Cytogenetic Investigations in Immunological and Gastrointestinal Diseases and Malformations in Children

PhD Thesis

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Abbreviations

CD	Coeliac Disease
CSI	Centromere Separation Index.
DNA	Deoxyribonucleic acid
EMA	anti-endomysium antibody
ID	Immunodeficiency
IgA	Immunoglobulin A
KCl	Potassium-chloride
PCD	Premature Centromere Division
SCE	Sister Chromatid Exchange
SGA	small for gestational age

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INTRODUCTION

Walther Flemming was the first to identify chromosomes inside cell nuclei in 1877. He also investigated mitosis, the process of cell division. His discovery served as the start point of cytogenetics. It was the early 20th century when the karyotype (the set of chromosomes) was recognized as the carrier of genes but it became clear only by the mid 1950s that the karyotype of man included 46 chromosomes. The first report of human numerical abnormalities of chromosomes was the discovery of trisomy 21 in Down syndrome in 1959. With the aid of chromosome banding techniques introduced in the late 1960s not only could chromosomes be differentiated but structural changes such as deletion, translocation, etc. could also be identified. Further advances led to the technique of fluorescent in situ hybridization (FISH) in the 1980s when fluorescently labelled probes were hybridized to chromosome preparations.

Development of molecular genetics compared to cytogenetics was much faster. It started in 1944 when Avery showed that deoxyribonucleic acid (DNA) is the hereditary material and took only 3 decades to perform the first prenatal DNA diagnosis. In 1990 the first successful human gene therapy was reported. In the same year the human genome project (identification of all the approximately 20,000-25,000 genes in human DNA and determination of their base pair sequences) begun and was completed by 2003.

Mitosis is the process of eukaryotic cell division that leads to two identical daughter cells. Chromosomes are replicated during the S phase and condense into compact structures during prophase of cell division. Each of them consists of two identical sister chromatids held together by the centromere. In prometaphase and metaphase chromosomes are aligned along the metaphase

plate of the spindle apparatus at the equatorial plane in the midline of the cell. Spindle fibers are bound to the kinetochores (structures associated with the centromere of each chromosome) and during anaphase the centromeres divide and the separated sister chromatids are pulled to the opposite poles by spindle fibers. By the end of cell division two daughter cells are produced with the same genetic component as the parent cell. (**Fig. 1.**)

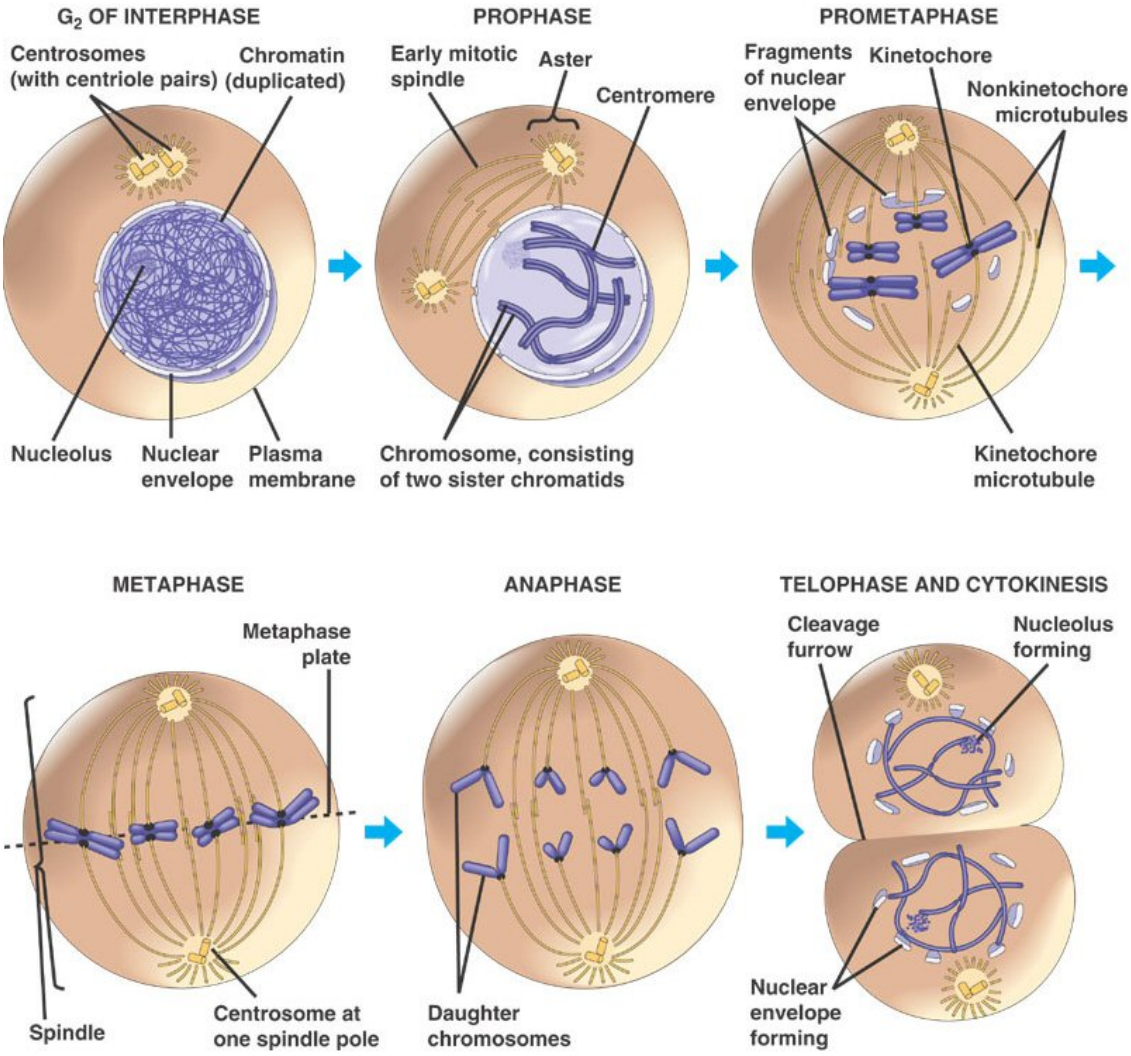

Fig. 1. Mitosis (from http://royaleb.wordpress.com/2009/04/27/mitosis/)

To prepare a standard karyotype any population of dividing cells providing metaphase cells is required. Most of the time blood is the sampled tissue as lymphocytes can easily be induced to proliferate, but other sources of tissue e.g. skin (cultured fibroblasts), bone marrow, amniotic fluid, chorionic villi, etc. also can be used when appropriate.

Routine technique for karyotype preparation – brief summary

1. A sample of blood is drawn and coagulation is prevented by addition of heparin.

2. Mononuclear cells are purified from the blood by centrifugation through a dense medium that allows red cells and granulocytes to pellet, but retards the mononuclear cells (lymphocytes and monocytes).

3. The mononuclear cells are cultured for 3-4 days in the presence of a mitogen like phytohemagglutinin, which stimulates the lymphocytes to proliferate madly.

4. Mitosis is arrested in metaphase by a solution of colchicine. At the end of the culture period, when there is a large population of dividing cells, the culture is treated with colchicine, which disrupts mitotic spindles and prevents completion of mitosis. This greatly enriches the population of metaphase cells.

5. The lymphocytes are harvested and treated briefly with a hypotonic solution. This makes the nuclei swell osmotically and greatly aids in getting preparations in which the chromosomes do not lie on top of one another.

6. The swollen cells are fixed, dropped onto a microscope slide (forcing the chromosomes into a single plane) and dried.

7. Slides are stained after treatment to induce a banding pattern. When they are stained, the mitotic chromosomes have a banded structure that unambiguously identifies each chromosome of a karyotype. Without banding it is impossible to distinguish between among chromosomes.

8. Once stained slides are prepared, they are scanned to identify "good" chromosome spreads (i.e. the chromosomes are neither too long nor too compact and are not overlapping), which are photographed.

9. The photomicrograph is cut apart and the individual chromosomes are arranged into a definitive karyogram. Alternatively, a digital image of the chromosomes can be cut and pasted using a computer. If standard staining was used, the orderly arrangement is limited to grouping like-sized chromosomes together in pairs, whereas if the chromosomes were banded, they can be unambiguously paired and numbered.

The pictures below show the schematic structure of a chromosome (**Fig. 2a**) and non-banded chromosomes as they are seen on the slide. (**Fig. 2b**).

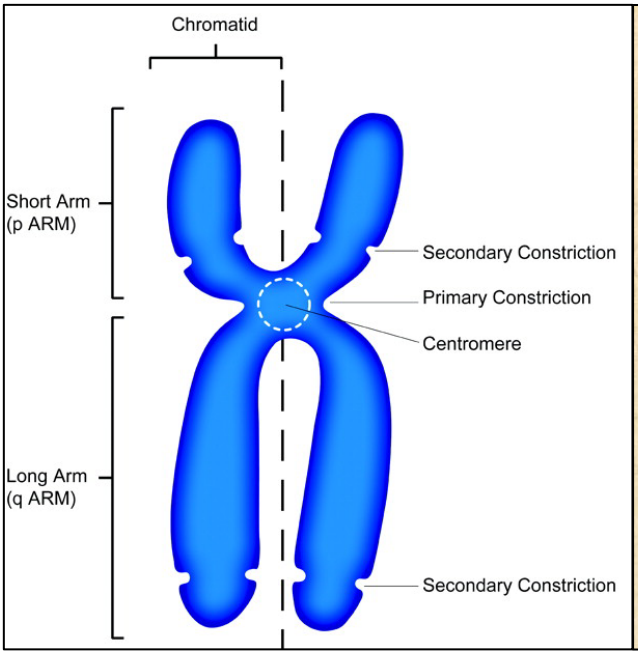

Fig. 2a.

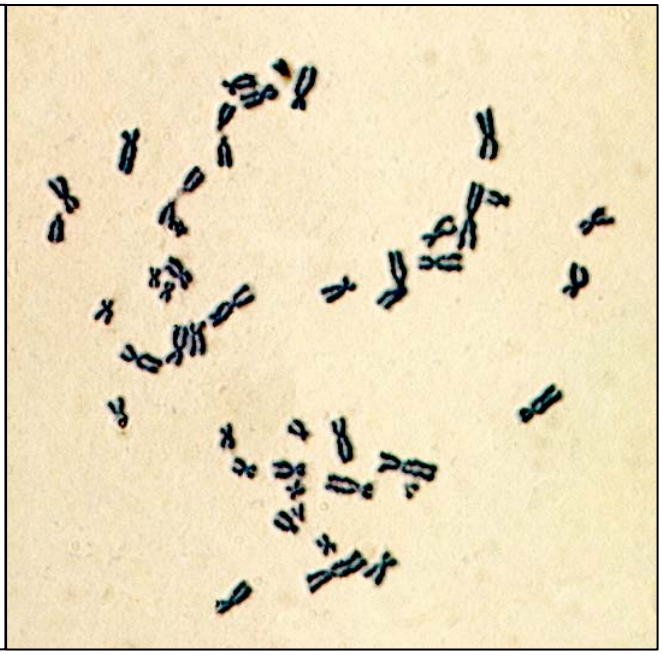

Fig. 2b.

Metacentric chromosomes  are characterized by their centromere lying in the median resulting in two well defined arms with similar length; in submetacentric chromosomes  as the centromere is slightly shifted towards the short arms the difference between the arms is more apparent. In acrocentric chromosomes  the centromere is close to one end of the chromosome.

Based on the size and shape of the chromosomes of a somatic cell human chromosomes are divided into 7 groups and sex chromosomes (**Table 1.**). Autosomes are numbered from largest to smallest, except that chromosome 21 is smaller than chromosome 22. (**Fig. 3.**)

Table 1. Human chromosome groups

Group	Chromosomes	Description
A	**1-3**	Largest, 1 and 3 are metacentric but 2 is submetacentric
B	**4, 5**	Large submetacentric, submetacentric with two arms very different in size
C	**6-12, X**	Medium-sized, submetacentric – difficult to distinguish one from another
D	**13-15**	Medium-sized acrocentric with satellites
E	**16–18**	Small, 16 is metacentric but 17 and 18 are submetacentric
F	**19, 20**	Small metacentrics
G	**21, 22, Y**	Small acrocentrics with satellites. Y has no satellites.

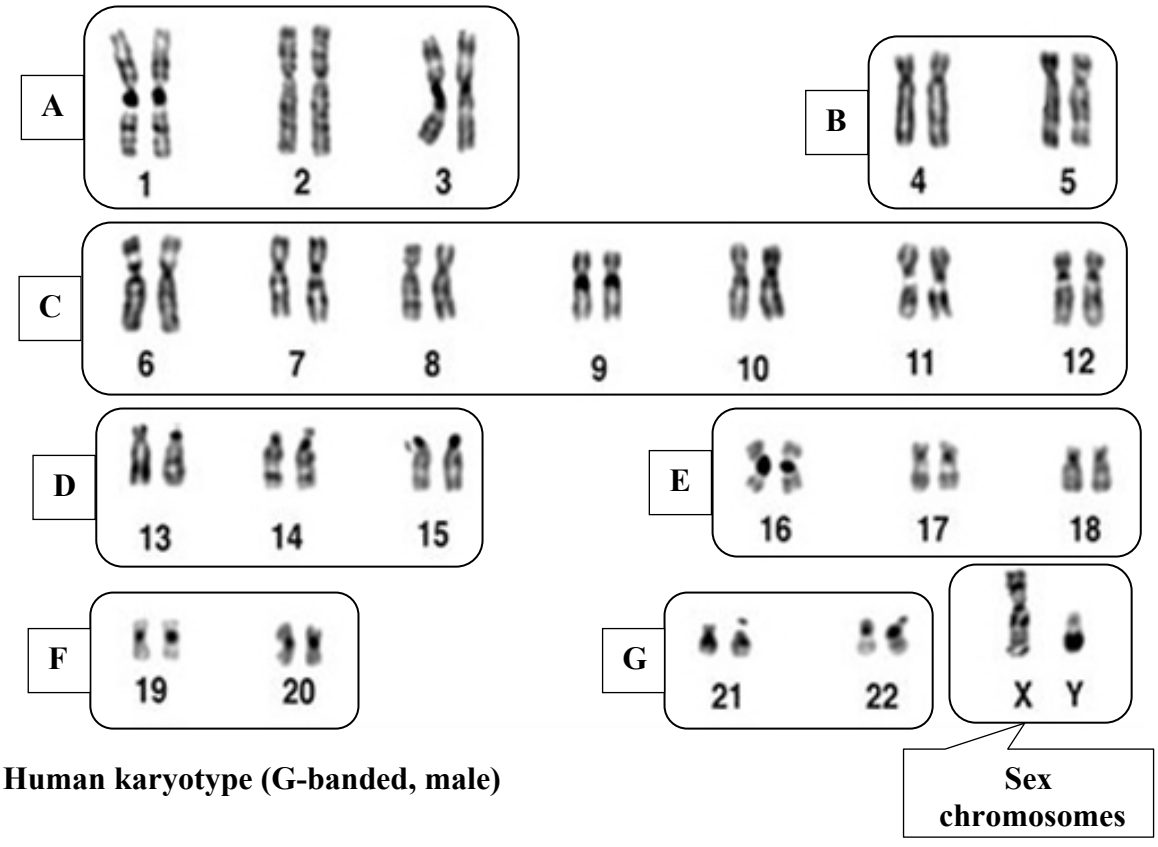

Fig. 3. Human karyotype (G-banded, male)

Centromere separation

Existence of a sequence

It is a crucial point of cell division when sister chromatids forming chromosomes are separated at the centromere and they move to daughter cells. Aisenberg was the first to report in 1935 that this separation has a fixed order (Aisenberg 1935). In the late 1960s during routine diagnostic examination of human chromosomes Méhes noticed that at the end of metaphase centromeres do not separate at the same time. It was striking that chromosomes 2, 17 and 18 separated earlier and the acrocentric chromosomes did not show any signs of division when all others had already separated. His observation in lymphocyte cultures of 20 healthy subjects was submitted in 1973 but it was published only in 1975 (Méhes 1975). A similar observation was made by Vig and Wodnicki (Vig and Wodnicki 1974). In the following years several articles were published by these working groups. Later on other teams joined this research, and centromere separation was investigated not only in different human cells but in other species (e.g.: Chinese hamster, frogs, plants) as well. Subsequent reports confirmed the original observation. As a result of this research the normal sequence of centromere separation was determined in humans.

Assessment of Centromere Separation

For the investigation of centromere separation a meticulous analysis of several hundreds or thousands of mitoses is required. Centromere separation can be assessed best on traditionally stained, non-banded chromosomes on the one hand, meanwhile on the other hand G-banding is necessary for the individual chromosomes to be clearly identified. One can imagine how difficult and time consuming this process is to find late metaphases with clear separation of chromosomes which can be still identified by their G-banding patterns. As the chromosomes do not expand at the

same level on the slides fine tuning with the micrometer knob is also essential and lengthens time for analysis. Beyond the previously mentioned difficulties subjectivity in the assessment of centromere division also contributes to complexity when determining the separation order.

There are 2 methods used for the assessment of centromere separation:

1. **Premature Centromere Division** (PCD) introduced by Méhes is used to determine the susceptibility to too early (premature) separation. During analysis only mitoses showing not more than 3 complete separations are taken into account. Complete separation is considered to be present when no connection between the sister chromatids can be seen on microscopic examination. The number of separations for an individual chromosome in a large series is divided by the value calculated based on a "random" separation order. The result reflects the tendency for premature separation. ("Calculated value" is the total number of separations observed in 100 mitoses divided by 23.)

2. Vig introduced a scoring system called the **Centromere Separation Index** (CSI).

   By this method

   - the centromeres that had not divided at all were given a score of 0,

   

   - those that had just begun to separate scored as 1,

   

   - and the ones showing clear separation, i.e. no connection between the sister chromatids, were scored as 2.

   

The scores obtained were pooled for individual pairs of chromosomes. These totals were then divided by the highest value in the series, providing relative values (CSI) against a given value of 1 for the earliest separating pair. The higher the CSI for a chromosome, the earlier was its position in the centromere separation sequence.

Based on repeated observations in different laboratories and despite all the difficulties in the assessment of centromere separation it is now proven and accepted that during metaphase in human mitotic cells chromosomes do not separate randomly.

- Chromosomes 2 and 18 are the first to separate,
- followed by chromosomes 4, 5, X, 12, 3 and 17.
- Chromosomes 1, 7, 8, 9, 11, and 16 separate late
- and the large acrocentrics (13-15) are the last ones in this order.

The more marginal the position of a certain chromosome in the separation sequence the more accurately can its position be determined. On the other hand, when assessing chromosomes in the middle of the separation order uncertainty due to subjectivity has to be taken into account.

Consistency of Separation Sequence

When investigating separation sequence in different living beings sequences were found to be species-specific.

Based on findings published so far the following factors have NO INFLUENCE on the separation order:

length of chromosome,

position of centromere,

size of heterochromatic part,

time or temperature of incubation,

type of medium used,

colchicine treatment,

type of hypotonic solution or process,

drugs,

radiation,

or gender.

The role of aging has not been unravelled yet. Premature centromere separation of chromosome X associated with aneuploidy has been reported in aged men and women (Fitzgerald et al. 1975; Fitzgerald and McEwan 1977).

Based on species specificity and consistency it is highly likely that the sequence of centromere separation is a genetically determined, species-specific phenomenon unalterable by exogenous factors known to date. This assumption is supported by observations in families where dominantly inherited abnormal separation sequence could be found (Méhes and Bajnóczky 1981).

Separation Sequence and Disorders – brief summary

It is well known that factors affecting cell division may cause chromosome injury and may disrupt chromosome pairing or migration. Chromosome aberration arising from this may serve as a basis for a latent or manifest disease which may be inherited in certain cases.

1) Aneuploidy

Alteration of centromere separation sequence may lead to non-disjunction causing aneuploidy. When investigating trisomic children and their parents Méhes reported (Méhes 1978) a higher frequency of early separating G group chromosomes in Down syndrome (trisomy 21) and too late or absent separation of early separating chromosome 18 in 2 mothers of children with Edwards syndrome (trisomy 18). Chromosome 21 – which is normally among the last in the separation sequence – was also reported (Fitzgerald et al. 1986) as the first to separate in a clinically normal 28-year-old woman who had three conceptuses with Down syndrome and one normal child.

2) Structural abnormalities

Too early or too late separation of chromosomes may occur with structural chromosomal abnormalities. Not only out-of-phase separation in subjects with chromosome deletion (13q14) (Méhes and Bajnóczky 1982) and translocation (3p;19q) (Méhes and Kosztolányi 1990) were reported but late separating fused D-group chromosomes (D/D fusions) as well (Méhes et al. 1991).

3) Carcinogenesis

Chromosome aberrations play a crucial role in carcinogenesis. According to previously published papers altered centromere separation sequence may be an indicator for mutagenic effect therefore may play a role in carcinogenesis. Attention was called to the relationship between PCD,

aneuploidy, mutagenic agents, chromosome breakage, immunological changes and malignancy by Bühler (Bühler et al. 1987). Altered centromere separation sequence was shown in several types of malignancy.

In **Table 2.** disorders with premature centromere division are listed.

Table 2. Disorders with premature centromere division manifestation

Disorder	References
Roberts' syndrome (and subgroup)	> 100 cases
Aneuploidy	Fitzgerald *et al.* 1975, 1986; Méhes *et al.* 1978; Bajnóczky and Méhes 1988
Spontaneous abortions	Rudd *et al.* 1983; Murthy and Prabhakara 1990; Bajnóczky and Gardó 1993
Translocation carrier	Madan *et al.* 1987
Subfertility	Rudd *et al.* 1983; Gabarrón *et al.* 1986
Ambiguous genitalia	Miller *et al.* 1990
Pseudohermaphroditism	Rangnekar *et al.* 1990
Megaloblastic anemia	Heath 1966; Bamezai *et al.* 1986
Tuberous sclerosis	Scappaticci *et al.* 1988
Leukaemias	Sandberg 1980; Shiraishi *et al.* 1982; Gallo *et al.* 1984; Vig 1984; Littlefield *et al.* 1985
Burkitt's lymphoma	Zhang 1986
Nasopharyngeal carcinoma	Zhang 1986
Bladder cancer	Berrozpe *et al.i* 1990
Révész syndrome	Kajtár and Méhes 1994
Paracoccidioidomycosis	Freire-Maia *et al.* 1994
Fanconi's anaemia	Méhes and Bühler 1995
Ataxia teleangiectasia	Méhes and Bühler 1995
Alzheimer's disease	Moorhead and Heyman 1983; Kormann-Bortolotto *et al.* 1993

NB: References for these articles are not listed in the chapter References on **Page 50.**

Joining the cytogenetic team lead by Professor Méhes Károly as a medical student initially I started in vitro chromosome studies. After graduation I participated in developing a computer based objective method and in cytogenetic investigations in clinical patients with various disorders. After starting my training in gastroenterology mainly patients with gastroenterological diseases were chosen as subjects for such investigations. This thesis is a summary of studies and achievements on this topic.

Aim of this thesis

1. To investigate whether ”normal” centromere separation sequence can be influenced or altered by an extrinsic factor such as vanadate which is well-known to affect cell division;

2. to introduce an objective method for analyzing centromere separation;

3. to investigate chromosomal abnormalities and premature centromere separation in children with congenital immunodeficiencies;

4. to investigate chromosome fragility and premature centromere division in children with coeliac disease;

5. and to investigate chromosomal abnormalities and associated congenital abnormalities in newborns with gastrointestinal malformations.

STATISTICAL ANALYSIS

All statistical analysis was performed using Excel or the Statistical Package for the Social Sciences (SPSS) for Windows 7.5, 8.0 and 11.5 softwares. Statistical significance of the differences between groups was evaluated using the Fisher’s exact or Chi-square or Student’s *t*-test when appropriate. Differences were considered to be statistically significant when probability values were smaller than 5% (i.e. $P<0.05$).

INVESTIGATIONS

THE EFFECT OF VANADATE ON THE CENTROMERE SEPARATION SEQUENCE

(Based on the article published in *Acta Biol Hung* in 1993.)

INTRODUCTION

The existence of a "normal" sequence of centromere separation in human mitoses has repeatedly been described (Méhes and Bajnóczky 1981; Vig 1981a). Too early or too late separation may lead to trisomy or monosomy of the given chromosome (Fitzgerald 1987; Méhes 1978; Vig 1983) therefore alteration of the centromere separation sequence may lead to aneuploidy and may be an indicator of chromosome instability. This raises the question of whether this phenomenon can be influenced by mutagenic factors. To our knowledge only a few studies have dealt with this problem so far. Singh and Miltenburger (Singh and Miltenburger 1977) and Miltenburger et al. (Miltenburger et al. 1980) examined the effect of cyclophosphamide and isoniazid on the centromere separation sequence in Chinese hamster spermatogonia and bone marrow cells, respectively, and found no alteration after exposure to these drugs. At the same time, Bajnóczky et al. (Bajnóczky et al. 1980) demonstrated significant changes of the centromere separation sequence in lymphocytes of in vivo prednisolone-treated infants. No increase of chromatid or chromosome gaps, breaks, rearrangement figures, dicentrics or sister chromatid

exchanges were observed in these babies. This is of special importance because Bühler et al. (Bühler et al. 1987) claimed that similarly to structural aberrations and sister chromatid exchanges premature centromere divisions are also indicators of chromosome instability. A similar conclusion was drawn by Goswami et al. (Goswami et al. 1990) when examining the victims of the gas explosion in Bhopal.

In this study we made an attempt to analyze the centromere separation sequence in human lymphocytes exposed to vanadium, a well-known cytotoxic agent.

MATERIALS AND METHODS

Lymphocyte cultures from a healthy 23-year-old male subject with normal karyotype were cultivated for 72 hours in McCoy 1A medium. Mitotic arrest was made by adding 0.125 µg/ml colchicine for the last 2 hours, potassium-chloride (KCl) was used for hypotonic shock, and the fixed cells were stained with Giemsa.

To examine the effect of vanadate, 5% $NaVO_3$ was given to the cultures in various amounts and for various times in the following combinations:

Vanadate concentration (µmol/ml)	**Exposition (hours)**			
0.51	2.5			
1.02	2.5			
2.56	2.5	3	4	6
5.12	2.5	4		

Since vanadate concentrations above 2.56 μmol/ml and exposures of more than 4 hours proved to be so toxic that the preparations contained no evaluable mitoses (grey boxes), only the lowest and highest exposures allowing normal mitoses were further investigated (red margins). Simultaneous cultures of the same blood sample not treated with vanadate served as controls.

Mitotic indices were determined by counting at least 2,000 cells from 5 different fields and expressed as number of mitoses at any stage per 1,000 cells.

In order to see the separation more clearly, the chromosomes were not banded. This meant that only the main groups and the most characteristic individual chromosomes could be identified. The sequence of centromere separation was characterized by the centromere separation index (CSI) discussed previously (**Page 8**.) with mathematical correction. By this method a score of 0 was given to the centromeres that had not divided at all, those that had just begun separation scored 1, and those showing clear separation were scored 2. The scores obtained were pooled and divided by the elements of the group of chromosomes. These totals were then divided by the largest number in the series; thus relative values (CSI) against a given value of 1 for the earliest separating pair were gained. The higher the CSI for a chromosome, the earlier was its position in the separation sequence.

RESULTS

The findings in the three groups, i.e. low and high vanadate exposures and control, are summarized in **Table 3.** As shown by the figures, the mitotic indices were significantly lower in the vanadate-treated cultures than in the untreated controls (red box). Apart from 2 gaps, no breaks and other structural aberrations were found in any of the slides examined.

Table 3. Mitotic index and centromere separation index (CSI) values in control lymphocyte cultures and in those of slight and intensive vanadate exposure

	Control	Vanadate-treated cultures	
		0.51 µmol/ml 2.5 hours	2.56 µmol/ml 4 hours
Mitotic index (1/1000)	49.5	26.0[A]	24.5[A]
CSI			
Number of mitoses	95	91	93
Chromosome or group			
1	0.11	0.15	0.08
2	1.00	0.99	1.00
3	0.47	0.51	0.47
4-5	0.61	0.60	0.58
6-X-12	0.50	0.56	0.52
13-15	0.00	0.01	0.01
16	0.02	0.08	0.03
17-18	0.97	1.00	0.93
19-20	0.45	0.50	0.44
21-22-Y	0.00	0.01	0.02

[A] $p < 0.001$ against control.

The differences in CSI values of the three groups are statistically not significant.

The relative CSI values for both the slight and heavy exposures are almost identical with those of the control group. Since individual chromosomes could not be identified, only the mean values of chromosome groups are given, which demonstrate very similar tendencies of separation. As in the controls, in the cells treated with vanadate chromosomes 2, 17 and 18 also showed very early division, whereas chromosomes 1 and 16 and the acrocentrics were the last to separate. This sequence corresponds to the findings of several earlier studies from different laboratories.

DISCUSSION

Vanadate is regarded as an inhibitor of cell development and division, especially of chromosomal movement (Cande and Wolniak 1978; Klein et al. 1989). The decreasing mitotic index values reflected such an inhibition in the present study as well.

At the same time, vanadate did not alter the sequence of centromere separation. These findings have at least two implications:

1. Different vanadium compounds may have different effects on cell metabolism and division (Galli et al. 1991). The present data show that also Na-vanadate is toxic as far as slowing down the division of cultured lymphocytes and lowering their mitotic rate.
2. It has been shown in previous studies that varying factors of cell culture and preparation, such as temperature, medium, culture time, colchicin, hypotonic shock, and calcium, do not alter the sequence of centromere separation (Belcheva et al. 1978; Méhes 1978). The fact that even toxic levels of vanadate were ineffective in this respect provides further evidence for the suggestion that the centromere separation sequence is scarcely influenced by environmental factors but rather is a species-specific, genetically determined phenomenon.

Since our recent observation suggested a possible new aspect of out-of-phase centromere separation causing aneuploidy (Méhes and Kosztolányi 1992), on-going analysis of possible exogenous and endogenous factors influencing the separation sequence seems to be required.

CYTOGENETIC INVESTIGATIONS IN CHILDREN WITH CONGENITAL IMMUNODEFICIENCIES

(Based on the abstract published in *Monatsschr Kinderheilk* in 1993.)

INTRODUCTION

In previous studies it has been reported that congenital immunodeficiencies (ID) are associated with a higher frequency of structural chromosome aberrations (Blumel and Eibl 1992; Conley et al. 1986; Jaspers et al. 1988; Wegner et al. 1988). In addition, a few syndromes combine ID with fragility of chromosomes - ataxia telangiectasia, Bloom's syndrome (Rosin and German 1985) and Nijmegen breakage syndrome (Weemaes et al. 1981). Beyond centromeric instability of certain chromosomes somatic recombination of the arms of these chromosomes and a marked tendency to formation of multibranched configurations were also registered in the recently reported ICF (Immunodeficiency, Centromeric heterochromatin instability and Facial anomalies) syndrome (Maraschio et al. 1988). As discussed before centromeres do not separate at the same time during late metaphase/early anaphase of cell division. Previous studies have demonstrated that a species specific order exists in centromere division: chromosomes 18 and 2 are the first ones to separate followed by 4-5, X, 12, 3, and 17. Chromosomes 1, 7, 8-9, 11 and 16 separate late, whereas the large acrocentrics (13-15) are the last in the sequence. Alteration in this sequence may cause non-disjunction leading to aneuploidy and can be a possible indicator of chromosome-instability as well.

The aims of this study were:

- To investigate numerical and structural abnormalities of chromosomes and premature centromere division in children with various type of ID.

- To determine if there is any correlation between classic features of chromosome instability (i.e. break, gap, sister chromatid exchange, dicentric, ring formation, etc.) and previously uninvestigated "out of phase" centromere separation.

PATIENTS AND METHODS

"Routine" lymphocyte cultures of 12 patients with ID (**Table 4.**) were analyzed and compared to healthy controls (n=6). These lymphocyte cultures were prepared according to the method described previously (**Page 3**.).

Table 4. Patient demographics

No.	Gender	Age	Associated symptoms	Type of immunodeficiency (ID)
1	M	7 months	Rec. pneumonia and otitis	Hypogammaglobulinaemia
2	M	1 year	Rec. furunculi and diarrhoea	Hypogammaglobulinaemia
3	M	14 months	Rec. bronchitis	Hypogammaglobulinaemia
4	M	18 months	Rec. bronchitis and pneumonia	IgA deficiency
5	F	18 months	Rec. pneumonia, atopy, epilepsy	IgA deficiency
6	M	19 months	Rec. pneumonia and pyuria	IgA deficiency
7	F	3 years	Rec. bronchitis	Hypogammaglobulinaemia
8	M	15 months	Perinatal sepsis, rec. infections	Variable ID
9	F	6 years	Rec. pneumonia, otitis	Variable ID
10	M	6 years	Rec. osteomyelitis	Septic granulomatosis
11	M	9 years	Rec. bronchitis, otitis	Hypogammaglobulinaemia
12	M	11 years	Rec. infections, bronchiectasia	Variable ID

The following abnormalities/features were recorded in at least 60 mitoses of each individual.

- Hypo-, Hyperdiploidy
- Gap
- Breaks
- Structural abnormalities (dicentric, ring formation, translocation)
- Sister Chromatid Exchange (SCE) rate
- Premature Centromere Division (PCD)

Beyond the classic features of chromosome instability, like structural abnormalities and SCE, attention was focused on PCD.

When analyzing data all efforts were made to be as objective as possible, therefore a simplification was made in PCD analysis. The centromere separation sequence was not determined in each mitosis, only the percentages of those metaphases are given in which 3 or more completely separated centromeres could be seen. Based upon literature and our previous data, fewer than 4% of routine lymphocyte cultures contain such metaphases.

RESULTS

As **Table 5.** shows, the frequencies of chromosome breaks, SCE and PCD were similar in most of the children with ID compared to healthy controls. However, in patients 7 and 9 (with hypogammaglobulinaemia and variable ID respectively) significantly higher numbers of structural aberrations, SCE and PCD were found (red boxes).

To rule out the possible effect of a recent viral infection, drug effect or technical artefact during lymphocyte preparation these investigations were repeated after 4-6 months, and the findings

were similar again. In these 2 children the classical features of chromosome instability and PCD were parallel.

It is well-known that subjectivity can not be eliminated when analyzing centromere separation. However, in these 2 patients not only PCD was noted. Frequent separation of the large acrocentric chromosomes, which are known to be among the last ones in centromere division, also was observed. Separation of these D-Group chromosomes is not seen normally.

Table 5. Chromosome breaks, sister chromatid exchange (SCE) and premature centromere division (PCD) in patients with immunodeficiency (ID) and in controls

Patients	Analyzed mitoses	Chromatid break/cell	Chromosome break/cell	SCE	Mitoses ≥ 3 PCD (%)	PCDs per mitosis of chr. 13-15.
Controls (n=6)	86	0,009	0,004	4,1 (2,1-6,0)	2,2 (1-4)	0,004
1.	70	0,014	0,014	4,6	4,2	0
2.	85	0,012	0	3,7	2,3	0
3.	76	0	0	-	2,6	0
4.	100	0,02	0,01	6,1	5,0	0,01
5.	70	0	0	4,4	2,8	0
6.	60	0,017	0	3,9	6,7	0
7.	66	0,061*	0,03	9,7	13,7*	0,182*
	100	0,1*	0,04*	11,6*	19,0*	0,210*
8.	100	0,01	0	-	2,0	0,10
9.	80	0,025	0,037*	8,5	22,5*	0,137*
	100	0,06*	0,02	7,9	17,0*	0,06*
10.	64	0	0,016	3,6	1,6	0
11.	75	0,013	0,013	4,5	4,0	0,013
12.	60	0,017	0	5,8	3,4	0,017

*p< 0,05

Mitoses ≥3 PCD (%): Percentages of metaphases with 3 or more complete PCD

PCDs per mitosis of chr. 13-15.: Number of PCDs of chromosomes 13-15 per mitoses

DISCUSSION

Based on the present findings it is important to stress that PCD - ignored as "artefact" previously - can be recognized effortlessly in "routine" chromosome preparations. This observation has a significant impact on future research.

Howard et al. (Howard et al. 1985) reported centromeric instability of chromosomes 1 and 16 with variable ID suggesting a new syndrome which was later supported - and complemented with chromosome 9 - by the findings of Valkova et al (Valkova et al. 1987). The most frequent symptoms of this syndrome are facial dysmorphism, mental retardation and recurrent infections secondary to variable ID. Later the name ICF (Immunodeficiency, Centromeric heterochromatin instability and Facial anomalies) syndrome was assigned (Maraschio et al. 1988). The most striking characteristic chromosomal anomalies in this syndrome are multibranched configurations and breakage of chromosomes 1, 9 and 16 at the centromeric region.

In this study neither ICF nor any other specific syndrome could be identified in any of the 12 children with ID. Two out of 12 children with ID showed signs of chromosome instability. This could be demonstrated not only by the classic methods but by the high percentages of metaphases showing PCD, as well. These data and our previous findings in Fanconi anaemia and ataxia teleangiectasia suggest that well-marked aberrant separation sequence and too early separation can be considered as a sign of chromosome instability (Méhes and Bühler 1995). Therefore analysis of centromere separation should be integrated when investigating chromosome instability.

OBJECTIVE ANALYSIS OF CENTROMERE SEPARATION

(Based on the article published in *Hum Genet* in 1996.)

INTRODUCTION

The existence of a genetically determined sequence of centromere separation in human mitotic chromosomes seems to be established. Disturbance of this "normal" pattern may lead to nondisjunction, causing aneuploidy (Méhes 1978; Vig 1983). Of special interest is premature centromere division (PCD), which has been implicated in the causation of aneuploidies in aged men and women (Fitzgerald et al. 1975), in malignancies (Vig 1984) and in various conditions related to chromosomal instability (Bühler et al. 1987; Méhes and Bühler 1995). PCDs and puffing of the centromeres are regarded as characteristic features of Roberts syndrome and, although recent findings suggest heterogeneity of this condition (Allingham-Hawkins and Tomkins 1995), the evaluation of PCDs has been found applicable in the prenatal diagnosis of Roberts syndrome (Stioui et al. 1992). In spite of the increasing number of such observations, the pathogenetic role of altered centromere separation and PCD has failed to achieve a widespread acceptance, mainly because of the criticism that visual evaluation of the separation grade of a centromere (i.e. the sister chromatids) under the light microscope cannot be accurate and objective. Here we describe a simple computer-based method for the precise measurement of centromere distances within chromosomes by means of image analysis and public software that makes an objective and exact staging of centromere division possible.

MATERIALS AND METHODS

Routine Giemsa-stained chromosome preparations of blood lymphocyte cultures were analyzed as follows: Well-spread metaphases were selected in a NIKON Microphot FXA microscope using 400x primary magnification. The microscope was completed with a charge-coupled device camera (model C 72; Dage-MTI, Michigan City, Ind., USA). Appropriate fields were captured and transferred to a Macintosh Quadra computer installed with the public NIH Image Version 1.55 program. After identification of individual chromosomes on the grey scale digital image, the density slice option was selected. Within this, a line perpendicular to the longitudinal axis of the chromosome, exactly across both sister centromeres, was drawn on the monitor image and the density histogram (plot) of this line was created in each case by the software. Centromere separation distance could be obtained directly by measuring the length between the 2 density peaks representing the 2 centromeres. The distance between the separating centromeres could be given in pixels or in micrometers after setting the pixel/ micrometer ratio.

Images were also analyzed in the binary (black and white) option. By using binary transformation of the image, the separation of the sister chromatids became even clearer, although the fine density differences corresponding to the centromere structure stained with Giemsa and the background grey level were lost. Thus, both grey scale and binary functions of the software gave interesting information concerning the stage of centromere division.

RESULTS AND DISCUSSION

Some 15-20 mitoses of 5 healthy subjects (3 males) with normal karyotypes were analyzed. In each mitosis, the centromere separation distances of the individual chromosomes were compared with each other and aligned according to their separation grade. The measurement was quick and objective and resulted in a "normal" centromere separation sequence that was similar to that observed in earlier studies (Méhes and Bajnóczky 1981; Vig 1981b), i.e. chromosomes 2, 18, 17, 4, 5 and X divided early, whereas chromosome 16 and the acrocentrics were the last to separate.

The advantages of this method are obvious in the determination of PCDs in the mitoses of a patient with Fanconi anaemia. In this case, in addition to completely intact and partially divided chromosomes, PCD of the large acrocentrics is the most conspicuous finding (**Fig. 4.**). As shown by the histograms, a single density peak can be found over the centromere of the intact chromosome (**Figs. 4, 5a**); two separate peaks with an obvious connection are seen in the case of partial centromere separation (**Figs. 4, 5c**), whereas a complete PCD is characterized by a density curve declining to the background level between the two peaks (**Figs. 4, 5b**).

The method is simple, quick and relatively inexpensive, provided that an image analysis system is available. It may also be utilized in retrospective analyses of old slides, e.g. in family investigations, in which centromere anomalies are sought as possible signs of chromosome instability.

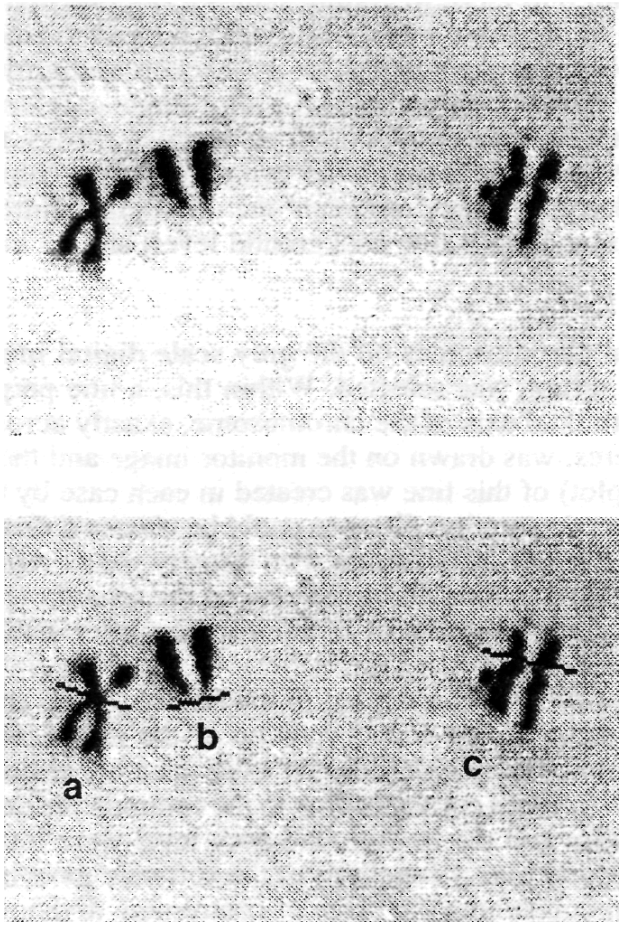

Fig. 4. Raw *(top)* and processed *(bottom)* digital images from a partial metaphase of a child with Fanconi anaemia with different degrees of centromere separation, ***a*** Intact centromere; ***b, c*** seemingly complete separation in a large acrocentric and in a submetacentric chromosome. *Lines* of the density histograms are drawn across the centromeres in ***a-c.***

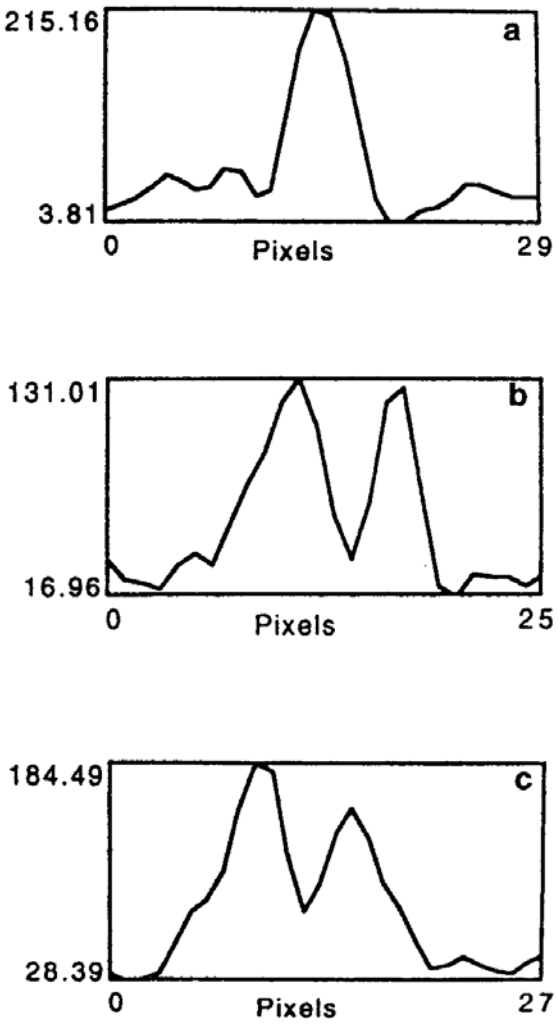

Fig. 5a-c. Cross centromere density histograms of the chromosomes in Fig. 1. *x* Distance in pixels, *y* density grade (grey scale levels from 0 to 255). In chromosome a (***a***), one high peak corresponding to the dense intact centromere is present. In chromosome b (***b***), 2 peaks separated from the background grey level represent 2 divided chromatids. Chromosome c (***c***) also has 2 peaks but with an overlapping of the density profiles; this indicates a latent connection between the 2 chromatids. Numerically, the lowest density values between the dividing centromere peaks proved to be 37 in ***b*** (the background level) and 72 in ***c***.

CHROMOSOME FRAGILITY AND PREMATURE CENTROMERE DIVISION IN COELIAC DISEASE

(Based on the article published in *Gyermekgyógyászat* 2002 and the abstract published in *J Pediatr Gastroenterol Nutr* 2004.)

INTRODUCTION

The frequent association of coeliac disease (CD) with type 1 diabetes mellitus and other autoimmune diseases is well known. This and the increased risk of malignancy in CD (Holmes et al. 1989) raised the question of the possible role of genetic instability as an underlying condition. The results of previous studies regarding chromosome instability in treated and untreated coeliac patients are still controversial (Fundia et al. 1996; Fundia et al. 1994; Kolacek et al. 1998; Kolacek et al. 2001). Premature centromere division (PCD) – the early separation of centromeres – was found to be a possible marker for chromosome instability by Méhes and Bühler (Méhes and Bühler 1995). The aim of our study was to investigate chromosome fragility and PCD in patients with CD.

PATIENTS AND METHODS

Spontaneous and bleomycin-induced chromosome fragility and PCD were determined in 48- and 72-hour peripheral blood lymphocyte cultures of 22 patients with CD. In all cases diagnosis of

CD was established by ESPGHAN criteria (1990). The male/female ratio was 12/10, ages were 3-19 yrs (mean: 12.5), and duration of disease was 0-13 yrs (mean: 5.9) in this cohort. Similarly prepared cultures of 18 healthy individuals (M/F ratio=9/9) served as controls. Simultaneously anti-endomysium antibody (EMA) was determined and a questionnaire regarding the diet, febrile illness, vaccination and exposure to conditions causing chromosome fragility was obtained. According to this questionnaire no febrile illness, vaccination or exposure to conditions causing chromosome fragility was registered within 6 weeks prior the investigation in any of the subjects. To estimate the effect of gluten-free diet, newly diagnosed patients (n=2) and non-compliant patients (n=5) were also enrolled.

Routine peripheral blood lymphocyte cultures with an incubation time of 48 and 72 hours were prepared according to the method described previously (Page 3.). Bleomycin-treated cultures were prepared as routine cultures, with addition of 30 μg/ml bleomycin for the last 5 hours of incubation. A G-banded slide (resolution of approximately 400 bands) was made in each subject for routine karyotyping. The remaining slides were Giemsa stained only and the following abnormalities were registered: chromatid and chromosome breaks, rearrangements, and dicentric and ring chromosomes.

On final analysis only the percentages of mitoses with aberrations were registered irrespective of their nature or number per mitoses. This part of the study was in accordance with the bleomycin test described previously (Hsu et al. 1989; Székely et al. 2001). Cell divisions demonstrating more than 3 PCD (PCD>3%) were also registered. For the assessment of these abnormalities 50-100 mitoses were evaluated in all subjects at 48 and 72 hours of incubation time in routine and bleomycin-treated cultures, respectively. Frequency of chromosome aberration and PCD was compared in coeliac patients and controls.

RESULTS

All the karyotypes were normal, i.e. no chromatid or chromosome breaks, rearrangements, or dicentric or ring chromosomes were identified. The number of spontaneous chromosome aberrations in routine cultures was found to be very low (between 0 to 3% in each patient) and no differences were seen between coeliac patients and controls (inside red boxes, $p>0.10$, not significant). Though frequencies of mitoses with chromosome aberrations were significantly higher in bleomycin-treated cultures compared to routine cultures in both patient groups (e.g. 48 hours: 42.2% vs. 0.38%^{A} and 72 hours: 34.7% vs. 0.1%^{C}, respectively, $p<0.05$) there was no significant difference between coeliac and control patients in spontaneous and bleomycin-induced chromosome fragility (**Table 6.**).

Table 6. Percentages of mitoses with chromosome aberrations in routine and bleomycin-treated cultures (%, mean ± SD)

	Routine		Bleomycin treated	
	48 hours	**72 hours**	**48 hours**	**72 hours**
Coeliac (n=22)	0.38±0.74 ^{A}	0.1±0.29 ^{C}	42.2±11.68 ^{A}	34.7±16.56 ^{C}
Control (n=18)	0.25±0.46 ^{B}	1.2±1.23 ^{D}	40.6±9.02 ^{B}	35.3±9.97 ^{D}

A,B,C,D = $p<0.05$

The frequency of PCD was similarly between 0-5%, in both the coeliac and control patients, although with a high rate of 8-23% in 4 coeliac patients (**Fig. 6. and Fig. 7.**).

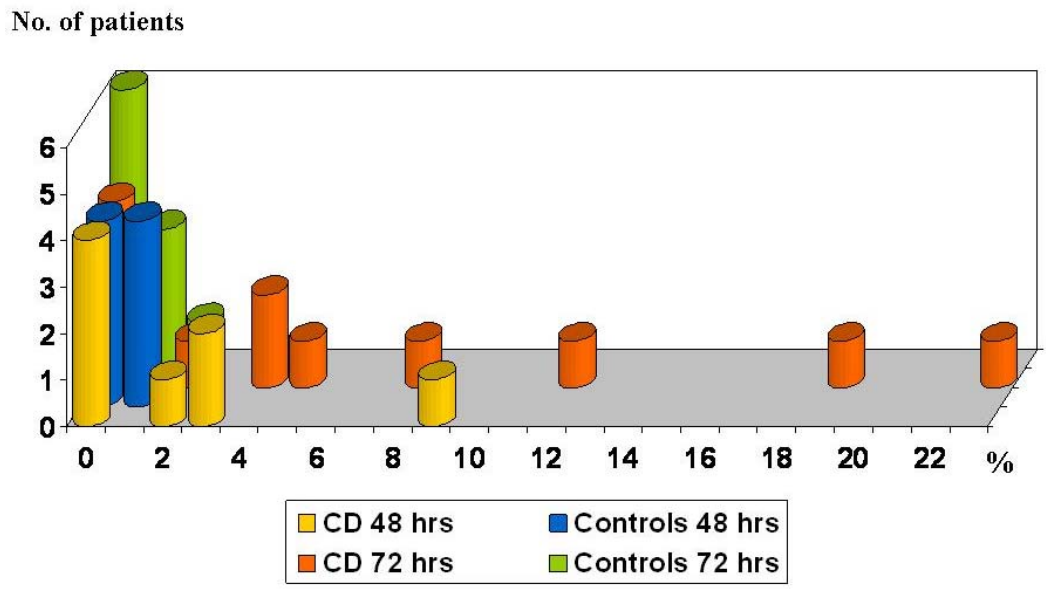

Fig. 6. Percentages of mitoses with more than 3 PCD in routine cultures

CD: Coeliac disease PCD: Premature centromere division

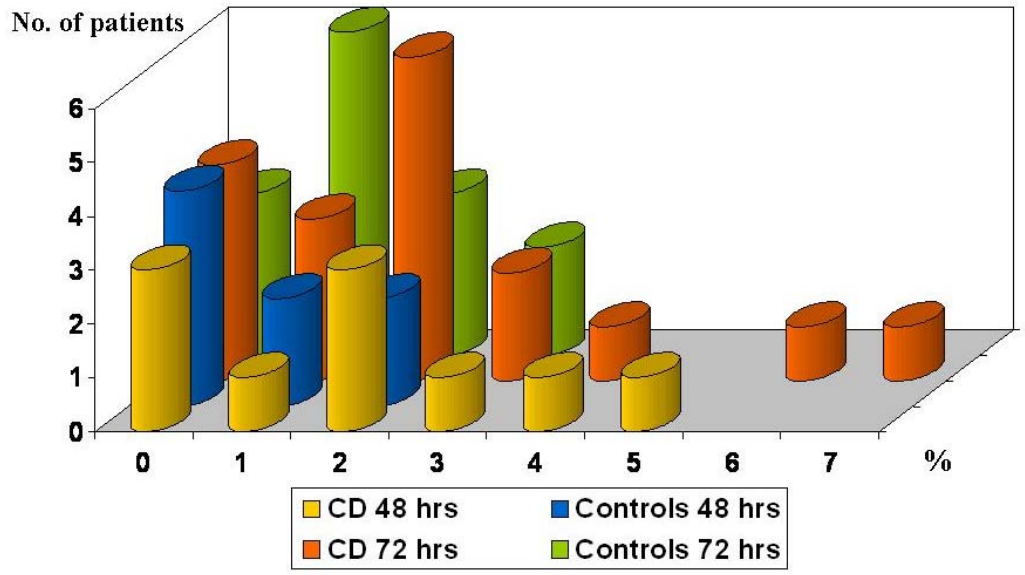

Fig. 7. Percentages of mitoses with more than 3 PCD in bleomycin-treated cultures

DISCUSSION

Irrespective of the method applied, no differences in the rate of chromosome breakage were found between coeliac patients and controls. Fragility could not be correlated with diet, duration of gluten enteropathy, or age or sex of the patients. The fragility tests showed no chromosome instability in CD. The significance of unusually high frequencies of PCD (8-23%) observed in a few patients is not clear, but the phenomenon deserves further studies from the aspect of the relation of gluten enteropathy to malignancies.

Our results are in contrast with previous studies and support the findings of Kolacek et al (Kolacek et al. 1998) but do not show any correlation between the duration of diet and fragility. The same correlation can not be excluded between PCD and diet although our findings are not consistent. Among the 4 patients showing unusually high frequencies of PCD 2 adhered to a strict gluten-free diet, one was non-compliant and one was newly diagnosed with CD, i.e. not having gluten-free diet at all. It is evident that failure of "repair mechanism" is responsible for chromosome breakage while SCE, telomeric association and PCD – considered as manifestations of chromosome instability – have different underlying mechanisms (Matsuura et al. 2000). Therefore it is possible that these different features of instability may manifest themselves differently in the same condition, as suggested by Fundia (Fundia et al. 1994).

Despite all these equivocalities it is highly likely that classic gluten enteropathy – in contrast to ulcerative colitis (Cottliar et al. 2000a) and chronic pancreatitis (Cottliar et al. 2000b) – is not necessarily associated with chromosome instability. Elucidation of the relationship between CD and malignancy can be expected from studies of patients and families affected by both conditions, focusing on carcinogenesis and PCD (Méhes 2000a; Méhes 2000b).

ANALYSIS OF GASTROINTESTINAL MALFORMATIONS, ASSOCIATED CONGENITAL ABNORMALITIES, AND INTRAUTERINE GROWTH IN NEONATES

(Based on the article published in *J Pediatr Gastroenterol Nutr* in 2002.)

INTRODUCTION

Gastrointestinal malformations often require surgical intervention and intensive care, therefore challenging the paediatrician and paediatric surgeon. The fact that gastrointestinal malformations are frequently associated with other congenital disorders may aggravate the difficulties in diagnosing and treating affected infants (Muller et al. 1994; Passarge and Stevenson 1993; Tulloh et al. 1994). In contrast with other malformations, congenital anomalies of the gastrointestinal tract have been scarcely at all investigated. We report an analysis of gastrointestinal malformations, associated congenital abnormalities, and their relation to intrauterine growth in neonates treated in our neonatal intensive care unit.

PATIENTS AND METHODS

Among the 4,241 newborn infants treated during the 14-year period between 1987 and 2000 in the Neonatal Intensive Care Unit of the Department of Paediatrics, Faculty of Medicine, University of Pécs, 278 (6.55 %) had gastrointestinal malformations. Neonates with Hirschsprung disease were excluded from the study because not all of them were treated in the neonatal intensive care unit. Patients with pyloric stenosis also were excluded because this malformation typically does not manifest itself in the neonatal period. In the remaining 241 neonates with gastrointestinal malformations (5.68%), we recorded associated congenital abnormalities, chromosomal abnormalities verified by 150-to 400-band karyotyping (performed in all the newborns with multiple congenital abnormalities), malformation syndromes and associations, gestational age, and birth weight. As defined in previous studies, newborns with birth weights below the gestational age-specific 10^{th} percentile were classified as small for gestational age (SGA).

RESULTS

In 241 neonates, excluding patients with Hirschsprung disease and pyloric stenosis, 304 gastrointestinal malformations were found. **Table 7.** shows their distribution.

Table 7. Distribution of gastrointestinal malformations

Diagnosis	Total	SGA	Isolated	SGA	Multiple	SGA
Oesophageal atresia	57	30	30	16	27	14
Anal atresia	43	15	17	3	26	12
Cleft lip and palate	40	11	11	4	29	7
Duodenal atresia	40	14	13	8	27	6
Malrotation of small bowels	23	4	1	1	22	3
Omphalocele	17	3	7	1	10	2
Small-bowel atresia	16	7	6	2	10	5
Gastroschisis	11	8	6	5	5	3
Rectal atresia	7	3	0	0	7	3
Ductus omphaloentericus persistens	6	1	3	0	3	1
Malposition of the anus	6	2	1	0	5	2
Mesenterium ileocommune	6	0	0	0	6	0
Volvulus	6	1	1	0	5	1
Extrahepatic biliary atresia (gall bladder agenesis)	3	1	2	1	1	0
Hirschsprung disease*	3	2	0	0	3	2
Intestinal duplication	2	0	2	0	0	0
Liver malformation	2	0	0	0	2	0
Tracheo-oesophageal fistula	2	0	2	0	0	0
Abdominal hernia*	1	0	0	0	1	0
Brachioesophagus	1	0	0	0	1	0
Cloaca duplex	1	0	0	0	1	0
Colonic atresia	1	0	0	0	1	0
Duodenal stenosis	1	0	0	0	1	0
Oesophageal stenosis	1	0	1	0	0	0
Gastric duplication	1	1	1	1	0	0
Gastric hypoplasia	1	0	0	0	1	0
Jejunal stenosis	1	0	1	0	0	0
Malrotation of colon	1	0	1	0	0	0
Neurointestinal dysplasia	1	0	1	0	0	0
Pyloric stenosis*	1	1	0	0	1	1
Rectum duplex	1	0	0	0	1	0
Vesicointestinal fissura	1	0	0	0	1	0
Total	**304**	**104**	**107**	**42**	**197**	**62**

* Registered as associated anomaly only
SGA: small for gestational age

Gastrointestinal malformation alone was found in 108 patients, whereas in 133 cases it was observed as one of multiple anomalies. Among these 133 patients, a specific syndrome could be identified in 27 cases and a specific association and 9 **(Table 8).**

Table 8. Distribution of multiple congenital anomalies

Syndrome, association	Number of patients
Down	13
VACTERL	8
Patau	4
Edwards	3
Pierre-Robin	2
CHARGE	1
Cornelia de Lange	1
Opitz G	1
Roberts	1
Short rib-polydactyly	1
Treacher-Collins	1
Nonclassified	97
Associations (total)	9
Syndromes (total)	27

The prevalence of gastrointestinal malformations in the infants with the "classic" autosomal trisomies was: trisomy 21 (n=13), 9 with duodenal atresia, 2 with oesophageal atresia, 2 with omphalocele, 4 with malrotation of bowels, and one with cleft lip and palate; trisomy 18 (n=3), 2 with oesophageal atresia, one with omphalocele, and one with anus atresia and malposition of the anus; and trisomy 13 (n=4), 3 with cleft lip and palate and one with liver malformation.

Using cytogenetic investigations and evaluation of major and minor congenital anomalies, no classified syndromes or associations could be diagnosed in 97 patients. **Table 9.** shows the associated anomalies in these patients.

Table 9. **Associated anomalies in children with gastrointestinal malformations without classified syndrome or association**

Associated malformation	Number of patients
Bone	44
Heart	40
Urogenital	31
Face	7
Ear	6
Central nervous system	5
Eye	4
Lung	4
Nose	3

An unexpectedly large number of skeletal disorders exceed the numbers of heart and urogenital malformations. When birth weight values were plotted against the local percentile chart, 161 of the 241 (66.8%) fell below the fiftieth percentile, suggesting a lower than expected birth weight in neonates with gastrointestinal malformations (**Fig. 8.**).

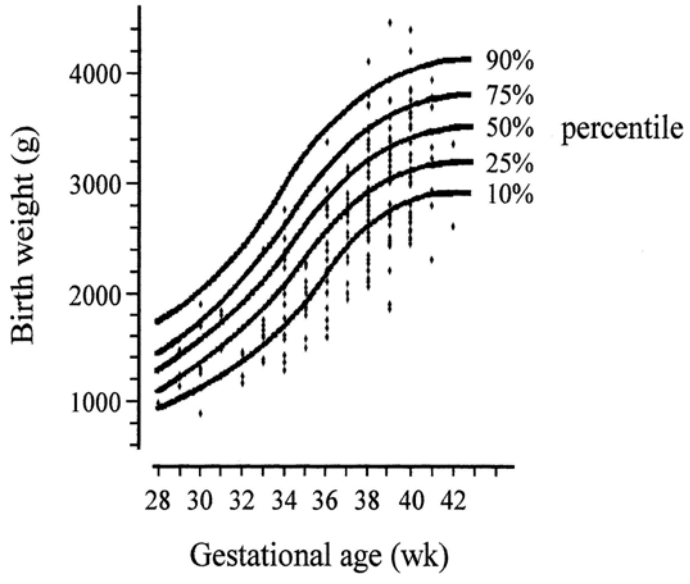

Fig. 8.
Distribution of birth weight according to gestational age in newborns with gastrointestinal malformations

Compared with the normal population, the frequency of intrauterine growth retardation was significantly increased ($P < 0.001$) (**Fig. 9**). The proportions of patients who were SGA in the isolated and multiple malformation groups were 38.9% and 30.8%, respectively.

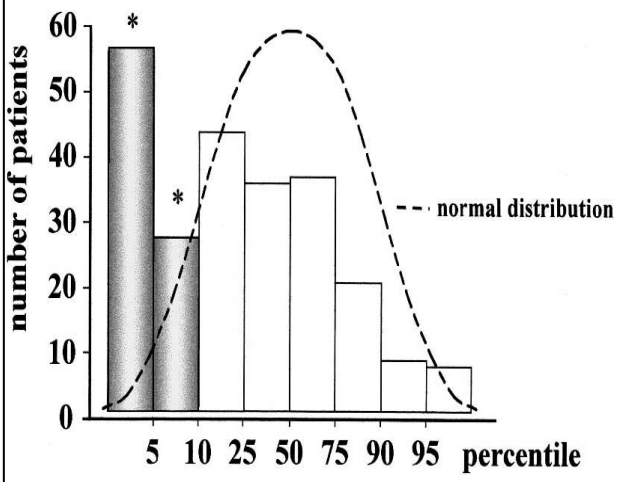

Fig. 9.
Distribution of birth weight percentiles in newborns with gastrointestinal malformations.

The numbers of patients below the gestational age specific 10th percentile (dark columns) are significantly higher than expected.
*P < 0.0001.

DISCUSSION

Because ours is a tertiary centre, no epidemiologic data could be calculated from the 4,241 newborns treated in the neonatal intensive care of our department. The distribution of gastrointestinal malformations in this study was similar to that reported in the literature (Schoetzau et al. 1997; Taeusch et al. 1991), with the exception of cleft lip and palate. This common anomaly occurred less frequently than expected in our study. The reason for this may have been that newborn infants with isolated cleft lip or palate usually do not require intensive care.

Ruszinkó and Nagymányai (Ruszinkó and Nagymányai 1986) found gastrointestinal malformations in 4.2% of Hungarian infants with chromosomal abnormalities. In trisomy 21 they found oesophageal and duodenal atresia, in trisomy 18 they found accessory liver, and in trisomy 13

they found cleft lip and palate.

Torfs et al. (Torfs et al. 1995) investigated the prevalence of oesophageal atresia, tracheo-oesophageal fistula, and oesophageal atresia with tracheo-oesophageal fistula in more than 1,000,000 births. The total prevalence rate was 2.82 in 10,000 live and stillbirths. No secular trend or marked seasonal variation could be demonstrated. The mean birth weight of the affected infants was below that of the normal population, but the difference was not significant. The lowest birth weights were in cases of trisomy and oesophageal atresia with tracheo-oesophageal fistula. Trisomy 21 was found in newborns with oesophageal atresia and in those with oesophageal atresia with tracheo-oesophageal fistula, but not in infants with tracheo-oesophageal fistula only. On the contrary, trisomy 13 occurred only in patients with tracheo-oesophageal fistula, but not with oesophageal atresia or oesophageal atresia with tracheo-oesophageal fistula. Trisomy 18 was seen in association with all 3 defects. The proportion of cases with additional midline defects or VATER/VACTERL associations was similar for all 3 types of oesophageal defects, suggesting a common developmental pathogenesis.

Gilbert and Nicolaides (Gilbert and Nicolaides 1987) found chromosomal abnormalities in 54% of 35 fetuses with antenatally diagnosed omphalocele. Seventeen fetuses had trisomy 18, one had triploidy, and one other had an XXY sex chromosome constitution. Additional malformations were found in 73% of the cases, the most frequent being congenital heart defect (47%). Although the incidence of additional abnormalities and chromosomal defects in this study differed substantially from the figures reported in the paediatric literature, the incidence in the surviving infants did not differ.

Concerning cardiac and urogenital abnormalities, our current findings support those of previous reports. However, we observed skeletal malformations in almost half the infants with nonclassified multiple anomalies. This may stimulate a thorough search for bone disorders in infants with gastrointestinal malformations.

In this study, more neonates were SGA in the group with gastrointestinal malformation than were those in the normal population. According to earlier studies, patients with gastrointestinal malformations had smaller birth weights than expected, but the difference was not significant. Khoury et al. (Khoury et al. 1988) investigated the association between intrauterine growth retardation and congenital malformations in general. They found intrauterine growth retardation in 22.3% of the 13,074 newborns with major structural abnormalities. Among isolated defects, only pyloric stenosis, congenital hip dislocation, and polydactyly were not associated with excess intrauterine growth retardation. Frequency of intrauterine growth retardation increased with the number of defects in infants with multiple malformations (20% in infants with 2 defects and 60% in infants with 9 or more defects.)

In an earlier survey, Méhes also found a positive correlation among major congenital malformations, multiple minor anomalies, and intrauterine growth retardation (Méhes 1988).

As to specific anomalies, Blake et al. (Blake et al. 1993) found mean birth weight and length at less than tenth percentile in patients with CHARGE association. They also found postnatal growth retardation.

Mapstone et al. (Mapstone et al. 1986) found postnatal growth deficiency in 45% of patients with VATER association, 64% of these growth-deficient patients had severe cardiac defects. Mean birth weight and length were below the normal but were still within normal ranges.

The relationship between intrauterine growth and malformations was most thoroughly investigated in the case of chromosomal aberrations with or without heart malformations (Kramer et al. 1990; Méhes 1982; Pueschl et al. 1976). These data support a relationship between low birth weight and intrauterine growth retardation on the one hand, and congenital malformations on the other. This can be explained easily in cases with chromosomal abnormalities: trisomy or monosomy of hundreds of genes affects metabolic and endocrine processes in morphogenesis. Simultaneously with incorrect differentiation of organs, altered fetal circulation or central nervous coordination may

lead to inadequate growth and development. However, this is not a sufficient explanation in patients with isolated gastrointestinal malformations, because an isolated cleft lip, anteponated anus, or duplication of the small bowel has no direct connection to intrauterine growth (as the foetus is fed via the umbilical cord). Gastrointestinal malformations are often complicated by skeletal anomalies and intrauterine growth retardation. The association among these disorders requires further investigation. However, from a practical point of view, this association should be considered in the treatment of affected patients.

Discussion and Practical Consequences of the Studies

1. The effect of vanadate on the centromere separation sequence

Vanadate is regarded as an inhibitor of cell development and division, especially of chromosomal movement. The decreasing mitotic index values in the present study also referred to such an inhibition. At the same time, vanadate did not alter the sequence of centromere separation.

Practical consequences

These findings have at least two implications:

A. Different vanadium compounds may have different effects on cell metabolism and division. The present data show that Na-vanadate also is toxic in that it slows the division of cultured lymphocytes and lowers their mitotic rate.

B. It has been shown in previous studies that various factors of cell culture and preparation, such as temperature, medium, culture time, colchicine, hypotonic shock, and calcium do not alter the sequence of centromere separation. The fact that even toxic levels of vanadate were ineffective in this respect provides further evidence for the suggestion that the centromere separation sequence is hardly at all influenced by environmental factors but rather is a species-specific, genetically determined phenomenon. On-going analysis of possible exogenous and endogenous factors influencing the separation sequence seems to be required.

2. Cytogenetic investigations in children with congenital immunodeficiencies

Two out of 12 children with ID showed signs of chromosome instability. This could be demonstrated not only by the classic methods but the high percentages of metaphases showing PCD, as well.

Practical consequences

These findings have at least two implications:

A. Based on the present findings it is important to stress that PCD – ignored as "artefact" previously – can be recognized effortlessly in routine chromosome preparations. This observation has a significant impact on future research.

B. These data and our previous findings in Fanconi anaemia and ataxia teleangiectasia suggest that well-marked aberrant separation sequence and too early separation can be considered as a sign of chromosome instability (Méhes and Bühler 1995). Therefore analysis of centromere separation should be integrated when investigating chromosome instability.

3. Objective analysis of centromere separation

Introduction of a simple digitalized image analysis system – by combining light microscopy and a PC based image analyzer software – is described that makes an objective and exact staging of centromere division possible.

Practical consequences

The method is simple, quick and relatively inexpensive, provided that an image analysis system is available. It offers an accurate and objective way to determine centromere division. It may also be utilized in retrospective analyses of old slides, e.g. in family investigations, in which centromere anomalies are sought as possible signs of chromosome instability.

4. Chromosome fragility and premature centromere division in coeliac disease

Irrespective of the method applied, no differences in the rate of chromosome breakage were found between coeliac patients and controls. Fragility was not correlated with diet, duration of gluten enteropathy, or age or sex of the patients. The fragility tests showed no chromosome instability in CD. The significance of unusually high frequencies of PCD (8-23%) observed in a few patients is not clear, but the phenomenon deserves further studies from the aspect of the relation of gluten enteropathy to malignancies.

Practical consequences

Our results support the findings of Kolacek et al (Kolacek et al. 1998) but did not show any correlation between the duration of diet and fragility. The same correlation can not be excluded between PCD and diet although our findings are not consistent. It is possible that these different features of instability may manifest themselves differently in the same condition as suggested by Fundia (Fundia et al. 1994).

Despite discrepancies it is highly likely that classic gluten enteropathy is not necessarily associated with chromosome instability. Two years later our suggestion was confirmed by Kolacek et al (Kolacek et al. 2004). They found that frequency of chromosome aberrations in peripheral

blood lymphocytes in CD patients decreased significantly on a gluten-free diet. According to their opinion genomic instability is a secondary phenomenon, possibly caused by chronic intestinal inflammation.

Continuing our investigation in children with CD we found 2 girls with 45,X/47,XXX karyotype (Méhes et al. 2007). Coeliac disease associated with sex chromosome abnormalities – especially with Ullrich-Turner syndrome – has been reported before (Ságodi et al. 2006), but this type of mosaicism in CD has not been published so far.

These findings have at least two implications:

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A. Screening for CD in patients with Turner syndrome should be performed and small bowel biopsy is recommended in positive cases. Undiagnosed and untreated CD has a much higher risk of malignant lymphoma and other neoplasms compared to that in CD patients following a strict gluten-free diet; in addition, untreated CD may impair growth hormone therapy in the affected patients.

B. Cytogenetic investigation is warranted in CD patients if there is any clinical suspicion, especially in girls with short stature.

5. Analysis of gastrointestinal malformations, associated congenital abnormalities and intrauterine growth in neonates

The distribution of gastrointestinal malformations in this study was similar to that reported in the literature. The frequency of intrauterine growth retardation was significantly increased in patients with gastrointestinal malformations compared with the normal population. Analysis of associated anomalies found that an unexpectedly large number of skeletal disorders exceeded numbers of heart and urogenital malformations (45.4%, 41.2% and 31.9%, respectively).

Practical consequences

Gastrointestinal malformations are often associated with intrauterine growth retardation and mostly complicated by skeletal anomalies. The association among these disorders – not published before – requires further investigation. However, from a practical point of view, skeletal anomalies – beyond cardiac and urogenital disorders – should be sought in newborns with gastrointestinal malformations.

SUMMARY

According to previous observations chromosome aberration, immunodeficiency, malformation, malignancy, reproductive loss, altered growth and development do not exist on their own only. Instead various degrees of overlap exists among them in certain cases (Méhes and Kosztolányi 2004). The relationships among these conditions are displayed in **Fig. 10.**

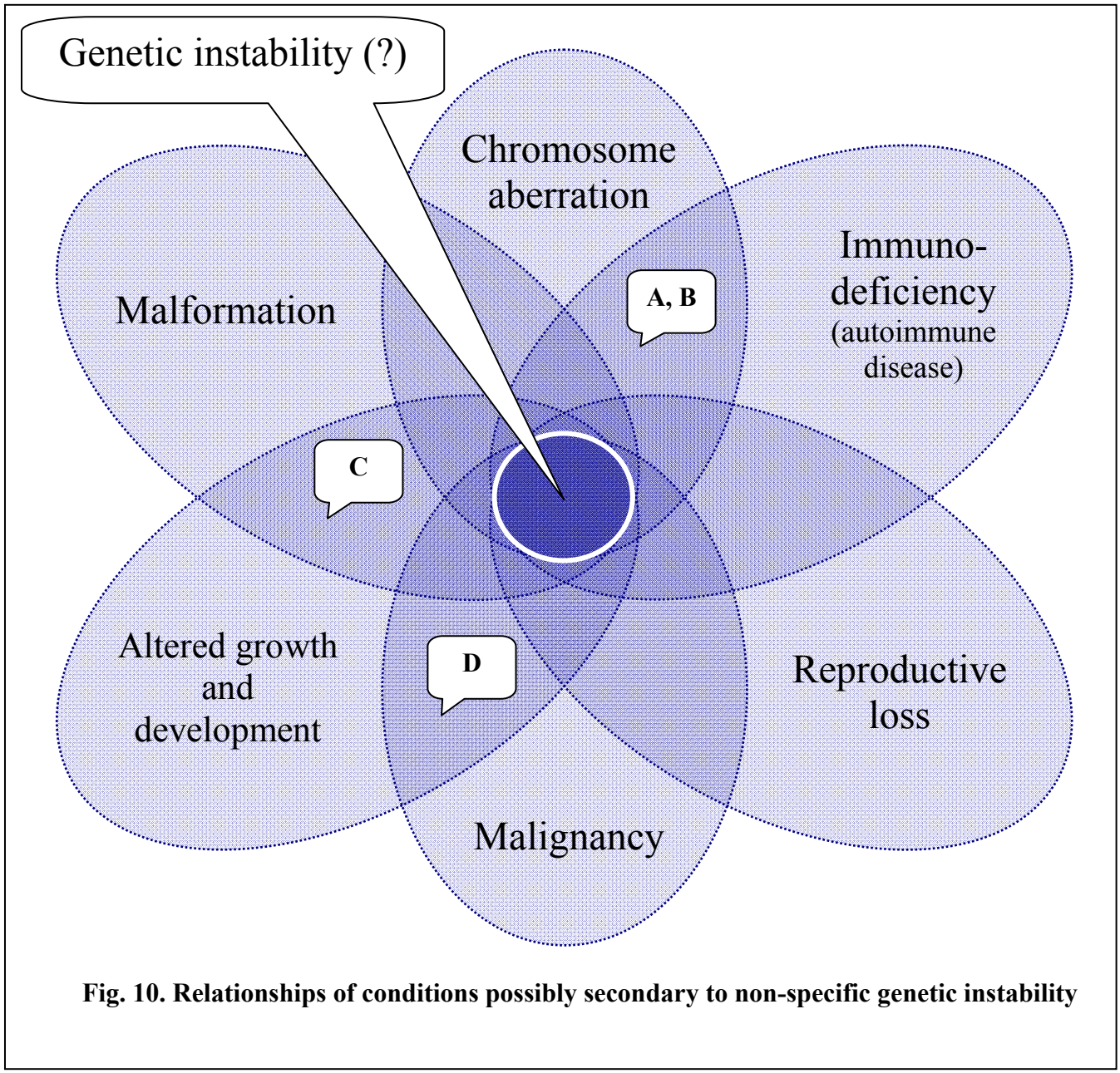

Fig. 10. Relationships of conditions possibly secondary to non-specific genetic instability

Most likely non-specific genetic instability may serve as a basis for these conditions resulting in different clinical manifestations (centre with white margin in **Fig 10.**). This hypothesis is supported not only by several references cited above but by the findings and results of this thesis, as well.

Referring to **Fig. 10.** contribution was made to the following areas marked by capital letters:

A. Overlap between chromosome aberrations and immunodeficiency,

B. Overlap between chromosome aberrations and coeliac disease (autoimmune disease),

C. Overlap between malformations and intrauterine growth retardation.

With respect to the overlap of malignancy and malformation (**D** in **Fig. 10.**), in a systematic analysis that we undertook (not discussed in this thesis), when prospectively screening children with malformations and their relatives for malignancies we found significantly more malignancies in grandparents of malformed children compared to controls, raising the possibility of increased risk for malignancy at a higher age (Hadzsiev et al. 2006).

Despite widespread investigations analysing the role of centromeres in cell division there is little knowledge about regulation of centromere separation. The processes controlling separation order are almost unknown. Further studies on this topic would enable us to understand the mechanisms of this process.

On a final note it is important to stress that experimental and clinical investigations presented in my thesis show that even difficult and exciting problems can be investigated by simple methods providing data for further research.

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