemented diet at three time points.** n = 15 females/diet group. Data are presented as mean ± SEM. Two-way ANOVA followed by Tukey multiple comparison tests were used to assess the difference between the two diet groups at each time point. Asterisk indicates a significant diet effect between females fed a FA supplemented and control diets for 8, 16, and 24 weeks ($p < 0.001$); and indicates a time effect among the groups.

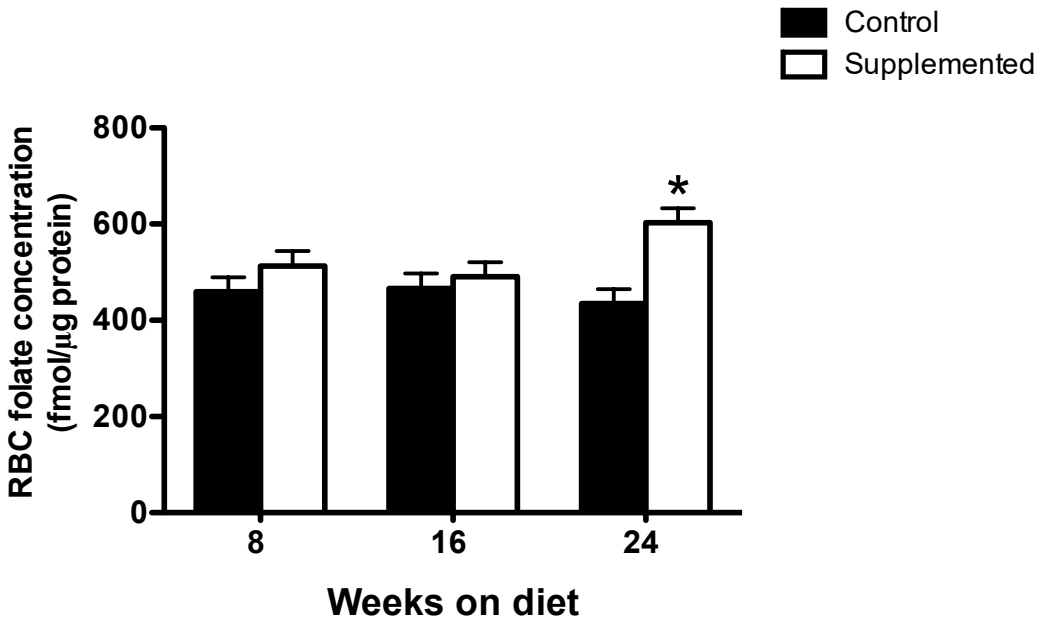



**Figure 3. 6 Effect of dietary folic acid intake on RBC folate in female mice fed a folic acid control or supplemented diet at three time points.** n = 15 females/diet group. Data are presented as mean ± SEM. Two-ways ANOVA followed by Tukey multiple comparison tests were used to assess the differences between the two diet groups at each time point. Asterisk indicates a significant diet effect between females fed a FA supplemented and control diets for 24 weeks ($p < 0.001$).

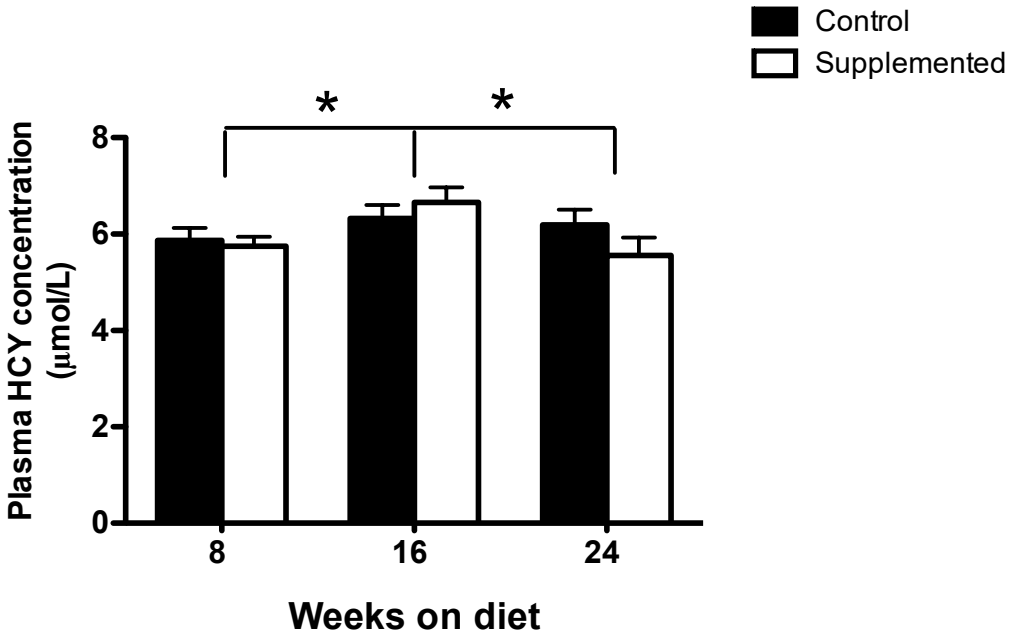
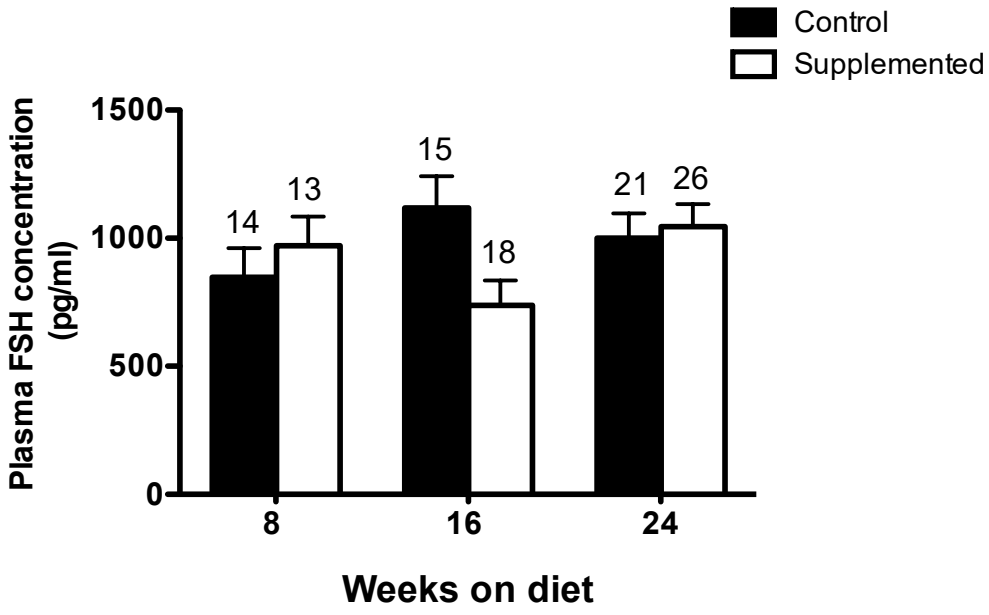



**Figure 3. 7 Effect of dietary folic acid intake on plasma homocysteine in female mice fed a folic acid control or supplemented diet at three time points.** n=20 females/diet group. Data are presented as mean±SEM. Two-way ANOVA followed by Tukey multiple comparison tests were used to assess the difference between the two diet groups at each time point. Asterisk indicates a significant time effect between groups. $p$ = 0.017 for 8 vs.16 weeks; $p$ = 0.006 for 16 vs. 24 weeks.

A




B

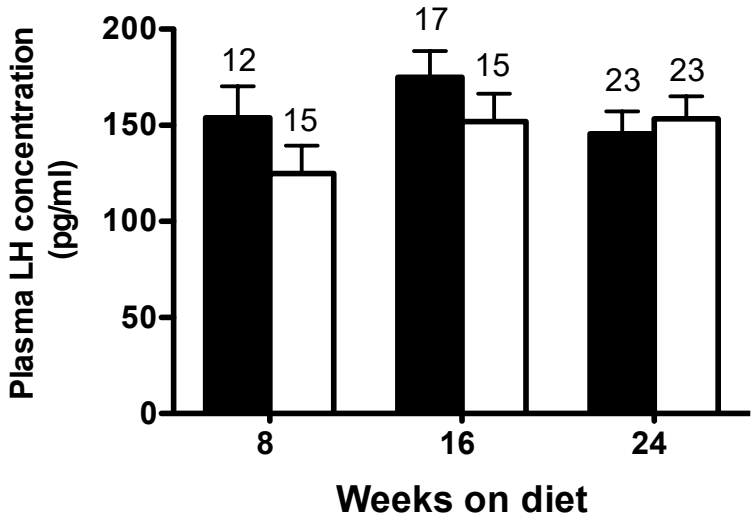



**Figure 3. 8 Effect of dietary folic acid on plasma gonadotropin hormones in female mice fed a folic acid control or supplemented diet at three time points. (A)** Effect of FA diets on plasma follicle stimulating hormone (FSH) concentration. **(B)** Effect of FA diets on plasma luteinizing hormone (LH) concentration. Numbers above bars represent number of females analysed. Data are presented as mean±SEM. Two-way ANOVA was used to assess the differences between the two diet groups at each time point.

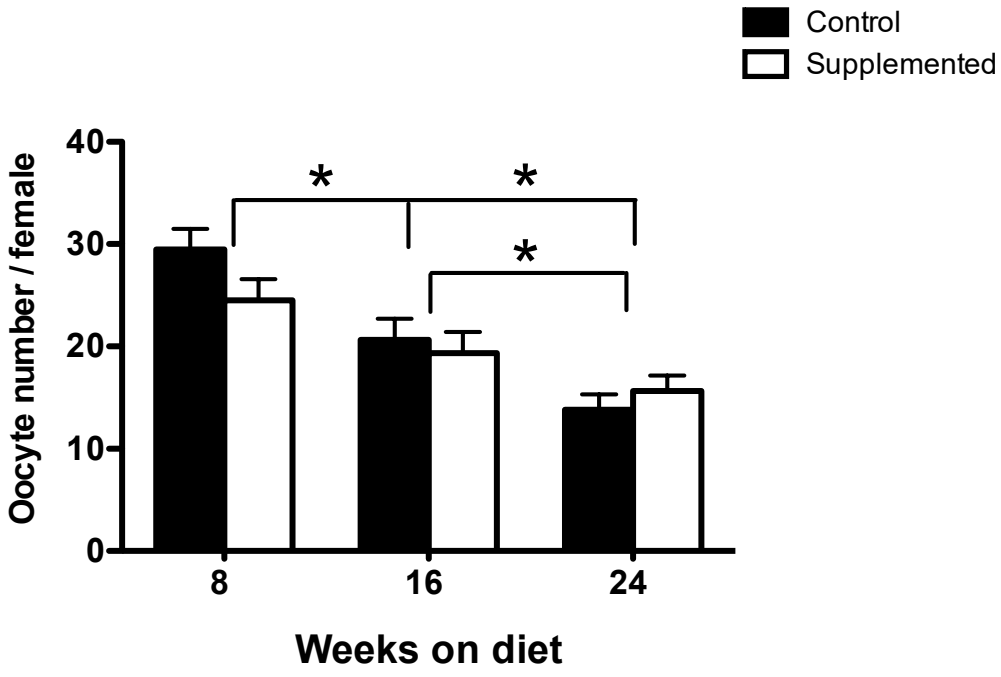



**Figure 3. 9 Effect of dietary folic acid intake on the number of oocytes ovulated at three time points.** Data are presented as mean±SEM. Two-way ANOVA followed by Tukey multiple comparison test were used to assess the difference between the two diet groups at each time point. Asterisks indicate a significant time effect between groups. $p = 0.002$ for 8 vs. 16 weeks; $p < 0.001$ for 8 vs. 24 weeks; $p = 0.010$ for 16 vs. 24 weeks.

**Table 3. 1** Effect of dietary folic acid intake on MII oocyte aneuploidy at three time points

| Weeks on diet | Diet (n=No. of females) | Total oocytes No. | No. of oocytes/female | Total MII | Total MII analysed | <20 (%) | 20 (%) | >20 (%) | PSCS (%) | PA (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 8 | Control (n=21) | 619 | 29.4 | 360 | 185 | 80 (43.2) | 39 (21.1) | 66 (35.7) | 101 (54.6) | 22 (11.8) |
| | Supplement (n=21) | 490 | 24.5 | 182 | 91 | 37 (40.7) | 19 (20.8) | 35 (38.5) | 49 (53.8) | 8 (8.8) |
| 16 | Control (n=20) | 413 | 18.2 | 89 | 29 | 9 (31) | 5 (17.2) | 15 (51.7) | 14 (48.3) | 11 (37.9) |
| | Supplement (n=20) | 387 | 20.2 | 88 | 46 | 24 (52.2) | 10 (21.7) | 12 (26.1) | 30 (65.2) | 14 (30.4) |
| 24 | Control (n=38) | 525 | 13.8 | 214 | 111 | 68 (61.3) | 10 (9) | 33 (29.7) | 42 (37.8) | 8 (7.2) |
| | Supplement (n=38) | 595 | 15.6 | 184 | 85 | 49 (57.6) | 21 (24.7)* | 15 (17.6) | 41 (50) | 3 (3.5) |

* Asterisk indicates a significant diet effect between females fed FA control and supplemented diets ($p = 0.005$).
Data are presented as mean±SEM for no. of oocytes; Two-Way ANOVA followed by Tukey multiple comparison test were used to compare the mean no. of oocytes/female.
For hypoploidy (< 20), euploidy (= 20), hyperploidy (> 20), PSCS and PA, data are presented as percentage; Chi-square test was used to compare the hypoploid, euploid and hyperploid, PSCS and PA frequencies between the two diet groups at each time point.

**Table 3.2** Effect of dietary folic acid intake on the normal MII oocytes with or without PSCS in old female mice

| Diet | No. of normal oocytes | 20 No PSCS (%) | 20+PSCS (%) |
|---|---|---|---|
| Control | 10 | 8 (80) | 2 (20) |
| Supplemented | 21 | 15 (71.5) | 6 (28.5) |

Data are presented as percentage; Chi-square test was used to compare the PSCS frequency in normal MII oocytes between the two diet groups at 24 weeks ($p$ = 1.000).

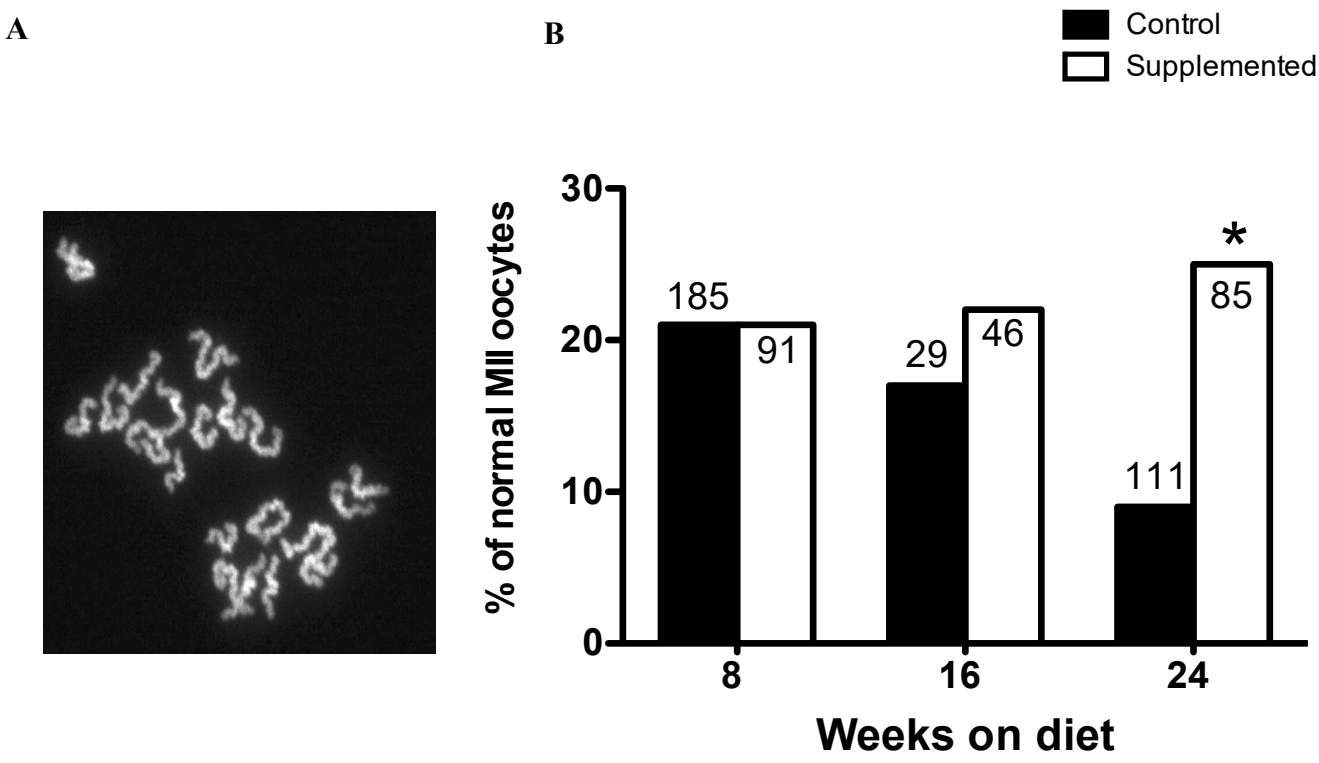



**Figure 3. 10 Effect of dietary folic acid intake on euploidy frequency in MII oocyte in female mice fed a folic acid control or supplemented diet at three time points. (A)** An example of the cytogenetic analysis of mouse metaphase II oocytes, n = 20 dyads. Chromosomes were stained with a Diamond Antifade Mountant with DAPI, oil immersion at 100× **(B)** Percentage of normal MII oocytes containing 20 chromosomes. Data are represented as a percentage. Chi-square test was used to assess the differences between the two diet groups at each time point. The asterisk indicates significant difference between the control and supplemented groups at 24 weeks ($p$ = 0.005). The number over the bar indicates the number of MII oocytes analysed.

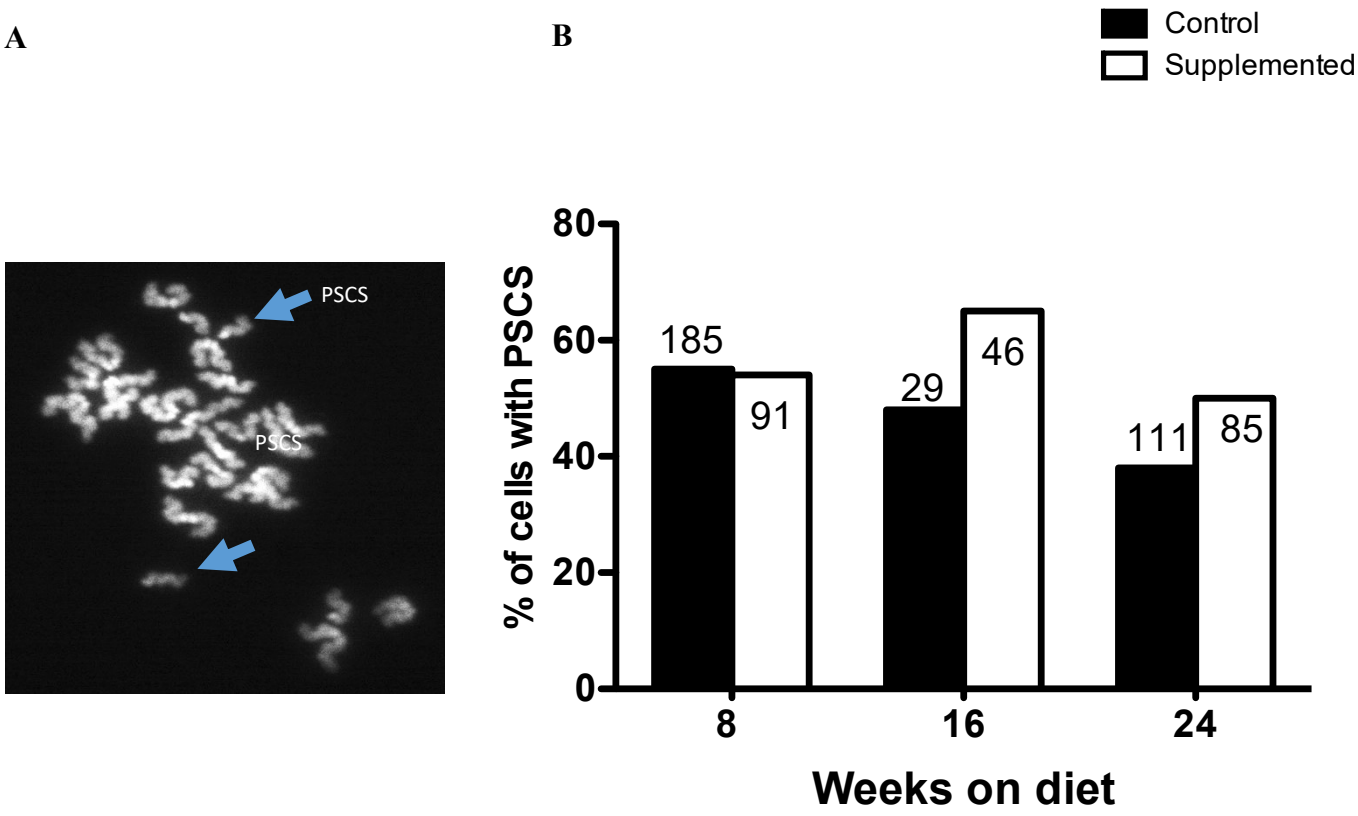



**Figure 3. 11 Effect of dietary folic acid intake on premature sister chromatid separation (PSCS) frequency in MII oocytes in female mice fed a folic acid control or supplemented diet at three time points. (A)** Example of the cytogenetic analysis of mouse metaphase II oocytes, n = 20 dyads + 4 chromatids, arrows indicate the PSCS of one dyad into two single chromatids. Chromosomes were stained with a Diamond Antifade Mountant with DAPI, oil immersion at 100× **(B)** Percentage of MII oocytes with PSCS. Data are represented as a percentage. Chi-square test was used to assess the differences between the two diet groups at each time point. Numbers over bars indicate the number of MII oocytes analysed.

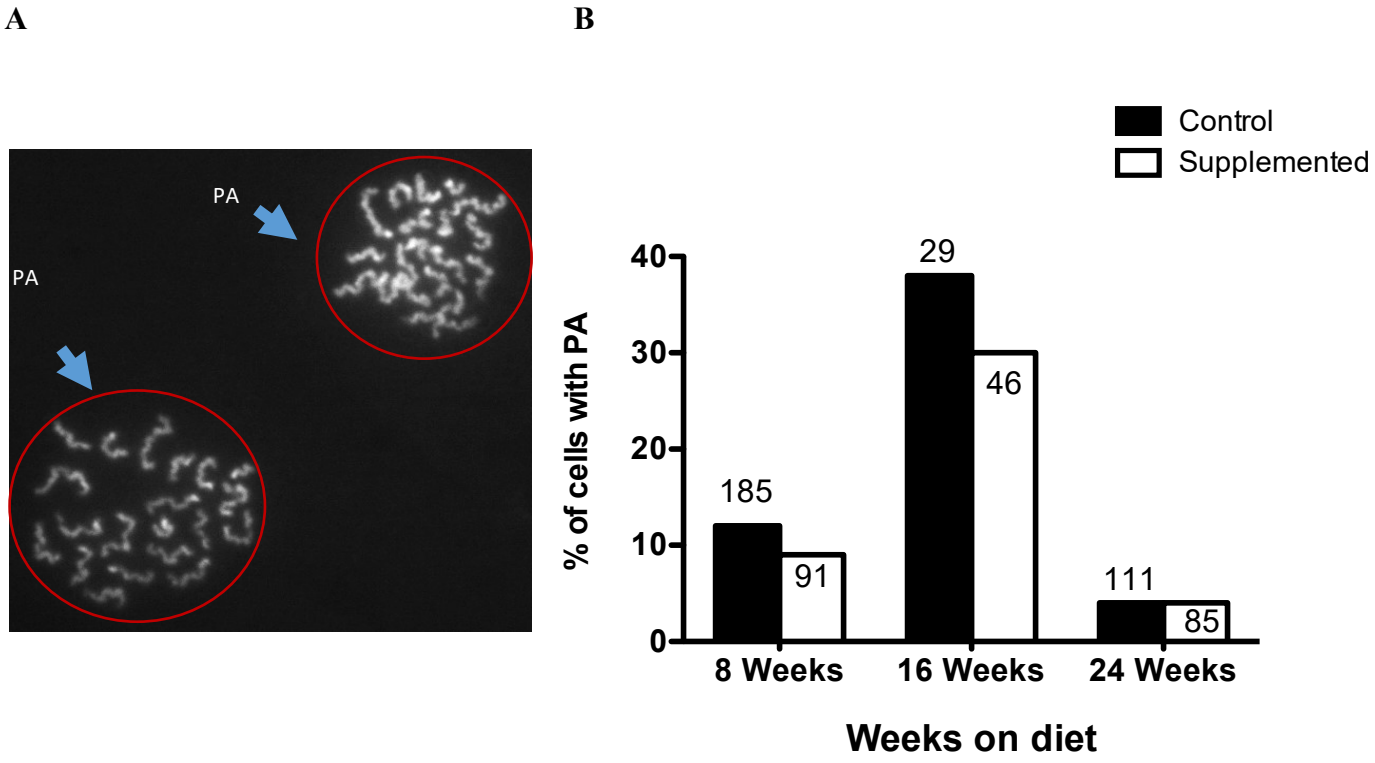



**Figure 3. 12 Effect of dietary folic acid intake on premature anaphase (PA) frequency in MII oocytes in female mice fed a folic acid control or supplemented diet at three time points. (A)** Example of the cytogenetic analysis of mouse metaphase II oocytes, n = 40 chromatids, arrows indicate the PA separation into two groups each of 20 chromatids. Chromosomes were stained with a Diamond Antifade Mountant with DAPI, oil immersion at 100× **(B)** Percentage of MII oocytes with PA. Data are presented as a percentage. Chi-square test was used to assess the differences between the two diet groups at each time point. Numbers over bars indicate the number of MII oocytes analysed.

### 3.7 Discussion

Here for the first time, we provide evidence that supplementation with FA over a long duration reduced female germ cell aneuploidy associated with advanced maternal aging. We examined the effect of dietary FA supplementation in a model of premature maternal age induced oocyte aneuploidy, namely Bub1-heterozygous female mice. The dominant negative mutation used to disrupt the *Bub1* gene kinase activity in mouse oocytes demonstrates the important role of Bub1 in controlling chromosome segregation in oocytes. The transgenic mouse model used in this study is characterized by: 1) oocytes with a high frequency of aneuploidy and PSCS; 2) an oocyte aneuploidy incidence that increases with advanced maternal age and that is passed to zygotes; and 3) early loss of fertility (Leland et al., 2009). Our results show that old female $Bub1^{+/m}$ mice fed a FA supplemented diet had significantly (2-fold) higher proportion of normal oocytes (25%) compared to the control group (9%). Of note, the FA supplemented diet had no effect on euploidy frequency in young and middle-aged females.

To date, no study has directly examined the effect of FA intake on oocyte aneuploidy in humans and there have been only a limited number of studies that examined the effect of FA on oocyte quality and maturation in female germ cells in animal models. An *in vivo* study reported that three-week old Cr1:CD1 female mice fed a FA deficient diet (0 mg/kg) for ~two months did not affect oocyte meiosis and did not increase the frequency of oocyte abnormalities as indicated by the percentage of abnormal spindle formation compared with females fed a control diet (2.4 mg/kg FA) (Tsuji et al., 2016). *In vitro* culture of porcine oocytes exposed to a FA supplemented medium (10 ng/ml) for 44 hours was shown to improve oocyte maturation and embryonic development, as indicated by a higher number of blastocyst cells compared with oocytes cultured in a control medium (1 ng/ml) (Kim et al., 2009). Recently, in human colon cells, it was reported

that folate deficiency induced SAC dysfunction by dysregulating the expression of core SAC components including *BUB1,* the main SAC protein in the mouse model, which contributed to a significantly higher frequency of chromosome misalignment and missegregation, and spindle abnormality (Guo et al., 2017). We propose that the same mechanism may be involved in our model *in vivo*, that FA supplementation might maintain the expression of SAC proteins including Bub1 which maintains a proper chromosome alignment and segregation and results in a higher number of normal oocytes.

Dietary intervention studies have reported that the incidence of aneuploidy either decreased or was not detected in aged female mice. Selesniemi et al, reported that no hyperploidy was detected in MII oocytes from 12 months old female C57BL/6 mouse maintained on caloric restriction diet versus ~5% hyperploidy in non-caloric restricted 12-month old females (Selesniemi et al., 2011). So, dietary intervention might represent a suitable intervention by which to reduce the effect of maternal aging on oocyte aneuploidy.

We assessed the incidence of PSCS between the FA control and supplemented diets in young, middle-aged and old females. Our result showed that FA supplementation had no effect on the incidence of PSCS in all young, middle-aged and old females. Oocytes from old female C75BL/6 mice maintained on a caloric restriction diet for 12 months reported no difference in the PSCS incidence when comparing the caloric restricted and the age matched control groups (Selesniemi et al., 2011), which agreed with our results that dietary intervention had no effect on PSCS incidence in oocytes. Since we observed a protective effect of FA supplementation on oocyte aneuploidy in old females, we further analysed the incidence of PSCS in normal MII oocytes in old females. PSCS might pass from oocyte to embryo and could cause embryo aneuploidy (Mailhes et al., 1998). Our results showed that of the normal MII oocytes 20% had a

complete set of chromosomes (20 chromosomes) but with PSCS in old females fed a FA control diet. Similarly, 28.5% of oocytes with a normal chromosome number had PSCS in old females fed a FA supplemented diet. PSCS frequency was shown to increase in aged mouse oocytes, as might be expected. Because of the limited models of *in vivo* aging mouse that assess the incidence of PSCS, we used the postovulatory aging model to compare PSCS incidence in oocytes with aging. Postovulatory aging of 6-12-week old ICR mouse oocytes indicated that the proportion of oocytes 25 hours post ovulation with PSCS was 23.5% compared to 13.1% and 2.8% of oocytes at 20 and 15 hours of ovulation respectively. Our results showed a significant decrease in PSCS incidence over time. It should be noted that the postovulatory aging model is difficult to compare to our model of *in vivo* aging but few other studies have examined the effect of aging on PSCS.

In both human and mouse studies, the individual variation in both aneuploidy and PSCS incidence is usually not reported. Pooling of oocytes is the primary strategy used when assessing the presence of oocyte aneuploidy and PSCS because of the nature of the cytogenetic analysis. As such, it is not possible to determine the individual variation in these outcomes. Unpublished data from our mating study showed on average ~8% of oocytes in old Bub1 female mice are normal

In both human and mouse studies, the individual variation in both aneuploidy and PSCS incidence is not reported. As pooling of oocytes is the strategy that used to assess the presence of aneuploidy and PSCS which represents the mean of the result and that could mask the individual variation. Unpublished data from our mating study showed ~8% of the oocytes are normal regardless of diet.

A premature anaphase II (PA) phenomena was observed during the cytogenetic analysis of MII oocytes. PA appears as two groups of single chromatids. Because the lack of *in vivo* aging mouse model that assess the presence of PA, we used the postovulatory model to compare the

incidence of PA associated with aging. Postovulatory aging of mouse oocytes showed that the PA frequency increases with aging. Postovulatory aging of 8-12-week old ICR mouse oocytes leads to a higher degree of PA. The proportion of oocytes with PA at 23 hours post ovulation was 69.4% compared to 8.7% of oocytes ovulated at 17 hours (Mailhes & Marchetti, 1994b). Another study by the same investigator confirmed that postovulatory aging of 6-12-week old ICR mouse oocytes led to a higher degree of PA as the proportion of oocytes with PA at 25 hours post ovulation was 17.5% compared to 0.8% and 0.6% of oocytes ovulated at 20 and 15 hours respectively (Mailhes et al., 1998). Also, PA predisposed MII oocytes to aneuploidy as hyperploidy was detected in one-cell zygotes, and the frequency of hyperploidy increased with increasing time post fertilization (Mailhes et al., 1998). Furthermore, Steuerwald et al., reported a 44% incidence of PA at 24 hours post ovulation compared to 3.5% of PA in oocytes at 19 hours post ovulation in 8-12 weeks ICR mouse (Steuerwald et al., 2005). The degree of PA observed in our study varied between the time points as follows: young females had about 8-12%, middle-aged females 30-38%, and old females 3-7% for both supplemented and control FA diets respectively. Our analysis showed that the PA frequency appeared to decrease over time which contrasts with what has been published previously. The previous reported studies that discussed above were performed in ICR mouse using the postovulatory strategy for oocytes aging, and our study was done *in vivo* using C57BL/6 mouse. In the postovulatory aging strategy, the mice were not chronologically old but the oocytes, which are the main model, were aged *in vivo*. However, in all cases the effect of PA in oocytes was assessed after ovulation. The different experimental designs may be a contributing factor for the different results. Alternatively, since PA occurred at a fairly low frequency at all ages, our study may have been underpowered to determine any true differences among the groups.

We also evaluated the effect of FA supplementation on oocyte yield obtained from young, middle-aged and old female. The oocyte number ovulated by each female did not differ between FA control and supplemented diets at any timepoint. We did observe a significant gradual decline in the number of ovulated oocytes as the females aged, which agrees with previous published results. (Fu et al., 2014; Merriman et al., 2012; Selesniemi et al., 2011; Tarin et al., 2002) reported similar results showing a declining number of ovulated oocytes with advanced maternal age in CD1 and C57BL/6 mice. We also observed that 12-month old C57BL/6 females had almost no oocytes (the mean oocyte number was 2.19±1.1 /female) (Appendix III). This decline in ovulation with aging might be due to depletion of the ovarian primordial follicle reserve as shown in humans and mice (Faddy et al., 1992; Kevenaar et al., 2006).

The concentrations of gonadotropin hormones (FSH and LH) were measured to assess the effect of FA supplemented diet on gonadotropins. This was a point of interest for us since high levels of gonadotropins contribute to the incidence of oocyte aneuploidy in humans and mice (Roberts et al., 2005; Xu et al., 2011). Our results showed that FA supplementation had no effect on gonadotropins in young, middle-aged and old females. In humans, an age-related increase in gonadotropins throughout the reproductive life in regularly menstruating women has been reported, and elevated gonadotropins are considered to be endocrine biomarkers for aging associated with diminished fertility (Ahmed Ebbiary et al., 1994; MacNaughton et al., 1992). An increase in FSH concentration in reproductively old women might be caused by a diminished feedback loop from the ovary explained by decreased inhibin B secretion; inhibin B suppresses pituitary FSH secretion, which correlates with the depletion of the follicular pool in older women (Klein et al., 1996). A case-control study reported that mothers of children with Down syndrome (DS) have significantly increased FSH concentration compared to control mothers, suggested that

the increase in FSH concentration in DS mothers might play a causal role in chromosome 21 nondisjunction (van Montfrans et al., 1999). In different mouse strains, e.g. CD1 and C57BL/6, plasma FSH and LH concentrations were found to be elevated in old females (21-20 months) compared to young females (2-4 months) (Collins et al., 1980; Parkening et al., 1980). Furthermore, higher concentrations of FSH and LH are found to contribute to a higher incidence of oocyte aneuploidy in humans and mice (Roberts et al., 2005; Xu et al., 2011). Evidence from human and animal studies suggested that gonadotropins might increase the aneuploidy rate because of their effects on microtubules, which may have a role in altering meiotic spindle (Dursun et al., 2006). Our result agrees with the mouse results that reported no change in FSH concentrations between younger (6 week) and older (9 months) CD1 female mice (Fu et al., 2014). It has been hypothesized that in aged animals the hypothalamus cannot tightly regulate the secretion of gonadotropins from the anterior pituitary. This dysregulation of hormone secretion causes increase in FSH which in turn might affect the follicle pool in the ovary (Qiao et al., 2014). So, we had hypothesized that supplementation with FA might protect the hypothalamic-pituitary system from the damage caused by aging by maintaining lower FSH which in turn decreases the damage in oocytes in ovary and increasing the eggs number. However, we reported no change in both FSH and LH plasma concentrations in young, middle-aged and old female fed FA supplemented diet.

Plasma, RBC folate and plasma HCY are biomarkers of folate status and one-carbon metabolism. In general, plasma folate is considered an indicator of recent folate intake, while RBC folate is an indicator of long-term folate status since it reflects the amount of folate accumulated in RBCs during erythropoiesis (Bailey et al., 2015). It has been reported that mothers of DS individuals have lower circulating folate and RBC folate concentrations compared to mothers of

non-DS individuals (Mohanty et al., 2012; Pandey et al., 2013; Takamura et al., 2004). Additionally, plasma HCY concentration was found to be higher in mothers of DS individuals (Bosco et al., 2003; da Silva et al., 2005; James et al., 1999; Takamura et al., 2004; Wang et al., 2007). Our analysis showed that plasma folate concentration was higher in all young, middle-aged and old females fed the FA supplemented diet compared to those fed the control diet. Of note, a time-dependent decrease in plasma folate was detected as females aged. RBC folate concentration was higher in old females fed FA supplemented diet compared to females fed a control diet. No change in RBC folate was observed as females aged. Also, we did not observe a change in HCY concentration between FA control and supplemented diets at any age nor did we observe an age-associated increase in HCY despite the lower folate status. Plasma HCY concentration in our study was higher in middle-aged females compared to young and old females, an unexpected result as HCY usually increases with age. If our observation of an age-dependent decrease in circulating folate is confirmed in humans, lower folate status, as observed in mothers of DS individuals, could be contributing to a higher risk for a DS affected pregnancy. This may also suggest that older mothers have a higher need for FA supplementation before pregnancy.

It has been reported that folate intake from food was significantly lower in mothers of DS individuals (James et al., 1999; Meguid et al., 2008; Santos-Reboucas et al., 2008). Also, lack of maternal FA supplementation during the preconception period might be a risk factor for chromosome 21 non-disjunction in aged oocytes (Hollis et al., 2013). In addition to folate, it has been reported that maternal nutritional status for other nutrients has an effect on oocyte quality. For example, deficiency of biotin, which is an important vitamin that regulates several physiological processes such as development of the embryo, growth and metabolism leads to a 40% increase in the frequency of abnormal oocytes compared to mice fed a control diet (Tsuji et

al., 2015). Furthermore, zinc deficiency and maternal metabolic disorders such as obesity and diabetes were shown to affect oocyte development and quality (Gu et al., 2015).

It is well known that maternal aging affects oocyte quantity and quality (Hassold & Hunt, 2001). In this study, we showed that supplementation with FA in old female mice increases the proportion of normal oocytes. Investigating the mechanism(s) underlying the protection of FA supplementation against increased aneuploidy incidence in oocytes in old female mice would be important to understand. FA is transported into cells by three major folate transport systems: the PCFT, RFC and FR. The expression of RFC was found to be high in the ovary of hens (Jing et al., 2009). Also, RFC1 is expressed in the cumulus-oocyte complex in mice (Kooistra et al., 2013). Recently, there is evidence showing that folate is actively transported into growing mouse oocytes via folate receptors, such as the FOLR2 isoform (Meredith et al., 2016). Together these data suggest that oocytes require active folate-mediated one-carbon metabolism, at least transiently. Folate transportation is activated for short periods of oocyte growth when an oocyte is about to reach full size and then stops. It is hypothesised that this transported folate supports the final stage of oogenesis and/or is stored to provide endogenous folate that is needed to support early embryogenesis (Meredith et al., 2016).

It is known that DNA methylation in mammalian oocytes occurs primarily after birth and increases during oocyte maturation (Nashun et al., 2015; Reik et al., 2001). *De novo* DNA methylation occurs in mouse oocytes during oogenesis and throughout maturation, when the oocyte is about to reach full size (Hiura et al., 2006; Lucifero et al., 2004). The activation of folate-mediated one-carbon metabolism is needed to increase the pool of SAM, the major methyl donor, to establish oocyte methylation (Waterland et al., 2006). Alteration of SAM metabolism can disrupt DNA methylation in the oocyte by depleting SAM or increasing the level of metabolites

that act as DNA methyltransferase inhibitors. An improper methylation of centromeric DNA has been associated with defective centromeric function and chromosome missegregation in somatic cells (Zingg & Jones, 1997). Additionally, James et al suggested that abnormal methylation of pericentromeric DNA was associated with abnormal segregation of chromosome 21 and may underlie the association between FA and DS (James et al., 1999). Recent studies reported that the methylation status at CpG dinucleotide sites on the extra chromosome of trisomy 21 is altered (Alves da Silva et al., 2016); and hypomethylation and hypermethylation at CpG dinucleotides have been detected in trisomy and monosomy cases (Denomme et al., 2016). We suggest that one mechanism by which FA supplementation might improve oocyte aneuploidy is by increasing the methylation capacity which leads to proper chromosome segregation.

Another possible mechanism that could explain the FA-mediated reduction in oocyte aneuploidy in old females is that FA supplementation might improve mitochondrial function. It is well known that mitochondrial function deteriorates with aging due to a decline in mtDNA copy number and ATP content, and increased mtDNA mutation rate in several mammalian species, which in turn affects oocyte quality, fertilization ability, embryo development and overall female fertility (Chan et al., 2005; Keefe et al., 1995; Simsek-Duran et al., 2013). For more details on mitochondrial dysfunction in aged oocytes refer to Chapter 1, Section 1.2.4. An oocyte requires sufficient energy in the form of ATP during maturation and after fertilization to support several events including resumption of meiosis, spindle formation and cellular division (Eichenlaub-Ritter, 2012).

It has been shown that folate deficiency resulted in mtDNA disorders and reduced mtDNA content in somatic tissues, which might lead to reduced expression of genes encoding important components of the electron transport chain (Ormazabal et al., 2015). Also, it has been reported

that older rats (12 months of age) fed a control and a supplemented FA diet (2, 8 mg/kg) for 20 weeks had significantly 2.2 and 2.3-fold lower mtDNA 'common' 4.8 kb deletions in somatic cells (specifically liver) when compared with rats fed a deficient FA diet (0 mg/kg) (Crott et al., 2005). From the previous reports, we can infer that FA supplementation over a long period of time might improve mitochondrial function, which is known to deteriorate with female reproductive aging, and in turn improve oocyte quality in old females.

Possibly, FA supplementation might support DNA repair mechanisms that are active during oogenesis. DNA repair is critical for maintenance of oocyte quality and therefore essential for female fertility (Winship et al., 2018). Failure of DNA repair during meiotic recombination in oocytes can lead to chromosome missegregation and aneuploidy, which affects embryo development and overall female fertility (MacLennan et al., 2015). It has been shown that the efficiency of primordial follicle oocytes to repair DNA double strand breaks declines with maternal aging. In humans and mice, the proportion of DNA double strand breaks increased significantly from 19% and 32.6% in young oocytes to 63.6% and 58.5% in aged oocytes (Titus et al., 2013). Also, the expression of key DNA double strand break repair genes was shown to decline in aged human and mouse oocytes (Titus et al., 2013). Adequate folate intake is required for nucleotide synthesis and DNA repair (Stover, 2009). As such FA supplementation might mitigate the effect of aging on oocyte aneuploidy through supporting DNA repair in old oocytes.

### 3.8 Strengths and Limitations of the Study

A point of strength in this study was the use of $Bub1^{+/m}$ female mice as an experimental model of premature oocyte aneuploidy that induces aneuploidy at relatively younger age, which could not have been achieved using naturally aged mice. Also, the ability to analyse chromosomes and assess the incidence of aneuploidy, PSCS, and PA is another point of strength.

One of the limitations of the study is the missing intervention with FA deficiency. It would be interesting to assess the effect of FA deficiency on aneuploidy incidence. Therefore, the study design in this chapter reflects what was observed in human in Canada where is no folate deficiency in the general population (MacFarlane et al., 2011). In human study, clinical trials assess the different fertility parameters between women who had a normal folate status and women are given a supplemental FA. Also assessing the effect of FA deficiency in our mouse model would be time and money consuming and use of more females which we want to avoid using more of alive animals. Another limitation is the small number of oocytes that we got from the old females where we observed the effect. We had to double the number of old females to get as close as possible to the targeted oocyte number for analysis. Also, the study would be more strengthen if it involves mating to assess if the effect that we observed in old females would also be seen in the embryos. Therefore, a mating study using the $Bub1^{+/m}$ mouse model is ongoing to assess the effect of FA supplementation before and during pregnancy on embryos.

**3.9 Conclusion**

In conclusion, we examined the effect of dietary FA supplementation on maternal age-induced oocyte aneuploidy *in vivo* using $Bub1^{+/m}$ female mice. Our results showed that a FA supplemented diet (8 mg/kg) resulted in a significant increase in normal oocytes in old female mice that were 24-week on diet in comparison to a control diet. Our data suggest that FA supplementation may mitigate the effect of maternal aging on oocyte aneuploidy and could improve oocyte quality and fertility.

# Chapter 4: Investigating the Effect of Dietary Folic Acid Intake on Chemically Induced Aneuploidy in Wild Type

# Chapter 4
## Investigating the Effect of Dietary Folic Acid Intake on Chemically Induced Aneuploidy in Wild Type Female Mice


### Abstract

Oocyte aneuploidy, the presence of extra or missing chromosomes, arises as a result of chromosome segregation errors during oogenesis. Aneuploidy is a major cause of pregnancy loss and birth defects in humans. Segregation errors and therefore aneuploidy increases with advance maternal age. Folic acid (FA), an essential B vitamin, is required for DNA synthesis, chromosome stability and methylation reactions which are fundamental processes for female reproductive physiology. In this chapter, we examined the effect of dietary folic acid deficiency (0 mg/kg), adequacy (2 mg/kg) and supplementation (8 mg/kg) on oocyte aneuploidy induced by colchicine as a model for studying age-induced aneuploidy in C57BL/6 mice. We report that colchicine treatment in these mice led to the arrest of a high proportion of oocytes (68%) in metaphase I relative to mice in the colchicine untreated control group (0%). As such only 32% of metaphase II oocytes were available for analysis. Whether FA has an impact on oocyte aneuploidy cannot be drawn from this preliminary study due to the low number of oocytes analyzed at metaphase II. Our results indicate that the C57BL/6 inbred mouse strain may be particularly sensitive to colchicine-induced MI arrest. Future studies will be required to determine the appropriate time between colchicine treatment and oocyte harvest to maximize the isolation of MII oocytes.

## 4.1 Hypothesis

FA deficiency will enhance oocyte aneuploidy whereas FA supplementation will reduce oocyte aneuploidy induced by colchicine in wild type (C57BL/6) mice.

## 4.2 Study Rationale

The meiotic inhibitor colchicine inhibits microtubule polymerization, disrupts spindle formation and induces aneuploidy in female germ cells (Marchetti et al., 2016). Microtubules function to assemble and separate chromosomes to the spindle poles in preparation for cell division (Field et al., 2014). The two primary colchicine-dependent mechanisms of action, the inhibition of microtubule polymerization and spindle formation, are similar to observations that maternal aging severely impacts microtubule growth and organization in mouse oocytes (Nakagawa & FitzHarris, 2017). This makes colchicine a suitable chemical model for studying aging induced oocyte aneuploidy. Studies showed that FA deficiency is associated with chromosome aneuploidy in somatic cells (Ni et al., 2010; Wang et al., 2004) and our results (Chapter 3, Section 3.6.7) showed that FA supplementation was associated with improved oocyte aneuploidy in old $Bub1^{+/m}$ mouse oocytes. As such, we proposed that colchicine-induced aneuploidy in oocytes represents a model of aging to assess aneuploidy incidence in oocytes and that dietary FA intake will modify colchicine-induced oocyte aneuploidy.

## 4. 3 Introduction

Microtubule drugs (also known as spindle poisons) such as colchicine, paclitaxel, vinblastine or oryzalin from chemically diverse groups have the ability to change microtubule properties (Marchetti et al., 2016). Because of the ability of microtubule drugs to target and change microtubule dynamics, they have been used successfully in different medical, agricultural and research applications. Clinically, microtubule-targeting agents have been used in chemotherapy treatment for different types of cancer and tumors due to their anti-angiogenic and anti-vascular

effects. Also, microtubule-targeting agents have been recently used to treat neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Field et al., 2014).

In several mammalian models, microtubule disrupting agents have been used to induce aneuploidy in germ cells; they interfere with the proper function of the meiotic spindle and other aspects of chromosome segregation. For example, numerous chemicals including colchicine, benomyle, griseofulvin, vinblastine sulfate and triaziquone administered *in vivo* by subcutaneous injection, i.p. injection, or oral gavage have been identified to induce aneuploidy in female germ cells (Mailhes & Marchetti, 1994a).

Colchicine is a natural lipid soluble alkaloid with a chemical formula N-(5,6,7,9, tetrahydro-1,2,3,10, tetramethoxy-9oxobenzo[α]heptalen-7-yl) acetamide (Figure 4.1). Colchicine is extracted from two plants of the lily family: the leaf of meadow saffron plant (*Colchicum autumnale*), and the glory lily plant (*Gloriosa superba*). *Colchicum* is the Latin name of the meadow saffron plant, derived from the name of the ancient district of Colchis on the eastern shore of the Black Sea (Ben-Chetrit & Levy, 1998). Colchicine has been used for centuries for the treatment of acute gout arthritis and later for other conditions including Behcet's syndrome, Sweet syndrome, scleroderma, sarcoidosis, amyloidosis and Familial Mediterranean Fever (FMF). The use of colchicine to treat acute gout and FMF was approved by the US Food and Drug Administration (FDA) in 2009 (Slobodnick et al., 2015). Colchicine can be administered either intravenously (IV) in an IV solution of 0.5 mg/ml, or orally as tablets containing 0.6 or 1.0 mg. The recommended oral dosage of colchicine for FMF ranges from 1.2 to 2.4 mg/day for adults and from 0.3 to 1.8 mg/day for children adjusted to body weight. For the treatment of acute gout, the recommended dose is 1.2 mg followed by a single dose of 0.6 mg one hour later (Finkelstein et al., 2010).

The main route of exposure of colchicine is via oral administration. After oral administration, colchicine is rapidly absorbed from the gastrointestinal tract. The plasma concentration peaks 0.5 to 3.0 hours after ingestion (Finkelstein et al., 2010). Colchicine undergoes hepatic first-pass metabolism, which reduces its bioavailability, resulting in 25-50% reaching circulation (Ben-Chetrit & Levy, 1998). After absorption, colchicine is metabolized by intestinal and hepatic P450 cytochrome (CYP3A4) by demethylation of the three methoxy groups in the molecule. There are multiple routes to eliminate colchicine from the body. P-glycoprotein regulates the tissue transportation of colchicine and 16-50% of absorbed colchicine is excreted via the biliary tract in the liver and kidneys to limit gastrointestinal absorption (Niel & Scherrmann, 2006). Biliary excretion is the main route of colchicine elimination. Urinary excretion accounts for 5-20% of total colchicine administrated (Niel & Scherrmann, 2006). In addition, colchicine has been detected in feces, in cord blood from newborns of mothers treated for FMF and in breast milk (Ben-Chetrit & Levy, 1998).

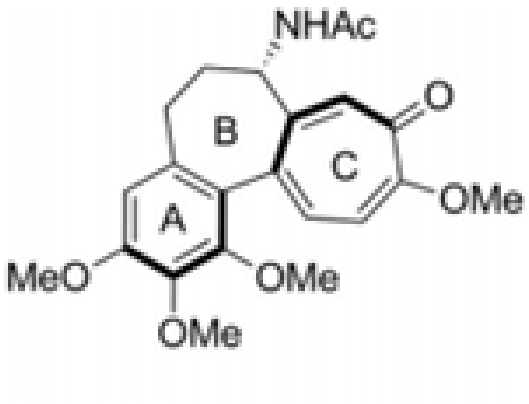



**Figure 4. 1** Molecular structure of colchicine

### 4.3.1 Mechanism of Action of Colchicine

Colchicine is one of the oldest microtubule drugs that changes and blocks the microtubule network. Administration of colchicine causes disruption of the microtubule cytoskeleton leading to impairment of protein assembly in the Golgi apparatus, decreased exocytosis and endocytosis, altered cell shape, decreased cell motility and mitotic arrest. Organ failure or death can occur as a result of the accumulation of the pathological effects after administration of an overdose of colchicine (Finkelstein et al., 2010).

Colchicine is a known microtubule destabilizer; the inhibition of microtubule polymerization is the main mechanism of action of colchicine at the cellular level. Microtubules are widely distributed protein filaments that constitute the cytoskeleton of all eukaryotic cells. They are long rigid polymers characterized by their dynamic behavior which have the ability to switch between elongation, pause, and shortening periods continuously (Field et al., 2014). They are critical for many cellular processes including morphogenesis, cell proliferation, migration, motility, intracellular trafficking, and chromosome segregation during mitosis and meiosis (Niel & Scherrmann, 2006).

Structurally, each unit of a microtubule is composed of α and β tubulin heterodimers, each of which consists of approximately 450 amino acids; the α and β tubulin chains share ~40% sequence homology (Downing & Nogales, 1998; Little & Seehaus, 1988). Under certain conditions, α and β subunits form stable heterodimers, then tubulins polymerize in a head-to-tail fashion to form protofilaments. The number of protofilaments in mammals is 13 and varies from 8 to 19 in other species (Dostal & Libusova, 2014). The protofilaments assemble together to form a microtubule. Microtubule dynamics alternate between growth, through the addition of more tubulin subunits to the plus end of microtubules, and shortening, through the removal of tubulin subunits from the minus end of microtubules (Dostal & Libusova, 2014). The arrangement of

microtubules varies among different cells. For example, the microtubule arrays and centrioles are responsible for initiating the bipolar spindle in somatic cells, while in mammalian oocytes the centrioles are missing, and the bipolar spindle is established from a microtubule organization center (MTOC) (Marchetti et al., 2016).

Colchicine blocks microtubule assembly and polymerization. Each colchicine molecule binds to a non-polymerized α and β tubulin subunit preventing it from incorporating into the polymer leading to the prevention of microtubule elongation and polymerization. As such, colchicine prevents the formation of the spindle in dividing cells. The A and C rings of the colchicine molecule are important for high affinity binding while the B ring links the other two rings (Marchetti et al., 2016). Depolymerization of microtubules leads to spindle abnormalities and cell division disruption, and as a consequence results in the occurrence of aneuploidy (Finkelstein et al., 2010)).

### 4.3.2 Colchicine Induces Aneuploidy in Female Germ Cells

Colchicine has the ability to enhance aneuploidy in mammalian oocytes as reported in mice and hamsters (Hummler & Hansmann, 1985; Mailhes & Yuan, 1987b; Mailhes et al., 1990; Pligina et al., 2017; Sugawara & Mikamo, 1980; Tease & Fisher, 1986). The ability of colchicine to induce aneuploidy in oocytes depends on several variables including: route of administration, dose selection, treatment time, treatment duration before cell harvesting, and animal age (Mailhes & Marchetti, 1994a). Among several mouse studies, colchicine was administrated either via i.p. injection or through oral gavage (Mailhes et al., 1990). Doses of colchicine ranging from 0.1 to 6.0 mg/kg have been used to induce aneuploidy in mouse oocytes (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990; Tease & Fisher, 1986; Pligina et al., 2017). A dose of 0.1 mg/kg colchicine administrated by i.p. did not significantly induce aneuploidy in MII mouse oocytes (Mailhes & Yuan, 1987; Mailhes, 1988), whereas doses between 0.2 to 0.4 mg/kg induced

a significant increase in aneuploidy incidence when administrated by the i.p. route (Tease & Fisher, 1986; Mailhes, 1988; Pligina et al., 2017). Colchicine doses that exceeded 2.0 mg/kg administrated via i.p. injection were shown to arrest oocyte maturation and result in the production of only MI oocytes (Mailhes & Yuan, 1987; Pligina et al., 2017). However, a dose of 4.0 mg/kg was reported to significantly increase oocyte aneuploidy when administrated orally (Mailhes et al., 1990). The time of colchicine treatment was shown to influence the proportion of aneuploid mouse oocytes. In a study when colchicine was injected either shortly before HCG (2 to 4 hours), at the same time as HCG, or shortly after HCG (2 to 4 hours), the oocyte aneuploidy frequency was maximal when colchicine was injected at the same time as HCG (Mailhes & Yuan, 1987). Colchicine sensitivity also differs between young and old mice. Old mice were more sensitive to MI arrest at low doses of colchicine (0.25 mg/kg) compared to young mice (Tease & Fisher, 1986).

## 4.4 Materials and Methods

### 4.4.1 Experimental Design and Diets

All female mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care (CACC), as described in the CACC Guide to the Care and Use of Experimental Animals (Olfert et al., 1993). The study was approved by the Health Canada Ottawa Animal Care Committee. Female mice were housed as trios in plastic, HEPA-filtered cages and maintained under 12 hours light/dark photoperiod at room temperature of (22 ± 2 ºC) and with standard humidity (40% - 60%). Mice had *ad libitum* access to food and water.

The experiment was designed to examine the effect of FA deficiency or supplementation on oocyte aneuploidy induced by colchicine (Figure 4.2). Three-week old C57BL/6 female mice (total of 96 mice) were obtained from Charles River Laboratories, Inc. Canada, and upon arrival females were acclimatised for a week before given the diet intervention. Female mice were fed one of three FA-defined diets based on the AIN-93G formula (Dyets, Inc.; Bethlehem, PA). The

diets contained 0 mg FA/kg (deficient), 2 mg FA/kg (control) or 6 mg FA/kg (supplemented) (Dyets, Inc.; Bethlehem, PA). The deficient diet (0 mg FA/kg) represents an inadequate dietary FA intake. The control diet (2 mg FA/kg) represents an adequate dietary FA intake for growing and pregnant rodents as recommended by the American Institute of Nutrition (Reeves et al., 1993). The control diet is also analogous to the recommended dietary allowance (0.4 mg FA/day) for women who could become pregnant. The supplemented diet (6 mg/kg FA) is 3-fold the control diet (1.2 mg/day) and approximates the combined FA intake from FA fortified foods in Canada and prenatal FA supplements available in the Canadian market. Four groups of female mice were fed one of three FA-defined diets and maintained on the diet for six weeks to ensure that tissue folate status reflected the given experimental diet. The three experimental dietary intervention-colchicine treated groups had a sample size of n = 28. A fourth group was fed a FA control diet (n = 12) but was not treated with colchicine to provide a negative control for the colchicine experiment and to provide a baseline oocyte aneuploidy. The rationale for the experimental sample size was based on a power calculation to generate a sufficient number of oocytes for the aneuploidy analysis. If it is assumed that each female ovulates ~25 oocytes, 28 females per group would ovulate a total of 700 oocytes. Considering that oocytes are lost during the preparation and a number of oocytes will not be analysed because of chromosome over-spreading and clumping, we assumed that we will collect 200 oocytes per group for aneuploidy analysis. Based on Mailhes & Yuan, 1987b, 0.2 mg/kg colchicine at the same time as HCG induces aneuploidy in 8% of oocytes, so 8% aneuploidy in 200 oocytes will be 16 aneuploid oocytes. Assuming aneuploidy is doubled because of the FA deficient diet, representing 16% aneuploidy in 200 oocytes will be 32 aneuploid oocytes (Chi square $p$ = 0.02). Assuming aneuploidy is decreased by 2/3 because of FA supplementation, so 3% aneuploidy in 200 oocytes will be 6 (Chi square $p$ = 0.01). Each female

was weighed weekly from the time of the FA intervention at 4-weeks of age until the day of necropsy.

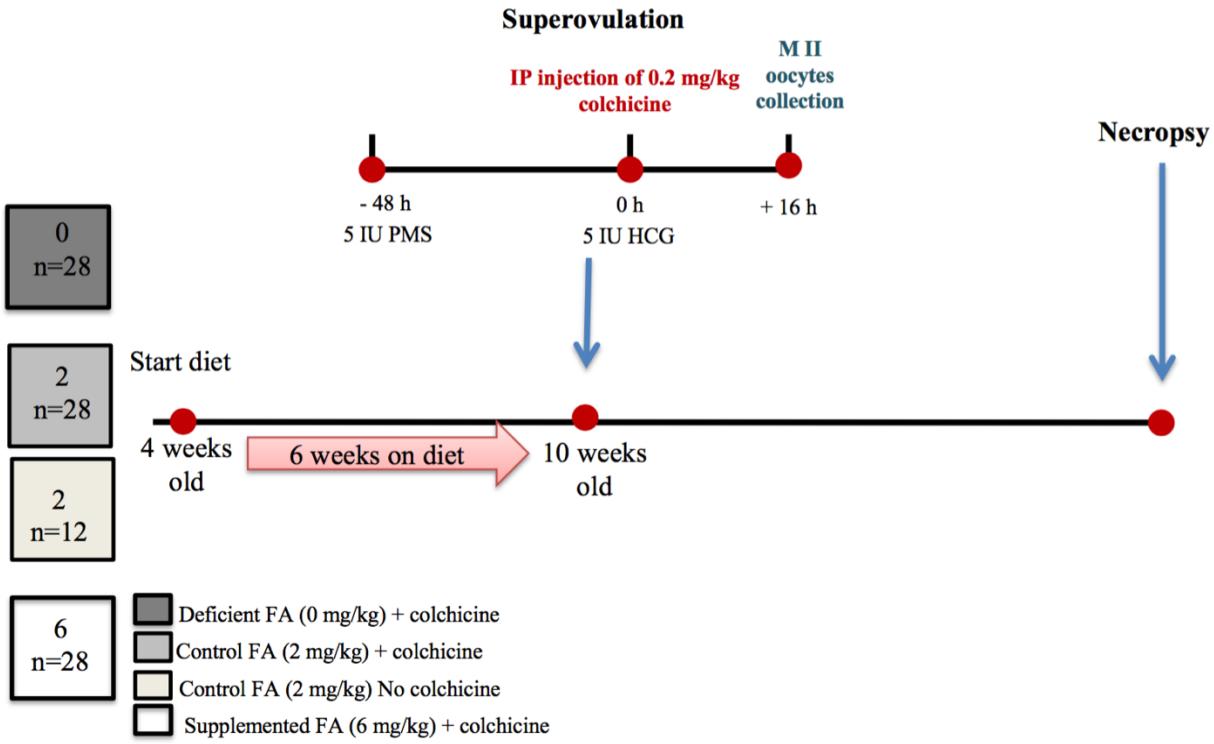



**Figure 4. 2 Animal study design.** Female mice were fed a FA deficient, control or supplemented diet from 4-weeks of age for 6 weeks. Females were superovulated by i.p. injection with 5 IU of PMS followed 48 h later by 5 IU of HCG to stimulate ovulation. A dose of 0.2 mg/kg colchicine was injected i.p. simultaneously with HCG. Oocytes were harvested 16 h relative to HCG injection.

### 4.4.2 Superovulation and Colchicine Administration

Female mice were superovulated by i.p. injection with (5 IU) pregnant mare serum gonadotrophin (Merck Folligon; Intervet) followed 48 h later by an i.p. injection (5.0 IU) of human chorionic gonadotrophin (HCG; Sigma-Aldrich) to stimulate ovulation as described by Mailhes & Yuan, 1987a. Colchicine (Lot #. SLBQ813v, Sigma-Aldrich) solution was prepared in distilled water and administrated by i.p. injection (0.2 mg/kg) administered simultaneously with HCG. The concentration of the colchicine stock solution was 5 mg/ml, and the working solution was prepared fresh before injection (Mailhes & Yuan, 1987b). Females were euthanized under isoflurane 16 h after the administration of HCG and colchicine by cardiac puncture followed by cervical dislocation (Figure 4.2).

### 4.4.3 Tissues Collected at Necropsy

Oocytes were collected and processed for the metaphase II chromosome analysis. Blood was collected by cardiac puncture into EDTA coated Vacutainer® tubes. Blood was centrifuged at 1000× *g* at 4ºC to separate red blood cells (RBCs) and plasma. Plasma was aliquoted for HCY and folate analysis and stored at -80°C until analysis.

### 4.4.4 Cytogenetic Analysis of MII Oocytes

At each necropsy, a group of seven females was euthanized and their oocytes were pooled for the cytogenetic analysis. Oocytes were processed for the metaphase II chromosome analysis to assess the presence of aneuploidy as described previously (Mailhes & Yuan, 1987a). Refer to chapter 3, section 3.4.6 Cytogenetic Analysis of MII Oocytes for detailed assay.

### 4.4.5 Folate Extraction from RBCs

Folate extraction from RBCs was done in a subset of samples (n = 5/diet) as described by Molloy & Scott, 1997. Refer to chapter 3, section For the young and old females, ~70% and 50%

respectively of the targeted number of MII oocytes (200) to analyse for the presence of aneuploidy were achieved. However, the middle-aged females had some technical issues such as losing oocytes during the fixation process which led to a loss of ~80% of the targeted oocyte number. for the detailed assay.

### 4.4.6 Plasma and RBC Folate Measurement

Plasma folate and folate extracted from RBCs (as described in chapter 3, section 3.4.7) were measured for a subset of samples (n = 5/diet) using the *Lactobacillus casei* (*L. casei*) microbiological assay as described by (Molloy & Scott, 1997). Refer to chapter 3, section 3.4.8 Plasma and RBC Folate Measurement for detailed assay.

### 4.4.7 RBC Protein Content Measurement

Since folate in tissues is protein bound, RBC folate concentrations were normalized to total protein content. The modified Lowry assay was used to quantify the protein contents in a given sample as described in Bensadoun & Weinstein, 1976. For the detailed assay, refer to chapter 3, section 3.4.9 RBC Protein Content.

### 4.4.8 Homocysteine Analysis

plasma HCY is a biomarker for functional folate status, as it indicates the ability of cells to remethylate homocysteine to form methionine (Kopp et al., 2017). Plasma HCY was analyzed using the Advia Centaur XP immunoassay (Siemens Canada), as described by the manufacturer. Refer to chapter 3, section 3.4.10 Homocysteine (HCY) Analysis for the detailed assay.

## 4.5 Statistical Analysis

Statistical analyses were performed using SigmaPlot 12.5 software. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance. A non-parametric test was applied to data that did not meet the parametric assumption. Statistical significance was tested using a *t*-test to evaluate the differences between the colchicine-treated and untreated females for number of

ovulated oocytes and HCY analysis. One-way analysis of variance (ANOVA) followed by multiple comparison procedures Dunnett's method were used to evaluate the significant differences between the colchicine-treated females fed FA deficient and supplemented diet versus the females fed FA control diet for body weight, weight gain, plasma and RBC folate analysis and number of ovulated oocytes. ANOVA on Ranks (Kruskal-Wallis One Way Analysis of Variance on Ranks) followed by pairwise multiple comparison procedures (Dunn's method) was used to evaluate the significant differences in plasma HCY concentrations among the FA diet groups. Chi-square test was used to compare the aneuploidy frequency between the colchicine-treated and non-treated groups. Statistical differences were considered significant if $p < 0.05$. Data is presented as mean±standard error of mean for body weight, weight gain, plasma and RBC folate, plasma HCY and number of ovulated oocytes, and as a percentage for aneuploidy incidence.

### 4.6 Results

A comparison between colchicine untreated females fed a FA control diet and all colchicine-treated females fed either FA deficient, control and supplemented diets was done for the HCY analysis and the number of ovulated oocytes to examine the biological activity of colchicine. To examine the effect of diet on colchicine-induced oocyte aneuploidy, only the groups treated with colchicine were compared.

#### 4.6.1 Body Weight and Weight Gain

Body weight did not differ significantly among females fed FA deficient, control or supplemented diets ($p = 0.101$). Total weight gain did not differ significantly among the females fed FA deficient, control or supplemented diets ($p = 0.532$) (Figure 4.3).

#### 4.6.2 Plasma folate

Colchicine-treated females fed a FA deficient diet had significantly lower plasma folate concentration compared to colchicine-treated females fed a control FA diet ($p < 0.001$).

Colchicine-treated females fed a supplemented FA diet had significantly higher plasma folate concentration compared to colchicine-treated females fed a control FA diet ($p < 0.001$) (Figure 4.4). Plasma folate analysis was not done for colchicine untreated females.

### 4.6.3 RBC folate

Colchicine-treated females fed a deficient FA diet had significantly lower RBC folate concentration compared to colchicine-treated females fed a control FA diet ($p < 0.001$). Colchicine-treated females fed a supplemented FA diet had similar RBC folate concentration compared to colchicine-treated females fed a control FA diet ($p = 0.218$) (Figure 4.5). RBC folate analysis was not done for colchicine untreated females.

### 4.6.4 Plasma Homocysteine

Plasma HCY concentration did not differ significantly among the colchicine-treated and untreated females ($p = 0.088$). Colchicine-treated females fed a FA deficient diet had significantly higher plasma HCY concentration compared to the colchicine-treated females fed FA control and supplemented diets ($p < 0.05$) (Figure 4.6).

### 4.6.5 Number of Superovulated Oocytes

The number of oocytes ovulated by each female did not differ significantly among colchicine-treated and untreated females ($p = 0.392$). The number of ovulated oocytes did not differ significantly among colchicine-treated females fed either a FA deficient, control, or supplemented diet ($p = 0.542$) (Figure 4.7).

### 4.6.6 Aneuploidy Incidence in MII oocytes

Results for the cytogenetic analysis for MII oocytes are presented in (Table 4.1). The incidence of hyperploidy was significantly higher in colchicine-treated females in comparison to untreated females ($p < 0.001$). The proportion of normal (haploid) oocytes is significantly higher in colchicine untreated females in comparison to colchicine-treated females ($p < 0.001$). The

incidence of aneuploidy in MII oocytes among colchicine-treated females fed the FA deficient, control or supplemented diets could not be assessed due to a low number of ovulated oocytes released in the MII phase; the majority was unexpectedly in MI.

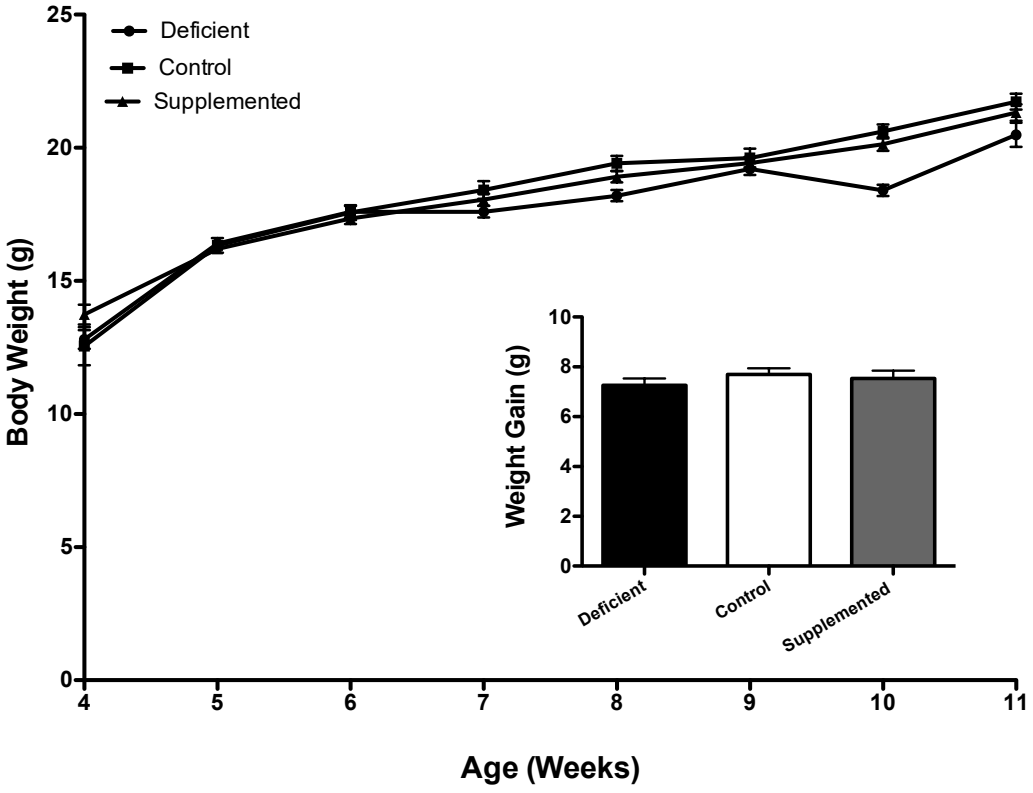



**Figure 4. 3 Body weight and overall weight gain of female mice fed folic acid defined diets.** For body weight: circle, deficient diet; square, control diet; triangle, supplemented diet. Inset, total weight gain from weaning. Black bar, deficient diet; white bar, control diet; gray bar, supplemented diet. Data are presented as mean ± SEM. One-way ANOVA was used to assess the differences between the diet groups.

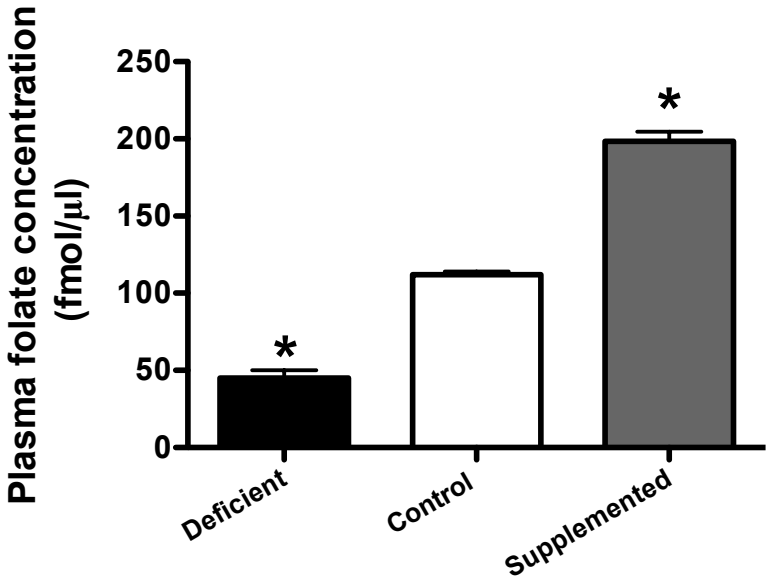



**Figure 4. 4 Effect of dietary folic acid intake on plasma folate.** n = 5 females/diet group. Data are presented as mean ± SEM. One-way ANOVA followed by Dunnett's multiple comparison test were used to assess differences among the diet groups vs. control group. Asterisks indicate significant effect between females fed a deficient FA diet vs. females fed a control diet ($P < 0.001$); and between females fed a supplemented FA diet vs. females fed a control diet ($P < 0.001$).

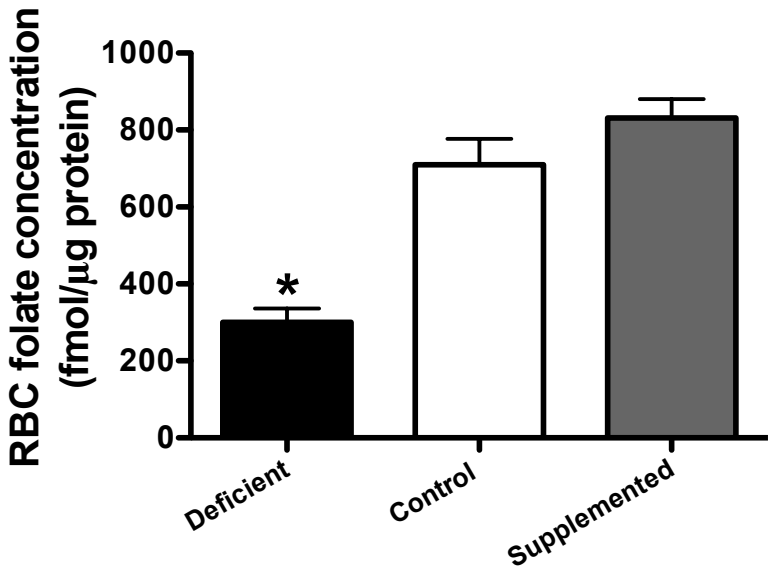



**Figure 4. 5 Effect of dietary folic acid intake on RBC folate.** n = 5 females/diet group. Data are presented as mean ± SEM. One-way ANOVA followed by Dunnett's multiple comparison test were used to assess differences among the diet groups vs. control group. Asterisk indicates significant effect between females fed a deficient FA diet vs. females fed a control diet ($P < 0.001$).

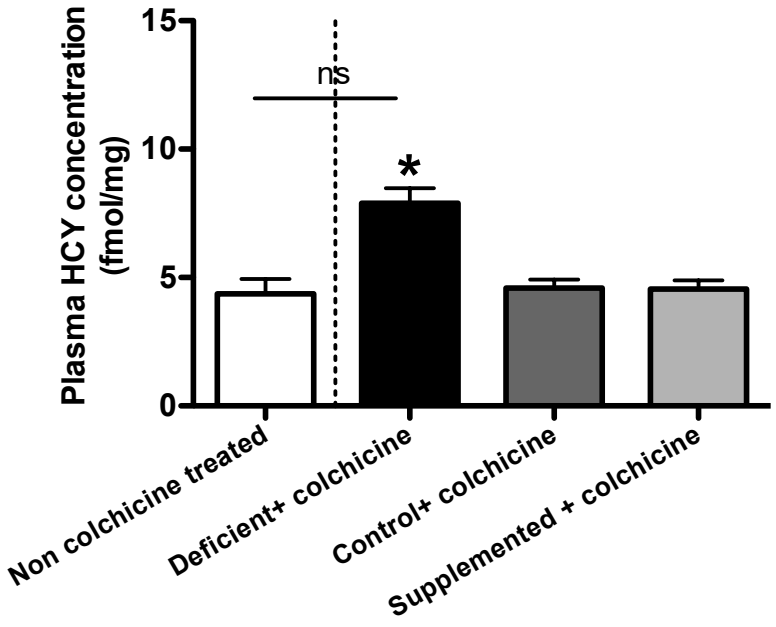



**Figure 4. 6 Effect of dietary folic acid intake on plasma homocysteine.** *t*-test was used to compare colchicine non-treated (n = 12) and treated (n = 77) females; (ns) no statistically significant difference between colchicine treated and non-treated females. Kruskal-Wallis One-way ANOVA on Ranks followed by pairwise multiple comparison procedures (Dunn's method) was used to compare colchicine treated females fed different FA diets. n = 28 for each of the diet groups treated with colchicine. Data are presented as mean ± SEM. Asterisk indicates a significant effect among females fed a deficient FA diet vs. females fed a control and supplemented diets ($P < 0.001$).

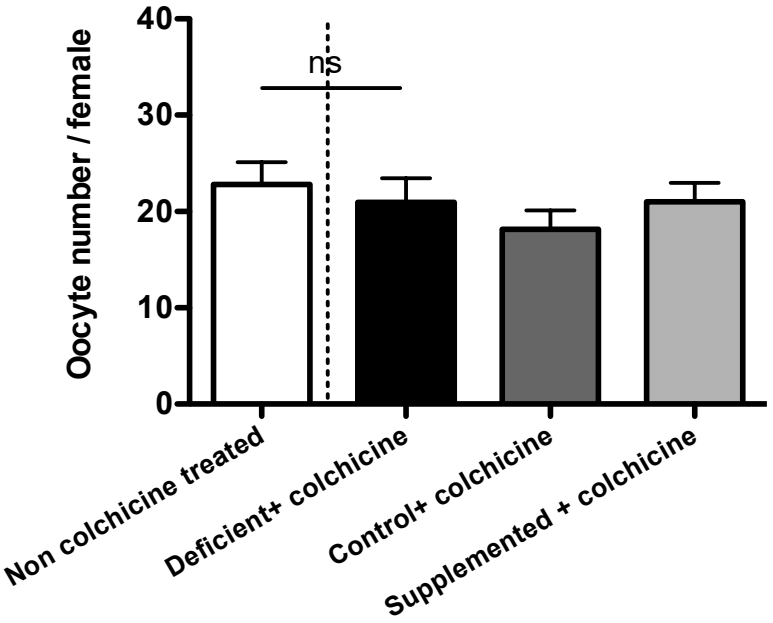



**Figure 4. 7 Effect of dietary folic acid intake on number of oocytes ovulated by each female.** *t*-test was used to compare colchicine non-treated (n = 12) and treated (n = 77) females; (ns) no statistically significant difference between colchicine treated and non-treated females. One-way ANOVA was used to compare colchicine treated females fed different FA diets. n = 21 females for deficient diet; n = 28 females for control and supplemented diets. Data are presented as mean±SEM.

**Table 4. 1** Effect of dietary folic acid intake and colchicine treatment on MII oocyte aneuploidy

| Treatment | Diet | No. of mice | Total oocytes No. | No. of oocytes/ mouse | No. of oocytes analysed | | | No. of MII oocytes analyzed | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total | MI (%) | MII (%) | Hypoploid (%) | Haploid (%) | Hyperploid (%) |
| **No colchicine** | Control | 12 | 274 | 22.8±2.2 | 94 | 0 (0) | 94 (100) | 17 (18) | 77 (82)* | 0 (0)* |
| **Colchicine** | All colchicine-treated diets | 77 | 1518 | 20±1.2 | 296 | 202 (68.2) | 94 (31.7) | 51 (54.3) | 18 (19.1) | 25 (26.6) |
| **Colchicine** | Deficient | 21 | 440 | 20.9±2.4 | 75 | 49 (65.3) | 26 (34.6) | 15 (57.7) | 5 (19.2) | 6 (23) |
| | Control | 28 | 490 | 18.1±1.9 | 100 | 74 (74) | 26 (26) | 14 (53.8) | 3 (11.5) | 9 (34.6) |
| | Supplemented | 28 | 588 | 21±1.9 | 121 | 79 (65.2) | 42 (34.7) | 22 (52.3) | 10 (23.8) | 10 (23.8) |

* Asterisks indicate a significant treatment effect between colchicine-treated and all non-treated oocytes in haploidy and hyperploidy frequencies ($p < 0.001$) using Chi-square test

Data are presented as mean±SEM for no. of oocytes/female; One-Way ANOVA was used to compare the mean no. of oocytes/female.

For MI, MII, hypoploidy (< 20), euploidy (= 20), hyperploidy (> 20), data are presented as percentage.

## 4.7 Discussion

The objective of this study was to investigate whether dietary FA deficiency or supplementation affects oocyte aneuploidy induced chemically by colchicine, an antimitotic drug. Colchicine is a microtubule destabilizer that binds to tubulin, inhibits microtubule polymerization and blocks spindle formation (Marchetti et al., 2016). Colchicine was shown to induce aneuploidy in female germ cells in mice (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990; Tease & Fisher, 1986) and Chinese and Djungarian hamsters (Sugawara & Mikamo, 1980) (Hummler & Hansmann, 1985). It has been reported in naturally aged female mice that aging affects microtubule dynamics through altering microtubule-kinetochore interactions resulting in multipolar spindle and defective kinetochore-microtubule attachment which predispose oocytes to segregation errors that lead to aneuploidy during meiosis (Nakagawa & FitzHarris, 2017; Shomper et al., 2014). Therefore, we hypothesized that colchicine treatment could represent a model for studying age-induced oocyte aneuploidy.

In this study, we showed that colchicine does indeed induce aneuploidy, but in this case, also arrested oocytes at MI, which limited our ability to assess MII oocyte aneuploidy as intended. A colchicine dose of 0.2 mg/kg was chosen because it had been previously shown to significantly induce aneuploidy in mouse oocytes and, in those studies, did not arrest oocytes in MI (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990; Pligina et al., 2017; Tease & Fisher, 1986). Tease & Fisher used F1 female mice (C3H/HeH x 101/H) aged between 9-12 weeks and used a colchicine dose of 0.25 mg/kg administrated 3 hours post HCG injection. Mailhes et al. used CD1 female mice aged between 8-12 weeks and colchicine was administrated at the same time as HCG via i.p. injection at a dose of 0.2 mg/kg; oocytes were harvested 16-17 h post HCG administration (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990). Recently, Pligina et al, used F1 female mice (CBA x C57BL/6) aged 6-8 weeks and used a colchicine dose of 0.2 mg/kg

administered via i.p. injection at the same time as HCG injection and oocytes harvested 17 hours after HCG administration (Pligina et al., 2017). In this study we used C57BL/6 female mice aged 10 weeks and colchicine was administrated in a dose of 0.2 mg/kg via i.p. injection at the same time as HCG administration and oocytes harvested 16 hours post HCG injection. Our experimental conditions were most similar to what was reported previously by (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990 ; Pligina et al., 2017). When we compared aneuploidy incidence (presented as hyperploidy) between colchicine-treated and untreated females we found that hyperploidy incidence in colchicine-treated females was significantly higher (26.5%) compared to untreated females (0%). This indicates that the colchicine dose we used was biologically active and had the ability to induce aneuploidy in oocytes.

However, the main objective of our study was to examine the effect of FA diets on aneuploidy frequencies induced by colchicine in MII oocytes. Our results showed that the effect of different FA diets on MII oocyte aneuploidy induced by colchicine could not be detected due to the low proportion of ovulated MII oocytes. Only ~35% of oocytes in the deficient and supplemented groups and 26% of oocytes in the control group were MII oocytes. The remaining oocytes were arrested at MI. This reduced the overall number of MII oocytes available for aneuploidy analysis resulting in a study that was underpowered to detect diet-induced differences in the frequency of MII oocyte aneuploidy. Looking from this perspective, our results contrast with those of previously published reports that used colchicine to induce aneuploidy in different mouse and hamster strains as shown in (Table 4.2). In mouse studies, three different strains, F1 C3H/HeH x 101/H, CD1, and F1 CBA x C57BL/6, were used to study oocyte aneuploidy induction by colchicine. In all of these studies, only a small proportion (0 to 4%) of oocytes were blocked at MI whereas 67% to 100% of oocytes were released to MII. Also, colchicine induced 0 to 15.6%

hyperploidy incidence in MII oocytes (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990; Pligina et al., 2017; Tease & Fisher, 1986). Other rodent species have shown consistent results with these mouse studies. Colchicine–induced oocyte hyperploidy was 8.15% in Chinese hamsters (Mikamo & Sugawara, 1980; Sugawara & Mikamo, 1980) and 11.7% in Djungarian hamsters (Hummler & Hansmann, 1985). In contrast to all of these studies, we observed that about 68% of the colchicine-treated oocytes were arrested at MI, which is 17 times higher than previously reported. Conversely, only about 32% of colchicine-treated oocytes released to MII, a 2 to 3 times lower proportion than previously reported.

Colchicine has the ability to block microtubule assembly and polymerization and inhibit spindle formation (Marchetti et al., 2016). It was reported that certain chemicals such as griseofulvin and vinblastine sulfate known to damage the spindle fibers have the potential to arrest oocytes in MI (Mailhes & Marchetti, 1994a). In the cases where these chemicals were used, the time between HCG administration and oocyte harvest must be extended to allow oocytes to overcome an MI block and advance to MII (Mailhes & Marchetti, 1994a). When griseofulvin was given at the same time as HCG, MI oocytes represented 83.4% and 6.3% of oocytes when they were harvested at 17 hours and 23 hours post HCG, respectively (Marchetti & Mailhes, 1994). Based on these previous observations, we assume that an extended time between ovulation and oocyte harvest may be required to avoid the high frequency of oocytes arrested at MI.

Colchicine dose can also have an effect on MI arrest. Colchicine given in high doses (higher than 0.3 mg /kg i.p.) was shown to arrest more than 50% of mouse oocytes at MI (Mailhes et al., 1990). The dose of 0.2 mg/kg colchicine administrated via i.p. injection, as used in our study, was shown to arrest a maximum of 4% of mouse oocytes at MI in comparison to 50-98% of oocytes blocked at MI when 0.4 and 0.5 mg/kg of colchicine doses were used (Mailhes & Yuan,

1987b; Mailhes et al., 1990). In our study, the effect of 0.2 mg/kg of colchicine was similar to that of 0.4 and 0.5 mg/kg doses in terms of blocking oocytes at MI. A possible explanation for the high proportion of oocytes blocked at MI in our study is differences in the susceptibility to colchicine of the mouse strain we used (C57Bl/6). The previously reported studies used F1 hybrid mouse strains (Pligina et al., 2017; Tease & Fisher, 1986) and the CD1 outbred stock (Mailhes et al., 1988; Mailhes & Yuan, 1987b; Mailhes et al., 1990). We used an inbred mouse strain, C57BL/6, which had not previously been reported in studies in which colchicine was used to induce oocyte aneuploidy. The genetic variation in F1 and outbred mice might make them less susceptible to colchicine. In contrast, the inbred mouse strain we used was appears to be much more susceptible to colchicine treatment. Pligina et al reported that 100% of oocytes released to MII and no oocytes were arrested at MI (0%) when they used an F1 hybrid resulting from the intercross of two inbred strains (CBA x C57BL/6) with similar experimental conditions as we used. This difference in strain susceptibility could possibly explain the high proportion of oocytes being blocked at MI since all other experimental variables were similar.

It should be noted that any differences in the proportion of MI and MII cells in this study were not due to a difference in the number of ovulated oocytes. While a colchicine dose that exceeded 2.0 mg/kg resulted in fewer ovulated oocytes per female (Mailhes & Yuan, 1987b), we did not observe any differences when comparing colchicine treated and non-treated females. Also, we did not observe any differences in the number of ovulated oocytes among the FA diet groups.

To ensure that any differences among FA diet groups were a result of folate intake and not in response to the colchicine treatment, plasma HCY concentrations were measured in both colchicine-treated and non-treated females. Plasma HCY is a measure of functional folate status and the lack of difference between the non-treated and treated females indicates that colchicine

did not meaningfully influence one-carbon metabolism. Among the colchicine-treated females, plasma and RBC folate were reflective of FA intake. Plasma folate is a sensitive indictor of recent folate intake (Matejcic et al., 2017). Females fed a FA deficient and supplemented diet had significantly lower and higher plasma folate concentrations, respectively, compared to females fed the FA control diet. RBC folate concentrations were also measured since it reflects tissue folate status and is usually considered a marker of long-term status (De Bruyn et al., 2014). Females fed a FA deficient diet had significantly lower RBC folate concentrations in comparison to females fed a FA control diet, whereas the RBC folate in supplemented females was numerically, but not significantly, higher. This reflects a ceiling above which tissues cannot accumulate additional folate from circulation. In addition, plasma HCY is a biomarker for functional folate status, as it indicates the ability of cells to remethylate homocysteine to form methionine. As such, high plasma HCY is an indication of folate deficiency (Kopp et al., 2017). We report that among the FA diet groups, females fed a FA deficient diet had significantly higher plasma HCY concentrations, as would be expected.

**4.8 Strengths and Limitations of the Study**

A point of strength of this study was the use of a mouse model in which aneuploidy incidence in oocytes can be assessed in 10-week old mice compared to the *in vivo* aging model which can take up to 12 months to asses aneuploidy (Merriman et al., 2012). Another point of strength is the study design including the control group in which mice were untreated with colchicine to provide a negative control for the colchicine treatment and to provide an assessment of baseline oocyte aneuploidy. Also, the analysis of folate status and functional biomarkers of folate metabolism was critical for the interpretation of findings.

A limitation of the study was that the colchicine untreated group was not injected with saline. However, it should be noted that females were injected with colchicine at the same time as

HCG, so our colchicine untreated females were given an i.p. injection of HCG meaning that they were exposed to a similar stressful intervention (injection) as the colchicine treated group. Another limitation in the study was not having a diet-matched colchicine- untreated group for each FA diet, except for the sufficient diet (2 mg/kg). However, given that we were not concerned about a diet X colchicine effect on oocyte aneuploidy, we felt that this was an efficient approach to reduce the number of mice required for the experiment. A critical limitation was using the C57BL/6 mouse strain which had not previously been used to study aneuploidy induction in oocytes, and which appears to be more sensitive to colchicine compared to other strains.

**4.9 Conclusion**

In conclusion, the aim of this study was to examine the effect of FA intake on oocyte aneuploidy induced by colchicine in MII oocytes as a model for studying age-induced oocyte aneuploidy. Our model, however, resulted in a low proportion of ovulated MII oocytes and a large proportion of oocytes blocked at MI, which limited our power to detect any effect of FA on colchicine-induced oocyte aneuploidy. We propose that different mouse strains vary in their tolerance to colchicine-induced arrest of oocytes at MI, since the effect of 0.2 mg/kg colchicine in the C57BL/6 strain had a similar effect as 0.4 and 0.5 mg/kg doses in other strains, CD1 and F1 hybrid strains (Mailhes et al., 1990 ; Mailhes & Yuan, 1987b; Pligina et al., 2017; Tease & Fisher, 1986). The data indicate that the C57BL/6 mouse strain is much more sensitive to colchicine exposure.

**Table 4. 2** Results for i.p. colchicine administration in different mouse and hamster strains

| Colchicine dose (mg / kg) | Species | Strain | Age (weeks) | Type of administration | Time of administration relative to HCG (h) | No. of oocytes analyzed | % of MI oocytes | % of MII oocytes | % of hyperploid oocytes | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.25 | Mouse | F1 (C3H / HeH x 101 / H) | 9-12 | NI | 3 | 241 | 4.1 | 68.6 | 11.2 | Tease and Fisher (1986) |
| 0.25 | Mouse | F1 (C3H / HeH x 101 / H) | 50-56 | NI | 3 | 77 | 30.9 | 24.7 | 15.6 | Tease and Fisher (1986) |
| 0.2 | Mouse | CD-1 | 8-12 | i.p. | 0 | 462 | 0 | 100 | 7.8 | (Mailhes & Yuan, 1987b) |
| 0.2 | Mouse | CD-1 | 8-12 | i.p. | 0 | 220 | NI | NI | 9.5 | (Mailhes et al., 1988) |
| 0.2 | Mouse | CD-1 | 8-12 | i.p. | 0 | 504 | 0.3 | 99.7 | 7.3 | (Mailhes et al., 1990) |
| 0.2 | Mouse | F1(CBA x C57BL/6) | 6-8 | i.p. | 0 | 150-160 | 0 | 100 | 0 | (Pligina et al., 2017) |
| 3 | Chinese hamster | NI | 20-32 | i.p. | 10 h preovulation | 40 | NI | NI | 7.5 | (Mikamo & Sugawara, 1980) |
| 3 | Chinese hamster | NI | 20-32 | i.p. | 10 h preovulation | 342 | NI | NI | 8.8 | (Sugawara & Mikamo, 1980) |
| 3 | Djungarian hamster | NI | 8-14 | i.p. | 5 | 137 | NI | NI | 11.7 | (Hummler & Hansmann, 1985) |

Footnote: (NI) Not Indicated

# Chapter 5: General Discussion

# Chapter 5
# General Discussion

## 5.1 Thesis Results Summary

The main objective of the research presented in this thesis was to investigate the effect of FA supplementation on female germ cell aneuploidy associated with advanced maternal age. Establishing whether a FA supplemented diet could mitigate aneuploidy incidence in oocytes using different experimental mouse models could shed light on the causal role of FA, if there is any, in the protection of oocytes from aneuploidy. In this final chapter, the major findings regarding the effect of a FA supplemented diet on maternal aging-associated oocyte aneuploidy will be discussed. The main results demonstrate that old $Bub1^{+/m}$ female mice fed a supplemented FA diet for 24 weeks had a lower oocyte aneuploidy incidence due to a doubling of the number of normal oocytes compared to females fed a control FA diet. Bub1 is a key protein kinase in the SAC mechanism that controls the chromosome segregation process. A defect in Bub1 function leads to the generation of misaligned chromosomes at the metaphase plate, presence of premature sister chromatid separation, and the occurrence of oocyte aneuploidy, which are phenotypes that are associated with advanced maternal aging (Leland et al., 2009; McGuinness et al., 2009; Tsurumi et al., 2004; Yin et al., 2006).

It has been reported that the majority of meiotic errors in female germ cells occur during the first meiotic division (Hassold & Hunt, 2001). In our study, FA intervention was initiated when oocytes were meiotically arrested at prophase I. The duration of this arrest takes years in humans and months in mice (Clift & Schuh, 2013). During this long arrest period, oocyte quality can be affected by multiple endogenous factors including the degradation of cohesion subunits, misregulation of key SAC proteins, and mitochondria dysfunction, as reported in both human and mouse studies (Simsek-Duran et al., 2013; Steuerwald et al., 2001; Tsutsumi et al., 2014). It might

be that FA intervention during mouse adulthood mitigates the effect of maternal aging on oocyte aneuploidy by maintaining some or all of these endogenous mechanisms that are negatively affected by maternal aging, refer to Figure 2.3. Since adequate folate intake is required for proper nucleotide synthesis and cellular methylation reactions (Stover, 2009), we hypothesized that FA supplementation during the adulthood of female mice would reduce the adverse effects of maternal aging on specific mechanisms known to lead to a high incidence of segregation errors and therefore oocyte aneuploidy.

The main observation in this thesis, that old Bub1$^{+/m}$ female mice fed a supplemented FA diet for 24 weeks had a lower oocyte aneuploidy incidence through doubling of the number of normal oocytes compared to females fed a control diet, FA supplementation during the adult life of old Bub1$^{+/m}$ female might indeed maintain chromosome stability and proper chromosome segregation during MI. Similarly, a study in somatic cells recently reported that folate deficiency induced the dysregulation of *BUB1* expression which contributed to a significantly higher frequency of chromosome misalignment and missegregation, and spindle abnormality (Guo et al., 2017). The same mechanism could be proposed to play a role in our model *in vivo*, such that FA supplementation maintains the expression of SAC proteins including Bub1 which supports proper chromosome alignment and segregation and leads to a higher frequency of normal oocytes in old females.

### 5.2 Implications for Female Reproductive Health

There is an increasing trend in the Canadian population to delay childbearing to increasingly older ages. This occurs for several mostly social or economic reasons including: personal development, attainment of higher levels of education and income, and career and relationship stability (Tough et al., 2007). According to Statistics Canada, the proportion of the live birth rate has decreased for younger women and increased for older women over the last two plus decades

(between 1993 and 2013). 54.4% of all Canadian births in 2013 were attributed to mothers aged between 30 to 49 years, up from 39.6% in 1993. Similarly, 3.5% of all births in 2013 were born to mothers 40 years and older, compared to 1.3% in 1993. In addition, first births to mother aged 35 to 39 years has increased from 5.4% in 1993 to 10.7% in 2013 and from 0.7% in 1993 to 2.2% in 2013 for mothers aged 40 years and older (Statistic Canada, 2016). Given that age-associated oocyte aneuploidy increases with maternal age, these changing dynamics have real implications for female fertility and pregnancy outcomes.

With increasing age comes increasing degrees of infertility. In Canada, like many other countries, infertility rates are indeed increasing. The prevalence of infertility was estimated to be 8.5% in 1992 (Dulberg, 1993) but had increased to 15.7% by 2010; this change means that almost one in six couples have infertility issues (Bushnik et al., 2012). As a consequence, the use of assisted reproductive technologies in Canada has also dramatically increased from 7,884 cycles in 2001 (Gunby et al., 2005) to 33,092 cycles in 2017 (Gunby, 2017). Among the Canadian provinces, Quebec and Ontario cover a limited number of IVF cycles. In Ontario, the system allows a woman under the age of 43 to take advantage of one covered IVF cycle in her lifetime, whereas Quebec's offers a sliding scale of tax credits (Government of Canada, 2017). As such, it is imperative that we identify modifiable determinants of infertility to reduce the emotional and economic burdens it causes.

Despite the benefits of ART in helping infertile couples conceive children, it has been shown that ART is associated with negative impacts on offspring development. For example, children born using ART have an increased risk of birth defects including: low birth weight, preterm birth, small for gestational age, stillbirth, perinatal mortality and admission to a neonatal intensive care unit (Buckett et al., 2007; Chen & Heilbronn, 2017; Hansen et al., 2013; Liberman et al., 2017).

In mice, it has been reported recently that ART use was associated with increased embryonic loss and developmental delay (Rahimi et al., 2019). A high incidence of aneuploidy has been reported in the abortuses of infertile patients who conceived pregnancy using ART (Lathi & Milki, 2004; Nayak et al., 2011) and this high incidence of aneuploidy was associated with advance maternal age (Kushnir & Frattarelli, 2009; Spandorfer et al., 2004). What is not clear is whether these adverse effects are due solely to the ART or to the underlying causes of infertility. Either way, interventions that can improve ART outcomes and offspring development are needed.

The association between FA supplementation and female fertility outcomes has been investigated in both the general population and in women undergoing ART treatment. Among women in the general population, FA supplement intake before and during pregnancy was associated with higher pregnancy rates, increased mean birth weight, and reduced rates of miscarriage and preterm birth (Czeizel et al., 1994 ; Czeizel et al., 2010). Similarly, in women undergoing ART treatment, FA supplementation was associated with better quality and maturation of oocytes, better embryo quality, and higher fertilization (Berker et al., 2009; Czeizel et al., 2010; Gaskins et al., 2014a; Szymanski & Kazdepka-Zieminska, 2003; Westphal et al., 2006). Also, FA supplement intake was associated with reduced embryonic developmental delay and loss in mice undergoing ART (Rahimi et al., 2019). Since the proportion of women who delay pregnancy until increasingly older ages has increased and subsequently more couples are having infertility issues and pursuing ART interventions, there is concern that the incidence of oocyte aneuploidy and consequent disorders in the offspring will also increase.

About 63.5% of Canadian population have a folate status which suggests that their FA intake is potentially at or above the tolerable upper intake level (1 mg/day) (MacFarlane et al., 2011). Based on the current folate status of the Canadian population, FA supplementation was an

important consideration in our study. FA fortification has been a successful public health intervention for reducing the incidence of NTDs. The higher folate status of the population may also prove beneficial for reproductive aged women in terms of their reproductive success. In our study, we showed that FA supplementation (8 mg/kg) has reduced the effect of aging on oocyte aneuploidy by significantly increasing the proportion of normal oocytes in old female mice fed the supplemented FA diet for 24-week. Also, a similar level of FA supplementation (8 mg/kg) was shown to be beneficial for mice undergoing ART by reducing embryonic developmental delay and loss (Rahimi et al., 2019). It should be noted that the 8 mg FA/day supplementation is comparable to the combined FA intake from both fortified foods and over-the-counter prenatal supplements.

It is well known that folate deficiency has negative effects on genome stability, human pathologies and female fertility. On the other hand, the potential beneficial effects or risks associated with FA supplementation remain unclear. Since mandatory fortification with FA was initiated in the late 1990s, the mean FA intake within the Canadian population has increased (MacFarlane et al., 2011). Therefore, it is important to investigate any potential benefits or risks associated with higher intakes of FA on human pathologies in general and female fertility specifically. High doses of FA could affect folate metabolism and may result in unmetabolized FA in circulation (Smith et al., 2008). Increased concentrations of circulating FA has been proposed to lead to DHF accumulation. Accumulation of DHF can potentially inhibit thymidylate biosynthesis and MTHFR. Inhibition of MTHFR could result in reduced methionine synthesis and lower 5-methyl-THF production, leading to decreased remethylation of homocysteine, as well as decreased nucleotide biosynthesis (Smith et al., 2008). Therefore, impaired nucleotide synthesis may lead to increased mutation rates, genome instability, and reduced cell proliferation (Steegers-Theunissen et al., 2013).

Additionally, there is a concern regarding a negative interaction when supplemental FA intake is combined with vitamin $B_{12}$ deficiency. High FA intake can mask the symptoms of vitamin $B_{12}$ deficiency (Morris et al., 2007). The enzyme MTR, a vitamin $B_{12}$ dependent enzyme, is responsible for transferring a methyl group from 5-methyl-THF to homocysteine to form methionine and recapitulate THF (Stover, 2004). As such, vitamin B12 deficiency diminishes MTR activity and results in accumulation of 5-methyl-THF. The accumulation of 5-methyl-THF depletes the relative concentrations of other forms of folate such as THF and inhibits nucleotide synthesis (Stover, 2009). Case studies have suggested that high dose FA can rescue vitamin B12 deficiency anemia by supporting THF production and in so doing masks vitamin B12 deficiency allowing for the more serious symptom of deficiency, namely irreversible neurodegeneration to progress. High FA intake and vitamin $B_{12}$ deficiency has been associated with cognitive decline (Morris et al., 2007).

High FA intake has been associated with an increased risk of some type of cancers including prostate and colorectal cancers (Figueiredo et al., 2009; Mason et al., 2007). However, these potential concerns remain largely hypothetical and require further investigation. High maternal FA intake was also reported to have adverse effects on offspring development and health in mice. High maternal FA supplemented diets containing 40 mg FA/kg, 20-fold the control FA diet (2 mg/kg) for rodents, has been associated with embryonic delay and growth retardation particularly in early development at embryonic day 10.5 (Pickell et al., 2011). Similar adverse effects have been observed when maternal FA intake in mice reaches 10-fold the control diet (20 mg FA/kg) including embryonic delay and growth retardation in addition to altered brain development and impaired memory in offspring (Bahous et al., 2017). In summary, over-consumption of FA may have negative health consequences.

Based on our results, it is important that women of advanced maternal age consume an adequate diet of FA in order to reduce the effect of aging on oocyte aneuploidy, improve oocyte quality and overall fertility. The natural consequence of this is a reduction in the emotional and financial costs of infertility and ART treatment, especially given that FA represents a promising and economical method to improve oocyte quality and aneuploidy. More studies will have to be conducted to further explore the beneficial effect of FA supplementation in women of advanced maternal age on their reproductive and offspring health. FA supplementation could be an appropriate intervention method to reduce aneuploidy and improve the quality of oocytes leading to better reproductive outcomes.

Based on the major findings in this thesis, in which a modest supplementation reduced the risk of oocyte aneuploidy, and taking into consideration the need to balance benefits and risks of higher FA intakes, a possible recommendation is that women of child bearing age consume an adequate diet of FA and lower dose supplement containing 400 µg FA. This recommendation would potentially reduce the risk of oocyte aneuploidy and aligns with the Health Canada recommendation for FA intake for NTD prevalence. It is recommended that all women of childbearing age and could become pregnant need to take a multivitamin with 0.4 mg of FA every day in addition to dietary folate intake from a varied diet (Health Canada, 2013).

Canada is one of the countries that mandates the fortification of white flour, and other enriched cereals and cornmeal with FA. The goal of fortification, implemented in1998, was to increase the average daily consumption of FA for women of childbearing age by about 100-150 µg FA (Colapinto et al., 2011; De Wals et al., 2007). Since fortification, the prevalence of DS birth in Canada between 2005 to 2013, has remained stable averaging around 15.8 per 10,000 total birth (Public Health Agency of Canada, 2017). Despite the trend in delaying childbearing to older

age, the prevalence of DS birth has not increased in the time between 2005-2013. The primary reason for a lack of increasing risk for DS birth is likely to be due to the prenatal diagnosis of chromosome anomalies and the subsequent decision of pregnancy terminations. However likely this is, it is difficult to show definitively that this is the case given the difficulty in capturing early terminations in the Canadian congenital anomaly surveillance system. Based on our study design and results, we might assume that the prevalence of DS birth decreased after fortification with FA, but because of pregnancy terminations of DS conceptus, we cannot tell if the prevalence of DS pregnancies relative to maternal age has decreased due to higher FA intake or has stabilized due to more frequent terminations.

Because of the lack of data on the surveillance of DS birth in Canada before the FA fortification period, we examined infant death rates from chromosome anomalies as a proxy. Based on data from Statistics Canada, the infant death rates from chromosomal anomalies including DS did not change between 1974 and 2102 (Statistics Canada, 2016), which encompasses the periods before and after fortification. However, in countries that do not fortify with FA, such as Europe (Food Fortification Initiative), the prevalence of DS birth has also been stable (22 per 10,000 birth) between 1990 to 2009 (Loane et al., 2013) despite a rise in the average maternal age in the European population over time and an increase in the number of pregnancies affected by trisomy 21. An increase in the use of prenatal screening and terminations of DS pregnancies have likely influenced the detection of the effect of maternal age on aneuploidy pregnancies and resulted in a relatively stable prevalence of the DS birth.

Based on our results, it is important that women of advanced maternal age consume an adequate diet of FA in order to reduce the effect of aging on oocyte aneuploidy, improve oocyte quality and overall fertility. The natural consequence of this is a reduction in the emotional and

financial costs of infertility and ART treatment, especially given that FA represents a promising and economical method to improve oocyte quality and aneuploidy. More studies will have to be conducted to further explore the beneficial effect of FA supplementation in women of advanced maternal age on their reproductive and offspring health. FA supplementation could be an appropriate intervention method to reduce aneuploidy and improve the quality of oocytes leading to better reproductive outcomes.

### 5.3 Strengths and weaknesses

**Strengths:** One of the strengths of this research is the use of three experimental mouse models to study the effect of FA intake on oocyte aneuploidy. The three models represent the effect of maternal aging on oocyte aneuploidy via different strategies.

A specific strength was the use of the experimental model $Bub1^{+/m}$ female mouse to study the effect of FA supplementation on aneuploidy incidence. $Bub1^{+/m}$ is a key protein kinase in SAC mechanism that regulates chromosome segregation (Marchetti & Venkatachalam, 2010). $Bub1^{+/m}$ mouse model represents a failure of SAC mechanism that was shown to be negatively affected by maternal aging. By demonstrating that this model is responsive to FA supplementation, we are not only demonstrating a causal link between FA intake and oocyte aneuploidy, but we are identifying a specific aging-associated mechanism that is folate-responsive. Further studies can explore the relationship between FA intake and SAC dysfunction as it relates to oocyte aneuploidy.

**Weaknesses:** A weakness of the studies presented in this thesis is the use of a single inbred mouse strain, C75BL/6, for the studies. For the *in vivo* aging mouse study (Appendix III), aging the mice for ~12 months led to such a major reduction in ovulated oocyte number (mean = ~3 oocytes/female) which reduced the ability to examine the effect of FA supplementation. The number of ovulated oocytes was remarkably lower than previously observed in other *in vivo* aging studies that used outbred and F1 mouse strains. In the colchicine induced aneuploidy study, the

effect of FA intake could not be analysed because the majority of oocytes were arrested at MI, making the number of oocytes available for analysis to low o determine an effect of FA. Again, this was in contrast to other studies in which other strains, outbred or F1 crosses, did not demonstrate oocyte arrest.

Another weakness is that the cytogenetic technique used to assess aneuploidy required pooling of oocytes from ~8 females which limited our ability to assess the inter-mouse variability of oocyte aneuploidy. This limitation could be overcome through the implementation of a mating study to assess the effect of FA supplementation directly on embryos, which would allow for an assessment of aneuploidy within individual pregnancies.

**5.4 Future Directions**

The results of this study highlight the effects of FA supplementation on oocyte aneuploidy associated with advanced maternal age using the Bub1$^{+/m}$ female mouse as an experimental model. My main results demonstrate that old Bub1$^{+/m}$ female mice fed a supplemented FA diet had a lower oocyte aneuploidy incidence due to a doubling of the number of normal oocytes compared to mice fed the control diet. In future studies we would like to investigate the effect of the supplemented FA diet on embryonic aneuploidy in old Bub1$^{+/m}$ female mice to confirm if the effect of FA supplementation on the mother is translated to the offspring. A similar study design will be employed to assess the effect of FA supplementation on embryo development and aneuploidy in Bub1 females fed the FA diets before and during pregnancy. A group of Bub1$^{+/m}$ female mice will be fed a control FA diet (2 mg/kg), and the other group will be fed a supplemented FA diet (8 mg/kg) for 24 weeks. The females will then be mated with males and embryo development assessed at embryonic day 10.5-15. Several end points will be analysed including implantation sites, resorption rate, and embryonic viability including developmental and morphological changes. I propose that FA supplementation will improve the number of viable embryos.

Furthermore, it will be beneficial to investigate the potential mechanisms that may explain the positive effect of FA supplementation on oocyte aneuploidy in old Bub1$^{+/m}$ females. RNA-sequencing in oocytes would be one way to start exploring the transcriptomic changes that are altered by FA supplementation to identify candidate functional pathways that may be related to improved oocyte aneuploidy.

In chapter 4, we performed a study to assess the effect of FA intake on oocyte aneuploidy induced by colchicine, a known meiotic inhibitor that inhibits microtubule polymerization and spindle formation. The preliminary results showed that a high proportion of oocytes were arrested at MI leaving a low number of MII oocytes for analysis which made the study underpowered to detect a diet effect. I would propose a follow-up study using a similar experimental design but extending the time between the induction of superovulation and the collection of oocytes. I would suggest harvesting the oocytes over a time course up to 23 h based on a study in which another meiotic inhibitor, griseofulvin, was shown to achieve the collection of more than 90% of MII oocytes (Marchetti & Mailhes, 1994).

**5.5 Alternative Approaches**

We have used the Bub1$^{+/m}$ mouse model, which represents a dysfunction of SAC mechanism. This mouse model was an excellent model to study aneuploidy incidence; however, it would be useful to use other mouse models that demonstrate dysfunction in the other pathogenic mechanisms proposed to be negatively affected by maternal aging such as mitochondrial dysfunction. It is well known that mitochondrial function deteriorates with maternal aging due to a decline in mtDNA copy number and ATP content, and increased mtDNA mutation rate, which in turn affects oocyte quality, fertilization ability, embryo development and overall female fertility (Chan et al., 2005; Keefe et al., 1995; Simsek-Duran et al., 2013). The mouse model, Senescence-accelerated mice (SAM), exhibits mitochondrial dysfunction and oocytes from these mice

demonstrate chromosome misalignment at both MI and MII, which predispose oocytes to missegregation and therefore aneuploidy (Liu and Keefe, 2002). The SAM model therefore represents a good model to study effect of FA on the mitochondrial dysfunction mechanism, especially given that approximately 40% of folate one-carbon metabolism occurs in the mitochondria (Stover & Field, 2011; Tibbetts & Appling, 2010).

Another approach would be to employ a transgenerational FA intervention. Generally, the epidemiological studies look at only the maternal diet, and demonstrate associations between impaired maternal folate metabolism and DS birth (Hollis et al., 2013). In fact, it should be taken into consideration the potential transgenerational contribution of folate metabolism to the risk of a DS birth. The majority of DS cases originate from a maternal MI error (Hassold & Hunt, 2001). Examining the effect of dietary FA intake for at least two generations, grandmother and mother, is therefore important. Sufficient dietary folate intake of the grandmother before and during pregnancy is required for proper embryo development, including the fetal ovary and the progression of fetal oocytes to MI. Also, as shown here, the maternal FA diet before and during conception may be required for appropriate MII progression. Based on this, it would be of interest to study the effect of transgenerational FA intake throughout two generations to assess the effect on the third generation (oocytes and embryos). Since most aneuploidy accurse at MI during the maternal fetal life and it is irreversible, a transgenerational intervention with FA may help reduce MI errors and MI derived aneuploidy.

**5.6 Conclusion**

In conclusion, this thesis highlights the importance of maternal FA intake on oocyte aneuploidy associated with advanced maternal aging. It demonstrates that FA supplementation has a beneficial effect on mitigating oocyte aneuploidy in old female mice suggesting that FA

supplementation is an important and feasible intervention method to reduce the effect of maternal aging on aneuploidy in oocytes.

# Appendix I
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# Appendix II

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# Appendix III

## Investigating the Effect of Dietary Folic Acid Supplementation on Oocyte Aneuploidy Associated with Advanced maternal Age in Naturally Aged Wild Type Female Mice

### Purpose of the study

The purpose of this study was to investigate whether dietary FA supplementation reduces oocyte aneuploidy that is associated with advanced maternal age.

### Hypothesis

Aged female mice fed a FA supplemented diet will have fewer aneuploid oocytes compared to female fed a control FA diet.

### Experimental design

Two experimental groups of WT female mice (C75BL/6) (total of 52 mice) were used in this experiment. At weaning (21±1 day), the first group (n = 26) was fed a control diet contained 2 mg / kg FA whereas the second group (n = 26) was fed a FA supplemented diet (8 mg/kg). Females were fed their diet for almost one year (~48 weeks). At the end of the intervention period, females were superovulated.by i.p. injection with PMS (7.5 IU) followed 48 h by an injection of HCG (5.0 lU) to induce ovulation. Then females were euthanized 16 h after the administration of HCG and oocytes were collected for MII cytogenetic analysis to assess the present of aneuploidy.

### Results and conclusion

No result was obtained from this study as the aneuploidy incidence could not be assed due to the low number of ovulated oocytes (mean oocytes/female was 2.19±1.1 in the control group and 3.16±1.6 in the supplemented group). The basis for this study design was a study done by Tease and Fisher by which they used F1 mouse (C3H / HeH x 101 / H) up to 12 months old. The mean oocytes number was 7.6±0.8 per female (Tease & Fisher, 1986). In naturally aged CD1 female mice the mean oocyte number in 12 months female was 5.4±0.5 (Merriman et al., 2012).

We conclude the difference in number of oocytes at this advanced age might be due to different responses to aging among different mouse strains.

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