The association of the C677T ^{5,10}*methylenetetrahydrofolate reductase* variant with elevated maternal serum α-fetoprotein and complications of pregnancy.

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Abstract

Statement of problem: We have shown that the C677T ^{5,10} *methylenetetrahydrofolate reductase* (MTHFR) variant is associated with elevated maternal serum α-fetoprotein (MSAFP), the most common screening test for neural tube defects (NTD). Therefore, past contradictory studies of NTDs and C677T MTHFR may have been biased because of changes in case populations after prenatal diagnosis and termination of pregnancy. Further, an unexplained elevation of MSAFP is known to increase the risk for later pregnancy complications. Is the C677T MTHFR variant a predisposing genetic variant for both NTDs and later complications of pregnancy?

Methods: A retrospective study of women with pregnancies resulting in NTD outcome and women with unexplained elevations of MSAFP was undertaken. Women and their partners were genotyped for the C677T MTHFR allele. Couples with a pregnancy resulting in a NTD outcome were compared to couples whose pregnancy outcome did not involve. Couples with unexplained elevations of MSAFP who did and did not have later complications of pregnancy were also compared. Allele frequencies for all groups were then compared against the previously established Manitoba population allele frequency (based on 977 consecutive newborn metabolic screening bloodspots). A review of all studies of NTDs and association with the C677T MTHFR variant was

undertaken to determine if the association between the variant and MSAFP is a source of bias. NTD incidence was examined before and after folic acid food fortification introduced in Canada in 1999.

Results: There is an increase in the allele frequency of the C677T MTHFR variant in parents with an unexplained elevated MSAFP followed by later complications of pregnancy. The C677T MTHFR variant is also a contributing genetic factor to NTDs worldwide. The incidence of NTDs in Manitoba has decreased by 37% since food fortification with folic acid was introduced.

Conclusions: The C677T MTHFR variant is a contributing genetic factor to both later complications of pregnancy after an unexplained elevation of MSAFP and to NTDs. This variant is folate sensitive and folic acid fortification has reduced the incidence of NTDs.

List of abbreviations

AFP α-fetoprotein

CI confidence interval

FFQ food frequency questionnaire

HCG human chorionic gonadotrophin

HSC Health Sciences Centre (Winnipeg)

HELLP syndrome: H= hemolytic anemia EL= elevated liver enzymes LP= low platelet count

ICDC international classification of disease codes

IUD intrauterine death

IUGR intrauterine growth restriction/retardation

LCL lower confidence limits

MI myocardial infarction

MMSSP Manitoba Maternal Serum Screening Programme

MOM multiples of the median

MSAFP maternal serum α-fetoprotein

MTHFR ^{5,10}*methylenetetrahydrofolate reductase*

MTR *methionine synthase*

MTRR *methionine synthase reductase*

NCSS Number Cruncher Statistical System

NO nitrous oxide

NTD neural tube defects

OR odds ratio

PEMT phosphoethanolamine methyl transferase

PIH pregnancy induced hypertension

RBC red blood cells

RR relative risk

SAH S-adenosylhomocysteine

SAM S-adenosylmethionine

SD standard deviation

TA termination of pregnancy

TDT transmission disequilibrium test

THF tetrahydrofolate

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Introduction

It has long been recognized that women who have a well balanced diet generally have better pregnancy outcomes compared to those who do not (1). Even so, a good diet is no guarantee of a good pregnancy outcome. Although the "rule" of good diet=good outcome generally holds, some women who follow this "rule" correctly will have serious complications during pregnancy. These can include the mother developing high blood pressure and associated complications, having difficulty in delivery of the placenta, having a baby that has not grown properly, or delivering a normal baby several weeks too early. Sometimes, the complication of pregnancy means a baby with a life threatening malformation. In contrast, other women who are not so careful about their diet can often deliver a healthy baby with relative ease. This apparent contradiction can, in part, be understood by examining the unique genetic predisposition to complications that an individual woman has, the environment in which she lives, and the environment her body provides for her developing child. In short, complications of pregnancy are usually the net result of multiple factors.

Multifactorial traits are those that require both a polygenic predisposition and an environmental trigger interacting together. Multifactorial causes are thought to underlie most complications of pregnancy. Studying multifactorial traits is difficult because they represent the sum of "a large sample of chaotic elements" (2). Yet, the study of multifactorial traits is important because continuous traits (those that can be measured as a normal distribution of type) are almost invariably multifactorial in origin (3).

The maternal serum α-fetoprotein screen test (MSAFP) was the first prenatal screening test of the mother's blood to be used to assess fetal well-being in mid pregnancy (4). Initially MSAFP testing was used to detect open defects in the spine and brain caused by failure of the neural tube to close in early development. When a baby has an open neural tube defect (NTD), measurably higher amounts of a common fetal blood protein, α-fetoprotein, are absorbed into the mother's circulation. Mothers with higher amounts of α-fetoprotein are offered fetal assessments with ultrasound that can result in diagnosis of a NTD in midpregnancy.

By providing detailed ultrasound assessments for all mothers with an elevation of MSAFP, physicians can diagnose 85% of babies with a NTD in midpregnancy. This is an advantage for parents because it allows them to either request a termination of the pregnancy (TA) or to prepare for the birth of a child with special needs. As experience with the MSAFP test grew, early detection of other malformations (such as ventral wall defects) became possible (5).

The majority of NTDs are considered to be multifactorial. A diet high in a wide variety of vitamins and other nutrients results in better outcome. Important clues about the cause of NTDs may lie in variations of individual ability to metabolize vitamins. Of all the vitamins that have been examined for connections to NTDs, folate, a B vitamin common in fruits and vegetables, has shown the greatest potential in offering clues to the multifactorial nature of NTDs (6). There is currently intense interest in studying the enzymes involved in folate metabolism. Results from the Medical Research Council Vitamin Study show that women who supplement their diet with folic acid (an artificial

form of folate) periconceptionally are 50-70% less likely to have a baby with a NTD (7). There is also a growing body of evidence suggesting that adequate folic acid intake may prevent other malformations in addition to NTDs (reviewed in the Background). Folic acid may also prevent a host of other complications of later pregnancy. Individual differences in the metabolism of folate may be the major source of genetic factors predisposing embryos to the development of NTDs and possibly to many other complications of pregnancy (8).

It had been previously hypothesized that a subgroup of women with one or two copies of the common, but inefficient, version of one of the important enzymes in folate metabolism, C677T *methylenetetrahydrofolate reductase* (MTHFR), combined with low folate intake, would be at increased risk for NTDs (9). Understanding the role of the C677T MTHFR variant as a predisposing genetic factor, combined with the environmental factor of low dietary intake of folate, might explain why some women have NTDs while others do not even when all of them appear to have adequate dietary intakes of folate. There is presently extensive literature on the topic of the C677T MTHFR variant and NTDs. The literature is contradictory. Some studies show an association and others do not (reviewed in detail in Chapter 4). Manitoba has an excellent system for studying NTDs because there is near full ascertainment of the NTD cases in the province and Manitoba has universally available MSAFP screening. The provincial screening programme is coordinated through a single location at Winnipeg's Health Sciences Centre.

Our preliminary pilot study (detailed in Chapter 1) consisted of the first retrospective study comparing the C677T MTHFR allele frequency in women who had a NTD affected fetus with a previously established population frequency (10). This study, utilizing 977 anonymous consecutive blood spots from the provincial neonatal screening programme, showed that 36% of Manitoba newborns have one C677T MTHFR allele and 7% have two. As these newborns represent the same population as the offspring of the pregnant women in the study, this cohort can be considered an appropriate and representative population control group. The women whose pregnancies were affected by NTDs were also compared to a cohort of women who had an elevation of MSAFP, but who delivered a healthy baby. The unexpected result was that the women with unexplained elevations of MSAFP and normal babies at midtrimester scan (our controls), were the group found to have a significantly higher allele frequency than the population frequency for the C677T MTHFR variant (see Appendix 1). The mothers of babies with NTDs also had an apparently higher allele frequency, but the result was not statistically significant (perhaps due to small sample size).

MSAFP screening is the primary screening tool for NTDs in many countries. MSAFP screening is 85% effective in detecting NTDs, with higher rates of sensitivity for anencephaly and lower rates for spina bifida (11). Could an association of the C677T MTHFR variant with an elevated MSAFP screen test mean that a woman with a fetus having both the C677T MTHFR variant and spina bifida is more likely to have the fetal defect ascertained by screening and then choose have the pregnancy terminated? Conversely, could mothers of fetuses with spina bifida, but without the C677T MTHFR

variant, be more likely to screen negative and avoid prenatal detection? Would this cause a bias that would skew the allele frequency of groups of individuals affected by NTDs (and their parents) depending on whether or not prenatal screening had taken place? This was the first important question we raised.

Pregnant women with unexplained elevations of MSAFP, whose fetuses do not have a NTD (or other congenital anomalies), and whose initial ultrasound scans are normal, are still at increased risk for complications such as small-for-dates babies, premature delivery, and poor placental function (12). Thus, women with an elevation of MSAFP represent a population of special clinical concern. Many of these complications have their own distribution of increasing severity (13). One example is hypertensive disorders of pregnancy. These can range from pregnancy induced hypertension (PIH) that disappears with delivery to preeclampsia with its characteristics of severe hypertension (blood pressure > 170/110 mmHg) and associated pathological effects (14, 15). This continuum suggests these complications are modulated by many factors.

It has not been previously possible to distinguish those women with an elevation of MSAFP who will have complications later in pregnancy from those who will not. No preventive therapy can be offered beyond close monitoring to try to detect complications early. A method of predicting the women at highest risk would be a valuable clinical tool. Is there a difference in the C677T MTHFR allele frequency in parents with and without complications of pregnancy after an unexplained elevation of MSAFP? This was our second important question.

In 1999, the Canadian government began fortifying the Canadian diet with folic acid in order to prevent NTDs. Fortification increases the average daily intake by about 50% across the entire population. We do not currently know if this is enough or too much fortification. Similarly, it is not known what fraction of the population will benefit the most or be the most likely to be adversely affected. We do not even know precisely why there may or may not be a benefit to fortification. Since the C677T MTHFR variant is both folate sensitive and so common in the population, it represents an excellent place to begin to examine these unknowns.

Understanding why some women have complications of pregnancy after an elevation of MSAFP requires an understanding of the context in which the elevated result occurs. One of the important factors to be discovered is whether or not the introduction of folic acid fortification in food has reduced the incidence of NTDs in Manitoba. This is our third important question.

Summary of Introduction

This thesis explores three interrelated questions. 1) Is the C677T MTHFR variant a genetic contributor to NTDs independently of a positive or negative MSAFP result? 2) Is there a difference in the C677T MTHFR allele frequency in parents with and without complications of pregnancy after an unexplained elevation of MSAFP? 3) Have we seen a reduction in NTD incidence and in the incidence of other complications of pregnancy in Manitoba since folic acid fortification of food began?

Background

Neural Tube Defects

Description of NTDs

NTDs result from a failure of the rising and fusing of the neural folds during the earliest stages of development. In humans, neural tube closure begins in the third week after conception, often before a woman knows that she is pregnant. When a region or regions of the neural tube fails to close normally, the result is a NTD. A NTD can range from a mild dysraphism of the spinal column to complete failure of development of the entire central nervous system. Defects that leave the spine or brain untouched and only involve the covering tissues can almost always be repaired successfully after birth. Open defects are those that leave some part of the neural tube open and exposed, except perhaps for a thin membranous covering. Failure of differentiation of part of the central nervous system results from open defects. Further, the brain and spine often fail to develop normally when not enclosed in associated outer structures even if they have initially differentiated appropriately.

There are three general classifications of open NTDs. Anencephaly occurs when the brain region of the neural tube fails to close. The result is an opening where the brain and skull should have developed. An encephalocele occurs when part of the sealing of the neural tube fails and a hole exists in the head through which brain, or the tissue protecting the brain, protrudes. Spina bifida occurs when the lower portion of neural tube fails to close leaving an opening in the spinal region. The most severe form

of NTD, generally referred to as cranioraschisis, results in the entire nervous system being left open and failing to develop (16, 17).

Due to the high morbidity and mortality associated with NTDs, prenatal diagnosis and elective termination of an affected pregnancy has become the option chosen by 80% of families faced with a NTD pregnancy. Prenatal diagnosis is usually made via fetal assessment with ultrasound followed by amniocentesis, if necessary. Other options depend on the severity of the defect and its location. Where the brain has been severely affected, death of the baby soon after delivery is virtually inevitable. Closing the defect at birth and providing supportive care is offered for those with spina bifida. During development, the spinal cord of fetuses with spina bifida is often tethered at the defect site. As the spine grows, the tethered cord does not and it pulls the brain into the top of the spinal column. This changes the shape of the brain. This shape change is known as the Arnold Chiari malformation. This also can block the free flow of cerebral fluid and cause hydrocephalus. This change in brain shape is detectable by fetal ultrasound assessment and is considered diagnostic of spina bifida (18, 19). Where the choice is to continue the pregnancy, the baby must be monitored for hydrocephalus and early delivery, often by caesarean section, is sometimes the best option. Care of infants born with spina bifida usually includes installing a shunt to relieve hydrocephalus soon after delivery.

Most individuals with spina bifida will undergo multiple surgeries as they grow in order to treat problems with scoliosis, urinary and fecal incontinence, sexual dysfunction, and mobility issues. Blockage of the shunt and infections from surgery can be life threatening (20, 21). Latex allergy is often a problem. Many affected individuals

do not survive to adulthood. In addition, while these children are generally of normal intelligence, they often struggle academically with learning disabilities likely related to hydrocephalus or shunt problems (21).

Controversial experimental surgical closure of the defect *in utero* has shown some promise. Children who have had this surgery avoid shunting for hydrocephalus although that is true of 14% of children born with spina bifida in any case. The experimental surgery has thus far been limited to lower and smaller lesions that might not have required shunting. Children who have had this surgery also have better general motor functioning than otherwise expected. However, they still have characteristic bladder control abnormalities. The surgery is high risk for both mother and fetus (22, 23).

Worldwide prevalence of NTDs

Except for heart defects, NTDs are the most common form of major congenital malformation with a birth prevalence of 1-5/1000 births worldwide (reviewed in Chapter 4). There are large geographic, regional and ethnic differences in prevalence. There is clear evidence from family and twin studies that there is a strong genetic component to the occurrence of NTDs. The percentage of chromosomal syndromes associated with NTDs is small. The process of twinning may itself increase risk (24). NTDs are more common among women of certain Hispanic subpopulations, and among populations in Ireland, China, and the United Kingdom, especially Wales (25). In Canada, there is an east/west gradient with the highest incidence rates being in the

Maritimes (24) .Temporal differences, both short term, as in seasonality effects, and long term changes in prevalence over time, have been observed. Season of conception, gender, parity, mother's age, socioeconomic status, urban versus rural location, maternal diabetes, smoking, alcohol use, obesity, and hyperthermia have also been associated with increased risk for NTDs. Family or personal history of multiple miscarriages, stillbirths, and NTD increase individual risk (24, 25). In addition, there are several drugs that interfere in some fashion with folate metabolism that also increase the risk of NTDs. These include a broad spectrum of anticonvulsants such as valproic acid, drugs used for treating cancer such as methotrexate, and certain anesthetic agents (24).

Prevention of NTDs using folic acid

The first clue that NTDs might be related to diet came from examining the Dutch famine winter of 1944. There was a doubling of the incidence of spina bifida among those conceived during the famine. The quality of nutrition was later investigated by gynecologist ED Hibbard in the 1960s and pediatrician RW Smithells in the early 1980s, among others (26, 27). A series of studies soon pointed to multivitamins, folic acid in particular, as having the ability to prevent NTDs (28). The most widely cited study was published in 1991 (7). It found that supplementing with 0.4 mg of folic acid per day is capable of preventing 70% of NTDs in women with a past history of NTDs and 50% of all NTDs in the general population. Widespread periconceptional supplementation with folic acid has great promise for preventing the majority of isolated NTDs (6, 29).

In 1973, the Food and Drug Administration in the USA allowed an increase in the amount of folic acid in standard multivitamins. The increase in folic acid coincided with a drop in the prevalence of NTDs from 20/10,000 births prior to 1973 to 13/10,000 births after 1973. Between 1995 and 1998, when periconceptional folic acid supplementation was recommended, and nationwide educational programmes were initiated, the prevalence of NTDs dropped to 10/10,000. However, since half of all pregnancies are unplanned, the population was not getting the maximum benefit from the new knowledge about folate. Mandatory food fortification began in the USA on January 1, 1998 and prevalence has since dropped to below 5/10,000 births (30).

Trends in Canada have paralleled those in USA. Fortification in food prepared by Canadian manufacturers that was also to enter the US market resulted in increasing amounts of folic acid entering Canadian food by 1998. On January 1, 1999, fortification in Canada became mandatory. The push for fortification came from the manufacturing industry in order to facilitate manufacturer compliance with US standards. The measure was widely supported by the medical profession.) Since fortification began in Canada, there has been a significant drop in NTDs in Nova Scotia. Rates there have dropped from 26/10,000 to 11/10,000 births (31). In Ontario, rates have dropped from 17/10,000 to 6/10,000 births (32).

Embryological explanations for NTDs

Development is generally considered to be a series of bifurcating choices for developing cell types. For example, the ectoderm is changed to two new types of tissue,

presumptive neural epithelium and presumptive epidermal cells. In classical embryological terms, the ectoderm is considered to be on a pathway to forming epidermis unless "induced" to form neural tissue. A signal that induces this alternate pathway of development comes from the dorsal lip of the blastopore during early gastrulation while mesoderm and endoderm is being tucked inside the embryo and ectoderm is expanding to cover the whole exterior. The primary neural induction signal spreads over half of the ectoderm. This changes in response to the induction signal. It begins expressing new gene products unique to neural tissue. The other half of the ectoderm does not receive the induction signal. It therefore follows the default programme, which is to change gene expression to form the epidermis. Thus, there are two new tissues arising out of one earlier tissue. By continually repeating this process throughout the embryo, and in many different tissue types, the entire embryo forms (33, 34).

NTDs are most commonly thought to occur due to failure of the sides of the neural tube to rise or a failure of the neural tube margins to seal, or some combination thereof. There are multiple explanations of why this failure or failures might occur, but no one explanation is generally accepted.

The neural epithelium forms a thick flat plate, which then subdivides into multiple embryonic neural tissue types as it folds and seals (35). Many of the differentiations change the mechanical properties of the cells. The cells change shape and the nature of their adhesion to their neighbours and therefore change the region of the neural plate they reside within (36). The failure of any differentiation from the initial step of primary neural induction to the end of neural tube closure can result in a NTD. The

relative timing of the different zones of differentiation is also critically important to development. For example, if the inner region of the plate has faster development compared to the outer edges of the plate, the edges won't meet at a time when they are competent to bind to each other. Such localized differentiations are required in the multi-site closure hypothesis. This hypothesis includes separate gene(s) expressed at each closure site during neurulation (17). Also, if one zone of the neural plate takes up too small or too large a portion of the whole plate, the mechanical properties of the entire plate are changed such that the plate cannot fold properly and roll up to close. In general, the earlier the failure of differentiation, the more severe the neural tube defect (33, 37).

NTDs can be roughly classified as syndromal or multifactorial. Multifactorial NTDs, which represent about 70% of all NTDs, occur due to a combination of environmental factors and genetic predisposition. Syndromal types of NTDs are those that are part of a single genetic defect, as in the autosomal recessive Meckel-Gruber syndrome, or as part of larger chromosomal changes such as trisomy 18 (16). Non-genetic factors can cause syndromal type NTDs as well. Maternal alcoholism is one example of a non-genetic risk factor (38).

Folates, homocysteine and the embryology of NTDs

Studying the place of folates in embryological functioning has provided important clues about the etiology of NTDs. Folic acid is an artificial form of folate that is readily converted into the metabolically active derivatives of folate such as $5'$-

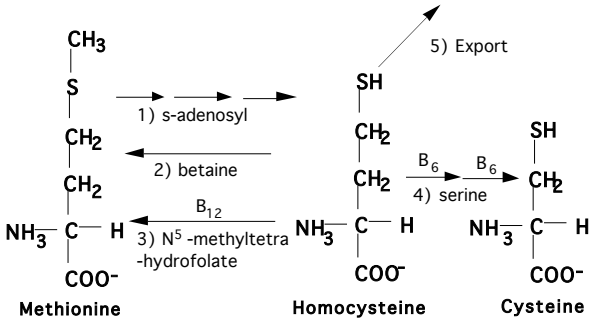
$^{10'}$methylenetetrahydrofolate. Folates, including folic acid, provide a critical methyl group for the creation of a high energy methylating intermediate. This methylating intermediate is needed in enormous quantities for proper construction and functioning of DNA, proteins, and lipids. As developing embryos are rapidly creating new cells, they are especially sensitive to reduced levels of folates. Ectoderm and early neural epithelium tissue may be the most sensitive of all embryonic tissues. Any reduction in the availability of folates can cause a NTD even if the rest of the embryo develops normally. This idea is supported by the observation that neural plate epithelium expresses extremely high levels of messenger RNA for folate receptors when compared to other embryonic tissues such as the developing heart (39).

Neural epithelium is also extremely sensitive to homocysteine. Homocysteine concentrations are normally inversely proportional to the concentration of folate derivatives (Fig. 1). Homocysteine is converted to methionine. The enzyme *S-adenosylmethionine synthase* (also known as *methionine adenosyltransferase*), joins a methionine to an adenosine triphosphate by linking the sulfur of the methionine to the 5' carbon of the ribose unit. This forms the high energy compound S-adenosylmethionine (SAM). SAM is the single most important methylating reagent in the cell including, but not limited to, the methylation of lysine, arginine, and histidine in post-translational modifications of proteins. Critical methylations of DNA, RNA, and lipids also require SAM (40).

SAM is converted back to S-adenosylhomocysteine (SAH) when the methylation reaction occurs. Several different enzymes catalyze this methylating reaction depending on which compound in the cell requires a methyl group. The product, SAH, is a

feedback inhibitor of all the enzymes that create SAM and can therefore be regarded as a potentially toxic waste product. The ratio of SAM to SAH in the cell is tightly regulated in each cell type. In normal adult rat liver, SAH exists at 13 μmol/g while SAM is normally at 60 to 90 μmol/g (41). In the rat embryo, the difference is even more profound with SAM concentration being 42 times higher than SAH in some tissues (42). After the methylation reaction has occurred, the adenosyl group is normally stripped off of SAH leaving homocysteine. Homocysteine must be quickly condensed to cysteine via the pathway called transsulfuration, or recycled back to methionine (remethylation), or exported from the cell, to avoid causing elevated levels of SAH and the resulting inhibiting activity of SAH on the generation of SAM (see Fig. 1 and 2).

Figure 1: The structural and enzymatic relationships between methionine, homocysteine, and cysteine and the cofactors involved.

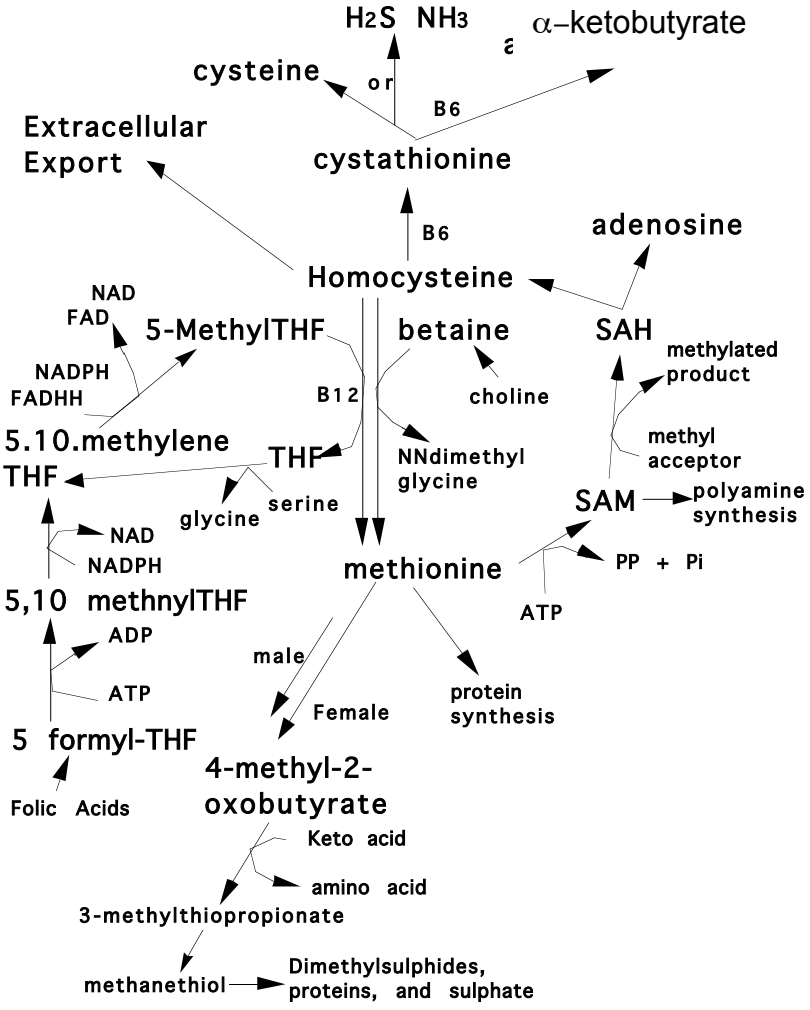


Arrows represent a single enzymatic reaction. Adapted from (43).

In cultured rat embryos, exposure to a 3 millimoles concentration (added to the culture media) leads to a drop of the SAM/SAH ratio from 42 to 3.6 (42). Any buildup

of homocysteine in the cell results in reversal of the hydrolase reaction that changes SAH to homocysteine. This causes an increase in SAH levels. This will in turn inhibit the production of SAM, reducing methylation reactions throughout the cell and impeding normal cellular functioning. Very small rises of less that four μmol/L of homocysteine can cause NTDs in chick embryos (39).

Figure 2: Basic pathways of homocysteine metabolism. Adapted from (42-44).



There are differences between human males and females in terms of metabolism of methionine (44). Females are more likely to shunt off a portion of the available methionine to 4-methyl-2-oxybutrate in the presence of increased homocysteine. This is because the enzymes in this pathway appear to be enhanced by estrogen. It is unlikely there is any gender based effect at the time of neural tube closure because the embryo is not yet producing significant amounts of its own estrogen and testosterone at that early stage. However, at later stages of pregnancy, there may be survival advantages for one gender over another. The enhancement offered by estrogen may explain the epidemiological differences in prevalence of males versus females among fetuses with NTDs.

The human embryo/fetus has both less efficient transsulfuration and remethylation abilities when compared to the adult (41, 45). Yet, the human embryo/fetus simultaneously requires a much higher SAM/SAH ratio in spite of this dual "impairment". Maintaining the high SAM/SAH ratio in the embryo/fetus can only be accomplished by lowering maternal homocysteine levels. Increasing both transsulfuration and remethylation in the mother, which in fact occurs, can do this. As well, estrogen related compounds enhance removal of methionine from the cycle, which enhances remethylation of homocysteine. Perhaps the dramatic physiologic drop in homocysteine during normal pregnancy of 50% or more (46, 47) evolved as a protective mechanism for nurturing a hyperhomocysteinemia susceptible embryo by allowing it to export excess homocysteine if required.

Even though high levels of homocysteine are toxic to the embryo, the embryo still needs to import some homocysteine as an essential amino acid. This may explain why women who have enzyme variants that increase homocysteine later in pregnancy have been shown to have fewer cases of intrauterine growth restriction (IUGR) among their babies (48). The embryo may also control how much homocysteine it imports from the mother. Women with homocystinuria due to cystathionine beta-synthase deficiency appear to have normal pregnancy outcomes (49, 50) indicating that high homocysteine levels in the mother are not necessarily a problem for the embryo.

Methylation Failures and NTDs

Cow serum must be supplemented with methionine in order to prevent NTDs in rat embryos grown *in vitro* on cow serum during the period of neural tube closure (51). Cow serum is low in methionine and causes low SAM levels in rat embryos. This indicates a critical period of increased requirement for methionine during neural tube closure in rats. This critical period corresponds to the previously noted increased need for folates in neural plate tissues. It would appear that homocysteine is embryotoxic in a general sense. However, it does not directly cause NTDs. This hypothesis is supported by recent research (52). Rather, it is the loss of folate derivatives and reduced SAM that cause NTDs.

Rat embryos grown on low methionine cow serum, and with NTDs, have been shown to reduce amounts of two posttranslational methylations of specific proteins in the neural tissue. Methylation of histidine to form a trimethyl compound was reduced by up to 56%. Methylation of arginine to form a dimethyl compound was reduced by

42%. This effect was not seen in normally developing, but methionine deprived, heart tissue from the same embryo. Methylation of histidine and arginine was normal in embryos that were methionine supplemented and that subsequently developed normally without NTDs (53).

Why is this specific decrease in methylation restricted to rat embryo neural tissue? NTDs may represent a borderline case of SAM deficiency. Too great a deficiency results in death of the embryo. A borderline deficiency affects only embryonic neural tissue because this is the only tissue with elevated SAM demands during the critical period of neural tube closure, at least in rats.

In adult rats, SAM provides the methyl group for a brain specific *phosphoethanolamine methyl transferase* (PEMT) enzyme, which preferentially methylates polyunsaturated fatty acids. Most polyunsaturated fatty acids used in the adult are produced in the liver and transported through the blood stream to wherever they are needed (54). PEMT activity also occurs at very low levels in some other tissue types. There is a brain specific PEMT enzyme required because polyunsaturated fatty acids cannot pass the blood brain barrier, yet they accumulate in brain tissue (55). This brain specific PEMT has been isolated in newborn rat brain tissue (56). Recent research has shown that PEMT activity in the adult liver regulates homocysteine levels (57). Thus it is not implausible to suggest that there is a form of PEMT in developing neural tissue that creates polyunsaturated fatty acids in embryonic neural tissue and that this enzyme may also regulate homocysteine in the neural tissue. Certainly changes in homocysteine levels result in changes in brain membrane composition in chick

embryos. Increases in homocysteine levels result in decreases in the levels of polyunsaturated fatty acids. The likely mechanism is through inhibition of PEMT by elevations in SAH levels (55).

The PEMT enzyme of neonatal rat brain is different from the adult rat version in the liver. The neonatal PEMT has a high affinity for SAM and methylates four times faster than the adult liver version. Its concentration also increases 2-fold from neonatal days 5 to 20, a period of rapid rat brain growth (56).

There are PEMT2 knockout mice. (PEMT2 is the version of PEMT normally expressed only in adult liver.) These knockout mice develop normally, from a behavourial perspective, if given choline supplements, because extra phosphotidylcholine is produced via the alternate pathway of phosphorylation of choline (58). They do have some anatomical brain abnormalities even though their behaviour is normal (58).

There is an extremely high demand for neural lipids during early development. The proportion of the neural plate that is made up of cell membrane increases dramatically during neural plate formation and neural tube closure. Individual neural plate cells are both elongating and rapidly dividing while the total neural plate volume remains relatively constant (60, 61). This indicates there would be a high demand on any embryonic version of PEMT that might be present in early neural tissue. Normally some of the choline stored in yolk platelets is used for producing phosphorylated choline derivatives such as sphingomyelin (62). Some of the choline is also converted to betaine, which then acts as a methyl donor for methylation of homocysteine to methionine. This then increases available SAM. In addition, some limited amounts of

phosphotidylcholine are normally produced by PEMT during gastrulation and neurulation. However, the betaine pathway is not active until the end of neurulation (62). This could then explain why neural tissue is so sensitive to reduced availability of folate derived SAM. SAM is only available via the folate pathway. SAM is also being used up by high affinity PEMT for production of phosphotidylcholine first. Other required methylations such as methylations of histidine and arginine in proteins, cannot be completed because of the lack of methyl groups. What evidence is there for an effect on differentiation of reduced availability of methylated forms of histidine and arginine?

Cell State Splitters and NTDs

There are three components of the cytoskeleton that are normally methylated using SAM. These are actin, α, and β tubulin, and neurofilament L. Contractile function of myosin and a highly conserved 3-methylhistidine residue that is actively methylated by SAM during neural tube closure regulates actin. Reduced methylation results in failure of these cytoskeletal elements to localize in the basal and apical ends of the cells (63). Failure of appropriate localization of the cytoskeletal elements of neural tissue could result in mechanical failures of cell contractions and movements and thereby cause NTDs. Therefore, reduced methylation of cytoskeletal elements is an ideal candidate for reduced methylation causing changes in differentiation that affect the developing neural plate, which then leads to a NTD. This requires a direct connection between the cytoskeleton and differentiation. While no one model of differentiation is accepted universally, there is a model of neural tissue differentiation

that is based on cytoskeletal functioning. This model is called the "cell state splitter model" (34).

Figure 3: Embryonic metabolic pathways affecting SAM/SAH levels.

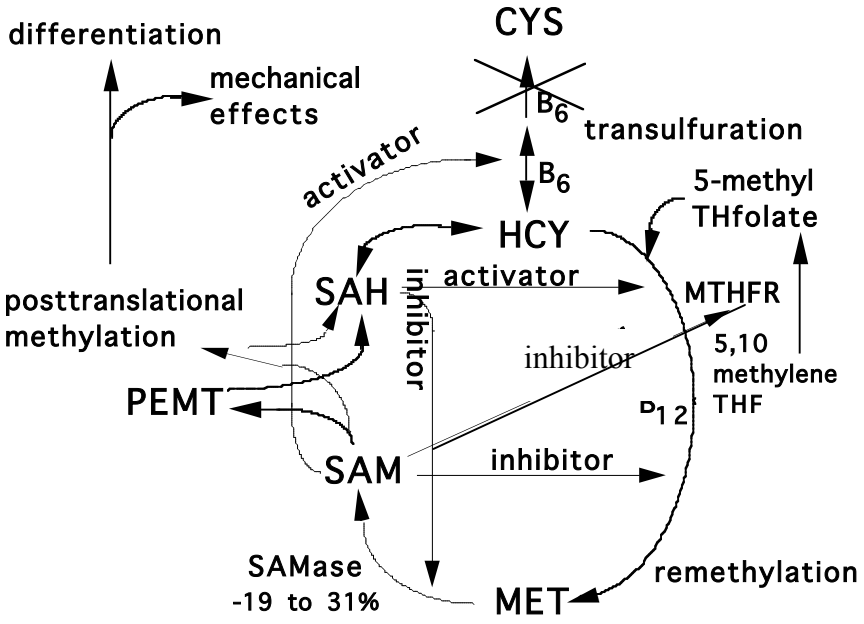
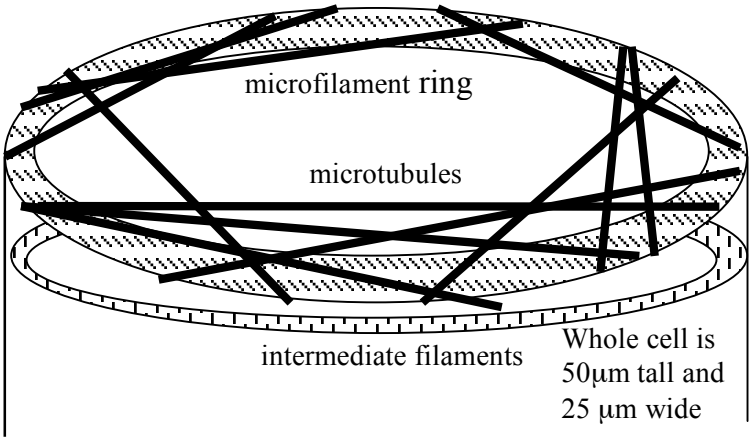


Figure 4: The apical end of a single ectoderm cell with three cytoskeletal elements forming the bistable organelle, the cell state splitter.



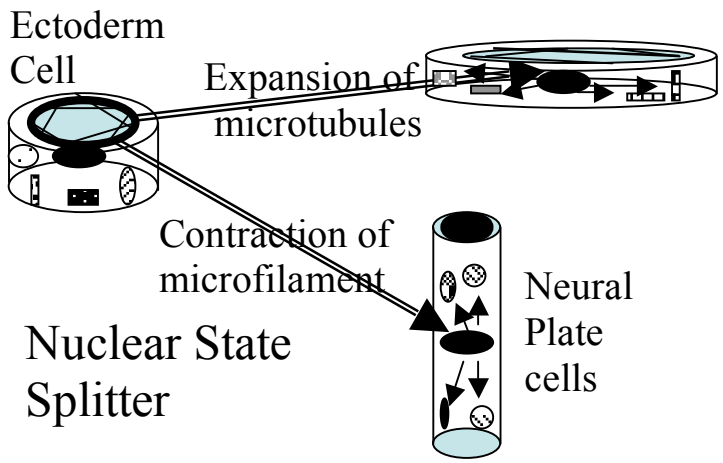
The cell state splitter model of differentiation postulates that the combination of actin, microtubules, and intermediate filaments, seen at the apical end of the neural plate cells using electron microscopy (64), forms a bistable organelle that is responsible for sensing and then propagating a contraction or expansion wave across presumptive neural epithelium (Fig. 4). Many waves of contraction have been observed in the open neural plate of the developing axolotl (*Ambystoma mexicanum*) (35). The axolotl ectoderm also has its own unique contraction wave and a corresponding expansion wave. The ectoderm contraction wave begins at the dorsal lip of the blastopore at the same time and place that primary neural induction is known to occur. Its trajectory is limited to the same region of the ectoderm that is affected by classical primary neural induction, i.e. the presumptive neural plate. After this contraction wave has ended, an expansion wave begins at the bottom of the embryo and travels upward over the region of ectoderm that forms presumptive epidermis. This expansion wave ends at the border

of the newly forming neural plate. Both these observed contraction and expansion waves are hypothesized to begin propagating due to a specific mechanical signal (34).

In the case of the axolotl's ectoderm contraction wave, the invaginating pharyngeal endoderm touches the underside of the ectoderm inducing the contraction wave. This particular wave travels over that entire presumptive neural developmental field, moving from cell to cell and thereby propagating the induction signal. The newly forming neural plate cells narrow and elongate. The neural plate thereby exert mechanical pull on the remaining ectoderm tissue in the lower half of the ectoderm. This starts an expansion wave over the remainder of the ectoderm carrying a different signal in this region (34, 65).

The cell state splitter model proposes that while each individual cell is contracting or expanding, a simultaneous chemical signal is sent to the nucleus indicating that the cell has participated in a wave (66). The nucleus responds to the expansion or contraction wave signal with changes in gene expression that allow differentiation into the next appropriate cell type (Fig. 5). Trajectory of the waves is influenced and limited by simple mechanical forces in cell sheets. The final stage of differentiation of an individual cell is the preparation of a new cytoskeletal structure capable of responding to and propagating the next wave (35).

Figure 5. Schematic of the cell state splitter.



Schematic of the determination signal sent by the cell state splitter in response to an external signal to expand or contract. The determination signal is then acted upon by the nucleus resulting in changes in expression and differentiation. Expansion of microtubules is accomplished by polymerization. Contraction is of the microfilament ring.

Contraction and expansion waves provide the spatial and physical component that is missing from most other embryological models (33). The classical primary neural induction model is unchanged except that the inducer is a mechanical signal sensed and propagated by cytoskeleton to adjacent cells. The cytoskeleton is specified in this model as that which transduces the mechanical induction signal into a chemical signal. The nucleus senses and responds to the transduced chemical signal. Contrary to the classical model, there is no default state for a tissue type that is not induced. Instead, there is an active change from a previous tissue type to one of two new cell types. The change occurs as a response to which of two types of waves the individual cell actively participates in. Although there is direct correlation between the timing and spatial components of the waves for all the inductions of all presumptive tissue types that

appear during axolotl gastrulation, a direct cause and effect relationship remains to be proven (65).

If the cell state splitter model is correct, any malfunction of microfilament function would cause NTDs by preventing contraction waves and thereby preventing localized differentiations in the open neural plate. The reduced methylations of critical regulatory sites may be the underlying mechanism behind multifactorial NTDs. Failures of differentiation may occur because malfunctioning cytoskeletal elements cannot propagate the contraction wave. These cytoskeleton malfunctions could be due to the reduced level of posttranslational methylation in apical cytoskeleton that has been observed in rat embryos with NTDs (63). In the embryo affected by a NTD, this particular failure is confined to embryonic neural tissue. The neural tissue is most affected because of its higher SAM requirement relative to other tissues. The higher requirement is due to PEMT activity creating new membranes during neural plate formation and closure. This failure (or combination of failures) is circumvented by folic acid supplementation because increasing folate increases SAM levels and allows proper methylation of the apical cytoskeletal elements of neural epithelium. Any variant enzyme that affects SAM availability is a candidate for the genetic predisposition portion of multifactorial NTDs. Reduced folate intake or increased homocysteine becomes the environmental triggers for NTDs.

NTDs insensitive to increased folates

Mutations or syndromic conditions that result in the failure of expression of any of the proteins required for cell to cell signaling, cytoskeleton to nucleus signaling, or

the subsequent differentiations would also cause NTDs. These types of NTDs would not be folate sensitive. Such mutations or syndromes could account for the 30% of NTDs that are not prevented by folic acid supplementation.

Hyperhomocysteinemia and NTDs

Hyperhomocysteinemia in the amniotic fluid was first noted in women who had pregnancies resulting in a fetus with NTDs (67, 68). Hyperhomocysteinemia has also been associated with increased rates of early pregnancy loss, IUGR and perinatal death, severe early onset preeclampsia, recurrent spontaneous abortion, and *abruptio placentae*. NTDs may represent only part of a spectrum of adverse effects due to impaired methylation, the majority of which are fatal to the embryo or fetus (69). Another possibility for the negative effects of hyperhomocysteinemia is its enhancement of thrombotic events that may be associated with specific congenital malformations such as limb reduction and gastroschisis and with placental malfunctions such as Breus mole (69).

The observations that hyperhomocysteinemia is associated with NTDs, and that folate supplementation reduced the frequency of NTDs, encouraged many researchers to begin examining the enzymes of folate metabolism for genetic variants. These variants are being actively studied for both changes in homocysteine metabolism and for association with NTDs. The first important finding was an increased allele frequency of a C677T variant of the folate-metabolizing enzyme, *methylenetetrahydrofolate reductase* in Dutch families affected by spina bifida. The

variant found more often in these Dutch families has reduced activity in the presence of low folate (9).

Methylenetetrahydrofolate reductase

Characteristics and function of MTHFR

Methylenetetrahydrofolate reductase (MTHFR) is a 77-kDa cytoplasmic enzyme. The gene for MTHFR has been localized to chromosome 1p36.3. There is evidence of imprinting effects in this region of the chromosome as well as results suggestive that the gene itself is affected by imprinting with the paternally inherited allele preferentially expressed in early pregnancy (70, 71). This enzyme is responsible for catalyzing NADPH linked reduction of $N^{5,}N^{10,}$methylenetetrahydrofolate to form $N^{5,}$methyltetrahydrofolate (5-methylTHF). The reaction is bidirectional *in vitro*, but under physiological conditions, it is unidirectional. The reaction produces 5-methylTHF. 5-methylTHF is then utilized in the production of methionine from homocysteine (see Fig. 2,3,6) (72).

MTHFR has been reasonably well characterized. The porcine equivalent has been evaluated using tryptic proteolysis. The results indicate that the enzyme has separate active and regulative regions. The C terminal 37 kDa fragment binds both SAM and SAH. SAM is an inhibitor and SAH is an activator indicating this is the regulatory region. The N-terminal region contains a binding site for FAD and NADPH. This region is highly conserved across widely divergent species including *Escherichia coli, Caenorhabditis elegans,* and *Saccharomyces cerevisiae*. There is a hydrophilic region

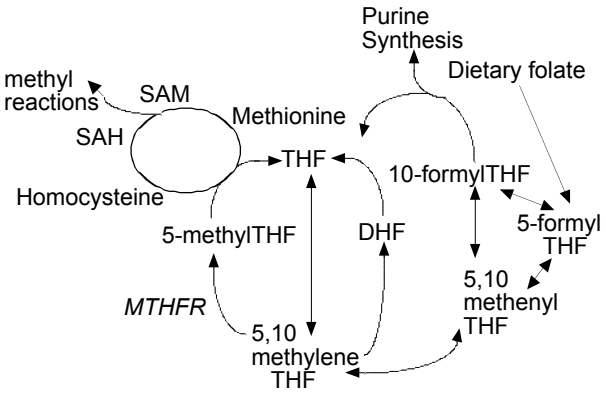
that bridges the catalytic and regulatory regions of the enzyme. Mutations in the N terminal catalytic region that cause severe deficiency have been documented (73).

The C677T MTHFR variant

The thermolabile variant of MTHFR was first described in 1988 in patients with vascular disease (74). Thermolabile MTHFR is different from the wild type MTHFR because of a single mutation. There is a C to T codon substitution at nucleotide 677 in exon 4. This substitution results in the conversion of an alanine to a valine in the catalytic region (76-77).

The variant is considered thermolabile because, *in vitro,* when transiently heated from 37°C to 46°C, it loses more than 70% of its activity. The normal enzyme loses only about half. Thermolability is also evident if the same mutation is introduced in the *E. coli* enzyme. The mechanism for the reduced activity has not been precisely documented in humans, but in *E. coli* the mutation causes the bacterial enzyme to readily lose its flavin cofactor. A similar inability to maintain the binding of the NADPH in the human version is the likely cause of the reduced activity (77, 78). Even under normal conditions, the C677T MTHFR variant has only 50% of the activity of the normal enzyme. The effect is codominant for enzyme activity. Heterozygotes have homocysteine levels between the levels of the homozygotes for the normal and the variant enzyme (76, 79).

Figure 6: Folic acid cycling with MTHFR.



THF = tetrahydrofolate, DHF = dihydrotetrafolate, SAM = S-adenosyl methionine, SAH = S-adenosyl homocysteine Adapted from (80).

The C677T MTHFR variant is particularly important as a cause of mild hyperhomocysteinemia. The elevation in serum homocysteine is present from childhood in homozygotes who also have diets that are low in folate or who have plasma folate levels that are <15.4 umol/L. There is no similar correlation with either low B_{12} or low B_6 (cofactors of homocysteine metabolism), although the use of multivitamins has been shown to lower homocysteine more than folate alone. When plasma folate levels are above the median values, no elevation in serum homocysteine occurs. The mild homocysteine elevation that occurs in a folate deficient homozygote is much less clinically than would be expected with a 50% reduction in this enzyme's activity. This indicates there are additional controls, beyond folate availability, on phenotypic expression (81, 82). The C677T MTHFR variant is thrombophilic because of its activity in increasing the levels of thrombophilic homocysteine in serum (82). In pregnancy, the C677T MTHFR variant is only important in determining homocysteine

levels if a woman is not taking multivitamins. If she is taking prenatal multivitamins, her homocysteine levels are not increased (83).

Worldwide allele frequency of the C677T MTHFR variant

The C677T MTHFR allele frequency varies considerably in different populations worldwide. It is most common among Caucasian populations bordering the northern Mediterranean coast with a gradient of decreasing frequency moving northward in Europe. Homozygosity frequencies of 10-12% occur in Spain and Italy while they are 4% in Finland. The pattern appears to approximately match average dietary folate intake with regions of high average folate intake having higher frequencies of the variant (84). This suggests that among Caucasians there may be an advantage to carrying the variant if folate levels are high and a disadvantage if folate levels are low or that survival disadvantages that would select against the variant are not present when folate is abundant in the diet.

The variant is rare among African Blacks with less than 2% of the population being heterozygous. Homozygosity is virtually unheard of. A strong negative association between the C677T MTHFR variant and sickle cell trait may indicate negative selection in Africans (85). Among Asians, the patterns appear to vary as much as among Caucasians by region, but this has not been well characterized (86).

In North America, the allele frequencies of the C677T MTHFR variant reflect the frequency of the populations' ethnic origins. Hispanic Americans have very high frequencies, Non-Hispanic Caucasians have intermediate frequencies, and American

Blacks have the lowest frequencies. Aboriginal groups in both North and South America show wide variation in allele frequency depending on their ancestral group (87-89).

In Canada, Quebec has the highest allele frequency of the C677T MTHFR variant with homozygosity frequencies of 11% while Alberta has a frequency of only 5.8% (86). Quebec was settled by French Canadians and has the highest proportion of individuals from Greek and Italian background compared to any other province. Most original settlers of Alberta are from northern Europe (86, 90). In the definitive study for Manitoba of 977 newborns, the frequency of homozygosity is 7% and 36% of Manitobans are heterozygous (10). Manitoba has the second highest population of original French Canadians outside of Quebec, and a large Metis (French/Aboriginal) population. The intermediate value found within Manitoba is not unexpected (see Appendix 13).

The C677T MTHFR variant and NTDs

As previously noted, Van der Put et al., (9) were the first to report an association between the C677T MTHFR variant and NTDs. The frequency of this common variant was found to be higher in individuals with NTDs, and their parents, than in a control group. Since then, some studies have confirmed this observation, while others have not. Inadequate or unsuitable control groups and small study size have been the most frequently discussed contributors to these conflicting results (91).

Associations between the C677T MTHFR variant and other complications of pregnancy

Reduced folate or increased homocysteine has been associated with several maternal complications of pregnancy (92, 93). It is also associated with fetal malformations other than NTDs (94). Thus, the C677T MTHFR variant, with its effect of raising homocysteine when folate intake is low, has also been examined for association with these complications (91). The variant has been positively associated with preeclampsia (95) and gestational hypertension (96). Some studies found no association between the C677T MTHFR variant and IUGR (97, 98).

There have been contradictory results for association between the C677T MTHFR variant and unexplained early loss and stillbirth, retained placenta, post partum hemorrhage and abruptio placenta. It is likely that folate supplementation is an important preventive measure for avoiding late pregnancy loss in the presence of the variant. Researchers who found no association did not control for the use of multivitamins, whereas those that had positive results found the association only among women not taking multivitamins. Not controlling for folate intake is a major flaw of many studies (99-101).

The C667T MTHFR variant may contribute to pregnancy complications by several proposed mechanisms. Most proposed mechanisms relate to increased circulating homocysteine and interference with vascular remodeling during trophoblast invasion of the spiral arteries and the replacing of maternal endothelium with fetal cells. One mechanism proposed is that homocysteine becomes a contributor to oxidative

stress on endothelial cells lining blood vessels in the placenta, thereby interfering with trophoblast function indirectly. Metabolic interactions between homocysteine and S-nitrosoalbumin and nitric oxide may result in reduced trophoblast migration because nitrous oxide is a mediator of migration (15). Alternatively, reduced nitrous oxide may result in reduced vascular tone (102). Increased levels of total plasma homocysteine are associated with an increased risk of thrombosis due to interactions between the reactive sulfhydryl moiety of homocysteine and molecules involved in coagulation cascades. This may result in thrombotic damage to portions of the placental circulation (103).

There is some evidence that the C677T MTHFR variant does not directly affect maternal homocysteine levels in pregnancy (83). The C677T MTHFR variant may affect fetal homocysteine only. The development of the placenta involves multiple differentiations of trophoblast cells. Placentas of women with preeclampsia, for example, have reduced expression of endothelial antigens and improper expression of adhesion molecules (15). The C677T MTHFR variant might be associated with placental malfunction via faulty methylation affecting differentiation in the placenta in the same manner proposed previously for failure of neural cell differentiation.

Folate supplementation has been shown in some studies to reduce the rate of cleft lip and cleft palate (94). As in the case of NTDs and the C677T MTHFR variant, the literature is contradictory. Undoubtedly there will have to be many more such studies before this question is resolved.

A single study on the association between the C677T MTHFR variant and Down Syndrome created a lay media sensation. James et al., (104) found a 2.6 odds ratio (OR) for a Down Syndrome child in mothers with one or two C677T MTHFR alleles.

Subsequent publications show the same pattern of contradictory results that exists in the case of NTDs with some studies confirming the original finding of an association and while others do not (70, 106-108).

The C677T MTHFR variant and other diseases

C677T MTHFR has been associated with vascular disease especially with atherosclerosis as related to heart attack and stroke (108-110). The variant is also associated with thrombophilic conditions especially venous thrombosis (111). The variant seems to be protective against certain cancers (112, 113) while increasing risks for others (112, 114, 115). One difficulty with evaluating any role of the variant with cancer is that, in addition to folate intake as a confounding factor, alcohol use and smoking status change the effects of the variant. For example, the variant protects against colorectal cancer except among alcohol drinkers where it increases the risk for this cancer (116). Smokers with the variant and low folate intake are at greatly increased risk for lung cancer. The risk was also higher in smokers with the variant, who had adequate folate intake, but the increase was lower (117). Any condition that may be related to elevated homocysteine could potentially be indirectly associated with the C677T MTHFR variant, because of its propensity for elevating homocysteine when folate intake is low.

Other MTHFR variants

There are several other variants of MTHFR. The A1298C MTHFR variant is another common variant that decreases efficiency. The decrease is not as much as that created by the C677T MTHFR (118). As with the C677T MTHFR variant, there is a large and contradictory literature on the effects of other variants of MTHFR on the risk for pathology such as NTDs and vascular disease. Some may interact with the C677T MTHFR while others may not. There are also other sequence changes of MTHFR itself. Some of these cause severe deficiency and significant elevations of homocysteine. Compound heterozygosity, as in C677T MTHFR and A1298C accounts for some of the variability of elevations of homocysteine found with the C677T MTHFR variant (119).

Interactions with other variant enzymes

Numerous other genetic variants have been suggested as possible candidates for causing folate sensitive NTDs. Other candidate enzymes include, *betaine-homocysteine methyltransferase* (exon 6-R239Q), transcobalamin (TCN2) (I23V, G94S, P259R, S348F, G776C, and R399Q), *methionine synthase* (MTR) (A2756G*), methionine synthase reductase* (MTRR) (A66G, C524T), *cystathionine beta-synthase* (-5697 (GT) STR, C1080T, C699CT, 844ins68*), glutamate carboxypeptidase II* (GCPII) (C1561T), and reduced folate carrier RFC-1 (A80G) (81, 120-125).

Some of these polymorphisms increase homocysteine and others decrease it. Some interact with folate and other B vitamins while others do not. Combinations of some variants interact synergistically to produce unexpectedly large increases in homocysteine (126). Other combinations can be protective; lowering homocysteine

despite the presence of a variant normally associated with increased homocysteine. These other variants may provide some explanation for the apparently contradictory results of the C667T MTHFR variant association studies in different populations.

Maternal serum α-fetoprotein screening (MSAFP)

α-fetoprotein

α-fetoprotein (AFP) is the most common protein in fetal circulation after albumin. AFP is a glycoprotein. It has a molecular weight of 68,000 daltons. It is initially produced by the fetal yolk sac and later by fetal liver. The function of AFP is unknown. It reaches a maximum concentration (3.0-4.0 g/L) at 12-15 weeks gestation and then gradually decreases to term. AFP is also found in the amniotic fluid and it enters the maternal circulation. Levels in maternal serum start to rise above pre-pregnant levels at about 13 weeks gestation, continue to rise until approximately 30-32 weeks gestation (about 250 mg/L) and then gradually decline. Fetal plasma concentration increases to a maximum between 13-14 weeks of gestation. Maternal serum levels peak at about 30 weeks. After birth, maternal and infant AFP rapidly decline (127).

Significant elevations of amniotic and maternal serum AFP have been shown to be associated with spina bifida and other forms of open NTDs since 1973 (128). The usefulness of MSAFP for prospective screening of open NTDs was first suggested in 1977 (4). It is now offered in many places around the world as a screening test. It has also proven its usefulness in screening for other complications of pregnancy such as abdominal wall defects (129).

MSAFP screening

The level of MSAFP that is considered clinically relevant is affected by several factors including ethnicity and variations of practice within individual laboratories. Black women have MSAFP levels about 10-15% higher compared to Caucasians, Asian women have levels 4% lower and Aboriginal women have levels about 4-5% higher (130). MSAFP is measured in 'multiples of the median' (MOM) for gestational age. Every laboratory determines its own medians by measuring the concentrations of MSAFP in collections of normal pregnant women from their own populations. Cut off values vary somewhat between centres to achieve appropriate sensitivity (detection rate) and specificity (which impacts on false positive rates). Triple testing, by adding measurements of human chorionic gonadotropin (HCG) and estriol increases both sensitivity and specificity for Down Syndrome and, to a lesser extent, for NTDs. For example, a combination of elevated MSAFP and low estriol indicates anencephaly and is useful for ruling out twin pregnancies (131). The improved sensitivity is particularly important for screening for chromosome anomalies.

Gestational age also affects the accuracy of MSAFP testing. Testing before 15 weeks or after 22 weeks decreases sensitivity (132, 133), although some more recent research disputes this earlier conclusion (134). There are also ethical concerns about delaying diagnosis of Down Syndrome (which is done using amniocentesis) beyond the gestational age when a termination of pregnancy can be offered (135). One US survey found that only 10% of laboratories offer MSAFP for Down Syndrome screening after 22 weeks (136).

For every 10,000 women whose pregnancies are screened, approximately 400 (4%) will have an elevation of MSAFP (137). Fetal assessment with ultrasound is normally recommended for these women. Of the 400, about 8 women will have a baby with spina bifida. Other abnormalities such as anencephaly, gastroschisis, and omphalocele also cause elevated MSAFP. In total, only about 16 of the 400 women with an elevation of MSAFP will actually have a baby with an abnormality. Twins (15%), overestimation of dates (10%), and early fetal death, are common causes of an elevated MSAFP. Other explanations include uterine bleeding, placental anomalies, and certain rare conditions (e.g. congenital nephrosis). About 240 of the 400 women with an elevation of MSAFP (60%) have false positive tests for NTDs and no other explanation is found for their positive result. Women who do not have an explanation for their elevation of MSAFP are at increased risk for low birth weight, prematurity, impaired fetal growth, perinatal deaths, oligohydramnios, and maternal hypertensive disorders of pregnancy (12).

In Manitoba, the MSAFP test has been offered on a voluntary basis to all pregnant women since 1985. (See Chapter 5 for exact numbers on how many women choose MSAFP.) It is normally drawn between 16 and 20 weeks, but it is available for later gestational ages of 21 to 24 weeks. In Manitoba, an MSAFP test is considered positive if the MOM is ≥2.3 (elevated and therefore indicating an increased risk for NTDs) or ≤0.25 (low). After a positive test, women are offered fetal assessment by ultrasound, usually in Winnipeg, and possibly amniocentesis. If no cause is found for the elevation of MSAFP, women are invited to return for up to two more ultrasound assessments

because of their increased risk for later complications. Triple testing with HCG and estriol was added to the Manitoba screening programme in 1999 and are used with MSAFP and maternal age to derive specific risks for Down syndrome and trisomy 18 (135).

Summary of Background

In summary, there are two interacting observations. Elevated MSAFP is associated with certain complications of pregnancy, especially NTDs. The C677T MTHFR variant is associated with the same complications of pregnancy in at least some studies, but not in others. What remains to be clarified is the relationship between the C677T MTHFR variant and elevations of MSAFP to subsequent complications of pregnancy and whether this has created a bias in previous C677T MTHFR and NTD studies.

Methods

Detailed Methodology

Ascertainment of Cases and Controls

Pregnancies resulting in NTDs were ascertained using three sources described below. This combination of active and passive surveillance ensured that early terminations (from 14-20 weeks gestation) and early intrauterine deaths were included in the total number of NTDs (138).

i) MSAFP Screening Programme

The primary source of information for this study was the outcome records of the Manitoba Maternal Serum Screening Programme (MMSSP). MSAFP screening has been available on a limited basis since November 2, 1983 and was offered Province wide by 1985. As of April 1, 1999, the programme has been expanded to include triple testing with HCG and estriol. The programme keeps detailed records of outcomes of all screen positive mothers.

ii) Section of Genetics and Metabolism

Babies born with NTDs and their family members (often including the extended family) are usually referred to the Section of Genetics and Metabolism at the Health Sciences Centre in Winnipeg for genetic counseling. This section provides all genetic counseling services within the province. The database was searched several times

during the course of the studies. All files from the database related to NTDs were pulled and crosschecked against MSAFP outcome files.

iii) Department of Biochemistry and Medical Genetics

NTD cases have also been tracked by the University of Manitoba's Department of Biochemistry and Medical Genetics (until 1999 the Human Genetics Department) for research purposes. These research files came via the Province of Manitoba's Congenital Anomalies Registry, the Section of Metabolism and Genetics, and the MSAFP screening programme and through governmental sources.

1) Contacting NTD Study Cases and Controls

Mothers of NTD affected cases were contacted at their last known address by letter (Appendix 2a). The letter was from the geneticist with whom they initially had contact with during the fetal assessment period and, for most families, during post-termination counseling and/or counseling during a subsequent pregnancy. The letters these mothers received included an explanation of the study (Appendix 3a) with a consent form and a self-addressed, postage paid return envelope.

Women who did not respond to the letter were contacted by telephone to confirm the letter had arrived and to request a response. This step was taken to increase response rates and to reduce non-responder bias (139). Those who requested more information were also contacted by telephone. All aspects of the study were approved in advance by the University of Manitoba's Research Ethics Board (Appendix 4).

Control mothers were also contacted initially by letter (Appendix 3a). This letter was from Dr. Cheryl R. Greenberg and contained an introduction to the study that was identical to the one received by case mothers and a similar consent form with postage paid return envelope (Appendix 3b). Control mothers who did not respond to the letter were also contacted by telephone to confirm that the letter had arrived and to request a response. Those who requested additional information were also contacted by telephone.

MSAFP Study: Contact of Cases and Controls

The same methodology was used for contacting the cases and controls for this study (Appendix 5a,b, 6a,b). Ethics approval was also provided by the Faculty of Medicine Research Ethics Board (Appendix 7).

Questionnaires

All mothers who agreed to be in the study were sent two questionnaires in a single package by mail. The first questionnaire was a detailed analysis of family medical history (Appendix 8). The second questionnaire was a food and supplement intake survey (Appendix 9). The package also included a postage paid envelope for return. A telephone number was provided to participants so that they could contact researchers as questions arose. A pager number was also provided so that participants could receive immediate assistance outside of regular office hours.

Family History Questionnaires

i) Ethnicity

During preliminary work testing the questionnaires, it was noted that Manitoba individuals often cite one ethnicity, for example that of their surname, to claim as their own while ignoring other ethnic contributions to their background. (See (140) for commentary on this problem when dealing with Caucasian populations.) To avoid missing ethnic data, mothers were also asked to specify the ethnic background of each of the grandparents of their baby. The results were then compared to the ethnicity stated for the parents. In cases where there was a conflict between the parents' stated ethnicity and the grandparents' stated ethnicity, the ethnicity was determined from that of the grandparents.

ii) Geographic location of participants

Geographic location within the province was determined for each woman. Participants were divided into regions. The divisions were City of Winnipeg (within the perimeter boundary), a southern town/city outside of Winnipeg and large enough to have a hospital with an obstetrical service, a southern village/town without an obstetrical service, a northern town/city large enough to have an obstetrical service, a northern village/town without an obstetrical service, and a First Nations Community. The north/south boundary chosen for the province was the bottom tip of the two central lakes, Lake Manitoba and Lake Winnipeg. Address was based on mailing address.

iii) Medical history of family members

The questionnaire was based on the standard form used by the Section of Genetics and Metabolism. This form is mailed out to new patients prior to a first genetics counseling session. It was modified slightly for the purposes of this study. The conditions mothers were specifically asked to comment on were: any type of blood clotting problem (example phlebitis, cancer, chromosome abnormality (e.g. Down syndrome), cleft lip or palate, diabetes, infertility (sterility), heart conditions, heart attacks, high blood pressure, high cholesterol, miscarriages or still births, malformations or birth defects, obesity, spina bifida, stroke, vascular problems.

Information from the questionnaires was used to construct a pedigree. All medical conditions noted for any family member were also recorded in a database that could be rapidly searched by subject (141). As the NTD was listed as part of the history, it was impossible to be blinded to which family was case and which was control in the NTD and C677T MTHFR association study (Chapter 1). To minimize bias, the returned questionnaires were analyzed in the same manner each time in a random mix of cases and controls. In the second study of elevated MSAFP and C677T MTHFR (Chapters 2, 3), this potential for bias was removed and there was no way to identify case and control while compiling the family history data.

General categories were prepared for each group of relatives in this database. In each group, the age of the individuals (except for cousins and aunts and uncles where the numbers were very high) as well as any additional information was recorded. If a person was deceased, the age at time of death was used for the analysis.

iv) Family medical history data analysis

The data were analyzed by first listing all reported medical conditions/risk factors in all families. Totals were tallied for each category of relative (aunts, grandparents etc.) and for cases and controls as a group. Occurrence rates for the total case and control groups were calculated for each condition and in each degree of relative. Statistical significance was calculated for any result with a difference of 2% or more. Disorders that could be categorized in groups (such as 'all cancers') were then collected and analyzed in the same manner.

Data on all heart attacks and strokes were examined for associated risk factors. Each individual was initially given a score of 1. From that risk a lifestyle, related health condition, and age risk adjustment was made (Table 1 and (110, 142). After the risk factors adjustment, the case and control groups were compared.

v) Accuracy of reported history

Information about women and their partners was recorded at the time of the MSAFP pre-fetal assessment counseling session. This record was used to check the information provided by the parents in the study questionnaire for accuracy as well as to look for recall bias in the case mothers compared to control mothers.

<u>Food frequency questionnaires</u>

The second questionnaire was designed to allow evaluation of current total folate intake from both natural folate and synthetic folic acid. Dietary folate was determined

using a semi-quantitative food frequency survey. The survey was based on established methodology (143, 144), and using modified sample food frequency questionnaires (FFQs) (145-148). These were modified slightly to include some additional items commonly consumed in Manitoba such as wild rice and game meats. Some names of foods were changed to those colloquially used in Manitoba. The survey had several places for "other" entries so mothers could add anything they ate that was not mentioned by the survey (Appendix 9).

Folate values for each food consumed were determined from one of two sources. All vegetables, fruits, dairy, and meats or meat alternate values were taken from the USDA National Nutrient Database (149). This database is superior to others because it is based on bioavailable folate as well as several serving sizes and preparation methods.

The United States began fortification of certain cereal grain products in 1998, including rice and pasta (150). The USDA database does not differentiate synthetic from naturally occurring folate. As dietary intake was being calculated for both the time of the pregnancy and the time of the survey, two values were required, often with one before fortification and one after. Accordingly, grain group pre-fortification values were obtained from two different sources. In the case of processed and packaged cereal foods (example 'Special K'), values used were those provided by the manufacturer where available. Where manufacturer's data was unavailable, another standardized reference was utilized (150).

Table 1: Computation of lifestyle and age risk adjustments for myocardial infarct (MI) and stroke.

Adjustment to risk scores	**Stroke (110)**	**MI (148)**
initial score	number of individuals	number of individuals
gender	subtract ten years from age for women	subtract ten years from age for women
age	after age 55 multiply result by 2 for each decade. Subtract number of individuals to get age adjustment	after age 55 - 64 multiply result by 1.2/decade. Subtract number of individuals to get age adjustment
smoking	multiply #smokers by 2 for each decade after age 20 that an individual smoked. subtract number of actual smokers from result to get smoking adjustment	multiply #smokers 1.56 for each decade after age 20 that an individual smoked. subtract number of actual smokers from result to get smoking adjustment
coronary artery disease	add one to score for each person with CAD	add one to score for each person with CAD
hypertension	multiply #hypertensives of by 3.1, subtract original # and add remainder to total	multiply #hypertensives of by 2.1, subtract original # and add remainder to total
diabetes	multiply # diabetics by 2.9, subtract original #, and add to total	multiply # diabetics by 1.5, subtract original #, and add to total
atrial fibrillation	add 1/individual	add 1/individual
high cholesterol	add 1/individual	add 1/individual
MVP or valve replacement	add 6/individual	do nothing
heart disease	add 1/individual	add .2/individual
migraines	add .1/individual	do nothing

For our survey, vegetables were assumed to be either fresh (as in lettuce) or boiled (as in peas) depending on how they are commonly consumed in Manitoba based on information provided by 'Peak of the Market' a province wide vegetable growers association located in Manitoba. Values were assigned accordingly. Fruits were assumed fresh and raw unless the mothers noted otherwise. Meats were assumed to be cooked by roasting and to be mixed cuts with trimmed fat. Poultry values were assumed to be mixed white and dark meat portions with skin attached. Caribou and deer meat did not have values from the USDA database so the values for beef were substituted. Adjustments were made for vegetable preparation based on the answers provided reducing folate values by half for cooking.

The dietary survey was scored with the researcher blinded as to whether or not the respondent was a case or a control. Data were prepared using a spread sheet programme (141, 151). The programme summed total intake over a one month period and divided it by 30 to give a typical daily intake value. Zero scores were given for rarely or never consumed foods. Some mothers commented about eating certain fruits only when they were 'in season'. Pregnancy stage was determined and "in season" foods were only included if the season corresponded to the stage of pregnancy under study (First trimester for the NTD study, third trimester for the MSAFP study.)

There were additional questions at the end of the survey asking mothers if they were presently taking a vitamin supplement and what type and brand it was. They were also asked if they had taken any supplement before becoming pregnant or after they found out they were pregnant. Mothers who reported taking supplements

periconceptionally were asked why they decided to take supplements. Those who reported beginning during pregnancy were asked at how many weeks past their first missed menstrual period did they begin supplementing and why. Values for the periconceptionally period, pregnancy, and post pregnancy were adjusted to account for multivitamin use.

Values of synthetic folic acid consumed in supplements were included separately. As vitamins are standardized, there was little discrepancy between brand types. For most women the value for folic acid was 400 μg. A value of 800 μg was used for prenatal vitamins in the calculations. Values for 1999 pregnancies were increased to 1000 μg as an increase to the level of folate in prenatal vitamins reached the local store shelves at the end of 1998.

Identical methodology was used for B_6 and B_{12} values. Additional values for B_{12} and B_6 were added to the dietary values for women who reported supplementing. For regular multivitamin supplements, the value for B_{12} was 9 μg. For B_6 the value was 3 mg. For prenatal vitamins, values for B_{12} were 12 μg and for B_6, 6mg.

Biochemical Analysis

Mothers were sent requisition forms by mail (along with forms for the fathers where mothers reported the fathers had agreed to participate, see Appendix 10). Parents were instructed to take the requisition to their doctor's office or laboratory of their choice. Instructions on the requisition requested that three tubes of blood were to be collected. Two tubes were for biochemical analysis and the third tube was for later

DNA extraction and banking. These were forwarded to the Health Sciences Centre in Winnipeg (HSC), usually on the same day. The requisition for the blood samples included instructions that for the total plasma homocysteine and serum folate testing, samples were to be spun and serum separated at the draw site if the blood could not be delivered to HSC for analysis the same day.

The biochemical analyses were preformed in the Dept. of Clinical Chemistry, HSC. Biochemical parameters measured were total plasma homocysteine, red blood cell folate, and serum folate in mothers using established methodology (152, 153). Total plasma homocysteine was also measured in fathers.

DNA was extracted according to previously established methods (126, 154). PCR analysis for the presence of the C677T MTHFR substitution and the A2756G MTR substitution was carried out according to standard procedures as follows. The PCR reaction was carried out in a final volume of 100 μL, containing approximately 100ng of genomic DNA, 100 pmol of each primer VD-1 and VD-2, 2.5 units of Taq polymerase (Gibco), 200 μM dATP/dCTP/dGTP/dTTP, 1.5 μM MgCl and 1x PCR buffer. Amplification, carried out on a Perkin-Elmer 9600 thermocycler, consisted of an initial denaturation step at 94^{o}C for 1 minute followed by 30 cycles at 94^{o}C for 30 seconds, 55^{o}C for 1 minute at 72^{o}C, followed by a final extension at 72^{o}C for 10 minutes. For MTHFR 15 μL aliquot of each PCR reaction was digested with Hinfl for 1 hour on a 10% polyacrylamide minigel at 200V for 45 to 60 minutes. The gel was stained with ethidium bromide and photographed. The presence of the T variants was

indicated by the absence of the HinfI restriction site. The A2756G MTR variant created a HaeIII restriction site.

Statistical Analysis

Parametric data were analyzed with χ^2, *t* test for difference between means, and Fisher's exact test as appropriate (two tailed unless otherwise stated). All data not normally distributed were analyzed using the nonparametric "Mann-Whitney Rank Sum Test" with Yates correction for continuity used to determine *p* values. Software for linear regression and discriminant function analysis was initially SPSS (1997) however all statistical analyses were redone after the third year of the project using NCSS (2001) (155). For linear regression, the results of the biochemical analysis, genotype analysis and dietary intake survey were examined for associations. The mother's location within the province (based on address at time of MSAFP), smoking (both at time of pregnancy and at time of survey), and gender of the baby of participants were examined for cases and controls both nonparameterically and via a numerical assignment for discriminant function analysis. The discriminant function analysis included age, gravidity, parity, smoking at time of pregnancy, previous pregnancy loss, MSAFP, weeks of gestation and mother's weight at the time of MSAFP test, presence of the C677T MTHFR variant (and A2756G MTR where available), gender of baby, mother's and father's biochemical parameters, and FFQ results for folate, B_{12} and B_6.

The fact that several participants declined to participate in some part of the study meant that the final dataset analyzed had missing values. All statistical analyses were

done in three ways. First, all data sets analyzed comprised only those with all the pertinent data points. Second, the analyses were completed with all the missing data points filled in by "dummy" variables, either a mean value or a median value depending on whether or not the value was for a parametric or a nonparametric value. Third, the data were examined allowing the NCSS programme to deal with missing data points automatically, meaning that in some analyses some portion of the partial responses were excluded and some were included depending on how the programme functioned. There was no significant difference in the results when the three outcomes were compared except that, in some multivariate analyses, the lack of data points resulted in the programme giving warnings about lack of convergence with datasets where only those with complete data were used, but not where data with missing values had medians and means inserted. Results where there was lack of convergence were excluded. Which of the three results was reported in each of the chapters was based on clarity and is detailed in each chapter. There were no instances where an individual result was statistically significant using one method of analysis and not statistically significant using another.

Author's contributions

First drafts of request for ethics approval were prepared originally by Cheryl R Greenberg for the NTD Study and by the author for the MSAFP Study and then revised as needed and submitted with CR Greenberg and Bernard N Chodirker. The author also assisted extensively with all grant applications for funds, both successful and otherwise.

Review of the records, definition of case and control and decision to include or exclude any given file was done by the author although there was occasional consultation with CR Greenberg, Jane A Evans, Karen MacDonald and BN Chodirker on some specific cases. Margaret Coggrave did all Congenital Anomalies Registry searches. BN Chodirker did all the searches of the MSAFP database.

Determination of inclusion criteria for women in this study were prepared by the author subject to approval of JA Evans and CR Greenberg, with input from BN Chodirker on several specific individuals and Carol E Schneider on some details.

Pregnancy outcomes were prepared by M Coggrave although approximately 15% of file outcomes were completed in a preliminary fashion for use in the study by the author and results were later confirmed by M Coggrave.

The family history questionnaire was prepared by the author using the HSC Genetics Department family history questionnaire as a template detailed below. The dietary intake questionnaire was developed by the author beginning with a standardized semi-quantitative food frequency questionnaire (FFQ). The survey was then modified to include common items in the general Manitoba population and the Aboriginal population. (Lorraine Tobacco, of Thompson MB, was instrumental in making the survey more appropriate for First Nations' people by providing recipes for traditional dietary items such as bannock.)

The dietary survey was also designed by the author to determine multivitamin and supplement intake periconceptionally, during pregnancy and present use. The survey was tested for readability and utility by individuals not involved in the study, Caroline Ben Ari, Lana Hunstad (then grade seven), Alan Hunstad (then grade nine), Richard

Gordon, Connie Gaszener, and Vanessa Gaszener (then grade eleven). Analysis of the survey required developing a spreadsheet from a Macintosh computer based Access programme for ease of data entry and analysis of total intake, which was prepared by the author (141). About one quarter of the data entry was completed by summer students Christine Mesa and Christina Richmond working under the supervision of the author, the balance of the data entry and all analysis was completed by the author

The survey was validated by comparing the intake reported to maternal serum folate and red blood cells folate results. Initially, all statistical analysis was completed by Lawrence Erdile, but in the final two years of the study, statistical analysis was completed by the author with some methodological consultation with Thomas Hassard.

Biochemical analysis was completed in the HSC clinical chemistry laboratory. DNA analysis was completed in the laboratory of CR Greenberg by Rupinder Singal assisted by C Mesa and C Richmond except for a few that were completed in the HSC laboratory. Development and design of the laboratory requisition forms was completed by CR Greenberg and Marilyn Henke for the NTD Study and subsequently modified by the author and M Henke to improve clarity and better detail handling and billing procedures. The author and CR Greenberg together handled coordination of the outside laboratory collection sites and administrative details. Gerhard Dyck developed the billing procedure for outside laboratories and assisted with modifications of the requisition form in order to facilitate billing.

Summary of Methodology

This work consisted of five parts

1) Selection of cases and controls according to study design

2) Construction of the study assessment tools for dietary intake and family history as well as collection and analysis of the data

3) Biochemical and genotyping sample collection and laboratory analysis

4) Statistical analysis of all data.

5) Interpretation of results

Except for the actual laboratory analysis portion, the author was the primary contributor under the supervision of JA Evans and CR Greenberg with the assistance of the Advisory Committee and others.

Chapter 1: NTD Study Results: The C677T *5,10methylenetetrahydrofolate reductase* variant is associated with unexplained elevations of maternal α-fetoprotein in pregnancy.

Abstract

Introduction: The two common enzyme variants of homocysteine metabolism that have been tested by others for association with folate sensitive multifactorial NTDs are the C677T variant of *5,10-methylenetetrahydrofolate reductase* (C677T MTHFR) and the A2756G variant of *methionine synthase* (A2756G MTR). We hypothesized that these two variants are contributing genetic factors to multifactorial NTD incidence in Manitoba.

Methods: The frequency of the two variant alleles was compared in two groups ascertained after an elevation of MSAFP. One group was comprised of women whose pregnancy resulted in a NTD outcome (cases) and their partners. The second group was women with unexplained elevation of MSAFP (study controls) and their partners. The allele frequency for the C677T MTHFR variant in the Manitoba population had been previously established at q = 0.2497 by genotyping of 977 Manitoba newborn screening blood spots and this group was used as a population control. The C677T MTHFR allele frequency of both cases and study controls was compared to the population controls.

Results: Unexpectedly, we found that the C677T MTHFR variant is associated with unexplained elevations of MSAFP (study controls) (OR 1.64, 95% CI 1.06–2.54, p=0.012). Although homozygosity for the C677T MTHFR variant has been associated with third trimester placental anomalies and other complications of later pregnancy, this is the first report of elevations of MSAFP with no NTD being associated with increased allele frequency of the C677T MTHFR variant.

Introduction

NTDs are one of the more common and severe congenital malformations. Seventy percent of NTDs are preventable by taking folic acid periconceptionally (7). Folic acid provides a critical methyl group needed for normal protein, lipid and DNA synthesis during development. Some babies with NTDs, and/or their parents, may not effectively metabolize folic acid because they have variant alleles of the enzymes involved. This would result in a genetic predisposition to NTDs when folate intake is low (8).

Many previous studies have documented an association between the C677T MTHFR variant and NTDs (91). Many of these studies did not attempt to provide as near to complete ascertainment of all NTDs within a population as is possible. Also, the baseline allele frequency of the C677T MTHFR variant in their "general" population, as opposed to their controls, was unknown. Similarly, there have been several recent studies of the A2756G MTR variant and its possible association with NTDs. These have also had contradictory results (reviewed in Chapter 4). We hypothesized that both the C677T MTHFR and the A2756G MTR variants would be more common among

women and their partners with a NTD pregnancy outcome. This preliminary pilot study began to test the feasibility of a larger study to explore this hypothesis.

Methods

Case and control selection

Pregnancies resulting in babies with NTDs were ascertained from multiple sources. Inclusion criteria for the study group were) multifactorially inherited, isolated (i.e. no other malformations and a normal or presumed normal karytope), and elevated MSAFP at 16-24 weeks gestation with no other anomalies. Inclusion criteria for study controls were based on an unexplained elevation of MSAFP with a healthy baby after delivery. All MSAFP files were reviewed forward numerically from the file number of a NTD case until two control matches were found. Matching was based on mother's birth year (±3 years), mother's parity at screening, and the gender of the baby. After approval of the research protocols by the University of Manitoba Health Research Ethics Board (Appendix 4), all women ascertained were invited to participate by letter. Detailed methodology of study protocols and procedures can be found in the Methods section.

Results

Participants

Manitoba Maternal Serum Screening Programme began in 1983 and, by 1999; MSAFP levels had been determined in 130,409 pregnancies. A total of 169 fetuses/infants with NTDs were ascertained during this period. Thirty-nine cases of NTDs were associated with other anomalies, 28 cases were screen negative NTDs, and these were excluded from the study. The remaining 102 cases formed the study group. Twenty-eight cases and 38 study controls completed the dietary survey. Twenty-five cases and 32 study controls provided blood for the biochemical and genotype analyses. Twenty partners of cases and 19 partners of study controls also provided blood samples. The study population is summarized in Table 2.

Table 2: Study Participation

	Cases:	**Study Controls:**
Total # of eligible mothers	102	183*
Agreed to participate	40 (39%)	53 (29%)
Declined to participate	19 (19%)	29 (16%)
Lost to follow-up	25 (25%)	56 (31%)
No response	18 (18%)	45 (25%)
Results from those who agreed to participate	**40 Total**	**53 Total**
Family history completed	29 (73%)	38 (72%)
Dietary survey completed	28 (70%)	38 (72%)
Mother's biochemical, molecular testing	26 (65%)	33 (62%)
Father's biochemical, molecular testing	20 (50%)	19 (37%)

*21 study controls were missing due to a lack of a suitable match. 1 case couple tested only for the C677T MTHFR variant.

Questionnaire and biochemical results

The validity of our dietary survey was demonstrated by linear regression analysis. Consistent with known homocysteine metabolism, a negative correlation existed

between serum homocysteine and red blood cell folate (p = 0.002) and serum folate (p = 0.029) at time of study. High intake of combined folate and synthetic folic acid by the FFQ at time of study was associated with a high RBC folate in both cases and study controls (p =0.002). The nonparametric analysis of location within the province, smoking patterns, and gender of baby of participating parents revealed no significant differences between cases and study controls except that mothers of a NTD baby were more likely to report having quit or reduced smoking since delivering a baby with a NTD. Discriminant function analysis did not reveal any statistically significant novel associations.

Study control babies had mean birth weight of 3371 g (range 1906-4775g) with delivery at a mean gestational age of 39 weeks (range 33 to 41.5 weeks). Demographic analysis of the cases and study controls yielded no significant differences between the groups. There were no significant differences in dietary intake of folate B_{12}, or B_6 or in the biochemical parameters between case and study control mothers either at time of pregnancy or at time of biochemical analysis (Table 3 and 4).

There were no statistically significant differences in the frequencies of any disorder in the immediate families of the cases or controls. Specifically, there was no statistically significant evidence of differences in the frequencies of cancer and vascular disease between case and control families.

Table 3: Demographic data on cases and study controls:

	Case Mothers (N= 25)			**Study Control Mothers (N = 32)**		
	Mean	Median	Range	Mean	Median	Range
maternal age (years)	27.8±5.22	28	19-39	29.3 ±4.6	29	20-37
gravidity	1.96 ±1.31	1	1-6	1.88 ±1.13	2	1-6
parity[1]	0.76 ±1.20	0	0-5	0.72 ±0.81	1	0-3
prior loss[2]	0.20 ±0.58	0	0-2	0.16 ±0.72	0	0-4
weight (kg)[3]	76 ±19	76	44-111	69 ±17	65	37-110
MSAFP in MOM	5.5 ±3.8	4.8	2.3-19.0	3.9 ±4.4	2.65	2.3-25.3
gestational age in wks[3]	17 ±1.9	16.5	15-23.5	17 ±1.6	16.5	15-20.5

[1]The zero parity refers to the fact that majority of babies with NTDs did not survive to term. [2]Refers to a miscarriage or stillbirth in a pregnancy that occurred before the pregnancy examined for the study. [3]Refers to the time of the elevation of MSAFP.

Table 4: Dietary and biochemical parameters in cases and controls

			Cases			**Study Controls**	
Parameter	**Minimum/ Normal**	**N**	**mean**	**Range**	**N**	**mean**	**range**
folate[1]	0.4 mg/day	28	0.21	0.19 - 1.24	37	0.38	0.10 - 1.90
B_{12}[1]	0.24 mg/day	28	0.38	0.05 - 0.94	37	0.55	0.05 - 1.34
B_6[1]	1.6 mg/day	28	1.77	0.59 - 3.99	37	1.80	0.50 - 4.33
serum folate nmol/L	7.0 – 28	25	31.1	15.4 - 45	28	28.2	18.1 - 45
RBC folate nmol/L RBC	430 - 1250	25	1267	634 - 2033	30	1242	784 - 1904
maternal homocysteine μmol/L	4.5 - 13.0	25	8.6	4.6 - 13.9	31	9.2	5.1 - 17.6
paternal homocysteine μmol/L	4.5 - 13.0	18	9.7	7.5 - 13.6	18	9.5	5.3 - 14.3

[1]Intake was estimated at time of conception and including supplemental sources of synthetic folic acid. There were no statistically significant differences between cases and controls.

Genotype Results

There is no established baseline frequency for the A2756G MTR variant in Manitobans. The results for the A2756G MTR variant were similarly limited with power ranging from only 8-19% and so no conclusions can be drawn from this sample. Results are summarized in Table 5. For comparisons between cases and controls the power of the analysis was ≤ 20% indicating our sample size was too small and should have been in the range of 150 cases in order to get a true negative result.

The allele frequency for the C677T MTHFR variant in the Manitoba population was previously established at q = 0.2497 (10). The study control parents had an allele frequency of q = 0.3558, (OR 1.64 95% CI 1.06-2.54, p=0.012), significantly higher than the population controls. All other analyses were negative, but the results were hampered by low sample numbers, reducing power. Results are summarized in Table 6.

Table 5: Results of MTR genotyping

Subjects	**N**	**A/A**	**A/G**	**G/G**	**A**	**G**
Control parents	50	39 (78%)	9 (18%)	2 (4%)	87 (87%)	13 (13%)
Control mothers	32	23 (72%)	7 (22%)	2 (6%)	53 (82%)	11 (18%)
Control fathers	18	17 (94%)	1 (6%)	0 (0%)	35 (97%)	1 (3%)
Case parents	44	27 (59%)	15 (33%)	2 (4%)	69 (78%)	19 (43%)
Case mothers	25	13 (52%)	10 (40%)	2 (8%)	36 (72%)	14 (28%)
Case fathers	19	14 (74%)	5 (26%)	0 (0%)	33 (87%)	5 (13%)

Table 6: Results of MTHFR genotyping

Subjects	**N**	**C/C**	**C/T**	**T/T**	**C**	**T**	**OR**
Manitoba population	977	557 (59%)	352 (36%)	68 (7%)	1466 (75%)	488 (25%)	-
control parents	52	19 (37%)	28 (56%)	4 (8%)	66 (65%)	36 (35%)	OR 1.64 (1.06-2.54) *p*=0.012*
control mothers	32	12 (36%)	17 (55%)	3 (9%)	41 (64%)	23 (36%)	OR 1.67 (0.97-2.91) *p*=0.066†
control fathers	19	7 (37%)	11 (58%)	1 (5%)	25 (66%)	13 (32%)	OR 1.56 (0.75-3.21) *p*=0.267‡
case parents	46	23 (50%)	18 (41%)	5 (9%)	64 (70%)	28 (30%)	OR 1.13 (0.81-2.12) *p*=0.291
case mothers	26	13 (50%)	10 (38%)	3 (12%)	36 (69%)	16 (31%)	OR 1.36 (0.75-2.45) *p*=0.430^{+}
case fathers	20	10 (50%)	8 (40%)	2 (10%)	28 (70%)	12 (30%)	OR 1.317 (0.67-2.58) *p*=0.588$^{\sim}$

T/T=homozygote for thermolabile variant, *statistically significant. C/C=normal type, C/T=heterozygote for thermolabile variant, †power 45% recommended sample size 36, ‡power 20%, recommended sample size 78, $^{+}$power 18%, recommended sample size 90, $^{\sim}$power 8% recommended sample size 262.

Discussion

This is the first case control study of C677T MTHFR allele frequency that compares well matched case and study control groups with a previously established baseline population frequency. Our original hypothesis was that the frequency of the variant alleles, C677T MTHFR and the A2756G MTR, would be significantly different between cases and study controls. We also predicted that the allele frequency of the C677T MTHFR variant in our study controls would be comparable to the previously established baseline frequency in the Manitoba population.

We found instead that, in the parents of NTD cases, there was a moderately higher allele frequency of the C677T MTHFR variant (OR 1.13), consistent with previous

reports in the literature. However, this difference was not significant when compared to the frequency in the population of Manitoba (p= 0.291). This is likely due to small sample size as power was low. We estimate we would need 250 cases in order to have sufficient power to confirm whether this was really a negative result.

Our study controls (unexplained elevations of MSAFP and normal pregnancy outcome) had significantly higher frequency of the C677T MTHFR variant than our Manitoba population. We hypothesize our study controls are not representative of the general Manitoba population with respect to the allele frequency of the C677T MTHFR variant. An elevation of MSAFP, without fetal anomaly, is associated with numerous adverse outcomes in later pregnancy. These include placental pathological changes, small-for-gestational-age babies, hypertension developing into preeclampsia, HELLP syndrome, low birth weight, fetal death, preterm delivery, placental abruption, placenta previa, and complications of the third stage of labor (92).

The C677T MTHFR variant has been tested for association with some of these complications. Most recently, this variant has been associated with placental vasculopathy, preeclampsia, gestational hypertension with or without proteinuria, intrauterine growth restriction, and late fetal loss (68). This variant may cause complications of late pregnancy because of its thrombophilic nature, especially when homocysteine levels are higher due to low folate intake. Several complications of pregnancy are associated with genetic thrombophilia (156).

For the A2756G MTR variant, our case mothers had a lower frequency (q = 0.3200) compared to our study control mothers (q = 0.1719). However, as the analysis

is limited by low power due to small size, the Manitoba population frequency for this variant has not been established, and as our study control group is not representative of that population, we cannot interpret this result.

As the C677T MTHFR variant has now been found to be associated with elevated MSAFP, a selection bias in past studies should be considered. Studies limited to live born MSAFP screened children with spina bifida (157), may have been influenced by prenatal ascertainment. Babies with spina bifida and the C677T MTHFR variant may have higher rates of positive MSAFP screens compared to babies with spina bifida, but without C677T MTHFR. This would mean babies with spina bifida and the C677T MTHFR variant would be more likely to be diagnosed prenatally and their mothers would be more likely to undergo a termination of pregnancy. These babies would not be available to take part in studies of liveborn spina bifida survivors and this would result in negative skewing of any study using only liveborn survivors to examine associations between the C677T MTHFR variant and NTDs.

Conclusion

In this preliminary pilot study, we did not find an association for either the C677T MTHFR variant or the A2756G MTR variant with NTDs; however, small case population size hampered our efforts. We did find evidence that the C677T MTHFR variant is associated with unexplained elevations of MSAFP with normal outcome. This unexpected finding indicates that the C677T MTHFR variant may be associated with complications of later pregnancy that are common in women with elevations of

MSAFP without fetal anomaly. This is the first report of unexplained elevations of MSAFP associated with increased allele frequency of the C677T MTHFR variant (see Appendix 1).

Chapter 2: The C677T methylenetetrahydrofolate reductase variant and third trimester obstetrical complications in women with unexplained elevations of maternal serum alpha-fetoprotein.

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Short title: C677T MTHFR and elevations of MSAFP

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Abstract

Introduction: The C677T MTHFR variant has been associated with the same third trimester pregnancy complications as seen in women who have elevations of MSAFP. We hypothesized that these women with third trimester pregnancy complications and MSAFP elevations would have an increased frequency of the variant compared to an abnormal study control group (women with MSAFP elevations without pregnancy complications) as well as to normal population controls.

Methods: Women who had unexplained elevations of MSAFP in pregnancy were ascertained retrospectively. The frequency of the C677T MTHFR variant among

those women with unexplained elevations of MSAFP who had experienced later pregnancy complications was compared to that of women with unexplained elevations of MSAFP without complications. Both were also compared to that of the previously established Manitoba frequency.

Results: Women who had complications of pregnancy and an unexplained MSAFP elevation had a higher allele frequency for the C677T MTHFR variant (q=0.36) compared to women with MSAFP elevations and normal pregnancy outcomes (q=0.25, OR 1.73 95% CI 1.25-2.37, *p*=0.030). The frequency was also higher than that of the population controls (q= 0.25, OR 1.70 95% CI 1.11-2.60, *p*=0.007). The frequency in women with MSAFP elevations without pregnancy complications was not significantly different from that of the population controls (*p*=0.410).

Conclusion: Women with unexplained elevations of MSAFP and who experience complications in later pregnancy are more likely to have one or two alleles of the C677T MTHFR variant.

Introduction

Significant elevations of amniotic fluid and MSAFP have been shown to be associated with spina bifida and other NTD. The province of Manitoba, Canada offers province-wide midtrimester MSAFP screening to all pregnant women as part of the MMSSP. After exclusion of fetuses with congenital malformations, twins, pregnancies with overestimation of gestational age, early fetal death and other fetal complications, the cause for the MSAFP elevations remains unknown in about half of this group of women (158).

It has been recognized that some pregnant women with unexplained midtrimester elevations of MSAFP are at increased risk for later complications such as intrauterine growth retardation (IUGR), premature delivery, complications in the third stage of labor, and poor placental function (12). Previously, it has not been possible to distinguish those women with elevation of MSAFP who are at increased risk of complications later in pregnancy from those who are not.

In a small pilot study of 32 couples (see Chapter 1), we found that both women who had an unexplained elevation of MSAFP and a normal midtrimester fetal ultrasound, and their partners, had a significantly increased C677T MTHFR frequency compared to Manitoba newborns (RR 1.42, 95% CI 1.08-1.85, p = 0.012, two tailed, see Appendix 1). The newborn study that examined 977 anonymous consecutive neonatal screening blood spots showed that 36% of Manitoba newborns were heterozygous and 7% were homozygous for C677T MTHFR (10) (q=0.25). As these newborns essentially represent the same population as the offspring of the pregnant women in the study, this cohort can be considered an appropriate and representative population control group. Subsequently, on evaluation of the pregnancy outcomes of our pilot study women, we noted that, among eight women who had gone on to experience complications of pregnancy, the OR for having the C677T MTHFR allele was 2.3 times higher than in the Manitoba population. However, the result was not statistically significant (p=0.151, two tailed). A power analysis showed low probability (<0.3) of detecting a difference with our sample size, and that we needed a minimum of 30 women with complications and 30 control women without to do so with any reliability. After removal of these women with pregnancy complications from the

sample, the remaining women had a similar C677T MTHFR variant frequency as the population controls (RR 1.83, 95% CI 0.887-3.735, p = 0.117) although the power was low (0.38) in this sample as well. Further investigations of the C677T MTHFR variant and its association with pregnancy complications among women with unexplained elevations of MSAFP were therefore undertaken.

Increased total plasma homocysteine alters placental function and has been associated with the same complications that are associated with unexplained elevated MSAFP (68, 103, 159-161). The C677T MTHFR variant has also been associated with complications of pregnancy in some, but not all, studies (83, 97, 162). C677T MTHFR may therefore contribute to complications of pregnancy by elevating serum homocysteine. Poor placental functioning could result in both an unexplained elevation of MSAFP and complications of pregnancy. We therefore hypothesized that women with third trimester pregnancy complications and MSAFP elevations (cases) would have an increased frequency of the variant compared to the Manitoba population (population controls) or women with MSAFP elevations without pregnancy complications (study controls). The negative effects of C677T MTHFR are presumed to be preventable by increasing folic acid intake to compensate for the inefficiency of the enzyme. If this is indeed the case, this group of women could potentially avoid complications by taking folic acid supplements.

Methods

Ascertainment and recruitment of study population

All pregnant women in Manitoba are eligible for routine serum screening (MSAFP, and, as of May 1999, human chorionic gonadotrophin and estriol) through the voluntary MMSSP. Women who have an abnormal result are referred to the HSC in Winnipeg for genetic counseling, followed by a fetal ultrasound assessment. The MMSSP obtains pregnancy outcomes for all women with a positive screen. In Manitoba, an elevation of MSAFP is defined as 2.3 MOM or greater.

Candidates for inclusion in this study were women with an unexplained MSAFP elevation (i.e. not due to fetal anomalies, incorrect dates, previously unrecognized fetal demise, or multiple gestation) with either a normal or an abnormal pregnancy outcome. After appropriate approvals had been obtained from The University of Manitoba Health Research Ethics Board (see Appendix 7), review of the screening records began in 1999 and took three years. For a study using a two step consent to participate methodology administered by mail, the expected response rate (after excluding lost to follow-up) would be 20% (163). Our goal was 1000 invitations. We anticipated this would result in approximately 120 participants. This would be double the minimum suggested power of our pilot study. To increase our response rate further, we added telephone follow-up for invited potential participants who were non-responders (139).

All screening records from 1995-1999 were reviewed, accounting for 783 invitations. Records for 2000-2002 were reviewed systematically as outcome information on each pregnancy became available to MMSSP. Records for 1990-1994 were then reviewed systematically in order to bring the total up to 1000. If a woman had more than one pregnancy with an elevation of MSAFP screened by the MMSSP,

only the first such pregnancy encountered in the retrospective review was used for the study. Previous or subsequent pregnancies were not included.

Women with preexisting conditions known to influence pregnancy outcome such as essential hypertension, genetic conditions such as myotonic dystrophy or sickle cell anemia, uterine abnormalities, thrombophilic disorders requiring medication, or drug or alcohol abuse were excluded. Women with anomalies of the cord or placenta (such as an umbilical cord knot or a stillbirth attributed to a cord accident), that were judged to be unrelated to the placental anomalies of interest as defined below, were also excluded. Mothers of babies with major congenital anomalies were excluded. A small number of women who had relinquished their babies for adoption or whose babies were placed in foster care were also excluded because of ethical concerns about contacting them about their pregnancy.

Women who met the inclusion criteria were divided into two groups for analysis. Cases were defined as women with pregnancies complicated by one of the complications previously shown to be associated with an unexplained elevation of MSAFP at midtrimester (12). These include: intrauterine growth restriction (IUGR) ($<10^{th}$ percentile), PIH, preeclampsia, eclampsia, postpartum hemorrhage, retained placenta requiring manual delivery, abruptio placenta, premature delivery (<36 weeks gestation or requiring specialized neonatal care for prematurity) and unexplained fetal demise. Study controls were women with normal outcomes, which were defined as those with delivery at term ≥36 weeks gestation), no complications of pregnancy, a normal placenta and a healthy baby. Definition of complications was based on ICDC-9 codes (164) in the MMSSP outcome charts for each patient, which are then confirmed later by chart review for all those with a positive MMSSP result. The previously reported newborn study provided the population control group data (10).

All women ascertained as having unexplained MSAFP elevations and who fit the inclusion criteria above, were invited by letter to participate. Women who agreed to participate in the study were mailed the appropriate questionnaires and requisitions.

Study Questionnaires

The questionnaire included a semi-quantitative food frequency questionnaire (FFQ) based on standard methodology, but modified to suit Manitoba residents and previously validated for this population by biochemical analysis during the pilot study. The survey included questions on vitamin supplement intake to determine periconceptional or prenatal supplementation as well as current use of vitamins**.** Dietary intake of folate and folic acid from supplements, and intake of the cofactors B_{12} and B_6, were calculated from the FFQ for intake both during pregnancy and at the time of the study. A correction of an additional 0.1 mg for folic acid fortification that began in Canada in 1998 was included for pregnancies that began after fortification (165). The analyses of the FFQ were completed with the researcher (NB) blinded as to the status of the mothers. (Identical FFQs were used for cases and controls and contained no information about pregnancy outcome. Case or control status was determined only after the final FFQ result.)

Laboratory Analysis

Total plasma homocysteine, red blood cell folate, and serum folate were determined using established methodology (152, 153). DNA was also extracted from whole blood and MTHFR C677T genotyping was performed using previously established methodology (126, 154). Genotyping and biochemical analyses were performed blind.

Statistical Analysis

χ^2 analysis (one tailed unless otherwise noted) was used for allele frequency. Comparisons between case and study controls were undertaken using MMSSP, FFQ and biochemical data. Parametric data were analyzed with the Student's *t* test for difference between means with Bonferroni correction for multiple comparisons. Data not normally distributed were analyzed using the nonparametric Mann-Whitney Rank Sum Test. Linear regression was used to test the validity the dietary survey. A multivariate analysis was undertaken and included age, smoking, maternal weight at the time of MSAFP testing, presence of C677T MTHFR, gender and weight of infant, biochemical parameters, and FFQ results for folate, B_{12} and B_6, both at the time of the survey and during the pregnancy. In order to avoid convergence due to the large number of variables that we wished to examine, and the relatively small numbers of participants, the analysis was completed by breaking the variables into subsets of six variables at a time. Variables with the higher association scores from these analyses were then combined for further testing in various combinations using stepwise multiple linear regression. In addition, linear regression analysis of each continuous variable with genotype results was performed. Corrections for multiple comparisons were included. Software used was NCSS Statistical Systems for Windows (155).

Results

Participation Rates

Nine hundred and ninety four women were identified as eligible (342 cases and 652 controls). Of the 590 women successfully contacted, 130 (22%) agreed to participate (56 cases and 74 controls). Four hundred and two women were lost to follow-up because their addresses were out of date and an updated address could not be

located. Family members of two women (both controls) informed us that they had died in unrelated events. Cases were 1.5 times more likely to choose to participate than controls and this difference was significant (p=0.030). There was no difference in the proportions of cases and controls that were lost to follow-up (p=0.157). We had anticipated a 20% response rate and we achieved 24%.

Genotype Results

Genotypes were available for 54 cases and 73 controls for this analysis. One case sample was lost at the collection site. One control and one case sample of DNA failed to amplify. Results are summarized in Table 7. The allele frequency for the C677T MTHFR variant in the Manitoba population had been previously established to be q=0.25. Women who had complications of pregnancy and an unexplained MSAFP elevation had a higher allele frequency for the C677T MTHFR variant (q=0.36) compared to women with MSAFP elevations and normal pregnancy outcomes (q=0.25, OR 1.73 95% CI 1.25-2.37, p=0.030). The frequency was also higher than in the population controls (q= 0.25, OR 1.70 95% CI 1.11-2.60, p=0.007). The frequency in women without pregnancy complications and MSAFP elevations (study controls) was not significantly different from that seen population controls (p=0.410).

Biochemical results

Heterozygotes and homozygotes for C677T MTHFR had lower average values (p=0.019) for serum folate than those who did not have the variant (29 nmol/L and 26 nmol/L respectively versus 33nmol/L). None of the women was deficient in either serum folate (defined as <7.0 nmol/L) or red blood cell folate (defined as <430 nmol/L

RBC) Averages values were 1261 nmol/L RBC for those without the variant, 1219 nmol/L RBC for heterozygotes, and 1340 nmol/L RBC for homozygotes. Heterozygotes and homozygotes for C677T MTHFR had lower average values (p=0.027) for serum folate than those who did not have the variant (9.0 nmol/L and 8.2 nmol/L respectively versus 7.6 nmol/L). Results by case and control are summarized in Table 7.

Table 7: Comparison of allele frequency of C677T MTHFR between cases, study controls, and population controls.

Subjects	**C/C (%)**	**C/T (%)**	**T/T (%)**	**Comparing to study controls*** OR (95%CI)	**Comparing to population*** OR (95%CI)
cases N=54	21 (39)	27 (50)	6 (11)	1.727 (1.25-2.37) (p=0.033)	1.70 (1.11-2.60) (p=0.007)
study controls N= 73	40 (55)	30 (41)	3 (4)	~	0.98 (0.46-1.55) (p=0.410)
population N = 977	557 (57)	352 (36)	68 (7)	0.98 (0.46-1.55) (p=0.410)	~

*χ^2 comparison of allele frequency (total T and C) in each group, one tailed. Cases: women with unexplained elevations of MSAFP who had subsequent complications of pregnancy, (C=69, T=39) Controls: women who had unexplained elevations of MSAFP and no subsequent complications (C=110, T=36) Population controls were 977 newborns (C=1466, T=488) (10). C/C=normal type, C/T=heterozygous for thermolabile variant, T/T=homozygous for thermolabile variant.

Validity of surveys

Seven cases and one study control declined to fill out their dietary surveys. Multivariate analysis was performed with both the women whose values were missing being excluded and with mean values were inserted in the multivariate analysis for these eight women. The results for both analyses were very similar. Therefore, the final

analysis was conducted using the dummy variables to allow the women's data for other points to be included. The validity of the dietary survey was demonstrated again for this study by linear regression analysis. Consistent with known homocysteine metabolism (166), a negative correlation existed between serum homocysteine and both red blood cell folate (p=0.005) and serum folate (p<0.001). Higher intake of dietary folate (including synthetic folic acid from supplements) as reported by the FFQ for the time of study was associated with higher serum folate (p<0.001) and higher red blood cell folate (p=0.017).

Comparisons of specific data items available in the women's MMSSP charts at the time of pregnancy with the data reported in the surveys showed excellent agreement for every item examined indicating women answered questions accurately. Women who reported smoking (as a quantitative value from 0-3 based on ½ packs/day smoked) showed a negative correlation with serum folate (p=0.006) consistent with accurately reporting their smoking habits (167).

Analysis of FFQ survey, Family History, and MMSSP data

The ethnicity of the infants born to the case mothers (based on the ethnicity reported for the infants' grandparents) was 84% Caucasian, 5% Aboriginal. Mixed ethnicity was reported for 11% of the infants with one parent Caucasian and the other parent Aboriginal, or rarely Black or Asian. The ethnic distribution was the same for controls and is typical for the Manitoba population (90, 130). There were also no significant differences between cases and controls with respect to their place of residence within the province (such as rural versus urban address). There were no

differences in the frequencies of vascular disease and cancers or in the risk factors and lifestyle choices made by grandparents.

There were no significant differences in dietary and supplemental intake of folate, B_{12}, or B_6, or in the biochemical parameters of case and control mothers. There was no difference in the percentage of cases and controls that reported taking prenatal vitamin supplements during pregnancy (37/48 (77%) cases and 55/72 (76%) controls) or taking vitamin and/or folate supplements periconceptionally (17/48 (35%) cases and 25/72 (35%) study controls).

After the presence or absence of the C677T MTHFR variant, the largest difference between the cases and controls in the *t*-tests was the average birth weight of the babies (3364g for controls versus 2770g for cases, p=0.001). Given that cases, by definition, included small for gestational age infants, premature babies, and women with fetal deaths, this result was not unexpected (Table 8 and 9).

There was a trend towards higher mean weight for mothers who had complications at the time of MSAFP test in the individual comparisons, but this was not significant after correction for multiple comparisons (Table 2.2). The multivariate analysis did not reveal any unexpected associations, but it did show the importance of maternal weight as a variable (p=0.024). This was also not unexpected given that some of the complications we were examining are associated with obesity (168). Even after controlling for women's weight in the multivariate analysis, the higher frequency of C677T MTHFR among cases remained significant (p=0.046). There was no association between weight and MTHFR status (p=0.679).

Table 8: Comparison of the parametric characteristics of women with unexplained elevations of MSAFP according to those with and without complications of pregnancy

Characteristic	**Mean cases (SD)**	**Mean controls (SD)**	***p* value**
MSAFP result in MOM	2.78 (±0.62)	3.16 (±3.76)	0.398
weeks gestation at draw date	17.1 (±1.61)	16.9 (±1.40)	0.432
μg/folate/day in pregnancy[3]	1216 (±915)	1010 (±892)	0.206
μg/folate/day at time of study[3]	557 (±341)	523 (±498)	0.588
erc folate (nmol/L RBC)	1234 (±289)	1208 (±317)	0.632
serum folate (nmol/L)	32.3 (±5.80)	32.3 (±5.71)	0.956
serum homocysteine (μmol/L)	7.8 (±2.26)	8.4 (±2.80)	0.246
μg B_{12}/day in pregnancy[3]	12.4 (±5.37)	13.4 (8.31)	0.488
μg B_{12}/day at time of study[3]	8.9 (±12.26)	8.6 (±10.83)	0.899
mg B_6/day in pregnancy[3]	8.4 (±9.69)	7.2 (±9.01)	0.461
mg B_6/day at time of study[3]	6.0 (±13.35)	5.5 (±11.10)	0.792
mother's age at delivery (years)	31 (±4.19)	30 (±5.19)	0.251
babies weight at birth in grams	2770 (±964)	3364 (±482)	<0.001[1]
mother's weight in Kg	76 (±17.26)	69 (±16.09)	0.013[2]

[1]This value is statistically significant. [2] This value is not significant after Bonferroni correction for multiple comparisons. See discussion. [3]Data was skewed due to a small number of women in both groups megadosing. When these women were removed from the analysis the result remained nonsignificant. MOM=multiples of median

Table 9: Comparison of the nonparametric characteristics of women with unexplained elevations of MSAFP according to those with and without complications of pregnancy

Nonparametric Characteristics (e.g. yes/no)	Median Cases (95% LCL of mean)	Median Controls (95% LCL of mean)	*P* value
diabetes in pregnancy (0=no, 1=yes)	0 (0,0)	0 (0,0)	0.207
maternal smoking, (0= nonsmoke 3=half pks/day increments)	0 (0,0)	0 (0,0)	0.721
gender of baby (1=boy, 0=girl)	0 (0,1)	1 (0,1)	0.352
parity	1 (1,2)	0 (1,2)	0.254
previous miscarriages	0 (0,1)	0 (0,1)	0.203
previous case pregnancy	0 (0,0)	0 (0,0)	0.472

Discussion

Unexplained elevations in MSAFP are known to be associated with an increased risk for complications of pregnancy (12) Others have reported that presence of the C677T MTHFR variant in pregnant women with low folate intake is associated with increased risk for pregnancy complications (12, 100, 169, 170). The unique finding of this study is an increase in the frequency of the C677T MTHFR variant among women with normal folate intake, who went on to have complications of pregnancy after an unexplained elevation of MSAFP (Table 7).

Retrospective studies, such as this one, have limitations. Participant recruitment in this retrospective study was based on the addresses in the MMSSP files. Those no longer at their last recorded address were often lost to follow-up. The study may therefore be biased in favour of women in stable personal situations that did not require

that they move or change their names. However, any such bias would affect both case and control groups equally and we found no significant difference between the numbers of case and control women lost to follow up.

The case and study control groups included women at various stages of their childbearing years. No case subjects had a previous or subsequent pregnancy with an unexplained elevation of MSAFP and a normal outcome. In addition, four case subjects had a previous or subsequent pregnancy with complications after an elevation of MSAFP. Only one woman recruited as a control subject had a previous or subsequent pregnancy with an unexplained elevation of MSAFP and complications. She was a heterozygote for C677T MTHFR. If the case versus control classification had been based on whether or not a woman had *ever* had a pregnancy with an unexplained elevation of MSAFP followed by complications, the association found would have been stronger (q=0.3636, OR 1.72, 95%CI 1.27-2.61, p=0.006).

The lack of folate deficiency in this population was unexpected, given previous research which showed that 23.6% of Newfoundland and Labrador women are folate deficient at their first prenatal visit (171). As study was retrospective, we did not have data on levels during pregnancy. The majority of our study participants took prenatal vitamins, but only 35% took periconceptional supplements. It has recently been shown that the C677T MTHFR variant does not affect maternal serum homocysteine levels in pregnancy among women who take prenatal multivitamins (83). Also, a recent prospective study shows that there is no difference in homocysteine levels at midtrimester between women who later develop preeclampsia and those who do not (172).

We therefore suggest that the negative effects of the C677T MTHFR variant, combined with low folate intake, are more likely to occur in early pregnancy before women began taking prenatal vitamins. We suspect that reduced methylation capacity of individual cells may have interfered with cell proliferation in the placenta as originally suggested by Eskes (68). As is the situation with NTDs, lack of folate deficiency by current definitions in a non-pregnant woman may not indicate that her folate intake is adequate for pregnancy. This would especially be true for women with the C677T MTHFR variant. Reexamination of the current definition of what constitutes a normal biochemical result for folate intake for women of child bearing age should be undertaken to clarify this (see Appendix 12).

We attempted to divide our cases into smaller groups by type of pregnancy complication (Table 10). We also separated IUGR by itself and IUGR associated with hypertensive disorders of pregnancy. Most of the groups lacked power for statistical analysis due to small numbers. However, there was one statistically significant result. Normotensive women whose fetus had IUGR (N=12) had a higher frequency of the C677T MTHFR variant compared to the population controls (q=0.33, OR 2.58 95% CI, 1.78-3.73, p=0.013). Homozygosity for the C677T MTHFR variant is associated with IUGR in women who do not take vitamin supplements according to one large study of Canadian women (48, 98). Our findings are in agreement with this result as only 3/12 (25%) women took supplements. We found this effect in a group of combined heterozygous and homozygous women.

Table 10: Types and numbers of complications of pregnancy in case women.

Types of Complication(s):	**N**	**CC**	**CT**	**TT**	**C**	**T**	**OR**	***p*[1]**
Manitoba Population (10)	977	557	352	68	1466	488	~	~
1. retained placenta and/or post partum hemorrhage	10	4	4	2	12	8	2.00	0.200[3]
2. all IUGR	17	6	9	2	21	13	1.87	0.117[3]
2a. normotensive IUGR	11	2	7	2	11	11	3.00	0.015
3. placental abnormalities	7	3	3	1	9	5	1.67	0.539[3]
4. unexplained preterm delivery	8	3	5	0	11	5	1.37	0.854[3]
5. all hypertensive disorders of pregnancy[3]	19	11	7	1	29	9	0.93	0.994[3]
5a. those in #5 with IUGR	6	4	2	0	10	2	0.60	0.742[2,3]
5b those in #5 excluding IUGR	13	7	5	1	19	7	1.11	1.000[3]

C/C=normal type, C/T=heterozygous for thermolabile variant, T/T=homozygous for thermolabile variant. C = number of C alleles in this groups. T = the number of T alleles. [1]χ^2 analysis based on C versus T as compared to the Manitoba population except where noted otherwise, [2]Fisher's exact test. [3]includes pregnancy induced hypertension, preeclampsia and eclampsia. [3]Power was ≤ 20% and this negative result may be due to small sample size.

In conclusion, using a retrospective case/control study, we have found that women with unexplained MSAFP elevations who have complications in later pregnancy are more likely to have the C677T MTHFR allele. Our results could not determine whether C677T MTHFR predisposes a woman to having an elevation of MSAFP level (as we did not compare the C677T MTHFR frequency in women with and without an elevation of MSAFP), but do indicate that having one or more copies of this variant predisposes such screen positive women to having complications in later gestation. It remains to be seen if other risk factors can be identified which can more accurately define this high risk group.

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Chapter 3: Increased frequency of the C677T *methylenetetrahydrofolate reductase* variant in couples with unexplained elevations of maternal serum α-fetoprotein and pregnancy complications.

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Abstract

Introduction: We have shown that women with MSAFP elevations followed by third trimester pregnancy complications have an increased allele frequency for the C677T MTHFR variant. We hypothesized that the paternal contribution to fetal genotype is also important. Thus, we compared the frequency of mating types in couples having an unexplained elevation of MSAFP with the mating types expected for the Manitoba population.

Methods: Women and their partners who had had unexplained elevations of MSAFP in pregnancy were ascertained retrospectively and genotyped for the

C677T MTHFR variant. Mating types were compared with those expected for the Manitoba population under Hardy-Weinberg equilibrium. The frequency of the C677T MTHFR variant among partners was also compared to that of the previously established Manitoba newborn allele frequency.

Results: Analysis of mating types showed skewing towards heterozygous couples and away from couples without C677T MTHFR alleles (p=0.031). Partners of women with complications had a higher frequency of C677T MTHFR (q=0.5000, OR 3.00, 95% CI 1.53-7.83, p=0.011) compared to the Manitoba population.

Conclusion: Both partners in a pregnancy with an unexplained elevation of MSAFP are more likely to have one or two copies of the C677T MTHFR variant. Partners of women with complications of pregnancy after an unexplained elevation of MSAFP are also more likely to have one or two alleles for the variant when compared to the newborn population.

Introduction

Previously, we have shown that women who had an unexplained elevation of MSAFP and later developed complications of pregnancy had a higher allele frequency for the C677T MTHFR variant (q=0.36) compared to women with MSAFP elevations and normal pregnancy outcomes (q=0.25, OR 1.73 95% CI 1.25-2.37, p=0.030) (173). The frequency was also higher than in newborn population controls (q= 0.25, OR 1.70 95% CI 1.11-2.60, p=0.007). The frequency in women without pregnancy

complications and MSAFP elevations (study controls) was not significantly different from that seen in newborn population controls (p=0.410). The newborn study that examined 977 anonymous consecutive neonatal screening blood samples and showed that 36% of Manitoba newborns were heterozygous and 7% were homozygous for C677T MTHFR (10). We hypothesized that paternal contribution to fetal genotype is also important. We, therefore, compared mating types in couples with an unexplained elevation of MSAFP with the mating types expected for the Manitoba population.

Methods

The details of this study population have been described previously (173). Briefly, study controls were women who had an unexplained elevated MSAFP followed by a normal pregnancy outcome, defined as delivery at term (≥36 weeks gestation), no complications of pregnancy, a normal placenta and a healthy newborn (see Chapter 2). Cases were defined as pregnancies complicated by one or more of the following after an unexplained MSAFP elevation: infants who were small for gestational age at birth (<10th percentile for gestational age at delivery), pregnancy induced hypertension, preeclampsia, postpartum hemorrhage, retained placenta requiring manual delivery, placental abruption, premature delivery (<36 weeks gestation or requiring specialized neonatal care for prematurity) or unexplained fetal demise. Definition of complications was based on International Classification of Disease Codes ICDC-9 in MMSSP outcome charts for each patient (164).

After approval of the research protocols by the University of Manitoba Health Research Ethics Board, all women ascertained as having unexplained elevations of MSAFP and who fit the inclusion criteria above were invited to participate by letter. Forty of the 147 women who participated in the study, also agreed to ask their partners to participate. When women agreed to ask their partners, a second consent to participate form was provided to each woman for her partner to review. Genotyping and biochemical analyses were performed with the laboratories blinded as to case or control status of participants as previously described (173).

The Hardy-Weinberg distribution of types of mating pairs expected in Manitoba was calculated based on the previously determined newborn C677T MTHFR frequency and then compared with the mating types of couples using a χ^2 Fischer's exact test. The allele frequency of the C677T MTHFR in fathers was compared to that in the Manitoba population. Fathers were divided according to their partner's case or control status and the two groups were compared to each other and compared to the Manitoba population. Fisher's exact test or χ^2 analysis (one sided), as appropriate, was used for allele frequency with NCSS Statistical Systems for Windows (155).

Results

Twenty-four of 40 partners agreed to participate. One partner asked to be included in genotyping even though his wife declined to participate in the genotyping portion of the study leaving 23 couples for analysis. There was a statistically significant skewing towards both parents being heterozygous for the C677T MTHFR variant and there was

a large decrease in the number of non C677T MTHFR homozygote pairings compared to that expected (p=0.031, Table 11).

When partners of women with complications were compared to the Manitoba newborn population, there was a significantly higher frequency of C677T MTHFR (q=0.5000, OR 3.00 95%CI 1.53-7.83, p=0.011). Among partners of women who had no complications of pregnancy, the difference was not significant (q=0.2857, OR 1.20, 95% CI 0.56-2.78, p=0.455) (Table 12). No significant difference was detected between partners of women with complications and partners of women without complications (OR 2.5, p=0.227). However, with our small sample size, there was only an 11% chance of finding a significant difference assuming an OR 2.5.

Table 11: Observed versus expected genotypes among couples with a positive MSAFP screen pregnancy;

Mating Type~	Formula	Expected*	N expected	N observed
AA x AA	p^4	0.3250284	7 (30%)	1 (4%)
AA x Aa	$4p^3q$	0.4217588	10 (43%)	11 (46%)
AA x aa	$2p^2q^2$	0.0684096	2 (9%)	1 (8%)
Aa x Aa	$4\ p^2q^2$	0.1368192	3 (13%)	8 (33%)
Aa x aa	$4pq^3$	0.0443844	1 (4%)	2 (8%)
aa x aa	q^4	0.0035996	0 (0.4%)	0 (0%)
		Sum=1.00	Sum=23	Sum=23

p=0.031[1] *Based on the Manitoba population (10) of 977: p = 0.755058, q = 0.264942, see Table 2. 1,[1]one tailed χ^2 Fisher exact test (aa x aa excluded from analysis). Mating type refers to couples. Therefore, AA (paternal genotype) x Aa (maternal genotype) and Aa (paternal genotype) x AA (maternal genotype) couples are combined into one group.

Table 12: Comparison of genotypes of MTHFR in the Manitoba population to that of women and their partners with an elevation of MSAFP, in total and in those with and without complications.

Subjects	N	C/C	C/T	T/T	Alleles	*OR (95%CI)*	*p*
Manitoba Population (10)	977	557 (57%)	352 (36%)	68 (7%)	C = 1466 T = 488	-	-
Partners of women with elevated MSAFP	24	9 (39%)	12 (52%)	3 (9%)	C = 30 T = 18	1.80 (1.26-1.42)	0.036[1]
Partners of women with complications	10	2 (30%)	6 (60%)	2 (10%)	C = 10 T = 10	3.00 (1.53-7.83)	0.011[1]
Partners of women with normal pregnancy	14	7 (50%)	6 (43%)	1 (7%)	C = 20 T = 8	1.20 (0.56-2.78)	0.455[1]

N= total number of subjects. C/C=normal type, C/T=heterozygous for thermolabile variant, T/T=homozygous for thermolabile variant. [1]one tailed χ^2.

Discussion

In this study, our significant finding is an association between unexplained elevations of MSAFP when both parents are heterozygotes for the C677T MTHFR variant. This confirms the result that we found in a smaller pilot study of this group. Our second important finding is an increase in the frequency of C677T MTHFR in partners of women who go on to have a complication of pregnancy after an elevation of MSAFP compared to the newborn population.

The mechanism behind increased complications with the C677T MTHFR variant has been attributed to increased homocysteine causing thrombosis in the placenta or to changes in early placental development due to inhibition of methylation reactions (68). However, it has recently been shown that C677T MTHFR does not affect maternal serum homocysteine levels in pregnancy among women who take prenatal multivitamins (83) as 12/24 (50%) of the women in our study did. Therefore, it is unlikely that the affect of C677T MTHFR we have found occurred due to elevated homocysteine levels within the placenta in later pregnancy.

Only 2/24 (8%) of the women in our study (one who had complications and one who did not) reported taking multivitamins or folic acid in the first four weeks after conception. Therefore, it seems more likely that having one or two C677T MTHFR alleles interferes with placental development in early pregnancy, before women begin taking prenatal vitamins. The C677T MTHFR mutation significantly affects enzyme activity in term human placentas (174). There is high expression of reduced folate carrier and folate binding proteins in early placenta with expression highest within the trophoblast cells surrounding blood lacunae (175, 176). Human cytotrophoblast cells show higher rates of apoptosis when cultured in reduced folate and folate free media when compared to other tissues (177). Fetal cytotrophoblastic cells therefore have higher folate requirements and would be more likely to be adversely affected by reduced MTHFR efficiency. Inhibition of normal fetal trophoblast differentiation combined with reduced invasion by fetal cytotrophoblastic cells during transformation of the spiral arteries and increased apoptosis may be present in fetuses with the C677T MTHFR variant when folate levels are low. Failure of physiologic transformation of the

spiral arteries is more common in women with preeclampsia and IUGR (two instances of complications that we evaluated) (178).

There is evidence that the gene for MTHFR is affected by imprinting with paternal inheritance contributing to gene expression in early pregnancy (26, 179). If the paternal gene is the only gene expressed in early placental development, then a fetus with a paternally inherited C677T MTHFR allele would be at increased risk for abnormal placentation. Alternatively, the most significant genotype may be a C677T MTHFR homozygous fetus (as has been observed for neural tube defects) (180). Maternally and paternally inherited C677T MTHFR alleles would both contribute directly if a homozygous fetal genotype is the "at risk" genotype. Maternal genotype may also contribute indirectly through an environmental effect of lower folate for either of these situations (120). Which genotype is contributory merits further analysis.

In conclusion, using a retrospective case/control study, we have found evidence that increased allele frequency for C677T MTHFR in both parents is associated with both an unexplained elevation of MSAFP and with pregnancy complications after such an elevation.

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Chapter 4: A meta-analysis of association studies of the C677T *methylenetetrahydrofolate reductase* variant and neural tube defects.

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Abstract

Introduction: Many studies have reported an association between the C677T MTHFR variant of methylenetetrahydrofolate reductase (C677T MTHFR) and neural tube defects (NTDs). Other similar studies have not found such an association. Recently, we found an independent association between the C677T MTHFR variant and an elevation of maternal serum α-fetoprotein (MSAFP) in pregnancy. MSAFP screening is the primary screening tool for NTDs in many countries. We hypothesized that the association of MSAFP screening and the C677T MTHFR variant may have biased previous studies of this variant and NTDs. Further, if this bias was controlled for, contradictory results might be clarified and

the actual role of the variant as a possible genetic contributors to NTDs could be better determined.

Methods: We used χ^2 analysis to compare the allele frequency of the C677T MTHFR variant in NTD affected cases, and in their parents, with the allele frequencies of large control groups collected in different countries. A multivariate analysis was undertaken to evaluate any possible bias created by MSAFP screening. The analysis controlled for other genetic contributors, different allele frequencies of the C677T MTHFR variant in various ethnic groups, folate intake, and disparities in medical care.

Results: We analyzed data from 22 association studies in 11 countries, 4 ethnic groups from the United States, and 2 regions of Canada. We combined these data with the C677T MTHFR allele frequencies from the control data of 66 association studies.

Conclusions: This meta-analysis shows that MSAFP screening *is* a source of bias in association studies of the C677T MTHFR variant and NTDs. When this bias is accounted for, the C677T MTHFR variant, combined with a diet low in folate, is shown to be a contributing factor to NTD prevalence worldwide.

Introduction

Van der Put *et al.*, (9), were the first to report an association between the C677T MTHFR variant and NTDs. The frequency of this common variant was found to be higher in Dutch individuals with spina bifida, and their parents, than in a Dutch control group. Since then, subsequent studies that have confirmed this observation, but other studies have not. Inadequate or unsuitable control groups and small sample size have been the most frequently discussed contributors to the contradictory results (91).

We have previously found that there is another independent association between heterozygosity for the C677T MTHFR variant in parents and elevations of MSAFP in pregnancy (Table 7). MSAFP screening is the primary screening test for NTDs in many countries. MSAFP screening is 85% sensitive in detecting NTDs, with higher rates of detection for anencephaly and lower rates of detection for spina bifida (4). Previous C677T MTHFR and NTD association studies have had case groups ascertained from different sources. For example, case groups have included only live born survivors with spina bifida (181), or only samples from fetuses whose mothers had undergone amniocentesis (182). We hypothesized that MSAFP screening could have biased previous association studies in countries where MSAFP screening and termination of affected pregnancies is available. We examined this possibility through a systematic review and statistical analysis of previously reported C677T MTHFR and NTD association studies.

Methods

Ascertainment of C677T MTHFR and NTD association studies

PubMed and Google online search systems were used to collect studies of association between the C677T MTHFR variant and NTDs. Multiple search terms were utilized in many different combinations with different spellings. The terms used included (but were not limited to) "spina bifida," "MTHFR," "C677T," "NTD," "folate", "folic acid," "MSAFP," "alpha," "fetoprotein," "prenatal diagnosis," "anencephaly," "encephalocoele", with both United Kingdom and American spelling variations. References in articles were also reviewed. Google searches led to databases of conference proceedings and abstract collections, which were searched using the same terms. In addition, PubMed, Google and any other subsequent database encountered from them were searched, using the names of authors who had previously published on the topic, and names of their institutions and affiliated research groups. Grant applications were not systematically reviewed although several research proposals were identified that were then searched for subsequent publications by the authors and, in some situations, the authors were directly contacted.

Publications from research groups' most recent study (or in some cases the study that had the highest number of individual cases) were used to avoid counting the same case twice. Where publications did not include full information on the exact numbers of heterozygotes versus homozygotes, the authors were contacted directly or related publications where the data might be included were examined.

Ascertainment of matched population controls and determination of OR

The same methodology was used to collect other reports of the C677T MTHFR allele frequency in normal population controls. Using the previously established methodology (91), normal control groups were pooled to create larger country-specific control groups. Some studies were confined to a specific ethnic or regional subgroup, for examples Black South Africans (183). For these studies, allele frequencies specific to that subgroup were determined. Accordingly, Canadian data were classified into two groups, Quebec, and other regions of Canada excluding Quebec (184, Table 13-15, also see Appendix 13).

Separate pooled allele frequencies for USA's "American Blacks", "Hispanic" and "non-Hispanic Caucasians", and "others" (largely Asians) were estimated by pooling respective data from multiple studies within the United States (Table 15). Specific ethnic mixes were required as controls for some C677T MTHFR and NTD association studies. For example, if the study group was 85% Caucasian and 15% Black, a control group that was 85% Caucasian and 15% Black was generated. Any mixed control group was generated beginning with the ethnic group that had the lowest numbers. A sub-sample of the largest ethnic group was prepared with a proportionately correct number of heterozygotes and homozygotes for the C677T MTHFR variant (Table 15).

The method of case ascertainment was determined in each NTD association study (Fig. 7). Studies were grouped into one of five methods of case ascertainment. These were: Type I- prenatally ascertained before 24 weeks gestation, Type II- full population with complete or nearly complete ascertainment of all NTD cases for both screened and

unscreened pregnancies, Type III- unscreened whole populations with complete or nearly complete ascertainment of all NTDs, Type IV- mixed screened and unscreened NTD survivors, and Type V- screened NTD survivors only (Table 16). Where a study had included mothers' and/or fathers' genotypes, the method of case ascertainment group was

based on that of the affected offspring.

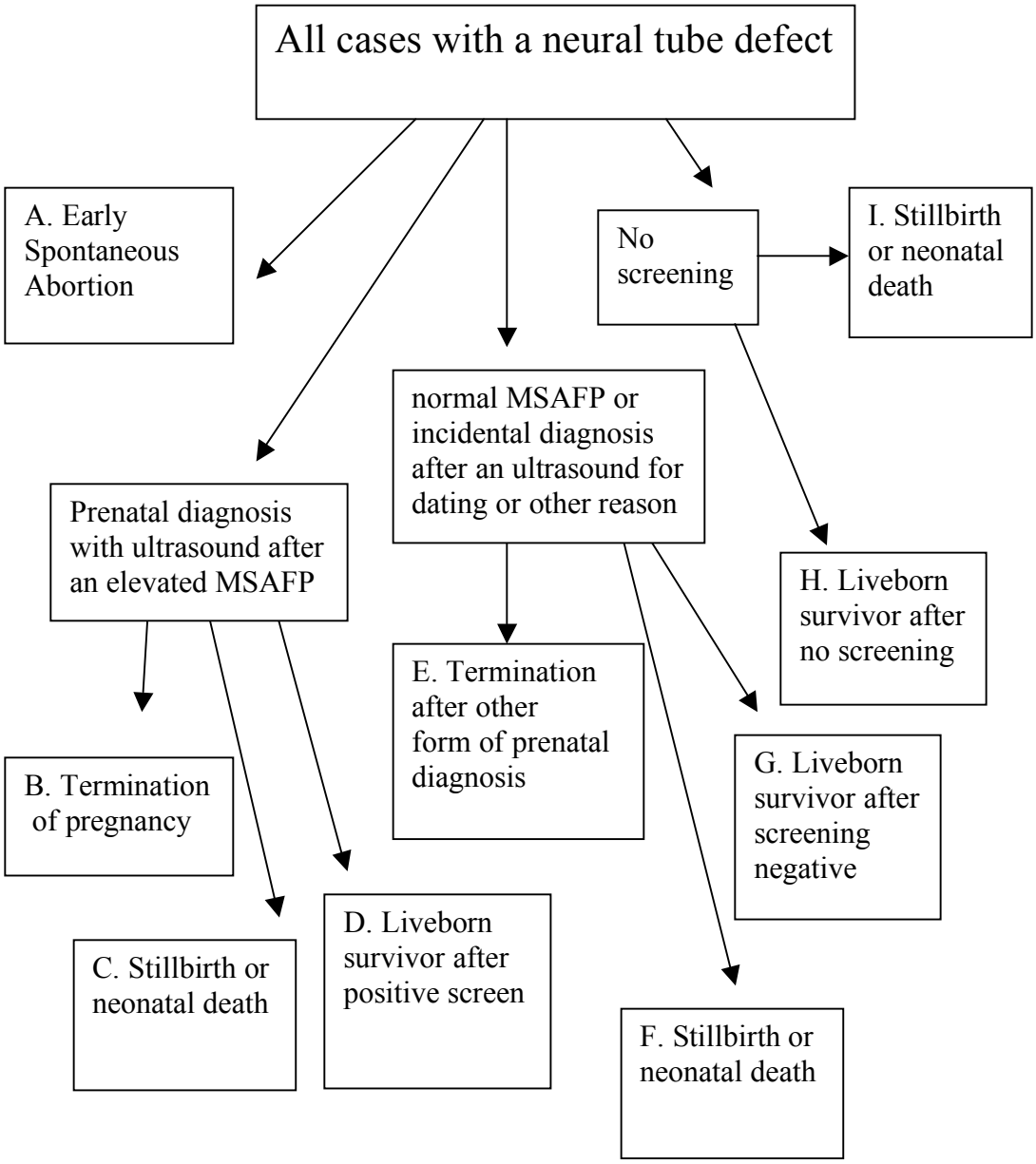

Figure 7: Potential outcomes for a pregnancy with a fetus affected by a neural tube defect.

Table 13: Worldwide distribution of the MTHFR C677T variant allele by NTD study for countries with a single predominant ethnic group.

Country/Ethnicity/Region	N	C/C	C/T	T/T	Freq	Ref.
Pooled Irish Controls	1518	650	708	160	0.339	(185-188)
Affected Cases	218	82	95	41	0.406	(180)
Mothers	218	80	108	30	0.385	(180)
Fathers	218	83	109	26	0.369	(180)
Pooled German Controls	2676	1410	1003	263	0.289	(189-195)
Affected Cases	161	60	76	25	0.391	(191,194)
Mothers	110	62	65	21	0.486	(191,194)
Pooled UK Controls	2190	1019	924	247	0.324	(75, 196-205)
Affected Cases	49	28	14	7	0.286	(204)
Mothers	42	23	15	4	0.274	(204)
Fathers	34	18	14	2	0.265	(204)
Pooled Italian Controls	3406	1064	1687	655	0.440	(86, 95, 109, 205-211)
Affected Cases	203	62	89	52	0.475	(207)
Pooled French Controls	416	181	191	44	0.335	(86, 166, 212, 213)
Affected Cases	43	19	21	3	0.314	(213)
Pooled Norway Controls	820	416	329	75	0.292	(214-216)
Affected Cases	28	12	15	1	0.304	(214)
Pooled Turkish Controls	269	151	98	20	0.257	(217-219)
Affected Cases	91	39	48	4	0.308	(218)
Mothers	72	26	36	10	0.389	(218)
Fathers	20	6	12	2	0.400	(218)
Affected Cases	56	22	29	5	0.348	(217)
Mothers	49	26	20	3	0.265	(217)
Pooled S.Africa Blacks	208	166	42	0	0.101	(183, 220)
Mothers	53	42	11	0	0.104	(183)
The Netherlands Pooled Controls	1758	861	751	146	0.299	(9, 12, 169, 204)
Affected Cases	55	22	26	7	0.364	(221)
Mothers	70	46	39	16	0.507	(221)
Fathers	60	48	42	10	0.517	(221)
Affected Cases	31	25	6	0	0.097	(204)
Mothers	36	30	6	0	0.083	(204)
Fathers	30	25	5	0	0.083	(204)

Table 14: Canadian results of pooled control groups.

Country/Ethnicity/Region	N	C/C	C/T	T/T	freq	Ref.
Pooled Canadian. Quebec residents only. Controls	1836	833	679	160	0.272	(56, 97, 222-225)
Affected Cases	56	19	26	11	0.429	(223)
Mothers	62	24	27	11	0.395	(223)
Pooled Canadian excluding Quebec residents. Controls	1836	833	679	160	0.272	(10, 63, 86, 226, 229)
Mothers	26	13	10	3	0.308	(228)
Fathers	20	10	8	2	0.300	(228)
Affected Cases	161	96	60	5	0.217	(228)

Statistical Analysis

An OR for each NTD study was calculated and the allele frequencies were compared using χ^2 analysis. Studies were then grouped by the method of case ascertainment as it related to MSAFP screening. For each method of case ascertainment, the heterogeneity of the groups was determined and, where types did not have significant heterogeneity (defined as $p>0.025$), a combined relative risk (RR) was calculated.

Four other potentially confounding variables were considered in a logistic regression analysis. These included; 1) Other genetic and environmental contributors (39). NTD prevalence (births and still births) by region or ethnicity was used a proxy for this value. 2) Ethnic and regional differences in the C677T MTHFR allele frequencies as determined from our control groups. 3) Average national folate intake for women of childbearing age. 4) Access to prenatal diagnosis and termination of pregnancy, determined by the percentage of pregnancies with NTDs that ended in terminations. Multiple linear regression analysis was used to compare OR of cases, with folate intake, control allele frequency, NTD prevalence, and percentage of terminations as numerical

variables. Method of case ascertainment was treated as a categorical variable. The same analysis was then carried out for OR of mothers and OR of fathers. Software used was NCSS (155).

Table 15: Results for American mixed ethnicity control populations.

Country/Ethnicity/Region	N	C/C	C/T	T/T	freq	Ref.
Pooled USA White Controls	6998	3438	2761	799	0.311	(86, 126, 157, 224, 229-235)
Pooled USA Black Controls	891	670	206	15	0.132	(86, 231, 232, 234, 236)
Affected Cases	111				0.311	(237)
Mothers	164				0.354	(237)
Fathers	127				0.317	(237)
Affected cases	42				0.179	(238)
Pooled USA Hispanics Controls	625	180	318	127	0.458	(86, 125, 181, 232, 241)
Affected Cases	234	55	114	65	0.521	(181)
Mothers	214	41	112	61	0.547	(181)
Fathers	99	31	46	22	0.455	(181)
Pooled Puerto Rican Controls	528	233	249	46	0.326	(240-242)
Affected Cases	31	10	18	3	0.387	(240)
Mothers	37	7	23	7	0.500	(240)
Fathers	36	16	18	2	0.306	(240)
94.3% Hisp., 5.6% White	596	174	302	120	0.455	
Affected Cases	19	3	17	4	0.658	(239)
Mothers	87	23	50	14	0.448	(239)
85% White, 15 % Black	4546	2384	1702	460	0.288	
Affected Cases	71	40	24	7	0.268	(182)
83% White, 17 % Black	4080	2155	1519	406	0.286	
Affected Cases	41	17	15	7	0.354	(243)
49%H,41%W,4%B,6%Other	800	324	354	122	0.373	
Affected Case	214	73	100	41	0.445	(232)

Table 16: Determination of method of case ascertainment (using Fig. 7).

Study Location	Ref.	Groups[1]	Type	Ascertainment
Ottawa, Canada	(226)	A,B,E	1	spontaneous abortions
Manitoba, Canada	(228)	B,C,D	1	elevated MSAFP screen positive
France	(213)	B,E	1	assorted prenatally diagnosed
Atlanta USA	(243)	A,B,E	1	assorted prenatally diagnosed
Puerto Rico	(240)	B,C,D,E	1	assorted prenatally diagnosed
Birmingham USA	(244)	B,C,D,E	1	Amniocentesis
Germany	(194)	B,C,D,E,F,G	2	live born & prenatal diagnosis
Texas USA	(239)	B,C,D,E,F,G	2	live born & prenatal diagnosis
California USA	(232)	B,C,D,E,F,G	2	live born & prenatal diagnosis
Turkey	(217)	H	3	unscreened population
South Africa	(183)	H	3	unscreened population
Ireland	(180)	H	3	unscreened population
Turkey	(218)	H	3	unscreened population
Texas USA	(245)	G,H	4	screen negative & unscreened
Quebec Canada	(222)	G,H	4	screen negative & unscreened
North Carolina USA	(246)	G,H	4	screen negative & unscreened
Italy	(207)	G,H	4	screen negative & unscreened
Netherlands[2]	(222)	G,H	4	screen negative & unscreened
United Kingdom	(204)	G	5	live born after prenatal screening
Netherlands	(204)	G	5	live born after prenatal screening
Norway	(214)	G, E	5	live born after prenatal screening
Iowa USA	(238)	G	5	live born after prenatal screening

[1]See Fig. 7 for categories. [2]while MSAFP screening is now widely available and accessed in the Netherlands, the cases used in this study were born 1990 or earlier when MSAFP screening was not as widely available.

Results

A total of 44 C677T MTHFR and NTD association studies in 14 countries were ascertained. After eliminating those with insufficient or overlapping data, 22 studies from 11 countries remained for the analysis (Table 13-15). All but two (238, 246) included a full accounting of the numbers of homozygotes and heterozygotes. Allele frequency for the two other studies was available. Eighty-eight additional studies were used for

preparing control groups. Three methods of case ascertainment did not have significant heterogeneity and did have significant relative risk (RR) (Types II, IV and V). These are detailed in Table 17.

One American study (157) was not included in the analysis because individual results were not specified. Data from two Mexican studies (248, 249), were not included. Mexico has widely differing C677T MTHFR frequencies of 0.350-0.586 by region and ethnicity (250, 251). Thus, it was not possible to generate a reliable matched population control. The same is true of a small Brazilian study (87). A Chinese study (252) was also excluded because appropriate Chinese control groups were not available.

χ^2 analysis of association

χ^2 analysis of association showed that when a case group that is made up exclusively of screened survivors is considered, the OR drops significantly. Cases taken from screened and unscreened populations in various combinations show significantly increased ORs (Table 18).

Table 17: Summary of data used in multivariate analysis.

Ref.	Country	Type[8]	C677T Freq[9]	OR Case	OR Mother	OR Father	NTDs/ 10/000 births	% TA	Folate[21]
226	Canada[1]	1	0.276	0.625	~	~	10.00[11]	51.00[15]	249[22]
228	Canada[1]	1	0.276	~[10]	1.043	1.317	10.00[11]	63.75[17]	249[22]
213	France	1	0.335	0.907	~	~	10.00[12]	77.80[11]	391[23]
243	USA[2,3]	1	0.285	1.465	~	~	10.05[13]	9.00[15]	200[24]
240	Puerto Rico[4]	1	0.326	1.324	2.097	0.839	16.00[15]	15.00[18]	344[24]
244	USA[2,4]	1	0.288	0.612	~	~	10.06[13]	45.83[15]	200[24]
194	Germany	2	0.289	1.607	1.431	~	12.40[12]	18.57[12]	246[23]
239	USA[2,3]	2	0.453	1.127	0.995	~	14.70[19]	15.63[19]	185[24]
232	USA[2,3,4,5]	2	0.445	1.227	~	~	13.80[13]	38.00[15]	185[24]
217	Turkey	3	0.275	1.419	1.427	~	30.10[16]	1.00[15,16]	185[15]
183	South Africa[7]	3	0.101	~	1.605	~	35.50[15]	1.00[15]	185[15]
180	Ireland	3	0.339	1.310	1.250	1.167	14.45[12]	1.00[12]	260[23]
218	Turkey	3	0.275	1.279	1.845	1.932	30.10[16]	1.00[15,16]	185[15]
245	USA[4]	4	0.470	1.291	1.430	0.988	13.80[13]	15.63[15]	185[23]
222	Canada[6]	4	0.356	1.356	1.534	~	14.00[14]	63.75[17]	249[22]
246	USA[2]	4	0.322	0.998	1.215	1.028	10.00[13]	21.00[15]	210[24]
207	Italy	4	0.440	1.153	~	~	6.60[12]	35.00[12]	400[26]
221	Netherlands	4	0.297	1.365	1.285	1.065	11.30[12]	21.81[12]	238[23]
204	UK	5	0.324	0.297	0.788	0.752	15.90[12]	69.07[12]	289[23]
204	Netherlands	5	0.299	0.289	0.245	0.449	11.30[12]	21.81[12]	238[23]
214	Norway	5	0.292	1.057	~	~	10.75[20]	81.00[20]	195[25]
238	USA[2,3,4]	5	0.322	0.481	~	~	10.00[13]	24.00[13]	210[24]

TA = termination of pregnancy. 1. Canada excluding Quebec; 2. USA, Non Hispanic whites; 3. USA Blacks; 4. USA Hispanic whites; 5. USA others; 6. Canada, Quebec only; 7. Rural South African Blacks; 8. see Table 14; 9. detailed in Tables 13-15; 10; ~ = not available; 11. (254); 12. (255):13. (256); 14. (257); 15. based on information provided by authors in original publication; 16. (258); 17. based on information provided by the Manitoba Maternal Serum Programme; 18. estimated from 13 and 15; 19. (259); 20. Estimated from (260, 261); 21. micrograms per day includes folic acid supplementation; 22. (164, 262); 23. (84); 24. (263); 25. (264); 26. estimated from 15, 24; 27. based on biochemical data (217).

Table 18: Combined relative risks by method of case ascertainment.

Group	OR Affected Cases	OR Mothers Affected	OR Fathers Affected	OR Combined
Type I	~	~	~	~
Type II	1.426 $p<<0.001$	1.293 $p=0.010$*	no data	1.376 (cases and mothers) $p<<0.001$
Type III	1.279 $p=0.004$	1.169 $p=0.082$*	1.222 $p=0.053$*	~
Type IV	1.527 $p<<0.001$	1.377 $p<<0.001$	1.322 $p=0.010$	1.475 $p<<0.001$
Type V	0.653 $p=0.003$	0.541 $p=0.003$	0.512 $p=0.005$	0.541 $p<<0.001$

Type 1 = prenatal ascertainment only, Type II = full population, Type III = unscreened population only, Type IV = screened and unscreened, Type V = screened survivors only, ~ indicates significant heterogeneity ($p\leq0.025$) prevented combining study numbers. *not statistically significant

Multivariate Analysis

Initial multivariate analysis failed when all variables were used due to small sample size. To increase sample size, we substituted mothers OR into 2/22 rows where those rows were missing for the affected case OR (see Table 17). We then completed multiple regression analysis with all variables (with method of case ascertainment as a categorical variable). The result was that level of folate ($p=0.001$) and percentage of terminations ($p<<0.001$) were significant contributing factors, but method of case ascertainment, frequency of the C677T MTHFR allele and prevalence of NTDs were not. We did the analysis again with method of case ascertainment as a numerical variable (assuming increasing probability of screening negative by method of case ascertainment), the results were the same except that method of case ascertainment became a significant contributing factor ($p=0.020$) in addition to folate intake and percentage of terminations.

Discussion

Our original hypothesis was that the OR of affected individuals and their parents is biased if MSAFP screening and termination of affected pregnancies is occurring within a population selected by researchers for study. Our results identify the C677T MTHFR variant as one contributing genetic factor to NTDs and one that is folate intake dependent, as originally hypothesized by van der Put *et al.* (9). Further, the independent association of the C677T MTHFR variant with the common screening method for NTDs, has created a bias in allele frequency of affected survivors. Each method of case ascertainment had a particular profile due to the effects of this screening bias.

Type I: Prenatally diagnosed NTD

This group consists of studies with the case groups being samples of fetuses with NTDs. The source of the fetal sample varied between studies. Thus, this group cannot be regarded as a homogenous one. This was confirmed by the analysis of heterogeneity, which was significant ($p \leq 0.0001$). The Canadian study from Ottawa (226) drew cases from a pool of both spontaneously aborted fetuses and products of conception following termination of pregnancy. The allele frequency for the C677T MTHFR variant for this group was reduced at OR 0.625. Thus, C677T MTHFR variant may be protective in early pregnancy or preconception, by preventing karytope abnormalities from occurring in the first place and/or by preventing the severest forms of NTDs commonly lost in early pregnancy. Alternatively, the effect may be one of folate rescue or allowing affected

pregnancies to survive to midtrimester. The Ottawa study (226) showed reduced allele frequency for the C677T MTHFR variant among newborns, suggesting there is also a selective loss of fetuses with the variant allele that survived to midtrimester. This observation requires further research.

The Georgia study (243) consisted of fetuses that had survived long enough to undergo amniocentesis. The OR for this group is 1.49 indicating either a survival bias, such as the one suggested by the Ottawa study, or an increased likelihood of undergoing amniocentesis. Both factors may be present. The French group (213) is difficult to assess because France's more liberal abortion laws preclude drawing conclusions as to the gestational age of the fetuses involved from the information provided. Our Manitoba study (228), while not statistically significant, would seem to indicate that each parents' contribution is equally important in fetuses with NTDs who screen positive in MSAFP testing. The Puerto Rican study (240) appears to directly contradict this result. However, there were no details in this publication indicating how or when in gestation a prenatal diagnosis occurred or whether ultrasound was also used as a screening tool.

Type II: Full population

These association studies are the closest to full ascertainment of the population of those affected by NTDs. There was no significant heterogeneity within the group and the combined RR was 1.376 ($p<<0.001$). The German study (194) had only 7% of cases with anencephaly and encephalocoeles, and 93% liveborn survivors indicating a large number of missing fetuses. Half the pregnancies that resulted in terminations were missing in the California population (232). Type II illustrates that screening and termination of affected

fetuses may be removing some C677T MTHFR alleles from the populations studied, but not enough to reduce the OR to one. The presence of the allele increases the risk for a NTD.

Type III: Unscreened population

These association studies examined survivors in areas where no screening is available to the general population and termination of pregnancy is difficult or impossible to obtain. The combined affected case RR of 1.279 was significant (p=0.005). This group provides the strongest evidence that C677T MTHFR is associated with NTDs. Unscreened populations would have no bias in method of case ascertainment due to MSAFP screening although case detection would be affected by any survival bias.

Type IV: Screened neg and unscreened mixed

These association studies occur where prenatal care and MSAFP screening are not uniformly available or, as in the case of the Dutch study (221), became available part way through the period during which the cases were ascertained in. The case groups were made up of a mixture of screened and unscreened live born individuals. The combined RR 1.527 was significant (p<<0.001) supporting the association of the C677T MTHFR allele and NTDs.

Type V: Liveborn after screening

This group is made of studies from countries where MSAFP screening and termination of affected pregnancies is readily available to the entire population and the NTD case method of case ascertainment is restricted to liveborn survivors. The study of Iowan Americans was done in a region with a statewide, government funded, MSAFP screening programme with state supported prenatal clinics. For this group, prenatal care and access to MSAFP screening is close to universally available (238). The United Kingdom group (204) is from the original home of MSAFP screening. These UK affected cases all had a family history of NTD making a choice in favour of screening by parents even more likely. This set of association studies provides the best evidence of a bias created by the association of the C677T MTHFR variant with elevated MSAFP. The group is made up of the babies with NTDs who would have been most likely to screen negative using MSAFP and thereby avoid a termination of pregnancy. The combined RR is 0.653 ($p << 0.001$). The single OR over one is from Norway (214), a country where ultrasound was widely used as a screening tool in addition to MSAFP, during the period when most cases were ascertained (265). Prenatal ascertainment of NTDs in Norway would not depend primarily on a positive MSAFP screen test and would be less subject to any screening bias this might create.

Comment on control groups

Botto and Yang (91) reduced the possibility of bias in small groups through control group pooling. In this analysis, "convenience sampling" is defined as a group of healthy individuals who were collected in some nonsystematic or unspecified fashion that may or may not represent the general population. (See (266), for one such example.) Pooled

control data were only taken from studies using healthy individuals from the general population such as volunteer blood donors, newborn data and healthy individuals. (Details of control groups are provided in Table 14.) While this type of pooled control group must still be approached with caution, the larger the combined sample size, and the more studies involved, the less the likelihood that the control group does not reflect the true population frequency.

Transmission disequilibrium tests

Four research groups applied the transmission disequilibrium test (TDT) to their populations. Two American studies did not find an association between C677T MTHFR and NTD in 36 family trios (157) and 58 parent/child pairs (246). A German study (194), applied TDT in 98 family trios and 31 parent-offspring pairs and failed to find significance. In contrast, the Irish group (180) applied TDT to 217 heterozygous parents and found a nonsignificant transmission disequilibrium (transmitted:nontransmitted 121:96, p=0.100). Their hypothesis is that homozygosity in the affected is the "at risk" genotype (i.e. the genetic mechanism is principally recessive) and therefore the TDT is not the most appropriate for measuring an effect. Further, when they utilized a test for possible homozygosity in the affected offspring, the result was significant (transmitted:nontransmitted 82:57, p=0.040). The Irish study's case group was made up of unscreened individuals, whereas both the German and the two American study case groups consisted of survivors from areas where MSAFP screening is available. Therefore, these last three studies are potentially biased by the removal, through MSAFP

screening, of fetuses with both C677T MTHFR and NTDs. We propose that the Irish study provided the most accurate result as this population is not screened.

Implications for Trisomy 21 and C677T MTHFR studies

We have shown a survival disadvantage for fetuses with NTDs because the variant is associated with higher maternal serum screen positive results. Survival advantage and the C677T MTHFR variant have already been examined with respect to conceptions with Trisomy 21 (70, 179). Biochemical screening for Trisomy 21 relies, in part, on low MSAFP (135). Fetuses with both Trisomy 21 and C677T MTHFR may be more likely to be missed (false negative) during screening and this possibility must be considered in any study of C677T MTHFR and chromosomal disorders.

Conclusion

This analysis shows that MSAFP screening *is* a source of bias in association studies of the C677T MTHFR variant and NTDs. When this bias is controlled for, the C677T MTHFR variant combined with a diet low in folate, is shown to be a contributing factor to NTD prevalence worldwide.

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Chapter 5: Changes in incidence of neural tube defects in Manitoba after folic acid fortification.

Abstract

Introduction: On January 1, 1999, food fortification became mandatory in Canada and an estimated average of 100 additional micrograms per day was added to the diet of Canadians. Fortification is expected to result in a reduction in the incidence of NTDs in Manitoba. We wished to determine if a drop in incidence of NTDs has occurred.

Methods: All files relating to NTDs ascertained in Manitoba were also reviewed. The results were used to calculate NTD incidence before and after the introduction of fortification.

Results and Conclusions: There has been a 37% drop in the incidence of all NTDs province wide in Manitoba since the introduction of folic acid fortification.

Introduction

Beginning in 1996, food fortification of flour, and related food items, for USA markets resulted in an increase in the amount of folic acid being consumed by American women. By late 1997, Canadian women were also getting increased levels of folic acid via the fortification of food that was being distributed in both Canada and the USA. By January 1, 1999, fortification also became mandatory in Canada. The amount of additional folic acid reaching each woman is an estimated average of 100 micrograms per day (267). If folic acid fortification at these levels is preventing NTDs, there should be a

measurable drop in the incidence of NTDs in Manitoba when the periods before and after fortification are compared.

Methods

NTD incidence/10,000 pregnancies

All pregnancies resulting in NTDs in Manitoba were ascertained using a combination of active and passive surveillance modes, including early terminations from 14-20 weeks gestation and early intrauterine deaths (138). The three sources of information are pregnancy outcome files for the MMSSP, Section of Genetics files where all families in the province with a history of NTDs are referred for counseling, and the Manitoba Congenital Anomalies Registry before it was discontinued. Incidence of NTDs was determined for before and after fortification in the more commonly reported form of NTDs per 10,000 births and stillbirths for the whole province of Manitoba. The year in which a pregnancy was classified was determined by expected date of delivery when pregnancies did not end in a live birth at term.

Statistical Analysis

χ^2 analysis and Students two sample *t* test for differences between the mean were utilized as appropriate (with Bonferroni correction for multiple comparisons). Where *t*-tests of differences between the mean involved proportions, the data were transformed using the inverse sine of the square root to restore normal distribution. Software used was NCSS Statistical Systems for Windows (155).

Results

NTD incidence rate/10,000 pregnancies before and after fortification

The number of all reported NTDs (screened and unscreened) per 10,000 total births (births and stillbirths) has dropped significantly since fortification began. Before fortification, there were 75 NTDs cases in 78,110 total pregnancies. After fortification, there were 43 NTDs in 70,913 total births. The difference was significant at p=0.015. This can be expressed as an incidence of 9.70/10,000 (±2.65) pregnancies before fortification versus 6.05/10,000 total births after fortification (±1.40). This represents a drop of 37% drop in the incidence of NTDs/10,000 total births in Manitoba since fortification was introduced (Table 19 and Fig. 8).

Table 19: NTDs/10,000 births and stillbirths diagnosed province wide by year

Year	All NTDs province wide[1]	Live Births and Still Births	NTDs/10000 pregnancies
1993	17	16447	10.34
1994	15	16208	9.25
1995	12	15870	7.56
1996	12	15195	7.90
1997	19	14390	13.20
1998	6	14201	4.23
1999	10	14421	6.93
2000	7	14194	4.85
2001	10	14106	7.10
2002	10	13991	7.15

[1]All NTDs diagnosed within Manitoba including spontaneous abortions and terminations and NTDs associated with multiple congenital anomalies. [2]For 1993-1998, Manitoba Perinatal Surveillance Report (268). 1999-2002 figures are approximate, based on an average of 0.0074% of all pregnancies resulting in a still birth over six years (calculated from the birthrate (269) and pregnancies rate (268) for 1993-1998).

Figure 8: Plot of NTDs /10,000 births and stillbirths by year for 1993-2002

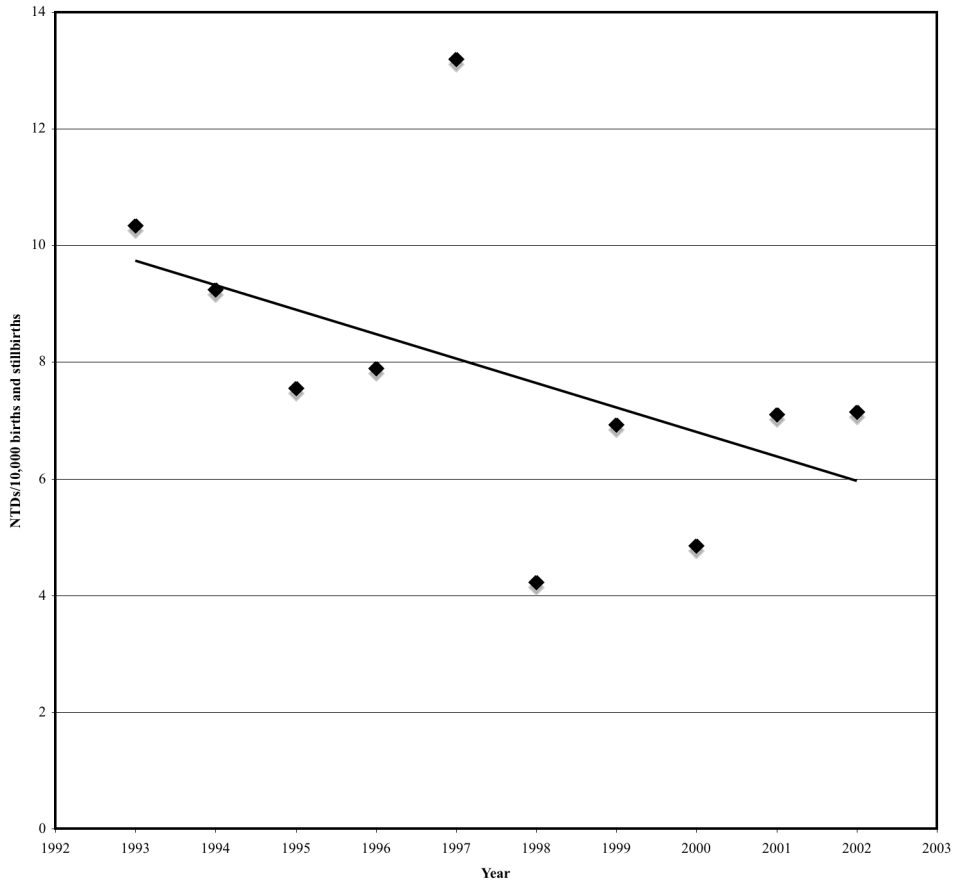

Table 20 NTDs by test results and types.

Year	Total	Simple	Complex	Elevated MSAFP	False Neg	No test
1993	14	11	2	11	2	1
1994	15	12	1	12	3	0
1995	9	8	1	8	1	0
1996	19	17	2	15	3	1
1997	13	10	3	9	2	2
1998	5	4	1	4	0	1
1999	9	8	1	4	2	3
2000	9	6	3	8	0	1
2001	9	7	0	8	0	1
2002	5	5	0	2	1	2

Table 21: False negative screened pregnancies by type of NTD

Year	Complex	Simple	Covered	Total
1993	0	0	2	2
1994	1	0	2	3
1995	1	0	0	1
1996	1	0	2	3
1997	1	0	1	2
1998	0	0	0	0
1999	0	0	2	2
2000	0	0	0	0
2001	0	0	0	0
2002	0	0	1	1

Discussion

NTD incidence in Manitoba

The most significant finding of this review is there has been a 37% drop in the incidence of NTDs/10,000 total births in Manitoba. This decrease coincides with the introduction of folic acid fortification of food. This result is not unexpected, given the

reduction in NTD incidence in other provinces. There has been a significant drop in NTDs in Nova Scotia where rates have dropped from 26/10,000 to 11/10,000 and in Ontario where rates have dropped from 17/10,000 to 6/10,000 since fortification began (31, 32). Manitoba appears to have had a lower incidence to begin with and so has benefited less from fortification. This may be due to differences in diet or ethnicity of Manitobans compared to Ontario and Nova Scotia residents or it may reflect differences in ascertainment of all NTD cases. Also, the Nova Scotia and the Ontario study did not include data for a five-year period since fortification began. Their data included only the first two years post fortification. The Manitoba data for 1998 and 1999 indicates a lower incidence in those years when compared to 2001 and 2002. If Manitoba trends apply to Ontario and Nova Scotia, these two provinces should also have seen a slight increase in subsequent years that would lower their average reduction in incidence of NTDs since 1999 and making their results closer to that seen in Manitoba. A seven-province study of this possibility is currently in progress.

Types of NTDs before and after fortification

"False negative" NTD is something of a misnomer. False negative NTDs included in our analysis were pregnancies where a NTD was present, but MSAFP was normal. "False negatives" included closed defects that are not expected to be detectable by MSAFP, and multiple congenital anomalies, often associated with chromosomal abnormalities. Therefore, in a percentage of these complex NTDs, the reduction of MSAFP related to trisomy 18 or 21 and a tendency for a higher MSAFP due to the NTD.

The resulting MSAFP for a complex NTD would then often be normal might balance out, leaving the screen result within the normal range.

The incidence of all complex and closed NTDs/10,000 births and stillbirths before and after fortification appeared to have dropped as well but, small sample size prevents analysis (Table 21). The incidences of complex and closed NTDs may not be affected by fortification. Conversely, there may in be a reduction in both complex and covered NTDs, but there is not enough power to detect this because the numbers are small. Further research is required to clarify which possibility is correct.

Conclusion

There has been a 37% drop in the frequency of all NTDs in Manitoba since the introduction of folic acid fortification.

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Discussion:

Comments on Methodology

NTD and MSAFP study participation rates

There were three types of participants when the NTD pilot study results are included within the larger MSAFP study of women with an elevation of MSAFP: i) women whose babies were diagnosed as having a NTD after an elevation of MSAFP: ii) women who were classified as cases for the MSAFP study because they had an unexplained elevation of MSAFP and later pregnancy complications; iii) women classified as controls for the MSAFP study who had elevations of MSAFP and no complications.

In the NTD study, 40/77 (52%) of those successfully contacted participated. The women who had had a previous NTD affected fetus were offered fetal assessment in all subsequent pregnancies and 100/106 (94%) had sought fetal assessments for subsequent pregnancies. Therefore, these women were much less likely to be lost to follow-up (23% for NTD study cases versus 40% for MSAFP cases and controls). The women were likely also more motivated to participate. Women who had lost a pregnancy to a NTD outcome had a strong desire to avoid a second loss. Some had a need to resolve emotional issues relating to the lost pregnancy. In 1986, the Health Sciences Centre initiated a programme of grief counseling and memorial services for families after a pregnancy loss under HSC social worker Susan Dolinsky. Women contacted prior to the implementation of grief counseling were far more likely to express concerns about unresolved issues or far more likely to refuse to participate based on negative experiences they had while in the

hospital. Women who had a NTD outcome were more likely to have had repeated contacts with their physician and were therefore more likely to agree to their physician's offer to participate in the research study. There were no significant differences in the percentages of women who agreed to participate in one part of the study, but not the other, in any of the three groups.

Many past negative studies have had an inadequate numbers of controls and/or small numbers of participants. Also, in our study, the greatest increase in the C677T MTHFR allele frequency was due to large numbers of heterozygotes whereas some negative studies excluded heterozygotes from consideration or included them with controls (206). Heterozygotes for the variant who have low folate intake have increases in homocysteine to levels between those of heterozygotes and those who do not have the variant. Thus excluding them, or including them with controls, may bias results. Small studies would not be able to reveal the small increases in risk that we have found. Nonetheless, given the high frequency of the C677T MTHFR variant, many women within a general population could be adversely affected even if the relative risk is too low to be detected in a small study. This study has the advantage of starting with a group of women already known to be at increased risk for complications, and of having a large control population, and a well-established population frequency for the variant, thereby increasing the sensitivity compared with much of the previous research.

Involving fathers

Among the fathers, 24 fathers were ascertained through their partners. One father asked to be included in genotyping even though his wife declined to participate in the

genotyping portion. The participation rate for fathers was 60%. Twenty case fathers of NTD affected fetuses also agreed to be involved, a participation rate of 65%. The practice of asking the women to invite their partners appears to have been a successful way to recruit fathers without involving fathers who no longer had contact with their partners.

Table 22: Comparisons of participation rates in NTD, and MSAFP studies among cases and controls.

	NTD affected cases	**Complications after ↑MSAFP**	**No complications after ↑MSAFP**
potential participants	106	342	652
participation rates from all potential participants	31 (23%)	66 (19%)	80 (12%)
lost to follow-up	25 (23%)	137 (40%)	266 (40%)
declined	50 (47%)	203 (59%)	346 (53%)
declined genotyping	6/31 (19%)	11/66 (17%)	6/80 (8%)
declined surveys	2/31 (6%)	6/66 (9%)	1/80 (1%)

Genotype results

Genotypes were available for 53 cases and 73 controls for this analysis. One case sample was lost and, for one control and one case, the DNA failed to amplify. This study established that it is possible to collect samples for DNA and serum analysis from across the province using the preexisting collection system. External laboratories were fully cooperative. Only 21/168 (total with father's samples plus two repeats) or 13% of participating laboratories billed for the service with charges ranging between $8-$40 (most commonly $25).

Analysis of dietary survey and family history questionnaires

Infants born to the mothers in both the case and control groups were 84% Caucasian, 5% Aboriginal and 11% mixed with one parent Caucasian and the other parent Aboriginal, or, in a few cases, Black or Asian. This distribution is typical for the Manitoba population (90, 130). More specific analysis of the Caucasian couples with respect to their ethnic origin did not indicate a high incidence of assortive mating, reflecting the heterogeneous and multicultural nature of the Manitoba population. There were no significant differences between cases and controls by their place of residence within the province (such as rural versus urban address).

The 126 women who agreed to provide a blood sample to the study had their homocysteine levels evaluated and 93/126 (74%) women also had their serum folate and red blood cell folate determined. None of the women was deficient in either serum folate (<7.0 nmol/L) or red blood cell folate (<430 nmol/L RBC). Four women (all controls) had homocysteine values greater than the normal upper reference value in Manitoba of 13 μmol/L at 17.7, 16.4, 14.3, and 14.1. Among the fathers, the range for homocysteine was 7.5-15 μmol/L. Four fathers (three cases and one control) had homocysteine values over 13 μmol/L, at 13.7, 14.3, 14.3, 15 umol/L, respectively and these differences were not significant.

Part way through the study, the HSC laboratory stopped offering the serum and red blood cell folate testing. Our own analysis showed that all of the women we examined had serum folate and red blood cell folate within the normal range even if their homocysteine levels were elevated and their dietary survey indicated that their intake

might be inadequate. Our study results agree with Booth *et al.,* 1998 (270) who concluded that the best way to determine deficiency of folate for women of childbearing age is an elevated serum homocysteine and not by testing red blood cell or serum folate levels.

The validity of the dietary survey was demonstrated repeatedly during the study by linear regression analysis. Consistent with known homocysteine metabolism, a negative correlation existed between serum homocysteine and both red blood cell folate and serum folate. Heterozygotes and homozygotes for the C677T MTHFR variant had higher average values for homocysteine than those who did not have the variant. Higher intake of dietary folate (including synthetic folic acid from supplements) as reported by the FFQ for the time of study was associated with higher serum folate and a trend to higher red blood cell folate, but this was not significant. *P* values in all of the validation tests were significant at ≤ 0.0001. There were no significant differences in dietary and supplemental intake of folate, B_{12} and B_6, or in the biochemical parameters of case and control mothers. There was no difference in the percentage of cases and controls who reported taking prenatal vitamin supplements (77%). Our dietary survey is therefore an appropriate and sensitive tool for measuring folate intake. We did not validate the survey for either B_{12} or B_6 intake.

Comparisons of specific data items available in the women's MMSSP charts (such as prenatal vitamin supplement use) at the time of pregnancy, with the data reported in the surveys, showed excellent agreement for every item examined. Another significant check of the family history data was to specifically examine 40 of the family history

surveys for items that were asked for by the person counseling the mothers at first contact (e.g.: birth defects in the family). There was also excellent agreement between reported data and the MMSSP files. Women who reported smoking had a significantly negative correlation with serum folate (p=0.017) and RBC folate (p=0.033) consistent with accurately reporting their smoking habits (271). We are therefore confident of the accuracy of the answers women gave us to the survey questions.

We were unable to find any difference in the frequency of heart attacks and strokes between case and control group for either the NTD study or the MSAFP study. We had expected, based on research linking elevated homocysteine and vascular disease, to see a higher frequency among families of cases. Our methodology may not have been sensitive enough to detect a difference. It may be that the control women who chose to participate did so because they were attracted to the heart disease aspect of the study outlined in the invitation. This may have skewed our family history results. If women with a family history of heart disease were more likely to choose to participate, any differences between the frequency of heart attacks in case and control families would be obscured.

Comments on Additional Results

Complications after unexplained elevations of MSAFP

Manitoba women have been offered the opportunity to have MSAFP screening, if they wish to, since 1985. On May 1, 1999, the MMSSP changed to triple testing of maternal serum by adding measurements of estriol and human chorionic gonadotrophin. While these changes were introduced to improve screening for chromosomal anomalies,

they were also expected to improve the efficiency of NTD screening, especially by reducing false positives. (A false positive is defined as a woman who tests as over ≥2.3 MOM but, whose fetus does not have a NTD.)

There are two types of false positives that require follow-up in the MMSSP. The first type of false positive is the woman who tests as initially positive, but follow-up reveals an explanation for the positive result unrelated to the presence of a NTD. The best example would be in the case of twins. When there is a multiple gestation, there are two babies contributing AFP to the mother's blood and therefore the levels initially test as elevated. After correcting for the presence of twins, the result is often converted to a negative screen result. The second type of false positive is the woman whose screen is positive, but fetal assessment neither shows a NTD nor provides any other explanation for the elevation. We defined this as an unexplained elevation of MSAFP. Women with unexplained elevations of MSAFP are at increased risk for complications of later pregnancy and are therefore monitored more carefully as their pregnancy progresses than are women with a normal MSAFP (92).

There are three ways in which the introduction of triple testing was expected to improve overall screening efficiency. First, triple testing means the addition of total human chorionic gonadotropin (HCG) and estriol to improve interpretation of the initial screening result. For example, twins normally do not have reduced estriol levels. In contrast, this combination is common in the presence of anencephaly (131). Women with incorrect dates can be more readily identified because all three markers are either higher or lower than expected. The additional information from the triple test allows for better

initial interpretation of the reason for the positive result and the possibility of ultrasound assessment of gestational age or diagnosis of the presence of twins, without a trip to Winnipeg for a full fetal assessment. The effect on the MMSSP should be an increase in the percentage of women who have an initial elevation of MSAFP result, which is then converted to a negative result.

Second, when triple screening was initiated, improvements in computerization were introduced and the MMSSP was also able to change from using gestational ages estimated to each one half week, to the actual day of gestational age. This should increase the accuracy of the initial MOM and reduce the number of unexplained false positives that are actually dating errors. This should result in a reduction in the percentage of women with initial elevation of MSAFP results.

Third, improved MSAFP testing methodology was introduced with triple testing. The actual MSAFP test is preformed at Cadham Laboratories located in Winnipeg. Cadham is supplied with the test by Amersham Biosciences. The test used before 1999 was a radioimmunochemical type. The results were determined using a gamma radiation counter. In 1999, the test was altered from radiation to chemoilluminescent. This improves dynamic working range. There have also been improvements in several quality control procedures by both Cadham and Amersham. These improvements are expected to narrow the +2 standard deviation from the mean portion of the normal distribution curve from 9-10% of the total length of the curve to 5%. In other words, the normal MOM range has been reduced (R Thompson, senior scientist, Cadham Laboratory, personal communication). Improved clinical sensitivity is the expected outcome. We have

completed a preliminary analysis that indicates this may well be the case, but this requires further research.

We attempted to compare the frequency of complications after an unexplained elevation of MSAFP before and after fortification. Given the association of these complications with an enzyme variant sensitive to folate intake and common in the population, we expected to see a drop in the incidence of either elevations of MSAFP or complications following an unexplained elevation of MSAFP, or some combination of both.

Unfortunately, there were too many files missing the final outcome data to be able to examine 2000-2002 thoroughly. The percentage of women who had complications of pregnancy compared to the total population fluctuated up and down from 1993-1995 and then dropped to the lowest rates in 1998 and 1999. This may indicate a trend to less case type complications. If this trend remains consistent in subsequent years, this may reflect the effect of fortification, but there are insufficient data to draw any conclusions. The changes in the maternal serum screening due to triple screening, while improving clinical utility of the screen test by improving sensitivity and specificity, have confounded our attempts to examine an effect of folic acid fortification on complication frequency after an elevation of MSAFP.

Detection of NTDs and abdominal wall defects

During the analysis of NTD incidence in Manitoba, we also tracked the incidence of other defects. Types of fetal anomalies were divided into three broad categories; 1)

NTDs, those with and without other anomalies; 2) abdominal wall defects (including gastroschisis, omphalocele or unspecified abdominal wall defects); 3) other anomalies that resulted in exclusion of the pregnancy from the MSAFP review, but which are not normally considered to be associated with an elevated MSAFP (i.e. heart defect, cleft lip and palate). There has been no decrease in the number of NTDs being diagnosed after an elevation of MSAFP screen result (p=0.113) even though, with dropping incidence, the actual number detected would be expected to drop (Table 23). This is likely due to improvements in screening due to the introduction of triple screening in 1999.

Table 23: Unexplained elevations of MSAFP – Outcomes

Year	Unexplained elevation of MSAFP	Controls	Cases (% of unknown)	Not classified	Case with Fetal death	% of total pop.
1993	211	141	69 (32.70%)	1	2	0.423
1994	205	138	63 (30.73%)	4	4	0.382
1995	182	111	67 (36.81%)	5	2	0.416
1996	226	155	67 (29.65%)	4	9	0.433
1997	231	154	73 (31.60%)	0	4	0.498
1998	203	137	53 (26.12%)	13	6	0.366
1999	155	103	48 (30.96%)	4	5	0.335
*2000	166	27	36	103	4	-
*2001	144	13	8	123	0	-
*2002	94	7	7	80	0	-

*Insufficient outcome data to calculate cases versus controls.

There has also been a parallel increase in both the number and proportion of abdominal defects diagnosed by an elevation of MSAFP (Table 24). Before triple screening, the percentage of elevated MSAFP tests later diagnosed as an abdominal wall defect was 0.80 (±0.10). After triple screening this mean percentage rose to 1.87

(p=0.005). There are three explanations for this observation. First, as with NTDs, there appears to be an increased ability of maternal serum screening to detect these defects with the introduction of triple screening. Second, there has been an overall increase in the incidence of abdominal wall defects in Manitoba. Third, there are an increasing number of cases of abdominal wall defects that are being rescued by folate fortification so that they reach midtrimester where they are being diagnosed by screening, whereas previously they would have been lost as early spontaneous abortions. This observation should be studied further.

Table 24: Fetal abdominal wall defects diagnosed by elevations of MSAFP.

Year	**Abdominal wall defect with ↑MSAFP** (gastroschisis, omphalocele or unspecified abdominal wall defect)
1993	3 (0.72%)
1994	3 (0.71%)
1995	4 (0.94%)
1996	4 (0.89%)
1997	4 (0.85%)
1998	3 (0.71%)
1999	8 (2.21%)
2000	7 (1.79%)
2001	3 (4.05%)
2002	8 (2.74%)

A2756G MTR results

For the variant A2756G MTR, our case mothers had a lower frequency (q = 0.3200) compared to our control mothers (q=0.1719). However, as the analysis is limited by low power due to small sample size, the Manitoba population frequency for this variant has

not been established, and our control group is not representative of that population, we could not interpret the result.

Table 25: various A2576G MTR variant studies.

Study	Ethnicity	OR Mothers	OR Fathers	OR babies	Population
(272)	Hispanics	1.19	~	1.86	whole
(120)	Caucasians	2.16	~	1.18	Mixed
(204)	Caucasians (Dutch)	1.207	0.864	1.577	Mixed
(204)	Caucasians (English)	1.00	2.43	1.36	Screen neg
(273)	Caucasians (Italians)	0.510	0.710	0.623	Screen neg
(176)	Caucasians	~	~	0.97	Screen neg
(222)	Caucasians Quebec	0.301	~	0.76	Screen neg
(246)	Alabama	~	~	1.6	Screen pos
Chapter 1	Caucasians	2.26	1.38	~	Screen pos

When we examine our study in the context of other similar studies worldwide, the results are interesting. Using the same methodology as in the C677T MTHFR analysis, (Chapter 4) the results suggest a similar pattern of possible selection bias exists. In cases that were previously screened, the variant is less common, except among the English where the opposite result is observed. In cases where at least a portion was not screened, the results show increased presence of the variant. However, small numbers of studies and limited controls are a problem in this analysis. There has not been the explosion of interest in this variant that has occurred with the C677T MTHFR variant. Further analysis of the A2576G MTR variant, especially after determining population frequencies might well show this variant is also contributor to the prevalence of NTDs. The hypothesis that homozygosity for C677T MTHFR in the fetus acts with some other maternal genetic

effect(s), including *methionine synthase* (MTR), to produce an important percentage of the NTDs that occur in the world is supported by this analysis although further research is required to confirm this. Such research must taken into account the possibility of a screening bias created by this variant (see Table 26).

Comments on mechanisms

We have previously commented on the mechanisms that may underlie NTDs in the Background. There are several possible explanations for the mechanism underlying the association between elevations of MSAFP and C677T MTHFR. Increased levels of total plasma homocysteine are associated with an increased risk of thrombosis (12). Potentially due to interactions between the reactive sulfhydryl moiety of homocysteine and molecules involved in coagulation cascades. One hypothesis is that the unique physiology and anatomy of the placenta may make it especially vulnerable to the detrimental effects of homocysteine causing vascular disruptions in the placenta (68). However, it has recently been shown that C677T MTHFR does not affect maternal serum homocysteine levels in pregnancy among women who take prenatal multivitamins (83). Therefore, it is unlikely that the affect of C677T MTHFR is within the placenta in later pregnancy among the participants in our study, the majority of who took prenatal supplements once their pregnancy was recognized, but did not take periconceptional supplements.

It seems more likely that having one or two C677T MTHFR alleles interferes with placental development in early pregnancy, before women begin taking prenatal vitamins. Inhibition of normal fetal trophoblast differentiation by a mechanism similar to the one

suggested previously (see Background and Chapter 3 for more details) for the cause of NTDs could result in reduced invasion by fetal cytotrophoblastic cells during transformation of the spiral arteries. Increased apoptosis may also be a factor with dying cells creating regions of necrosis within the placenta. Both may be more likely to be present in fetuses with the C677T MTHFR variant when folate levels are low. One immediate consequence of this explanation is that prenatal vitamin supplements will not adequately protect women if taken only after pregnancy is recognized. As is the case for NTDs, periconceptional supplementation or folic acid fortification may be necessary for women to have the full benefit of additional folate. We did see some preliminary evidence in the MSAFP of a trend to reduced frequency of complications compared to the total population since the introduction of folic acid fortification. This trend requires confirmation.

If the paternal gene is the only gene expressed in early placental development (70, 71), this would mean a paternally inherited C677T MTHFR allele would be the most vulnerable. Alternatively, the most vulnerable genotype may be a C677T MTHFR homozygous baby as has been observed for NTDs (180) and confirmed by our own results from the NTD review (in Chapter 4). Maternally inherited C677T MTHFR would contribute directly to a homozygous at risk fetal genotype. Maternal genotype may also contribute indirectly through an environmental effect of lower folate for either of these potentially at risk fetal genotypes (120). This would explain our observations of an increased frequency of T alleles in both parents.

There is a correlation between physiologically relevant levels of total plasma homocysteine and likelihood of thrombosis. This may possibly be due to interactions

between the reactive sulfhydryl moiety of homocysteine with molecules involved in coagulation cascades and/or by metabolic interactions with levels of S-nitrosoalbumin and nitric oxide (274, 275). Variants of other genes for metabolically related enzymes (such as G894T *nitric oxide synthase*) may also interact with the C677T MTHFR variant. Nothing in this research either discounts or proves this possibility and it merits further exploration.

Only some thrombophilic complications of pregnancy related to hyperhomocysteinemia in women who do not take prenatal vitamins or to thrombophilia in general can be attributed to the C677T MTHFR variant. It is therefore important to look for synergistic interactions between the C677T MTHFR variants and other genetic polymorphisms (92). Once other contributing factors to genetic thrombophilia have been studied for their contribution to elevated MSAFP and complications of pregnancy, it may become appropriate for all women with an unexplained elevation of MSAFP and complications to undergo thrombophilic screening.

Summary of significant results

In this work, the first significant finding is an association between unexplained elevations of MSAFP when both parents have one or two alleles for C677T MTHFR. This confirms the result that we found in a smaller pilot study of this group. The second important finding is an increase in the frequency of C677T MTHFR in partners of women who go on to have a complication of pregnancy after an elevation of MSAFP compared to the newborn population. The third finding is that there is a potential bias in studies of

NTD and C677T MTHFR because of the independent association between MSAFP and C677T MTHFR and termination of affected pregnancies after a positive screen test.

Future Research

1) We were unable to detect any change in the frequency of complications of later pregnancy after an unexplained elevation of MSAFP due to lack of full outcomes for the period after fortification began. It is expected that the frequency of many complications of pregnancy, beyond those associated with malformations such as NTDs, will be reduced. This should be examined.

2) Folic acid is currently available to women by both supplementation and by food fortification. More research is required to determine how to improve periconceptional supplementation rates and to determine if fortification at current levels is adequate. It is of note that among the few women who indicated they were supplementing periconceptionally for a first pregnancy, several stated they were doing so because their pharmacist recommended it. The potential role of pharmacists has not been well considered and may well be underestimated. One area of research to explore is the potential role of pharmacists in encouraging periconceptional supplementation. The generally poor level of periconceptional supplementation found in this study indicates current methods are ineffective.

3) MTHFR is only one of several enzymes directly involved in folate metabolism. The C677T MTHFR variant is only one variant of this enzyme. Research into other variants of MTHFR and related enzymes should be undertaken. This should include the larger family of folate receptors as some variants of these are known to exist and there are likely more.

4) Some types of folate receptors are expressed in different concentrations in different tissues and there is variability in the affinity for folate. Of particular interest are folate receptors that may be expressed only in particular stages of development. Another potentially fruitful study is to examine women for auto antibodies to folate receptors and how different variants of folate receptors or variation in the immune system may be involved (276).

5) The hypothesis in the Background section of a proposed high affinity PEMT variant expressed specifically during open neural plate stage would be a similar embryologically specific protein. These patterns of embryologically specific protein expression require further study. These proteins may also have variants that affect development under specific environmental effects or interact with variants of "housekeeping" genes such as the C677T MTHFR.

6) The A2756G MTR issue, which prevents analysis of the results of our work, illustrates the need for developing high quality, appropriate population samples for determining

allele frequency. While there have been some excellent steps towards providing a comprehensive population based allele frequencies for the C677T MTHFR variant (87), no other variant allele has been given similar attention. Studies of variants cannot proceed without a good population frequency with which to compare results. Transmission disequilibrium tests can be useful in determining if a variant merits further research on the scale of ascertaining large samples for determining population allele frequencies.

7) We did not test for B_{12}. However, there has been some clinical indication that B_{12} deficiency in pregnancy may be relatively common in Manitoba (Micheil Innes, personal communication) as has already been noted in the literature for other regions other than Manitoba (277-279). It would be valuable to validate the dietary survey for B_{12} and test women in Manitoba for B_{12} intake. As B_{12} is a cofactor for *methionine synthase,* increasing folic acid alone may not reduce the incidence of either neural tube defects or later complications of pregnancy as much as a combination of B_{12} and folic acid. The same may also be true of the other cofactor involved in homocysteine metabolism, B_6 and other metabolically related nutrients such as riboflavin (280, 281). Examination of intake patterns and enzyme variants for metabolizing these related vitamins could increase our ability to prevent complications of pregnancy.

8) The possible effect of folic acid on the prevalence of abdominal wall defects requires exploration. It is important to determine if the increase in abdominal wall defects detected because of an elevation of MSAFP is due to better survival rates with fortification or due to improvements in screening technology.

9) We have shown that maternal serum screening is affected by the presence of the C677T MTHFR variant. MASFP screening is used not only for NTDs. It is also used to screen for chromosomal abnormalities and defects such as abdominal wall defects. The MSAFP test is also only one of several screen tests currently in use. The discovery of this variant's effect on screening raises several possibilities. Are other variants similarly creating screening biases? Have these other potential biases appeared in the examination of the association between various conditions of concern with other variants? Are other screening tests causing similar biases?

10) We attempted to study the extended families of parents of babies with NTDs. We suspect that these families are also at increased risk for heart attack and stroke. As the variant may be protective against cancer, perhaps these families also have a reduced risk for cancers. Unfortunately, our methodology appears to have lacked the necessary sensitivity. Nonetheless, this avenue of study is worth continued exploration. If these families are at increased risk, pregnancy and childbirth is an ideal time to begin lifestyle changes in young couples that could serve to reduce this risk in future.

Conclusion

This thesis set out to explore three interrelated questions. 1) Is the C677T MTHFR variant a genetic contributor to NTDs independently of a positive or negative MSAFP result? 2) Is there a difference in the C677T MTHFR allele frequency in parents with and without complications of pregnancy after an unexplained elevation of MSAFP? 3) Have we seen a reduction in NTD incidence and in the incidence of other complications of pregnancy in Manitoba since folic acid fortification of food began?

We have shown the C677T MTHFR variant is an independent contributor to NTDs through our meta-analysis. According to our results, the increase is modest, but significant worldwide, accounting for approximately 10% of the genetic contribution to cases of multifactorial NTDs. We agree with the conclusions of the TDT of the Irish research group, that the C677T MTHFR homozygote fetus is the "at risk" genotype for NTDs (180). This variant is folate sensitive and folic acid fortification will reduce the prevalence of NTDs. We have shown a significant (37%) decrease in the incidence of NTDs in Manitoba since the introduction of folic acid fortification in Canada an observation that supports our conclusion.

We have shown that an increase in the allele frequency of the C677T MTHFR variant exists in those couples where an unexplained elevation of MSAFP is followed by later complications of pregnancy. The 'at risk' individual appears to be either a fetus with a paternally inherited C677T MTHFR allele or a homozygous fetus. There may be an environmental effect due to the maternal C677T MTHFR allele as well. The increased frequency of C677T MTHFR was not found among parents who did not have a

complication of pregnancy and so a different mechanism likely underlies their unexplained elevation of MSAFP. The effect is probably not due to any elevation of homocysteine in later pregnancy because a substantial proportion of the women in this study took prenatal vitamins. The effect is likely present in early pregnancy before women begin taking prenatal vitamins. Preliminary indications from our review of the MMSSP indicate there may be a trend favoring the hypothesis that folic acid fortification is reducing the frequency of unexplained elevations of MSAFP followed by complications of pregnancy, but this remains to be confirmed.

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*Alexander Matthew Hunstad was born after a normal labor and delivery on October 16, 2004 weighing 6lbs, 7oz. His doting Savtah was among those present to greet him.

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Appendices

Appendix 1: Am J Hum Genet 67(4) Suppl 2: 417

2351

Association of thermolabile 5,10-methylenetetrahydrofolate reductase (C677T) with elevated maternal serum alphafetoprotein and normal pregnancy outcome. *N.K. Bjorklund*[1]. J.A. *Evans*[1,2,3], *CR. Greenberg*[1,2],

1) Biochemistry and Med. Genetics; 2) Pediatrics and Child Health, University of Manitoba; 3) Community Health Sciences, University of Manitoba, Winnipeg, Manitoba, Canada.

Introduction: We compared the frequency of the ^{5,10}methylenetetrahydrofolate reductase C677T thermolabile variant (tMTHFR) in three groups: 1) parents of babies with isolated neural tube defects (NTD), 2) parents of babies whose mothers had elevated midtrimester MSAFP with normal outcome, 3) large unselected newborn population in Manitoba, Canada. Methods: Cases and controls were selected on the basis of elevated MSAFP with NTDs and with normal outcomes respectively. They were studied with respect to tMTHFR, dietary and supplemental intake of folate, B_{12}, B_6, urban/rural location, ethnicity, baby's gender, mother's age and parity. Collaborators in our department had previously established the baseline allele frequency of tMTHFR in Manitoba (0.2497) by the analysis of 977 anonymous consecutive newborn blood spots collected for routine neonatal screening. Results: Twenty-five case and 32 control mothers, and 19 case and 18 control fathers participated. When compared to tMTHFR in the Manitoba population, cases did not differ significantly (OR 1.18, p=0.640). However, we found that controls did differ significantly from the baseline population frequency (OR 1.42, p=0.012). Conclusion: Compared to the Manitoba population, our cases had elevated tMTHFR that, while not significant, was consistent with most literature reports. This is, however, the first report that suggests elevated MSAFP in the absence of a NTD outcome is associated with a higher frequency of tMTHFR. Elevated MSAFP is also associated with adverse outcomes in later gestation including prematurity, preeclampsia, placental abnormalities and growth retardation. Our results are suggestive of a possible role for tMTHFR in all these complications of later pregnancy. Prospective analysis of tMTHFR in all mothers with elevated MSAFP and non-NTD outcomes, including third trimester complications, is being pursued.

Appendix 2a Case

Section of Genetics & Metabolism
FE229 - Community Services
Bldg. Health Sciences Centre 820
Sherbrook Street Winnipeg, MB
R3A 1 R9 Phone: (204) 787-2494
FAX: (204) 787-1419

As you may recall, I saw you in 19XX as one of the genetic specialists involved in your care when it was identified that your baby was affected with a neural tube defect. Recently my colleague Dr. Cheryl Greenberg and myself have received approval to proceed with a study investigating one possible cause of neural tube defects. I am sending you a summary of this study and I would encourage you if at all possible to participate. If you would like to participate in this study, please complete and return the attached form in the enclosed envelope or by FAX. If you would like further information please contact me by phone at 787-3395 (or e-mail jevans@ms.umanitoba.ca).

Thank you for your attention to this program.

Yours
Sincerely,

Dr. Jane Evans, B.Sc., Ph.D.,
FCCMG Professor, Division
of Human Genetics

Appendix 2b Control

Section of Genetics & Metabolism FE229 - Community Services Bldg. Health Sciences Centre 820 Sherbrook Street Winnipeg, MB R3A 1 R9 Phone: (204) 787-2494 FAX: (204) 787-1419

January 31, 2000

Dear,

As you may recall, in 19XX you were referred for a fetal assessment when your maternal serum AFP test came back as being higher than expected. The subsequent fetal assessment revealed no abnormalities and we understand your baby is fine. As you may be aware, the Health Sciences Center is a teaching and research center as well as a tertiary health care center. My colleague Dr. Jane Evans and myself have received approval to proceed with a study investigating a severe birth defect known as a neural tube defect. Your baby does not have a neural tube defect but we are inviting you to participate in this study as a "control". Complete information on the study is enclosed. If you would like to participate in this study, please complete and return the attached form in the enclosed envelope or by FAX. If you would like further information please contact me by phone at 787-2494 (or e-mail greenbrg@cc.umanitoba.ca.).

Thank you for your attention to this program.

Yours
Sincerely,

Dr. Cheryl Rockman Greenberg
MDCM, FRCPC, FCCMG
Director, Metabolic Services
Clinical Geneticist, Section of Genetics and Metabolism Depts of Pediatrics & Child Health and Human Genetics

Appendix 3a Case

"HOMOCYSTEINE METABOLISM AND NEURAL TUBE DEFECT" STUDY

STUDY INVESTIGATORS:
Dr. Cheryl R. Greenberg 787-2494
Dr. Jane Evans 789-3395
Departments of Human Genetics
and Pediatrics & Child Health

INFORMATION FOR PARENTS - STUDY GROUP

Since 1985 in Manitoba we have offered pregnant women a screening test called MSAFP to determine if they are at increased risk to have a child with certain types of congenital malformations including neural tube defects. You opted to have this test and it and subsequent investigations revealed that you were carrying a baby with a neural tube defect. Later, you were offered counseling at the Genetics Clinic at the Health Sciences Centre to discuss the cause of this problem, its likelihood-of recurrence and possible methods of prevention in future pregnancies. As you may recall being told, neural tube defects result from the failure of closure of the spinal canal very early in a baby's development. It is believed that both genetic factors (factors in the baby's genetic blueprint) and nongenetic factors (factors in the environment, external to the baby) contribute to this congenital malformation. Recently a new relationship has been suggested between a lower than average level of the vitamin folic acid in the mother's blood and an increased risk for the baby to have a neural tube defect. The level of folic acid in our bodies is determined by the type of foods we eat and whether or not we take vitamins that contain added folic acid. Folic acid is converted to different active forms in the body. The level of one of the active forms of folic acid called 5,10 methylene-tetrahydrofolate (5,1 O-MTHF) is controlled by several important genes. A gene is a unit of heredity composed of the chemical molecule DNA. One gene important in regulating the level of 5,10-MTHF is known as MTHFR. A genetic error in MTHFR has been shown to be associated with an inability to convert 5,10 MTHF to other active forms. This can lead to an increased level of the amino acid known as homocysteine. Recent studies have shown that parents of children with neural tube defects may have an altered form of MTHFR that can lead to both increased levels of plasma homocysteine and an increased risk for neural tube defects. Increased homocysteine has also recently been associated with an increased risk for cardiovascular disease and stroke.

As you have had a child with a neural tube defect, we are asking if you would like to participate in a study to determine whether or not you have a

higher level of homocysteine than normal and whether you carry a variant form of MTHFR. If you agree to this study, we will:

1) collect information from you on any history of heart attacks or stroke in yourself, your brothers or sisters, parents, aunts, uncles and cousins, and draw up a family tree.
2) Record information about your diet during pregnancy and about vitamin supplementation.
3) perform the following laboratory investigations on a blood sample:
    a. measure plasma homocysteine level
    b. perform DNA analysis for the variant form of MTHFR

These blood tests will required 5-10 mls of blood collected from a vein into a blood collection tube using standard sterile technique. Withdrawing blood from a vein in this fashion poses little risk to yourself except for mild discomfort or possible bruising at the blood taking site.

If you are found to have an elevated homocysteine level or a variant of MTHFR, then further laboratory investigations and nutritional counselling regarding folic acid supplementation will be offered to you and other family members. You will have already received information regarding the importance of taking folic acid supplements - prior to any future pregnancy and continuing them for the first three months once pregnancy is confirmed. participation in this study is entirely voluntary.

You may withdraw from this study at any time, even after signing this form and this will, in no way, affect the medical care you will receive. No records bearing your or your child's name will leave this institution and your child or yourself will not be identified in any medical publication. Further information may be obtained from the local investigators of this study.

Please check off one box and return in enclosed envelope or FAX. Thank you.

☐ I DO NOT WISH TO PARTICIPATE IN THIS STUDY AT THIS TIME.

☐ I WISH TO PARTICIPATE IN THIS STUDY. PLEASE CONTACT ME.

☐ I WOULD LIKE FURTHER INFORMATION. PLEASE CONTACT ME.

Parents: _____

Date:_____

Appendix 3b Control

PATIENT INFORMATION - COMPARISON GROUP

"HOMOCYSTEINE METABOLISM AND NEURAL TUBE DEFECT" STUDY

STUDY INVESTIGATORS:
Dr. Cheryl R. Greenberg 787-2494
Dr. Jane Evans 789-3395
Departments of Human Genetics
and Pediatrics & Child Health

Since 1985 in Manitoba we have offered pregnant women a screening test called MSAFP to determine if they are at increased risk to have a child with certain types of congenital malformations including neural tube defects. You opted to have this test and your MSAFP level was high. Subsequent investigations revealed that your baby had no obvious congenital malformations. As you may recall being told,

neural tube defects result from the failure of closure of the spinal canal very early in a baby's development. It is believed that both genetic factors (factors in the baby's genetic blueprint) and nongenetic factors (factors in the _environment, external to the baby) contribute to this congenital malformation. _Recently a new relationship has been suggested between a lower than average level of the vitamin folic acid in the mother's blood and an increased risk for the baby to have a neural tube defect. The level of folic acid in our bodies is determined by the type of foods we eat and whether or not we take vitamins that contain added folic acid. Folic acid is converted to different active forms in the body. The level of one of the active forms of folic acid called 5,10 methylene-tetrahydrofolate (5,1 O-MTHF) is controlled by several important genes. A gene is a unit of heredity composed of the chemical molecule DNA. One' gene important in regulating the level of 5, 10-MTHF is known as MTHFR. A genetic error in MTHFR has been shown to be associated with an inability to convert 5,10 MTHF to other active forms. This can lead to an increased level of the amino acid known as homocysteine. Recent studies have shown that parents of children with neural tube defects may have an altered form of MTHFR that can lead to both. increased levels of plasma homocysteine and an increased risk for neural tube defects. Increased homocysteine has also recently been associated with an increased risk for cardiovascular disease and stroke.

During your participation in our Maternal Serum AFP Screening Program you were identified to have an increased maternal serum AFP. Although your baby was not identified to have a neural tube defect, we are asking you to participate in this study as a "control" to provide a comparison to a study

population of parents with children with neural tube defects. If you agree to this study, we will:

1) collect information from you on any history of heart attacks or stroke in yourself, your brothers or sisters, parents, aunts, uncles and cousins, and draw up a family tree.
2) Record information about your diet during pregnancy and about vitamin supplementation.
3) perform the following laboratory investigations on a blood sample:
   a. measure plasma homocysteine level
   b. perform DNA analysis for the variant form of MTHFR

These blood tests will required 5-10 mls of blood collected from a vein into a blood collection tube using standard sterile technique. Withdrawing blood from a vein in this fashion poses little risk to yourself except for mild discomfort or possible bruising at the blood taking site.

If you are found to have an elevated homocysteine level or a variant of MTHFR, then further laboratory investigations and nutritional counselling regarding folic acid supplementation will be offered to you and other family members. You will have already received information regarding the importance of taking folic acid supplements - prior to any future pregnancy and continuing them for the first three months once pregnancy is confirmed. participation in this study is entirely voluntary.

You may withdraw from this study at any time, even after signing this form and this will, in no way, affect the medical care you will receive. No records bearing your or your child's name will leave this institution and your child or yourself will not be identified in any medical publication. Further information may be obtained from the local investigators of this study.

Please check off one box and return in enclosed envelope or FAX. Thank you.

☐ I DO NOT WISH TO PARTICIPATE IN THIS STUDY AT THIS TIME.

☐ I WISH TO PARTICIPATE IN THIS STUDY. PLEASE CONTACT ME.

☐ I WOULD LIKE FURTHER INFORMATION. PLEASE CONTACT ME.

Parents: _____

Date:_____

Appendix 4

UNIVERSITY OF MANITOBA
FACULTY COMMITTEE ON THE USE OF HUMAN SUBJECTS IN RESEARCH

NAME: Dr. C Greenberg

DATE: December 15/97
REFERENCE: E97 341

YOUR PROJECT ENTITLED:

Protocol Title: Homocysteine Metabolism and Neural Tube Defects

Approval of study Information for Parents and Consent Form - study Group (undated)

Patient Information and Consent Form - Comparison Group

HAS BEEN APPROVED BY THE COMMITTEE AT THEIR MEETING OF:

Approved by Dr. G. Grahame on behalf of the committee on December 13, 1997

COMMITTEE PROVISOS OR LIMITATIONS:

Approved as per your letter dated December 5, 1997

You may be asked at intervals for a status report. Any significant changes of the protocol should be reported to the Chairman for the Committee's consideration, in advance of implementation of such changes

**THIS IS FOR THE ETHICS OF HUMAN USE ONLY. FOR THE LOGISTICS OF PERFORMING THE STUDY, APPROVAL SHOULD BE SOUGHT FROM THE RELEVANT INSTITUTION, IF REQUIRED.

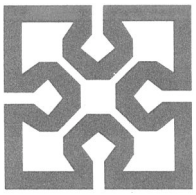
Sincerely yours,

THE UNIVERSITY OF MANITOBA

Gordon R. Grahame, M.D., Chairman,
Faculty Committee on the Use of
Human Subjects in Research
GRHjtk

Inquiries should be directed to Theresa Kennedy
Telephone: 789-3255 Fax: 789-3942
E-mail: kennedy@bldghsc.lan1.umanitoba.ca

Appendix 5a Case



Health Sciences Centre

Section of Genetics and Metabolism
FE229- Community Services Bldg.
Health Sciences Centre 820
Sherbrook Street Winnipeg, MB R3A
1R9 Phone: (204) 787-4803 FAX:
(204) 787-1419

June 10, 2003

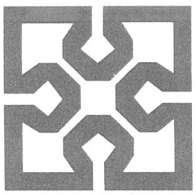
Dear Mrs.

As you may recall, in 1997, it was identified that your maternal serum AFP result was elevated. Your baby appeared to be normal and healthy at a midtrimester fetal assessment. I am a genetic specialist and the codirector of the Manitoba Serum Screening program. Our MSAFP program routinely follows the pregnancy outcomes of all mothers who have an MSAFP screen test. Subsequently, our follow up program was informed that you had a complication in later pregnancy of the type more likely to occur in women with an elevated MSAFP and normal fetal assessment at mid pregnancy. Recently my colleagues Drs. Cheryl Greenberg, Jane Evans and myself, have received approval to proceed with a study investigating one possible cause of these complications of later pregnancy. I am sending you a summary of this study and I would encourage you to participate if at all possible. If you would like to participate in this study, please complete and return the attached form in the enclosed envelope or by FAX. If you would like further information about this study please contact us at the numbers provided in the enclosed Participant Information and Consent Form or return the form marked in the appropriate section and the study coordinator will contact you.

Thank you for your attention to this program.
Yours Sincerely,

Dr. Bernie Chodirker, MSc, MD, FRCPC, FCCMG Clinical Geneticist,
Section of Genetics and Metabolism Departments of Pediatrics & Child Health and
Biochemistry & Medical Genetics

Appendix 5b Control



Health Sciences Centre

Section of Genetics and Metabolism
FE229- Community Services Bldg.
Health Sciences Centre 820
Sherbrook Street Winnipeg, MB R3A
1R9 Phone: (204) 787-4803 FAX:
(204) 787-1419

June 10, 2003

Dear Mrs.

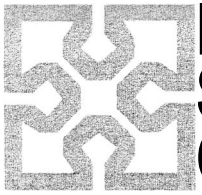
As you may recall, in 2002, you were referred for a fetal assessment when it was identified that your maternal serum AFP was elevated. The subsequent fetal assessment revealed no abnormalities and we understand your baby is fine. As you may be aware, the Health Sciences Centre is a teaching and research center as well as a tertiary health care center. Recently, my colleagues Dr. Cheryl Greenberg, Dr. Jane Evans and myself have received approval to proceed with a study investigating one possible cause of these complications of later pregnancy. You did not have any of the complications we are studying in your pregnancy, but we are inviting you to participate in this study as a "control". Complete information on the study is enclosed. If you would like to participate in this study, please complete and *teturn* the attached form in the enclosed envelope or by FAX. If you would like further information, contact numbers where the study coordinator and the study's doctors can be reached are included in the Participant Information and Consent Form. Thank you for your attention to this program.

Thank you for your attention to this program.

Yours Sincerely,

Dr. Bernie Chodirker, MSc, MD, FRCPC, FCCMG Clinical Geneticist,
Section of Genetics and Metabolism Departments of Pediatrics & Child Health and
Biochemistry & Medical Genetics

Appendix 6a Case



Principal Investigator:

Dr. Bernard Chodirker Section of
Metabolism and Genetics
FE229 820 Sherbrook St.
R3A 1R9

Co-Investigators:
Dr. Cheryl Greenberg Section of
Genetics and Metabolism
FE229 820 Sherbrook St.
R3A 1R9

Dr. Jane Evans
Dept. of Biochemistry and Medical Genetics
University of Manitoba 336 BMS Bldg- 770
Bannatyne Ave. RJE OW3

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your doctor, mends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of the Study

This human research study is being conducted to study complications of the third trimester of pregnancy in women who have had an elevated MSAFP screen test and whose baby appeared normal at a mid pregnancy ultrasound. You are being asked to take part in this study because you have previously had a third trimester complication following a positive MSAFP screen test. A total of approximately 2000 women will be asked to participate in this study.

Since 1985 in Manitoba we have offered pregnant women a screening test measuring the level of alpha-fetoprotein in the maternal blood known as MSAFP in order to determine if they are at increased risk to have a baby with certain types of malformations including neural tube defects (such as spina bifida and anencephaly). Since our program began, researchers in larger centers have found that women who have an elevated MSAFP result, but whose baby appears normal at a mid-pregnancy ultrasound, are more likely to have complications later on in their pregnancy.

These complications include a smaller baby, an early delivery, high blood pressure, or problems with bleeding or delivering the placenta after the baby is born. You opted to have this test and your MSAFP level was high. Subsequent investigations

revealed that your baby had no obvious malformations, however, you went on to have at least one of the complications mentioned above in the third trimester. It is believed that both genetic factors (factors in the baby's or mother's genetic blueprint) and non-genetic factors (factors in the environment) contribute to these complications of later pregnancy.

Recently a new relationship has been suggested between a lower than average level of vitamins, especially folic acid, in the mother's blood and an increased risk for complications of later pregnancy. The levels of these vitamins in our bodies are determined by the foods we eat and whether or not we take vitamin supplements. The vitamin folic acid is converted to different active forms in the body. The presence of other vitamins, specifically B_6 and B_{12}, are also important in contributing to the necessary active forms of folic acid.

The level of one of these active forms of folic acid, called ^{5,10,}methylene-tetrahydrofolate (^{5,10,}MTHF) is controlled by several important genes. A gene is a unit of heredity composed of the chemical molecule DNA. One gene important in regulating the level of ^{5,10,}MTHF is known as MTHFR. A genetic error in MTHFR has been shown to be associated with an inability to convert ^{5,10,}MTHF to other active forms. This can lead to an increased level of the amino acid known as homocysteine. Recent studies have shown that pregnant women who have higher levels of homocysteine in their blood are more likely to develop complications of later pregnancy. Such women may also be more likely to have an altered form of MTHFR (tMTHFR). This can lead to higher levels of homocysteine in the blood. Increased homocysteine in the blood has been associated with increased incidence of blood clotting disorders in pregnant and in non-pregnant women as well as in men. It has also been associated with an increased risk for cardiovascular disease and stroke.

Study Procedures

If you agree to participate in this study, we will:

1) collect information from yourself on any history of blood clotting disorders, heart attack or strokes, cancer and other dieseases in yourself, your brothers or sisters, parents, aunts, uncles and cousins and your baby's father's family and draw up a family tree.

2) record information about your diet during pregnancy and about vitamin supplementation.

3) perform the following laboratory tests on a blood sample:

   a. measure plasma homocysteine

   b. measure the amount offoiate in your blood

   c. perform DNA analysis for the variant form of MTHFR (tMTHFR)

4) store your DNA extracted fTom your blood sample for possible analysis of other genetic variants in the future related to complications of pregnancy, especially blood clotting disorders.

These blood tests will require 15 mls (1 tablespoon) of blood collected from a vein into blood collection tubes using standard sterile technique. We do not require a blood test fTom your child or any other relative. It is not necessary for you to come to the Health Science Center in Winnipeg for this test. We can arrange the blood test in laboratory of your choice including the laboratory of your own family doctor. You may choose to come to the Health Sciences Center for the blood test if that is more convenient for you. It should not take more than a few minutes for the blood test once you are at the laboratory. There is no charge to you for this blood test.

Mothers who have taken part in the dietary survey section of other related research projects in the past have told us that it normally takes one half hour or less to complete the form. The family history survey normally takes an hour to complete but this varies depending on the family. If your family is large or you wish to ask other relatives for information it may take you longer than this. If you do not have information available about your family history (for example, if you are adopted) you may still choose to participate. Information for the family history portion of the study requires you to provide only a first name for your relatives and we will not contact any relative for confirmation or share your results with a relative or any other individual without your permission.

You do not have to come to the Health Sciences Center to complete the survey. Both surveys will be mailed to you to complete in your home at your own pace. The surveys can be returned by mail and you will be also receive a postage-paid envelope for that purpose. If English is not your first language, we can arrange for translation services for you. If you require special assistance in completing the survey it will be made available to you through the study's coordinator at your request

Risks and Discomforts

Withdrawing blood from a vein in the fashion required for this study poses little risk to you except for mild discomfort or possible bruising at the blood-taking site.

Benefits

By participating in this study as a case you will be providing information to the study doctors that may show an association between diet and genetic variants and the risk for complications in the third trimester of pregnancy. We hope the information learned from this study will benefit women with complications of the third trimester of pregnancy and their babies.

There mayor may not be direct medical benefit to you from participating in this study. The possible benefit to you in choosing to participate is that any nutritional deficiency of vitamins B12, B6, and folic acid that you may have will likely be identified. You will then be otlered nutritional counseling that will allow you to correct such a deficiency. If you are found to have an elevated homocysteine level in your blood, this will also be identified. You will receive, if you wish, counseling on treating and monitoring of elevated homocysteine in order to reduce any potential increased risk for disease. If you are identified as having the variant MTHFR (tMTHFR) you will be provided with counseling about the consequences, treatments, or risks, if any. Your family history will be reviewed for the study. If there is a pattern that identifies a genetic risk for other types of disorders, you will be informed and offered genetic counseling.

Participation

Your participation in this study is entirely voluntary. If you are willing to participate in only part of the study, for example you wish to have the blood test but not complete the dietary survey, you may choose to do so. You may withdraw from all or part of this study at any time, even after signing this form, and this will, in no way, affect the medical care you will receive. The researchers may decide to remove you from the study if funding is stopped or other information becomes available. There are no risks to you if you choose to stop your participation.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include the Manitoba Medicine Faculty of Medical Research Ethics Board.

Questions

You are free to ask any questions that you may have about this study and your rights as a research participant. If any questions come up during or after the study contact the study doctors, Dr. Bernie Chodirker or Dr. Cheryl Greenberg at (204) 787- 4803 or 787-2711 respectively or the study coordinator Natalie Björklund at (204) 789-3392 (or through her pager at 932-7275). For questions about your rights as a research participant, you may contact the University of Manitoba Faculty of Medicine Research Ethics Board

at (204) 789-3389. Do not sign this form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent:

I have read this consent form. I have had the opportunity to discuss this research study with Drs. Chodirker or Greenberg and Natalie Bjorklund. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I have been given a copy of this consent torm. I understand that my participation in this research study is voluntary and that I may choose to withdraw from this study at any time. If so, there will no change in my medical management. I understand that no personal information will be marked on my stored DNA sample but researchers will use an anonymous coding system. I may request that my DNA sample be removed from the DNA bank and destroyed at any time.

As my DNA may be stored for as long as twenty years, for the following statements I am indicating my choices as to the long term handling and storage of my DNA, specifically:

1.1 give my consent for the DNA sample extracted ftom my blood to be used in the search for causes of high MSAFP and complications of the third trimester, but my DNA will be discarded once the initial results of the MIHFR analysis are available.	Yes	No
2.If my DNA is destroyed, I understand that if I want further genetic testing to be done in the future I will need another blood test taken.	Yes	No
3.The DNA obtained ftom me may be stored for 20 years so that further testing may be perfonned with respect to MSAFP and complications of the third trimester of pregnancy	Yes	No
4.I wish to be recontacted regarding the results of any new tests that are pertonned on my DNA in future.	Yes	No
5.Samples may be used in this laboratory or sent to other laboratories for research on other genetic diseases after all identifying information has been removed.	Yes	No
6. Prior to my death, members of my family are allowed access to my stored DNA only if! give written permission.	Yes	No
7. My first-degree relatives will be allowed access to my stored DNA after my death.	Yes	No

I freely agree to participate in this study. I understand that information regarding my personal identity will be kept confidential but that confidentiality is not guaranteed. I authorize inspection of my medical records by University of Manitoba Faculty of Medicine Research Ethics Board

By signing this consent form I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature: Date:

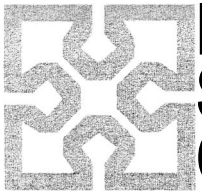
Signature of person explaining the study:

I do not wish to participate in this study. Please do not contact me again in regards to this study. _____

I may be interested in this study but I wish more information first. Please call me at _____.
The best time to reach me is at _____

Name: _____

Appendix 6b Control



Principal Investigator:
Dr. Bernard Chodirker Section of
Metabolism and Genetics
FE229 820 Sherbrook St.
R3A 1R9

Co-Investigators:
Dr. Cheryl Greenberg Section of
Genetics and Metabolism
FE229 820 Sherbrook St.
R3A 1R9

Dr. Jane Evans
Dept. of Biochemistry and Medical Genetics
University of Manitoba 336 BMS Bldg- 770
Bannatyne Ave. RJE OW3

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your doctor, mends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of the Study

This human research study is being conducted to study complications of the third trimester of pregnancy in women who have had an elevated MSAFP screen test and whose baby appeared normal at a mid pregnancy ultrasound. A total of approximately 2000 women will be asked to participate in this study.

Since 1985 in Manitoba we have offered pregnant women a screening test measuring the level of alpha-fetoprotein in the maternal blood known as MSAFP in order to determine if they are at increased risk to have a baby with certain types of malformations including neural tube defects (such as spina bifida and anencephaly). Since our program began, researchers in larger centers have found that women who have an elevated MSAFP result, but whose baby appears normal at a mid-pregnancy ultrasound, are more likely to have complications later on in their pregnancy.

These complications include a smaller baby, an early delivery, high blood pressure, or problems with bleeding or delivering the placenta after the baby is born. You opted to have this test and your MSAFP level was high. Subsequent investigations revealed that your baby had no obvious malformations and you went on to have a healthy baby without complications.

Recently a new relationship has been suggested between a lower than average level of vitamins, especially folic acid, in the mother's blood and an increased risk for complications of later pregnancy. The levels of these vitamins in our bodies are determined by the foods we eat and whether or not we take vitamin supplements. The vitamin folic acid is converted to different active forms in the body. The presence of other vitamins, specifically B_6 and B_{12}, are also important in contributing to the necessary active forms of folic acid.

The level of one of these active forms of folic acid, called ^{5,10,}methylene-tetrahydrofolate (^{5,10,}MTHF) is controlled by several important genes. A gene is a unit of heredity composed of the chemical molecule DNA. One gene important in regulating the level of ^{5,10,}MTHF is known as MTHFR. A genetic error in MTHFR has been shown to be associated with an inability to convert ^{5,10,}MTHF to other active forms. This can lead to an increased level of the amino acid known as homocysteine. Recent studies have shown that pregnant women who have higher levels of homocysteine in their blood are more likely to develop complications of later pregnancy. Such women may also be more likely to have an altered form of MTHFR (tMTHFR). This can lead to higher levels of homocysteine in the blood. Increased homocysteine in the blood has been associated with increased incidence of blood clotting disorders in pregnant and in non-pregnant women as well as in men. It has also been associated with an increased risk for cardiovascular disease and stroke.

Study Procedures

If you agree to participate in this study, we will:

1) collect information from yourself on any history of blood clotting disorders, heart attack or strokes, cancer and other dieseases in yourself, your brothers or sisters, parents, aunts, uncles and cousins and your baby's father's family and draw up a family tree.

2) record information about your diet during pregnancy and about vitamin supplementation.

3) perform the following laboratory tests on a blood sample:

   a. measure plasma homocysteine

   b. measure the amount offoiate in your blood

   c. perform DNA analysis for the variant form of MTHFR (tMTHFR)

4) store your DNA extracted fTom your blood sample for possible analysis of other genetic variants in the future related to complications of pregnancy, especially blood clotting disorders.

These blood tests will require 15 mls (1 tablespoon) of blood collected from a vein into blood collection tubes using standard sterile technique. We do not require a blood

test fTom your child or any other relative. It is not necessary for you to come to the Health Science Center in Winnipeg for this test. We can arrange the blood test in laboratory of your choice including the laboratory of your own family doctor. You may choose to come to the Health Sciences Center for the blood test if that is more convenient for you. It should not take more than a few minutes for the blood test once you are at the laboratory. There is no charge to you for this blood test.

Mothers who have taken part in the dietary survey section of other related research projects in the past have told us that it normally takes one half hour or less to complete the form. The family history survey normally takes an hour to complete but this varies depending on the family. If your family is large or you wish to ask other relatives for information it may take you longer than this. If you do not have information available about your family history (for example, if you are adopted) you may still choose to participate. Information for the family history portion of the study requires you to provide only a first name for your relatives and we will not contact any relative for confirmation or share your results with a relative or any other individual without your permission.

You do not have to come to the Health Sciences Center to complete the survey. Both surveys will be mailed to you to complete in your home at your own pace. The surveys can be returned by mail and you will be also receive a postage-paid envelope for that purpose. If English is not your first language, we can arrange for translation services for you. If you require special assistance in completing the survey it will be made available to you through the study's coordinator at your request

Risks and Discomforts

Withdrawing blood from a vein in the fashion required for this study poses little risk to you except for mild discomfort or possible bruising at the blood-taking site.

Benefits

By participating in this study as a control you will be providing information to the study doctors that may show an association between diet and genetic variants and the risk for complications in the third trimester of pregnancy. We hope the information learned from this study will benefit women with complications of the third trimester of pregnancy and their babies.

There may or may not be direct medical benefit to you from participating in this study. The possible benefit to you in choosing to participate is that any nutritional deficiency of vitamins B_{12}, B_6, and folic acid that you may have will likely be identified. You will then be otlered nutritional counseling that will allow you to correct such a deficiency. If you are found to have an elevated homocysteine level in your blood, this will also be identified. You will receive, if you wish, counseling on treating and monitoring of elevated homocysteine in order to reduce any potential increased risk for

disease. If you are identified as having the variant MTHFR (tMTHFR) you will be provided with counseling about the consequences, treatments, or risks, if any. Your family history will be reviewed for the study. If there is a pattern that identifies a genetic risk for other types of disorders, you will be informed and offered genetic counseling.

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Questions

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Statement of Consent:

I have read this consent form. I have had the opportunity to discuss this research study with Drs. Chodirker or Greenberg and Natalie Bjorklund. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I have been given a copy of this consent torm. I understand that my participation in this research study is voluntary and that I may choose to withdraw from this study at any time. If so, there will no change in my medical management. I understand that no personal information will be marked on my stored DNA sample but researchers will use an anonymous coding system. I may request that my DNA sample be removed from the DNA bank and destroyed at any time.

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1.1 give my consent for the DNA sample extracted ftom my blood to be used in the search for causes of high MSAFP and complications of the third trimester, but my DNA will be discarded once the initial results of the MIHFR analysis are available.	Yes____ No_____
2.If my DNA is destroyed, I understand that if I want further genetic testing to be done in the future I will need another blood test taken.	Yes ____ No_____
3.The DNA obtained ftom me may be stored for 20 years so that further testing may be perfonned with respect to MSAFP and complications of the third trimester of pregnancy	Yes____ No ___
4.I wish to be recontacted regarding the results of any new tests that are pertonned on my DNA in future.	Yes ____ No ___
5.Samples may be used in this laboratory or sent to other laboratories for research on other genetic diseases after all identifying information has been removed.	Yes ____ No ___
6. Prior to my death, members of my family are allowed access to my stored DNA only if! give written permission.	Yes ____ No ___
7. My first-degree relatives will be allowed access to my stored DNA after my death.	Yes ____ No ___

I freely agree to participate in this study. I understand that information regarding my personal identity will be kept confidential but that confidentiality is not guaranteed. I authorize inspection of my medical records by University of Manitoba Faculty of Medicine Research Ethics Board

By signing this consent form I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature: Date:

Signature of person explaining the study:

I do not wish to participate in this study. Please do not contact me again in regards to this study. _____

I may be interested in this study but I wish more information first. Please call me at _____.

The best time to reach me is at _____

Name: _____

Appendix 7

THE UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

A112 - 753 McDermot Avenue
Winnipeg, Manitoba
Canada R3E 0W3

APPROVAL FORM

Principal Investigator: Dr. C. Greenberg

Protocol Reference Number: H2000:138
Date: November 6, 2000
Protocol Ref # H2000:138

Protocol Title: Is There An Association Between MSAFP and Genetic Thrombophilia in the Third Trimester of Pregnancy?

The following are approved for use:

- Research Proposal
- Letter to Participants
- Informed Consent Form (Retrospective Case) dated October, 2000
- Informed Consent Form (Retrospective Control) dated October 25, 2000
- Informed Consent Form (Prospective Case) dated October 25, 2000
- Informed Consent Form (Prospective Control) dated October 25, 2000

The above was approved by Dr. A. Katz, Chair, Health Research Ethics Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated October 25, 2000. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba.

This approval is valid for one year only. A study status report must be submitted annually and must accompany your request for reapproval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

[redacted]

Alan Katz, MB., Ch.B., MSc., CCFP, FCFP.
Chair,
Health Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255 / Fax: (204)789-3942

Appendix 8

Family History

The section will allow us to construct a family tree based on the health history of the individuals in your extended family. Please answer all of the questions as completely as possible. If you do not know all the information, please tell us whatever you do know. If any person we ask about is deceased please note that on the form. If you have the information, please include the age they were when they died and the cause of their death. Rates of the variant MTHFR enzymes vary widely between different ethnic populations. It will help us if you can share as much information as possible about ethnic backgrounds of family members. If you, or anyone else in your extended family, was adopted and you simply cannot provide any information just leave it blank. If you have lost contact with someone we ask about through divorce or other family difficulties and you can't answer because of this, simply leave it blank or write "Don't know". All information is kept strictly confidential and we will not contact any family member except through you. If there is any pattern of inheritance (ie familial cancers, recessive metabolic disorders) not already dealt with in your genetics or MSAFP file, this history will be reviewed by a geneticist. You may also be contacted and referred to the appropriate genetic specialist if it is deemed appropriate. Otherwise you will not be contacted about the results of the family history. If you have any questions, problems, or concerns you can reach the **Study Coordinator, Natalie Björklund**, by paging her at **1-204-932-7275** from 8:00 am to 10:00 pm weekdays and Sundays (**Friday**, 8:00 am **until** 4:00 pm).

Examples of conditions we are especially interested in are as follows:

Any type of blood clotting problem (example phlebitis)
Cancer (specify type if possible)
Chromosome abnormality (i.e. Down syndrome)
Cleft Lip or Palate
Diabetes
Infertility (Sterility)
Heart conditions
Heart attacks
High Blood Pressure
High Cholesterol
Miscarriages or still births
Malformations or birth defects
Obesity
Spina bifida and anencephaly
Stroke
Vascular problems

If there are other health problems in your family you think are important for us to consider please include them as well.

Smoking: If any family member we ask about smokes or has smoked in the past please let us know and if possible give us an idea of how much and how long he or she has smoked.

Today's date: _____
Baby's first name: _____ (This is the baby for which you had an elevated MSAFP that resulted in your being invited into the study. If the baby is not born yet, please put in your due date instead.)
Baby's birth date: _____ or Baby's Due Date: _____
Baby's health problems if any:

_____
_____
_____
_____
_____

Parents:
Your first name: _____
Your age at baby's birth/due date: _____
Your age today: _____
Your ethnic background: _____
Smoking history: _____
Health problems, if any: _____

_____
_____
_____
_____
_____

Father's first name: _____
Father's age at baby's birth/due date: _____
Father's age today: _____
Father's ethnic background: _____
Smoking history: _____
Health problems of Father of baby if any: _____

_____
_____
_____
_____
_____

Is any part of the your family related to the father's family?

Have either you or the baby's father had a relationship with a different partner that resulted in a miscarriage, termination, or stillbirth? Please explain.

Brothers and Sisters of baby:

First Name: Age (Today): Health Problems if any:

1) _____ ____ _____

2) _____ ____ _____

3) _____ ____ _____

4) _____ ____ _____

5) _____ ____ _____

6) _____ ____ _____

Half brothers and sisters of baby:

First Name: Age (Today): Health Problems:

1) _____ _____ _____

2) _____ ____ _____

3) _____ ____ _____

4) _____ ____ _____

If any of the brothers and sisters are adopted or foster children please note that.

Grandparents:

Grandfather (your father)
First Name: _____ Age: _____
Health Problems if any: _____
_____
_____
_____
_____
Smoking history: _____
Ethnic background: _____

Grandmother (your mother)
First Name: _____ Age: _____
Health Problems if any: _____
_____
_____
_____
_____
Smoking history: _____
Ethnic background: _____

Grandfather (father's father)
First Name: _____ Age: _____
Health Problems if any: _____
_____
_____
_____
_____
Ethnic background: _____
Smoking history: _____

Grandmother (father's mother)
First Name: _____ Age: _____
Health Problems if any: _____
_____
_____
_____
_____
Ethnic background: _____
Smoking history: _____

Uncles and Aunts:

Your sisters and brothers

	Name:	Age:	Health Problems if any:
1)	_____	_____	_____
2)	_____	_____	_____
3)	_____	_____	_____
4)	_____	_____	_____
5)	_____	_____	_____
6)	_____	_____	_____
7)	_____	_____	_____
8)	_____	_____	_____

Father's sisters and brothers

	Name:	Age:	Health Problems:
1)	_____	_____	_____
2)	_____	_____	_____
3)	_____	_____	_____
4)	_____	_____	_____
5)	_____	_____	_____
6)	_____	_____	_____
7)	_____	_____	_____
8)	_____	_____	_____

If any of the brothers and sisters are adopted or were foster children please note that.

Mother's Side Only First Cousins of baby:

	Name:	Parent:	Age:	Health Problems if any:
1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)				
11)				
12)				

If any of the brothers and sisters are adopted or foster children please note that.

Do any of these people have a health problem you are concerned about or feel we should know about? If so please identify them and the health problem.

Father's Side Only First Cousins of Baby:

	Name:	Parent:	Age:	Health Problems if any:
1)	_____	_____	____	_____
2)	_____	_____	____	_____
3)	_____	_____	____	_____
4)	_____	_____	____	_____
5)	_____	_____	____	_____
6)	_____	_____	____	_____
7)	_____	_____	____	_____
8)	_____	_____	____	_____
9)	_____	_____	____	_____
10)	_____	_____	____	_____
11)	_____	_____	____	_____
12)	_____	_____	____	_____

If any of the brothers and sisters are adopted or foster children please note that.

Do any of these people have a health problem you are concerned about or feel we should know about? If so please identify them and the health problem.

Appendix 9

Dietary Survey

Our dietary survey normally takes about half an hour to complete. Please answer the questions as they come up without spending too much time on any one question. We have tried to include just about every kind of food people normally eat in Manitoba but we may have missed something, especially if you are on a special diet or a diet not typical of most Manitobans. To assist us in our study, you will see numerous places to add in anything we may have missed. Please feel free to use them. If you think something you wrote needs to be explained to make it clearer to us, please add your comments. If you only eat certain foods when they are in season, (watermelon only in summer, tangerines and eggnog over the winter holidays) please note that on the form beside the food.

Vegetables:

I eat at least one or more serving of each of the following vegetables
(please check appropriate column).

	per day			*per week*			*per month*		*rarely*	*never*
	4+	2-3	1	4+	2-3	1	2-3	1		
Alfalfa sprouts										
Artichoke										
Asparagus										
Avocado										
Bamboo shoots										
Basil, fresh										
Bean sprouts										
Beans, green										
Beans, yellow wax										
Beets										
Beet greens										
Bokchoy										
Broccoli										
Brussel Sprouts										
Cabbage										
Carrots										
Cauliflower										
Cilantro										
Corn										
Celery										
Chinese Cabbage										
Cucumber										
Eggplant										
Eggplant (Chinese)										
Garlic (1 clove)										
Green onions										
Jicama										
Kohlrabi										
Leeks										
Lettuce, butternut										
Lettuce, green leafy										
Lettuce, red leafy										
Lettuce, Iceberg										
Lettuce, Romaine										
Lettuce, Other										
Mixed seed sprouts										

1

Mushrooms										
Okra										
Onions										
Parsley, fresh										
Parsnips										
Peas, green										
Peas, snow										
Peppers, hot fresh										
Peppers, green										
Peppers sweet red										
Peppers sweet yellow										
Peppers, other										
Potatoes										
Radishes										
Rutabaga										
Squash Acorn										
Squash Hubbard										
Squash Spagetti										
Squash Summer										
Spinach										
Swiss Chard										
Sweet Potatoes										
Tomatoes										
Turnip										
Turnip Greens										
Water Chestnuts										
Yam										
Zuchinni										
Other Vegetables (please specify)										
1)										
2)										
3)										
4)										
5)										

Cooking methods affect the amount of certain vitamins that are present in vegetables. We want to correct for that possibility in our study. Please list the ten most common vegetables you eat and check what form you normally buy them in.

Vegetable	Fresh or Raw	Canned	Frozen	Dried
1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)				

Do you cook any of the vegetable listed as purchased in fresh or raw form before you eat them? If so, circle which ones. 1 2 3 4 5 6 7 8 9 10

Fruits

I eat at least one or more serving or cup of juice of each of the following fruits (please check appropriate column).

	per day			*per week*			*per month*		*rarely*	*never*
	4+	2-3	1	4+	2-3	1	2-3	1		
Apples										
Apricots										
Bananas										
Blackberries										
Blueberries										
Boysenberries										
Berry Juice Punches										
Cantaloupe										
Cherries										
Choke Cherries										
Cranberries										
Currants										
Dates										
Figs										
Grapefruit										
Grapes, green seedless										
Grapes, red										
Grapes, concord										
Guava										
Honey Dew										
Kiwi										
Mango										
Mixed Fruit Punches										
Nectarine										
Oranges, navel										
Oranges, regular										
Oranges Mandarin										
Oranges, other										
Papaya										
Peach										
Pear										
Pineapple										
Plantain										
Plums										
Pomegranate										
Prunes										
Raisins										
Raspberries										
Rhubarb										
Strawberries										
Tangerine										
Watermelon										
Others (please specify)										
1)										
2)										
3)										
4)										

Please list the ten most common fruits you eat and check what form you normally buy them in.

Fruit	Fresh or Raw	Canned	Frozen	Dried
1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)				

Do you cook any of the fruits listed as purchased in fresh or raw form before you eat them? If so, circle which ones. 1 2 3 4 5 6 7 8 9 10

Grains:

I eat at least one or more serving of each of the following grains (please check appropriate column).

	per day			*per week*			*per month*		*rarely*	*never*
	4+	2-3	1	4+	2-3	1	2-3	1		
1 slice of:										
White bread										
60% whole wheat										
100% whole wheat										
Rye bread										
Oatmeal bread										
Seven grain bread										
Pumpernickel										
Other mixed bread										
Pita (large 1/2)										
Pita (1/2 whole wheat)										
Bagel, plain										
Bagel, whole wheat										
Bagel, other										
Bannock										
Donut										
Muffin, regular										
Muffin, English										
Cake										
Other pastry										
2 or more:										
Whole wheat crackers										
Rye crackers										
Soda crackers										
Cheese crackers										
Other crackers										
Granola bar										
1 to 1/2 cup of:										

Barley (pearl or pot)										
Buckwheat or kasha										
Bulgar										
Cornmeal										
Millet										
Brown rice										
White rice										
Wild rice										
Tabouleh										
Pasta										
Pasta (whole grain)										
Perogies										
Egg noodles										
1 serving of:										
Cream of Wheat										
Shredded wheat										
Corn flakes										
Cherrios										
Raisin Bran										
Bran flakes										
Granola										
Oatmeal										
Red River Cereal										
Seven grain cereal										
Wheaties										
Fruit Loops										
Alphabets										
HoneyComb										
Shreddies										
Other (please specify)										
1)										
2)										
3)										
4)										
5)										
Others grain sources										
1)										
2)										
3)										
4)										
5)										
Herbal/Health Grain Foods as supplement										
Flax Seeds (tsp)										
Wheat Germ (tsp)										
Wheat Bran (tsp)										
Others (Please Specify)										
1)										
2)										
3)										
4)										
5)										

Milk and Dairy Products:

I eat one or more serving of each of the following milk and dairy products each (please check appropriate column).

	per day			*per week*			*per month*		*rarely*	*never*
	4+	2-3	1	4+	2-3	1	2-3	1		
1 glass of										
Milk										
Milk shake										
Milk with fruit										
Yogurt drinks										
Diet or meal replacement shakes (specify brand)										
Hot chocolate										
Eggnog										
Goat's milk										
Soy milk										
1 bowl of										
Creamed soups										
Cheese soups										
Pudding										
Custard										
slice of:										
Cheese, Hard (ie cheddar)										
Cheese, Parmesan										
Cheese, Feta										
Cheese, wrapped slices										
Cheese sprea(serving)										
1 serving of:										
Cottage cheese										
Yogurt										
Ice cream or ice milk										
Others (please specify)										
1)										
2)										
3)										
4)										
5)										

Meat and Vegetarian Alternates:

I eat one or more serving of each of the following meat or alternate (please check appropriate column).

	per day			*per week*			*per month*		*rarely*	*never*
	4+	2-3	1	4+	2-3	1	2-3	1		
1 serving of: (approx. equal to one 1/4 pound cooked hamburger patty in weight)										
Beef										
Pork										
Venison (Deer)										
Rabbit										
Caribou										

Elk										
Chicken										
Goose (domestic)										
Goose (wild)										
Lamb										
Fish										
Seafood										
Turkey										
other meat (please specify)										
1)										
2)										
3)										
4)										
5)										
Hot-dog										
2 slices of luncheon meat (ie bologna)										
1/2 cup meat alternate										
Vegetarian protein										
Lentils										
Chickpeas										
Beans										
Tofu										
Soybeans										
Peanut Butter										
Egg										
other (please specify)										
1)										
2)										
3)										
4)										
5)										

Pregnancy and vitamin supplements change some of the blood tests that we are doing for this study and we need to account for these changes in our results. Some participants will be doing this questionnaire while they are still pregnant, soon after the elevated MSAFP because of which they were invited into the study (prospective participants). Other participants will be doing this questionnaire at some time after the pregnancy with an elevated MSAFP result (retrospective participants). It is possible that some retrospective participants may be pregnant again with their next child after an elevated MSAFP result in the previous pregnancy. To allow us to distinguish which situation you are in, please answer the following questions.

Are you pregnant now? Yes___ No___
If yes, your due date is _____

If yes, have you had an elevated MSAFP test in this pregnancy?
(Please check the appropriate answer.)
Yes, this is why I was invited into the study_____
No, the result for this pregnancy is normal _____
I don't know the result yet, as it is too early_____
Yes, but this is not the pregnancy for which I was invited into the study._____

I have had more than one pregnancy with an elevated MSAFP. _____
For the questions below, I am referring to the pregnancy with the baby that was due/born on _____

Please consider the pregnancy for which you were invited into the study.

Did you change your diet before becoming pregnant? Yes___ No___
If yes, in what ways did you change your diet? What did you eat more of? Less of?

How far along were you (weeks past your last missed period) when you discovered you were pregnant?

Did you change your diet during the pregnancy after you found out you were expecting? Yes___ No___

If yes, in what ways did you change your diet? What did you eat more of? Less of?
1st trimester:

2nd trimester:

3rd trimester

Can you think of any other way you that you have changed your diet as reported above since you were pregnant?

Vitamin Supplements:
1) Have you taken any kind of vitamin supplement in the last three months?
Yes_____ No _____
(If no skip to the next numbered question.)

A multivitamin? Yes_____ No _____
If yes please list the brand names where possible. (ie Centrum Forte, One a Day)

How often do you take this multivitamin?

Do you take any single vitamin supplement (ie vitamin E) not including folate/folic acid which is included in a separate question below
Yes_____ No _____
If yes please list the type of single supplement.

How often do you take this single vitamin(s) supplement?

What dose do you normally take? (ie Vitamin C 50 mg per day)

2) Did you take a folic acid or folate supplement in the three months before you were pregnant? Yes_____ No_____
(If no skip to the next numbered question.)

How often did you take this supplement?

How many mg did you normally take?

Did your doctor prescribe this for you or did you choose this yourself?

If you chose it yourself, why?

For how many weeks into the pregnancy did you take the folic acid supplements?

3) Did you take any other vitamin supplement in the three months before you discovered you were pregnant? Yes_____ No_____
(If no skip to the next numbered question.)

If yes, what sort of vitamin supplement was it?

Please give the brand name if possible. (ie Materna)

Did your doctor prescribe this for you or did you choose this yourself?

If you chose it yourself, why?

4) Did you start taking a folic acid or folate supplement once you discovered you were pregnant? Yes _____ No _____
(If no skip to the next numbered question.)

If so, how many weeks after your first missed period did you start?

For how many weeks into the pregnancy did you take the folic acid supplements?

5) Did you start taking any other vitamin supplement once you discovered you were pregnant? Yes_____ No_____
(If no skip to the next section.)

If yes, what sort of vitamin supplement was it? (ie multi vitamin, vitamin C tablets)

Please give the brand name. (ie Materna)

Did your doctor prescribe this or did you choose this yourself?

If you chose it yourself, why?

Are you presently taking any kind of alternative/health food store type supplement? If so, what is it?

Did you take any kind of alternative/health type supplement while you were pregnant that you do not take now? If so, what was it?

If you have any comments, questions or concerns about the dietary questionnaire or if you have any additional information you think we should know, please add it below. Thank you for completing this questionnaire.

Appendix 10 **Information about the blood test**

Thank you for taking part in our study. If you have any questions, problems, or concerns you can reach the **Study Coordinator, Natalie Bjorklund,** by paging her at **1-204-932-7275** from 8:00 am to 10:00 pm weekdays and Sundays (Friday, 8:00 am until 4:00 pm). As you can see from the enclosed requisition form, we require a blood test using one needle insertion into a vein from which two tubes of blood will be taken. For the prospective study, (the study where the baby not yet born) two tubes of blood will also be taken from fathers. For those in the retrospective study (started after the baby was born), no blood is needed from the fathers.

To have this blood test completed, you should take it to the nearest convenient laboratory that regularly draws blood. You do not need to make an appointment to see your doctor for this test even if you choose to have the test done at his/her office. You may also choose to have the blood test at the Health Sciences Center. (If you go to any information booth at the main entrances to the HSC, they can direct you to the laboratory. An appointment for a blood test is not necessary at HSC. Please note the laboratory at the Children's Hospital is closed Thursday mornings.) If you don't know the location of a convenient laboratory, your own doctor's office can direct you, you may find one from the Yellow Pages under **Laboratories-Medical,** or you may find one by contacting the Study Coordinator for assistance.

You do not have to fast or otherwise restrict your intake of food or water prior to this blood test. If you normally take multivitamins, and have noted this on your dietary survey, then continue to do so at your usual time. If you normally do not take multivitamins, please do not take any just for the test.

You should not be charged in any way for this test. Most laboratories will charge nothing extra for taking a research study sample. A few labs may ask for a small fee in the range of $6.00 to $10.00 to cover their material or labor costs. You are not responsible for paying such a fee. The laboratory should forward the bill to the address supplied on the requisition form,

If you are presently outside of the province of Manitoba, the laboratory will have to ship the sample to us via courier. You should call the lab- in advance and have them contact the study coordinator so that she can arrange to have appropriate information and courier fees forwarded to the laboratory before you have your blood test. We will cover any fees associated with courier service. Courier service affects only laboratories located outside of the province of Manitoba.

The first tube of blood will be used to analyze the amount of the amino acid, homocysteine in your blood. The second tube is for a sample from which your DNA will be separated and analyzed within the limitations you outlined for us in the consent-to-participate form. No drug tests will be done on this sample. As we will not be testing your blood for anything else, we will not be able to tell you about any other type of medical condition or test result. You should see your own doctor if you require or wish to request any other kind of test. As this is a research study, we will not contact you about the results of this study until after the study is completed and the data analyzed. This may take from one month to a year or more. Also, we will not contact you if the results of your test indicate everything is normal. We will not forward the results of our tests to your doctor unless you specifically request that we do so.

If you wish to know your results and have not heard from us, you may contact to the Study Coordinator. Thank you again for taking part in our study.

Health Sciences Centre
MS-5, 820 Sherbrook Street
Winnipeg, Manitoba, Canada R3A
Telephone # (204) 787-1534

RESEARCH PROJECTS
LABORATORY TEST REQUISITION
FOR CLINICAL CHEMISTRY AND
HEMATOLOGY
Lab Contact: Marion Henke .
Telephone # (204) 787-4395

STUDY INFORMATION	PATIENT INFORMATION
Project Name: Elevated MSAFP and Genetic Thrombophilia in Pregnancy Location: FE-229 Community Services Bldg Telephone #: 787-2494 HSC Account #: Project #: Institution Code: GREHO722	HSC#: Name: DOB: Loc: FE-229 Community Services Bldg Dr.: Dr. C.R. Greenberg Date: Time:

TESTS REQUIRED	SPECIMENS REQUIRED
HCYE – Homocysteine	1 red top tube containing gel – Spin serum immediately after clotting. If tube does not contain gel, separate serum from cells. Serum is stable at 4^0C once separated from cells. 5ml
BANK – DNA Banking	1 lavender top tube (5ml)

LAB ACCESSIONING INSTRUCTIONS:

1. Accession tests and/or profiles as listed above. **If required, all samples processed as defined above can be stored at 4^0C over the weekend prior to shipping.**
2. Do not use regular requisition forms for this sample. This form MUST accompany the sample or it will not be processed properly.
3. If there is any charge for supplying a research study sample please forward a numbered bill marked "Payable upon Receipt" to: Natalie K. Björklund, MSAFP Study Coordinator, Depts of Biochemistry & Medical Genetics, University of Manitoba, 336 BMS Bldg, 770 Bannatyne Ave. Winnipeg MB, R3E 0WE. DO NOT bill the Health Sciences Centre.

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Appendix 11

University of Manitoba: Research Days 2000 poster abstract

GENETIC VARIATION AND MULTIFACTORIAL NEURAL TUBE DEFECTS Natalie K Biorklund, Jane A. Evans, Cheryl R. Greenberg_Dept. of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB

Introduction: Neural tube defects (NTDs) are common severe congenital malformations most often caused by multifactorial inheritance. The methylenetetrahydrofolate reductase (MTHFR) C677T thermolabile variant (tMTHFR) is associated with NTDs in most studies. Data on a variant of methionine synthase (MS) D919G are limited. The Manitoba frequency for tMTHFR has been established. We compared the frequencies of these variants in case and control populations. Methods: Cases and controls were ascertained by elevated. MSAFP with NTD and with normal outcome respectively. Ascertainment was complete. We controlled for confounders. Results: The frequency of tMTHFR in cases was lower (OR 0.83) than controls but not significantly (p=0.131). The frequency of MS *D919G* in cases was higher (OR 1.69) than in controls (p = 0.042). When compared to tMTHFR in the Manitoba population cases do not differ significantly (OR 1.18, p=0.640) but controls do (OR 1.42, p=O.O 12). Conclusion: The lower frequency of tMTHFR in cases compared to controls was unexpected. Compared to the Manitoba population cases have elevated tMTHFR that, while not significant, is consistent with most literature reports. tMTHFR is also associated with elevated MSAFP with normal outcome. Our findings are the first report that suggests elevated MSAFP is associated with tMTHFR. Elevated MSAFP is associated with adverse outcomes in later gestation such as placental abnormalities and growth restriction. Our results are suggestive of a possible role for tMTHFR in such outcomes. MS *D919G* is associated with NTDs when compared to high MSAFP normal outcome controls. We need to determine the general Manitoba frequency of this variant in order to assess the significance of our result as our control population may not be not representative of the general population.

Appendix 12

Text of: CMAJ. 2000;163:1129-30

Letters

Folic acid supplementation: more work is needed

Natalie K. Björklund, Jane A. Evans and Cheryl R. Greenberg

Department of Biochemistry and Medical Genetics; Departments of Biochemistry and Medical Genetics, Pediatrics and Child Health, and Community Health Sciences; Departments of Biochemistry and Medical Genetics, and Pediatrics and Child Health University of Manitoba Winnipeg, Man.

James House and colleagues recently reported that 25% of Newfoundland women had low or indeterminate red blood cell folate levels (< 420 nmol/L) at their first prenatal visit (1). Booth and colleagues reported that the reference values in use today were defined on the basis of absence of biochemical signs of folate deficiency (2). These values do not reflect recommendations for folic acid intake to prevent neural tube defects (3). Booth and colleagues found that for women of child-bearing age attempting to reach a folate intake of 400 μg/day, deficiency is best defined as a serum homocysteine value above 10 μmol/L or a red blood cell folate value below 615 nmol/L (2).

We recently completed a case–control study of 28 women with a previous pregnancy resulting in a neural tube defect and 38 matched controls with a normal pregnancy outcome. All mothers were ascertained to be screen positive by an elevated maternal serum-fetoprotein level between 1983 and 1999. We used a semiquantitative food frequency survey to measure dietary and supplemental intake of folate and vitamins B12 and B6. The dietary survey was later validated using biochemical results from 25 and 32 of the case and control mothers respectively. Linear regression analysis showed significant correlation between reported intake of folate and serum folate ($p = 0.018$) and red blood cell folate levels ($p = 0.002$), but an inverse correlation with serum homocysteine levels ($p = 0.029$). Analysis indicated consistent underreporting of actual vitamin intake (common in food frequency surveys).

We found no difference between case and control subjects in terms of intake or eating patterns. Even after correcting for underreporting, we found that in our case and control groups combined, 58% of women were not consuming enough folate, 46% were not consuming enough vitamin B6 and 28% were not consuming enough vitamin B12. Only 12% reported preconceptional supplementation whereas 82% reported supplementing after they became pregnant. There was no difference in preconceptional supplementation patterns after 1994, when preconceptional supplementation with folic acid was recommended (4). Although none of our mothers were folate deficient according to current reference values, 34% had serum homocysteine values in excess of 10 μmol/L and 10% had levels higher than 13 μmol/L, the current reference value. Our results, albeit in a much smaller and differently selected group, support the results of the Newfoundland study and indicate that vitamin B6 intake may also be suboptimal in many women of child-bearing age.

1. House JD, March SB, Ratnam S, Ives E, Brosnan JT, Friel JK. Folate and vitamin B12 status of women in Newfoundland at their first prenatal visit. *CMAJ* 2000;162(11):1557-9.
2. Booth CK, Clark T, Fenn A. Folic acid, riboflavin, thiamine, and vitamin B-6 status in a group of first time blood donors.*Am J Clin Nutr* 1998;68(5):1075-80.
3. Hall JG. Folic acid: the opportunity that still exists. *CMAJ* 2000;162(11):1571-2.
4. Canadian Task Force on the Periodic Health Examination. Periodic health examination, 1994 update: Primary and secondary prevention of neural tube defects. *CMAJ* 1994;151(2):159-66.

Appendix 13

http://jmg.bmjjournals.com/cgi/eletters/40/8/619#46,

Dear Editor

Wilcken *et al*, (2003), in "Geographical and ethnic variation of the 677C>T allele of the 5,10 methylenetetrahydrofolate reductase (MTHFR): finds from over 7000 newborns from 16 areas worldwide" showed that the TT genotype in Calgary, Alberta was present in 5.8% of newborns as compared to one previous report from Quebec of 11% [Infante-Rivard et al., 2003]. The authors did not explain why this difference was present, but they did state that the latter study was drawn from a selected population of infants over the 10th percentile, whereas their own study involved consecutive newborns.

We suggest that the difference in the frequency seen in Alberta and Quebec more likely reflects the ethnic origin of Albertans versus the Quebeçois. Most Quebec residents are descendants of immigrants from France and the province has a larger proportion of Greeks and Italians compared to other Canadian jurisdictions. Caucasians in Alberta are generally the descendants of Northern and Eastern Europeans with 50% of the population being descendents of immigrants from the UK and Ireland [Population by ethnic origin,1996]. French, Italy and Greece all have a higher frequency of C677T MTHFR than eastern and northern European countries. Therefore, it is not surprising that the Quebeçois also have higher frequencies than Albertans. There have also been five other C677T MTHFR association studies in Quebec that included small control groups variously selected with homozygote frequencies ranging from 11 to 16% [Deloughery et al., 1996, Christensen et al., 1997, 1999, Delvin 2000, Merouani et al., 2001.]

The largest single study of C677T MTHFR frequency was conducted using newborn screening filter paper cards here in Manitoba. Mogk et al (2000), genotyped 977 consecutive Manitoba newborns for the 677 C>T polymorphism and found the frequency of 677 C>T homozygotes to be 7%. The percentage of TT genotypes in Manitoba is 7% (36% heterozygotes). Manitoba has the second largest ethnically French population in Canada and has a substantial Metis (French/Aboriginal) population. We would therefore expect the frequency in Manitoba to be intermediate between that of Quebec and Alberta, as our data indicate. It is our opinion that both the 5.8% Alberta result and the 11% Quebec result are accurate estimates of the frequencies of this variant in these two ethnically different Canadian provinces.

References:

(1) Population by ethnic origin, 1996 Census, provinces and territories.2003a. Statistics Canada

(2) Christensen B, Arbour L, Tran P, Leclerc D, Sabbaghian N, Platt R, Gilfix BM, Rosenblatt DS, Gravel RA, Forbes P, Rozen R.5-21- 1999.Genetic polymorphisms in

methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet 84:151-157.

(3) Christensen B, Frosst P, Lussier-Cacan S, Selhub J, Goyette P, Rosenblatt DS, Genest J, Jr., Rozen R.1997.Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease. Arterioscler Thromb Vasc Biol 17:569-573.

(4) Deloughery TG, Evans A, Sadeghi A, McWilliams J, Henner WD, Taylor LM, Jr., Press RD.12-15-1996.Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. Circulation 94:3074-3078.

(5) Delvin EE, Rozen R, Merouani A, Genest J, Jr., Lambert M.2000a.Influence of methylenetetrahydrofolate reductase genotype, age, vitamin B-12, and folate status on plasma homocysteine in children. Am J Clin Nutr 72:1469-1473.

(6) Infante-Rivard C, Rivard GE, Yotov WV, Genin E, Guiguet M, Weinberg C, Gauthier R, Feoli-Fonseca JC.7-4-2002.Absence of association of thrombophilia polymorphisms with intrauterine growth restriction. N Engl J Med 347:19-25.

(7) Merouani A, Lambert M, Delvin EE, Genest J, Jr., Robitaille P, Rozen R.2001.Plasma homocysteine concentration in children with chronic renal failure. Pediatr Nephrol 16:805-811.

(8) Mogk RL, Rothenmund H, Evans JA, Carson N, Dawson AJ.2000b.The frequency of the C677T substitution in the methylenetetrahydrofolate reductase gene in Manitoba. Clin Genet 58:406-408.

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