THE GENETICS OF NEURAL TUBE DEFECTS AND TWINNING

by

Berdj Hratchia Garabedian
Department of Biology and The Centre for Human Genetics

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Gutta cavat lapidem non vi sed saepe cadendo.

A waterdrop hollows the stone not with force but by falling often.

ABSTRACT

Several investigators have suggested that "upper" neural tube defects (NTD's) - anencephaly, encephalocele, and thoracic spina bifida - are etiologically different from "lower" NTD's (lumbo-sacral spina bifida) This hypothesis was primarily based on the observations that the two types have different sex ratios and recurrence rates and that the NTD cases within one sibship are concordant for NTD type. In order to test this, the above figures were calculated in a sample of NTD probands from Montreal and Newfoundland. The findings were not consistent with the hypothesis. However, a previously unreported finding was observed: the frequency of twinning was significantly higher in the near relatives of upper NTD probands than in those of lower NTD probands or of controls. This curious association between upper NTD's and twinning may be explained by a familial factor predisposing to a delay early in development. This delay could also explain any differences observed in upper and lower NTD groups.

RESUME

Le fait qu'il existe une différence étiologique entre sujets atteints de malformation du tube neural (MTN) "supérieure" (anencéphalie, encéphalocèle et spina bifida thoracique) et ceux atteints de MTN "inferieure" (spina bifida lombo-sacré) a été proposé par plusieurs chercheurs. Cette hypothèse est fondée premièrement sur la différence du rapport masculin/féminin et du taux de récurrence qui a été observé entre les deux types et deuxièmement sur le fait que les cas de MTN dans une fratrie appartiennent à l'un des deux types. Afin de tester cette hypothèse, les valeurs ci-dessus ont été calculées dans un échantillon de sujets atteints de MTN provenant, l'un de Montréal et l'autre de Terre-Neuve Les résultats obtenus n'ont pas permis de confirmer cette hypothèse Cependant un fait non-signalé jusqu'à présent a été constaté la fréquence de naissances gémellaires est nettement plus élevé chez les proches parents de probants MTN supérieures que chez les proches parents de probants MTN inférieures ou les témoins Cette relation curieuse entre la MTN supérieure et les naissances gémellaires peut s'expliquer par un facteur familial prédisposant à un retard au début du développement. Ce retard pourrait également expliquer toutes les différences entre les deux groupes atteints de MTN.

I dedicate this Thesis to the one that has inspired me the most, Aris Zacharoff, my dear grandfather. He would have been so proud.

Γεια σου Παππουκα.

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INTRODUCTION

Twins have generally been thought of as a rare yet "normal" pregnancy outcome. Neural tube defects (NTD's), also considered to occur infrequently, are viewed as the end product of an "abnormal" course of developmental events. Although it is true that both of these phenomena are uncommon when compared to all pregnancy outcomes (about one in a hundred maternities and under two per thousand livebirths, respectively), it may be contended that the twinning process itself is the more abnormal of the two In fact, monozygotic (MZ) twinning can be considered a morphogenetic aberration, the most common one observed in the human (Jones,1988) and NTD's are among the most prevalent of congenital malformations.

Although MZ twinning usually gives rise to two perfectly healthy individuals, the initial destabilizing event (yet to be elucidated) that precipitated the split in the developing zygote is highly deviative. Dizygotic (DZ) twinning, a consequence of the independent release and subsequent fertilization of two ova, is deviative to a lesser extent. NTD's, on the other hand, arise merely from failure of neural tube closure perhaps due to a mild delay or other disturbance. Although the affected infant may be severely disabled, the pathogenesis may still be considered to be part of the normal spectrum, the abnormal result being created by a developmental threshold.

It is interesting that associations between NTD's and twinning have been made in various ways. The two do have many similarities, but differences also exist. They will first be discussed individually.

i) Neural tube defects (NTD's)

At around the third week of gestation in humans, the neural folds begin rising and fuse at their dorsal margins. Failure to do so before 28 days of gestation will result in an NTD. From experimental work in the mouse it is known that the neural folds elevate as a result of changes in the shape of neural plate cells, from columnar to apically-tapered (Copp *et al.*, 1990). Once the apices of the neural folds are apposed, fusion occurs along the dorsal midline. This is believed to occur via interdigitation of carbohydrate-associated cell surface projections (Sadler, 1978).

Fusion generally proceeds in a zipper-like fashion and in a cranio-caudal sequence [although there are exceptions in the cranial portion (Juriloff *et al.*, 1991)] so that anterior tube structures form first. An NTD at the most anterior region is called anencephaly (AN), more posteriorly, an encephalocele (EN), and most posteriorly, a spina bifida cystica (SB). If fusion fails to occur along the entire neural tube axis, the result is termed craniorachischisis. If multiple NTD's are present in the same individual, the most anterior NTD determines the proband's classification.

SB's are often characterized by a meningocele (a sac protruding from between the vertebrae and containing meninges) or

more commonly a myelomeningocele (a sac containing meninges and spinal tissue), the latter being a clinically more serious manifestation. Moreover, the more cranially the SB lesion is located, the more seriously compromised the affected individual will be since there is usually neural deficit caudal to the lesion.

Collectively, NTD's occur at a rate of under 2/1000 livebirths worldwide. This figure has varied enormously throughout time and between different populations, being as high as 1.0% in Belfast (Stevenson *et al.*, 1966), and lower than 0.01% in American blacks (Greenberg, 1983). The Sikh population is a distinct cultural and religious group in northern India that has one of the highest birth rates of NTD in the world (Verma, 1978). Interestingly, Sikh migrants do, in time, tend towards the incidence rates of the native population, but not completely. Since there usually is no outcrossing, this points to both a genetic and an environmental component in the etiology of NTD. Most NTD's are considered to be multifactorial in origin and an empirical recurrence risk of about 5% is given when counselling parents who have had an affected child. The sex ratio for NTD is less than one, the excess of females being more pronounced in the AN group (Elwood and Elwood, 1980).

Many epidemiological characteristics have been found to be risk factors for NTD, including maternal age of under 20 and over 35, primiparity, and low socio-economic status (Horowitz and McDonald, 1969)., yet none provide clues to its causation. Studies on season of conception have yielded different results according to the region and period involved, some studies showing certain months to be high risk periods (Fraser *et al.*, 1986; McKeown and Record, 1951) and others

showing no seasonal trends at all (Wehrung and Hay, 1970). There are secular trends for NTD birth prevalence. In Canada, the last thirty years have been a low cycle, the last peak being in the early 1960's (Elwood and Elwood, 1980). Maternal drinking water (Morton *et al.*, 1976, Dorsch *et al.*, 1984) and common cold (Kurppa *et al.*, 1991), tea (Seller, 1987a) and blighted potatoes (Sever, 1973) are among those possible causative factors which have been investigated, albeit with inconclusive results.

Nevertheless, a recent multi-centre trial (MRC, 1991) to assess the impact of periconceptional multivitamin and folic acid use by high risk mothers has yielded encouraging results in terms of prevention. The study concluded that folic acid supplements taken around the time of conception have a protective effect in women who have had a previous affected pregnancy. A similar prospective study has been initiated in Hungary to measure the efficacy of vitamin supplementation in all women, regardless of whether they have had an affected child or not (Czeizel & Rode, 1984). Preliminary results from the study have been equally encouraging (Czeizel & Fritz, 1989). However, a case-control study in the U.S. (Mills *et al.*, 1989) which also assessed the impact of vitamins on the general population did not find a significant decrease in the risk for having an infant with NTD. This finding may be due to the fact that the U.S. has a relatively low birth prevalence of NTD's and does not have those risk factors present in high prevalence populations that are counteracted with the use of vitamins.

There are a few reports of isolated NTD's having autosomal dominant (Farag *et al.*, 1986), autosomal recessive (Fineman *et al.*,

1982), or X-linked (Toriello *et al.*, 1980) modes of inheritance. NTD's may also occur as part of a syndrome, in which case the cause can be genetic (Meckel-Gruber syndrome, autosomal recessive), environmental [*e.g.* hyperthermia (Chance and Smith, 1978); valproate (Robert and Guiband, 1982)], mechanical [early amnion rupture, (Holmes *et al.*, 1976)], chromosomal, (*e.g.* trisomy 18), or unknown. All of these, however, are relatively rare. The non-syndromic, non-Mendelian NTD's form the majority of NTD's and will be the subject of this work.

Within this isolated NTD group, there is a subgroup in which the proband has additional malformations. These malformations are not a consequence of the NTD (as is the case with hydrocephalus and clubfoot). Characteristics of this subgroup are seemingly different from those of isolated NTD's. In particular, they do not have the geographic and ethnic variation, the female excess, or the decreasing prevalence in recent years which has been observed in the isolated form (Khoury *et al.*, 1982). However, these observations have been made on small sample sizes. Larger studies are needed to confirm this difference.

It is interesting that when these additional malformations do occur in the proband or in first degree relatives they are often of the schisis-type (e.g. some types of congenital heart defects, cleft lip with or without cleft palate, tracheo-esophageal fistula), reminiscent of the "schisis-association" described by Czeizel (1981) and the familial association observed by Fraser *et al.* (1982)

In the literature, NTD has historically been subdivided into AN and SB (which includes EN). It had been generally accepted that both

defects result from incomplete closure of the neural tube, but at different sites.

An alternate classification of NTD's has been recently suggested (Toriello and Higgins, 1985) on the basis of closure mechanism. 'upper' NTD's (AN, EN, and thoracic SB) versus 'lower' NTD's (lumbo-sacral SB). Uppers are believed to be etiologically different from lowers because the former may arise from defects in primary neurulation and the latter from defective canalization (secondary neurulation).

The process of primary neurulation generally consists of neural induction, elevation and apposition of the neural folds, followed by the fusion of these folds. The final event in this developmental phase is the closure of the posterior neuropore. Secondary neurulation occurs in the region caudal to the posterior neuropore and is an entirely different process. From the remnant of the primitive streak, mesenchymal cells emerge and condense. This mass of condensed cells then canalizes to form the caudal section of the neural tube (Copp *et al.*, 1990).

The Toriello and Higgins hypothesis is based on observations that recurrent cases of NTD within the same sibship are always concordant for type of NTD, and that spontaneous abortions and additional malformations are more common in the upper group.

Hall *et al.* (1988) followed up with a study and obtained similar results. In addition, they found markedly higher recurrence rates in the upper group. However, both of the populations studied (B.C. and Michigan) have a low NTD incidence. (N.B. Eastern Canada is a high NTD incidence area).

b) Twinning

There are two major types of twins: monozygotic (MZ) and dizygotic (DZ). MZ twinning gives rise to two genetically identical individuals. This occurs when the fertilized egg is somehow induced to split into two. DZ twinning is an altogether different phenomenon since it involves the fertilization of two ova by two sperm. The resulting individuals are no more genetically similar than siblings are (*i.e.* about 50% of genes in common).

Each fetus is normally surrounded by two membranes, the chorion, which develops from the trophoblast, and the amnion which lies inside the chorion and completely surrounds the fetus in a fluid bath (Bulmer, 1970). In MZ twinning, if division of the embryo occurs between the two-cell and morula stages, the twins will develop separate chorions and amnions. These would be indistinguishable embryologically from DZ twins and may have separate placentas if the embryos implant far away from each other or one fused placenta if they implant close to each other (as is also the case in DZ twins). If the division should occur after the differentiation of the trophoblast but before that of the amnion, monochorionic diamniotic MZ twins will result. Finally, if division of the embryo occurs after the differentiation of the amnion, monoamniotic monochorionic twins will arise (including conjoined twins). [See Appendix B for proportions of these types of twins and timing of their initiation].

The existence of a third type of twin has been suggested by Danforth (1916). Theoretically, this type of twin can arise in three different ways (Bulmer, 1970): 1) The ovum might divide before fertilization The daughter ova, once fertilized, would give rise to "uniovular dispermatic" twins which would be genetically very similar (due to identical maternal genes). 2) The secondary oocyte might divide equally (instead of unequally) during the second meiotic division. This would eventually give rise to two ova. Once fertilized, these would produce two individuals less alike than uniovular dispermatic twins but more alike than DZ twins. 3) The primary oocyte might divide equally during the first meiotic division. The resulting twins would be more alike than unrelated individuals but less alike than DZ twins.

Although there is some evidence of the above mechanisms in experimental mammals (Austin, 1961), it seems more likely that they give rise to mosaic individuals rather than twins (Bulmer, 1970). This also seems to be the case in humans (Race and Sanger, 1968).

The incidence of MZ twinning is quite constant throughout the world at approximately 3.5/1000 maternities (Bulmer, 1970), although secular (Bressers *et al.*, 1987; Nonaka and Miura, 1987) and seasonal fluctuations have been reported (Elwood, 1978). This type of twinning is occasionally familial (Shapiro *et al.*, 1979; Parisi *et al.*, 1983).

On the other hand, DZ twinning varies greatly between geographic regions, throughout time, and across the races (Bulmer, 1970). For example, it occurs at an average rate of 8/1000 in

Caucasoids, about twice that rate in Negroids, and at less than 4 per thousand in Mongoloid populations. At the two extremes, the Yorubas of Western Nigeria have the highest reported DZ twinning rates [5%] in the world (Nylander, 1978) and the Japanese have the lowest [0.2%] (Stevenson *et al.*, 1966). In general, twinning rates rise and then fall with increasing maternal age and parity, these effects being more marked in DZ twinning (Elwood, 1978). Familiality is often seen (Bulmer, 1970).

Sex ratio at birth for both MZ and DZ types shows an excess of females (Bulmer, 1970). This excess is more pronounced the later the twinning event has occurred. In other words, monoamniotic monochorionic MZ pairs and conjoined twins have the largest excess of females (James,1988).

c) Neural tube defects and twinning

Epidemiological similarities exist between NTD's and twinning. For example, declining secular trends for birth frequency of anencephalics and twins have been observed in Australia (Field and Kerr, 1974) and in Canada and the U.S. (Elwood, 1974). An exception to this association is the fact that the Yoruba people of Western Nigeria have very low anencephaly rates yet very high (DZ) twinning rates. However, these high rates have been suspected to be due to environmental factors indigenous to that region (Nylander, 1978).

Twins, especially MZ, have an increased overall malformation rate. These malformations include NTD's (Schinzel *et al.*, 1979),

cleft lip with or without cleft palate [CL/P] (Armendares and Lisker, 1975), cleft palate [CP] (Layde *et al.*, 1980) and congenital heart defects [CHD] (Burn and Corney, 1984). Possible reasons for this occurrence include destabilization or developmental retardation of one or both embryos (in the case of MZ twins only) as a result of the splitting event. This may make the embryo(s) more susceptible to a teratogenic insult. Alternatively, the physical constraints of the shared uterine environment may predispose to malformation (in MZ and DZ twins). Finally, if MZ twinning itself is a malformation, as previously noted, then the additional defects may be part of the MZ twinning spectrum (Bryan, 1986).

Any of the above are plausible explanations for the presence of malformations in the twins themselves. However they fail to explain why first degree relatives of twins have increased rates of NTD (Fraser and Hanson, 1984; Windham and Bjerkedal, 1984) and, conversely, why first degree relatives of probands with SB (LeMarec *et al.*, 1978), CL/P (Windham and Bjerkedal, 1984) and CP (Kramer *et al.*, 1982) have an excess of twins. They also cannot explain why near relatives of probands with CHD (Gauthier *et al.*, 1965), Klinefelter's syndrome (Nielsen, 1970), Turner's syndrome (Nance and Uchida, 1964; Nielsen and Dahl, 1976) and Down syndrome (Sharav, 1991) have an excess of twins.

Other curious associations between NTD and twinning (both types) include the relationship between NTD's and non-righthandedness in families, and that between twinning and non-righthandedness (Fraser, 1983; Boklage, 1981), and the finding that ovulation induction causes increases in NTD rate (Lancaster, 1987;

Cornel *et al.*, 1990; Milunsky *et al.*, 1990) and MZ twinning rate (Derom *et al.*, 1987). Also, several teratogens such as vitamin A, dimethyl sulfoxyde, urethan, and vincristine sulphate (Ferm and Hanover, 1969; Kaufman and O'Shea, 1978), as well as conditions of altered temperature and oxygen concentration (Stockard, 1921) have been observed to affect the MZ twinning rate in addition to causing certain types of developmental abnormalities in experimental mammals, including NTD's (Witschi, 1952).

Although these observations are, at most, indirect evidence of an association between twinning and NTD's (and perhaps other schisis-type defects and some chromosomal abnormalities), further study to investigate a possible familial/genetic relationship is warranted. Such a relationship may shed light on the etiology of both phenomena and on embryonic development in general.

d) Objectives of this study.

1) To test the Toriello and Higgins hypothesis (of distinct upper and lower NTD classes) on two populations with a high NTD incidence (namely, Montreal and Newfoundland).

2) To assess whether there is a familial association between NTD's and twinning by determining the twinning rate in the near relatives of probands with NTD's.

MATERIALS AND METHODS

Charts with family histories of probands with an isolated, non-syndromic NTD were obtained from the genetics units of The Montreal Children's Hospital (MCH) and the Janeway Child Health Center (JCHC) in St. John's, Newfoundland. These charts were classified according to the level of the NTD lesion in the proband. When the level of the lesion was not indicated or not stated in sufficient detail in the MCH genetics charts, patients' charts from the hospital's archives were retrieved for closer inspection of radiographs This had been done previously with the Newfoundland charts.

In order to ensure that the family and reproductive histories were as complete as possible, and to obtain information about the use of periconceptional vitamin supplements, questionnaires were sent out (see Appendix E) to the families of NTD probands born in 1970 or later (families of probands born earlier were very difficult to reach). Five respondents were contacted by phone to obtain further details about their reproductive outcomes.

NTD recurrence rates in sibs of NTD probands were calculated using the single incomplete ascertainment method (see Appendix C). Sex ratios in NTD probands were calculated by dividing the number of males by the number of females. Spontaneous abortion rates were obtained by counting fetal losses before 26 weeks of gestation and dividing by the total number of pregnancies, not including the proband.

Twinning rates were calculated by counting the total number of multiple maternities (almost all were twins) in the close relatives of NTD probands and dividing by the total number of maternities. Co-twins of the parents of the proband were not counted as uncle or aunt twins since they were members of the parental twin birth. Control rates for twinning were those obtained from families of probands with Mendelian disorders (tuberous sclerosis, albinism, muscular dystrophy, cystic fibrosis, and hemophilia). These families were seen at the same two hospitals and during the same time period as the NTD families (1950-1990 at the MCH, and 1980-1986 at the JCHC).

When data from the Montreal and Newfoundland samples seemed to be dissimilar, statistical tests were performed to determine whether they could be pooled (Heterogeneity test for second order interactions, as described by Plackett, 1962)

Charts of probands with isolated cleft lip with or without cleft palate (CL/P) and of probands with isolated cleft palate (CP) were also obtained from the MCH and twinning rates were calculated as above.

Because the zygosity of the twins was often not specified in the charts (especially in the case of parents, aunts, and uncles), the Weinberg method, which only requires the number of like-sexed and unlike-sexed twins, was used to get an estimate of the proportion of monozygotic twins in the sample (See Appendix D).

All P values were obtained using the chi-square analysis for contingency tables with Yates correction for continuity (Zar, 1984).

RESULTS - Part A.

Table 1A. NTD recurrence rates (R.R.) in sibs of NTD probands, sex ratios (S.R.) in NTD probands, and spontaneous abortion rates (S.A.) in mothers of NTD probands: Montreal data.

	# sibs	R.R. (%)	S.R.	(N)	S.A. (%)	N
ANENCEPHALY	89	2.2	0.87	(58)	21.4	187
ENCEPHALOCELE	35	5.7	0.14	(16)	21.2	66
UPPER SPINA BIFIDA	105	7.6	1.35	(47)	16.8	185
LOWER SPINA BIFIDA	228	6.1	1.11	(114)	10.2	381

Spina bifida cases were divided into 'upper' if the most cranial lesion occurred at the level of the eleventh thoracic vertebra (T11) or above, and 'lower' for lesions at T12 and below (according to Toriello and Higgins, 1985). Spontaneous abortion rates were calculated from reproductive histories to compare to previously reported rates.

Table 1B. NTD recurrence rates (R.R.) in sibs of NTD probands, sex ratios (S.R.) in NTD probands, and spontaneous abortion rates (S.A.) in mothers of NTD probands: Montreal data, upper versus lower NTD.

	# sibs	R.R. (%)	S.R.	(N)	S.A. (%)	N
UPPER NTD	229	5.2 *	0.86	(121)	19.4	438
LOWER NTD	228	6.1 **	1.11	(114)	10.2	381

Confidence intervals: *[2.4-8.1]; **[3.0-9.3]

Anencephaly, encephalocele, and upper spina bifida (SB) were grouped together as 'upper NTD' and lower SB was renamed 'lower NTD' (as per Hall, 1988. Classification III).

R.R. and S.R. were both higher in the lower NTD group, but not significantly (P=0.83 and 0.42, respectively). However, S.A. was significantly higher in the upper NTD group (P=0.0004).

Table 2. NTD recurrence rates (R.R.) in sibs of NTD probands and sex ratios (S.R.) in NTD probands: Newfoundland data.

	# sibs	R.R. (%)	S.R.	N
ANENCEPHALY	47	6.4	0.63	39
UPPER SPINA BIFIDA & ENCEPHALOCELE	60	3.3	0.47	56
UPPER NTD	107	4.7 *	0.53	95
LOWER NTD	78	1.3 **	0.66	63

Confidence intervals: *[0.7-8.7]; **[0.0-3.8]

R.R. was higher in the upper NTD group (P= 0.36, NS) and S.R. was higher in the lower NTD group (P= 0.61, NS).

N.B. Reproductive histories were not available to calculate spontaneous abortion rates as in the Montreal data (Tables 1A &B).

Table 3. NTD recurrence rates (R.R.) in sibs of NTD probands and sex ratios (S.R.) in NTD probands: Montreal and Newfoundland data combined.*

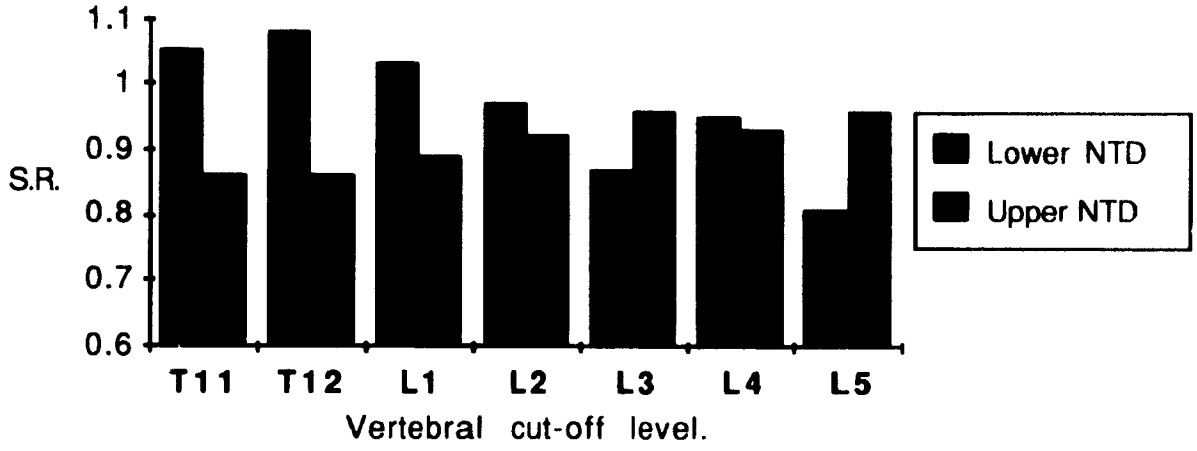
	# sibs	R R. (%)	S.R.	N
ANENCEPHALY	136	3.7	0.76	97
UPPER SPINA BIFIDA & ENCEPHALOCELE	200	6.0	0.65	119
UPPER NTD	336	5.1	0.70	216
LOWER NTD	306	4.9	0.92	177

* Since the two populations (Montreal and Newfoundland) had consistent findings, the data were combined.

R.R. was slightly higher in the upper NTD group, but this was far from being significant (P= 0.91). S.R. was higher in the lower NTD group (P= 0.19, NS).

Graph 1. Since the cut-off level between upper and lower NTD was arbitrarily assigned to T11 by Toriello and Higgins (the point at which neurulation ends and secondary neurulation begins is not known in humans), the cut-off level was shifted downwards one vertebral level at a time and sex ratios were re-calculated each time. The graph shows that the greatest difference in sex ratio between upper and lower NTD occurs at T12 (very close to the presumed T11 cut-off level).

Graph 1. Sex ratio (S.R.) and upper/lower NTD vertebral cut-off level (Montreal data only).



Graph 2. Cut-off level between upper and lower NTD was shifted downwards one vertebral level at a time as in the previous graph and recurrence rates were re-calculated. However, the greatest difference in R.R. occurred at L5, six vertebral levels down from the postulated cut-off level of T11.

Graph 2. Recurrence rate (R.R.) and upper/lower NTD vertebral cut-off level (Montreal data only).

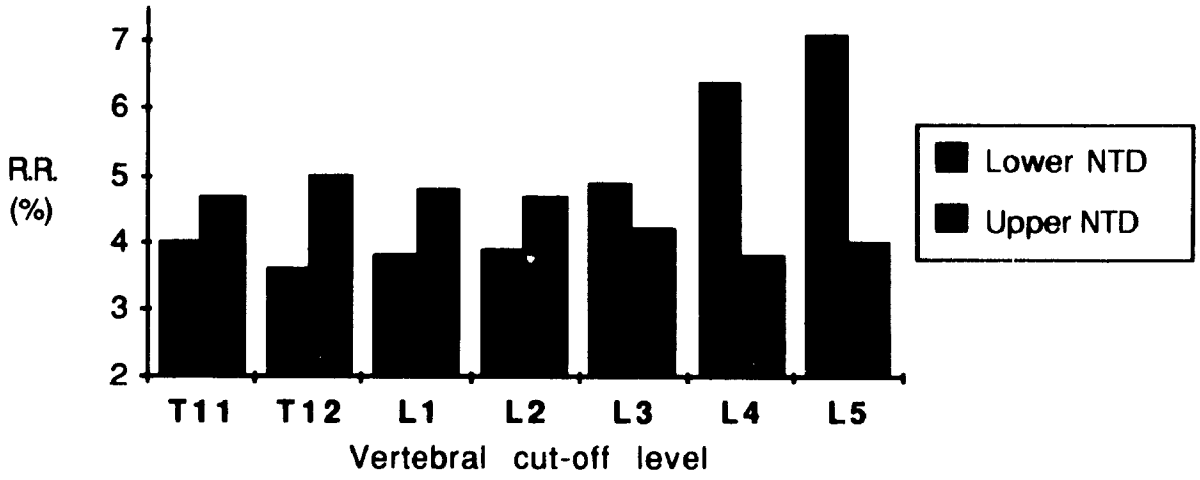


Table 4. NTD-related variables from various geographic areas.

Table 4A: Recurrence rates (%)

Region	N	Upper NTD	N	Lower NTD	Upp. vs. Low.	Reference
British Columbia (B.C.)	424	3.3	277	0.7	$P < 0.05$	Hall, 1988
Montreal (Mtl)	229	5.2	228	6.1	P= 0.83, NS	Present study
Newfoundland (Nfld)	107	4.7	78	1.3	P= 0.36, NS	Present study
Mtl + Nfld combined	336	5.1	306	4.9	P= 0.91, NS	Present study

Recurrence rate (R.R.) in upper NTD families from the B.C. study did not differ from that of the Mtl (P= 0.31), Nfld (P= 0.68), or Mtl + Nfld combined sample (P=0.31). R.R. in lower NTD families from the B.C. study did differ from those in the Mtl and the combined samples (P= 0.0014 and P= 0.0068, respectively), but not from that in the Nfld sample (P=0.83, NS).

Table 4B: Sex ratios

Region	N	Upper NTD	N	Lower NTD	Reference
B.C.	271	0.81	220	0.76	Hall, 1988
Mtl	121	0.86	114	1.11	Present study
Nfld	95	0.53	63	0.66	Present study
Mtl + Nfld combined	216	0.70	177	0.92	Present study
Nova Scotia	104	0.65	60	1.50	Winsor, 1988
South-east England	127	0.59	20	3.00	Seller, 1987
Western Scotland	248	'less than 1'	51	1.32	Drainer, 1991
Spain	194	0.96	125	1.27	M.Frias, 1986

Probands with upper NTD's consistently had a lower sex ratio than those with lower NTD's, except in B.C. where the opposite was true (not significantly, however. P= 0.83).

Table 4C: Sib concordance*

Region	N	%	Reference
B.C.	16	100	Hall et al., 1988
Michigan	9	100	Toriello & Higgins, 1985
Hungary	17	94	Török & Papp, 1991
Mtl	16	44	Present study
Nfld	11	64	Frecker et al., 1988
Mtl + Nfld combined	27	52	Present study
South-east England	38	82	Seller, 1990
Western Scotland	48	71	Drainer et al., 1991

* A measure of how often the type of NTD (i.e. upper or lower) in one sib is the same as in another affected sib.

Table 4D: Additional malformation rate (%) *

Region	N	Upper NTD	N	Lower NTD	P value	Reference
B.C.	112	22.3	225	6.7	< 0.0001	Hall, 1988
Michigan	40	22.5	164	3.6	< 0.0001	Toriello, 1985
Mtl + Nfld	129	7.8	185	4.3	0.31	Present study

* Since the Michigan study did not include anencephalic cases, they were excluded from the two other studies for the sake of comparison.

N.B. This rate is a measure of how often an NTD proband has an additional malformation (i.e. a malformation that is not a morphogenetic consequence of the NTD).

Table 4E: Spontaneous abortion rate (%)

Region	N	Upper NTD	N	Lower NTD	P value	Reference
B.C.	528	19.7	338	18.0	0.61, NS	Hall, 1988
Michigan	86	44.2*	262	18.7	< 0.0001	Toriello, 1985
Mtl	438	19.4	381	10.2	0.0004	Present study

* This very high rate may be an artifact of small sample size.

RESULTS - Part B.

Table 5. Twinning rates in near relatives of probands with NTD's: Montreal.

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	TOTALS
	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
NTD	**1.3 (227)**	**1.0 (405)**	**1.1 (450)**	**1.4 (1726)**	**1.4 (2217)**	**1.3 (5025)**
% like-sexed:	33.3	50.0	60.0	73.9	71.4	68.9
Upper NTD	**1.7 (118)**	**2.0 (198)**	**1.7 (234)**	**2.3 (838)**	**1.6 (1001)**	**1.9 (2389)**
% like-sexed:	50.0	50.0	75.0	82.4	86.7	80.0
Lower NTD	**0.9 (109)**	**0 (207)**	**0.5 (216)**	**0.7 (888)**	**1.2 (1216)**	**0.8 (2636)**
% like-sexed:	0	-	0	50.0	53.8	47.6
Controls	**1.4 (293)**	**0.7 (554)**	**1.1 (568)**	**1.0 (2194)**	**0.7 (2895)**	**0.9 (6504)**
% like-sexed:	75.0	0	50.0	59.1	50.0	52.7
NTD vs control					P= 0.03	P=0.02
Upp vs low				P= 0 009		P= 0.002
Upp vs control				P= 0.02	P= 0.02	P= 0.0002

Table 6. Twinning rates in near relatives of probands with NTD's: Newfoundland.

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	TOTALS
	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
NTD	**1.9 (159)**	**0.5 (182)**	**4.1 (319)**	**1.4 (1842)**	**1.5 (2101)**	**1.6 (4603)**
% like-sexed:	100	100	91.7	56.5	74.2	72.9
Upper NTD	**3.1 (96)**	**1.0 (104)**	**3.1 (193)**	**1.6 (1094)**	**1.3 (1155)**	**1.6 (2642)**
% like-sexed:	100	100	100	60.0	86.7	79.5
Lower NTD	**0 (63)**	**0 (78)**	**5.6* (126)**	**1.1 (748)**	**1.7 (946)**	**1.6 (1961)**
% like-sexed:	-	-	85.7	50.0	62.5	64.5
Controls	**0 (106)**	**0 (227)**	**1.4 (212)**	**1.6 (1161)**	**1.5 (1841)**	**1.4 (3547)**
% like-sexed:	-	-	66.7	73.7	55.6	63.3

* Lower NTD vs. controls, P= 0.07 (almost significant). However, this high twinning rate is probably due to the small sample size.
The apparently high twin rate in the controls does not vary significantly from expected population twinning rates (P= 0.13)

Table 7. Twinning rates in near relatives of probands with NTD's: Montreal and Newfoundland data combined.

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	TOTALS
	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
NTD	**1.6 (386)**	**0.9 (587)**	**2.3 (769)**	**1.4 (3568)**	**1.4 (4318)**	**1.5 (9628)**
% like-sexed:	66.7	66.7	82.4	65.2	72.9	71.0
Upper NTD	**2.3 (214)**	**1.7 (302)** 2?	**2.3 (427)**	**1.9 (1932)**	**1.4 (2156)**	**1.7 (5031)**
% like sexed:	80.0	66.7	88.9	71.9	86.7	79.7
Lower NTD	**0.6 (172)**	**0 (285)**	**2.3 (342)**	**0.9 (1636)**	**1.4 (2162)**	**1.2 (4597)**
% like sexed:	0	-	75.0	50.0	58.6	57.7
Controls	**1.0 (399)**	**0.5 (781)**	**1.2 (780)**	**1.3 (3355)**	**1.0 (4736)**	**1.1 (10051)**
% like sexed:	75.0	0	55.6	65.9	53 2	57.8
NTD vs control						P=0.02
Upp vs low				P= 0.02		P= 0.02
Upp vs control				P= 0.09		P= 0.0001

Table 8. Twinning rates in near relatives of probands with Cleft Lip/Palate (CL/P): Montreal.

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	TOTALS
	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
CL/P	**3.3 (368)**	**1.5 (811)**	**1.8 (728)**	**1.6 (3258)**	**1.5 (4807)**	**1.6*(9972)**
% like-sexed:	66.7	63.6	53.8	65.9	61.2	62.6

* vs. Montreal controls, P= 0.0002.

Table 9. Twinning rates in near relatives of probands with Cleft Palate (CP): Montreal.

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	TOTALS
	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
CP	**4.5 (154)**	**1.3 (383)**	**2.6 (305)**	**2.0 (1241)**	**1.4 (1864)**	**1.8*(3947)**
% like-sexed:	85.7	40.0	87.5	77.3	69.6	73.8

* vs. Montreal controls, P= 0.0002.

Table 10. Twinning rates (%): summary

	Probands	Sibs	Parents	Aunts & Uncles	First Cousins	Total	% MZ * *
NTD (N=386)	1.6	0.9	2.3	1.4	1.4	1.5	42
Upper NTD (N=214)	2.3	1.7	2.3	1.9	1.4	1.7	59
Lower NTD (N=172)	0.6	0	2.3	0.9	1.4	1.2	15
CL/P (N=368)	3.3	1.5	1.8	1.6	1.5	1.6	25
CP (N=154)	4.5	1.3	2.6	2.0	1.4	1.8	[illegible]
Controls (N=399)	1.0	0.5	1.2	1.3	1.0	1.1	15
Canadian twinning rate (Elwood, 1978):						1.1	~25

** using the Weinberg method (Appendix D).

Table 11. Recurrence rates (R.R.) of NTD's in families with twins and without.

Montreal:	Families w/ twins		Families w/o twins	
	R.R.(%)	(N)	R.R.(%)	(N)
NTD	7.7	(130)	4.6	(327)
Upper NTD	9.1*	(66)	3.1	(163)
Lower NTD	6.3	(64)	6.1	(164)

* P= 0.11

Newfoundland:	Families w/ twins		Families w/o twins	
	R.R.(%)	(N)	R.R.(%)	(N)
NTD	2.9	(69)	5.0	(101)
Upper NTD	2.4	(41)	7.1	(56)
Lower NTD	3.6	(28)	2.2	(45)

Combined:	Families w/ twins		Families w/o twins	
	R.R.(%)	(N)	R.R.(%)	(N)
NTD	6.0	(199)	4.7	(428)
Upper NTD	6.5	(107)	4.1	(219)
Lower NTD	5.4	(92)	5.3	(209)

Table 12. Effect of multi-vitamin supplementation on NTD and twinning rates.*

	Unsupplemented (N=520)	Fully supplemented (N=417)
NTD rate	4.4%	1.7%
Twinning rate	1.3%	0.2%

* Data from Seller and Nevin, 1984.

DISCUSSION

Part A: Neural tube defects.

The first objective of this work was to test the hypothesis that upper NTD's (those at T11 and above) and lower NTD's (those at T12 and below) are etiologically distinct congenital abnormalities in humans arising from defective neurulation and canalization, respectively (Toriello and Higgins, 1985). This hypothesis was originally put forth following the observations that affected sib pairs always had the same type of defect (*i.e.* upper or lower NTD), additional malformations occurred much more often in upper NTD probands, and spontaneous abortion rates were much higher in mothers of upper NTD probands.

Another investigator (Hall *et al.*, 1988) later supported the above hypothesis by observing significantly higher NTD recurrence rates in sibships of upper NTD probands. The 100% concordance of NTD type within sibships was also seen in this study sample. Also, other studies have shown that the sex ratio in upper NTD probands is low whereas lower NTD cases have a male excess (Martinez Frias *et al.*, 1986; Seller, 1987b; Drainer *et al.*, 1991). It should be noted that studies have long shown anencephalics, which are part of the upper NTD group, to have a marked female excess (Penrose, 1957; Elwood and Elwood, 1980).

The findings of this study differed in most instances from

those cited above, high spontaneous abortion rate in the upper NTD group when compared to the lower group (Tables 1B and 4E, P= 0.0004) being the only finding in agreement.

NTD recurrence rates were virtually equal in the upper and lower NTD groups (Table 3, P= 0.91, NS) and although the Newfoundland data (Table 2) showed a higher rate in the upper group as in the B.C. data (Table 4A), the difference was again not significant (P= 0.36) and may be due to small sample size. Perhaps the difference observed in the B.C. sample is somehow related to the fact that B.C. has a low incidence of NTD's.

Another variable measured, sex ratio (Table 3), showed a female excess in upper NTD's (0.70) and virtually no sex excess in lower NTD's (0.92) [but a slight male excess when compared to upper NTD's]. The difference in the two ratios was not statistically significant (P= 0.19) but the fact that upper NTD's have a sex ratio that is lower than that found in lower NTD's is consistent with findings from other studies (see Table 4B), the study from B.C. being the only exception.

Reorganization of the data, as seen in graphs 1 and 2, yields results that suggest that the vertebral level above and below which NTD's are designated as upper or lower may not be T11. Graph 1 suggests that T12 may be the cut-off level, whereas Graph 2 suggests that it may be as far down as the third to fifth lumbar vertebra. In fact, since the Toriello and Higgins paper it has been estimated [in the mouse by Copp and Brook (1989) and in the human

by Müller and O'Rahilly (1987)] that the position of posterior neuropore closure and therefore the end-point for neurulation lies near the level of S2. If so, and if an upper/lower NTD distinction is real, true lower NTD's represent a very small minority of NTD cases (in the Montreal sample, under 5%).

Sib concordance with respect to NTD type was 52% (Table 4C), strongly suggesting that upper and lower NTD's are, at least in this Montreal and Newfoundland sample, related congenital anomalies and not two distinct entities as previously hypothesized. Two studies since we reported this finding at the American Society of Human Genetics conference (Fraser *et al.*, 1989) have observed sib concordance values of less than 100%, namely 82% (Seller, 1990) and 71% (Drainer *et al.*, 1991), and serve as further evidence against the hypothesis by Toriello and Higgins. In total, 34 out of 113 sibships (30%) from Newfoundland, Montreal, south-east England, and western Scotland, all high incidence areas for NTD's, showed discordance for NTD type.

It should be noted that the 100% concordance (British Columbia, Michigan) and the 94% (16 of 17, the one discordant case possibly due to coincidence) reported by Török and Papp in 1991 (Hungary) were observed in sibships from regions having relatively low NTD birth frequencies. This may suggest that there is genetic heterogeneity, and that the type of NTD that can affect either the upper or lower neural tube occurs only, or most commonly, in high frequency areas for NTD's. Alternatively, there may have been faulty

case ascertainment in B.C. and Michigan, as suggested by Drainer *et al.* (1991). Also, as Frecker *et al.* (1988) proposed, the fact that the cut-off level may be more caudal would lead to concordant cases being misclassified as discordant.

NTD cases with additional malformations were included in this study only when they were added to the isolated NTD cases in order to determine what proportion of upper or lower NTD's they constituted (and to compare this to other studies). Although the occurrence of an additional malformation in the NTD proband was higher in the upper NTD group (anencephalics excluded), the difference was not statistically significant (Table 4D, P= 0 31) The other two studies, both from relatively low NTD incidence areas (B.C. and Michigan), showed significantly higher malformation rates in upper NTD probands.

To explain the conflicting observations from the various sources, one could attribute the apparent discrepancies (especially in recurrence rate profiles) to an inherent difference between high incidence and low incidence areas for NTD's. Areas with lower NTD frequencies such as B.C. and Michigan have a higher proportion of NTD's with additional malformations (especially in the upper type), which may be responsible for the discrepancies observed in the other values measured. Alternatively, a suggestion by Seller (1987b) and later by Copp and Brook (1989), originally made for sex ratio differences between upper and lower NTD's, may also be extended to reconcile the apparent differences in spontaneous

abortion, additional malformation, and sib concordance rates.

The suggestion is built around the critical assumption that female human embryos are developmentally retarded compared with males at the onset of neurulation [as is the case with female ct/ct mouse embryos (Seller and Perkins-Cole, 1987)]. This would predispose females to the earlier arising upper NTD's to a greater extent than males. Later in development the female developmental rate catches up to that of the males' (thus explaining the near-equal predisposition to lower NTD's). Seller goes on to say that, during lower NTD development, a slight growth retardation in some females may actually protect them from lower defects. Since canalization depends on the formation and coalescence of vacuoles within masses of cells, the faster developing males, it is postulated, might over-produce vacuoles during canalization. This would explain the occasional reports of a male excess in lower NTD's (Seller, 1987b; Drainer *et al.*, 1991).

Therefore, timing of developmental events may be the underlying cause for the observations made at birth. Developmental timing itself may be genetically programmed and passed on from one generation to the next. For example, in mice an H-2 associated gene governing either fast or slow cleavage has been demonstrated and other genes have been shown to modify this gene's expression (Goldbard and Warner, 1982). If this situation exists in humans, spontaneous abortion may be linked to genetically determined slower development which would predispose to high rates of fetal

loss and to the earlier arising upper NTD's. This slow development may concurrently lead to the high rate of other early-arising anomalies (the additional malformations) found in upper NTD probands.

This hypothesis would also explain why type of NTD concordance is usually high in sibships (an average of 77% from all studies). In general, a family will have similar developmental rates (thus yielding concordant siblings). However, some of the time, environmental influences (and/or segregation or epigenetic effects) will cause one of the two siblings to speed up or slow down development [*e.g.* food deprivation in mice prevents spina bifida (Copp *et al.*, 1988), folate supplements in humans reduce risk of NTD recurrence (MRC, 1991)], thus resulting in discordance.

Another line of evidence against the existence of two distinct types of NTD is Seller's (1990) finding that, of 10 cases of NTD that had arisen despite maternal periconceptional vitamin supplementation, 8 were upper and 2 were lower NTD's. The fact that both types arose suggests they have a common underlying factor. To assess whether this was true in the Montreal population also, questionnaires were sent out to families of NTD probands asking if NTD had recurred in the family and whether vitamins were taken periconceptionally (Appendix E). However, there were no recurrences.

Other variables looked at in this sample were NTD secular trends (decade intervals) and differences in paternal or maternal

family history of NTD. The data were divided up accordingly but no significant trends or differences were found.

Part B: Twinning and neural tube defects.

The second objective of this thesis was to determine whether a familial association exists between NTD's and twinning. Twinning rates were calculated in the near relatives of probands with NTD's. Twinning rates were also calculated in upper and lower NTD groups.

The total twinning rate was 1.3% in the Montreal NTD families and 1.6% in the Newfoundland NTD families (Tables 5 and 6). A heterogeneity chi-square analysis indicated that the Montreal and Newfoundland samples were homogeneous with respect to twinning rate ($P > 0.2$), so the two sets of data were pooled. The combined twinning rate was 1.5% (Table 7). The rates were significantly high in the Montreal group and the combined group ($P=0.02$) when compared to control families, but not in the Newfoundland group ($P=0.54$) due to an unusually high control twinning rate (which in turn may be due to a small sample size).

Only one group has previously reported a high twinning rate in family members of NTD probands (Le Marec *et al.*, 1978). This group first reported a significantly high rate of twins (3.9%) in the 310 parents of a sample of SB probands from France. The twin excess was attributable to the 5.2% rate in the mothers. Upon enlarging their sample size in a follow-up study, the same group reported

rates consistent with the above (Journel and Le Marec, 1989). Of the 836 parents of SB cases, 25 were twins for a rate of 3.0% Again, the significantly high rate was due to the rate in the mothers (4 5%). The twin rate in the fathers was only 1.4%, lower than the estimated 1.9% population rate for Brittany (not significantly)

Upon closer inspection of the Montreal and Newfoundland data, no difference was found between twinning rates in mothers and fathers, or in maternal and paternal relatives. However, an interesting difference was observed when twinning rates were calculated in the upper and lower NTD subgroups.

The near relatives of probands with upper NTD's had a significantly higher combined twinning rate than those with lower NTD's (P=0.02) and even higher (P=0.0001) than those with Mendelian diseases (*i.e.*, the controls). This difference was marked only in the Montreal data. The upper and lower groups from the Newfoundland data had identical rates (probably due to a small sample of parents of lower NTD probands).

The greatest difference in twinning rate between upper and lower NTD's was observed in the aunts and uncles (P=0 02), followed by the sibs (P=0.09). The same held true when upper NTD and controls were compared (P=0.09 and 0.13, respectively).

This is the first report of a high twinning rate in the families of upper NTD probands (first communicated by Fraser *et al.* in 1989 and later in 1990).

If developmental timing is indeed genetically transmissible as

mentioned earlier, then it can also explain the above findings. Stockard (1921) and Bulmer (1970) have suggested that early embryonic growth retardation and developmental arrest may be the cause of MZ splitting. However, the precise manner in which a delay can cause this is not known. Spiers (1982) has also suggested that growth retardation predisposes the fetus to congenital malformations Therefore, the reason twinning rates are high in upper but not lower NTD families may be because the former have the "slow" genes which cause early developmental retardation and predispose to upper NTD and twinning (probably MZ). The reason that the latter do not have high twinning rates may be because the delay manifests later in development, in time to upset lower neural tube formation but too late to have caused a twinning event.

High twinning rates were also found in the near relatives of probands with CL/P and CP (Tables 8 & 9)[This finding was reported by Fraser and Garabedian in 1991]. It should be noted that closure of the lip and the palate occur so late in development that it may seem unreasonable to expect a delay early in embryogenesis to have any effect. However, the actual critical period during which a delay takes effect can be much earlier than the morphogenetic event itself (Nora and Fraser, 1989). Closure of the palate, for example, occurs around day 60 post-conception in humans (Jones, 1988), but any prior developmental delay may have been sufficient in precipitating the eventual failure of palate closure.

It is well established that twins are more likely to have

congenital malformations than singletons (Jones, 1988) Knox (1970) suggests that NTD's can occur in one member of a twin pair as a result of uterine crowding effects ("fetus-fetus interaction"). In the light of this possibility, exclusion of NTD probands would give a truer estimate of twinning in NTD families, and perhaps in CL/P and CP families as well. However, the total twinning rates in Table 10 were not affected when the probands were excluded (*N.B* the numbers of probands were small relative to that of family members).

It can also be seen from the data in Table 10 that cousins have, in general, lower twinning rates than first and second degree family members in the groups where an association with twinning has been observed (*i.e.* upper NTD, CL/P, and CP). This is expected if a familial association is truly present. The fact that cousins do not exhibit a lower twinning rate only in the lower NTD group confirms the lack of an association between lower NTD and twinning.

The excess of twins in the upper NTD group seems to be due solely to like-sexed and presumably MZ twins (Table 10) This is also true in the CP families but not in the CL/P families, where there is no zygosity excess. It is difficult to explain this finding (however, it should be noted that the Weinberg method provides only an estimate of zygosity). An interesting hypothesis by Boklage (1987a) may provide the explanation. According to this hypothesis, MZ and DZ twinning share common mechanisms and may be more related than previously thought, especially if monovular dispermatic

twinning is a major form of DZ twinning.

Table 11 shows that, in the Montreal sample, NTD recurrence tends to be higher in families with history of twinning in near relatives of probands with upper NTD (P= 0.11, not quite significant). The Newfoundland sample shows no such association. Clearly, for counselling purposes, more studies are needed to assess the possibility that a family history of twinning is a risk factor for NTD, especially upper NTD.

Table 12 is interesting in that it shows an association between periconceptional vitamin use and reduction of both NTD and twinning rate (almost a three-fold and a greater than five-fold reduction, respectively). This was not significant (P= 0.13) but warrants further investigation. Tolarova (1987) has similarly found reduction of CP and CL/P incidence with periconceptional vitamin and folic acid use. This apparent prevention of the schisis-type abnormalities (or fusion malformations) and twinning leads one to postulate that they share underlying causes.

To summarize, NTD's are familially associated with facial clefts (Fraser *et al.*, 1982) and twinning (present study). Facial clefts are also familially associated with twinning (present study). Nonrighthandedness is familially associated with all three (Fraser and Rex, 1984) Perhaps they do share heritable causal elements as suggested by Boklage (1987b).

Part C: Conclusions.

This is the first time that a familial association has been reported between twinning (probably MZ) and NTD's (upper) A heritable delaying factor in early development may be the underlying cause of this association.

A similar hypothesis suggests retardation of development following delayed or anomalous X chromosome inactivation as the cause for NTD (Hall, 1986) and MZ twinning (Burn *et al*, 1986), thus explaining the excess of females in both groups However, this situation does not elucidate the etiology of NTD and MZ twinning in males.

Interestingly, a variety of human aneuploidies have also been known to predispose to NTD (Schinzel, 1984). Since aneuploid cells would be expected to divide more slowly than normal, this may also explain the high twinning rates in relatives of probands with aneuploidies. Perhaps the familial delay factors are the inherited balanced translocations (or a predisposition to non-disjunction is heritable).

Gedda and Brenci (1986) have suggested that abnormally modified embryonic forms of neural cell adhesion molecules may be responsible for the MZ twinning event. If these undergo enzymatic splitting of their sialic acid residues it would lead to reduced cell adhesion and subsequent cleavage of the embryo to produce MZ twins It is interesting that the same molecule is implicated in the

etiology of NTD's (Edelman, 1984), abnormal adhesion being the cause of faulty neurulation

Finally, Flannery (1987) proposes homeotic genes to have a possible role in the etiology of congenital malformations in general, and MZ twinning. To investigate the above possibilities any further, it is important first to confirm our findings in other populations. This would be a relatively simple undertaking since it mainly involves counting twins in the families of probands with various congenital malformations or chromosomal abnormalities.

SUMMARY

Both twinning and the failure of neural tube closure are common phenomena that arise early in human embryonic development (at the moment of conception in DZ twinning, before day 7 5 in MZ twinning, and before day 28 in NTD's). Associations between twinning and NTD's have been observed embryologically (*e g.* the high incidence of NTD's in twins) and epidemiologically (*e g.* similar secular trends).

To investigate this association from a genetic perspective, family histories were obtained from hospital charts of NTD probands and twins were counted in the close relatives (parents, sibs, aunts, uncles, and cousins). Controls used were cases that had Mendelian disorders and their families.

Because of the suggestion (Toriello and Higgins, 1985) that upper and lower NTD's (those above and below T11) are etiologically different since they have different sex ratios (S.R.) and subsequently (Hall *et al.*, 1988) because they were found to have different sib occurrence rates (R.R), families in this study were subdivided into upper and lower groups for purposes of analysis In addition to obtaining S.R. and R.R., twinning rates from the two groups were calculated and compared.

Differences in S.R. were found in agreement with those observed by various investigators. However, the R R difference

found by Hall *et al.* was not observed in this sample.

The results also showed significant differences in twinning rates, the upper group having roughly double the rate of the lower group (which had rates similar to the Mendelian controls and also to Canadian population twinning rates). The excess of twins in the upper group was of like-sexed pairs, presumably monozygotic twins.

Twinning rates were calculated in CL/P and CP families also. Again, significantly high rates were found.

These findings may be explained by: 1) Upper and lower NTD's are not etiologically different but represent the same defect occurring at different developmental stages. Early disruption of morphogenesis puts females at higher risk since they develop slower in the initial embryonic period when the upper neural tube is still fusing. 2) The disruption in morphogenesis is common to upper NTD's and twinning (and perhaps facial clefts) and is caused by a familial destabilizing factor (perhaps a 'slow' gene or genes) that manifests itself very early in development.

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APPENDIX A

Birth rate of NTD's (per 1000 births) in different geographic regions.

Region	Rate	Year(s)	Reference
British Columbia (BC)	1.6	1952-70	McBride, 1979
Montreal	3.3	1961-65	Horowitz & McDonald, 1969
Newfoundland	3.2	1976-83	Fraser et al. ,1986
U.S. *	1.3	1970	Yen et al., 1992
" "	0.6	1989	"
Atlanta, Ga.	2.0	1968	"
" "	0.6	1989	"
N.Ireland (Belfast)	8.7	1964-68	Elwood, 1973
" "	3.4	1980-86	Dolk et al., 1991
Ireland (Dublin)	6.6	1970-73	McCarthy et al., 1983
" "	4.0	1980-83	De Wals & Lechat, 1986
Wales (south)	7.6	1956-62	Laurence et al., 1968
Scotland (western)	5.2	1979	Connor, 1989
" "	2.4	1985	"
England (London)	3.0	1965-68	Carter & Evans, 1973
England (Liverpool)	2.1	1982-86	Dolk et al., 1991
France (Strasbourg)	1.2	1982-86	"
Netherlands	1.4	1981-86	"
Hungary	1.5	1979-81	Czeizel & Karig, 1984
Greece	1.5	1984	Lekea et al ,1988
N.India (Amritsar)**	8.0	-	Verma, 1978
N.India (Delhi)	4.7	-	"
S.India (Calcutta)	0.8	-	"
Singapore ***	0.8	1953-66	Stevenson et al ,1966
" "	1.5	1976-87	Tan & Ho, 1989
Australia (Western)	1 8	1966-81	Bower et al , 1984
BC (Sikhs)	2.9	1961,64,71&79	Baird, 1983
Singapore (Sikhs)	6.5	1953-66	Stevenson et al ,1966

* There is a decreasing east-to-west gradient in the U S (Greenberg, 1983), as observed in Canadian rates

** Large Sikh population.

*** One of the few places that has not experienced a decline in NTD birth rate.

APPENDIX B

Frequency of types of placentas and fetal membranes in twins and days of initiation of each type of twin.*

	Single chorion		Two chorions	
Zygosity	**Single amnion**	**Two amnions**	**Single placenta**	**Two placentas**
MZ	Rare	65%	25%	10%
DZ	-	-	40%	60%
Initiation**	Day 7-Day 7.5	Day 4-Day 7	Day 1-Day 4	

* adapted from Thompson et al. (1991), p.390.
** from James (1988).

APPENDIX C

The single incomplete ascertainment method (Fisher, 1934).

This method (also called the 'sib' method) assumes that each affected individual has a very small chance of being ascertained (therefore never more than one proband in a family). To obtain a recurrence rate, the proband is excluded and the number of affected sibs is counted and divided by the total number of sibs.

Examples	(Affected/total sibs)
●	(0/0)
● ●	(1/1)
● 0 0	(0/2)
● 0 ●	(1/2)
Total recurrence rate	(2/5) = 40%

APPENDIX D

The Weinberg method (Weinberg, 1909).

This method uses the number of like-sexed and unlike sexed maternities to obtain an estimate of the rate of monozygotic and dizygotic twinning in a population.

$$m = (L-U)/N \qquad d = 2U/N$$

m is the monozygotic twinning rate
d is the dizygotic twinning rate
L is the number of like-sexed maternities
U is the number of unlike-sexed maternities
N is the number of total maternities

APPENDIX E

Sample and results of questionnaire sent out in Montreal.

Dear Mrs. _____,

You may remember talking to Dr. _____ in 19__ about your baby with spina bifida and giving some details about your family. We are currently bringing our charts up to date and we would be most grateful if you would complete this form and return it to us as soon as possible.

1) Have you had any children since being interviewed in 19__?
No__ Yes____

a) If yes, please give their names, sex, and date of birth. Also tell us if they had any birth defects and if you were taking any vitamins around the time when you got pregnant.

Name	Sex	Date of Birth Year/Month/Day	Birth Defects (Please specify)	Vitamins
_____	___	_____	_____	_____
_____	___	_____	_____	_____

b) Please tell us if any of your children are half-brothers or half-sisters.

2) Have you had any stillbirths or abortions (spontaneous or induced) since being interviewed in 19__? No____ Yes____

a) If yes, please give the month of pregnancy they occurred in, the approximate date, and tell us if any birth defects were present. Also tell us if you were taking vitamins around the time when you got pregnant.

Month of pregnancy	Date	Birth Defects	Vitamins
_____	_____	_____	_____
_____	_____	_____	_____

3) Your present address and phone number: _____
_____

Your help would be very much appreciated.

Yours sincerely,
B. Garabedian
for Dr. F.C. Fraser,
Division of Medical Genetics,
The Montreal Children's Hospital.

Results:

104 questionnaires were sent out (English or French). 56 (54%) were completed and returned. Of the 48 families not heard from, 15 had changed place of residence and could not be located. There was no recurrence of NTD in the 55 sibs born after the probands (one child was born with "renal malformations"; mother had taken Nutrifer Plus). Periconceptional vitamin use was reported in 23 cases (42%). Vitamins: Materna (5 cases), Neotinic (6), Neotinic + folic acid (1), Nutrifer Plus (1), unspecified vitamins (9), unspecified vitamins + folic acid (1).

APPENDIX F

Type and/or level of NTD in probands and their affected sibs Montreal. *

*	Defect in proband	Defect in sib	Concordance
1	AN	Lumbar ME	No
2	AN	Low lumbar MM	No
3	AN	Lumbar MM	No
4	AN	Lumbo-sacral MM	No
5	AN	Mid-lumbar MM	No
6	AN	SB (L5-S3)	No
7	Cranial MM	Lumbar MM	No
8	Lumbar MM	Cervical MM	No
9	Occipital EN	Lumbar ME	No
10	Sacral SB	Sacral SB	Yes
11	AN	SBo C6,C7,T9,T10,T11 and MM L4 down	Yes
12	AN	SBo T1,2/ incomplete segmentation C2-C4	Yes
13	AN	Thoracic SB	Yes
14	AN	Occipital EN	Yes
15	AN	AN	Yes
16	Thoracic MM (T4-T12)	AN	Yes

LEGEND
AN= Anencephaly
EN= Encephalocele
ME= Meningocele
MM= Myelomeningocele
SB= Spina bifida
SBo= SB occulta

* for Newfoundland data, see Frecker (1988)