Modifiable Risk Factors During and After Women's Reproductive Years: From Prenatal Nutrition to Postmenopausal Exposures

by

Katharine Brieger

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Doctoral Committee:

Assistant Professor Kelly Bakulski, Co-Chair
Associate Professor Leigh Pearce, Co-Chair
Associate Professor Ana Baylin
Professor Donna Martin
Professor Bhramar Mukherjee
Research Assistant Professor Erin Ware

Katharine Brieger

kbrieger@umich.edu

ORCID iD: 0000-0002-7266-117X

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This dissertation examines modifiable risk factors during and after women's reproductive years. The focus is on the investigation of the timing of exposures in vulnerable population, ultimately to provide the possibility of modifying behavioral factors and exposures to promote health.

The first aim examines maternal prenatal vitamin use and supplemental folic acid intake during the first month of pregnancy for association with autism spectrum disorder in the Early Autism Risk Longitudinal Investigation, a pregnancy cohort enriched for autism outcomes. Prenatal vitamin use (59.7%) was not significantly reduced odds of autism (OR, 0.70; 95% CI, 0.32 to 1.53). High total folic acid supplementation (>1000 mcg, 9.4% vs 400-1000 mcg, 45.0%) was not significantly associated with odds ASD (OR, 1.64; 95% CI, 0.44 to 5.47). Future work will incorporate dietary sources of folic acid and maternal folate metabolism genetics.

The second and third aims transition to studying women in a pooled sample from the international Ovarian Cancer Association Consortium. The second aim examines the association between menopausal hormone therapy (MHT) use and ovarian cancer survival in over 6,000 post-menopausal women. Use of any hormone type (estrogen-only or estrogen + progestin) for at least five years prior to ovarian cancer diagnosis was associated with longer survival (HR, 0.80; 95% CI, 0.74 to 0.87), regardless of recency of use. Those who used MHT were also less likely to have any macroscopic residual disease at the time of primary debulking surgery (p_{trend} <0.01 for duration of MHT use). Residual disease mediated some (17%) of the relationship between

MHT and ovarian cancer survival. While there is evidence suggesting that MHT use after diagnosis is also beneficial for survival, a large clinical trial is needed to definitively establish the relationship.

The third aim more broadly explores factors that influence ovarian cancer survival. While studying over 9,000 women, this work contributes by identifying modifiable pro- and anti-inflammatory risk factors and their cumulative or joint effect on survival. Exposures of interest included environmental cigarette smoke, smoking history, BMI, physical inactivity, and use of acetaminophen, aspirin, NSAIDs, MHT, and alcohol. An inflammatory risk score was created based on a weighted sum of each factor's association with survival. The most important contributors to the score were smoking and BMI (pro-inflammatory, increased risk of death) and MHT use (anti-inflammatory, decreased risk of death). Risk of death was elevated with increasing inflammatory risk scores ($p_{trend} = 0.026$); there was significantly elevated risk of death for women with scores $\geq 90^{th}$ percentile compared to those with scores $<10^{th}$ percentile (HR, 1.29; 95% CI, 1.04 to 1.60). Likewise, the odds of residual disease were elevated among women in the with higher inflammatory score ($p_{trend} < 0.001$). Odds residual disease were significantly higher for scores $\geq 90^{th}$ percentile (OR, 2.11; 95% CI, 1.42 to 3.16). Approximately 28% of score's association with survival was mediated through residual disease. The findings highlight potential biology of disease progression and offer modifiable factors influencing survival. Future work should build upon these findings to investigate post-diagnosis exposures to enhance women's survival with ovarian cancer.

Together, these aims contribute to the understanding of modifiable risk factors during women's lives and affecting outcomes of public health significance.

Chapter 1 Introduction

Overview of dissertation

The goal of this dissertation was to develop projects that rigorously examined modifiable risk factors during and after a woman's reproductive years, with particular attention to the timing of modifiable exposures during the life course. I examined vulnerable groups of women: (1) women with a family member with autism spectrum disorder and (2) women surviving ovarian cancer. This dissertation is a comprehensive set of research projects, making using of multiple data types, longitudinal data, advanced analytic methods, and strong biological motivation for health impact and ultimately clinical intervention.

In the introductory chapter, I provide the relevant background to motivate the public health importance and the biological basis for these projects. For autism spectrum disorder, the introduction describes the prevalence of disease, current diagnosis and treatment guidelines, genetic risk factors, and environmental risk factors. For ovarian cancer, this chapter introduces ovarian cancer sub-types, risk factors for disease incidence, and factors influencing survival. The first aim (Chapter 2) uses an enriched-risk, longitudinal pregnancy cohort to study maternal prenatal nutrition and risk of their children developing autism spectrum disorder. In the second and third aims (Chapters 3 and 4), I use data from a large, international consortium of pooled case-control studies to examine factors that influence ovarian cancer survival. Finally, the

concluding chapter of the dissertation highlights the potential implications of the findings and the opportunities for further research (Figure 1-1.).

Autism spectrum disorder background

Clinical description

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by difficulty communicating and interacting with others and by repetitive behaviors and restricted interests. Individuals with ASD display a wide range of characteristics, abilities, and symptoms. The signs and symptoms usually present within the first two years of life and develop into impaired social functioning. Common co-morbidities include neuroinflammation [1-3] and immune dysregulation [4-6], allergic disorders [7,8], non-celiac food sensitivity [8,9], gastrointestinal problems [10,11], and seizure disorders [12-14].

The first description of what is now termed ASD was made by Leo Kanner in 1943 at Johns Hopkins, when he described 11 cases he called "autistic disturbances of affective contact" [15]. He noted many features still viewed as hallmark signs, including difficulty relating to people socially, trouble communicating, and unusual responses to the environment. Kanner described a resistance to change, stereotyped motor behavior, and atypical communication styles; he emphasized the deficiencies in social relations. Kanner also called attention to what he viewed as a lack of parental warmth and attachment, though he did not blame the parents; rather, he noted that they too were somewhat preoccupied and perfectionistic.

In the 1950's and 1960's, there remained an absence of any biomedical explanations for autism. Fraudulent child psychologist Bruno Bettelheim widely spread the idea that the etiology of autism was linked to parents who were emotionally unresponsive, often called the

"refrigerator mother" hypothesis [16]. The public's interpretation of Kanner's and Bettelheim's work led to unwarranted blaming of families, which stalled progress in finding biological causes of the disorder. Our current understanding of the clinical description of autism focuses on qualities of the child, not the parent. However, one recent, more subtle version of the parental involvement narrative has developed, in which parents are encouraged to seek therapeutic resources, schools, diets, etc., to maximize their child's potential for a full life.

Diagnostic criteria

Evidence slowly accumulated to support that autism was, in fact, a brain disorder present from infancy, requiring clinical diagnostic standards. In 1978, Michael Rutter proposed a landmark classification scheme that defined autism based on four essential criteria: (1) onset before age 30 months, (2) impaired social development, (3) delayed and deviant language development, and (4) insistence on sameness [17]. Rutter emphasized that the defining social and language development features were not a function of the child's intellect. He therefore recommended that cases also be described by their neurological status, medical status, and IQ level.

The condition was first formally included in the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders (DSM)* in 1980, in *DSM-III* [18], within the new class of Pervasive Developmental Disorders (PDDs). The PDDs included autistic disorder, Asperger's syndrome, Rett syndrome, child disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). Defining Asperger's syndrome has been a cause of substantial debate, with several competing definitions in addition to those provided in the *DSM* and the *International Classification of Diseases (ICD)* codes [19]. Hans Asperger first described

four children with difficulty with social integration in 1944 [20], unaware of Kanner's prior description of autism. The four boys described by Asperger demonstrated a lack of empathy, difficulty forming friendships, intense special interests, and clumsy movements, but with relatively normal overall intelligence and language. Rett syndrome is a severe disorder caused by a mutation in the X-linked *MECP2* gene [21]; this disorder primarily affects females and patients present with slow head growth, small hands and feet, scoliosis, growth failure, gastrointestinal disorders, and seizures [22]. Child disintegrative disorder is recognized by later onset of delays or even reversals in social and motor function [23]. Examining the closely related pervasive developmental disorders helps inform our current understanding of ASD comorbidities and of possible causes and treatments.

The diagnostic criteria have changed in each edition of the *DSM* and *ICD*. The *DSM-III* (1980 - 1994) criteria proved to be too stringent, since every feature had to be present to receive a diagnosis [24]. The *DSM-IV* (1994 - 2013) and *ICD-10* both adopted categorical diagnostic approaches, which gave essentially identical autistic disorder diagnoses in an international field trial [25]. The definition included 12 criteria grouped into three categories: social, communication-play, and restricted interests and behaviors. To receive a diagnosis, a patient needed to meet a minimum of six criteria, at least two of which had to be social, one of which had to be communicative, and one of which had to be behavioral. The next, most recent, update further broadened the scope by adopting our modern understanding of the disorder as a spectrum.

In the most current standards, *DSM-5* [26] (2013 - present), ASD encompasses those who would have previously been diagnosed with autistic disorder, Asperger's syndrome, or PDD-NOS. The ASD diagnostic criteria, adopted in 2013, fall into five major categories (with the first two being the core symptoms): (1) deficits in social communication and interaction, (2)

restricted, repetitive behavior and interests, (3) symptoms present in early development, (4) symptoms cause clinically significant impairment in social, occupational, or other domains, and (5) the symptoms are not fully explained by intellectual disability or global delay. The diagnoses should also be made with specifications about whether the patient has (a) accompanying intellectual impairment, (b) language impairment, (c) associated medical, genetic, or environmental factors, (d) another neurodevelopmental, behavioral, or mental disorder, or (e) catatonia. The range of individuals included in current case definitions means we should expect heterogeneous clinical presentations, as well as varied biological bases and responses to treatment.

ASD assessment tools can be broadly categorized as screening or as diagnostic. Initially, developmental screening tools are employed when there is any suspicion of a developmental delay, and many such screening exams are not specific to ASD. These tools include the Ages and Stages Questionnaires (ASQ), the Communication and Symbolic Behavior Scales (CSBS), Children's Communication Checklist [27], Social communication questionnaire [28], the Modified Checklist for Autism in Toddlers (M-CHAT), and the Screening Tool for Autism in Toddlers (STAT), among others. Non-specialists, including parents and pediatricians, can administer these tools. Children scoring out of normal ranges are then referred for in-depth testing by specialists who are trained in administering comprehensive diagnostic assessments. Diagnostic tools include the Autism Diagnosis Interview – Revised (ADI-R) [29], the Autism Diagnostic Observation Schedule (ADOS) [30], the Autism Observation Scale for Infants (AOSI), the Social Responsiveness Scale (SRS) [31], and many others. These tools are useful not only for diagnosis but also for assessing severity of symptoms in specific domains, but they are time consuming and many of them require a multidisciplinary team to conduct. In a recent review of 17 assessment

tools, the ADI-R and ADOS had the highest sensitivity and specificity for ASD [32]. The variety of diagnostic tools used in research studies can complicate the case-control definitions and the interpretation of resultant findings.

The nuances and diagnostic criteria have changed over the years, but the validity of the diagnosis and the disorder is not questioned. A current topic of much debate among both clinicians and researchers is whether the new *DSM-5* criteria, outlined above, are an improvement over *DSM-IV*, and whether the changes will alter diagnostic rates. Individuals who had previously received diagnoses of Asperger's or PDD-NOS would now receive the new diagnosis of ASD when re-evaluated. The new criteria are also stricter, with more specific symptoms required in each category to meet the criteria; over 80% of those meeting *DSM-IV* criteria would also being diagnosed under *DSM-5* [33], but that leaves nearly 20% of individuals without a diagnosis and potentially without special services. Additionally, the new social communication disorder (SCD) was created and will apply to those who do not have repetitive behaviors or restricted interests. Because there are not yet established treatments for SCD, children who do not meet ASD diagnostic criteria may no longer have access to insurance coverage for still-needed services. Another disadvantage of the new *DSM-5* is that the criteria no longer match those used in the *ICD-10* codes (as those of *DSM-IV* do), which complicates harmonization of large, international or longitudinal studies.

However, there are also important benefits to the new stricter criteria and the combining of autistic disorder, Asperger's and PDD-NOS. For research purposes, careful case definitions are critical to make unbiased prevalence and association estimates, and so a set of criteria that are more specific, even if less sensitive, has some advantages. The grouping together of autism with Asperger's and PDD-NOS improves agreement among clinicians evaluating the same individual,

which leads to less confusion for families (i.e., there will be no back-and-forth between labels of Asperger's and autism). The predictive validity of a subset of *DSM-5* behaviors has been demonstrated [34], which is useful in clinical and research settings where there is not sufficient time for a more complete assessment. Improvements are still needed to better diagnose ASD in females, those at an older age, and those with higher IQ, but overall the new criteria represent progress toward more consistent diagnoses.

Prevalence and cost

ASD is widely recognized as a major public health issue, affecting 1 in 59 children at age 8 in the U.S (16.8 children per 1,000) [35]. Over the last 30 years, the prevalence has increased markedly, up from 0.4-0.5 children per 1,000 before 1985 [36]. ASD is four times more likely to be identified in males than females (1 in 37 among boys and 1 in 151 among girls). Prevalence also differs by race and ethnicity, with the highest rates among non-Hispanic whites (17.2 per 1,000), followed by non-Hispanic blacks (16.0 per 1,000), Hispanics (14.0 per 1,000), and Asian/Pacific Islanders (13.5 per 1,000) [35]. The yearly cost burden of ASD is estimated at $268 billion [37] (for year 2015), which is roughly equivalent to cost estimates for diabetes [38], and greater than cost estimates for stroke or hypertension [39]. Based on current cost and prevalence trends, the cost of ASD is projected to grow to $461 billion per year by 2025 [37].

The steady increase in prevalence of ASD is in part attributed to the new diagnostic criteria; however, clinical recommendations, increased awareness, and other factors also play a major role. These other factors, including environmental and modifiable risk factors, contribute to the true increase in prevalence and are the focus of this dissertation. Beginning in 2006, the American Academy of Pediatrics has advised screening all children for ASD during routine

visits at 18 and 24 months of age. The financial costs and risk of misdiagnosis that come with universal screening [40] are outweighed by the benefits of identifying more cases at younger ages. Interestingly, there has been a concurrent decrease in the prevalence of intellectual disability diagnoses [41], indicating part of the rise in ASD may be that many cases were alternatively diagnosed with intellectual disability in previous decades. It is estimated that 26% of the increased autism caseload in California from 1992-2005 was due to the diagnostic change, between *DSM-III* and *DSM-IV*, which incorporated individuals previously diagnosed with mental retardation [42]. Clinicians are also aware of the funding and services that are available once a child receives a formal diagnosis of ASD, which may incline them to give a diagnosis in borderline cases. In cases with diagnostic uncertainty, 58% of Queensland psychiatrists and pediatricians erred on the side of diagnosis for special education funding purposes and 36% also diagnosed for "Carer's Allowance" [43]. Accounting for these changes in practices does not fully explain the increase in ASD. Using the California study's estimate that 26% of the caseload increase was due to diagnostic change, we are left with nearly three-quarters of the increase unexplained. Important environmental and biological factors may also contribute to the rise in ASD [44,45] and have been less studied than genetic contributions, in part because measurement is much more complicated. The ongoing environmental research will be discussed at length in the following sections.

Treatment

ASD patient treatment primarily focuses on psychosocial, behavioral, and communication interventions. Intervening at younger ages tends to lead to better outcomes [46,47]. There is substantial heterogeneity of treatment success, and methods that are highly effective in

some children may have little or no effect in others [48]. The major categories of treatment are social skills training, cognitive behavioral therapy, applied behavioral analysis, parent education, and medication. Social skills training involves teaching conversational skills, good sportsmanship, and principles for inferring the emotional or mental states of others [49-51]. In cognitive behavioral therapy, treatment goals typically focus on reducing the anxiety in children and adolescents with ASD [52-54]. Applied behavioral analysis takes advantage of scientific principles of behavior to directly modify aberrant behavioral patterns in those with ASD [55]. Successful long-term outcomes are possible with early behavioral analysis treatment [56-58]. While interventions at younger ages and more total treatment both positively affect development, maximizing effectiveness of interventions in a cost-effective and widely applicable manner remains challenging.

In addition to patient behavioral interventions, caregiver training is used. Studies of parent and caregiver training have yielded mixed results, which are difficult to meta-analyze due to the heterogeneity of training type and study design. Caregiver training has been associated with patient gains in general communication skills[59], number of words understood [60], and eye contact [60]. In other cases, there are little [61] to no [62] differences in outcomes for children whose parents underwent such training. Altogether, the effects of intensive patient treatment are larger than effects of parent-implemented studies.

Medication use among individuals with ASD is typically seen as second-line treatment to be used in conjunction with behavioral therapies [63,64]. In children, pharmacological treatment targets sleep problems (mirtazapine [65] melatonin [66]), irritability (risperidone [67] and aripiprazole [68]), and attention deficits (methylphenidate [69]). About 64% of children with ASD are on some form of medication, with 35% on two or more, despite the minimal evidence of its efficacy [70].

The only FDA-approved treatments for ASD are risperidone and aripiprazole, which treat the irritability symptoms. While other off-label options for clinicians exist, including selective serotonin re-uptake inhibitors [71] and naltrexone [72], there have yet to be any pharmacologic treatments proven to alleviate the core ASD symptoms of communication and social difficulties. Treatment options for ASD are improving, but incomplete, and thus prevention is a critical area for public health to address.

Genetics of autism

In addition to environmental factors, genetics plays an important role in ASD etiology. Evidence from twin and family studies suggests that heritability is at least 50% [73] and as high as 90% [74,75], but much of this genetic risk has not yet been elucidated. Monozygotic twin concordance rates are estimated at 60-70% [73,76] (though one cannot completely resolve the issue of a shared *in utero* environment) and recurrence risk in siblings is 18% [77]. Additionally, the undiagnosed relatives of patients often present with "broader autism phenotype", characterized by similar rigid thinking and behavior as well as social vulnerability [78]. In addition to inherited mutations [79], large effects are seen for *de novo* mutations and CNVs [80]. The genetic architecture of ASD is diverse and risk variants can be common or rare, inherited or *de novo,* and dominant/recessive or additive [81].

Some mutations associated with ASD have a large effect and are implicated in monogenic ASD (without the accumulation of numerous other common, low-effect variants). Among ASD cases, 3-5% also have single-gene disorders such as Fragile-X (mutations in *FMR1*), tuberous sclerosis (mutations in *TSC1* and *TSC2*), and neurofibromatosis (mutations in *NF1*) [82]. Other variants with large effects associated with ASD include activity-dependent

neuroprotector (*ADNP*), AT-rich interaction domain 1B (*ARID1B*), chromodomain helicase DNA binding protein 2 (*CHD2*), and synaptic Ras GTPase activating protein 1 (*SYNGAP1*) [83]. Single gene ASD cases are a small subset of the ASD population.

Genome-wide association studies of ASD: common variants, rare variants, and limitations

Apart from some of the example mentioned above, ASD risk is likely polygenic in most individuals, meaning that many variants each contribute a small portion to the overall risk [84,85]. Initial genetic studies were limited in their ability to detect small effect sizes of common variants [86] and were frequently interpreted in the context of prior knowledge or candidate genes. The findings that were successfully replicated include those for GABA A receptor beta 3 (*GABRB3*), oxytocin receptor (*OXTR*), reelin (*RELN*), serotonin transporter (*SLC6A4*), and contactin-associated genes (*CNTCAP2*) [87].

With increasing sample sizes, meta-GWAS studies, and whole genome sequencing, new genetic loci continue to be identified in ASD [88,89]. The largest meta-analysis (18,381 ASD cases, 27,969 controls) [90] identified five genome-wide significant loci, and an additional seven loci when leveraging GWAS results from schizophrenia, major depression, and educational attainment, which are known to have overlapping genetic architectures. The importance of the very large sample sizes is apparent, as even earlier in 2017 the Psychiatric Genomics Consortium's most recent and largest meta-GWAS of 14 independent cohorts (7,387 ASD cases, 8,567 controls) found no individual variants that met the accepted genome-wide significance threshold ($p \leq 5 \times 10^{-8}$)[91]. However, to assess the possibility of replicable and genuine associations among the highest-ranked associations, the authors then compared to summary association data from the Danish iPSYCH ASD GWAS (7783 cases, 11359 controls) and from

the deCODE/SEED ASD GWAS (1,369 cases, 137,308 controls) and found significant concordance in the direction of the effects, for the top 100 and top 70 markers in the two datasets respectively. Meta-analysis of the PGC and the iPSYCH data also gave rise to a single genome-wide significant association for rs1409313-T (OR = 1.12, $P = 1.058 \times 10^{-8}$), a marker within an intron of *CUEDC2*, encoding a gene in the ubiquitination-proteasomal degradation pathway and previously identified as associated with the social skills domain of autism [92]. The biological function of genes containing associated loci also helps us generate new etiologic hypotheses.

Several of the polymorphisms associated with risk of ASD are located in genes related to chromatin remodeling, RNA processing, and synaptic transmission, including neuroligins (*NLGN*) [93], SH3 and multiple ankyrin repeat domains (*SHANK*) [94], and neurexin (*NRXN*) [95]. Many of the synaptic gene variants identified for ASD have overlap with other neurodevelopmental and psychiatric disorders as well [96,97]. In a cross-disorder GWAS of ASD and schizophrenia together, 12 genome-wide significant loci were identified that were not previously reported in the PGC schizophrenia-only GWAS [91]. There was also significant genetic correlation observed between ASD and schizophrenia. The advent of whole-genome/whole-exome sequencing and of increasingly large consortia will continue to facilitate future discovery and provide biological insights relevant to ASD specifically and neuropsychiatric disease more broadly. In general, many genetic loci with small effect sizes are associated with increased ASD risk and these loci overlap with other psychiatric disorders.

Environmental risk factors

Neurodevelopmental sensitivity window

Neurodevelopment is a complex and tightly regulated process. The healthy progression of central nervous system development depends on both genetic encoding and environmental influence. In the human embryo, the outermost tissue layer, the ectoderm, gives rise to the neural plate in the third week of embryonic development [98]. By the fourth week, the neural plate wraps in on itself to become the neural tube, the anterior portion of which further divides to form a series of vesicles that become regions of the brain [98]. Neurogenesis, the productions of neurons from neural stem cells, occurs during embryonic weeks 4-17; synaptogenesis, the formation of synapses between neurons, occurs from embryonic week 18 to middle childhood; and experience-dependent synaptic pruning, the elimination of extra neurons and synaptic connections to improve efficiency, extends into young adulthood [99].

Disruption of this delicate process can cause abnormal brain and central nervous system development and contribute to neurodevelopmental disorders. Broadly speaking, the disorders can be classified either by the most prominent phenotype (e.g., motor disorders, communication disorders, etc.) or by the type of deleterious exposure or risk factor (e.g. neurotoxicant exposures such as fetal alcohol syndrome, genetic disorders such as Trisomy 21). Additionally, the disorders range in severity depending on the type and timing of disruptions. In fetal alcohol syndrome, for example, the teratogenic alcohol has effects that are both timing- and dosage-specific [100]. Likewise, other environmental disruptions to neurodevelopment can have a wide range of effects depending on the timing and dose.

Multiple lines of evidence point to the prenatal window as critical for ASD development. Exposure to teratogens, such as thalidomide and valproate [101], during the prenatal period was

associated with increased risk of ASD. In a rat model, teratogen exposure during pregnancy caused abnormal serotonergic neurons in offspring [102]. Neurologic abnormalities in size and connectivity are also identified very early, even before behavioral signs are present in children. At birth, children who go on to have ASD have smaller brains, but then there is excessive overgrowth from 6 to 14 months [103]. Likewise, functional neuroimaging of 6-month-olds has been shown to correctly predict diagnosis of ASD at 24-months, based on different patterns of connectivity [104]. Further study of both animals and humans is needed to elucidate critical timing and etiologic mechanisms of ASD pathology.

While some neurodevelopmental disorders can be traced to specific genetic, metabolic, immune, or chemical factors, others are considered multifactorial. ASD is one such multifactorial syndrome. Heritability estimates range from 50% to 90% [74], but there is also important is environmental contribution. Additionally, because many of the genetic studies have relied on twin and family studies, the *in utero* environment and early life exposures may have incorrectly contributed to heritability estimates. Though environmental contributions may indeed be smaller than the genetic contributions, it is crucial to study these potentially modifiable risks. Although no single environmental exposure or genetic change may be sufficient to cause ASD on its own, the combination of multiple environmental risks and genetics may substantially increase ASD risk. ASD is a complex disorder with both heterogeneous phenotypes *and* etiologies.

Many environmental factors have been well replicated in their associations to increased ASD risk. These include advanced parental age [105], prenatal infection [106], maternal diabetes [107], perinatal stress [108], environmental toxicants [109], and certain medications [110]. A few models have been proposed to connect the various environmental risks, including motifs centered on immune

dysregulation and zinc deficiency [111]. Identifying crucial time periods of vulnerability and at pathways prone to disruption has advanced biologic explanations for the environmental risks.

Parental age: maternal and paternal

Parental age is one important risk factor for many developmental disorders, including ASD. ASD risk is positively associated with maternal age [112], paternal age [113,114] or both [115]. Meta-analyses from several large epidemiologic studies have confirmed that advanced maternal age is a risk factor even when controlling for paternal age, and vice versa [116]. A 2016 population-based cohort study found that, in addition to increased age being associated with increased risk, young mothers (<20 years of age) were at increased risk of having a child with ASD, as were couples with a large disparity in age between the mother and father [117]. However, there may be unaccounted for confounding in the relationship between parental age disparity and risk of ASD, such as length of partnership and socio-economic, genetic and psychological factors. Examining parental ages separately (controlling for the partner's age), increased paternal age shows the stronger, more linear effect, whereas in women the effect is non-linear [118]. As men age, they transmit a higher number of mutations to their offspring, since small *de novo* mutations in sperm with subsequent cell divisions are increasingly common[119]. In women, however, all eggs are already present at birth, thus cell divisions do not accumulate as women age. The mechanisms through which maternal age increases risk are likely less direct; for instance, older women experience higher rates of pregnancy complications[120], birth complications[121], and maternal autoimmunity. Mean parental age and parental age difference are both important to ASD risk.

Perinatal exposures: infection, medication, environmental exposures

Neuroanatomical and epidemiologic studies suggest that the prenatal period is an important window of susceptibility and that the developing brain is acutely sensitive to the timing and type of environmental exposures [122,123]. Prenatal exposures for which there is the most robust evidence for association with ASD are maternal infection [124], maternal medication [125,126], air pollution [127,128] and pesticide exposure [129]. Mothers who have serious infections during pregnancy are at increased risk of having a child with ASD [106], particularly if they have multiple infections or the infections are bacterial[124,130]. Pathogens may have direct effects on the fetal brain [131], and infection can also affect the developing brain through changes in inflammatory pathways, as shown in rat models [132].

Maternal use of antidepressants [133,134], antiepileptics [135], acetaminophen [126], and some asthma drugs [136] may also increase risk of ASD in children. A major limitation of these studies is the unmeasured confounding due to genetics. Genetics for depression and ASD overlap [137] and maternal genetic predisposition to depression would predict both selective serotonin-reuptake inhibitor (SSRI) use and possibility of transmitting genes related to ASD. Recent studies of SSRIs suggest either null effects [125] or effects due to depression[138], not use of antidepressants.

Environmental toxicants, birth conditions, and maternal metabolic conditions may also influence risk of ASD. Prenatal exposure to air pollution is one of the most-studied environmental pollutants, but this is in part because of the availability of public data, since government agencies began tracking many air pollutants after the passage of the Clean Air Act in 1970. Studies report increased ASD risk with increasing particulate matter exposure [139,140], but the pollution data is limited in its spatial and temporal resolution, so there is the possibility of exposure misclassification. Studies outside the U.S. of nitrogen dioxide exposure [141] and air

pollution [142] have yielded null associations with ASD. Similar inconsistencies are seen in the literature examining pesticides [143,144] and endocrine-disrupting chemicals [143,145]. Study limitations that plague much of this research include that (1) laboratory and analytic choices affect measurements of compounds in maternal plasma and make replication difficult, (2) population-level data such as for air pollution rely on assumptions about how much time pregnant women spend at their residence, and (3) exposure to several pollutants can be tightly correlated so any findings of association may indicate only a proxy exposure.

All of the environmental risks discussed above indicate that the etiologic origins of ASD are related to exposures before, during, or immediately after birth. Postnatal exposure to vaccination is *not* associated with ASD. In 1998, Wakefield et al. published the now infamous case series [146] suggesting that the measles, mumps, and rubella vaccine (MMR), given first at 12 to 15 months of age, put children at increased risk of behavioral regression and developmental disorders. The flawed study was immediately refuted [147,148] and retracted in 2010 due to serious ethical and research misconduct. Tragically, the original paper had widespread influence and vaccination rates dropped [149]. The vaccination theory has been disproven by numerous studies, including a meta-analysis of five cohort studies (1,256,407 children) and five case control studies (9,920 children) [150]. There is no relationship between vaccination and ASD, or between components of the vaccine (thimerosal or mercury) and ASD. Although signs and symptoms of ASD often become increasingly pronounced around the age of MMR vaccination, the existing evidence suggests that the window of environmental susceptibility is earlier in development.

In summary, the complex etiology of ASD has only just begun to be approached through the lens of environmental risk. Exposures in the prenatal and perinatal period have been associated with ASD. Maternal and paternal factors, particularly age, are important. During

pregnancy, there are associations between ASD and many maternal factors. Environmental toxicants such as air pollution and pesticide exposure also increase risk. Finally, reviews of nutritional and toxic elements have been limited by study design but warrant further investigation and will be discussed in subsequent sections. There are many possible biological explanations for the associations with environmental factors, including oxidative stress pathways, neurotransmitter alterations, and non-causative associations, to name only a few. More precise exposure measurement, prospective design, timing of exposure measurement in relation to the critical period, and consideration of genetic interaction with exposures are critical to future research of these factors and clarifying public health messaging on susceptibility.

Nutrition and the role of folic acid

Micronutrients in neurodevelopment

Numerous micronutrients are critical for proper neurodevelopment, both for neurogenesis and for the subsequent elaboration of neural network connections [151]. Children with ASD have lower levels of many micronutrients in their tissues (primarily blood and urine) compared to neurotypical controls. These differences include, among others, lower levels of magnesium [152], zinc, vitamin B complex [153], vitamin D [154,155], and carnitine [156]. Because children with ASD often have abnormal eating patterns, these associations could be due to reverse causality and merit further study at early etiologic time points. The observed low tissue levels motivated further study of nutrients during pregnancy; now, specific maternal nutrient deficiencies during pregnancy have been identified as relevant to the etiology of ASD, including deficiencies in zinc, copper, iron [157], vitamin D [158-160], and vitamin B9 (folic acid) [161,162].

Vitamin D is a potent neurosteroid. The vitamin D – vitamin D receptor complex binds to vitamin D response elements in target genes, regulating expression. In rat models, prenatal vitamin D deficiency results in neuroanatomical and neurotransmission abnormalities similar to those observed in ASD, such as initial brain overgrowth, later slowed growth, and calcium signaling changes [163]. In a case-control analysis of children with ASD, paternal vitamin D receptor gene variants were associated with ASD, as was the child's cytochrome P450 2R1 (*CYP2R1*) genotype, which codes for the vitamin D hydroxylase that converts vitamin D to the circulating form, 25-hydroxyvitamin D [164]. Compared to controls, ASD cases were also more likely to have rare polymorphisms in the vitamin D receptor [165] and have lower serum 25-hydroxyvitamin D levels [166,167]. The differential effects of sex hormones on vitamin D metabolism may help explain the gender differences in ASD [155,158]. Ongoing research is evaluating the effectiveness of vitamin D supplementation for pregnant women and infants in reducing ASD risk. The remainder of the micronutrient background in this section focuses on folic acid intake and processing, but vitamin D provides an instructive example regarding the limitations and confounding factors in studying nutrients: specifically, accounting for genetics and timing are crucial for interpretation of results.

Folic acid intake and bioavailability

Folate, or vitamin B9, is an essential nutrient and is required for many biologic functions, including DNA repair, RNA synthesis, conversion of amino acids, and protection from oxidation [168]. Dietary folate is found in dark green vegetables, certain fruits, nuts, beans, dairy, meat, eggs, and seafood. Folic acid is an oxidized, synthetic form of B9 found in supplements and fortified grain products. Once consumed, folate and folic acid are both taken up by the small intestinal

mucosal cells (enterocytes), primarily by the proton-coupled folate transporter (PCFT) and to a lesser extent by the reduced folate carrier (RFC) (Figure 1-2.). Before folates perform biological functions, they are converted to bioavailable derivatives [169]. The intestinal mucosal cells rapidly convert dihydrofolate (DHF) first to tetrahydrofolate (THF) via dihydrofolate reductase (DHFR), then to 5,10-methylenetetrahydrofolate (5,10-MTHF) (folinic acid) via serine hydroxymethyltransferase (SHMT), and ultimately to the final active form, 5-methylenetetrahydrofolate (5-MTHF) via methylene tetrahydrofolate reductase (MTHFR) and release into circulation (Figure 1-2). Only this final form is bioavailable and capable of crossing the blood-brain barrier. Before entering the pathway described above, synthetic folic acid requires an additional (slow) reduction to DHF by DHFR. Because the intestinal DHFR enzyme has a more limited ability to reduce folic acid, humans rely on the liver for this reduction process. The limited conversion rate of folic acid is responsible for the occasionally high levels of unmetabolized folic acid seen in people who consume large quantities in supplement form. Additionally, about 20-70mg of folate can be stored in the liver, to be used in times of higher demand or fasting between meals. Total body folate availability is a combination of ingested folate, folic acid, and stored folate.

Conversions between forms of total body folate depend on intact function of the transporters (PCFT and RFC), the activity of the enzymes required (DHFR, SHMT, and MTHFR), and the required coenzymes, including riboflavin (vitamin B2), niacin (vitamin B3), pyridoxine (vitamin B6), zinc and serine. The multiple steps before folate is bioavailable highlight why deficiency (or over-abundance) could have several distinct causes in addition to lack of (or excessive) intake.

Differences in bioavailability between folates in food and folic acid supplements lead to the dietary folate equivalent (DFE) system [170]. Despite requiring one additional reduction step to be biologically active, the folic acid in supplements may be more bioavailable since natural folates are sensitive to high heat and oxidation, meaning that storage and processing of food affects their integrity. One DFE is 1 μg of dietary folate, but 1 μg of folic acid is 1.7 DFE; this is because about 85% of folic acid is available while only about 50% of folate in foods is [171]. However, foods have the benefit of containing additional essential co-factors and micronutrients involved in the folate cycle. Because there are so many contributing factors to the processing (both outside and inside the body) and ultimate absorption of folate, it is difficult to make blanket recommendations about preferred sources without knowing information about an individual's diet and about their ability to metabolize folate.

Folic acid fortification and recommendations

Fetal neural tube defects (NTDs) are caused by folate deficiencies early in pregnancy. A randomized trial was conducted in seven countries to determine if periconceptional supplementation with folic acid or other vitamins prevented NTDs, including anencephaly, spina bifida, and encephalocele. The study recruited over 1,800 women at high risk of having a pregnancy with a neural tube defect based on a previous affected pregnancy. The study found that folic acid had a significantly protective effect (RR 0.28, 95%CI 0.12-0.71) while other vitamins did not [172]. Though there had been prior studies hinting at these effects, this was the first well-designed, blinded trial, so only after these results were published in 1991 were public health measures put in place to ensure that women of childbearing age had adequate dietary folate or supplementation. A large body of evidence now supports that periconceptional folic acid intake

is associated with reduced first occurrence or reoccurrence of NTDs during pregnancy [173-175]. Recommended intakes are between 400μg (for most women) and 4000μg (for those at high risk due to a previous pregnancy with an NTD). Personal pre-pregnancy supplementation is not sufficient for many women, given that about half of U.S. pregnancies are unplanned [176].

Folic acid fortification was inspired by the evidence that it would prevent neural tube defects (NTDs). In 1998, a new United States Food and Drug Administration grain fortification requirement went into effect. The regulation requires the addition of folic acid to enriched breads, cereals, flours, corn meals, pastas, rice, and other grains. Since these recommendations have been put in place (and in some countries since fortification), there has been substantial reduction in NTD frequency, which is the only example of a congenital malformation that can be prevented consistently. However, the mechanisms and optimal dose for the protective effect of folic acid are still not agreed upon.

Fortified foods are now a major source of folic acid for Americans. Comparing pre- and post-fortification rates of NTDs in the U.S., four studies found decreases of 11-20% in anencephaly rates and decreases of 21-34% in spina bifida rates [177-180]. Fortification led to an even greater increase in folic acid intake than projected by the Food and Drug Administration; while they projected an increase of 100 μg/day, intake actually increased by closer to 200 μg/day, and total folate intake increase by over 300 μg/day [181]. In fact, with "super-fortified" foods such as some cereals (at 400 μg/ 30g cereal), a person could easily reach the Food and Drug Administration's safe upper limit (1 mg/day) in a large bowl of cereal [182]. Extremely high intake levels are possible when consuming both fortified foods and supplements. The processing systems can be overwhelmed and leave unmetabolized folic acid, particularly in individuals with any alternation in the metabolic cycle for folate. Additionally, high folic acid doses can mask

diagnosis of vitamin B12 deficiency (pernicious anemia) and have been associated with certain types of cancer, so many countries have not opted to fortify [183,184]. Overall, fortification has achieved the goal of decreasing NTDs, but monitoring and reevaluation of mandated amounts is warranted as new evidence emergences following this first cohort who has eaten fortified foods their entire life.

Folic acid during embryonic development

Folic acid is critical during *in utero* development, particularly for neurodevelopment and red blood cell production. Erythroblasts, the immature, nucleated red blood cell precursors, require folate, vitamin B12, and iron for successful proliferation and differentiation. Deficiency in folate causes erythroblast apoptosis and ultimately anemia from insufficient erythropoiesis [185]. In addition to preventing NTDs, folic acid may also help protect against congenital heart anomalies, orofacial clefts, miscarriages, pre-term birth, and other adverse outcomes [186]. Use of multivitamins (containing folic acid) near the time of conception is associated with reduced risk of heart defects and orofacial clefts in observational studies [187], though a randomized-controlled trial showed this effect only for heart defects [188]. Common adverse birth outcomes also are related to interpregnancy interval, which hints at the importance of nutrient depletion, since nutrient reserves are depleted after pregnancy [189,190]. In a retrospective cohort study, birth outcomes from 40,000 Australian mothers who had delivered three singleton births were assessed. Each woman had two interpregnancy intervals for comparisons both between and within mothers. With a traditional unmatched analysis, increased odds of preterm birth (1.41, 65%CI 1.31-1.51) and low birth weight (1.26, 95% CI 1.15 to 1.37) were observed for interpregnancy intervals of 0-5 months, compared to reference category of 18-23 months [191].

However, these associations were not observed when analyzing using a matched, within-mother design. There may be unmeasured confounders, such as socioeconomic factors, that affect both interpregnancy intervals and risk of preterm birth independently. A small case-control study (n=78) found that the effects of short interpregnancy interval and folate depletion can be mitigated by folic acid supplementation after the initial pregnancy [191,192], but these results need to be replicated in a larger cohort and in other populations. Though the precise mechanisms responsible for folic acid's effects are not yet understood, the critical nature of folate levels during early pregnancy for fetal health is well established.

Folic acid and ASD

Folic acid is necessary for normal fetal nervous system and red blood cell development. Maternal folate status during pregnancy may be a risk factor for ASD and current evidence supports that both genetic and dietary factors contribute to this risk [193], but the size and direction of these effects are variable. The largest folic acid study to date is a case-control study nested within a Swedish population-based cohort study of children born between 1996 and 2007 (n=273,107 mother-child pairs). All associations used healthy, neurotypical children as the reference group. Maternal multivitamin use (with or without additional iron or folic acid) was associated with lower odds of ASD with intellectual disability compared to those who did not use any of the three supplements (odds ratio=0.69, 95% confidence interval: 0.57-0.84). No associations were observed with either maternal iron supplementation or folic acid supplementation and ASD prevalence [194]. A major strength of this study was the sibling control analysis, as this allowed the authors to control for at least some of the unobserved confounders (general household environmental, maternal health behaviors) that could influence both maternal

supplement use and ASD in the child. However, there are still substantial confounders that cannot be fully controlled even within mother, as each pregnancy is different and past experiences may have influenced future behaviors. The findings that multivitamins decreased risk and that folic acid did not were surprising since existing evidence supports that the folic acid is the relevant compound within the multivitamins, but the study design had several limitations. First, there was not information on type, timing, and dose of multivitamins, so we cannot infer information about the critical period or nutrients. Secondly, the lack of overall association with folic acid may have been entirely due to the fact that folic acid supplementation was associated with *higher* risk in women who were admitted to the hospital during pregnancy; that is, hospital stay may be an important proxy for a confounder of the folic acid – ASD relationship, such as chronic disease, psychiatric illness, or acute infection.

Two other large cohorts also observed prenatal supplementation was associated with decreased ASD risk. First, a nested ASD case-control cohort study of 45,300 Israeli children found protective effects of multivitamins. Cases were all children diagnosed with ASD and the controls were a random sample of one-third of all live births in the same time period (2003-2007). Maternal use of multivitamins and use of folic acid before and during pregnancy were both associated with a lower likelihood of ASD, compared to children of mothers without those exposures (relative risk=0.39, 95% confidence interval: 0.30-0.50) [195]. The study was based on prescription records, as opposed to survey information, which is more accurate than retrospective data, but does not account for mothers who took non-prescription supplements. The study was also limited by the lack of a sibling control analysis, which meant the authors were unable to control for unmeasured confounders, including maternal characteristics, family environment, and other possible childhood factors. Finally, a case-control study within a prospective national birth

cohort in Norway (n=85,176) found that folic acid near conception (4 weeks prior to 8 weeks after) was associated with decreased odds of ASD (odds ratio=0.61, 95% confidence interval: 0.41-0.90). The large cohort and prospective data collection were major strengths of this study. However, an important limitation was the incomplete ascertainment of ASD cases, which could have biased the results in either direction. Overall, the three large cohorts found results consistent with protective effects of folic acid on ASD risk.

In contrast to the above studies, there is some evidence that very high intake of folic acid and high folate levels in maternal blood is linked to increased ASD risk. In the Boston Birth Cohort of 1,257 mother-child pairs, maternal multivitamin supplementation frequency demonstrated a "u-shaped" association with ASD risk. For each trimester of pregnancy, the lowest risk was in the group of mothers using supplements 3-5 times/week, and significantly increased risk was observed for mothers at both low (<2 times/week) and high (6-7 times/week). The same study found that high maternal plasma folate at birth ($\geq$60 nmol/L; normal = (4.5 – 43 nmol/L) was associated with a 2.5 times (95% confidence interval: 1.3-4.6) higher risk of having a child with ASD compared to folate levels at the mid-80th percentile [196,197]. These findings need replication but highlight the importance of studying the association between risk and a nutrient across all dosage levels, since the adverse outcomes are possible at both deficient and elevated levels.

Future research on maternal folic acid intake and ASD risk is warranted to improve recommendations and risk communication to women hoping to become pregnant. Questions about timing, type, and dose of supplement remain unanswered. In large Israeli, Swedish, and Norwegian cohorts, maternal prenatal supplementation was associated with decreased risk of ASD in the child, but optimal timing is still under investigation, as is the possibility of risk at the

high end of the intake range. The studies are heterogeneous in their measurement of exposure and outcome, so it is difficult to directly compare their findings. Many existing studies' conclusions are limited by a lack of appropriate timing of exposure windows (i.e. using retrospective recall to assess folic acid intake) and by confounding behaviors (e.g. overall diet quality). It may also be necessary to assess not just folic acid supplement history, but biological levels of folate, B12, and homocysteine, as well as gene variants involved in folate metabolism. Some studies have reported differential associations of folic acid supplementation on risk by ethnicity [198], which may be due in part to the differences in frequency of gene variants influencing metabolism. Another complicating factor, discussed above, is the difficulty in assessing the type, quality, and bioavailability of the different folate sources.

Folate metabolism genetics

The genetics of one-carbon metabolism relate to folate processing and thus potentially to ASD risk. The one-carbon metabolism cycle is essential for the processing of dietary folate and generating the metabolites required for DNA and RNA methylation. Among the genes coding for the enzymes required, several have common variants that affect the enzymes' production and efficiency, including reduced folate carrier 1 (*RFC-1*), dihydrofolate reductase (*DHFR*), 5,10-methylenetetrahydrofolate (*MTHFR*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*). The efficiency of the metabolic cycle is often measured in studies by using plasma homocysteine concentrations. Plasma homocysteine accumulates due to a deficiency at some point in the cycle [199]. Homocysteine concentrations tend to vary inversely with folate and other B-vitamins [200], since the vitamins facilitate the remethylation of homocysteine. (In fact, a reduction in homocysteine levels was observed in several countries after they adopted a food

fortification program[201,202].) In research moving forward, accounting for the amount of bioavailable folate in an individual may be possible by adjusting for polymorphisms affecting the one-carbon metabolism cycle

Studies of one-carbon metabolism began with candidate gene studies and later moved onto GWAS. The *MTHFR* 677C>T polymorphism is associated with lower plasma and red blood cell folate and with higher plasma homocysteine concentrations [203]. The study also confirmed that allele frequencies differed by race, thus genetic ancestry is important to account for in future studies. Another cohort study of 991 individuals investigated thirteen single nucleotide polymorphisms (SNPs) to cover a broader range of genes known to be involved in folate uptake and metabolism; these SNPs included folate hydrolase (*FOLH1*), folate polyglutamate synthase (*FPGS*), gamma-glutamyl hydrolase (*GGH*), methylene tetrahydrofolate reductase (*MTHFR*), and reduced folate carrier (*RFC1*). The *MTHFR* 677TT genotype was associated with increased plasma homocysteine and decreased plasma folate, *MTHFR* 1298A>C and *RFC1* intron 5A>G were associated with increased plasma homocysteine concentrations (17% higher for double mutant genotype A1298CC), and *FOLH1* 1561C>T SNP was associated with elevated plasma folate levels [199]. Because a handful of candidate SNPs cannot explain the observed variation in plasma folate and homocysteine, GWAS were conducted in multiple independent populations of different ancestries (Yoruban, Filipino, Hispanic, and European) [204-207]. The studies confirmed previous candidate gene findings in *MTHFR,* methionine synthase (*MTR*), and NADPH oxidase 4 (*NOX4*), and further identified SNPs in cystathione beta synthase (*CBS*), carbamoyl phosphate synthase 1 (*CPS1*), and methyl malonyl CoA synthase (*MUT*). A meta-analysis of GWAS in European individuals (n=44,147) identified 13 SNPs associated with total plasma homocysteine levels ($p< 10^{-8}$), explaining 5.9% of the variation in this population [208].

Folic acid-environment interactions

Folic acid interacts with other prenatal exposures to affect birth outcomes. These "environment-by-environment" interactions further highlight the importance of folic acid during pregnancy. For example, *in utero* exposure to bisphenol A (BPA), used in the manufacture of plastics, alters expression of sex hormones and alters epigenetic patterning during stem cell development; maternal supplementation with folic acid, though, negates these effects of BPA [209]. Exposure to organochloride pesticides is also associated with impaired neurodevelopment, evidenced in neonates by abnormal reflexes and in young child by developmental disorders [210]. A case-control study in California specifically examined associations between prenatal folic acid intake, prenatal pesticide exposure, and development of ASD. They found that the increased odds of ASD associated with pesticide exposure were attenuated by high folic acid intake during the first month of pregnancy [129]. It is likely that exposure to the persistent pollutants affects outcomes by impairing DNA methylation [211].

Similarly, certain types of air pollution and viruses each interact with other environmental factors to affect ASD risk. Exposure to increased levels of air pollutants during the first trimester of pregnancy is associated with increased ASD risk among mothers with low folic acid intake, but not among mothers with high folic acid intake, and this interaction is significant at least for NO_2 exposure [212]. As with pesticide exposure, it appears that folic acid mitigates the DNA methylation changes driven by air pollution exposure [212,213]. In conclusion, folic acid has widespread effects on birth outcomes, both directly and through interaction with genetics and other exposures. There is a critical gap in public health knowledge on additional exposures whose effects depend on folate levels, and this knowledge is vital to pregnant mothers.

Summary

ASD is a complex, costly, prevalent disorder. The condition is heterogeneous in both etiology and phenotype, which makes it particularly difficult to identify, treat, and research. Known risk factors include environmental factors, such as parental age and prenatal infection, as well as genetic risk, both inherited and *de novo*. Maternal nutrition, including folate status, is also thought to contribute to risk. Important gaps in knowledge about folic acid supplementation include (1) the importance of precise timing, (2) the optimal dose, and (3) consideration of a wider range (or simultaneous consideration) of exposures.

Ovarian cancer background

Statistics and tumor classification

Ovarian cancer is the least common gynecologic cancer, but the most fatal. In 2018, there were nearly 300,000 incident cases worldwide, with a relative five-year mortality rate of 47%[214]. It is primarily a post-menopausal disease, with a median age at diagnosis of 63 years [215]. A majority of women with ovarian cancer are diagnosed at late stages[216] when tumors have metastasized and prognosis is poor. In addition to prevention efforts, it is imperative to better understand the course of the disease and factors that could extend survival and improve quality of life.

Pathologic diversity of ovarian cancer is a key feature of the disease (Figure 1-3). The three main histopathologic types of ovarian tumors are germ cell, stromal, and epithelial, referring to the cell types from which the tumor develops. Germ cells tumors develop from the cells that produce the ova (egg), stromal tumors arise from cells producing connective tissue and hormones, and epithelial tumors originate from cells lining the outer surface of the ovary. Ovarian germ cell and stromal tumors are infrequent and represent approximately 5% of all primary ovarian tumors[216]. Although sex cord-stromal tumors present in a broad age group, the majority tend to present as a low-grade disease that usually follows a nonaggressive clinical course in younger patients. Furthermore, because the constituent cells of these tumors are engaged in ovarian steroid hormone production (e.g., androgens, estrogens, and corticoids), sex cord-stromal tumors are commonly associated with various hormone-mediated syndromes. The majority (85-90%) of malignant disease is of epithelial origin[216], and the focus of the research in this dissertation. Epithelial disease is further classified into histotypes. The five main histotypes are mucinous (<5%), endometrioid (~10%), clear cell (<5%), low-grade serous (<10%) and high-

grade serous (~65%)[217], and the remaining tumors are classified as Brenner tumors, borderline tumors, carcinosarcoma, or undifferentiated/unclassified.

Tumors are classified by stage and grade only after an examination of tissue samples obtained during surgery. Stage refers to macroscopic characteristics such as size and spread of tumor, while grade refers to microscopic characteristics and anomalies. Several staging criteria exist (

), including the American Joint Committee on Cancer/Tumor, Node, Metastasis (AJCC/TNM), the International Federation of Gynecology and Obstetrics (FIGO), and Surveillance Epidemiology and End Results (SEER) Program. The AJCC/TNM classifies a tumor's extent (size) (T), its spread to nearby lymph nodes (N), and whether it has metastasized (M) beyond nearby lymph nodes. Combinations of T, N, and M correspond to the FIGO stages I to IV. SEER stages describe the overall spread of the cancer as localized, regional, or distant. Grade is characterized as low or high (or occasionally intermediate) and refers to the level of tissue organization[218], or how "normal" the microscopic appearance is. For example, a low-grade tumor would have tissue with well-differentiated glands and papillae and cells exhibiting a typical nucleus and mitotic rate.

Risk factors

Ovarian cancer has both genetic and non-genetic risk factors[219]. A minority of ovarian cancers are associated with a familial predisposition[220], and include two highly penetrant syndromes. Hereditary breast-ovarian cancer syndrome is associated with germline mutations in the tumor suppressor genes *BRCA1* and *BRCA2*[221,222]. About 10-12% of women with ovarian cancer carrier mutations in these genes. Another ~2-3% carry mutations associated with

hereditary nonpolyposis colorectal cancer syndrome, namely germline mutations in mismatch repair genes[223]. There are also several moderate risk genes[224,225], many of which are shared with other cancer types (e.g. breast and prostate)[226], and genome-wide association studies have also identified greater than 30 susceptibility loci[220,227-229].

Environmental risk factors include a broad spectrum of exposures, including factors related to ovulation, medication use, and other health behaviors. Protective associations have been observed for factors that would imply decreased number of lifetime ovulatory cycles, including oral contraceptive use[230], breastfeeding[231], and parity[232]. Increasingly, evidence also points to a unifying theme of inflammation-related risk factors, including physical activity, body size, and diet, to name only a few. Finally, risk factors vary by histotype[233] and there is increasing emphasis on specifying the histotypes as potentially individual disease processes. For example, smoking is only a significant risk factor for mucinous type ovarian cancer[234] while endometriosis is most commonly observed in the history of women with clear cell, endometrioid, and low-grade serous ovarian cancers[235].

Clinical features: detection and treatment

Given the relative rareness of ovarian cancer, its few early warning signs, and lack of an understood pre-clinical phase, it does not meet criteria for routine screening in the general population. A large randomized trial indicated in the United Kingdom randomized women to multimodal screening (starting with CA125 concentration), transvaginal ultrasound screening, or to no screening, but did not find a significant mortality reduction in the primary analysis[236]. Unfortunately, symptom-based screening does not work particularly well, as the symptoms of ovarian cancer are also largely non-specific, including bloating, abdominal pain, difficulty

eating, and urinary urgency[237]. Pilot symptom-based screening programs have been largely unsuccessful, with a high rate of false positive results and health complications due to further follow-up tests[238]. By the time most women seek care, disease is already advanced; in fact, 70% of cases are diagnosed at stages III and IV, when disease has already spread to lymph nodes and/or other organs.

The standard of care for women diagnosed with ovarian cancer is an initial cytoreductive ("debulking") surgery followed by chemotherapy. Surgery, considered the cornerstone of treatment, is typically a lengthy process that attempts to remove as much tumor as possible. For most women, the surgery involves a total hysterectomy (removal of uterus), a bilateral salpingo-oophorectomy (removal of both fallopian tubes and both ovaries), and an omentectomy (removal of the omentum). Successful removal of all macroscopic disease is an important predictor of survival[239,240]. Chemotherapy typically consists of a platinum-based compound (e.g. cisplatin or carboplatin) and a taxane (e.g. paclitaxel or docetaxel). The platinum therapy directly damages DNA and limits cellular division and the taxanes halt mitosis and cause cell death. Chemotherapy regimens are individualized according to stage, grade, and other factors, but a typical course of treatment for a woman with advanced disease would be approximately six cycles (one to three weeks each) of the two drugs. More recently, bevacizumab, which appears to activate acquired immunity via expansion of B and T cells[241], is also sometimes added. Most chemotherapy is delivered intravenously or orally, though occasionally it is injected directly intraperitoneally. Even with optimal tumor reduction and chemotherapy, about 70% of advanced (stage III/IV) cases relapse[242].

Factors influencing survival

Once a person is diagnosed with ovarian cancer, additional factors are associated with their longevity. These may or may not overlap with etiologic (risk) factors for incident disease. Clinical guidance to improve survivorship is sorely needed. As discussed above, difficulties in detection, diagnosis, and treatment mean that overall survival of ovarian cancer is generally quite poor. The single most important prognostic factor is stage at the time of diagnosis, where more advanced stage confers poor prognosis[243-245]. Other factors influencing survival include age at diagnosis[246,247], comorbidities[243], histologic subtype[248,249], timing and type of chemotherapy[250], and the success of surgical debulking[251-253]. Unfortunately, other than chemotherapy and surgical results, these are non-modifiable at the time of diagnosis.

Ovarian cancer survival has also been associated with many behaviors and exposures that *are* generally modifiable. Health behaviors such as smoking[254] (worse prognosis) and physical activity[255] (better prognosis) exert effects. Obesity is associated with worse overall survival and progression-free survival[256]. Extended survival has been associated with many common medications, including metformin[257], statins[258 259], aspirin[260] and MHT[261-263]. Both pre-diagnosis[263-266] and post-diagnosis[261,262] MHT use is associated with increased survival, though additional investigation is needed to clarify the effects of timing, type, and duration. Post-diagnosis use of anti-inflammatory pain medications (aspirin and NSAIDs) has been associated with improved ovarian cancer survival as well[260]. These modifiable exposures and behaviors offer insight into possible mechanisms of disease progression and provide avenues for exploring interventions and ultimately updating recommendations for cancer survivors.

Summary

Given the grim prognosis for ovarian cancer, which is primarily diagnosed at advanced stages, further work is sorely needed to better characterize factors relevant to risk and survival of ovarian cancer.

A few key ideas are important to keep in mind when generating hypotheses and studying modifiable factors that could be related to both risk and survival. First, an exposure that increases risk does not necessarily negatively impact survival once diagnosed. For example, one could imagine that a certain risk factor could make it more likely that a woman develop cancer, but that it be of a less aggressive type and that her prognosis is better. Second, pre-diagnosis exposures and behaviors are often correlated to post-diagnosis exposures and behaviors[267,268]. This means that even if one is studying survival, and an exposure is only measured prior to diagnosis, the effect of the exposure may be due at least in part to the post-diagnosis exposure. Third, despite the previous point, one cannot assume that post-diagnosis behaviors remains the same; based on personal choice or clinical recommendation, many women do indeed successfully make major changes to their health behaviors. In this dissertation, I examine potentially modifiable pre-diagnosis exposures to contribute to our understanding of biological mechanisms influencing ovarian cancer survival and ultimately to improving the quality and length of life of women living with ovarian cancer.

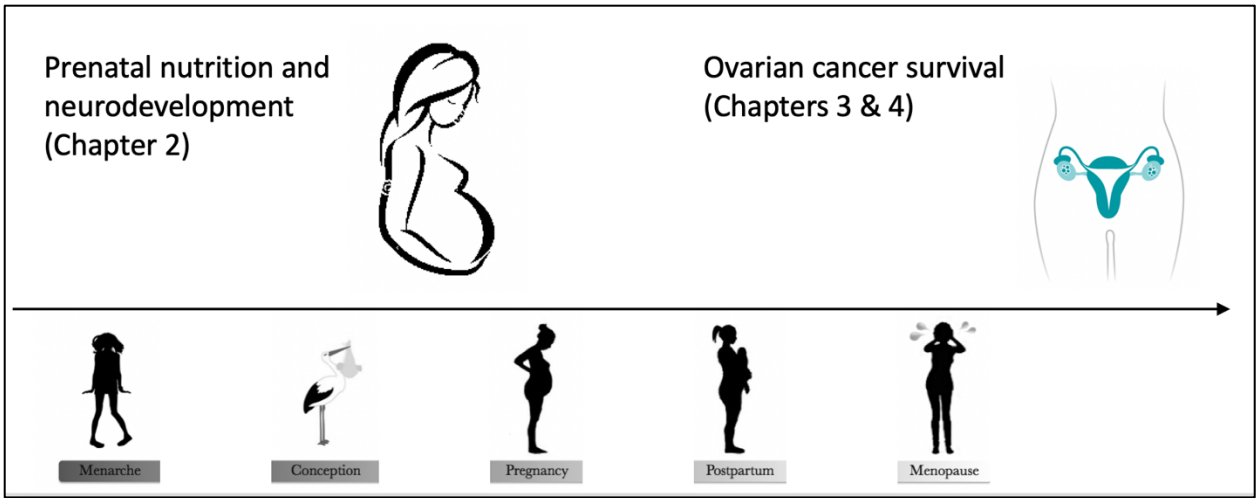

Figure 1-1. Conceptual timeline of dissertation work across a woman's life course.

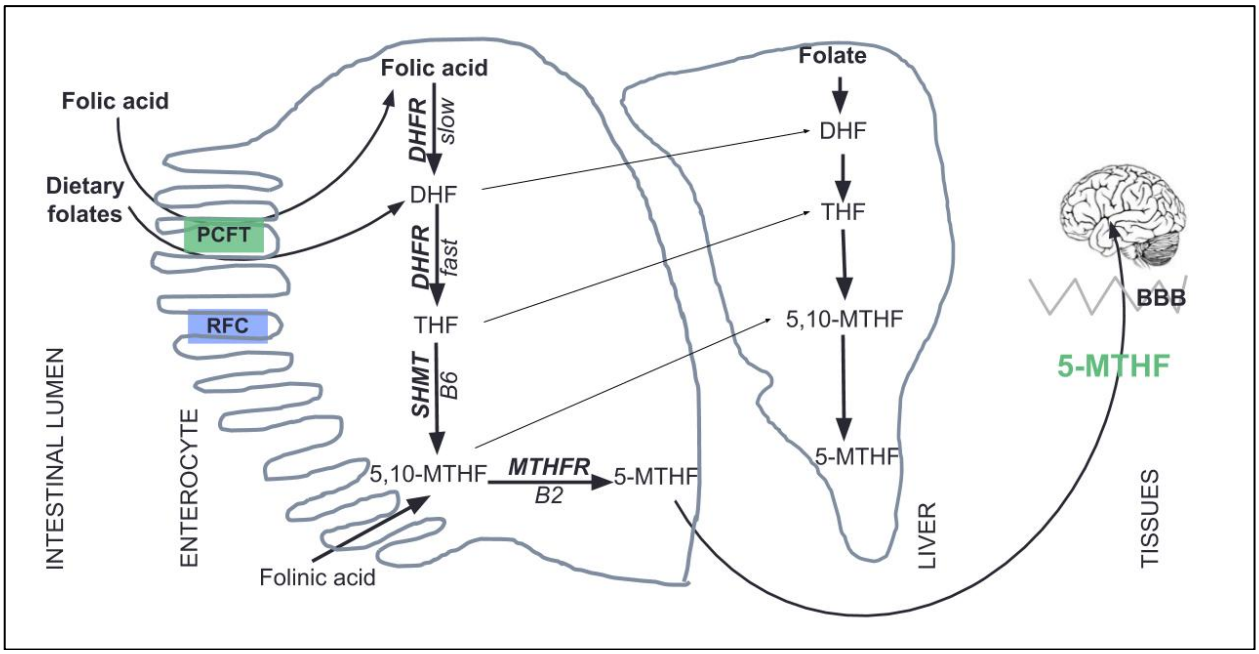

Figure 1-2. Conversion and activation of folate.
Folic acid and folate are both primarily taken up in the small intestine via the proton-coupled folate transporter (PCFT). Only the final product, 5-methylene tetrahydrofolate (5-MTHF) is metabolically active and able to cross to blood brain barrier (BBB). DHF=dihydrofolate, DHFR=dihydrofolate reductase, THF=tetrahydrofolate, SHMT=serine hydroxymethyltransferase, MTHF=methylene tetrahydrofolate, MTHFR=methylene tetrahydrofolate reductase. B2=riboflavin, B6=pyridoxal phosphate.

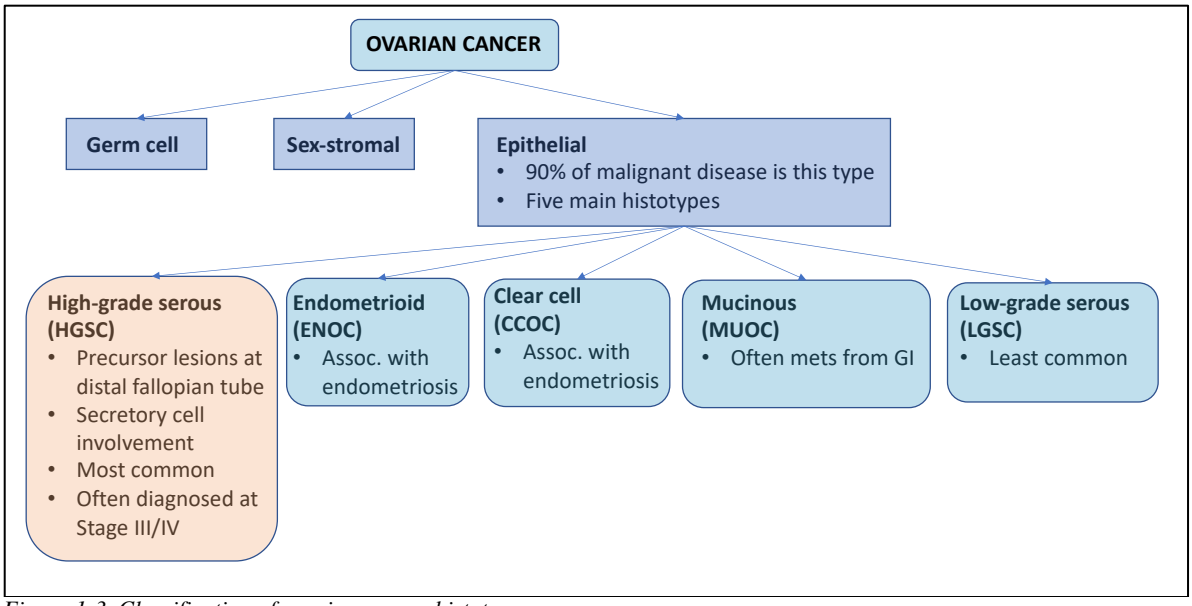

Figure 1-3. Classification of ovarian cancer histotypes.

Table 1-1. Staging criteria for ovarian cancer.

FIGO Stage	AJCC	SEER	Description
IA	T1a, N0, M0	Localized	One ovary, capsule intact
IB	T1b, N0, M0	Localized	Both ovaries, capsule intact
IC	T1c, N0, M0	Regional	Capsule ruptured, cancer on surface
IIA	T2a, N0, M0	Regional	Uterus, fallopian tubes
IIB	T2b, N0, M0	Regional	Bladder, sigmoid colon, rectum
IIIA1	T1/T2, N1, M0	Distant	Retroperitoneal lymph nodes
IIIA2	T3a2, N0/N1, M0	Distant	Small deposits in lining of upper abdomen
IIIB	T3b, N0/N1, M0	Distant	Moderate deposits (≤2cm) in the abdomen
IIIC	T3c, N0/N1, M0	Distant	Large deposits (>2cm) in the abdomen
IVA	any T, any N, M1	Distant	Fluid around the lungs
IVB	any T, any N, M1	Distant	Inside spleen/liver or outside peritoneal cavity

Chapter 2 Prenatal vitamin use and autism spectrum disorder

The association of prenatal vitamins and folic acid supplement intake with risk for autism spectrum disorder in a high-risk sibling cohort, the Early Autism Risk Longitudinal Investigation (EARLI).

Abstract

We examined maternal prenatal vitamin use or supplemental folic acid intake during month one of pregnancy for association with autism spectrum disorder (ASD) in the Early Autism Risk Longitudinal Investigation (EARLI), an enriched-risk pregnancy cohort. Total folic acid intake was calculated from monthly prenatal vitamins, multivitamins, and other supplement reports. Clinical assessments through age 3 years classified children as ASD (n=38) or non-ASD (n=153). In pregnancy month one, prenatal vitamin use (59.7%) was not associated with odds of ASD (OR=0.70, 95%CI: 0.32, 1.53). High total folic acid supplementation (>1000 mcg, 9.4% vs 400-1000 mcg, 45.0%) was also not associated with ASD (OR=1.64, 95%CI: 0.44, 5.47).

Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by persistent deficits in social communication and interaction, as well as restricted,

repetitive behaviors [269]. In 2018, ASD was observed to affect 1 in 59 children by eight years of age in the United States [35]. Perturbations to the delicate *in utero* neurodevelopmental process likely contribute to the development of ASD. While the etiology of ASD is at least in part genetic, several environmental factors have been associated with increased ASD risk including prenatal infection with fever [270-273], maternal diabetes [107], perinatal stress [108], chemical toxicants [109], and certain medications [110]. Understanding modifiable exposures associated with ASD has great public health potential, particularly for high-risk families.

Maternal prenatal nutrition is critical for proper neurodevelopment [157,159,160]. It is well-established that folic acid is crucial for neural tube development [173-175,274]. Pregnant women should consume at least 400 micrograms (mcg) folic acid or at least 600 mcg dietary folate equivalents [170,274] from dietary folates (naturally occurring in food), fortified foods (such as cereals), prenatal vitamins, multivitamins, and folic acid supplements. Compared to multivitamins, prenatal vitamins typically contain more folic acid (~800 mcg in prenatal versus 400 mcg in regular multivitamins) and iron (~28 mg versus 18 mg). Folic acid-specific supplements are prescribed in a range of doses, from 400 mcg to 4,000 mcg, with the highest doses often reserved for those with a previous pregnancy affected by a neural tube defect [275].

Evidence supports protective associations of prenatal vitamins [161,195,276] or folic acid [162,195,277] with ASD. However, the size and direction of the reported effects are variable [194,278]. Two studies observed supplementation versus very low levels was associated with protection, while very high folic acid intake (>1,000 mcg) or high maternal plasma folate levels (≥60.3nmol/L) were associated with increased odds ASD, suggesting a u-shaped pattern [196,197]. It is difficult to compare previous studies with pregnancy exposure periods defined in different and overlapping manners (periconceptionally, first trimester, all of pregnancy). Many studies queried supplement

use only once and are subject to exposure misclassification. Replication with prospective prenatal measures of intake doses, dietary and supplement sources, and precise timing is limited.

We evaluated the relationship between prenatal vitamin and folic acid intake (including timing, supplement type, and dose) during early pregnancy and odds of ASD recurrence in baby siblings of the EARLI cohort, a prospective, enriched-risk pregnancy cohort with detailed exposure measurements collected throughout pregnancy and child outcome evaluation at 36-months using a gold-standard clinical assessment protocol for ASD.

Methods

Study design and overall sample

EARLI follows families who already have one child (the proband) with clinically-confirmed ASD [279]. Mothers were enrolled by the 28th week of a subsequent pregnancy and 249 families were recruited from Southeast Pennsylvania (Drexel University), Northeast Maryland (Johns Hopkins University and Kennedy Krieger Institute), and Northern California (University of California, Davis, the MIND Institute, and Kaiser Permanente Division of Research). Informed consent was obtained from all participants and the Institutional Review Boards of all recruitment sites approved this study.

Prenatal vitamin and folic acid supplement assessment

At the first study visit, mothers were asked to recall supplement use (multivitamin, prenatal vitamin, or folic acid) for any previous month of pregnancy. Then, supplement use was

reported prospectively by monthly questionnaire. If a supplement was used, mothers were asked about the type, brand, and frequency of use. Average daily total supplemental folic acid intake was estimated from the total in the prenatal vitamins, multivitamins, and any other folic acid-containing supplements [276]. Each brand a mother reported was queried; if the brand was unknown then standard amounts found in that vitamin type were used. Total folic acid intake from supplements was then categorized into low (<400 mcg), adequate (400-1000 mcg), and high (>1000 mcg) based on the recommendations of the Centers for Disease Control [274]. Our primary exposure was prenatal vitamin supplement use (yes / no) during the first month of pregnancy.

ASD outcome assessment

Postnatal clinical evaluations were completed four times. Our primary outcome of interest was ASD at the 36-month visit using *DSM-5* criteria [269]. As sensitivity analyses, we considered continuous outcome scores on the Autism Observation Scale for Infants (AOSI) [280] at 12 months, the Autism Diagnostic Observation Scale (ADOS) [30] at 36 months, and the Mullen Scales of Early Learning (MSEL) at 36 months. As a sensitivity analysis, children were grouped into three outcome levels (ASD, non-typically developing, typically developing) according to the Baby Siblings Research Consortium (BSRC) criteria [281], based on definitions of elevated ADOS scores, low MSEL scores, or both.

Demographics and covariate ascertainment

Demographic information was collected at the maternal pregnancy interviews. The key covariates considered were maternal age (years), paternal age (years), maternal education

(bachelor's degree or higher), maternal smoking behavior (any), maternal body mass index (BMI) at the first pregnancy study visit, gestational age of the younger sibling at birth (weeks), and sex of the younger sibling.

Exclusion criteria for analytic samples

Of the 249 families enrolled, nine pregnancies did not result in live births. Ten of the remaining 240 pregnancies resulted in twins, and one twin from each family was randomly excluded. Next, 46 families were excluded due to missing ASD diagnosis at 36-months. One family was excluded for missing information for prenatal vitamins and total folic acid intake for the first trimester of pregnancy. Two families were excluded for missing maternal education level. The analytic sample was comprised of 191 mother-child pairs (Figure 2-3).

Statistical analyses

We compared demographic information and covariates for families in the analytic sample (n=191 families) to those excluded from the overall birth sample (n=49 families). For categorical variables (maternal race, maternal ethnicity, maternal education, maternal BMI classification, maternal smoking, prenatal vitamin use, prenatal folic acid intake, sibling sex), chi-square tests of proportions were used. For continuous variables (maternal and paternal age, gestational age, AOSI total score at 12 months, and ADOS comparison score at 36 months), t-tests were used.

We conducted bivariate analyses of parental demographics and child characteristics noted above by the primary exposure and outcome variables. We used multivariate logistic regression to estimate odds of ASD for children of mothers who took prenatal vitamins in month one

compared to those whose mothers did not, adjusting for (1) maternal education and (2) maternal education and child sex. Maternal education was a confounder (altered the relationship between prenatal vitamins and ASD by >10%). Child sex improved model performance by AIC.

Additional and sensitivity analyses

We performed several exposure sensitivity analyses. First, we used logistic regression to estimate the association of categorized total folic acid intake (low, adequate, and high) with ASD. Second, we used spline models to assess the relationship of total folic acid intake in month one of pregnancy to ASD outcomes. Third, we examined prenatal vitamin use across all months (one through nine) of pregnancy with a mixed effects logistic regression model, including a random intercept for each mother and a spline term for time.

We also conducted outcome sensitivity analyses, all measured at 36 months. First, we used logistic regression to assess BSRC groupings (ASD (n=36), non-typically developing (n=71), typically developing (n=77)). Next, we used a generalized linear model (with a negative binomial distribution and log link function) to assess the relationship between prenatal vitamin use and ADOS comparison score, normalized to allow comparison across ADOS modules [282,283]. Finally, we used a generalized linear model approach (with normality assumptions and a linear link function) to assess prenatal vitamin use with the MSEL t-score sum.

Comparison with prior studies' results

We conducted a literature review to identify relevant studies of prenatal nutritional supplements and ASD. We classified studies by exposure period (i.e. preconception, first

trimester, all of pregnancy) and by exposure type (folic acid, prenatal vitamins, multivitamins). We prioritized studies that examined the relationship between intake of supplements during the first or second trimester and ASD outcomes. From an initial 15 studies, two were excluded for exposure timing or for having only plasma measures [284,285], two were excluded for missing ASD outcomes [286,287], and one was excluded for only reporting on the interaction of folic acid with air pollution and not the direct effect of folic acid [212]. Thus, ten studies had tests in at least one supplement type: three of prenatal vitamins [161,276,278], five of multivitamins [194,195,197,277,288], and six of folic acid [162,194,195,277,288,289].

Results

Sample demographic characteristics

The families in the analytic sample did not differ from those excluded (Table 2-3). Similarly, the siblings excluded were comparable in terms of sex ratio and AOSI total score at 12 months (Table 2-3). In our analytic sample, maternal age at conception ranged from 21 to 44 (mean=33.59, SD=4.79) and paternal age ranged from 22 to 55 (mean=35.66, SD=5.98). Among mothers in the analytic sample, 60.2% reported White race, 17.3% reported Hispanic ethnicity, and 58.1% reported earning a bachelor's degree or higher (Table 2-1).

Distribution of prenatal vitamin and folic acid supplement use

Prenatal vitamin use in the first month of pregnancy was lower among mothers of ASD cases (52.6%) compared to mothers non-ASD children (61.4%, p=0.42,Table 2-1). Prenatal

vitamin use was significantly associated with maternal education, where a higher proportion of mothers with a bachelor's degree or higher (67.5%) took prenatal vitamins, relative to mothers with less education (44.2%, p=0.002). Additionally, prenatal vitamin use was associated with total folic acid category, where more mothers who took prenatal vitamins had adequate levels of folic acid (65.8% vs. 14.3%, p<0.001) (Table 2-1). Use of prenatal vitamins varied, but by the end of the first trimester, most women (89%) used prenatal vitamins (Figure 2-1).

Distribution of ASD outcomes in study sample

One fifth (n=38, 19.9%) of the children in our analytic sample were diagnosed with ASD at 36-months, according to *DSM-5* criteria (Table 2-1), as expected [77]. AOSI total score at 12 months was higher among cases (median=6.0, IQR: [4.0, 9.0]) compared to non-ASD controls (median=4.0, IQR: [2.0, 7.0], p=0.012). Mothers of ASD cases were less educated than those of non-ASD controls (44.7% with a bachelor's degree or higher compared to 61.4% of controls, p=0.092) (Table 2-1). Additionally, a higher proportion of mothers of cases were obese (50.0% versus 24.2%, p=0.005), based on pre-pregnancy BMI.

Association of prenatal vitamin use with ASD outcomes

Prenatal vitamin use in month one of pregnancy was not associated with ASD before adjustment (OR=0.70, 95%CI: 0.34, 1.43) or after adjusting for maternal education and child sex (OR=0.70, 95%CI: 0.32, 1.53) (Table 2-2, Figure 2-2).

Additional and sensitivity analyses

High folic acid intake (>1000 mcg) in month one of pregnancy compared to adequate (400-1000 mcg) was not significantly associated with odds of ASD (OR=1.64, 95%CI: 0.44, 5.47), adjusted for child sex and maternal education (Figure 2-2). In spline modeling of continuous total folic acid intake, we observed a suggestive increase in odds at the high end of intake values (Error! Reference source not found.). Across all months of pregnancy, a lower proportion of mothers of eventual ASD cases were taking prenatal vitamins (p=0.074) (Figure 2-1).

Sensitivity analyses using BSRC groupings revealed that the protective direction of prenatal vitamin use was only observed for ASD versus typically developing and not for non-typically developing compared to typically developing (Table 2-4). We observed slightly higher MSEL scores among children whose mothers took prenatal vitamins in month one of pregnancy (increase in t-score sum of 5.24, 95%CI: -8.10, 18.6) but this difference was not significant. There was no significant difference in ADOS comparison score between children whose mothers took prenatal vitamins and those whose mothers did not (Table 2-1).

Discussion

In the EARLI prospective, enriched-risk pregnancy cohort, we observed no significant association between prenatal vitamin intake in the first month of pregnancy and odds of ASD. However, the direction and magnitude of association was consistent with previous findings, though the confidence interval was wide. In these families at high risk of ASD, it is especially important to understand modifiable environmental risk factors. To our knowledge, this is only the second study reporting on prenatal vitamins' association with odds of recurrence in younger siblings. We observed a protective association of maternal education, consistent with prior

studies [290-292]. We also observed a higher rate of ASD among male children (sex ratio: 3.75 male to 1 female), consistent with the literature [293]. The higher rates of obesity among mothers of ASD cases was also consistent with the literature [294-296].

Prenatal vitamins taken during the first month of pregnancy had no significant association with ASD. The association (OR=0.70, 95%CI: 0.32, 1.53) was similar in magnitude and direction to previously reported, but with a wider confidence interval. Our precision of estimation was limited by sample size. Although statistical significance is an important consideration, observed effect sizes are valuable for clinical utility [297]. Mothers of children that later developed ASD were less likely to be taking prenatal vitamins throughout pregnancy; future studies should investigate combinations of time points and possible time intervals. These findings appeared specific to ASD, as prenatal vitamins did not show associations with other non-typical development in this sample. A previous study of prenatal vitamin use in the first month of pregnancy in a prospective U.S. high-risk sibling cohort found a protective OR of 0.50 (95%CI: 0.31, 0.81) for ASD recurrence [276]. Likewise, a U.S. case-control study of prenatal vitamin use in either the three months prior to pregnancy or the first month of pregnancy found an OR for ASD of 0.62 (95%CI: 0.42, 0.93) [161]. Given the magnitude of the association estimates observed in this and previous work, prenatal vitamin intake during early pregnancy could be a highly clinically useful preventative measure for ASD.

Several studies from other countries have examined associations between prenatal vitamin use and neurodevelopmental outcomes [194,195,277]. A key difficulty in comparing or meta-analyzing studies of supplements from other countries is that “multivitamin” and “prenatal vitamin” indicate different nutrient compositions by country [298]. Additionally, prescription and non-prescription products differ; in the U.S., prescription products typically contain higher levels

of folic acid, while non-prescription products tend to have higher levels of vitamins A and D, iodine, and calcium [299]. Studies define adequacy in different ways, such as using the CDC recommendation of 400 to 1,000 micrograms per day [275], the Institute of Medicine definition of at least 600 dietary folate equivalents per day [170], or defining their own cut point. Additionally, some of the largest studies of folic acid during pregnancy and ASD were conducted in countries without mandatory grain fortification, such as Norway [277], Sweden [194], and Israel [195]. Further work is still needed to understand the components or combinations of nutrients most related to the protection afforded by prenatal vitamins.

We noted that very high folic acid (>1000 mcg total supplemental from any source) relative to adequate (400-1000 mcg) was not associated with odds of ASD, though there was a suggestive trend that higher folic acid was associated with higher odds. At least one other study has noted a relationship between odds of ASD and high maternal folate levels [197], though these folate measures were taken at the time of delivery. There is also the possibility of confounding by indication in this highest supplemental intake category, since high doses of folic acid can be indicated for at-risk pregnancies, such as mothers who have already had a pregnancy resulting in a neural tube defect [275], other birth defects [300-302], or maternal anemia [303,304]. It is possible that the protection offered by prenatal vitamins is in part due to synergistic effects of the many component vitamins and minerals and that examining individual nutrients will not yield comparable results.

In addition to the prenatal vitamin association with ASD diagnosis, we tested the specificity of ASD continuous measures and subscales. MSEL scores at 36-months were not associated with prenatal vitamin use, though a trend was observed in the expected direction given that higher scores indicate more optimal mental development (motor skills, visual

perception, expressive and receptive language). ADOS comparison scores at 36-months were not difference among children whose mothers took prenatal vitamins.

One limitation of this study is the modest sample size, limiting the precision of effect estimates, and small numbers in strata prevented a sex-stratified analysis. There could also be residual confounding and confounding by indication; because prenatal vitamin use is associated with other health-conscious behaviors, there is some concern that other health-related behaviors were not measured or controlled for. Confounding by indication may have affected results for total folic acid, as women who are prescribed folic acid have a different set of risk factors.

In summary, prenatal vitamins may protect against ASD occurrence in younger siblings. If replicated, these findings have important implications for pregnant women. The prospective study design allowed for accurate exposure collection during the biologically relevant period, including details about type and dose that allow for specificity in examining risk factors. Additionally, detailed phenotyping of the children allowed for assessment of not just outcome classification but also markers of severity. Future work should examine other months of pregnancy, incorporate dietary nutrient information, and include maternal folate metabolism genetics.

Table 2-1. Characteristics of families in the Early Autism Risk Longitudinal Investigation.

	Case status			**Prenatal vitamin use month 1**		
	Non-ASD (n=153)	ASD (n=38)	p[1]	No (n=77)	Yes (n=114)	p[1]
Child characteristics						
Male sex (%)	71 (46.4)	30 (78.9)	0.001	36 (46.8)	65 (57.0)	0.213
Gestational age, weeks (mean (sd))	39.12 (1.45)	38.70 (1.51)	0.143	39.06 (1.18)	39.02 (1.62)	0.85
Missing (%)	23 (15.0)	6 (15.8)		14 (18.2)	15 (13.2)	
AOSI[2] total score, 12 months (median [IQR])	4.0 [2.0, 7.0]	6.0 [4.0, 9.0]	0.012	4.0 [2.0, 6.25]	4.0 [2.0, 8.0]	0.803
Missing (%)	9 (5.9)	1 (2.6)		5 (6.5)	5 (4.4)	
ADOS[3] comparison score, 36 months (median [IQR])	2.0 [1.0, 3.0]	7.0 [6.0, 8.0]	<0.001	2.0 [1.0, 5.0]	2.0 [1.0, 5.0]	0.354
Missing (%)	4 (2.6)	2 (5.3)		0 (0.0)	6 (5.3)	
ASD[4] case (%)	--	--	--	18 (23.4)	20 (17.5)	0.42
Parental characteristics						
Maternal age (mean (sd))	33.61 (4.91)	33.50 (4.31)	0.901	33.40 (5.15)	33.71 (4.54)	0.664
Paternal age (mean (sd))	35.85 (6.00)	34.92 (5.95)	0.394	35.12 (6.34)	36.04 (5.73)	0.3
Missing (%)	1 (0.7)	0 (0.0)		0 (0.0)	1 (0.9)	
Maternal race (%)			0.180			0.550
Asian	19 (12.4)	5 (13.2)		8 (10.4)	16 (14.0)	
Black, African American	16 (10.5)	5 (13.2)		10 (13.0)	11 (9.6)	
White	94 (61.4)	21 (55.3)		46 (59.7)	69 (60.5)	
Other/multiple	13 (8.5)	1 (2.6)		5 (6.5)	9 (7.9)	
Missing	11 (7.2)	6 (15.8)		8 (10.4)	9 (7.9)	
Maternal ethnicity (%)			0.538			0.966
Hispanic	24 (15.7)	9 (23.7)		15 (19.5)	18 (15.8)	
Non-Hispanic	115 (75.2)	25 (65.8)		54 (70.1)	86 (75.4)	
Missing	14 (9.2)	4 (10.5)		8 (10.4)	10 (8.8)	
Maternal education, bachelor's degree or higher (%)	94 (61.4)	17 (44.7)	0.092	34 (44.2)	77 (67.5)	0.002
Maternal BMI[5] (%)			0.005			0.274
Underweight	1 (0.7)	1 (2.6)		0 (0.0)	2 (1.8)	
Normal	63 (41.2)	14 (36.8)		27 (35.1)	50 (43.9)	
Overweight	48 (31.4)	4 (10.5)		25 (32.5)	27 (23.7)	

Obese	37 (24.2)	19 (50.0)		25 (32.5)	31 (27.2)	
Missing	4 (2.6)	0 (0.0)		0 (0.0)	4 (3.5)	
Maternal pregnancy behaviors						
Prenatal vitamin use, Mo. 1 pregnancy (%)	94 (61.4)	20 (52.6)	0.42	--	--	--
Folic acid, Mo. 1 pregnancy			0.662			<0.001
Low (<400 mcg)	71 (46.4)	16 (42.1)		65 (84.4)	22 (19.3)	
Adequate (400-1000 mcg)	69 (45.1)	17 (44.7)		11 (14.3)	75 (65.8)	
High (>1000 mcg)	13 (8.5)	5 (13.2)		1 (1.3)	17 (14.9)	
Prenatal smoking (%)			0.031			>0.99
No	122 (79.7)	25 (65.8)		57 (74.0)	90 (78.9)	
Yes	3 (2.0)	4 (10.5)		3 (3.9)	4 (3.5)	
Missing	28 (18.3)	9 (23.7)		17 (22.1)	20 (17.5)	

[1]Continuous variables tested with a t-test for difference in means and categorical variables tested with a chi-square test of equal proportions. Missing category excluded for categorical variables before testing for differences.

[2]AOSI = Autism Observation Scale for Infants. The AOSI assessment consists of 18 items and is administered to infants age 6 to 18 months. An AOSI total score of 13 or greater indicates a child is likely to go on to develop ASD.

[3]ADOS = Autism Diagnostic Observation Scale. This is a semi-structured interview conducted by a trained examiner to assess communication, social interaction, and play. It is valid for ages 12 months through adulthood. Higher scores indicate higher severity of autism symptoms; threshold level for a total score indicating ASD varies by module and age level. The comparison score is standardized across modules.

[4]ASD = autism spectrum disorder. Primary analyses will use diagnosis as defined by the *Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)*.

[5]pre-pregnancy self-report.

Table 2-2. Association of month 1 pregnancy supplementation with autism.

	Outcome		OR (95% CI)		
	ASD[1] (n=38)	Non-ASD (n=153)	Unadjusted	Adjusted[2] for education	Adjusted[2] for education and sex
PRENATAL VITAMINS					
No (%)	18 23.4)	59 (76.6)	1 (reference)	1 (reference)	1 (reference)
Yes (%)	20 17.5)	94 (82.5)	0.70 (0.34, 1.43)	0.81 (0.38, 1.68)	0.70 (0.32, 1.53)
FOLIC ACID					
CDC guidelines					
Adequate[3] (%)	17 (44.7)	69 (45.1)	1 (reference)	1 (reference)	1 (reference)
Low (<400 mcg) (%)	16 (42.1)	71 (46.4)	0.91 (0.43, 1.96)	0.75 (0.34, 1.67)	0.75 (0.32, 1.69)
High (>1000 mcg) (%)	5 (13.2)	13 (8.50)	1.56 (0.45, 4.79)	1.47 (0.45, 4.78)	1.64 (0.44, 5.47)

[1]According to *DSM-5* diagnostic criteria.
[2]Education refers to maternal education (less than bachelor's versus bachelor's degree or higher) and sex refers to child sex.
[3]Adequate intake used as reference category.

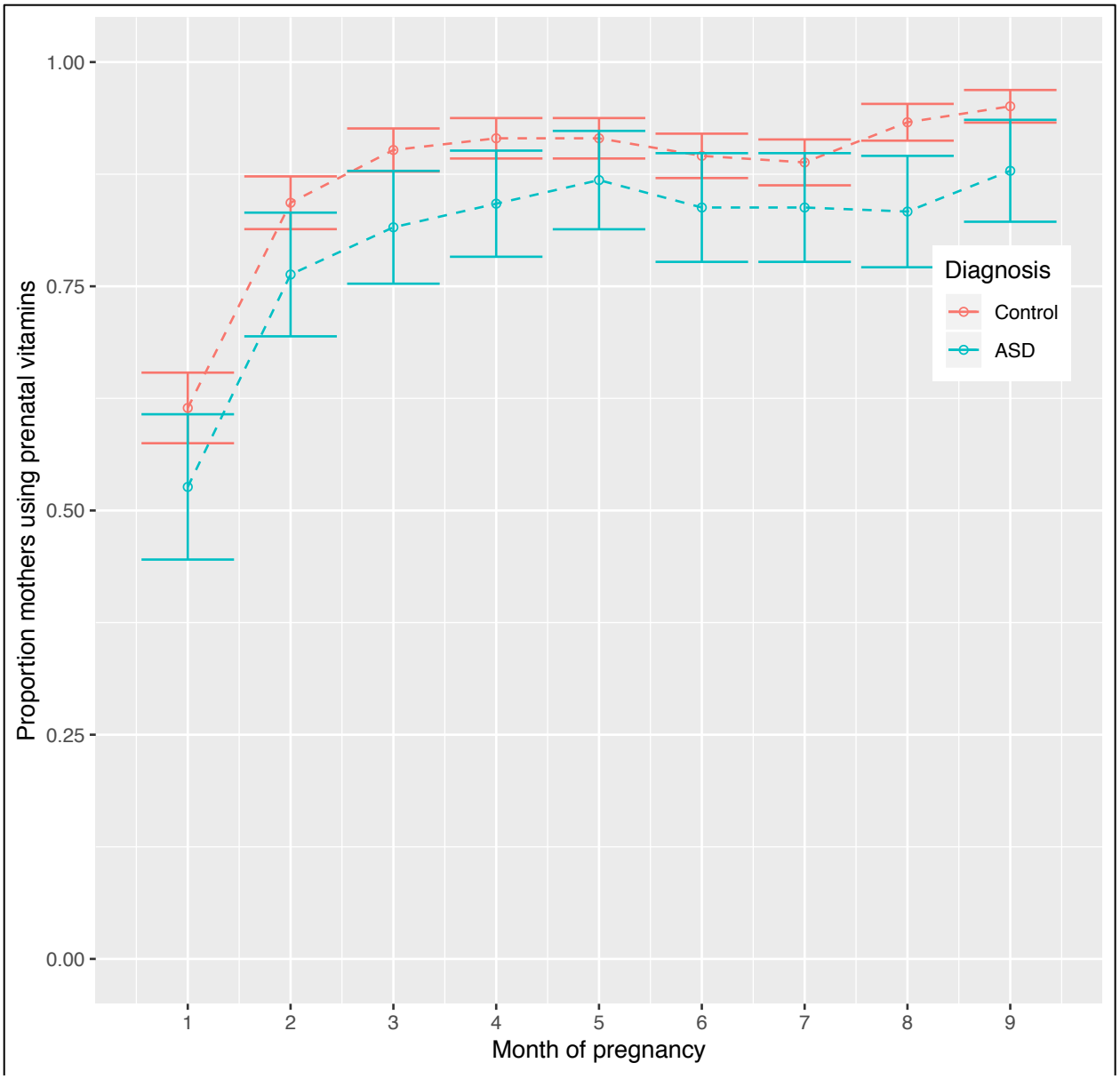

Figure 2-1. Prenatal vitamin use across pregnancy in 191 mothers in the pregnancy cohort.
Maternal use of prenatal vitamins increased steeply over the first trimester of pregnancy; by month 3 of pregnancy, 88% of mothers took prenatal vitamins. Across pregnancy, a lower proportion of mothers of eventual ASD cases (n=38) were taking prenatal vitamins compared to mothers of non-ASD controls (n=153). Errors bars represent the unadjusted standard error of the mean for proportions; SE=sqrt(p(1-p)/n).*

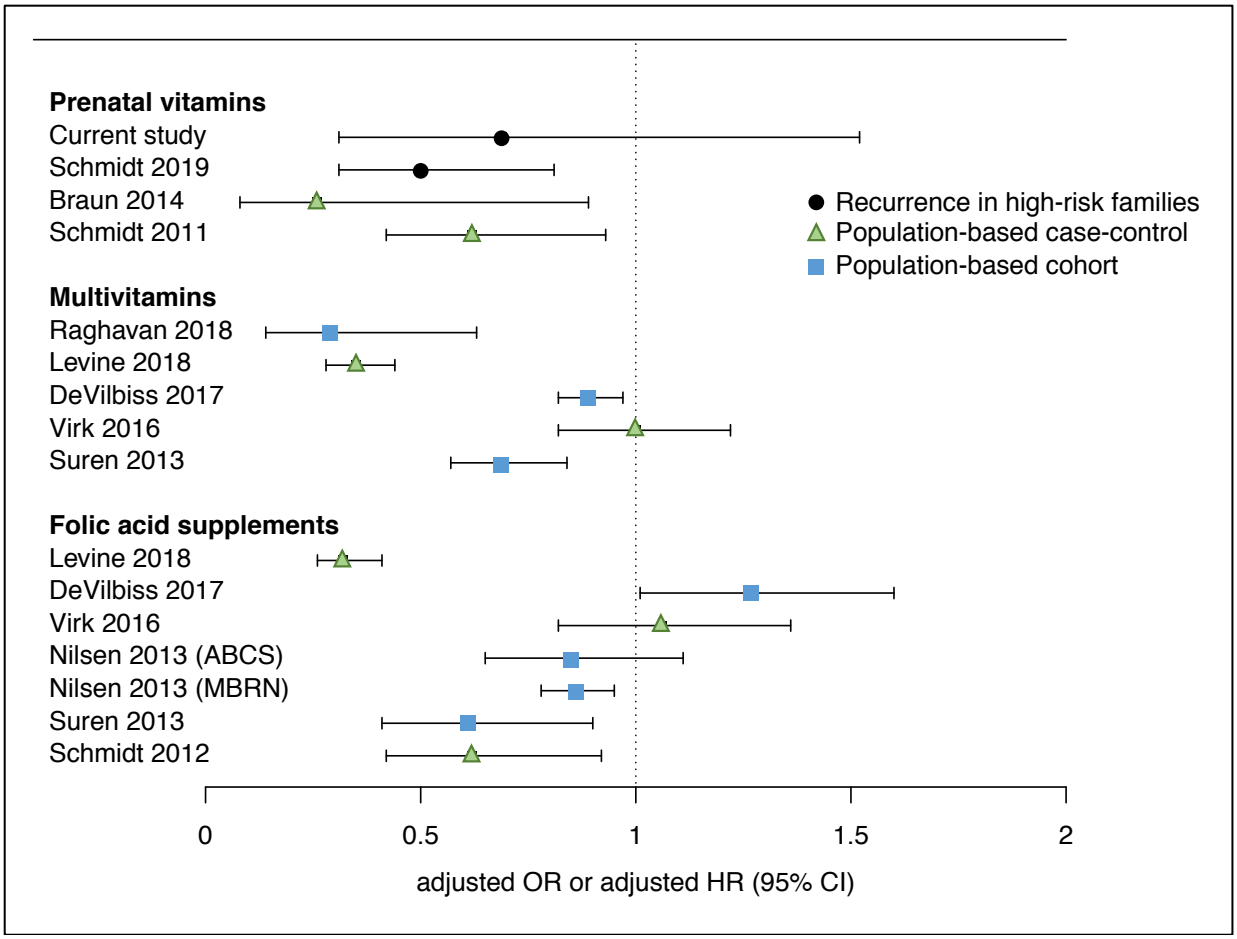

Figure 2-2. The protective association with prenatal vitamin use in prior studies.
Protective (though not significant) association of vitamin use in month 1 of pregnancy among mothers in the EARLI cohort is consistent in magnitude and direction when compared to prior studies, though the estimate is less precise. ABCS: Autism Birth Cohort Study; MBRN: Medical Birth Registry of Norway.

Supplemental materials

SUPPLEMENTAL METHODS

Genetic data collection

A total of 841 EARLI samples (mother, father, proband, sibling), and an additional 18 control samples, were genotyped on the Illumina HumanOmni5 + Exome array, which included an initial set of 4,641,218 single nucleotide polymorphisms (SNPs). The 18 control samples were comprised of five individuals from the CEPH (Northern and Western European) HapMap panel (nine samples, including technical replicates) and five individuals from the Yoruba HapMap panel (nine samples including technical replicates) [305]. Data cleaning steps included removing individual samples missing >3% of genotyping information (no samples removed), removing SNPs missing ≥5% of genotypes if minor allele frequency was >5% (removed 8,902 SNPs), removing SNPs with >1% of genotypes missing if minor allele frequency was <5% (removed 65,855 SNPs), checking for technical duplicates (removed 5 samples) and relatedness errors (removed 9 samples), and checking sex using X-chromosome markers. After data cleaning, 4,463,625 SNPs remained and 827 samples remained. EARLI data were then imputed to 1,000 Genomes Project v.5 reference panel [306] using Minimac3 [307]. Based on genetic clustering to reference populations, of the 827 samples, there were 520 European ancestry individuals, 138 Hispanic individuals, 102 African ancestry individuals, and 67 Asian ancestry individuals.

There were 241 families with information for the mother, the sibling, or both. We then extracted maternal and child genotypes at two SNPs in the *MTHFR* gene, C677T (rs1801133) and A1298C (rs1801131), using Plink1.9 [308].

SUPPLEMENTAL RESULTS

Genotype results

Genotype frequencies among mothers and children in the EARLI cohort were comparable to those observed in the 1000 Genomes reference panel. Minor allele frequencies among mothers (n=185 of the analytic sample) were 0.31 and 0.28 for *MTHFR* A1298C and C677T, respectively. For both SNPs, minor allele (risk) frequencies were greater than frequencies observed worldwide, but less than or equal to frequencies observed among Europeans. We observed departures from Hardy-Weinberg equilibrium in our sample, though tests among small samples of related individuals may not be valid and Type I error rates may be inflated. Linkage disequilibrium between these two SNPs is observed in European reference panels (4c); there are no individuals who are homozygous risk for both SNPs. Maternal C677T genotypes were 11 TT (5.9% homozygous risk), 83 CT (45% heterozygous), and 91 CC (49% homozygous reference). Maternal A1298C genotypes were 13 CC (7.0% homozygous risk), 88 (48% heterozygous) AC, and 84 AA (45% homozygous reference). Similar relative proportions were observed among child genotypes (n=172 of the analytic sample), though a slightly higher percentage of children (n=24, 16%) were homozygous risk at the C677T locus.

Bioavailable folate is affected by folate processing genetics, and optimal dosing may vary among mothers. Several common variants in folate transporters and conversion enzymes are associated with lower plasma folate levels [199,309,310]. In the methylene tetrahydrofolate reductase (*MTHFR*) gene, the 677C>T variant (rs1801133) reduces enzymatic activity by more than 50% in homozygous individuals [311]. A case-control study showed decreased odds of ASD with higher folic acid intake only when the mother had the *MTHFR* 677 "TT" variant [162].

Dietary folate results

Dietary folate intake was surveyed twice during pregnancy and the values remained relatively stable between these two time points (Supplemental Table 3). Values also did not differ significantly between those excluded from our analytic samples and those included. In our preconception analytic sample, 127 mothers filled out a dietary history questionnaire during the first half of pregnancy and 73 mothers during the second half of pregnancy. Total daily dietary folate intake was much lower (less than half) compared to the folic acid intake from supplements. Average total dietary folate in the first half of pregnancy was 437 mcg (SD=223), with an average of 248mcg from natural sources and 189mcg from synthetic (fortified) sources. In the second half of pregnancy, total dietary folate was remarkably similar. Among mothers in the post-conception analytic sample, average total dietary folate was 428mcg (SD=231), with 241mcg from natural sources and 188mcg from synthetic sources.

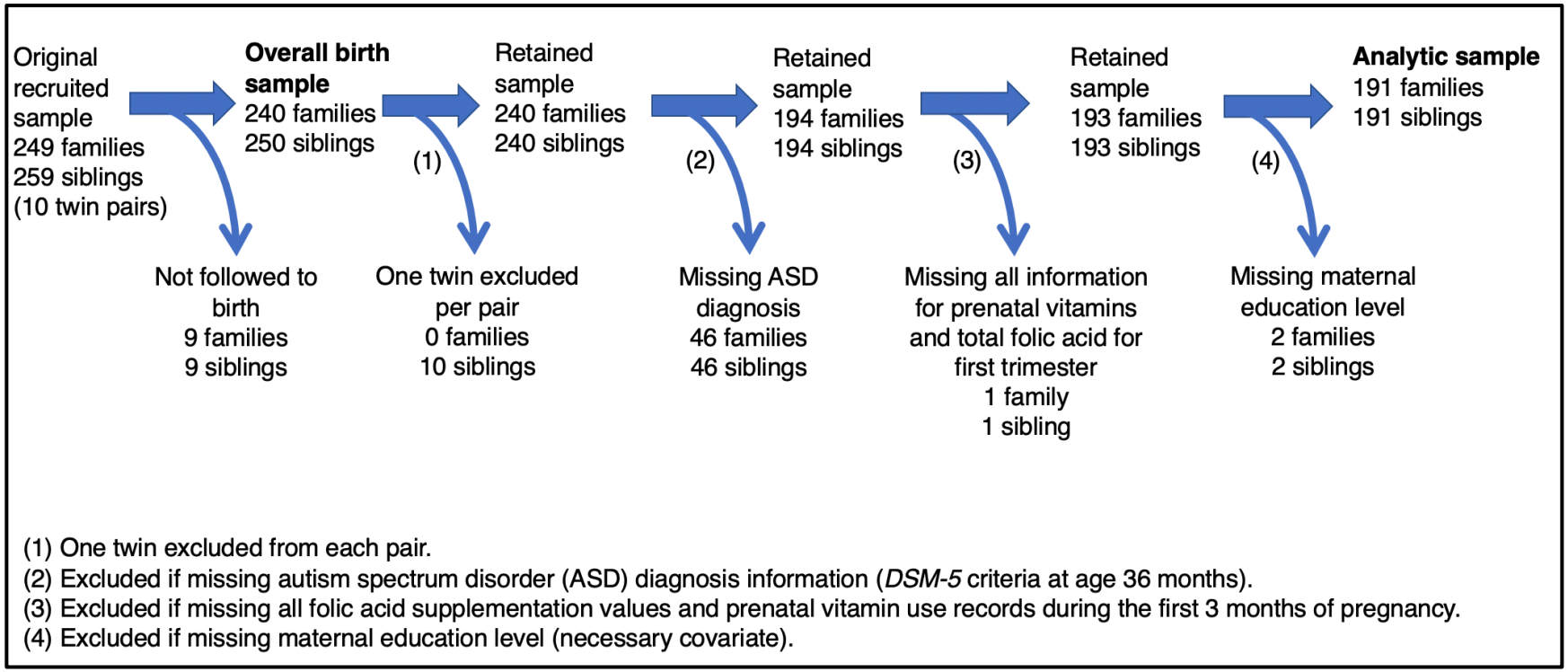

Figure 2-3. Prospective pregnancy cohort families in analytic samples.
*The original sample began with 249 mothers enrolled, including ten mothers pregnant with twins. Of these 249 pregnancies, nine were miscarried or lost to follow-up before a live birth. Families were excluded from the analytic sample according to the following criteria: (**1**) one twin from each pair was excluded, (**2**) if the sibling did not receive a 36-month evaluation for autism spectrum disorder, (**3**) if the mother was missing all information about folic acid-containing supplements (prenatal vitamins, folic acid, and multivitamins) for the first three months of pregnancy, and (**4**) if the mother was missing education level. The resulting analytic sample is comprised of 191 mother-child pairs.*

Table 2-3. Characteristics of mothers and children in overall and analytic sample.

	Overall	**Analytic**	**Excluded**	**p**[1]
N	240	191	49	
Child characteristics				
Male sex (%)	126 (52.5)	101 (52.9)	25 (51.0)	0.942
Gestational age, weeks (mean (sd))	38.99 (1.79)	39.04 (1.46)	38.70 (3.20)	0.378
Missing (%)	53 (22.1)	29 (15.2)	24 (49.0)	
AOSI[2] total score, 12 months (median [IQR])	4.0 [2.00, 8.00]	4.0 [2.00, 7.00]	4.0 [3.00, 8.00]	0.712
Missing (%)	35 (14.6)	10 (5.2)	25 (51.0)	
ASD[3] case (%)	41 (21.1)	38 (19.9)	3 (100.0)	0.008
Missing (%)	46 (19.2)	0 (0.0)	46 (93.9)	
Parental characteristics				
Maternal age (mean (sd))	33.51 (4.66)	33.59 (4.79)	33.23 (4.18)	0.636
Missing (%)	1 (0.4)	0 (0.0)	1 (2.0)	
Paternal age (mean (sd))	35.60 (5.85)	35.66 (5.98)	35.31 (5.25)	0.724
Missing (%)	8 (3.3)	1 (0.5)	7 (14.3)	
Maternal race (%)				0.756
Asian	29 (12.1)	24 (12.6)	5 (10.2)	
Black, African American	27 (11.2)	21 (11.0)	6 (12.2)	
White	143 (59.6)	115 (60.2)	28 (57.1)	
Other/multiple	22 (9.2)	14 (7.3)	8 (16.3)	
Missing	19 (7.9)	17 (8.9)	2 (4.1)	
Maternal ethnicity (%)				0.888
Hispanic	40 (16.7)	33 (17.3)	7 (14.3)	
Non-Hispanic	178 (74.2)	140 (73.3)	38 (77.6)	
Missing	22 (9.2)	18 (9.4)	4 (8.2)	
Maternal education, Bachelor's degree or higher (%)	137 (58.5)	111 (58.1)	26 (60.5)	0.911
Maternal BMI (%)				0.308
Underweight	3 (1.2)	2 (1.0)	1 (2.0)	
Normal	98 (40.8)	77 (40.3)	21 (42.9)	
Overweight	58 (24.2)	52 (27.2)	6 (12.2)	
Obese	69 (28.7)	56 (29.3)	13 (26.5)	
Missing	12 (5.0)	4 (2.1)	8 (16.3)	
Maternal pregnancy behaviors				
Prenatal smoking (%)				>0.99
No	173 (72.1)	147 (77.0)	26 (53.1)	
Yes	8 (3.3)	7 (3.7)	1 (2.0)	
Missing	59 (24.6)	37 (19.4)	22 (44.9)	
Estimated folic acid from supplements, Mo. 1 pregnancy				0.015
Low (<400mcg)	107 (45.3)	87 (45.5)	20 (44.4)	
Adequate (400-1000mcg)	100 (42.4)	86 (45.0)	14 (31.1)	
High (>1000mcg)	29 (12.3)	18 (9.4)	11 (24.4)	
Missing (%)	4 (1.7)	0 (0.0)	4 (8.2)	
Prenatal vitamin use, Mo. 1 pregnancy (%)	143 (60.6)	114 (59.7)	29 (64.4)	0.676
Missing (%)	4 (1.7)	0 (0.0)	4 (8.2)	

[1]Continuous variables tested with a t-test for difference in means and categorical variables tested with a chi-square test of equal proportions. Missing category excluded for categorical variables before testing for differences.
[2]AOSI = Autism Observation Scale for Infants. The AOSI assessment consists of 18 items and is administered to infants age 6 to 18 months. An AOSI total score of 13 or greater indicates a child is likely to go on to develop ASD.
[3]ASD = autism spectrum disorder. Primary analyses will use diagnosis as defined by the *Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)*.

Table 2-4. Association of supplementation with Baby Siblings Research Consortium groups.

Total supplemental folic acid in month 1 pregnancy	**Outcome[1]**			**OR[2] (95% CI)**	
	ASD	**Non-TD**	**TD**	**ASD**	**Non-TD**
N	36	71	77		
Prenatal vitamin use					
Yes (%)				0.93 (0.39,2.23)	1.92 (0.95,3.9)
No (%)				1 (reference)	1 (reference)
Folic acid					
Adequate[3] (%)	16 (44%)	30 (42%)	35 (45%)	1 (reference)	1 (reference)
Low (<400mcg) (%)	16 (44%)	36 (51%)	35 (45%)	0.75 (0.3,1.86)	1.02 (0.5,2.06)
High (>1000mcg) (%)	4 (11%)	5 (7.0%)	7 (9.1%)	1.25 (0.29,5.29)	0.80 (0.23,2.83)

[1]ASD = autism spectrum disorder. Non-TD = non-typically developing. TD = typically developing. Outcomes are defined according got the Baby Siblings Research Consortium (BSRC) criteria; 7 of the 191 children in the main analytic sample are missing BSRC grouping and are thus excluded from this analysis.

[2]Adujusted for maternal education and child sex

[3]According to CDC guidelines for pregnancy women. Percentages tabulated per outcome (column-wise)

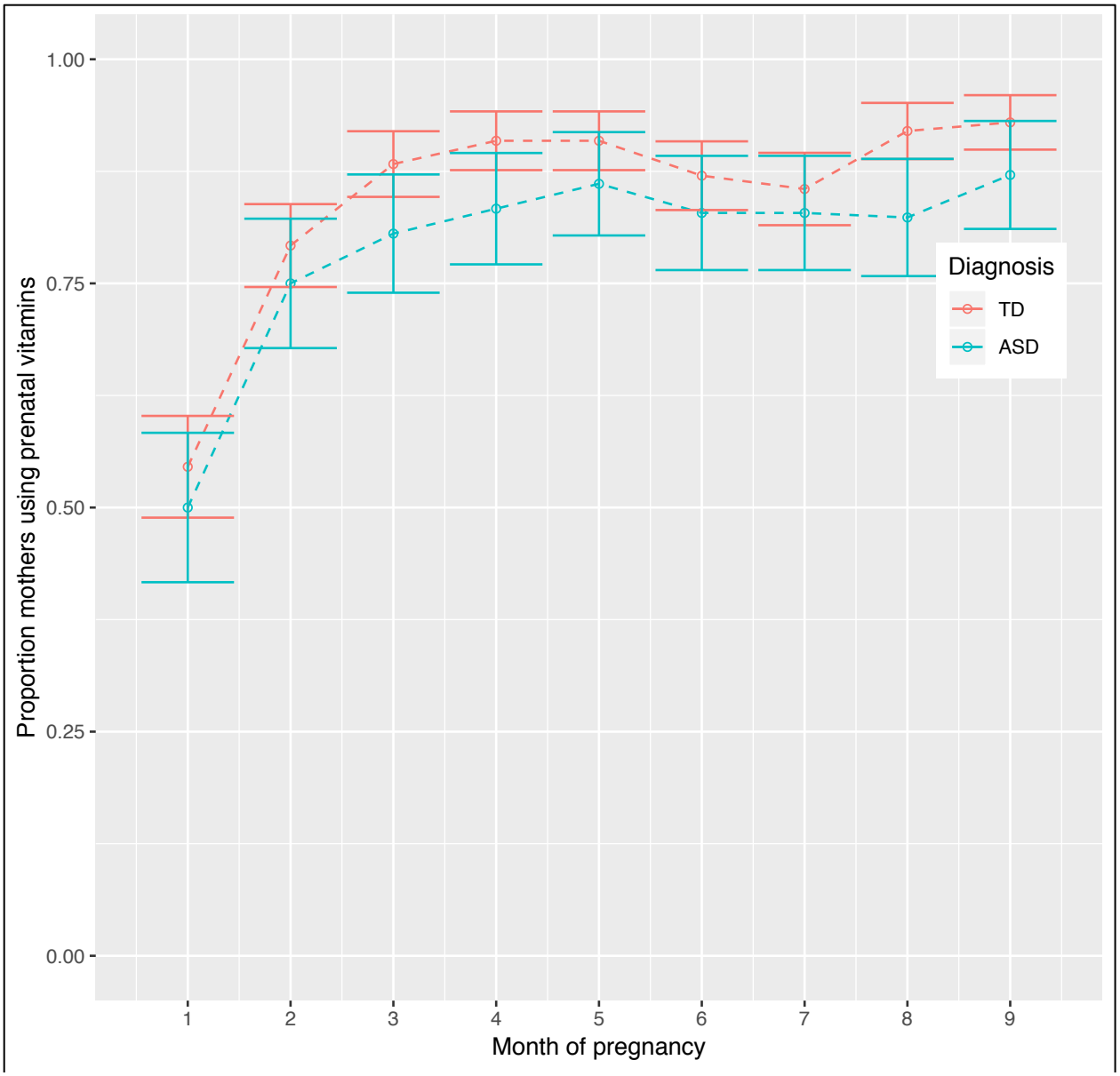

Figure 2-4. Prenatal vitamin use across pregnancy in 113 mothers in the pregnancy cohort.
Across pregnancy, a lower proportion of mothers of eventual ASD cases (n=36) were taking prenatal vitamins compared to mothers of those typically developing (TD, n=77). Errors bars represent the standard error of the mean for proportions; SE=sqrt(p(1-p)/n).*

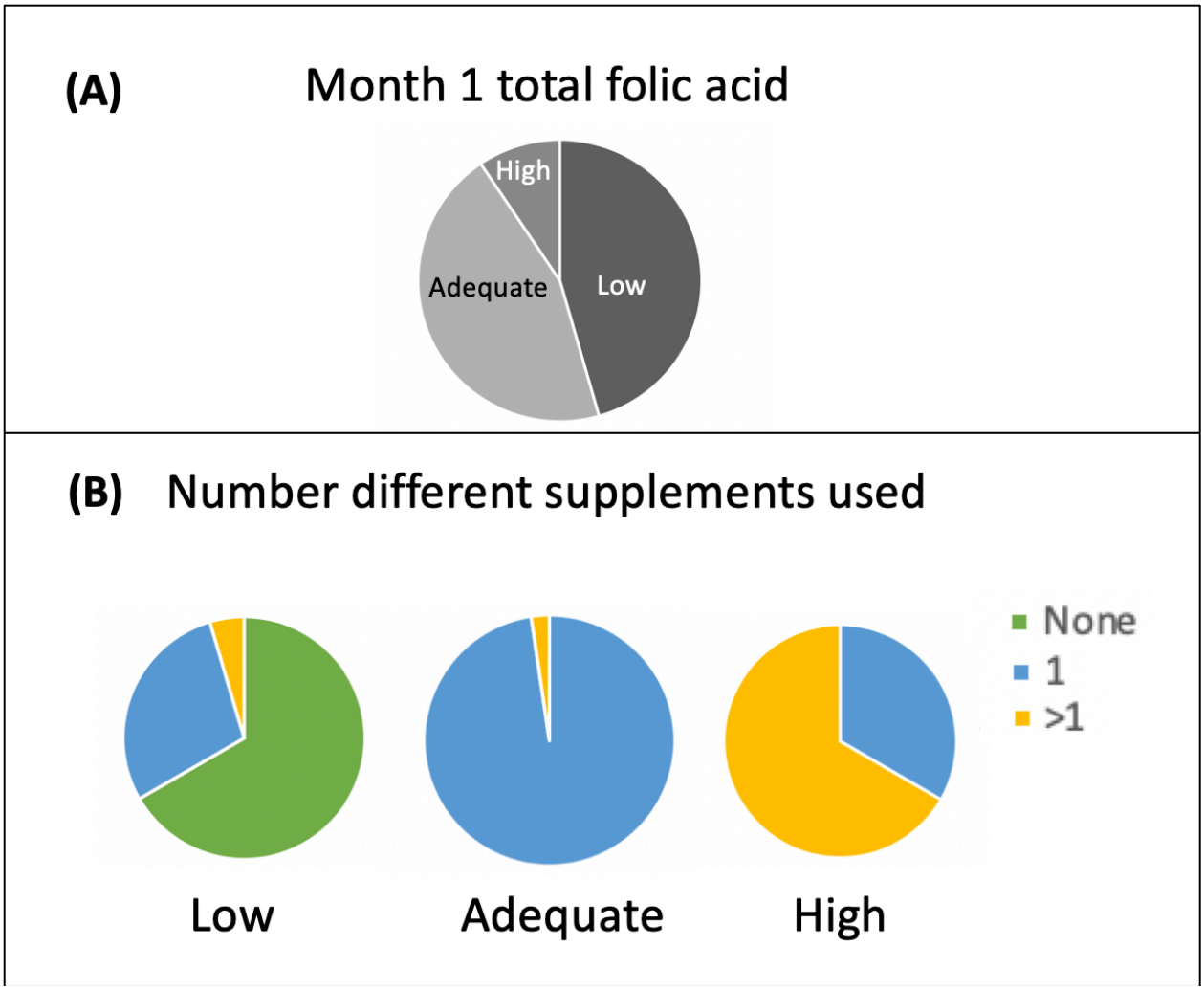

Figure 2-5. Prenatal vitamin use is not synonymous with high folic acid.
(A) Most mothers have adequate folic acid intake in month 1 of pregnancy. (B) The majority of mothers categorized as taking in high levels of folic acid were using multiple supplements, primarily a combination of prenatal vitamins and folic acid.

Table 2-5. Maternal dietary folate during pregnancy.

	Overall birth sample[1]
	N=240
<21 weeks pregnancy (N)	180
Missing	60
Total dietary folate[2], mcg (mean (sd))	435.56 (215.57)
Natural dietary folate[2], mcg (mean (sd))	247.10 (132.61)
Synthetic folate[2], mcg (mean (sd))	188.80 (119.57)
Dietary folate equivalents[3], mcg (mean (sd))	567.59 (289.29)
>21 weeks pregnancy (N)	108
Missing	132
Total dietary folate, mcg (mean (sd))	417.77 (219.92)
Natural dietary folate, mcg (mean (sd))	238.16 (133.51)
Synthetic folate, mcg (mean (sd))	179.97 (118.52)
Dietary folate equivalents, mcg (mean (sd))	543.60 (294.00)

[1] Overall birth sample includes all families with live births, 240 pregnancies of the initial 249 recruited into the study.
[2] Total dietary folate is the sum of natural (food) dietary folate, and synthetic (fortified) folate, which are each determined using information from the Nutrient Data System for Research on the foods reported.
[3] Dietary folate equivalents equal the micrograms of folate in food plus 1.7 times the micrograms of added (fortified) folic acid.

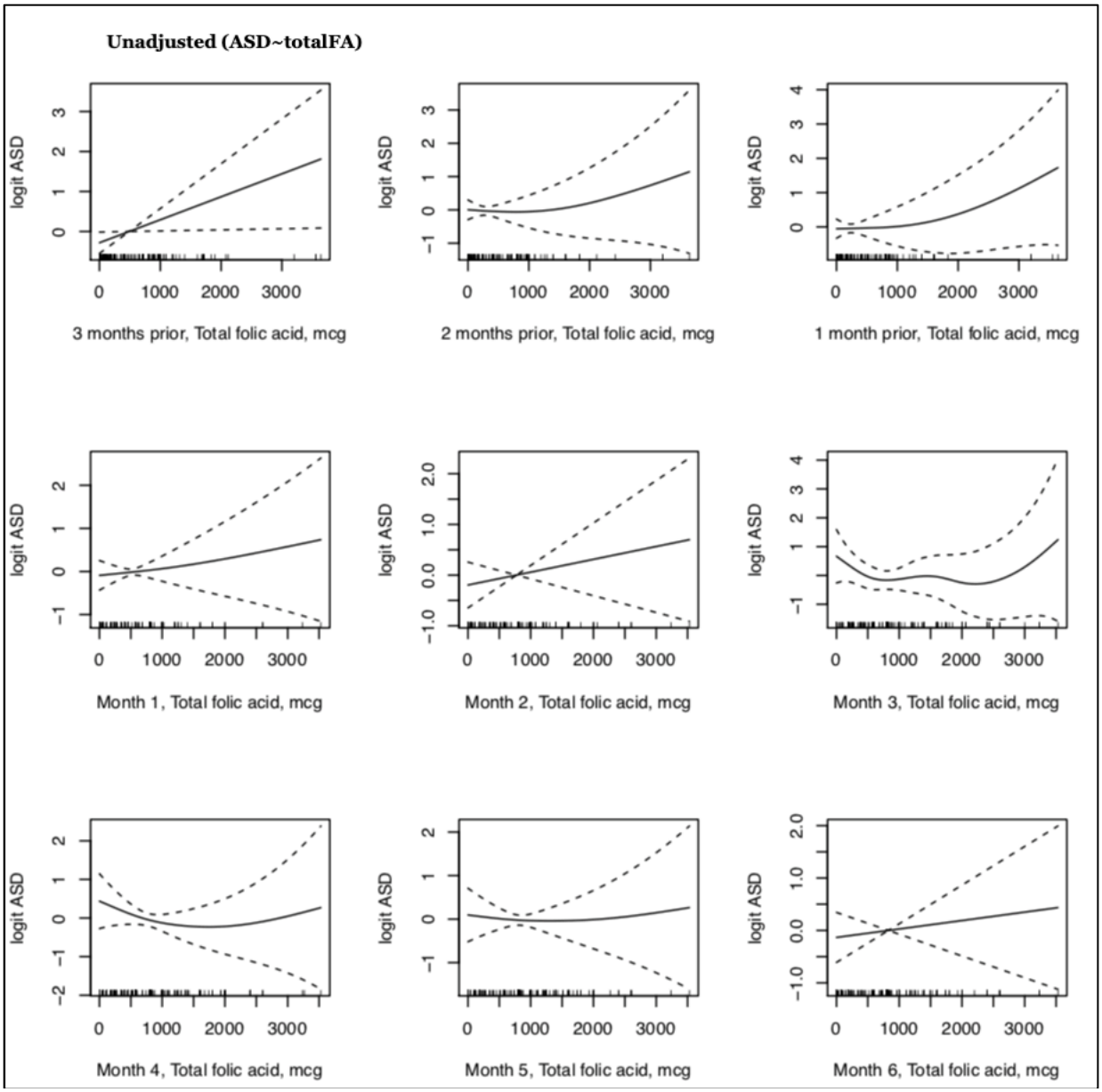

Figure 2-6. Spline modeling of relationship between total folic acid and autism risk.
We observe a possible relationship indicating increased risk ASD with high supplemental folic acid intakes.

Table 2-6. Bivariate characteristics by maternal genotype and child case status.

	Reference Genotype (AA) n = 79			**Variant (AC or CC)** n = 99		
	Control n=64	Case n=15	p	Control n=75	Case n=24	p
Child sex male (%)	32 (50.0)	11 (73)	0.179	32 (42.7)	20 (83.3)	0.001
Maternal education, ≥ Bachelors (%)	39 (60.9)	4 (28.6)	0.056	50 (66.7)	12 (52.2)	0.311
Folic acid month 1, mcg, mean (SD)	544 (560)	401 (400)	0.354	535 (531)	692 (885)	0.293

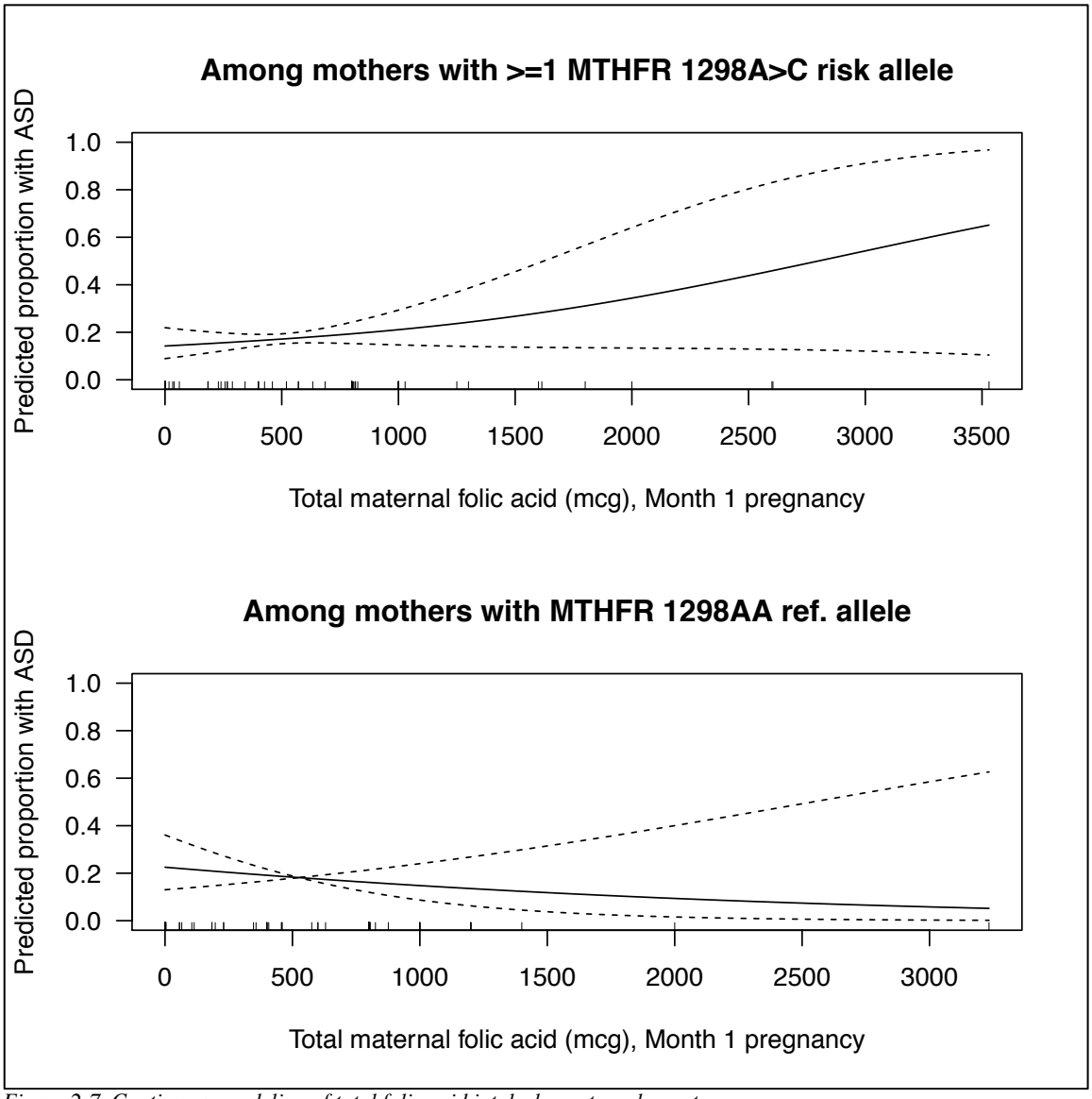

Figure 2-7. Continuous modeling of total folic acid intake by maternal genotype.
The increased risk ASD with high folic acid was more pronounced in children born to mothers with an MTHFR *A1298C risk allele present. Models above are adjusted for sex, maternal education, and maternal ancestry. Of genotyped mothers, 55% had at least one copy of the variant (CC or AC) and were compared to homozygous reference (AA, 45% of mothers).*

Chapter 3 Hormone therapy and ovarian cancer survival

The menopausal hormone therapy conundrum: a risk factor for ovarian carcinoma, yet a beneficial prognostic factor after diagnosis.

Abstract

Background: Current literature suggests menopausal hormone therapy (MHT) use pre- or post-diagnosis may be associated with improved ovarian cancer survival, but most prior studies have been limited by lack of hormone regimen detail and insufficient sample sizes. To address these limitations, a comprehensive analysis of 6,419 post-menopausal women with pathologically confirmed ovarian carcinoma was conducted to examine the association between MHT use prior to diagnosis and survival.

Methods: Data from 15 studies participating in the Ovarian Cancer Association Consortium were included. MHT use prior to diagnosis was examined by type (estrogen-only (ET) or estrogen+progestin (EPT)), duration, and recency of use relative to diagnosis. Cox proportional hazards models were used to estimate the association between hormone therapy use and ovarian cancer survival. Additionally, logistic regression and mediation analysis were used to explore the relationship between MHT use and residual disease following debulking surgery.

Findings: Use of ET or EPT for at least five years prior to diagnosis was associated with better ovarian cancer survival (hazard ratio, 0.80; 95% CI, 0.74 to 0.87). Among women with advanced stage, high-grade serous carcinoma, those who used MHT were less likely to have any macroscopic residual disease at the time of primary debulking surgery (p for trend <0.01 for

duration of MHT use). Residual disease mediated some (17%) of the relationship between MHT and ovarian cancer survival.

Interpretation: Pre-diagnosis MHT use for 5+ years was a favorable prognostic factor for women with ovarian cancer ($p<0.001$). While there is evidence to suggest that use of MHT after diagnosis is also beneficial for ovarian cancer survival, a large clinical trial would be needed to definitively establish the relationship. However, the findings presented here and those in the literature suggest that providers could consider MHT use for ovarian cancer survivors given the potential positive impact on quality of life and survival.

Introduction

Invasive epithelial ovarian cancers including ovarian, fallopian tube and primary peritoneal cancer (hereafter referred to as ovarian cancer) collectively account for more deaths than any other cancer of the female reproductive system in the United States, with a five-year survival rate of less than 50%[214]. There is clear evidence that menopausal estrogen-alone hormone therapy (ET) is associated with an increased risk of developing ovarian cancer[312-314]. The relationship between menopausal estrogen plus progestin therapy (EPT) and risk of ovarian cancer is less clear[313]. Additionally, the relationship between menopausal hormone therapy (MHT) use and survival may not be the same as the relationship with risk.

Pre-diagnosis MHT use and ovarian cancer survival has been examined in nine population-based studies[263-266,315-319]. Most observed a modestly inverse association, with hazard ratios ranging from 0.23[317] to 1.1[318] (Table 3-5), but protection was statistically significant in only one study (MHT use >5 years: HR, 0.79; 95% CI, 0.55 to 0.90)[265]. These studies were subject to one or more of the following important limitations: they (1) lacked information about duration of use; (2) did not distinguish between types of MHT use before diagnosis (i.e., ET and/or EPT); (3) had follow-up times of only a few years; (4) had an insufficient sample size to stratify by ovarian cancer histotype; and (5) lacked information about residual disease after debulking surgery. Many women use MHT for only a short period of time, thus missing duration information is an important weakness that may have masked effects in prior studies[320]. Rigorously evaluating the association between pre-diagnosis MHT use and ovarian cancer survival by hormone type, duration, survival time, residual disease and cancer histotype is essential to advance our understanding of disease prognosis.

In the present analysis from the Ovarian Cancer Association Consortium (OCAC), we followed 6,419 women with ovarian cancer for up to 26 years and investigated the association between pre-diagnosis MHT use and survival. We investigated duration, type and timing of MHT use in each of the main histological subtypes. A particularly important prognostic factor in ovarian cancer survival is residual disease after initial debulking surgery. Therefore, we also considered the potential relationship of MHT use with residual disease after surgery. Specifically, we evaluated whether some of the MHT effect on survival is indirect and flows through its influence on residual disease after primary debulking surgery.

Methods

Study populations and exclusion criteria

OCAC is an international, multidisciplinary collaboration of ovarian cancer research teams (http://ocac.ccge.medschl.cam.ac.uk/). This analysis used pooled ovarian cancer survival data from population-based (n=14) and clinic-based (n=1) OCAC studies (Table 3-6) conducted in the United States (n=9), Europe (n=4), and Australia (n=2). Institutional Review Board or comparable ethics approval was received by each study and informed consent was provided by all women. Post-menopausal women (as defined in each study) with pathologically confirmed ovarian carcinoma and survival time available (n=10,120) were considered for our analyses. Only those with invasive tumors, high-grade serous, low-grade serous, mucinous, endometrioid, or clear cell carcinomas, were eligible (i.e. mixed cell, undifferentiated, and non-epithelial cancers were excluded; n=1,260). We were interested *a priori* in the potential of a duration effect of MHT use and thus women were excluded if they were missing duration of MHT use (n= 2,212). Women missing data for stage at diagnosis (n=282), race/ethnicity (n=25), or time from

diagnosis to interview/study enrolment (n=13) were also excluded. There was no upper or lower age limit exclusion beyond the impact of excluding women who were pre-menopausal at diagnosis. Our final analytic sample was 6,419 ovarian cancer patients (Figure 3-1). Survival times and proportion of deaths were comparable between women excluded and those included.

Exposure and covariate assessment

Participants provided information on their history of MHT use prior to diagnosis via phone or in-person interviews (n=10 study sites) or self-completed questionnaires (n=5 study sites) (Table 3-6). MHT use was categorized as exclusive use of ET, exclusive use of EPT, use of both therapies, or use of unknown type. First, exclusive ET use was examined based on (1) total duration of ET use (never (reference category), >0 to <5, 5 to <10, 10+ years) and (2) recency of ET use (within the year prior to diagnosis, 1 to <5, 5+ years prior to diagnosis). There was no additional duration effect observed after 5 years and so the categories 5 to <10 and 10+ years were combined into one. Exclusive EPT use was examined in the same manner. The reference group for both the ET and EPT analyses was never use of any type of MHT. Next, total duration of any type of MHT use prior to diagnosis was examined (ET, EPT, both, or unknown type) with the same approach. BMI (kg/m^2) categories were assigned according to World Health Organization[321] definitions (underweight, BMI<18.5; normal weight, 18.5≤BMI<25.0; overweight, 25≤BMI<30; obese, BMI≥30), using the values reported for adult BMI one to five years prior to diagnosis. Duration of combined oral contraceptive use was coded as never, <1, 1 to <5, 5 to <10, or 10+ years. Parity was coded as 0, 1, or 2+ pregnancies. Education level was coded as less than high school, high school graduate, some college, college graduate, or graduate school. Stage was recorded as local (with no lymph node involvement), regional (direct

extension and/or local lymph node involvement), and advanced (distant sites and/or distant lymph nodes involved)[322].

Outcome assessment

Overall survival was recorded as either length of time (in days) from diagnosis to death or to date of last follow-up (for censored patients). All survival models incorporated left truncation time, accounting for the difference between date of diagnosis and date of patient interview, though there was little variability in delay to patient interview and so accounting for left truncation did not affect results. For a subset of women there was information on duration of progression-free survival (n=2,239) and presence/absence and size of residual disease after debulking surgery (n=2,056) (Table 3-6).

Statistical analysis

Overall survival models

Cox proportional hazards models with left truncation and right censoring were used to estimate the association (hazard ratio; HR, and associated Wald-type confidence intervals) of each hormone therapy exposure on ovarian cancer survival. The exposures were modeled as categories of duration of use and recency of use, as detailed above. Exclusive use of ET or exclusive use of EPT were first examined separately to determine their association with survival. Because the hazards for the two types of hormone therapies were not statistically significantly different and showed a similar magnitude of association, we combined types as an "any HT use" variable including unknown types of MHT, as these would have been either ET or EPT.

Important *a priori* variables included in all models were age at diagnosis (continuous), race/ethnicity, surgical stage at diagnosis, and OCAC study site. Sensitivity analyses adjusting for age in five- and ten-year categories did not materially alter the HR estimates for MHT. Education level adjustment in sensitivity analyses also did not influence HR estimates. The possible confounding effects of additional exposures prior to diagnosis were examined, including adult BMI, tubal ligation (yes/no), self-reported history of physician-diagnosed endometriosis (yes/no), hysterectomy (yes/no), combined oral contraceptive use, parity, prior primary cancer, family history of breast cancer (yes/no), and family history of ovarian cancer (yes/no) were considered, but none affected the association between MHT duration and survival (Table 3-9). Several of these variables were considered not because they would act as classical confounders but rather because they could have acted as proxy variables for an upstream effector (e.g. frequency of contact with the health system) influencing both MHT and survival.

The final models included covariate adjustment for age at diagnosis and race/ethnicity, and stratification by histotype, stage at diagnosis, and OCAC site. Stratification within the model for stage, OCAC site, and histotype allows for the baseline hazard function to vary between strata, while still using all women's information to estimate hazards for MHT; and does not assume that the risks from these three variables are multiplicative, but that within each stage or OCAC site or histotype, the relative hazards are the same for MHT and all the other variables that are adjusted for. Proportional hazards assumptions, tested using Schoenfeld residuals and the corresponding p-values, were found to not be violated in any models.

Following the same overall model structure described above, separate models for each histotype (high-grade serous, mucinous, low-grade serous, endometrioid, and clear cell) were fitted to estimate HRs for MHT duration. The adjusted survival curves presented (overall, and

high-grade serous) allow for visualization of survival curves based on the Cox proportional hazard results. Simulated survival curves allow for adjustment of covariates (age at diagnosis, race/ethnicity, histotype, stage at diagnosis, and OCAC site) by generating average survival curves based on subpopulations with different covariate values.

Discrete windows of clinical interest and progression-free survival

We tested discrete windows of clinical interest following diagnosis. Although the proportional hazards assumptions were not violated for MHT use prior to diagnosis in the Cox proportional hazards models described above, an additional model was fit allowing the data to be split into time intervals after diagnosis (0 to 2 years, >2 to 5 years, >5 to 8 years, >8 to 10 years, and greater than 10 years). This allowed us to assess subtle variation in HR estimates at all time points after diagnosis. Additionally, to assess the specificity of the protective effect of MHT, Cox proportional hazards model was fit for time to progression, treating progression of disease as the event of interest. Although ovarian cancer-specific mortality was not assessed, nearly all deaths within the first five years following diagnosis are likely to be related to ovarian cancer thus our time interval analysis provides insight into this question.

Residual disease in women with advanced stage, high-grade serous cancer

Among women with advanced stage (stage III or IV), high-grade serous carcinoma (HGSC, n=903), we examined possible mechanisms underlying the MHT-survival association, namely the association of MHT use with residual disease. We used logistic regression, investigating MHT use in those with and without macroscopic residual disease following primary debulking surgery. Mediation analysis was used to examine whether the relationship

between MHT use and survival was mediated by residual disease. In this analysis, the first step (mediator) model was residual disease regressed on MHT use and the covariates age, stage, histotype, education level, and race. The second step (outcome) model was modeled as survival regression on residual disease, MHT use, and the same set of covariates. Finally, mediation was assessed using 2,000 simulations to estimate the average causal mediated effect, the average causal direct effect, the total effect, and the proportion mediated, using the generalizable approach to causal mediation outlined by Imai et al. [323]. All statistical analysis was performed in *R*[324].

Results

The study sample to examine the associations between MHT use and ovarian cancer survival included 6,419 post-menopausal women from 15 sites in the OCAC (Figure 3-1**;** Table 3-6). A majority of the women had HGSC (68.4%) and most had advanced stage disease at the time of diagnosis (67.7%;Table 3-1). Exclusive EPT use (18.5%) was more common than exclusive ET use (14.2%). Most women (58.9%) did not use either type, and 212 (3.3%) used both ET and EPT (Table 3-1).

The median survival time among all included women was 5.4 years after diagnosis. ET and EPT use for at least five years were both associated with longer survival (Table 3-2). For exclusive ET users, lower mortality was observed for use of 5+ years (HR, 0.85; 95% CI, 0.75 to 0.96). For exclusive EPT users, the HR for use for 5+ years was similar (HR, 0.79; 95% CI, 0.70 to 0.89). Because the magnitudes of effect for ET and EPT were similar, all MHT types were combined for subsequent analyses. Significantly better survival was observed for those who had used any type of MHT for at least 5 years (HR, 0.80; 95% CI, 0.74 to 0.87) (Table 3-2). There

was a median survival of 5.75 years among women who had used MHT for 5+ years and 4.6 years for those who has not used any. The estimated hazards correspond to an estimated 4.7% fewer deaths overall (cumulative survival attributable risk) during the follow-up period if everyone had used MHT for at least five years.

An adjusted survival curve illustrates the apparent protective benefit of MHT use was restricted to women with 5+ years use compared to those who did not use MHT and that no benefit was observed for <5 years of use (Figure 3-2). Recency of MHT use did not affect the hazard ratio estimates. The association observed for all histotypes combined was also similar for individual histotypes, with the exception of endometrioid carcinomas, but was only statistically significant for HGSC (HR, 0.78; 95% CI, 0.71 to 0.86) (Table 3-3). Progression-free survival (time from diagnosis to first recurrence, documented by clinical, biochemical (e.g. serum CA125 levels) or radiological disease progression) was also better in those who had used MHT (Table 3-8).

To further explore the relationship between MHT use prior to diagnosis and survival, time-varying HRs were estimated. Although the proportional hazards assumptions were not violated for the survival model of MHT use, the additional analyses allowed for finer estimation of the protective association during particular windows of interest after diagnosis. Allowing the HR for MHT use to vary over time since diagnosis, the estimated effect was protective in all time intervals. MHT use was associated with reduced risk of death significantly in the first two years after diagnosis (HR, 0.72; 95% CI, 0.62 to 0.84) and in years 2 through 5 after diagnosis (HR, 0.86; 95% CI, 0.76 to 0.97) (Figure 3-3).

Stratification by stage at diagnosis for HGSC showed a positive association with prognosis at advanced stages (III/IV) (Table 3-7). Among women with advanced stage HGSC,

MHT use was associated with improved survival both in the women with and those without residual disease (Figure 3-4**;** Table 3-10). MHT use prior to diagnosis was associated with lower likelihood of residual disease at the time of debulking surgery among women with advanced stage HGSC. Of women with local (stage I, n=180) and regional (stage II, n=343) disease, only 2 women (2%) and 18 women (5.2%) respectively had residual disease after surgery, thus we cannot estimate ORs for MHT use in these strata. Among those with advanced disease (stage III/IV), MHT use was associated with significantly lower odds of having macroscopic residual disease relative to no macroscopic disease (p for trend = 0.009 for duration of MHT use), adjusted for age at diagnosis (

Table 3-4). Adjusting for OCAC site and race/ethnicity did not alter the trend. Residual disease partially mediated the relationship between long-term MHT use and survival. Among women with advanced HGSC, the proportion mediated was 0.17 (p=0.04).

Discussion

In this study, pre-diagnosis MHT use for at least five years was associated with better ovarian cancer survival, regardless of MHT type (ET or EPT) and recency of use relative to diagnosis. Other studies reported ever use of MHT to be associated with improved survival (Table 3-5), but this is the first study to report on the association of duration and recency of MHT use, type of MHT use, histotype, and residual disease after debulking surgery on survival outcomes.

Women with advanced HGSC who had used MHT prior to diagnosis were less likely to have macroscopic disease following primary debulking surgery. We estimated that about 17% of the survival improvement associated with MHT use could be due to the higher proportion of

MHT users with no residual disease. The mechanism of the association of MHT with residual disease is unclear. At least one previous study has noted that MHT use was associated with optimal debulking status[325]. One possibility is that MHT use prior to diagnosis alters the pattern of metastatic spread, such that the disease is easier to access or less adhesive to surrounding tissues and thus easier to resect. It has been reported that tumor tissue from sub-optimally debulked patients expressed molecular signatures consistent with increased stromal activation and lymphovascular invasion[326]. It is also possible that MHT use results in an anti-inflammatory milieu that is beneficial for resection. Particularly at high concentrations, estrogen has anti-inflammatory properties[327-329] in some tissues. A predictive gene expression signature, developed for likelihood of optimal debulking, suggested that there may be a subset of tumors for which the TGF-ß activated pathway stimulates epithelial to mesenchymal transition and activation of tumor associated fibroblasts[330], both of which would contribute to spread of tumor and difficulty in debulking. Prior studies have established a complex relationship between hormonal exposures, including hormone therapy[331-335], and inflammation that depends on multiple factors including the formula, dose, route of delivery, and other immune stimuli present. Furthermore, evidence also supports a mutually dependent relationship between inflammation and angiogenesis[336,337]. Immune cells stimulated during inflammatory reactions secrete cytokines such as IL-6, TNF-α and CXCR2 that promote neovascularization and thus potentially contribute to tumor establishment and growth. On the other hand, an anti-inflammatory environment would prevent this sequence. Mechanistic studies are needed to understand the relationship between MHT use, ease of debulking and survival. Mechanistic studies are also needed to investigate whether it is primarily women with estrogen-receptor negative cancers who are driving the protective association with MHT use; indeed, the current literature suggests avoiding MHT in women with

estrogen-sensitive histologic subtypes[338]. This may explain why the endometrioid subtype findings deviated from the other histotypes.

Pre-diagnosis use, as previously discussed and as demonstrated by this current study, appears to offer a survival benefit to women with ovarian cancer[263-266,317,319,325] (Table 3-5). The existing literature on post-diagnosis MHT use and ovarian cancer survival includes several population-based cohort studies[261,263,315,339,340] and two small randomized controlled trials[262,341]. The population-based studies were largely inconclusive, with small sample sizes and wide confidence intervals, but they all suggest reduced mortality in post-diagnosis MHT users[261,263,315,339,340,342,343].

Two randomized trials have indicated possible survival benefits of hormone therapy use[262,341] after surgical debulking of the ovarian tumor. A clinical trial in 1999[341] randomized women with ovarian cancer of any histotype to either conjugated estrogen or to no supplementation after debulking surgery. The women who received estrogen therapy had longer disease-free intervals as well as better overall survival. In a second study, Eeles et al.[262] randomized women who had been diagnosed with ovarian cancer within the previous 9 months to receive hormone therapy of provider's choice or none. The study observed a statistically significant beneficial effect of hormone therapy on overall survival (HR, 0.63; 95% CI, 0.44 to 0.90), but this likely reflects some of the general benefits of MHT on survival as this is not an ovarian cancer-specific survival estimate. However, no specific hormonal regimen was used, as individual clinicians with patients randomized to the treatment arm still had control over type, dose and duration.

Limitations of our results include the self-reported exposure measures, including MHT use and other covariates of interest. However, prior studies have documented good correlation

between self-report of hormone use and prescription records[344]. Although our analysis was restricted to women who were classified as post-menopausal at diagnosis, some may have used MHT before menopause occurred. To address this issue, we conducted a sensitivity analysis restricting the exposure to MHT use after the age of 50, as a proxy for post-menopausal use, and the results did not change. An additional limitation was the lack of information on MHT use post-diagnosis. We cannot exclude the possibility that pre-diagnosis use predicts post-diagnosis use, which is conferring part of the survival benefit. Finally, use of MHT could serve as a proxy for overall adherence to medical recommendations and treatment and access to specialist surgical practices. However, controlling for education, which would also be expected to correlate with these characteristics, did not affect the results.

We observed that the association of MHT use for five or more years prior to diagnosis was protective at all time points after diagnosis (significantly so in the first five years after diagnosis). Since the cause of death during this interval is most commonly ovarian cancer-specific and not from other causes, this suggests that the protection conferred by MHT use is at least in part due to cancer-specific protection. We also offer evidence that the relationship is partially mediated by the relationship between MHT use and optimal surgical cytoreduction.

The findings presented here and taken in context with the other literature on the topic (Table 3-5) suggest that MHT is beneficial with respect to ovarian cancer survival, particularly among women with HGSC. These findings are helpful to understand the biology of the disease, and ultimately our goal is to help women diagnosed with ovarian cancer to live both longer and with a higher quality of life. Post-menopausal symptoms, including severe vasomotor symptoms for some women, can negatively impact quality of life. Therefore, clinician and patient confidence in using MHT offers great potential benefit to women who have been diagnosed with

ovarian cancer. A large randomized clinical trial would help determine the impact of MHT on survival and quality of life for women living with ovarian cancer. Such a future trial could incorporate detailed mechanistic studies to better understand how MHT influences survival. Despite remaining unanswered questions, the current evidence should allow providers to at least discuss MHT use with ovarian cancer patients, with shared decision making regarding the potential benefits and limitations of therapy.

Table 3-1. Demographic and clinical characteristics of women in the survival analysis.

		Pre-diagnosis MHT use duration		
	Overall[1]	Never	<5 years	5+ years
N	6419	3784	1183	1452
Hormone therapy use (%)				
None	3784 (58.9)	3784 (100.0)	0 (0.0)	0 (0.0)
ET only	909 (14.2)	0 (0.0)	379 (32.0)	530 (36.5)
EPT only	1188 (18.5)	0 (0.0)	561 (47.4)	627 (43.2)
ET and EPT	212 (3.3)	0 (0.0)	62 (5.2)	150 (10.3)
Unknown +/- ET/EPT	326 (5.1)	0 (0.0)	181 (15.3)	145 (10.0)
Age at dx. (mean (SD))	62.67 (8.71)	62.36 (9.33)	60.78 (8.16)	65.00 (6.75)
Education (%)				
Less than high school	1135 (20.7)	760 (23.7)	177 (17.4)	198 (15.9)
High school graduate	1567 (28.6)	948 (29.6)	272 (26.7)	347 (27.8)
Some college	1325 (24.2)	745 (23.2)	265 (26.0)	315 (25.3)
College graduate	799 (14.6)	400 (12.5)	174 (17.1)	225 (18.1)
Graduate school	646 (11.8)	353 (11.0)	132 (12.9)	161 (12.9)
Race / ethnicity (%)				
Non-Hispanic white	5679 (88.5)	3308 (87.4)	1042 (88.1)	1329 (91.5)
Hispanic white	198 (3.1)	126 (3.3)	45 (3.8)	27 (1.9)
Black	101 (1.6)	72 (1.9)	15 (1.3)	14 (1.0)
Asian	249 (3.9)	146 (3.9)	51 (4.3)	52 (3.6)
Other	192 (3.0)	132 (3.5)	30 (2.5)	30 (2.1)
Histotype (%)				
Low-grade serous	245 (3.8)	134 (3.5)	47 (4.0)	64 (4.4)
High-grade serous	4393 (68.4)	2504 (66.2)	820 (69.3)	1069 (73.6)
Mucinous	373 (5.8)	255 (6.7)	65 (5.5)	53 (3.7)
Endometrioid	925 (14.4)	552 (14.6)	168 (14.2)	205 (14.1)
Clear cell	483 (7.5)	339 (9.0)	83 (7.0)	61 (4.2)
Stage (%)				
Local (FIGO I)	947 (14.8)	616 (16.3)	173 (14.6)	158 (10.9)
Regional (FIGO II)	1126 (17.5)	684 (18.1)	211 (17.8)	231 (15.9)
Advanced (FIGO III/IV)	4346 (67.7)	2484 (65.6)	799 (67.5)	1063 (73.2)
BMI category (%)				
Underweight	117 (2.0)	71 (2.1)	18 (1.6)	28 (2.0)
Normal weight	2684 (45.7)	1424 (42.0)	515 (45.9)	745 (54.5)
Overweight	1754 (29.9)	1026 (30.3)	339 (30.2)	389 (28.5)
Obese	1320 (22.5)	866 (25.6)	249 (22.2)	205 (15.0)
Family[2] cancer history (%)				
Breast cancer	1098 (17.6)	690 (18.9)	195 (16.8)	213 (15.0)
Ovarian cancer	329 (5.3)	203 (5.6)	61 (5.3)	65 (4.6)
Combined oral contraceptive use (%)				
Never	3127 (49.2)	2030 (54.2)	451 (38.6)	646 (44.9)

<1 year	590 (9.3)	313 (8.4)	142 (12.1)	135 (9.4)
1 to <5 years	1209 (19.0)	659 (17.6)	265 (22.7)	285 (19.8)
5 to <10 years	773 (12.2)	390 (10.4)	176 (15.1)	207 (14.4)
10+ years	656 (10.3)	356 (9.5)	135 (11.5)	165 (11.5)
Parity (%)				
0 births	1223 (19.1)	738 (19.6)	232 (19.6)	253 (17.4)
1 birth	858 (13.4)	525 (13.9)	157 (13.3)	176 (12.1)
2+ births	4324 (67.5)	2508 (66.5)	794 (67.1)	1022 (70.4)
Smoking (%)				
Never	2910 (52.9)	1803 (55.5)	495 (48.8)	612 (49.4)
Current	700 (12.7)	445 (13.7)	126 (12.4)	129 (10.4)
Former	1891 (34.4)	998 (30.7)	394 (38.8)	499 (40.2)

[1] The total N for certain variables reported does not total to 6,419 because of missing data. These included variables that were not confounders and thus not needed for covariate adjustment in final models, such as family history of cancer, education, and smoking.
[2] First-degree family members, i.e. sister or mother.

Table 3-2. Hazards ratios for menopausal hormone therapy use before diagnosis.

	Estrogen alone (ET)		Estrogen-progestin combined therapy (EPT)		Any menopausal hormone therapy	
MHT use	N [a]	HR (95% CI) [b]	N	HR (95% CI)	N	HR (95% CI)
None (ref)	3,784	1.0	3,784	1.0	3,784	1.0
<5 years	379	0.99 (0.86, 1.15)	561	1.01 (0.89, 1.14)	1,183	0.97 (0.88, 1.06)
5+ years	530	0.85 (0.75, 0.96)	627	0.79 (0.70, 0.89)	1,452	0.80 (0.74, 0.87)

[a] The three analyses have different total N's because the exclusive ET analysis excluded women who had ever used EPT, and the exclusive EPT analysis excluded women who had ever used ET. Users of unknown type were also excluded from this analysis.
[b] Hazard ratios (HRs) are adjusted for age at diagnosis and race/ethnicity, and stratified by histotype, stage at diagnosis, and OCAC site.

Table 3-3. Hazards ratios, by histotype, for menopausal hormone therapy use before diagnosis.

	Overall	High-grade serous	Mucinous	Endometrioid	Clear cell	Low-grade serous
N	6,419	4,393	373	925	483	245
MHT use	HR (95% CI)[a]					
None (ref)	1.0	1.0	1.0	1.0	1.0	1.0
<5 years	0.97 (0.88, 1.06)	0.94 (0.85, 1.04)	1.77 (1.04, 3.02)	0.99 (0.71, 1.37)	0.97 (0.62, 1.51)	0.98 (0.58, 1.66)
5+ years	0.80 (0.74, 0.87)	0.78 (0.71, 0.86)	0.66 (0.34, 1.26)	1.08 (0.81, 1.43)	0.83 (0.47, 1.48)	0.76 (0.47, 1.23)

[a] Hazard ratios (HRs) are adjusted for age at diagnosis and race/ethnicity, and stratified by histotype (overall analysis only), stage at diagnosis, and OCAC site.

Table 3-4. Odds ratios for residual disease based on use of menopausal hormone therapy use.

		Residual disease	OR[a] (95% CI)	p for trend
MHT use	N			
None (ref)	859	574 (66%)	1.0	
<5 years	239	146 (61%)	0.79 (0.58, 1.06)	
5+ years	290	171 (59%)	0.71 (0.54, 0.93)	0.009

[a]ORs are adjusted for age at diagnosis. Adjusting for OCAC site and race/ethnicity did not alter the trend for inverse association. Results are among women with advanced, high-grade serous carcinoma in the Ovarian Cancer Association Consortium (OCAC).

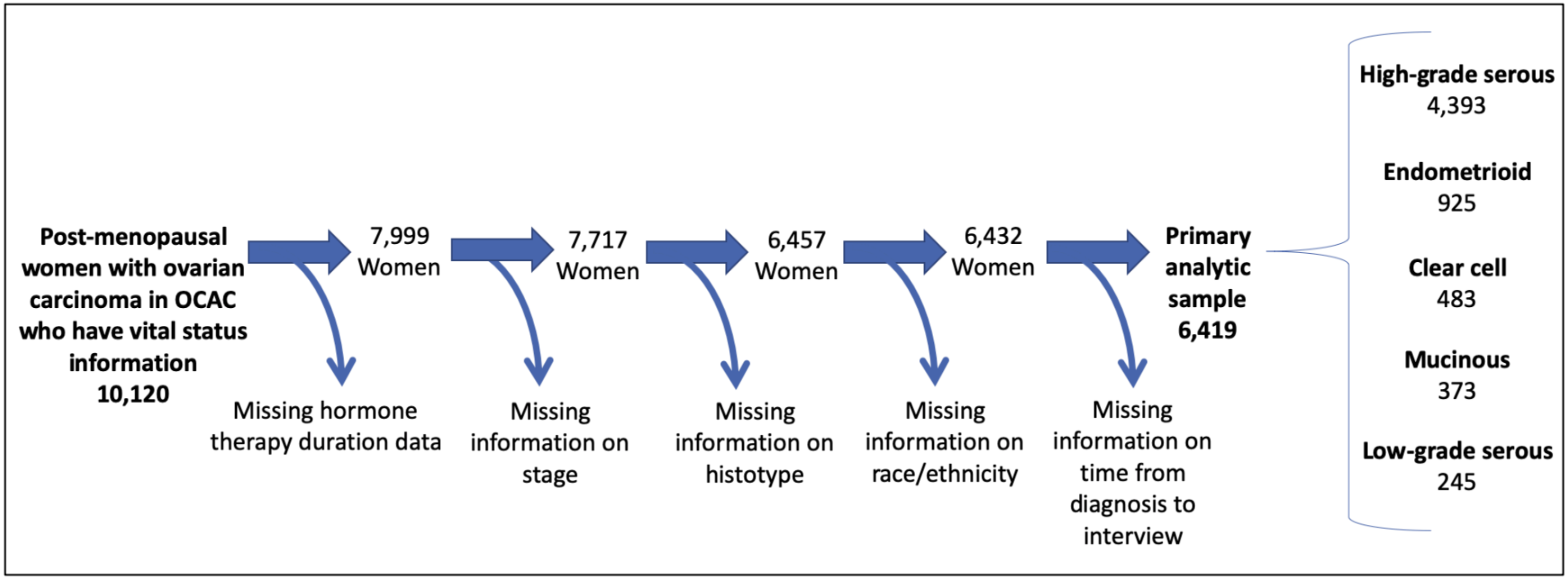

Figure 3-1. Study participants and exclusion criteria.
All women were from the Ovarian Cancer Association Consortium (OCAC). The population-based and clinic-based studies included were done in the United States (n=9), Europe (n=4), and Australia (n=2). Only post-menopausal women with ovarian carcinoma for whom survival time data was available were considered for this analysis. The exclusion stage "missing histotype" included exclude of those with mixed cell and undifferentiated tumors. Of the five histotypes high-grade serous was the most common (68% of cases) among these women, followed by endometrioid (14%), clear cell (7.5%), mucinous (5.8%), and low-grade serous (3.8%). Of the 6,419 women in our analytic sample, subsets with complete information were analyzed for progression-free survival (n=2,239) and for the association between hormone therapy and residual disease (n=2,056). Time of interview refers to time of study enrollment; for some studies this was an in-person interview and for some it was a self-administered questionnaire.

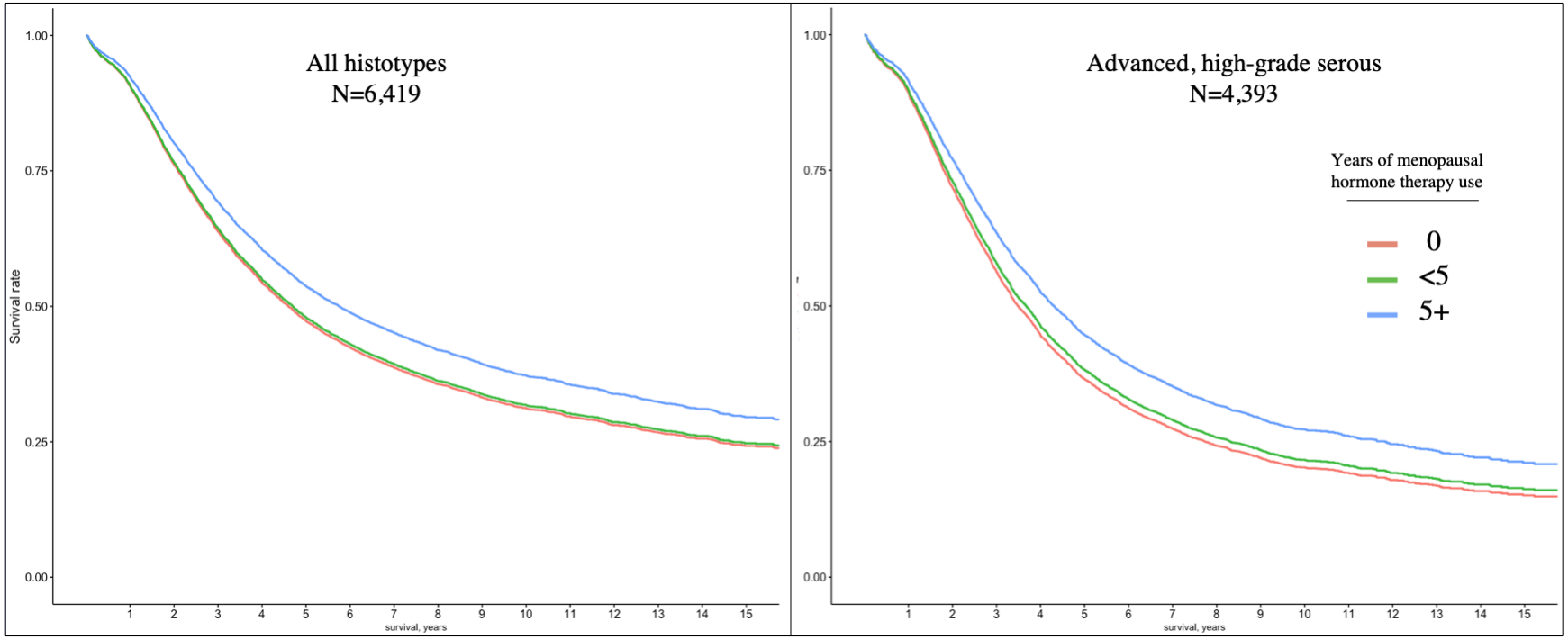

Figure 3-2. Overall adjusted survival stratified by years of menopausal hormone therapy use.
Adjusted survival curves among all women with ovarian cancer (n=6,419) and among women with advanced stage, high-grade serous cancer (n=4,393). The adjusted survival curves are generated from the hazard ratios estimated from a cox proportional hazards model of menopausal hormone therapy use and are adjusted for age at diagnosis, race/ethnicity, histotype (left panel only), stage at diagnosis, and OCAC site.

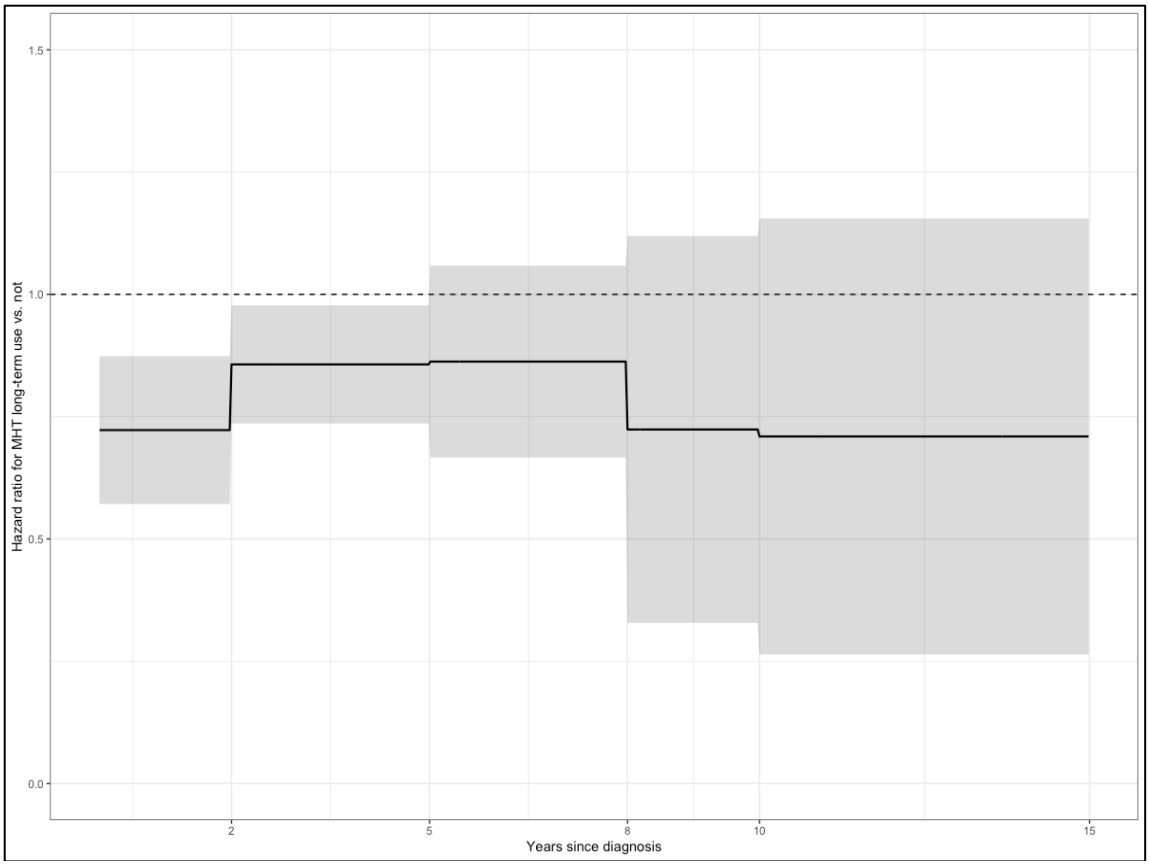

Figure 3-3. Estimated time-varying hazard ratio for menopausal hormone therapy use.
HR and 95% confidence intervals for use of menopausal hormone therapy (5+ years) relative to no use. In a Cox proportional hazard model allowing for interaction of the effect of menopausal hormone therapy use with time since diagnosis, the estimated effect is protective at all time points. Menopausal hormone therapy use is significantly protective in the first two years after diagnosis (HR = 0.72; 95% CI = 0.62, 0.84) and in years 2 through 5 after diagnosis (HR = 0.86; 95% CI = 0.76, 0.97).

Table 3-5. Published findings of menopausal hormone therapy and ovarian cancer survival.

Author, Year	HR (95% CI) overall survival for any HT use	Type	Study design	N, population	Notes
Pre-diagnosis					
Mascarenhas, 2006[263]	0.83 (0.65, 1.08)	Any	Cohort, follow-up on population-based cases	649, Sweden	Examination of different HRT preparations did not give different HRs
Wernli, 2008[318]	1.1 (0.85, 1.43)	Any	Case-control, population based	751, United States	
Nagle, 2008[264]	0.83 (0.64, 1.08)	Any	Cohort, population-based	676, Australia	
Zhang, 2012[317]	0.23 (0.03, 1.73)	Any	Cohort	195, China	
Hein, 2013[325]	75% (65, 86) 5-yr survival for HRT users; 43% (36, 52) for non-users	Any	Cohort	244, Bavaria	Women who had used HRT: younger, lower stage, more optimal debulking
Shafrir, 2016[265]	0.70 (0.55, 0.9)	Any	Cohort	1649, United States	
Kim, 2017[266]	0.79 (0.62, 1.01)	Any	Cohort, registry-based	1421, Canada	
Besevic, 2015[319]	0.80 (0.55, 1.16)	EPT	Cohort	1025, Europe	
	0.86 (0.54, 1.35)	ET			
Felix, 2015[316]	0.97 (0.68, 1.38)	EPT	Cohort, population-based	396, United States	Noted interaction between HRT type and histology
	1.09 (0.7, 1.68)	ET			
Post-diagnosis					
Eeles, 1991[261]	0.73 (0.44, 1.2)	Any	Cohort, retrospective	373, United Kingdom	No difference in disease-free interval
Ursic-Vrscaj, 2001[343]	0.67 (0.27, 1.62) (relapse)	Any	Case-control	72, Slovenia	
	0.90 (0.24, 5.08) (survival)	Any			

Mascarenhas, 2006[263]	0.57 (0.42, 0.78)	Any	Cohort, follow-up on population-based cases	649, Sweden	Examination of different HRT preparations did not give different HRs
Wen, 2013[339]	0.82 (0.48, 1.4) (relapse)	Any	Cohort, retrospective	144, China	
	0.67 (0.18, 2.5) (survival)	Any			
Eeles, 2015[262]	0.63 (0.44, 0.90)	Any	RCT	150, United Kingdom, Spain, and Hungary	Greater relapse-free survival, HR=0.67 (0.47, 0.97)
Power, 2016[315]	0.50 (0.23, 1.09) (<55yrs old)	Any	Cohort, retrospective	357, Manitoba, Canada	
	0.85 (0.43, 1.68) (≥55yrs old)	Any			
Li, 2012[340]	0.88 (0.35, 2.32)	EPT	Cohort, prospective	75, China	
Guidozzi, 1999[341]	0.97 (0.65, 1.18) (relapse)	ET	RCT	125, South Africa	Better overall survival (not significant)

Table 3-6. Ovarian Cancer Association Consortium study sites included in the analysis.

Site code	N (%)	Full study name	Country	Study design	Data collection	Diagnosis years	Residual Disease information
AUS	558 (8.7)	Australian Ovarian Cancer Study	Australia	Population-based	Self-completed questionnaire	2002-2006	Yes
CON	241 (3.8)	Connecticut Ovary Study	United States	Population-based	In-person interview	2002-2009	No
DOV	569 (8.9)	Diseases of the Ovary and their Evaluation	United States	Population-based	In-person interview	2002-2009	No
GER	97 (1.5)	Germany Ovarian Cancer Study	Germany	Population-based	Self-completed questionnaire	1992-1998	No
HAW	265 (4.1)	Hawaii Ovarian Cancer Study	United States	Population-based	In-person interview	1994-2007	Yes
HOP	407 (6.3)	Hormones and Ovarian Cancer Prediction	United States	Population-based	In-person interview	2003-2008	Yes
MAL	377 (5.9)	Danish Malignant Ovarian Tumor Study	Denmark	Population-based	In-person or telephone interview	1994-1999	No
MAY	638 (9.9)	Mayo Clinic Ovarian Cancer Case Control Study	United States	Clinic-based	In-person interview	1999-2008	Yes
NEC	879 (13.7)	New England Case-Control Study	United States	Population-based	In-person interview	1992-2008	Yes
NJO	118 (1.8)	New Jersey Ovarian Cancer Study	United States	Population-based	Telephone interview	2004-2008	No
OPL	478 (7.4)	Ovarian Cancer Prognosis and Lifestyle Study	Australia	Population-based	Self-completed questionnaire	2012-2015	Yes
POL	109 (1.7)	Polish Ovarian Cancer Case-Control Study	Poland	Population-based	In-person interview	2001-2003	No
UCI	221 (3.4)	UC Irvine Ovarian Cancer Study	United States	Population-based	Self-completed questionnaire	1995-2005	No
UKO	352 (5.5)	UK Ovarian Cancer Population Study	United Kingdom	Population-based	Self-completed questionnaire	2006-2007	No
USC	1110 (17.3)	Study of Lifestyle and Women's Health	United States	Population-based	In-person interview	1993-2005	No

Table 3-7. Hazard ratios for menopausal hormone therapy use by stage.

	High-grade serous HR (95% CI)
MHT use[a]	
Stage I (local)	N=205
<5 years	0.69 (0.32, 1.46)
5+ years	0.62 (0.28, 1.35)
Stage II (regional)	N=469
<5 years	0.83 (0.54, 1.30)
5+ years	0.72 (0.48, 1.08)
Stage III and IV (advanced/distant)	N=3719
<5 years	0.96 (0.86, 1.06)
5+ years	0.79 (0.71, 0.86)*

[a] Reference category of no use for all analyses. All high-grade serous histology.
[b] Hazard ratios (HRs) are adjusted for age at diagnosis and race/ethnicity, and stratified by OCAC site.
* Significant at a level of $p<0.001$.

Table 3-8. Menopausal hormone therapy use and progression-free survival.

	Progression-free survival
N[a]	2,150
MHT use	HR (95% CI)[b]
None (ref)	1.0
<5 years	0.94 (0.81, 1.09)
5+ years	0.94 (0.82, 1.09)

[a] Data on presence of progression and time to progression was only available for a subset of the women.
[b] Hazard ratios (HRs) were adjusted for age at diagnosis and race/ethnicity, and stratified by histotype, stage at diagnosis, and OCAC site.

Table 3-9. Menopausal hormone therapy use and overall survival in a fully adjusted model.

	Among women with complete information for all variables, N=4,044			
	Unadjusted	**Age and stage[1]**	**Primary model**	**Fully adjusted[2] model**
Variable included	--	Age at diagnosis, stage at diagnosis	Age, stage, race/ethnicity, histotype, OCAC site	
MHT use			HR (95% CI)[a]	
None (ref)	1.0	1.0	1.0	1.0
<5 years	0.96 (0.87, 1.07)	0.96 (0.86, 1.06)	0.96 (0.87, 1.07)	0.96 (0.87, 1.07)
5+ years	0.98 (0.89, 1.08)	0.80 (0.73, 0.88)	0.79 (0.71, 0.87)	0.79 (0.71, 0.88)

[1] Change in HR estimates was primarily driven by accounting for stage, though age confounded the relationship as well.
[2] Stratified by stage at diagnosis, OCAC site, histotype, and adjusted for age at diagnosis, race/ethnicity, BMI, education level, tubal ligation, endometriosis, hysterectomy, combined oral contraceptive use duration (never, <1 year, 1 to <5 years, 5 to <10 years, 10+ years), parity, family history of breast cancer, family history of ovarian cancer, smoking status (never, former, current).

Table 3-10. Hazard ratios stratified by macroscopic residual disease.

	HR (95% CI)	
MHT use	Residual disease [A] N=891	No residual disease N=497
None (ref)	1.0	1.0
<5 years	1.08 (0.86, 1.35)	1.07 (0.74, 1.53)
5+ years	0.89 (0.68, 1.17)	0.81 (0.54, 1.22)

[A] Among advanced stage, high-grade serous carcinoma

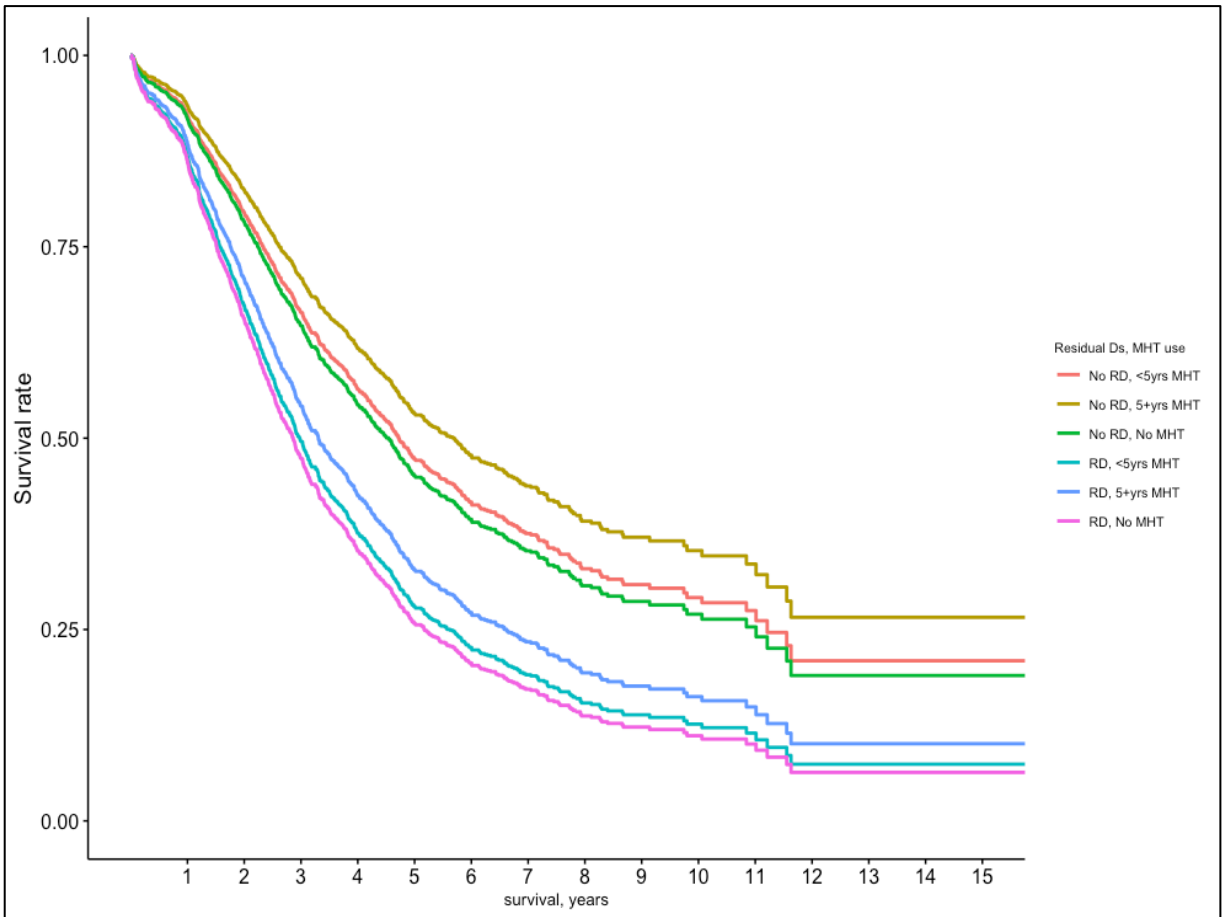

Figure 3-4. Adjusted overall survival curves by residual disease.
Results among advanced stage, high-grade serous carcinoma. Adjusted for age, race/ethnicity, and OCAC site.

Chapter 4 Inflammation and ovarian cancer survival

Pre-diagnosis pro-inflammatory risk factors decrease ovarian cancer survival

Abstract

Background: Ovarian carcinoma (cancer) risk is increased by several pro-inflammatory exposures. Inflammation may also be related to ovarian cancer survival. To identify modifiable pro- and anti-inflammatory risk factors and their cumulative effect on ovarian cancer survival, more research using larger sample sizes is needed.

Methods: The analysis used pooled data on 9,307 women with pathologically confirmed ovarian carcinoma from the Ovarian Cancer Association Consortium (OCAC). Anti-inflammatory exposures were acetaminophen use, aspirin use, NSAID use, menopausal hormone therapy use, and BMI, while pro-inflammatory exposures considered were physical inactivity, environmental cigarette smoke exposure, smoking history, and alcohol use. Multiple imputation was used to address data missingness. Using Cox proportional hazards models, each exposure was tested with survival and the associations were used to construct a weighted summary inflammatory risk score. The summary inflammatory risk score was assessed with survival using deciles of inflammatory score, with women in the lowest score decile as the reference. To test the relationship between inflammatory score and residual disease following primary debulking

surgery, logistic regression was used. Finally, the proportion of the inflammatory score's influence on survival that was mediated through residual disease was estimated.

Results: Women in the 50th to 90th percentile for score, had elevated risk of death compared to those below the 10th (HR, 1.15; 95% CI, 0.97 to 1.37) and women with scores ≥ 90th percentile had significantly elevated risk (HR 1.29; 95% CI, 1.04 to 1.60). Odds of residual disease were elevated among those from the 50th to 90th percentile for inflammatory risk score compared to those below the 10th percentile (OR, 1.30; 95% CI, 0.96 to 1.75) and were significantly higher for women with scores ≥ 90th percentile (OR, 2.11; 95% CI 1.42 to 3.16). Approximately 28% of the effect of inflammatory score on survival was mediated through residual disease.

Interpretation: Pro-inflammatory exposures prior to ovarian cancer diagnosis were related to increased odds of residual disease following debulking surgery and to decreased overall survival, while anti-inflammatory exposures had the opposite effect. The findings offer modifiable factors influencing survival and highlight residual disease in disease progression. Future work should build upon these findings to investigate post-diagnosis exposures that could enhance women's survival with ovarian cancer.

Introduction

Systemic and local inflammatory processes are related to the etiology of many important diseases, including autoimmune disease, cardiovascular disease, and cancers. Chronic inflammation can directly cause DNA damage[345-347], which is particularly relevant for cancer initiation and progression. Not surprisingly, ovarian carcinoma (cancer) risk is associated with

pro-inflammatory exposures, including talc powder application[348,349], smoking history[350], pelvic inflammatory disease[351-353], and endometriosis[349,354]. However, the impact of inflammatory exposures on ovarian cancer survival and the biologic mechanism(s) through which inflammation acts are less well known. Identifying modifiable inflammatory exposures associated with ovarian cancer survival is critical for affected women.

Pro-inflammatory exposures are associated with decreased ovarian cancer survival. For example, large body size[256], physical inactivity[255], and smoking[254] are associated with decreased ovarian cancer survival. Obesity, measured shortly before diagnosis, was associated with significantly decreased overall survival (increased risk of death, HR, 1.12; 95% CI, 1.01-1.25) and progression-free survival[256]. Women who reported no regular weekly physical activity had higher mortality than women who were active (HR, 1.34; 95% CI, 1.18-1.52)[255]. Cigarette smoking history at the time of diagnosis was associated with worse prognosis, particularly for current smokers at the time of diagnosis (HR, 1.17; 95% CI, 1.08-1.28)[254].

In contrast, anti-inflammatory exposures, such as green tea consumption[355], metformin use[257], statins use[258], aspirin use[260] and menopausal hormone therapy (MHT) use[261-263] are associated with extended ovarian cancer survival. Likewise, pre-diagnosis [263-266] and post-diagnosis[261,262] MHT use was associated with increased survival. The relationship between hormone therapy and inflammation depends on several factors[331-335], including the formula, dose, and route of delivery. At high concentrations, estrogen has anti-inflammatory properties[327-329] in many tissues, with evidence from inflammatory conditions such as arthritis and systemic lupus. In a subset of women in the prospective Nurses' Health Study diagnosed with ovarian cancer, post-diagnosis use of aspirin or nonsteroidal anti-inflammatory drugs (NSAID)s improved ovarian cancer-specific survival (aspirin HR, 0.68, 95% CI, 0.52-0.89; NSAID HR, 0.67, 95%

CI, 0.51-0.87)[260]. Finally, use of statins at the time of surgery for ovarian cancer was associated with longer progression-free survival (p=0.007)[259] and statin use within three years after diagnosis was associated with reduced risk of death from ovarian cancer (HR, 0.81; 95% CI, 0.72-0.90)[258]. Taken together, many commonly ingested anti-inflammatory exposures are relevant to ovarian cancer disease processes and survival.

Pre-diagnosis alcohol use, smoking history, environmental smoke exposure, BMI, physical inactivity, NSAID use, acetaminophen use, aspirin use, and MHT exposures have been individually associated with survival. However, a summary of the relative contribution of these exposures as well as the total contribution of inflammation is critically needed to better understand potential impacts on women currently diagnosed with ovarian cancer. Using data from the Ovarian Cancer Association Consortium (OCAC), a large, multi-national longitudinal cohort, we assessed individual exposure relationships with ovarian cancer survival and created a summary inflammatory risk score from inflammation-related exposures. We tested for an association between the summary inflammatory risk score and ovarian cancer survival. Additionally, residual disease after primary surgery was assessed as a potential mediator of the association between inflammation and survival. Together, this information gives important new insight into the role played by inflammation in ovarian cancer survival.

Methods

Study populations and exclusion criteria

This analysis uses pooled data from the OCAC, an international ovarian cancer collaboration (http://ocac.ccge.medschl.cam.ac.uk/). Women with pathologically confirmed ovarian carcinoma, vital status and menopausal status information (n=18,759 women from 30 sites) were considered for inclusion. Women were excluded if they were missing information

about histotype or stage at diagnosis or if they had a cancer that was not of one of the five main histotypes (low-grade serous, high-grade serous (HGSC), endometrioid, mucinous, or clear cell) (n=4,465).

The pattern of missingness among variables of interest (see below under exposure assessment) was investigated, and study sites with data available on at least four variables among NSAID duration, NSAID use duration, alcohol g/day, MHT months of use, physical inactivity, environmental smoke exposure, BMI, aspirin use, aspirin use duration, talc application, lifetime alcohol status, acetaminophen use, and acetaminophen use duration, were considered for inclusion (Figure 4-2). A total of 15 sites were selected as having sufficient data for analysis representing nine United States-based studies, one from Australia, one from the United Kingdom, two from Europe, and one from Japan. The primary analytic sample from these 15 studies consisted of 9,307 women, with data on residual disease following primary debulking surgery was available for 3,044 women.

Exposure assessment

Phone or in-person interviews were used to collect information from participants about their pre-diagnosis exposures. Modifiable exposures of interest included acetaminophen use, aspirin use, NSAID use, MHT use, BMI, physical inactivity, environmental cigarette smoke exposure, smoking history, and alcohol use (Table 4-2). Oral contraceptives (OCPs), history of pelvic inflammatory disease, endometriosis, and polycystic ovarian syndrome may be associated with survival and inflammation, but were not considered since there is no potential to modify them after diagnosis for most women. However, in sensitivity analyses we consider the impact of these exposures on the inflammation risk score and on survival. Covariates examined included

histotype, age at diagnosis, stage at diagnosis (local, no lymph node involvement; regional, direct extension and/or local lymph node involvement; advanced, distant sites and/or distant lymph nodes involved), education (less than high school, high school graduate, some college, college graduate, or graduate school), and race/ethnicity (Asian, Black, Non-Hispanic White, Hispanic White, other).

Outcome assessment

Survival was recorded as either length of time (in days) from diagnosis to death or to date of last follow-up (for censored patients). The presence/absence and amount of residual disease was recorded at six study sites among women with HGSC and was examined as a potential mediator of the inflammation-survival relationship.

Imputation

The goal of this analysis was to assess the value of a summary score for inflammation. To that end, we created a summary measure of modifiable inflammatory risk factors using exposure information from a women's history before diagnosis. Multiple imputation was used to address data missingness across sites to allow for a single data set to be used to create the summary score. Data were missing at random (by study site). We used multiple imputation via chained equations (*mice* package in *R*) to impute missing values iteratively and we generated 20 imputed datasets (Figure 4-1). All variables in the dataset were initially considered for imputation, including those that are not used in final models, to potentially improves imputation. Before imputing, we excluded variables deemed not useful for any predictor of interest (e.g. individual ID number), too highly collinear (e.g. grams of wine, in addition to grams of total alcohol), or

with a missingness of greater than 70%. These 20 imputed datasets were then split into a training and test set, where the individuals with residual disease measures were held out for testing; this resulted in an approximately 2:1 training:test ratio.

Risk score creation

On each imputed training set, we conducted Cox proportional hazards analysis estimated conditional hazard ratios. All inflammatory factors of interest and covariates were fit in one model, stratified by menopausal status and histotype. Factors are mutually adjusted because the summary score should account for conditional (not marginal) weights so as to avoid "over-counting" associated exposures. The parameter estimates across the imputed datasets were averaged to obtain a single point estimate for each coefficient. Modifiable factors with p-value $\leq$ 0.2 were included in the score. The more lenient criteria was selected to increase in variation after imputation. The beta coefficient estimates for individual exposures modeled in the Cox proportional hazards model were summed to create a weighted summary risk score[356] which was then applied to each individual woman in the test set. The score was divided into deciles to generate interpretable odds and risk ratios to compare those with the highest and lowest inflammatory risks.

Risk score application

We next applied our created summary inflammatory risk score in the test sets. Cox proportional hazards models were used to evaluate the association between the summary score and ovarian cancer survival. *A priori* important covariates were stage at diagnosis, histotype, age at diagnosis, and education level. Education level was included as a potential proxy for contact

with the medical system, which could influence health behaviors. We controlled for histotype in an overall model and also examined truly stratified models by histotype. We also tested for confounding by race/ethnicity.

Mediation via residual disease

We then performed a mediation analysis (*mediate* package in *R*) to test if the summary risk score was marginally associated with presence of residual disease following primary debulking surgery and if this association partially explained the association with overall survival. All mediation testing described was performed in the test data sets. The first (mediator) model used a logistic regression model for the binary outcome of residual disease (present vs. absent) regressed on the inflammatory risk score and covariates (stage, histotype, age, and education). The second (outcome) model was parametric survival analysis including residual disease, the score, and covariates. Mediation was then assessed using 2,000 simulations to estimate the average causal mediated effect, the average causal direct effect, the total effect, and the proportion mediated; confidence intervals were calculated using a quasi-Bayesian approximation. Finally, we pooled the estimated direct and indirect effects estimated from each test data set using Rubin's rule to pool estimates and standard errors to obtain the percent mediated by residual disease (Figure 4-1). All statistical analysis was performed in *R*.

Results

The study sample to examine the associations between inflammatory risk factors and survival of ovarian cancer survival included 9,307 women from 15 sites in the OCAC (Figure 4-1 and Table 4-1). A majority of the women had high-grade serous carcinoma (60.6%) and most had advanced stage disease at the time of diagnosis (62.5%; Table 4-1). The mean age at ovarian

cancer diagnosis was 57.6 years (SD = 11.3 years) and most women were post-menopausal at the time of diagnosis (72.2%). Physical inactivity was reported by 22% of the women. Regular use of acetaminophen, aspirin, and NSAIDs was reported by 18.3%, 18.2%, and 25.5% of women, respectively, and MHT use of at least five years was reported by 18.7% of women. Macroscopic residual disease was present in 46.1% of the 3,044 women with that information available (Table 4-1). All of these descriptive statistics are based on unimputed data.

Conditional hazard ratios (HRs) for each individual inflammatory factor were examined for potential inclusion in the risk score (Table 4-2). It was noted that risk factors were not strongly correlated with each other (Figure 4-3.). The strongest inflammatory risk factors for association with survival were (1) smoking (current vs. never HR, 1.15; 95% CI, 1.03 to 1.29; former vs. never HR, 1.05; 95% CI, 0.97 to 1.14) and (2) MHT use (HR, 0.92; 95% CI, 0.82 to 1.04), and former alcohol use (HR, 1.08; 95% CI, 0.97 to 1.22) (Table 4-3), but only the first was statistically significantly associated with ovarian cancer survival. These exposures as well as environmental cigarette smoke exposure and BMI were included in the risk score. Adjusting for additional covariates including oral contraceptive use, history of pelvic inflammatory disease, polycystic ovarian syndrome (PCOS), and endometriosis did not alter the associations between the inflammatory risk score and survival.

Pro-inflammatory factors (such as higher BMI and current smoking) increase the inflammatory score. Decile score (each individual's exposure was their quantile rank) were used in a Cox proportional hazards model to estimate the association with survival. Increasing inflammatory risk score was associated with decreased survival (p-for-trend=0.026). Women in the 50th to 90th percentile, compared to those below the 10th, had a 15% increased risk of death (HR, 1.15; 95% CI, 0.97 to 1.37). Women at and above the 90th percentile had a 29% increased

risk (HR 1.29; 95% CI, 1.04 to 1.60). Results were consistent in direction across histotype and strongest among women with endometrioid tumors (Table 4-5). Results were controlled for age, stage, menopausal status, and education level.

To evaluate the indirect effect of the inflammatory risk score on ovarian cancer survival via residual disease, mediation analysis was used (Table 4-4). Deciles of the inflammation summary score were used as the exposure of interest, with women in the lowest decile for score as the reference group, to examine the direct relationship between the inflammatory risk factors and residual disease. Analyses were controlled for age, stage, menopausal status, and education level. Odds residual disease were elevated among those with 50th to 90th percentile scores compared to those below the 10th percentile (reference) (OR, 1.30; 95% CI, 0.96 to 1.75). Odds were significantly higher for those at and above the 90th percentile for score (OR, 2.11; 95% CI 1.42 to 3.16) (Table 4-4). These findings are largely driven by smoking status and MHT use. Approximately 28% (95% CI, 13% to 92%) of the effect of the inflammatory risk score on survival was estimated to be mediated through residual disease (Table 4-4).

Discussion

There is substantial evidence to suggest that inflammation plays a role in ovarian cancer survival. The present analyses evaluated the joint effects of inflammatory exposures through a summary risk score on ovarian cancer survival in over 9,000 women in the OCAC. Women in the highest 10% of the risk score compared to those in the lowest 10% had a 29% increased risk of death, with follow-up extending greater than 10 years. Interestingly, 28% of the effect of the inflammation risk score was mediated through the amount of residual disease remaining after the primary debulking surgery. Women at and above the 90th percentile for score, had more than

double the odds of having residual disease present after initial debulking surgery. This suggested that inflammatory factors act at least in part by making primary sites more deeply invasive and adherent and/or new site initiation more likely.

This modifiable exposures before and following diagnosis of ovarian cancer that are critically linked to survival time. The magnitude and direction of the associated hazard ratios we observed are consistent with prior reports. In particular, we observed that pre-diagnosis smoking and higher BMI were associated with significantly decreased overall survival. Pre-diagnosis alcohol use and environmental cigarette smoke exposure were associated with marginally decreased survival. Pre-diagnosis MHT use of at least five years was associated with increased survival. We identified individual and cumulative modifiable exposures before diagnosis of ovarian cancer that are critically linked to survival time.

Previous work suggests mechanisms by which inflammatory factors impact cancer survival. Immune suppression is a hallmark of cancer that allows for tumor cells to evade removal by the immune system. The complex interplay between inflammation and the immune system is key to these processes. For example, tumors infiltrated by intraepithelial effector T cells predict better patient survival[357], while tumors infiltrated by immunosuppressive regulatory T cells confer poor prognosis[358]. Notably, there are two chemokines, CCL5 and CXCL9, associated with infiltration of solid tumors by effector T cells, which assist in destroying infected and cancerous cells[359]. A systemic immune-inflammation index[360], which integrates markers such as neutrophil, lymphocyte, and platelet count, also predicts overall survival and progression-free survival of ovarian carcinoma[361].

Metabolic factors may also contribute to this interplay among inflammation, residual disease, and survival. Ovarian cancer cells (and other abdominal cancer cells) home to the

omentum, draped over the stomach, and take up lipids which provide energy[362]. This insight also provides the potential therapeutic target of lipid metabolism and transport. Glycogen metabolism supplies energy for cancer growth and progression[363]. Additionally, the enzyme nicotinamide N-methyltransferase (NNMT) regulates methyl metabolism and has been linked to body composition regulation and obesity[364]. NNMT is highly expressed in the stroma surrounding ovarian cancer metastases. NNMT has important roles in regulation the epigenetic landscape; in this case, the NNMT expression leads to changes in gene expression that ultimately contribute to the conversion of normal fibroblasts to cancer-associated fibroblasts[365]. These findings support the further exploration of possible inhibitors of NNMT in cancer outcomes.

Our findings that MHT was beneficial for ovarian cancer survival was consistent with previous findings and proposed biologic mechanisms. Our previous findings in the OCAC showed a positive prognostic impact of MHT use of at least five years prior to diagnosis; this association may be partly explained with evidence that estrogen has anti-inflammatory properties[327-329,366], particularly at high concentrations. In addition to evidence that hormone status alters the course of many common inflammatory disease processes, there is also molecular evidence that activation of the estrogen receptor accelerates resolution phase of the inflammation in macrophagic cells[367].

On the other hand, smoking had a detrimental effect on survival. Cigarette smoke and of environmental cigarette smoke exposure have a pro-inflammatory role. Tobacco smoke exposure directly causes cellular changes that increase production of pro-inflammatory cytokines[368-370] and enhance recruitment of immune cells[371,372], not only in the lungs, but systemically. The association of former (but not current) alcohol use with decreased survival was somewhat surprising. However, because alcohol because alcohol has anti-inflammatory effects at low levels

and pro-inflammatory effects at high levels (once there is liver damage), a future, more comprehensive diet analysis will be informative.

There are a few limitations of our study. First, the high degree of exposure variable missingness necessitated imputation to create the inflammation risk score. Because certain variables were completely missing at some OCAC sites, we cannot rule out the possibility that imputation relied on the relationship between variables that ideally should have only been applied within site. However, the most likely introduction of bias due to poor predictive power for imputation models would have been a bias toward the null, so we remain confident in the validity of the direction and relative magnitudes of our conditional findings. We acknowledge that the score did not include all possible modifiable inflammatory factors. Finally, the summary score was created using data from all histotypes, while the mediation testing via residual disease is conducted only among women who had the measure reported. Future work may benefit from stratification by high-risk genotypes, such as *BRCA* mutations, to assess any differences in the effect of inflammation. These limitations are largely outweighed by the strength of the novel analytic approach, the large and diverse sample, and the proposed biologic mechanism.

Our findings not only highlight potential disease biology, but also offer potentially modifiable factors influencing survival. We found that modifiable, pre-diagnosis inflammatory risk factors are associated with ovarian cancer survival and that their effect acts partially though an influence on residual disease. Using worldwide consortium data extends the representativeness of these findings. Based on these promising findings, future work should continue to explore the role of inflammatory factors in ovarian cancer survival, using advanced methods to allow for summary of inflammation information and examining both pre- *and* post-diagnosis exposures. Because pre-diagnosis exposures and behaviors are often correlated to post-

diagnosis exposures and behaviors, the effect of a measured pre-diagnosis exposure may be due at least in part to the post-diagnosis exposure. Cohorts should aim to collect information about medications and behavior post-diagnosis to examine whether these relationships that we have found remain consistent with use after diagnosis. Important, many contributors to inflammation are modifiable, thus there is the potential for clinicians to make behavioral recommendations to enhance patient survival of ovarian carcinoma.

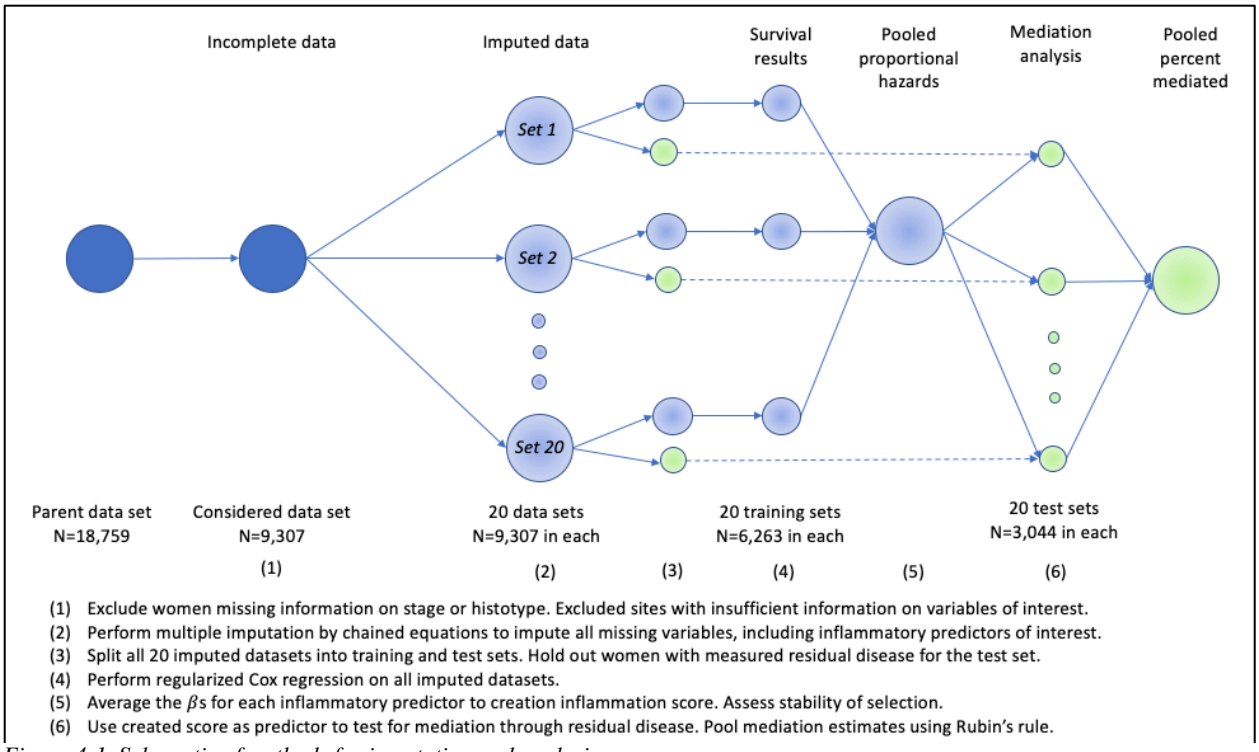

Figure 4-1. Schematic of methods for imputation and analysis.
Analysis of inflammatory contributors to overall survival of women with ovarian cancer, including imputation of missing variables and potential mediation through the presence of macroscopic residual disease after initial debulking surgery.

Table 4-1. Demographic and clinical information among women in analyses.

	Overall	Percent Missing
n	9307	
Histology (%)		
Clear cell	882 (9.5)	0
Endometrioid	1703 (18.3)	
High-grade serous	5644 (60.6)	
Low-grade serous	375 (4.0)	
Mucinous	703 (7.6)	
Stage (%)		0
Local	1750 (18.8)	
Regional	1740 (18.7)	
Distant	5817 (62.5)	
Age at diagnosis (mean (SD))	57.61 (11.30)	0
Post-menopausal (%)	6484 (72.2)	3.5
Education (%)		27.3
Less than high school	1051 (15.5)	
High school	1885 (27.9)	
Some college	1815 (26.8)	
College graduate	1134 (16.8)	
Graduate school	880 (13.0)	
Race/ethnicity (%)		6.7
Asian	532 (6.1)	
Black	235 (2.7)	
Hispanic White	289 (3.3)	
Non-Hispanic White	7389 (85.1)	
Other	238 (2.7)	
BMI 1 year prior to dx. (median [IQR])	25.5 [22.4, 29.8]	43.8
Physical inactivity (%)	1270 (22.0)	38.0
Acetaminophen regular use (%)	928 (18.3)	45.5
Aspirin regular use (%)	961 (18.2)	43.2
NSAID regular use (%)	1320 (24.5)	42.1
MHT 5+ years use (%)	788 (18.7)	54.8
Oral contraceptive use (%)	5097 (56.7)	3.4
Environmental cigarette smoke (%)	3939 (78.6)	46.1
Smoking status (%)		25.9
Never	3808 (55.2)	
Current	919 (13.3)	
Former	2174 (31.5)	
Alcohol use, lifetime (%)		49.8
Never	1897 (40.6)	
Current/ever	2110 (45.2)	
Former	664 (14.2)	
Macroscopic residual disease (%)	1402 (46.1)	67.3

Table 4-2. Description of examined variables.

Predictor	Description	Coding
Acetaminophen	Exposure of interest	Regular use (binary) Duration use (3 categories)
Aspirin	Exposure of interest	Regular use (binary) Duration use (3 categories)
NSAIDs	Exposure of interest	Regular use (binary) Duration use (3 categories)
Menopausal hormone therapy	Exposure of interest	Use of at least 5 years (binary) Months use (continuous)
BMI	Exposure of interest	Kg/m^2, 1 year prior to diagnosis (continuous)
Physical inactivity	Exposure of interest	Active/inactive (binary) Inactive = engaging in no regular, weekly aerobic activity
Environmental cigarette smoke	Exposure of interest	Ever exposure (binary) Years exposure (continuous)
Smoking	Exposure of interest	Never, former, current (3 categories) Packyears (continuous)
Alcohol	Exposure of interest	Never, former, ever (3 categories) Average g/day (continuous)
Age	Covariate	Age in years, at diagnosis (continuous)
Histotype	Covariate	5 categories
Stage	Covariate	3 categories
Education	Covariate	5 categories
Race/ethnicity	Covariate	5 categories
Residual ds. post-surgery	Potential mediator	2 categories (present, absent)
Outcome		
Survival		Days (continuous); left and right censoring

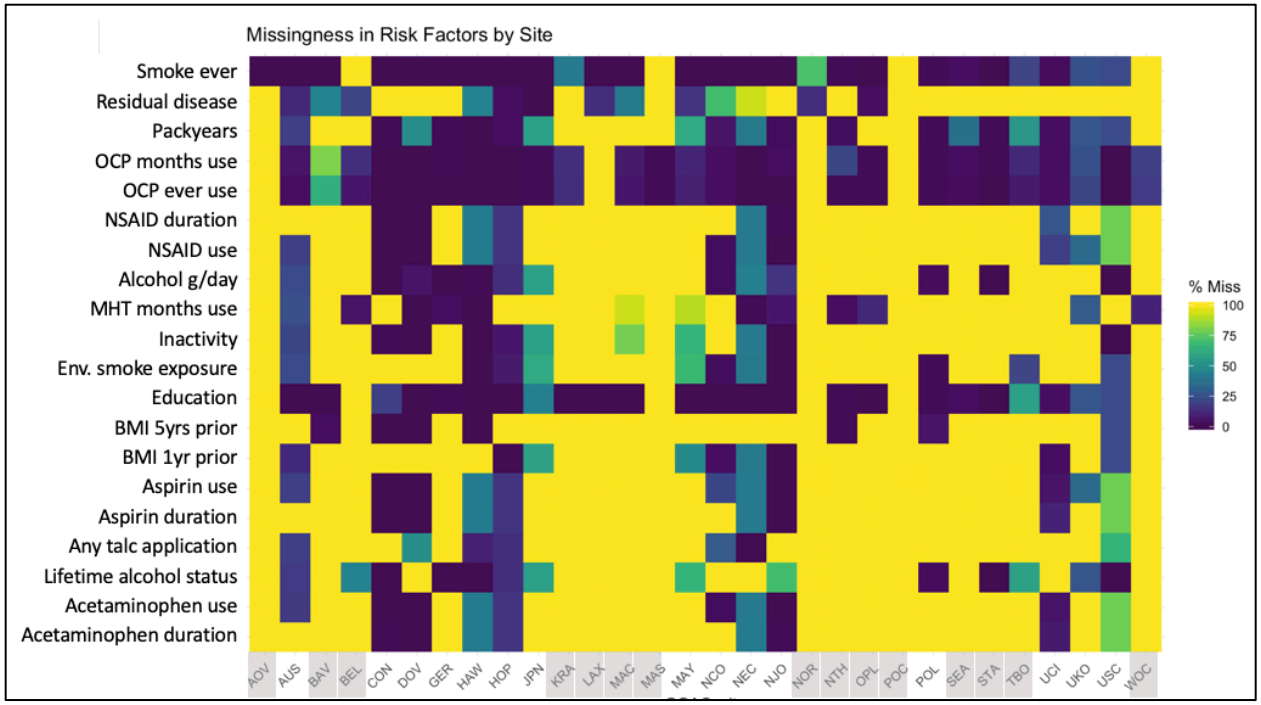

Figure 4-2. Missingness in risk factors by study site among women considered for inclusion.
14,294 women were initially considered for inclusion in analyses. Based on the pattern of overall missingness among predictors and covariate, 15 study sites were included, resulting in an analytic sample of 9,307 women. Criteria for site inclusion were having non-missing data for at least four of the variables not including, smoking and OCP variables. Sites with greyed out study acronyms along the bottom were excluded.

Table 4-3. Selected components for inflammation risk score.

	HR[1]	95% CI	p	Final model use
Age at diagnosis, years	1.02	(1.01, 1.02)	<0.01	Covariate
Stage regional vs. local	1.26	(1.07, 1.47)	<0.01	Covariate
distant vs. local	3.89	(3.41, 4.44)	<0.01	Covariate
Education (core)	0.95	(0.92, 0.98)	<0.01	Covariate
Physical inactivity	1.05	(0.95, 1.15)	0.33	--
Smoking, current vs. never	1.15	(1.03, 1.29)	0.01	Score component
former vs. never	1.05	(0.97, 1.14)	0.20	Score component
Environmental cigarette smoke	1.07	(0.96, 1.19)	0.20	Score component
BMI[A], +1kg/m^2	1.01	(1.00, 1.02)	0.09	Score component
Aspirin, regular use	0.99	(0.89, 1.10)	0.84	--
Acetaminophen, regular use	1.02	(0.91, 1.14)	0.74	--
NSAID, regular use	0.96	(0.87, 1.05)	0.37	--
MHT duration use ≥5 yrs.	0.92	(0.82, 1.04)	0.20	Score component
Alcohol use, ever vs. never	1.03	(0.94, 1.13)	0.53	Score component
former vs. never	1.08	(0.97, 1.22)	0.17	Score component

[1]From pooled estimates from 20 imputed data sets (training sets), modeled using cox proportional hazards for survival.
[A]BMI one year prior to diagnosis, or if missing then imputed using BMI 5 years prior and other variables.

Table 4-4. Inflammation risk score and survival mediated by residual disease.

Direct relationship between score and survival[1]			
Score percentile	HR[2]	95% CI	p
<10th (ref)	1.00	--	--
10th to <50th	1.06	(0.89, 1.25)	0.53
50th to <90th	1.15	(0.97, 1.37)	0.10
90th +	1.29	(1.04, 1.60)	0.02
			$p_{trend} = 0.026$
Direct relationship between score[2] and residual disease			
	OR[2]	95% CI	p
<10th (ref)	1.00	--	--
10th to <50th	0.96	(0.71, 1.29)	0.79
50th to <90th	1.30	(0.96, 1.75)	0.09
90th +	2.11	(1.42, 3.16)	<0.001
			p_{trend} <0.001
Mediated relationship between score and survival			
Proportion mediated	28%	(13%, 92%)	0.009

[1] Hazard ratio estimate from a Cox proportional hazards model of survival regression on deciles of score, stage at diagnosis, age at diagnosis, menopausal status, and education. The range of raw score values is from 0.05 to 0.54, where 0 is the null value.

[2] Logistic regression model for residual disease presence, adjusted for age at diagnosis, stage at diagnosis, menopausal status, and educational attainment.

Table 4-5: Direct relationship between inflammation score and survival by histotype.

Score percentile (ref=<10th)	**Overall** N=3043	**HGSC** N=1823	**Endometrioid** N=440	**Clear Cell** N=279	**Mucinous** N=160	**LGSC** N=108
			HR (95% CI)			
50th to <90th	1.15 (0.97, 1.37)	1.1294 (0.93, 1.37)	1.74 (0.86, 3.53)	1.19 (0.55, 2.55)	1.05 (0.34, 3.22)	1.33 (0.30, 4.63)
90th +	1.29 (1.04, 1.60)	1.29 (1.00, 1.65)	2.05 (0.93, 4.52)	1.30 (0.55, 3.09)	0.98 (0.28, 3.44)	2.70 (0.58, 12.5)
p_{trend}	0.026	0.056	0.063	0.285	0.670	0.123

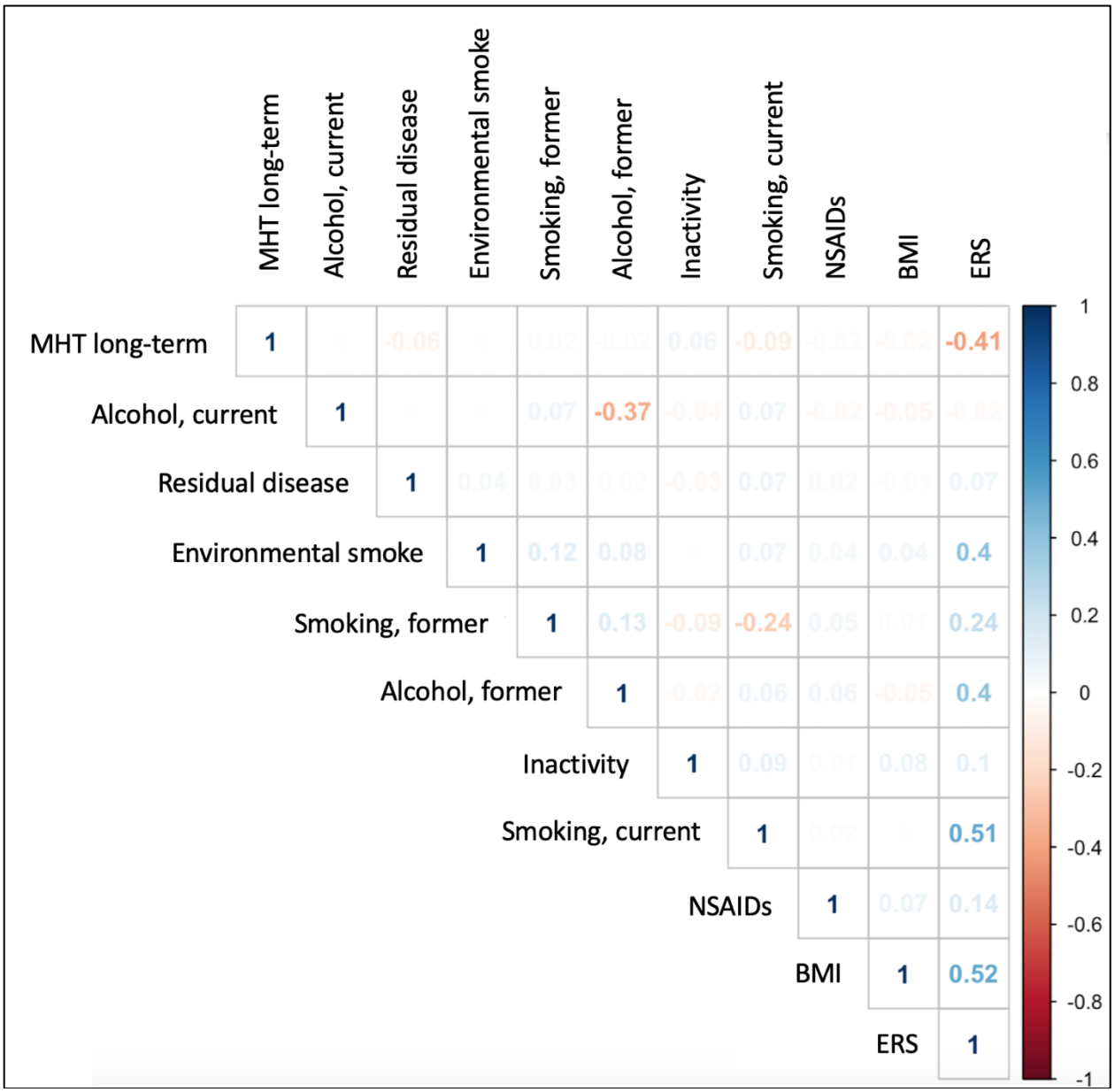

Figure 4-3. Correlations among inflammatory predictors and overall score.
Predictors that contribute the most to the inflammatory risk score (ERS) are also those that correlate most strongly with the score calculated for each individual. MHT use of greater than five years decreases the score (anti-inflammatory effect and increased survival), whereas smoking, alcohol, and BMI all increase the score (pro-inflammatory and decreased survival).

Chapter 5 Conclusions

Summary

The scope of this dissertation was broad and offered contributions to two main areas of public health: prevention of autism spectrum disorder and enhanced survival of ovarian cancer. Both areas were approached with particular attention to women's health and decision-making.

In Chapter 2, I examined prenatal nutrition in an enriched-risk pregnancy cohort and observed a protective association between prenatal vitamin intake in the first month of pregnancy and risk of ASD. Although these results were not statistically significant, they make important contributions to the cumulative evidence for prenatal vitamins' protective effect in early pregnancy; the consistency of observed effect sizes compared to prior studies provide clinical utility and confidence in the effect. These findings represented only the second study to date in a pregnancy cohort, which has the benefit of collecting high-quality information prospectively during pregnancy and following children from birth, in contrast to the numerous case-control studies in which all prenatal and early life exposure assessments are retrospective in nature.

In addition, the families in this cohort all had an older child with autism, indicating that they are at higher baseline risk of an occurrence of autism in subsequent children. For lengthy and time-intensive studies, this enriched-risk design is critical in obtaining enough cases for study. Beyond the logistical and economic practicality of such a cohort design, there is the added advantage of ultimately providing information to women and families who stand to benefit the

most. In these families at high risk of ASD, it is especially important to understand modifiable environmental risk factors.

Some of the most challenging work going forward is in the communication of risk-modifying behaviors to pregnant women. Many women receive messages about folic acid at a different time from when they are recommended to take prenatal vitamins. They may not know the various components of prenatal vitamins or the wide range of folic acid levels in vitamins and supplements. As discussed at length in the introduction, fortification of grain also has an important impact of women's folic acid intake, but this varies greatly depending on her diet and her ability to metabolize the different forms of folate and folic acid. Notably, very high levels of folic acid (particularly unmetabolized forms) are suggested to increase risk of breast and colon cancer. Because the prenatal period is already a vulnerable time both biologically and psychologically, messaging needs to be clear but also sensitive and without blame. This will require researchers, public health officials, and clinicians to work together in effectively conveying information to expectant mothers.

In Chapter 3, I turned to factors that influence ovarian cancer survival. I focused this project on menopausal hormone therapy (MHT) and found that pre-diagnosis MHT use for at least five years duration prior to diagnosis was associated with better ovarian cancer survival, regardless of MHT type and recency of use relative to diagnosis. While prior studies had reported on the protective potential of MHT, this was the largest study and the first to incorporate the effect of MHT use duration, recency, and type; tumor histotype and cancer stage; and residual disease after debulking surgery on survival outcomes. Women who had used MHT were less likely to have macroscopic disease following primary debulking surgery and approximately 17% of the survival improvement associated with MHT use could be due to the

higher proportion of MHT users with no residual disease. Although the mechanism of the effect of MHT on residual disease remains unclear, we hypothesized that MHT use prior to diagnosis could alter the pattern of metastatic spread, such that the disease is easier to access or less adhesive to surrounding tissues and thus easier to resect. MHT use could also provide and anti-inflammatory environment that is beneficial for resection. Although more mechanistic studies are needed to further elucidate the complex interplay between inflammation, immune reactions, and angiogenesis, we can start by examining other known pro- and anti-inflammatory exposures; this was the focus of the third aim of my dissertation. Future work also needs to more fully characterize ovarian cancers by estrogen-receptor type, as those with estrogen-sensitive histologic subtypes may not benefit from MHT.

Implementation of these findings is critical but not necessarily straightforward. First, a large randomized trial is needed to confirm the effect of post-diagnosis MHT use on survival and quality of life. Even ovarian cancer survivors with severe menopausal symptoms are not routinely encouraged to use MHT. This is in part due to the complicated changes in recommendations over the last several decades related to cardiac health and to other cancers, leading to both patient and provider reticence. Many observational studies in the 1990s had suggested that postmenopausal hormone therapy was associated with a decreased risk of coronary heart disease and with increased bone density[373,374], which meant that MHT was commonly prescribed as a preventive medication in older women at the time. Then, from 2002-2004, the Women's Health Initiative trials established that combined estrogen+progestin was associated with increased risk of breast cancer and of some cardiovascular disease[375] and that estrogen alone was associated with increased risk of stroke[334]. Therefore, MHT is no longer recommended as a prevention measure for cardiovascular disease in the general population.

However, this does not diminish the fact MHT still has important potential benefit for a certain subset of women, particularly ovarian cancer survivors who are at higher risk of death due to ovarian cancer than to cardiovascular disease or another gynecologic cancer. Understanding the complete picture and benefits and risks associated with different forms of MHT will enable clinicians to better provide tailored advice to women and help them in discussing their options.

In Chapter 4, I showed that several common pro-inflammatory exposures (e.g. smoking, high BMI) prior to ovarian cancer diagnosis are related to decreased overall survival and increased odds of residual disease, while anti-inflammatory exposures (e.g. MHT) had the opposite effect. Using a summary score to capture the cumulative effect of several exposures was a useful way to examine them simultaneously, and also allowed us to perform formal mediation analysis to assess how much of inflammation's effect on survival was via residual disease. Although the exposures included were by no means exhaustive, they captured many common behaviors, lifestyles, and medications; importantly, they were also all modifiable after diagnosis (alcohol use, hormone therapy use, BMI, smoking, and environmental smoke exposure).

There are several potential mechanisms by which inflammation acts to impact survival. In part, the effect of inflammation is due to the cross talk with other immune molecules. The clear impact of immune function has been demonstrated by work showing that tumors infiltrated by certain immune molecules predict longer patient survival[376]. Likewise, progression-free survival can be predicted by a combined immune-inflammation index that accounts for markers including neutrophil, lymphocyte, and platelet count[361]. Our findings contribute to this literature by offering a partial mechanism for inflammation's effect, via residual disease, and by offering modifiable exposures that could alter disease course. It is important to note that we studied only exposures *prior* to diagnosis, so future cohorts should collect post-diagnosis information as well.

That said, many of these behaviors and exposures are already known to be associated with survival in the general population; for example, quitting smoking is advisable for other cancers and cardiovascular disease. In light of this, it may already be appropriate to make behavioral recommendations to enhance patient survival of ovarian carcinoma.

Ultimately, with this dissertation and in future work, my goal is to provide evidence that will help clinicians and women together make informed decisions that enhance quality and length of life.

Recommendations for future studies

A few key themes are highlighted by the work presented in this dissertation. First, we see the importance of investigating timing of exposures that alter disease risk or progression. Vulnerable populations, such as women early in pregnancy and post-cancer diagnosis, represent a unique opportunity both to better understand the biology of health and disease and to target interventions where they will have the greatest impact for women. Future work in both autism spectrum disorder and ovarian cancer should focus on periods that offer the most potential improvement to patients' health.

Additionally, the research described in the previous chapters underscores the complex nature of studying exposures that have multiple sources. In the first project, we saw that folic acid intake is a combination of supplements (e.g. folic acid-specific supplements, prenatal vitamins), natural folate (e.g. spinach, oranges, beans), and dietary fortification (e.g. bread, pasta). The second project described the types of hormone therapy and the possible implications of pre- and post-diagnosis exposures. Finally, the third project created a summary score to model inflammatory exposures, in an effort to incorporate multiple sources of inflammation into a

single, testable model. Going forward, researchers should design and conduct studies that address these multiple sources of exposures or attempt to explain the relative contribution of the source under study.

Public health impact

Effectively changing health recommendations and policies to benefit women's health requires a strong understanding of the epidemiologic process of research. Once a public health problem has been recognized and described, as it has been for both autism and ovarian cancer, studies must be designed with particular hypothesis in mind, but also with an openness to the possibility of uncovering yet-unidentified risk factors. The translation of research findings into public health action requires continuous monitoring and testing. This process is particularly delicate when dealing with vulnerable populations such as pregnant women and cancer patients, as certain types of studies and treatments are unethical in these situations. However, these limitations are partially overcome with large, carefully designed observational studies and with replication of findings. Taken together, the projects in this dissertation represent a contribution to the literature and more broadly to public health describing exposure modifications that clinicians can discuss with women to improve health.

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